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ORIGINAL ARTICLE

Establishment of a novel double-monoclonal antibody sandwich enzyme-linked immunosorbent assay (ELISA): tool for human B7-H4 detection in autoimmune diseases

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Summary

B7-H4, one of the immunoregulatory proteins, plays an inhibitory role by inhibiting T cell proliferation and cytokine production. Nevertheless, the significance of soluble B7-H4 (sB7-H4) in autoimmune diseases is unclear. In our study, we developed two novel mouse anti-human B7-H4 monoclonal antibodies (mAbs) (clones 8D4 and 7E1) with utilities for flow cytometry, immunoblotting and immunofluorescence. We characterized 7E1 as a functional antibody with antagonistic activity, which could promote T cell proliferation and regulate cytokine production. Furthermore, based on the different epitope specificities, we established a novel enzyme-linked immunosorbent assay (ELISA) which could detect sB7-H4 sensitively and specifically. Using this ELISA kit, sB7-H4 was observed in a high proportion of autoimmune diseases patients. We found that the levels of sB7-H4 were significantly higher in patients with systemic lupus erythematosus (SLE), type I diabetes (T1D) and Graves' disease (GD). Together, sB7-H4 in human serum is regarded not only as a regulator of T cell activation but may also be a diagnostic marker of autoimmune diseases.

KEYWORDS

antibody, autoimmune disease, ELISA, sB7-H4

INTRODUCTION

B7-H4 is a member of the B7 family (1). It is a type I transmembrane protein which exhibits some homology with other members in the extracellular domain (2). B7-H4 mRNA has been reported to be detected in many tissues, but its protein is more limited in normal tissues, such as lung, pancreas and kidney (3–5).

While the receptor for B7-H4 is as yet unidentified, it consistently appears to exert negative regulatory functions by inhibiting the activation, proliferation and cytokine secretion of T cells in immune responses (2,6–8). Many studies reveal

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that aberrant expression of B7-H4 can affect the progression of tumors and autoimmune diseases (9). Given that B7-H4 is expressed by various cell types and, in humans, can shuttle between the cytoplasmic and nuclear compartments (10, 11), it may play different roles up to the cellular context. Thus, B7-H4 in different pathological conditions is promising to be a diagnostic marker. Besides the membrane-bound form, the soluble form of B7-H4 (sB7-H4) has been found to contribute to many diseases (12–15).

In this study, we successfully developed a series of mouse anti-human B7-H4 monoclonal antibodies (mAbs) and screened two novel versions (clone 8D4 and 7E1), which demonstrated great utility for flow cytometry and immunoblotting. The mAb 7E1 we characterized also showed the activated effect on T cells *in vitro*, which suggested its potential function for blocking the B7-H4 signal. We then established a sandwich enzyme-linked immunosorbent assay (ELISA) system based on the two mAbs with different epitopes. The ELISA system showed a higher degree of precision, stability and specificity in sB7-H4 detection, which provided a valuable tool to measure the concentration of sB7-H4 in human sera. We further investigated the levels of sB7-H4 in the patients with autoimmune diseases, including systemic lupus erythematosus (SLE), type I diabetes (T1D), type II diabetes (T2D), Graves' disease (GD), Sjögren's syndrome (SS) and rheumatoid arthritis (RA). Our research indicates that the expression of sB7-H4 can demonstrate a putative effect in autoimmune diseases, and may provide new guidance for effective interventions on clinical trials.

MATERIALS AND METHODS

Patients and samples

This study population comprised 406 patients, including 63 cases of SLE, 64 cases of T1D, 60 cases of T2D, 73 cases of GD, 71 cases of SS and 75 cases of RA. Seventy healthy individual healthy controls (HC) were also enrolled. Fasting venous blood was collected in a serum separator tube and centrifuged to isolate the serum. All subjects were collected from the First Affiliated Hospital of Soochow University, Suzhou, China. Informed consent was provided according to the protocol approved by the Ethics Review Board of the First Affiliated Hospital of Soochow University (approval number: 2018012).

Mice and cell lines

BALB/c mice were from the Animal Research Center of Soochow University. The institutional protocol for animal studies was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Soochow University. Chinese hamster ovary (CHO) cells and mouse myeloma cells (SP2/0 cell line) were from the American Type Culture Collection (ATCC, Manassas, VA, USA). CHO/Mock and CHO/B7-H4 cells were from the investigator's laboratory. All cells were cultured in RPMI-1640 containing 10% fetal bovine sera (FBS).

Generation of monoclonal antibodies

Generation of monoclonal antibodies were performed as previously described (16). Briefly, BALB/c mice (aged 6–8 weeks, female) were immunized with cells (1×10^7/mouse) pretreated by mitomycin (0.5 μg/ul). According to the classic methods (17), the booster injection was repeated three times every 21 days. The splenocytes were then harvested and fused with the SP2/0 in 50% polyethylene glycol (PEG). To select hybrid clones, the fusion cells were cultured by Dulbecco's modified Eagle's medium (DMEM) containing hypoxanthine–aminopterin–thymidine (HAT; Sigma, St Louis, MO, USA) and 15% FBS in 96-well plates. The hybrid clone supernatants were screened by flow cytometry to establish hybridoma cell lines. Finally, we used a protein G sepharose affinity column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) to purify the ascites antibodies.

Biological properties analysis

Immunoglobulin (Ig) isotypes were identified by mouse monoclonal antibody isotyping kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. The antibody specificity was investigated by flow cytometry. Indirect ELISA was used to determine the binding ability of antibodies to immobilized B7-H4Ig (R&D Systems, Minneapolis, MN, USA). Western blotting and indirect immunofluorescence were performed to show that the antibodies could recognize B7-H4 protein. All detailed protocols of these experiments were performed as previously described (18,19). Meanwhile, commercial B7-H4 antibodies MAB65761R (R&D Systems) and PA5-84187 (Invitrogen, Carlsbad, CA, USA) were used as the positive controls.

Additionally, the competition assays were performed by flow cytometry (performed as previously described) and ELISA (20). The protocols of competition assay by ELISA were as follows: one unlabeled anti-B7-H4 mAb was coated with carbonate buffer solution (CBS) in a 96-well plate overnight at 4°C. After blocking with 3% bovine sera albumin (BSA), purified human B7-H4 fusion protein were added and incubated at 37°C for 2 h. After washing, the plates were incubated with another biotin-labeled mouse anti-B7-H4 mAb

for 1 h at 37°C. The plate was then washed and incubated with horseradish peroxidase (HRP)-streptavidin (Sigma) at 1 : 10 000 for 1 h at 37°C. Tetramethylbenzidine (TMB; Sigma) was added after washing and followed stopped by 2 M sulfuric acid. The absorbance at 450 nm was measured by microplate reader (Bio-Rad, Hercules, CA, USA). Meanwhile, BSA was added to replace unlabeled or biotin-labeled antibody as a negative or positive control.

Cytokine and cell proliferation assay

CHO/B7-H4 cells (2×10^4/well) were pre-incubated into 96-well plates overnight. Peripheral blood mononuclear cells (PBMCs, 1×10^6/well) isolated from healthy controls were added with anti-CD3 mAb (100 ng/ml) and B7-H4 mAbs (1 μg/ml or 10 μg/ml) or anti-CD3 mAb plus isotype control antibody (mouse IgG, 10 μg/ml) at 37°C. After 72 h in co-culture, cell proliferation was investigated by cell counting kit 8 (CCK-8) (Mashiki-machi, Kiyushu, Japan). Meanwhile, supernatants were collected and cytokine concentrations were analyzed using a cytometric bead array (CBA) human chemokine kit (BD Biosciences, San Jose, CA, USA). The CBA kit can be used to quantitatively measure interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17, tumor necrosis factor (TNF)-α and interferon (IFN)-γ levels in a single sample.

Establishment of sB7-H4 ELISA

Mouse anti-B7-H4 (clone 8D4, 5 μg/ml) was coated with CBS in a 96-well plate overnight. After blocking with 3% BSA, purified human B7-H4 fusion protein was incubated at 37°C for 2 h. After washing, the plate was incubated with biotin-labeled mouse anti-B7-H4 (clone 7E1, 0.5 μg/ml) for 1 h at 37°C. The plate was then incubated with HRP-streptavidin at 1 : 10 000 for 1 h at 37°C. Tetramethylbenzidine (TMB, Sigma) was added after washing and followed stopped by 2 M sulfuric acid. The absorbance at 450 nm was measured by the microplate reader (Bio-Rad).

Precision, stability and specificity assay of sB7-H4 ELISA

Different concentrations of B7-H4-Fc fusion protein (10, 5 and 2.5 ng/ml) were tested to assess the intra-assay precision 20 times, and the same samples were tested 10 times for the interassay precision. To evaluate stability, the precoated plates were preserved for a long time (0, 10, 20 or 30 days) at 4°C and three known concentrations of sB7-H4 (10, 5 and 2.5 ng/ml) were detected. Moreover, the specificity of the ELISA was determined by other homologous proteins, including sB7-H1, sB7-H2, sB7-H3, sB7-H5 and sB7-H6.

Statistical analysis

Statistical analyses were performed by GraphPad (version 5.0). Soluble B7-H4 were non-normally distributed and presented as median + interquartile range (IQR). For independent samples, Student's *t*-test or a non-parametric Mann–Whitney *U*-test was used. One-way analysis of variance (ANOVA) or the Kruskal–Wallis test was performed for multiple comparisons. $P < 0.05$ was considered as the significant difference.

RESULTS

Identification of two novel anti-human B7-H4 mAbs

A large panel of mAbs against human B7-H4 (> 1500) was produced by different clones, and more than 250 clones showed positive binding by ELISA. We finally obtained 28 clones of anti-human B7-H4 mAbs (Supporting information, Figure S1) and two (clone 8D4 and 7E1) were selected for further validation. The isotypes of 8D4 and 7E1 were IgG1 κ and IgG2 κ, respectively. The specificity of the two mAbs was identified by indirect ELISA and flow cytometry; 8D4 and 7E1 could bind to immobilized B7-H4 Fc protein (Figure 1a) and CHO/B7-H4 transfected cells (Figure 1b). Moreover, immunofluorescence analysis indicated that 8D4 and 7E1 specifically recognized CHO/B7-H4 cells (Figure 1c). Western blotting showed that both 8D4 and 7E1 recognized the target protein bands of CHO/B7-H4 transfected cells and B7-H4 Fc, but not IgG protein or CHO/Mock cells (Figure 1d).

Promotion of cell proliferation and cytokine of co-cultured PBMC by 7E1

Cell proliferation was investigated after the co-culture of PBMC and CHO/B7-H4 cells. Compared with mouse IgG control, 7E1 promoted PBMC proliferation in a dose-dependent manner (Figure 2a). The production of TNF-α and IFN-γ significantly increased in the presence of 7E1 compared to the cells co-cultured with IgG, while secretion of IL-10 and IL-4 significantly decreased (Figure 2b–e). These results demonstrated that 7E1 was a functional antibody with antagonistic activity, which could promote T cell proliferation and regulate cytokine production by blocking B7-H4 co-inhibitory pathway in our experimental system.

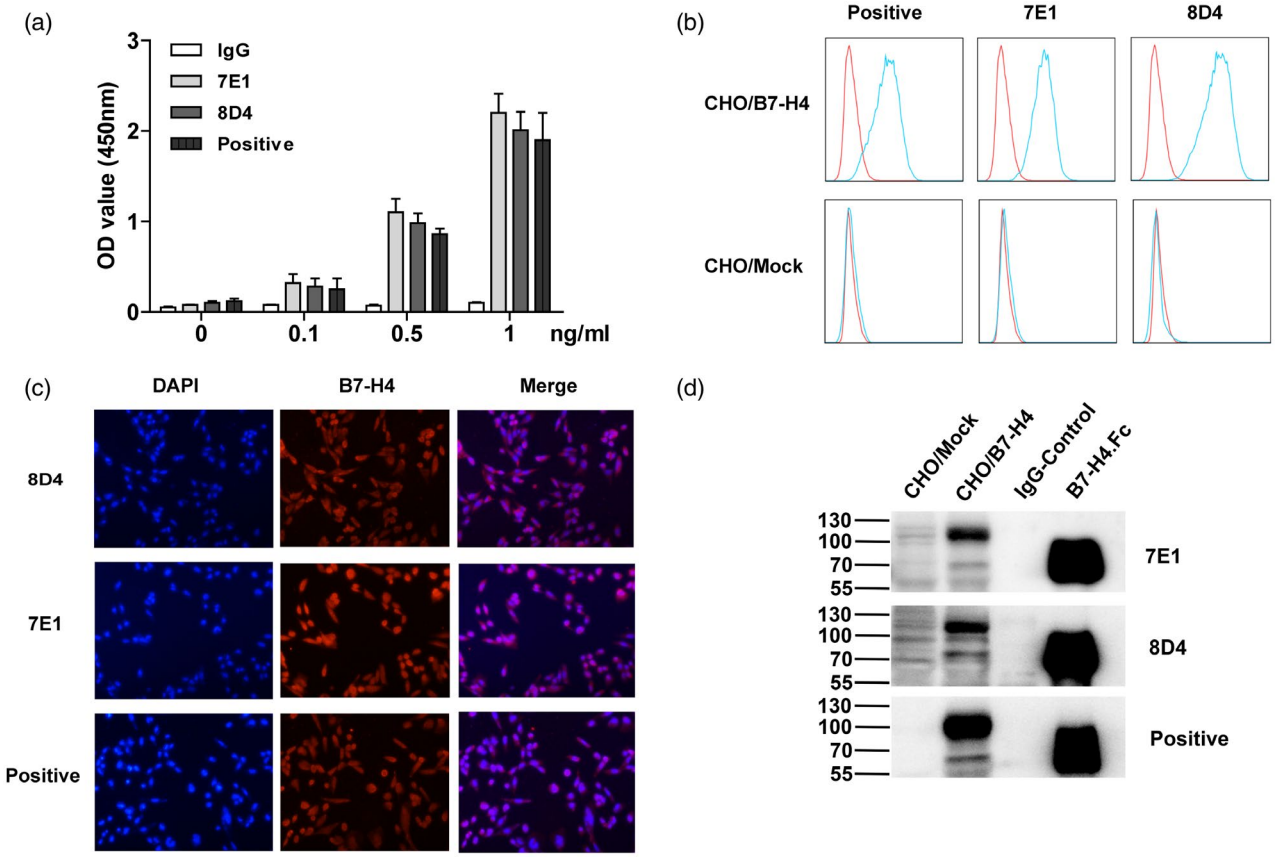

FIGURE 1 Characterization of B7-H4 monoclonal antibodies (mAbs). (a) Indirect enzyme-linked immunosorbent assay (ELISA) was used to determine the binding ability of antibodies to immobilized B7-H4 Fc. The results represent three independent experiments, and data are expressed as the mean of the absorbance at 450 nm. (b) The specificity of antibodies was investigated by flow cytometry. (c) Representative images of indirect immunofluorescence using B7-H4 mAbs. (d) Western blot showed the antibodies recognized B7-H4 protein.

8D4 and 7E1 recognized two different epitopes

The competitive binding assay by flow cytometry demonstrated that one unlabeled mAb could not inhibit the binding of the other biotin-labeled mAb (Figure 3a). ELISA revealed similar results: that one unlabeled mAb and the other biotin-labeled mAb could bind to different sites of B7-H4 Fc protein (Figure 3b). All the above results suggested that 8D4 and 7E1 recognized different epitopes of B7-H4.

Establishment of a novel sandwich sB7-H4 ELISA

By optimizing the working concentrations, a sensitive ELISA was established using 5 μg/ml 8D4 as the coating antibody and 0.5 μg/ml biotin-7E1 as the detecting antibody. The detectable limitation of B7-H4 Fc protein was 78–5000 pg/ml, with R^2 was 0.99574 (Figure 4a). Meanwhile, the ELISA system did not cross-react with other homologous protein, such as sB7-H1, sB7-H2, sB7-H3, sB7-H5 and sB7-H6 (Figure 4b–f). Thus, we successfully established a sandwich ELISA system that could detect sB7-H4 sensitively and specifically.

Precision and stability of sB7-H4 ELISA

Analysis of intra- and interprecision showed that the ELISA was very moderate, with only little variation among wells and plates [coefficient of variation (CV) % < 10%, Table 1]. In addition, there was no significant loss in signal intensity during different periods, including 0, 10, 20 and 30 days (CV % < 10%). The quality of the precoated ELISA plates was still stable after long-term storage, as it did not affect sB7-H4 detection (Table 2).

Application of sB7-H4 ELISA

CHO/B7-H4 cells can produce high levels of sB7-H4 in the supernatants. Using the ELISA system, we found that the levels of sB7-H4 in the supernatants increased in a time-dependent manner (Figure 5a). Furthermore, we investigated

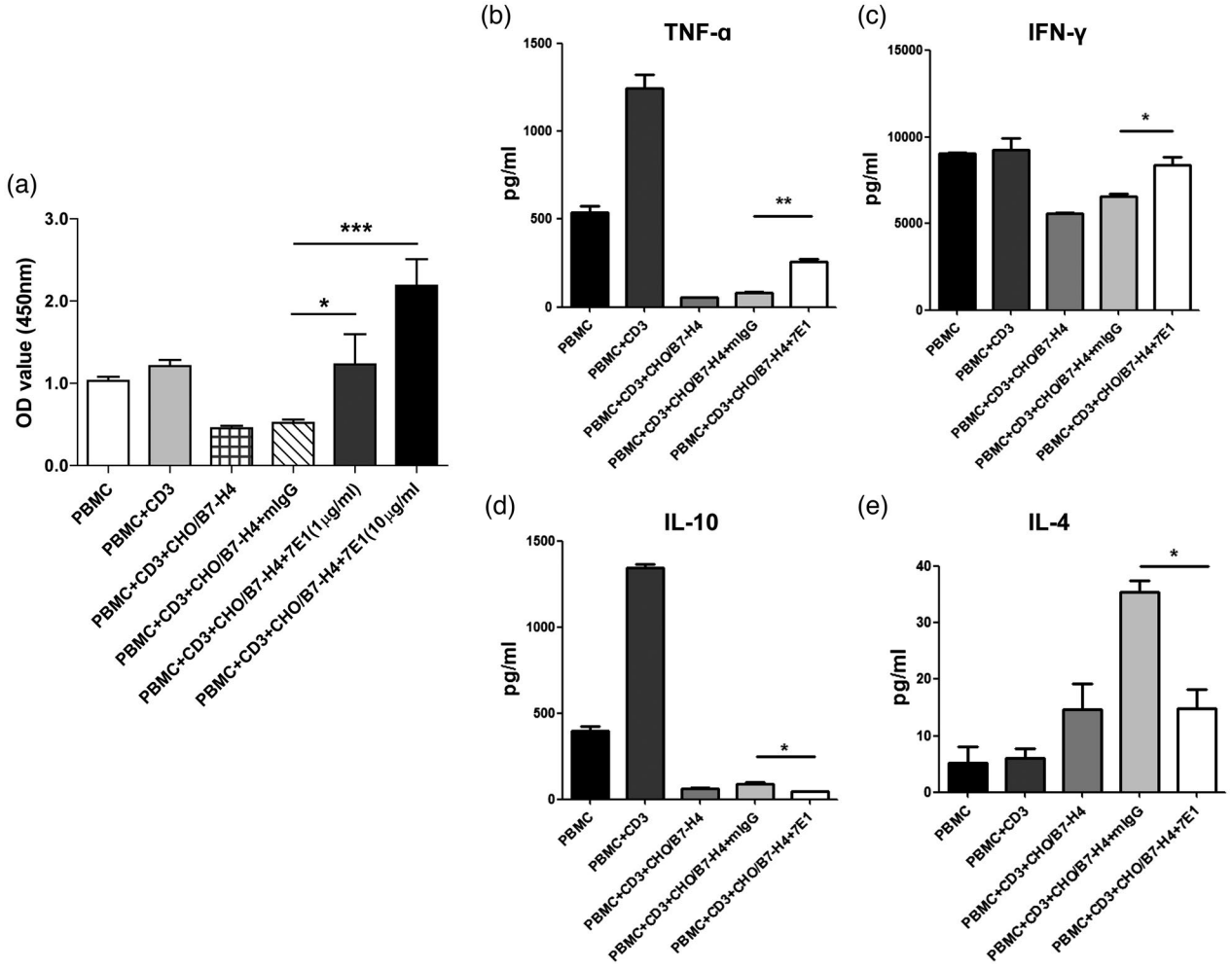

FIGURE 2 Function of 7E1 on peripheral blood mononuclear cell (PBMC) proliferation and cytokine production. (a) 7E1 promoted the proliferation of PBMC in a dose-dependent manner. (b,c) Compared with the cells co-cultured with immunoglobulin (Ig)G monoclonal antibodies (mAbs), the secretion of tumor necrosis factor (TNF)-α and interferon (IFN)-γ significantly increased in the presence of 7E1. (d,e) Compared with the cells co-cultured with IgG mAb, the secretion of interleukin (IL)-10 and IL-4 significantly decreased in the presence of 7E1. Data are representative of at least three independent experiments and the results are shown as mean ± standard deviation (s.d.) of triplicate wells. $*p < 0.05$, $**p < 0.01$ and $*** p < 0.001$

the levels of sB7-H4 in the sera of patients with autoimmune diseases. Our results showed that the ELISA kit was able to detect the concentrations of sB7-H4 in the sera of healthy donors and patients. The concentration of sB7-H4 was significantly higher in SLE (137.6 ± 114.3 pg/ml, $p < 0.0001$), T1D (79.78 ± 54.79 pg/ml, $p < 0.0001$) and GD (65.29 ± 42.55 pg/ml, $p = 0.0024$) patients when compared with healthy controls (49.49 ± 40.09 pg/ml, Figure 5b).

DISCUSSION

The major component of the immune balances is a series of co-stimulatory and co-inhibitory pathways engaged in modulating the host response (5,9). As a crucial co-stimulatory molecule, B7-H4 plays a negative role in regulating the adaptive immune response. Herein, we successfully developed two novel mouse anti-human B7-H4 mAbs and established a sandwich ELISA system for autoimmune disease detection. Our aim was to provide a valuable tool to evaluate the levels of sB7-H4 in the sera of individuals.

Most studies have reported that B7-H4 is associated with the adverse prognosis and high recurrence rate of tumors, which acts as a negative regulator in T cell-mediated anti-tumor immunity (4,7,21,22). Our recent work found that B7-H4 in metastatic lesions could predict poor prognosis in patients and promote the progression and metastasis of colorectal cancer (19). In addition, B7-H4 is engaged in the development and progression of autoimmunity. Chen's group found the presence of B7-H4 expression in endothelial cells and B cells in rheumatoid synovium tissues, suggesting that B7-H4 is involved in the neovascularization and associated with the pathological changes of RA (23). By influencing the regulatory T cells, B7-H4

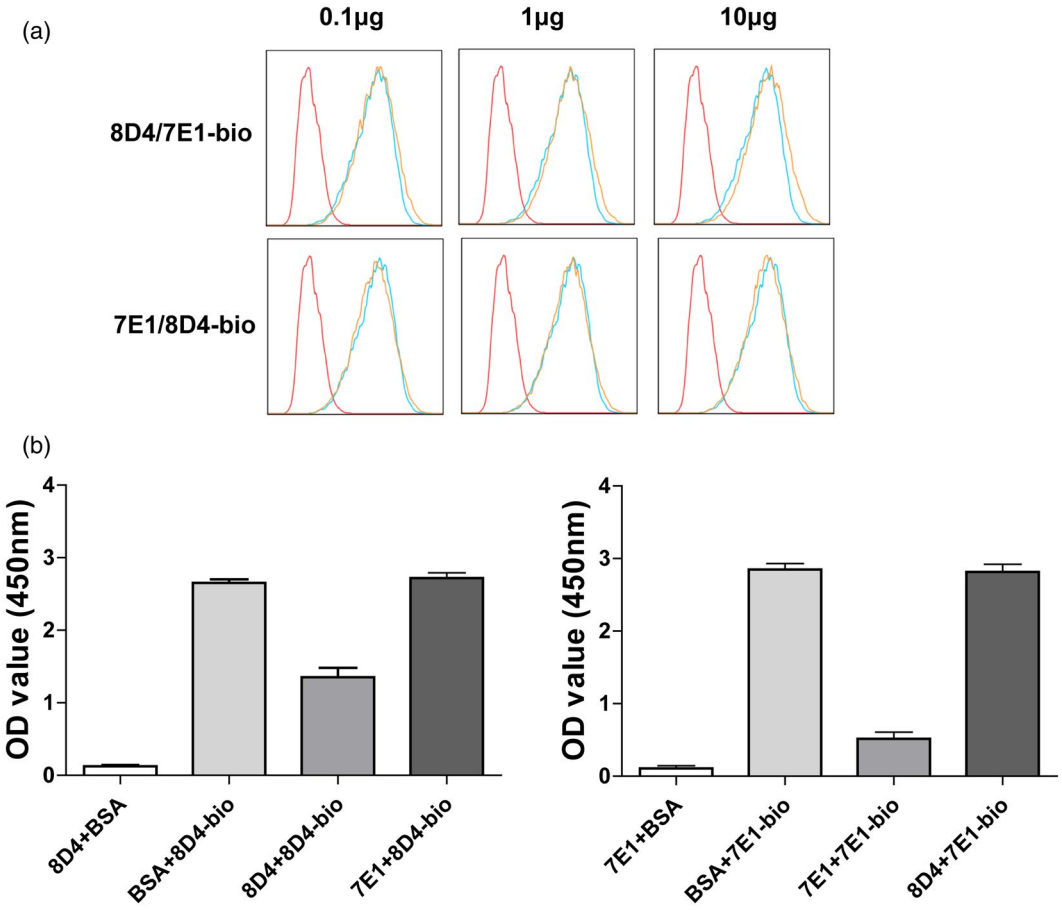

FIGURE 3 8D4 and 7E1 monoclonal antibodies (mAbs) recognized different epitopes. (a) Mutual competition assay by flow cytometry. Negative control (red lines): CHO/B7-H4 cells stained directly with biotin-immunoglobulin (Ig)G followed by streptavidin-phycoerythrin (PE). Positive control (blue lines): CHO/B7-H4 cells stained with biotin-B7-H4 mAbs. (b) Mutual competition assay by enzyme-linked immunosorbent assay (ELISA). The results represent independent experiments performed in triplicate, and data are expressed as the mean of the absorbance at 450 nm

can exert an immunosuppressive effect in a murine SLE model (24). In diabetes, enhanced expression of B7-H4 on the pancreatic islets exerted a protective function through $CD4^+$ and $CD8^+$ T cells and prolonged islet allograft survival after transplantation (25). These findings imply that B7-H4 is a novel molecule target for the therapy of autoimmune diseases.

During the past decades, immune checkpoint molecules provide effective guidance for potential clinical interventions and new targets for cancer treatment (26). Antibodies specific for B7-H4 in autoimmune diseases has been attracting more and more attention with the development of immunotherapeutic approaches (27,28). Using a B7-H4 antagonist antibody, Xiao *et al*. found that B7-H4 expressed on DC could suppress immune responses and thus aggravated the lupus model (24). While blockade of B7-H4 exacerbated experimental autoimmune encephalomyelitis (EAE) disease, the incidence and severity significantly reduced by treatment with B7-H4 Ig fusion protein (29). In this study, we obtained an anti-B7-H4 mAb (clone 7E1), which effectively promoted the proliferation of PBMC and regulated the production of cytokines, suggesting its potential function for blocking B7-H4 signal and enhancing T cell reactivity to various antigens. Thus, our antibody showed the activated effect on T cells, and it may be potentially useful as therapeutic agent in autoimmune diseases.

B7-H4 also exists in serum soluble form, which is cleaved by a metal endopeptidase from the membrane-bound form (30). The concentrations of sB7-H4 are increased and various, with subtypes in patients with ovarian cancer (31). The presence of serum sB7-H4 is also a negative prognostic indicator for multiple tumors and autoimmune diseases (32–35). In our study, we developed two B7-H4 mAbs with great biological properties. More importantly, 8D4 and 7E1 exhibited the different epitope specificities, which recognized the distinct antigenic epitopes on the B7-H4 molecule. Based on this, we successfully established a novel ELISA system with good precision, stability and specificity. The ELISA kit was approved to be competent for the detection of sB7-H4

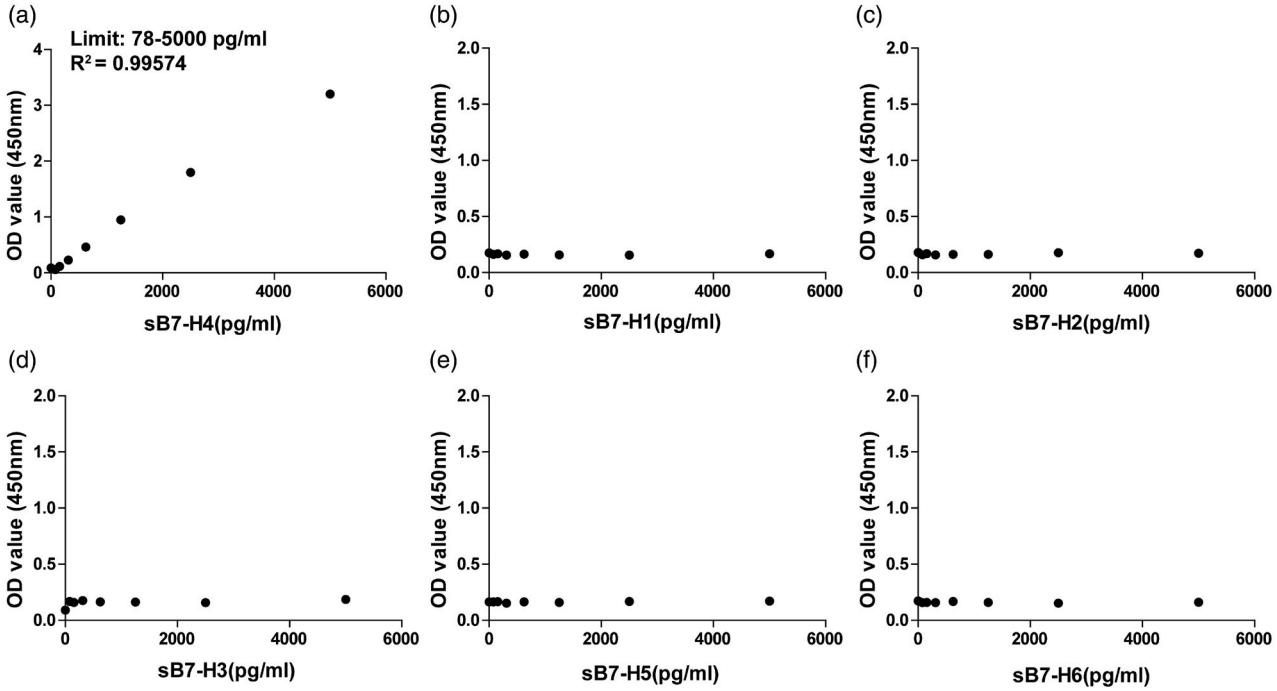

FIGURE 4 Establishment of sB7-H4 enzyme-linked immunosorbent assay (ELISA). (a) The unlabeled 8D4 was 5 μg/ml, the biotin-labeled 7E1 was 0.5 μg/ml. The twofold serial dilutions of the sB7-H4 immunoglobulin (Ig) protein starting from 5 ng/ml were detected by ELISA. R^2 represents the correlation coefficient. (b–f) The specificity of sB7-H4 ELISA. The proteins of sB7-H1, sB7-H2, sB7-H3, sB7-H5 and sB7-H6 were detected by ELISA. The results represent independent experiments performed in triplicate, and data are expressed as the mean of the absorbance at 450 nm

TABLE 1 Precision of the human sB7-H4 ELISA system

	Intra-assay precision			Interassay precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	10	10	10
$\bar{x}$	5.06	2.53	1.24	5.04	2.58	1.24
SD	0.23	0.12	0.07	0.24	0.23	0.09
CV%	4.57	4.64	6.04	4.72	8.75	7.66

Abbreviations: CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; s.d., standard deviation.

TABLE 2 Stability of the human sB7-H4 ELISA system

	5 ng/ml		2.5 ng/ml		1.25 ng/ml	
Time (day)	$\bar{x} \pm s$	**CV%**	$\bar{x} \pm s$	**CV%**	$\bar{x} \pm s$	**CV%**
0	5.10 ± 0.13	2.50	2.56 ± 0.08	3.27	1.25 ± 0.06	4.74
10	4.98 ± 0.16	3.15	2.51 ± 0.13	5.11	1.25 ± 0.04	3.42
20	4.90 ± 0.27	5.50	2.43 ± 0.19	7.94	1.21 ± 0.07	6.02
30	4.82 ± 0.37	7.76	2.38 ± 0.20	8.44	1.25 ± 0.10	8.33

Abbreviations: CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay.

in cell culture supernatants and serum samples. The kit's performance has been optimized and the lower limit of detection was 78 pg/ml, which demonstrated high relative sensitivity. Furthermore, we investigated the levels of sB7-H4 in the serum samples of healthy donors and patients of autoimmune diseases. We found the elevated sB7-H4

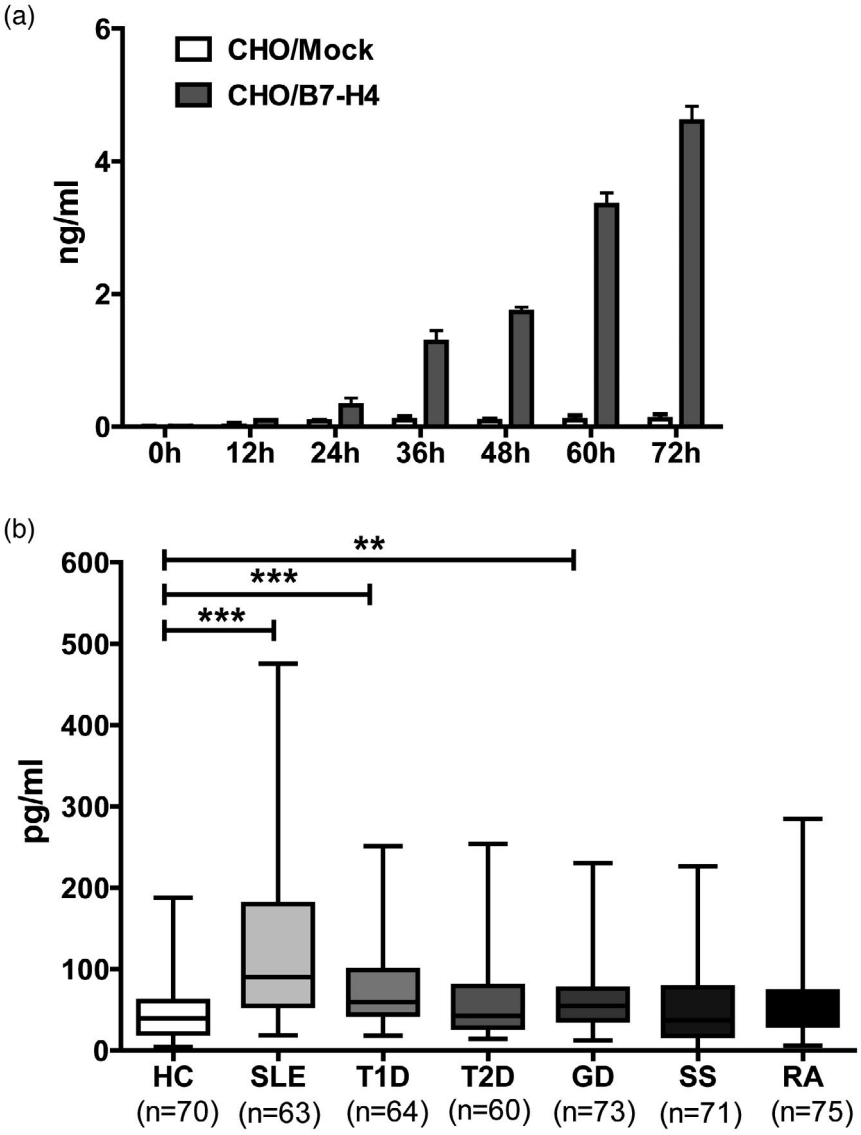

FIGURE 5 Application of sB7-H4 enzyme-linked immunosorbent assay (ELISA). (a) The sB7-H4 concentrations in the cell supernatants collected at different times were determined by ELISA. (b) The levels of sB7-H4 in sera from patients with autoimmune diseases, including systemic lupus erythematosus (SLE) ($n = 63$), type I diabetes (T1D) ($n = 64$), T2D ($n = 60$), Graves' disease (GD) ($n = 73$), Sjögren's syndrome (SS) ($n = 71$) and rheumatoid arthritis (RA) ($n = 75$). $^{**}p < 0.01$ and $^{***}p < 0.001$

levels in patients of SLE, T1D and GD, suggesting that the potential use of our ELISA system could contribute to distinguish the three autoimmune diseases from others.

This study population comprised 406 patients with various autoimmune diseases, as we attempted to collect enough samples during the past 5 years. Due to the limited proportion of patients, we were not able to collect more samples. We could not speculate precise answers when the sample size was not enough. Future studies with a larger population are required to elucidate the expression pattern and clinical significance of B7-H4 in autoimmune diseases.

In general, we developed two novel monoclonal antibodies against human B7-H4 and the biological properties were analyzed. We further established a novel sandwich ELISA for the detection of sB7-H4 in autoimmune diseases. Collectively, our results could promote the future application of B7-H4 antibodies in autoimmune diseases.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

X. C. and C. L. designed the study. S. D., H. Z., Y. G., Y. S., L. Z. and H. Z. performed the experiments. S. D. and C. L. analyzed the data. J. W. and X. Z. contributed new reagents. S. D. wrote the manuscript. All authors discussed the results.

ETHICS APPROVAL

All procedures performed in the studies were reviewed and approved by the Ethics Review Board of the First Affiliated Hospital of Soochow University, in accordance with the Helsinki Declaration of 1975, as revised in 2008.

DATA AVAILABILITY STATEMENT

The data sets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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