# A novel monoclonal antibody against human B7-1 protects against chronic graft-vs.-host disease in a murine lupus nephritis model

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**Abstract.** Lupus nephritis (LN) is the most common complication that causes mortality in patients with systemic lupus erythematosus. The B7-1/B7-2 and CD28/cytotoxic T-lymphocyte associated protein 4 co-stimulatory pathway serves a key role in autoimmune disease and organ transplantation. The aim of the present study was to generate and characterize a monoclonal antibody (mAb; clone 4E5) against human B7-1 and to investigate its potential use for the treatment of LN. The results demonstrated that the 4E5 mAb was successfully generated and able to recognize both human and mouse B7-1. After injection of this mAb into a mouse model with chronic graft-vs.-host disease (cGVHD)-induced lupus-like disease, the expression of CD21, CD23, CD80 and CD86 on B220+ B-cells in the spleen, and the concentrations of serum autoantibodies and urine protein, were decreased. Direct immunofluorescence analysis of the kidneys revealed that immunofluorescence of immune complex deposits was weaker in the 4E5-treated mice and electron microscopy analyses of renal tissues indicated that pathological injury of the kidneys of 4E5-treated mice was decreased compared with that in the model control mice. The results of the present study demonstrated that inhibition of the B7-1/CD28 co-stimulatory signaling pathway with the 4E5 mAb may represent a promising strategy to decelerate the progression of LN that is induced by cGVHD with potential for use in the treatment of other autoimmune diseases.

## Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease that is characterized by the production of autoantibodies and immune complexes that may affect the majority of organs in the human body (1). Lupus nephritis (LN) is the most common and serious complication of SLE and is the major cause of morbidity and mortality in patients with this condition (2). A previous survey demonstrated that at least 33% of patients with SLE also have a manifestation of significant glomerulonephritis (3).

The origins of SLE remain to be fully elucidated. It is generally thought that the imbalance between cellular and humoral immunity in patients with lupus is caused by a complex interaction between genetic and environmental factors and the abnormal function of T- and B-cells that ultimately destroy normal immune tolerance mechanisms. During the initiation of a normal immune response, T-cell activation, proliferation, differentiation, cytokine secretion and avoidance of apoptosis require at least two signals (4). These signals include a specific signal that is delivered by the antigen peptide presented by the major histocompatibility complex and a non-specific signal produced by the interaction of co-stimulatory molecules (5). Co-stimulatory pathways were first proposed and confirmed on the basis of a T-cell activation double signal theory (6).

B7, an important co-stimulatory molecule that is expressed on the surface of antigen-presenting cells (APCs), exists primarily in two forms, B7-1 and B7-2. The B7 receptor on the surface of T-cells is CD28/cytotoxic T lymphocyte antigen-4 (CTLA-4) and the interaction of B7 with CD28 promotes T-cell activation, proliferation and secretion of cytokines, as well as regulating T-helper ($T_H$)1/$T_H$2 cell differentiation and contributing to B-cell antibody production and isotype switching (7). The interaction of B7 with CTLA-4 inhibits T-cell activation and proliferation (5,8). A previous study demonstrated that

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the B7-CD28 co-stimulatory signal is associated with the development of SLE (9). Therefore, blocking or weakening of the induced signal may help reduce the associated pathological damage or the development of other immune-mediated diseases.

In this present study, a monoclonal antibody (mAb) against human B7-1 was generated and characterized. The potential efficacy against SLE-related pathologies was assessed using a lupus-like nephritis mouse model, a generally-accepted animal model induced by the injection of parental BALB/c lymphocytes into BALB/c x C57BL/J6 $F_1$ hybrids, leading to chronic graft-vs.-host disease (cGVHD) (10). This model has been previously verified and characterized as an SLE-like disease model that displays lymphoid hyperplasia, the formation of autoantibodies similar to those occurring in patients with SLE and an increased presence of renal pathologies mediated in part by immune complex formation and deposition (11).

## Materials and methods

*Cell lines*. The human B7-1-transfected L929 cell line (L929/B7-1) and mock-transfected L929 cells (L929/mock) were previously generated and used in this present study (12). Daudi and Raji cell lines were purchased from the American type culture collection. The cell lines were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (HyClone; GE Life Sciences) 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained at 37˚C in a humidified atmosphere containing 5% $CO_2$.

*Animals*. BALB/c mice (10; female; age, 6-8 weeks; weight, 25±2 g) and C57BL/J6 mice (10; male; age, 6-8 weeks; weight, 29±2 g) were purchased from the Department of Experimental Animals (Shanghai Institute of Biological Products, Ministry of Health of China). All mice were kept in specific pathogen-free animal facilities maintained at 18-22˚C and 50-60% relative humidity, with a 12-h light/dark cycle and provided *ad libitum* access to standard rodent chow and filtered water. All experiments were performed in accordance with the guidelines and approved by the Ethics Committee of Soochow University (Suzhou, China; approval no. 201912A341).

*Reagents and antibodies*. Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant and hypoxanthine-aminopterin-thymidine (HAT) selection medium were purchased from Sigma-Aldrich; Merck KGaA. The rabbit anti-mouse antibodies used were as follows: Mouse isotype immunoglobulin (Ig)G (cat. no. 555571), FITC-labeled anti-mouse granulocyte receptor 1 antigen (GR-1; $IgG_{2b}$; cat. no. 553127), FITC-labeled anti-mouse CD11b ($IgG_{2b}$; cat. no. 553310), phycoerythrin (PE)-labeled anti-mouse CD23 ($IgG_{2a}$; cat. no. 561773), PE-labeled anti-mouse CD21($IgG_{2b}$; cat. no. 552957), PE-labeled anti-mouse CD86($IgG_{2a}$; cat. no. 561963), PE-labeled anti-mouse CD80 ($IgG_{2a}$; cat. no. 561955), FITC-labeled anti-mouse CD11c ($IgG_1$; cat. no. 561045), PE-labeled mouse IgG (cat. no. 555988), PE-labeled rat anti-mouse IgG1 (cat. no. 562027) and allophycocyanin-labeled anti-mouse B220 ($IgG_{2a}$; cat. no. 561880). All antibodies were purchased from BD Pharmingen.

*Generation and characterization of anti-human B7-1 mAbs*. The hybridoma cell line from Shi *et al* (12) has been observed to secrete anti-human B7-1 antibodies. To generate anti-human B7-1, BALB/c mice were immunized with L929/B7-1 cells ($10^7$ cells in 100 $\mu$l PBS) that were pretreated with 10 $\mu$g/ml mitomycin (Sigma-Aldrich; Merck KGaA) for 2 h at 37˚C, by subcutaneous injection into the right flank in conjunction with a separate injection of 400 $\mu$l CFA. Mice then received three intraperitoneal injections of the cells/CFA at 1-week intervals. Mice were anesthetized (1% sodium pentobarbital; 50 mg/kg body weight) 4 days after the final injection and euthanized by cervical dislocation. The spleen was removed by opening the abdominal cavity and a single cell suspension of spleen cells prepared by mechanized homogenization with a syringe filled with PBS, filtration through a filter screen (200 mesh), centrifugation of the suspension (233 x g for 8 min at 4˚C) and removal of the supernatant. These cells were then fused with murine myeloma SP2/0 cells using standard protocols (13,14). The resulting hybridomas were subsequently cultured at 37˚C for 10 days in HAT selection medium. From the successful cultures, supernatants were evaluated for antigen (B7-1) recognition. In brief, L929/B7-1 cells were incubated with the supernatant at 4˚C for 45 min, then stained with PE-labeled rat anti-mouse IgG1 (1:200) at 4˚C for 30 min. FACS (Calibur system; BD Cell Quest Pro software; version 6.0; BD Biosciences) was used in order to analyze the stained cells. L929/B7-1 cells were also stained with PE-labeled rat anti-mouse IgG1 (1:200) as a negative control.

The specific 4E5 mAb was purified from BALB/c mouse ascites using Protein G-sepharose CL4B affinity columns (GE Healthcare) following the manufacturer's protocols. Female BALB/c mice were intraperitoneally injected with pristane (0.5 ml; Sigma-Aldrich; Merck KGaA) and 1 week later, mice were injected with a specific hybridoma line (intraperitoneally; $1x10^7$ total cells/mouse). Mice were then injected with an equal volume of a 1:1 (v/v) mixture of pristane and Freund's incomplete adjuvant. After a period of 1 week, which facilitated fluid accumulation in the abdomen (15), the ascites in each mouse were extracted with a syringe and processed using CL4B affinity column purification. The antibody was eluted with pH 2.8 glycine-hydrochloric acid mixture and the concentration was estimated spectrophotometrically. This was calculated as protein concentration (mg/ml)=[optical density at 280 nm $(OD_{280})x1.55]-(OD_{260}x0.76)$. The purified antibody was then conjugated with PE using standard protocols (16). Flow cytometry was subsequently used to verify the recognition of the B7-1 antigen by the clone 4E5 mAb with L929/mock, L929/B7-1, Daudi and Raji cells, as well as naive BALB/c mouse splenocytes, and performed as described earlier.

*Induction of the murine LN model of cGVHD and 4E5 antibody treatment*. Female BALB/c mice were mated with male C57BL/J6 mice to obtain $F_1$ generations. When $F_1$ hybrids reached the age of 6-8 weeks, the mice (all females selected) were randomly allocated into four groups: Normal control group (Mock, mice did not receive any treatment), model group (MG, cGVHD was induced in the mice), 4E5-treated group (4E5, cGVHD was induced in the mice and they were treated with 4E5) and a random IgG-treated group (IgG, mice cGVHD was induced in the mice and they were treated with

IgG), with 15 mice in each group. Excluding the Mock mice, all other F1 hybrids were used as recipients of female BALB/c donor cells.

To prepare the cells for each injection, two naive female BALB/c mice were anesthetized and euthanized using cervical dislocation. After death, the thymus, spleen and axillary lymph nodes of each mouse were recovered and single-cell suspensions were prepared by mechanical homogenization with a syringe filled with PBS, filtering through a filter screen (200 mesh), centrifuging the suspension (233 x g for 8 min at 4˚C) and removing the supernatant PBS. Then these single-cell suspensions of different organs from two mice were mixed together and counted [following standard treatments to remove contaminating erythrocytes as required (12)]. In each case, four individual intravenous injections were performed at 3-day intervals and a 100 $\mu$l volume of the solution, containing 5x10$^7$ cells/100 $\mu$l of the mixture of fresh donor cells, was injected into each recipient by tail vein. In the 4E5-treated mice, 4E5 mAb (10 mg/kg weight) diluted in PBS, was injected intravenously into the tail on days 15, 17, 19, 21, 44 and 74 after the first lymphocyte injection. This dose was selected based upon previous work reported by Shi *et al* (12). The IgG-treated mice were treated in parallel with mouse isotype IgG (BD Pharmingen; BD Biosciences). From 2 weeks after the first lymphocyte inoculation, mice received intraperitoneal anesthesia (1% sodium pentobarbital; 50 mg/kg body weight) and 100 $\mu$l of blood was drawn from the retro-orbital plexus every 30 days. Serum was isolated and frozen at -80˚C for subsequent analyses of autoantibodies. At the same time as the blood was collected, urine from each mouse was also collected, by pressing the mouse bladder and inserting a syringe into the urethra, for analysis of potential proteinuria. In all cases, urine and blood were collected at 10 am on the experimental day. At 12 weeks after the final inoculation, all mice were euthanized by intraperitoneal injections of 2% pentobarbital (120 mg/kg body weight) and death was verified using cervical dislocation. Tissues (including spleen and kidney) were harvested for subsequent analysis.

*Immune response evaluated by flow cytometry.* A total of 5 mice were selected randomly from each group at 3 weeks after the first lymphocyte injection and their splenocytes were isolated as mentioned above. After counting, distinct sets of 10$^7$ splenocytes were incubated with a specific fluorochrome-conjugated antibody (GR-1,1:500; CD11b, 1:500; CD23, 1:200; CD21, 1:200; CD86, 1:200; CD80, 1:200; CD11c, 1:500 or B220, 1:200) for 30 min on ice and washed in PBS (pH 7.4) for 30 min at room temperature. All samples were then analyzed using flow cytometry as mentioned above with analysis using Cell Quest Pro software (BD Biosciences). The major APC populations were macrophages, dendritic cells and granulocytes, which were pre-gated based on the APC activation markers GR-1, CD11b and CD11c in the present study.

*Anti-nuclear antibody (ANA) and anti-double-stranded DNA (anti-dsDNA) measurements.* To measure the levels of ANA and anti-dsDNA in isolated sera, immunofluorescent staining was performed using an ANA analysis kit (cat. no. YZB/Jing 1373-2009) and a daDNA analysis kit (cat. no. YZB/Jing 1372-2009, Beijing H&J NovoMed, Co., Ltd.) following the manufacturer's protocol. Sera were diluted (1:100 in PBS) and placed on cell-bearing glass slides containing ANA or dsDNA antigen for incubation (30 min at room temperature) in a humidified chamber. After being washed gently with PBS, the presence of any ANA or anti-dsDNA was determined using FITC-conjugated goat anti-mouse IgG antibodies with incubation for 30 min in the dark. Slides were washed again with PBS, air-dried and then sealed. Images were captured using a fluorescence microscope (Olympus Corporation). Levels of the autoantibodies were quantified indirectly using measurements of fluorescence intensity assessed in a blinded manner by three individuals using Image-Pro Plus software version 5.0 (BioRad Laboratories, Inc.). Intensities were then reported based using a semi-quantitative scale: Absent to mild (low) and moderate to very severe (high).

*Proteinuria measurement.* The fresh urine collected from each mouse was evaluated using Albustix urine dip sticks (Bayer AG), following the manufacturer's protocol. The extent of proteinuria was scored in a blinded manner by three individuals. Scores were reported as follows: Complexes absent (-), or present at mild (+), moderate (++), severe (+++) or very severe (++++) intensity.

*Histopathology.* A section from each mouse kidney dissected after mouse death was fixed in 10% buffered formalin for 2 h at room temperature and then embedded in paraffin. Sections (5 $\mu$m) were then prepared and stained with hematoxylin and eosin for 10 min at room temperature. The stained sections were then examined by light microscopy in a blinded manner.

A second section (100 nm) of each kidney was fixed in 2.5% glutaraldehyde buffer (pH 7.4) for 2 h at room temperature, washed twice with PBS for 15 min and dehydrated using gradient dilutions of acetone (70% acetone for 15 min, 80% for 15 min, 90% for 15 min and 100% twice for 10 min). Dehydration of samples was then performed using liquid CO$_2$. Samples were then sputter-coated with gold and examined using an H-800 transmission electron microscope (Hitachi, Ltd.) (17).

*Detection of immune complexes in kidney tissue.* The remaining parts of the kidney samples were frozen in optimal cutting temperature medium (Thermo Fisher Scientific, Inc.) and cut into 10-$\mu$m sections, which were air-dried and fixed in acetone at -20˚C for 20 min. After being washed in PBS, specimens were incubated with FITC-conjugated goat anti-mouse IgG (1:100 in PBS) for 30 min at 37˚C. Samples were then washed with PBS, air-dried and examined using a fluorescence microscope (Olympus Corp.). Each sample was scored for fluorescence intensity in a blinded manner by three individuals. Scores were reported as follows: Complexes absent (-), or present at mild (+), moderate (++), severe (+++) or very severe (++++) intensity.

*Statistical analysis.* All values are expressed as the mean ± standard deviation. The statistical significance of differences in values and frequencies between groups was evaluated using Student's t-tests (2 groups) or analysis of variance with Tukey's post-hoc test (>2 groups). All analyses were performed using SPSS 19.0 software (IBM, Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

## Results

*Generation and characterization of anti-human B7-1 mAbs.* Based upon previously established procedures (12), a novel mAb targeting human B7-1, 4E5, was generated (isotype IgG1). The flow cytometry results showed that cells were 97.8% positive in the L929/B7-1 line, 98.5% in the Daudi cell line and 98.7% positive in the Raji cell line, (Fig. 1). As the spleen contains several cell types, the spleen cells were only 46.9% positive. These results indicated that 4E5 mAb was able to recognize B7-1 not only on human cells (including L929/B7-1) but also on mouse splenocytes.

*4E5 mAb reduces the expression of activation markers on mouse splenocytes.* The expression of CD11b, CD11c and GR1 activation markers on APC cells was higher among the mouse splenocytes from the MG mice compared with that on cells from the Mock mice, indicating that the spleen cells were abnormally activated. By contrast, the frequency of the expression of each marker on cells from the 4E5-treated mice was significantly lower compared with that on cells from MG mice (Fig. 2, P<0.05). There was no significant difference in the expression levels of these markers between cells from MG mice and those from IgG control mice, suggesting that the activation of APC cells in the spleens of mice that received 4E5 was inhibited. The expression of the activation markers CD80 and CD86 on B220$^+$ B-cells from the 4E5-treated mice was also significantly lower compared with that on B-cells from MG mice (Fig. 3, P<0.05). These results suggested that 4E5 mAb may suppress immune responses that are normally induced by cGVHD.

*4E5 mAb treatment reduces the production of ANA and anti-dsDNA.* ANA and anti-dsDNA are cGVHD-induced lupus autoantibodies (18). To assess the severity of LN, the levels of each antibody in the sera were evaluated (Fig. 4). The results demonstrated that at 3 months after the initiation of the cell injections, 30% of the MG mice and 10% of the 4E5-treated mice exhibited high levels of ANA antibodies (P<0.05, Table I). At the 4-month time-point, 90% of the MG mice and 50% of the 4E5-treated mice exhibited high levels of ANA antibodies (P<0.05). At 3 months, 40% of the MG mice and 10% of the 4E5-treated mice exhibited high levels of circulating anti-dsDNA antibodies (P<0.05). At 4 months, 100% of the MG mice and 40% of the 4E5-treated mice expressed high levels of anti-dsDNA antibodies (P<0.05). No significant differences were indicated in the amount of serum ANA and anti-dsDNA antibodies at either time-point between the MG mice and IgG control mice. ANA and anti-dsDNA antibodies were not identified in the serum of the mock mice at any time-point.

*4E5 mAb treatment reduces the incidence of proteinuria.* Proteinuria is a marker of renal lesions, and in the present study, it was used as a criterion for the successful induction of the LN model. Analysis of the urine collected monthly following the initiation of lymphocyte inoculations indicated that proteinuria appeared later in the 4E5 treatment group and was less common compared with the frequencies in MG mice (Table II, P<0.05). At 3 months after initiation of inoculation,

50% of MG mice and none of the 4E5-treated mice exhibited high proteinuria (moderate to very severe; data not shown). The frequency of high proteinuria remained higher in MG mice (100%) at 4 months after initiation of the inoculations. By contrast, 50% of the 4E5-treated mice developed high proteinuria at this time-point. The frequency of proteinuria was not significantly different between the MG mice and IgG control mice and no proteinuria was present in the mock mice at any time-point.

*Effects of 4E5 mAb treatment on the formation of renal lesions.* Histological analysis revealed that kidneys from the MG mice exhibited a compensatory increase in the glomerular volume at 4 months after the initiation of inoculation. The analysis also revealed leukocytic infiltration in perivascular and internal areas, as well as endothelial and mesangial hypercellularity. By contrast, specimens from 4E5-treated mice exhibited slight histopathological changes, including glomerular enlargement and minor congestion of the glomerular vasculature (Fig. 5A). The IgG control mice also demonstrated a number of histological changes compared with mock mice.

*Effects of 4E5 mAb treatment on immune complex deposition.* Fluorescence analysis of kidney sections from the MG mice at 4 months following the initiation of the inoculations revealed a granular linear staining pattern of IgG deposits along the glomerular capillary loops, indicating that immune complexes were deposited in/on the loops of glomeruli. Although the fluorescence intensity of mice treated with 4E5 decreased significantly at the same time-point compared with MG group (P<0.05), no glomerulonephritis was observed and significant differences in the levels of immune complexes between the MG mice and IgG control mice were indicated at 4 months after the initiation of the inoculations (Table III; Fig. 5B). No immune complexes were observed in mock mice at 4 months after the initiation of the inoculations.

*Effects of 4E5 mAb treatment on renal ultrastructure.* TEM analysis demonstrated that in the kidneys of the MG and IgG control mice, electron-dense deposits were localized in subepithelial lesions of the glomerular basement membrane that were segmental and thickened. These deposits were also observed in visceral epithelial cells that exhibited effaced foot processes (humps). By contrast, in the glomerulus from 4E5-treated mice, the basement membrane layer was clear and intact and there were fewer, smaller 'humps' than in the MG group (Fig. 5C). No abnormal renal ultrastructure was indicated in the kidneys isolated from mock mice.

## Discussion

cGVHD, which is induced in recipient F1 hybrid mice following the inoculation of parental lymphocytes, resembles SLE with autoantibody production and glomerulonephritis (19). cGVHD is a sex-biased GVHD model in which the female donor makes the female host more vulnerable to SLE than a male (20-22). cGVHD has been revealed to be due to donor alloreactive CD4$^+$ T-cell activation of host B-cells (23) and is characterized by lymphocyte proliferation, the production of autoantibodies that resemble SLE
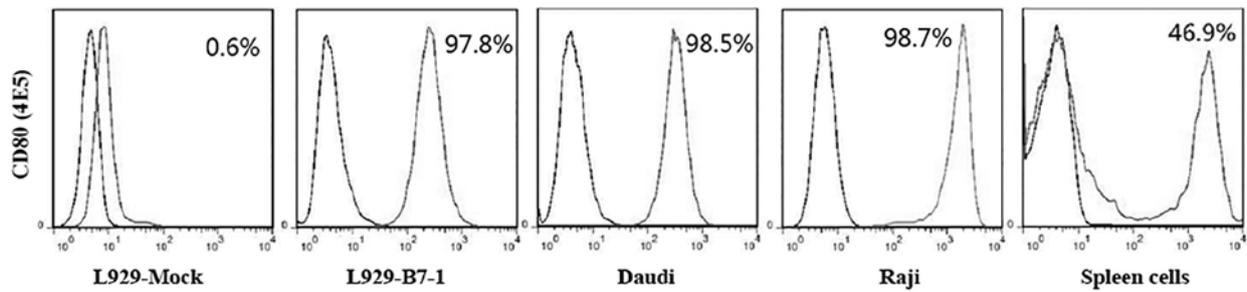
Figure 1. Histograms of cells stained with 4E5. Characterization of mouse anti-human B7-1 monoclonal antibody 4E5 using flow cytometry. Analyses suggested that 4E5 was able to recognize B7-1 on the L929 (B7-1), Daudi, Raji and mouse spleen cells, but not the L929-mock cells. Representative frequency plots are presented.
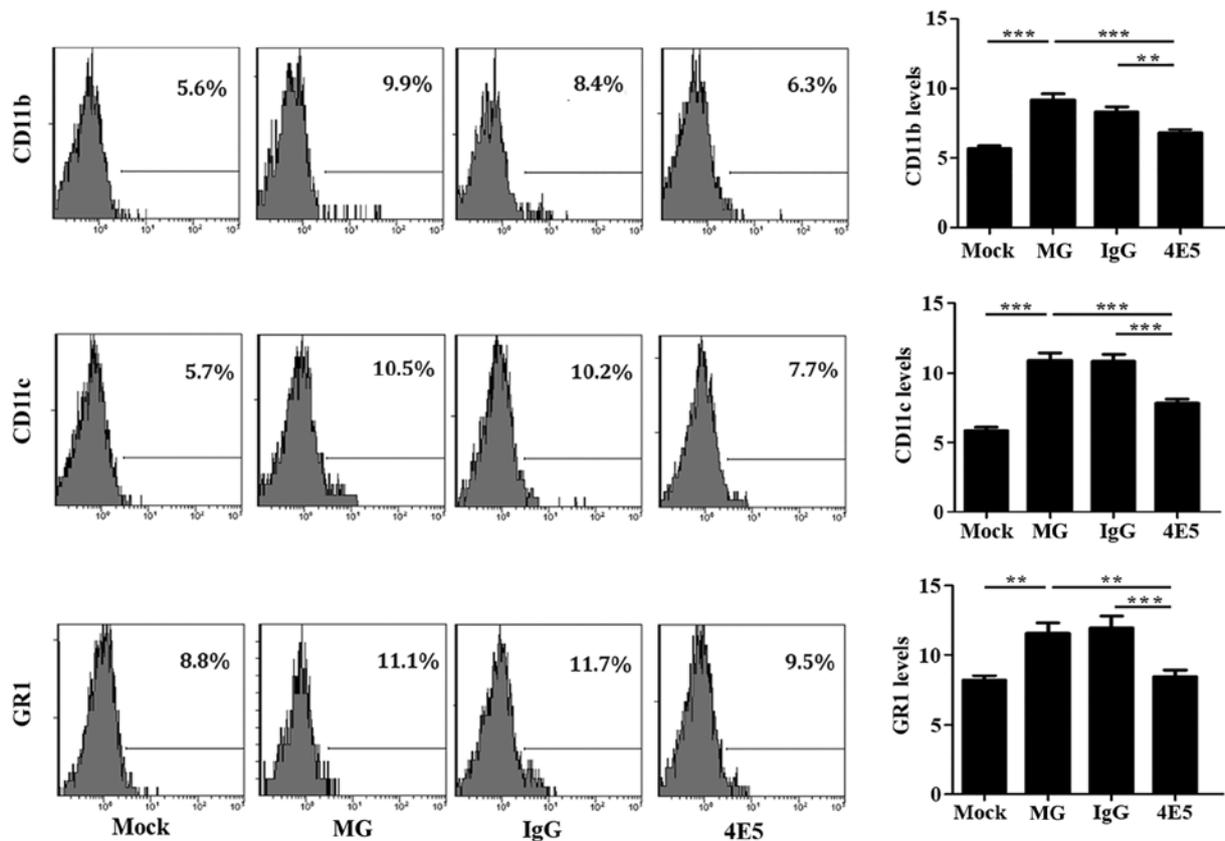


Figure 2. Effects of 4E5 on the activation marker expression levels in antigen-presenting cells. Expression of CD11b, CD11c and GR1 on isolated splenocytes was evaluated using flow cytometry. The expression of each of the markers on cells from 4E5-treated mice was significantly lower than that on cells of the model control mice. Representative histograms are provided. Values in the bar graphs are expressed as the mean ± standard deviation (n=5 per group at 3 weeks). **P<0.01, ***P<0.001. GR1, granulocyte receptor 1 antigen; Ig, immunoglobulin; MG, model group.

and serious renal pathology that is mediated by immune complexes. The pathological characteristics mentioned above are similar to those in human SLE; furthermore, it has a rapid onset (~2 months) and is easy to reproduce with a low cost (24). The most commonly used model of SLE worldwide is the MRL/lpr mouse model (25). These mice were produced by a series of complex hybridizations to the 12th generation from a number of different strains of mice, including LG/J, AKR/J, C3H/D and C57BL/6. The Fas gene of these mice became mutated and the lymphoproliferative gene appeared, leading to T-cell proliferation and lymph node swelling, so that the autoreactive lymphocytes are not

eliminated. The symptoms of this model are similar to those of human SLE, which is characterized by the presence of ANA, anti-dsDNA, anti-single stranded DNA and other auto-antibodies. The disadvantage of this model is that it is expensive to create, therefore it was not used in the present study. In future studies, this model should be used to confirm the results of the present study.

B7 molecules (including B7-1 and B7-2) are important co-stimulatory molecules, which may promote or inhibit T-cell activation, proliferation and differentiation, depending on the interactions with the receptors CD28 and CTLA-4 (26-29). B7-CD28 co-stimulatory signals serve important roles in SLE
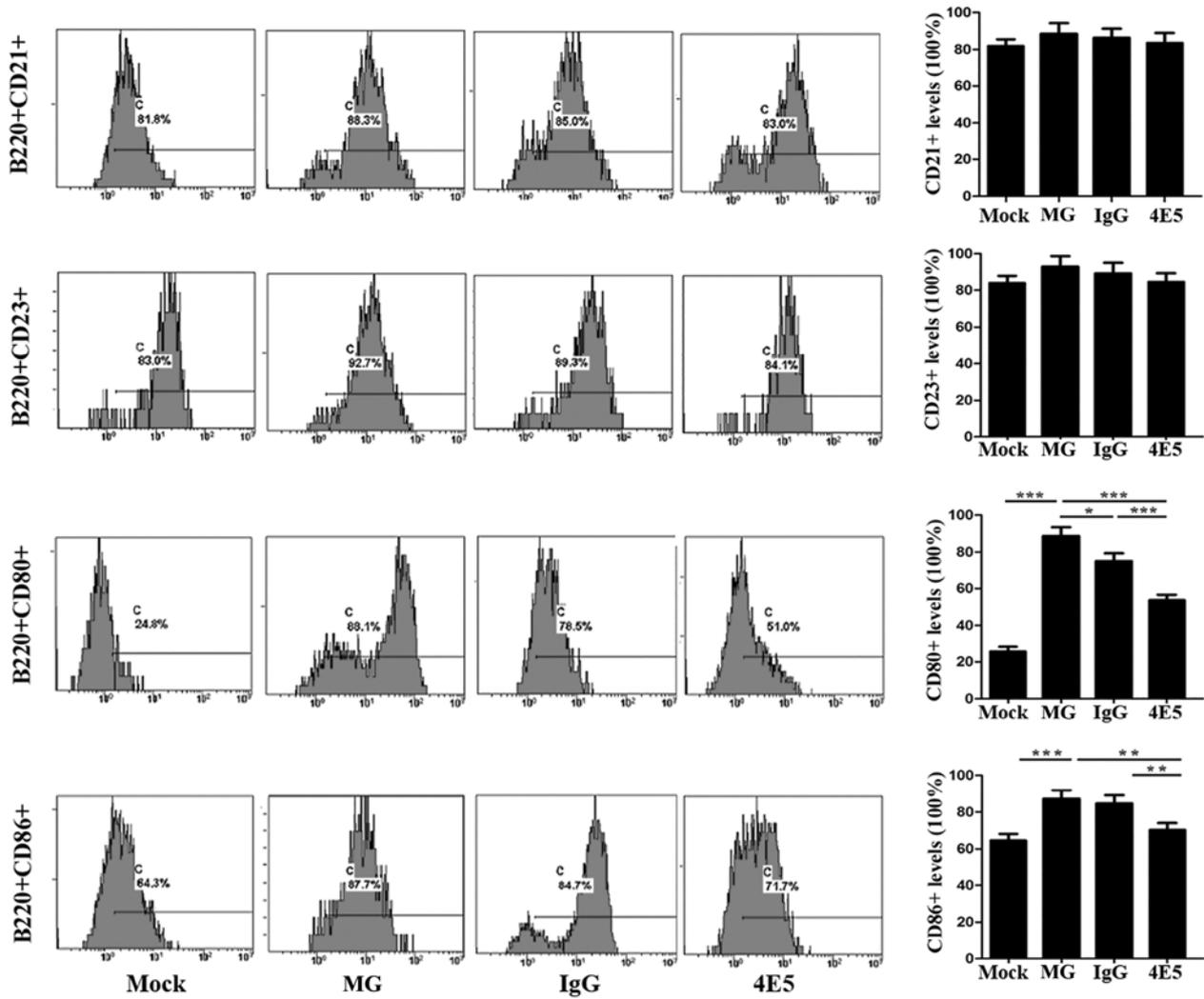
Figure 3. Effects of 4E5 on the expression of B220+ B-cells activation markers. Expression of CD21, CD23, CD80 and CD86 on B220+ B-cells from spleens were measured using flow cytometry. The expression of CD80 and CD86 on cells from 4E5 mice was significantly lower than on cells from MG mice. Representative frequency plots are provided. Values in the bar graphs are expressed as the mean ± standard deviation (n=5 per group at 3 weeks). *P<0.05, **P<0.01, ***P<0.001. Ig, immunoglobulin; MG, model group.
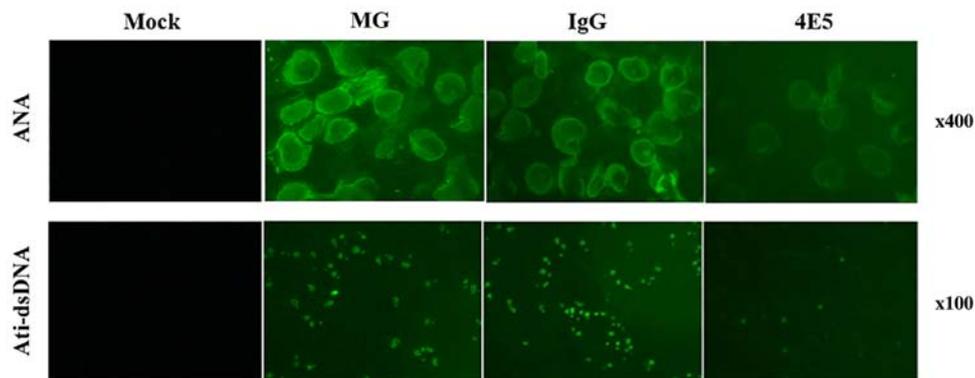


Figure 4. Effects of 4E5 on serum ANA and anti-dsDNA levels at 4 months after initial inoculation. Murine sera were collected monthly for evaluation of ANA (magnification, x400) and anti-dsDNA (magnification, x100) levels. Representative fluorescent images from sera of mice at the 4-month time-point after the initial inoculation are provided. dsDNA, double-stranded DNA; ANA, anti-nuclear antibody; MG, model group.

occurrence/development. It has previously been indicated that preferential expression of B7 on B-cells is essential for anti-DNA autoantibody production in patients with SLE (30).

Bijl *et al* (31) revealed that the expression of CD86 on CD19+ B-cells was increased and associated with disease activity, B-cell activation and levels of anti-dsDNA in patients with

Table I. Serum ANA and anti-dsDNA positivity at 3 and 4 months after initiation of inoculations.

| Time-point/autoantibody | Mock | MG | IgG-treated | 4E5-treated[a] |
|---|---|---|---|---|
| **3 months** | | | | |
| ANA | 0 (0) | 3 (30) | 4 (40) | 1 (10)[a] |
| Anti-dsDNA | 0 (0) | 4 (40) | 4 (40) | 1 (10)[a] |
| **4 months** | | | | |
| ANA | 0 (0) | 9 (90) | 9 (90) | 5 (50)[a] |
| Anti-dsDNA | 0 (0) | 10 (100) | 9 (90) | 4 (40)[a] |

[a]P<0.05 vs. MG group. Values are expressed as n (%) (total n=10 mice per group). ANA, anti-nuclear antibody; dsDNA, double-stranded DNA; Ig, immunoglobulin; MG, model group.

Table II. Comparison of proteinuria status in mice at 4 months after initiation of inoculations.

| Group | - | + | ++ | +++ | ++++ |
|---|---|---|---|---|---|
| Mock | 7 | 3 | 0 | 0 | 0 |
| MG | 0 | 0 | 4 | 5 | 1 |
| IgG-treated | 0 | 0 | 3 | 6 | 1 |
| 4E5-treated[a] | 1 | 4 | 5 | 0 | 0 |

[a]P<0.05 vs. MG group. Values are expressed as n (total n=10 mice per group). The proteinuria status was defined as follows: Absent, (-); mild, (+); moderate, (++); severe, (+++); and highly severe, (++++). Ig, immunoglobulin; MG, model group.

Table III. Comparison of fluorescence intensity in the mice at 4 months after initiation of inoculations.

| Group | - | + | ++ | +++ | ++++ |
|---|---|---|---|---|---|
| Mock | 5 | 5 | 0 | 0 | 0 |
| MG | 0 | 0 | 5 | 3 | 2 |
| IgG | 0 | 0 | 5 | 2 | 3 |
| 4E5[a] | 2 | 3 | 5 | 0 | 0 |

[a]P<0.05 vs. MG group. Values are expressed as n (total n=10 mice per group). The fluorescence intensity was rated as follows: Absent, (-); mild, (+); moderate, (++); severe, (+++); and highly severe, (++++). Ig, immunoglobulin; MG, model group.

SLE. The percentage of CD80^{+} cells in the large activated B-cell (CD19^{+}) subset of an SLE patient population was also significantly higher compared with subset populations from normal controls and patients with allergies (31). Folzenlogen et al (32) demonstrated that the B7 protein family may reflect immunologic dysregulation in patients with autoimmune diseases and may also indicate a state of increased B-cell activity and hypergammaglobulinemia that occurs during active SLE. Laurent et al (33) demonstrated that a CD28 blockade prevented the development of LN in NZB/NZW) F1 mice, which have typical lupus symptoms and are one of the most recognized animal models of lupus nephritis. In the present study, flow cytometry revealed that the expression of activation markers CD80 and CD86 on B220[+] B-cells in the MG group were significantly higher compared with those in the Mock group.

A number of therapies that block or activate co-stimulation molecules have been indicated to be efficient for the treatment of autoimmune diseases. Inhibiting the B7/CD28 co-stimulatory pathways using anti-B7 antibodies has been revealed to promote corneal allograft survival by inhibiting CD4^{+} T-cell secretion of interferon-γ, inhibiting further cellular immune responses and inflammatory reactions (34). Another study demonstrated that anti-CD80^{+} or anti-CD86 mAb infusion was effective in preventing GVHD-associated mortality by inhibiting donor CD4^{+} or CD8^{+} T-cell expansion in mice (35). Other avenues of investigation have examined the potential use of CTLA-4 as an immune checkpoint to inhibit T cell

activation (36). CTLA-4 Ig is a soluble protein that is composed of an extracellular portion of CTLA-4 and an $F_c$ fragment of $IgG_1$ (37). The essential component of Abatacept is CTLA-4 Ig, which was approved by the US Food and Drug Administration in December 2005 for the treatment of rheumatoid arthritis. Abatacept therapy has been demonstrated to be successful in murine SLE models and in early human clinical trials (38). In the previous study, it was observed that the clinical effects of Abatacept provided a possible benefit in patients with refractory disease, particularly articular or cutaneous involvement requiring medium- to high-dose corticosteroids (39). Previous studies have also indicated that CTLA4-Ig was more effective than cyclophosphamide in preventing glomerular sclerosis and tubular damage in murine SLE models (39,40).

In the present study, a mAb targeting the human CD80 molecule, 4E5, was successfully generated. This mAb was observed to not only recognize human CD80 on a variety of cells but also mouse splenocyte CD80. DNA sequencing analysis of the mouse CD80 complementary DNA has revealed that the mouse CD80 gene is closely associated with the human CD80 gene and shares a 63% identity in the protein-coding region (41). 4E5 was applied to the LN model that was induced by cGVHD and the present results demonstrated that 4E5 was able to prevent the development of cGVHD-induced lupus, as well as significantly inhibit immune-cell activation and autoantibody production. The onset of proteinuria, renal histopathologic changes and immune complex deposition in the kidneys were also inhibited. The expression of CD11b, CD11c
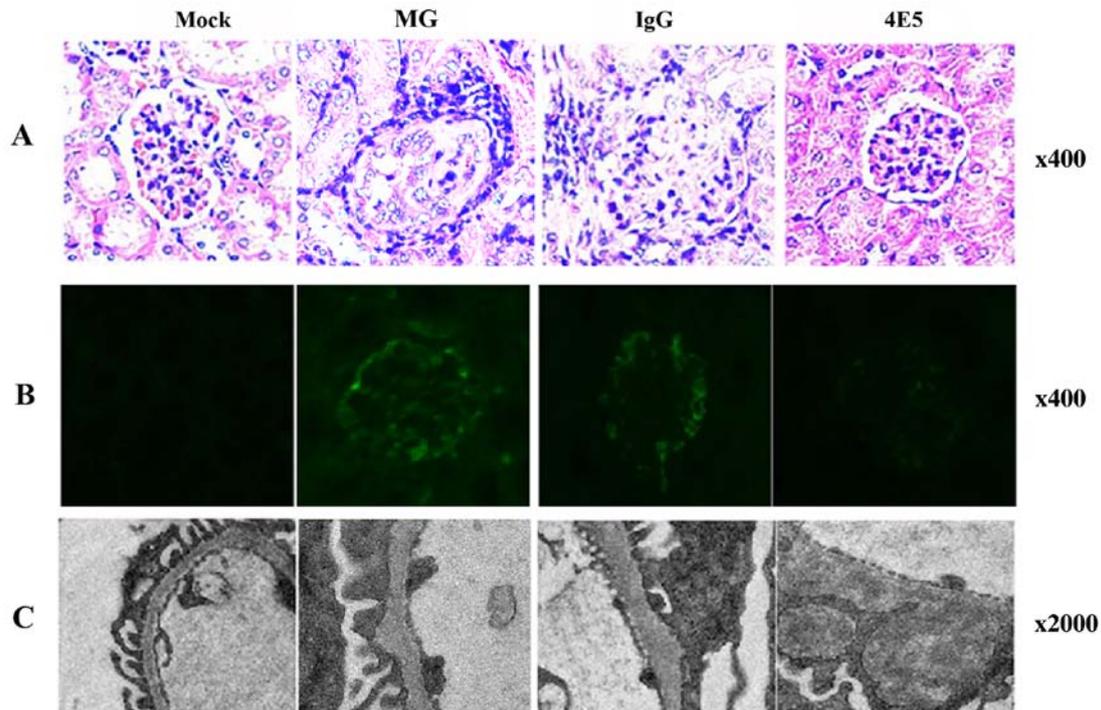
Figure 5. Analysis of kidneys at 4 months after initiation of inoculations. (A) H&E staining was used to evaluated the kidneys. The glomeruli of MG mice exhibited glomerular volume compensatory enlargement and leukocytic infiltration in perivascular and internal areas, as well as endothelial and mesangial cell hyper-cellularity. Only minor changes were noted in the organs of 4E5 mice when compared with mock mice (magnification, x400). (B) Representative fluorescence microscopy images of kidney sections from MG mice exhibited a granular linear staining pattern of IgG deposits along glomerular capillary loops; tissues of 4E5 mice had little-to-no staining intensity, suggesting minimal deposits (magnification, x400). (C) Representative transmission electron microscopy images revealing electron-dense deposits localized in subepithelial lesions of glomerular basement membrane in kidneys of MG mice; in glomeruli of 4E5 mice, the basement membrane layer was clear and intact, the 'humps' were less and smaller than MG group (magnification, x2,000). ANA, anti-nuclear antibody; dsDNA, double-stranded DNA; Ig, immunoglobulin; MG, model group.

and GR1 in spleen cells indicates the activation of macrophages, dendritic cells and granulocytes in the spleen respectively (12). In the present study, the activation of splenocytes (including macrophages, dendritic cells and granulocytes) in 4E5-treated mice was also significantly lower compared with that in the MG mice, suggesting that 4E5 exerted a suppressive effect during the early stages of immune activation.

Owing to their ability to promote the onset of SLE, B-cells are now considered to serve a key role in the pathogenesis of the disease by providing co-stimulatory signals necessary for T-cell activation, cytokine secretion and immune complex deposition. Treatments aimed at B-cells have therefore become a major therapeutic focus (42). In the present study, the expression of activation markers of B220+ B-cells CD86 and CD80 on cells in the 4E5-treated mice were lower compared with those on the cells of control mice, which may indicate a potential use in anti-B-cell therapy for treating SLE. Since T-B cell activation was inhibited and early immune responses were reduced in the cGVHD mice by treatment with the 4E5 mAb, the inhibition of CD80/CD28 signaling may be useful in B-cell-directed therapies against autoimmune diseases, including SLE. The model selected in this present study is characterized by the production of autoantibodies and immune complexes that are able to affect the majority of human organs (43). The autoantibodies are produced by the activated B cells, and thus, the present study focused on whether B cells were activated, while T cells were not assessed. However, the T-cell pathway is important and future research should

determine its role in SLE. Since proteinuria is a marker of renal lesions and a criterion for determining the induction of a successful LN model, the detection of proteinuria is regarded as an important index for the diagnosis and prognostic evaluation of LN. However, it is easily affected by the compliance of patients (44). A previous study (44) demonstrated that urinary albumin may be used to monitor renal damage due to the following: i) Albumin is the major component of urinary protein; ii) epidemiological data indicated that elevated levels of urinary albumin are associated with the risk of renal and cardiovascular disease; iii) according to the latest guidelines, the level of urinary albumin is one of the bases for the classification of chronic kidney disease. Overall, the determination of urinary albumin level is an important indicator for monitoring the development of nephropathy and should be performed in future experiments (45,46).

The primary cause of renal damage, LN, in patients with SLE is overproduction of pathogenic autoantibodies, including ANA and anti-dsDNA (18). The present study revealed that the production of ANA and anti-dsDNA in 4E5-treated mice was lower compared with that in the MG mice and that the extent of kidney damage, reflected by proteinuria, was also reduced. Although the present study demonstrated that B7-CD28 co-stimulatory signaling was associated with the development of SLE, inhibiting or weakening the signal may reduce the pathological damage associated with the disease. Further exploration of the roles of anti-CD80 antibodies in humans with SLE is required. A number of therapeutic

strategies for SLE are currently under investigation (47). The novel 4E5 mAb identified in the present study may be useful for immune therapies for the treatment of SLE, as well as the treatment of other autoimmune diseases and potentially in organ transplantation.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request

## Authors' contributions

YHQ and YX designed the experiments and drafted the manuscript. LJS, YZ, LHH, YYW, TMY and YK performed the experiments and analyzed the data. STZ analyzed the data. LJS, YX and STZ revised the manuscript and gave final approval of the version to be published . All authors read and approved the final manuscript.

## Ethics and approval and consent to participate

The present study was approved by the Ethics Committee of Soochow University (Suzhou, China; approval no. 201912A341).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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