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Preparation, characterization and application of anti-human OX40 ligand (OX40L) monoclonal antibodies and establishment of a sandwich ELISA for autoimmune diseases detection

Jingnan An[a,d], Sisi Ding[b], Xiaohan Hu[c], Lili Sun[b], Yanzheng Gu[a], Yunyun Xu[c], Yumin Hu[b], Cuiping Liu[a,*], Xueguang Zhang[b,**]

[a] Jiangsu Institute of Clinical Immunology, The First Affiliated Hospital of Soochow University, Suzhou, China
[b] Jiangsu Key Laboratory of Clinical Immunology, The First Affiliated Hospital of Soochow University, Suzhou, China
[c] Institute of Pediatrics, Children's Hospital of Soochow University, Suzhou, China
[d] Institute of Blood and Marrow Transplantation, Soochow University, Suzhou, China

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ABSTRACT

OX40L (CD252, TNFSF4), a type II transmembrane protein which like other tumor necrosis factor ligands, involved in the costimulation and differentiation of T cells, functions as a positive signal in immune response. To investigate the biological function of soluble OX40L (sOX40L), three functional anti-OX40L monoclonal antibodies (mAbs) 3D2, 3F7 and 2H3 were obtained by hybridoma technology. Besides, specificity of the mAbs was further demonstrated by ELISA, Western blot and Immunofluorescence experiments. We also developed a novel enzyme-linked immunosorbent assay (ELISA) based on two anti-human OX40L antibodies 3D2 and 3F7 with different epitopes. Using the ELISA system, we found that sOX40L in the sera of healthy donors increases in an age-dependent manner and that enhanced sOX40L expression in some autoimmune diseases especially in rheumatoid arthritis (RA) patients, suggesting the potential diagnostic significance of sOX40L in the autoimmune diseases. Together, these data demonstrate that the existence of circulating sOX40L in human sera might play an important role in immunoregulation.

1. Introduction

The activation of T cell is mediated not only by antigen stimulation through T cell receptors but also some costimulatory molecules to induce the costimulatory signals [1,2]. The costimulatory molecules of tumor necrosis factor (TNF) superfamily could play critical roles in modulating immune responses and inducing bidirectional signals [3,4]. OX40 (CD134) and OX40L (CD252), members of the TNF receptor superfamily (TNFRSF) and TNF superfamily (TNFSF), played important roles in T cells expansion and survival, has been found with aberrant expression in tumors, infectious, inflammatory and autoimmune diseases [5,6]. OX40L mainly expressed on the surface of antigen-presenting cells (APC), including B cells [7], dendritic cells (DCs) [8,9], macrophages [10], mast cells [11,12], T cells and endothelial cells [13] via its receptor OX40, which was expressed on the activated T cells [14]. OX40/OX40L signaling acts as a key role in the development and differentiation of some immunological cells, especially T cells.

The importance of OX40L in autoimmunity has been demonstrated in several studies [15]. Manku et al. found that OX40L may cause disease pathology in systemic lupus erythematosus (SLE) through enhance the B cell differentiation and proliferation [16]. OX40L was deadly to the polymicrobial sepsis patients [17]. Nordmark et al. observed that OX40L might cause the pathogenesis of primary Sjögren's syndrome (SS) in the immune system [18]. Besides its membrane bound isoform, soluble form of OX40L has previously been related to some autoimmune diseases.

Based on the above study, OX40L showed important activity in the autoimmunity system. The blockade of the OX40 and OX40L interaction by using anti-OX40L mAbs or OX40 fusion protein (OX40Ig) may be a promising therapy for the treatment of these diseases. To investigate this, we gained three novel mouse anti-human monoclonal antibodies and developed an ELISA system based on the two anti-

* Correspondence to: C. Liu, Jiangsu Institute of Clinical Immunology, First Affiliated Hospital of Soochow University, 708, Renmin Road, Suzhou, Jiangsu 215006, China.
** Correspondence to: X. Zhang, Jiangsu Key Laboratory of Clinical Immunology, First Affiliated Hospital of Soochow University, Suzhou 215006, Jiangsu, China.
E-mail addresses: liucuiping1980@126.com (C. Liu), xueguangzh@126.com (X. Zhang).

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OX40L mAbs 3F7 and 3D2 with different epitopes. Using this ELISA kit, we evaluated the level of sOX40L in human sera from healthy donors, which found the concentration increases in an age-dependent manner. We also measured the levels of sOX40L in the patients of autoimmune diseases, and found RA patients sera have a significant higher expression compared with those from healthy donors. These findings suggest that sOX40L may be used as a predictor for autoimmune disease diagnosis and therapy.

2. Materials and methods

2.1. Patients and controls

In this study, 94 RA patients, 26 SS patients, 63 SLE patients were enrolled in the study. For comparison, 107 healthy volunteers served as healthy controls (HC). Peripheral blood samples were collected from patients at the Rheumatology Department in the First Affiliated Hospital of Soochow University, Suzhou, China from 2013 to 2018. All of the patients fulfilled the 1987 American College of Rheumatology classification criteria. The study was approved by the Ethics Committee of first affiliated hospital of Soochow university. All participants had been informed and signed the consent for participation in this study.

2.2. Antibodies, cell lines and reagents

PE-conjugated mouse anti-human OX40L antibodies were purchased from Biolegend (San Diego, CA). Mouse anti-human CD3 mAb and biotinylated mouse IgG were all products from Immunotech (Marseille, France). Commercial recombinant OX40Ig protein was obtained from R&D (Minneapolis, MN). Hypoxanthine-thymidine (HT) and hypoxanthine-aminopterin-thymidine (HAT) were purchased from Sigma (St. Louis, MO). CBA human cytokine kit was purchased from BD Bioscience (San Jose, CA). Anti-OX40L antibody 3D2, 3F7 and 2H3 were produced in our laboratory. Sera levels of sOX40L were determined by ELISA developed in our laboratory with using 3F7 as a capture antibody and biotinylated 3D2 as a detecting antibody.

Mouse fibroblast L929 cells were stably transfected with empty vector (L929/mock cells) or human OX40L vector (L929/OX40L cells). RPMI-1640 medium and fetal bovine sera (FBS) were purchased from Gibco (Carlsbad, CA).

2.3. Generation of novel anti-human OX40LmAbs

L929/OX40L cells were used as immunogens. Female BALB/c mice were immunized with L929/OX40L cells pretreated by mitomycin. The injection was repeated three times every 21 days. After that, the spleen cells from the immunized mouse and myeloma cells (SP2/0 cell line) were fused in the presence of polyethylene glycol (PEG). The fusion cells were cultured with DMEM medium containing HAT and 15% FBS in 96-well plates to select the hybrid clones. The supernatants of hybrid clones were screened for the detection of antibodies using ELISA and flow cytometry. The hybrid clone-secreting antibodies recognized L929/OX40L cells but not L929/mock cells. Positive clones were subcloned and established a hybridoma cell line that secreted monoclonal antibody. Ascites antibodies were produced and purified by protein G sepharose affinity column (GE, Sweden).

2.4. Characterization of OX40L mAbs

Ig isotypes were identified by SBA Clonotyping System-HRP kit (Southern Biotech, Alabama) according to the manufacturer's instructions. Competition assay was performed to determine whether the antibodies recognize different epitopes of OX40L antigen. L929/OX40L cells were incubated with an unlabeled anti-OX40L mAb for 30 min at 4 °C, then the cells were washed and stained with another biotinylated anti-OX40L mAbs followed by streptavidin-PE for 30 min at 4 °C. The result was analyzed by flow cytometry. L929/OX40L cells were also stained directly with biotinylated mouse IgG followed by streptavidin-PE as a negative control or biotinylated anti-OX40L mAbs as positive control.

For the detection of anti-OX40L mAbs on OX40 and OX40L interaction, L929/OX40L cells were incubated with mAbs for 30 min at 4 °C. After washing for three times, the cells were reacted with OX40Ig fusion protein for 30 min at 4 °C. Then followed by adding PE goat anti-human IgG for 45 min at 4 °C. Meanwhile, L929/OX40L cells were directly stained with OX40Ig protein followed by PE-goat anti-human IgG (Fc) as a positive control or human IgG as a negative control. The stained cells were analyzed by flow cytometry.

Western blot assay was used to analyze the binding ability of the mAbs. Recombinant OX40LIg or L929/OX40L transfected cells containing high OX40L were separated on 10% non-reducing gels. L929/Mock and IgG protein were used as negative control. After transferring the membrane, washing with PBST (PBS containing 0.2% Tween 20) and blocking with 5% non-fat dried milk, the target bands were stained with two monoclonal antibodies 3D2 and 2H3 followed by HRP-labeled goat anti-mouse IgG mAb (Affinity Biosciences, OH). The membranes were visualized with Super ECL Reagents (Applygen Technologies Inc., Beijing). Commercial OX40L antibody PA5-34516 (Thermo Fisher Scientific, CA) was as a positive control.

A total of 1×10^4 cells were plated into a chamber of a Lab-Tek 8 chambers slide (Thermo Fisher, CA) overnight. Then cells were confluent to 60–80%, washed with PBS for three times, fixed with 4% paraformaldehyde (Fisher Scientific, CA) and permeabilized in 0.1% Triton X-100 (Sigma, MO) for 30 min at 4 °C. After washing three times and incubated with blocking solution (5% BSA in PBS), then incubated overnight with OX40L mAbs at 4 °C. Cells were washed three times and incubated with goat anti-mouse Alexa Fluor 594 (Thermo Fisher Scientific, CA) for 1 h in the dark. Finally, the cells were washed and incubated with DRAQ5 for 20 min at room temperature in the dark. The slides were washed and mounted with mounting medium (Agilent, CA). All matched samples were photographed using confocal microscopy with identical exposure times. Commercial OX40L antibody ab89896 (Abcam, CA) was as a positive control. The application of the OX40L mAbs was determined by an indirect ELISA. The detailed method was performed as described previously [19].

2.5. Proliferation and cytokine responses

Cell proliferation was determined using a Cell Counting Kit-8 (CCK-8, Mashikimachi, Japan). Briefly, PBMCs were isolated from healthy controls, and 1×10^5 cells were resuspended and stimulated with anti-CD3 (100 ng/ml) in the presence or absence of 3F7 (1 ng/ml or 10 ng/ml) at 37 °C, 5% CO_2. After 48 h or 72 h in culture, 10 μl CCK-8 were added to the cells and then cells were counted in a microplate reader (Bio-Rad, CA) for the absorbance at 450 nm.

The culture supernatants were collected, and the cytokine levels were measured by a CBA human cytokine kit. Briefly, 50 μl samples or standard samples were added to a mixture of 50 μl capture beads. 50 μl phycoerythrin conjugated detection antibodies were mixed at room temperature in the dark for 2 h, and then washed to remove unbounded antibodies. The stained cells were analyzed by flow cytometry and CBA software (BD Biosciences).

2.6. Establishment of sOX40L ELISA

Mouse anti-OX40L (clone 3F7, 5 μg/ml) was coated with sodium phosphate buffer in a 96-well plate (Corning, NY) overnight at 4 °C. The plates were blocked with 2% bovine sera albumin (BSA) in PBS at 37 °C for 2 h. Samples or purified human OX40L fusion protein were added and incubated at 37 °C for 2 h in duplicate. The plate was washed three times with TBST and then incubated with 0.5 μg/ml biotinylated mouse anti-OX40L (clone 3D2) for 1 h at 37 °C. All antibodies were titrated to

the optimal working concentrations. The plate was washed three times with TBST followed by incubation with HRP-streptavidin (Sigma, MO) at 1:7000 for 1 h at 37 °C. After washing with TBST for 10 times, the plate was incubated with TMB and stopped by concentrated sulfuric acid. The absorbance was measured by a microplate reader (Bio-Rad) at 450 nm. The minimal detectable concentration was 0.78125 ng/ml for sOX40L.

2.7. Stability, precision, specificity of sOX40L ELISA

To analysis the precision of the sOX40L ELISA system, five concentration of OX40L-Fc (50, 25, 12.5, 6.25 and 1 ng/ml) were tested 15 times. Meanwhile, the same five samples were detected in 10 separate assays. Five known -OX40LFc concentrations (50, 25, 12.5, 6.25 and 1 ng/ml) were detected to assess the variation of the ELISA system after the pre-coated plates were preserved for 0, 10, 20 or 30 days at 4 °C. Three plates were tested at the same time. The specificity of the ELISA was determined by other homologous proteins such as soluble B7H4-Fc, OX40-Fc, and PD-L1-Fc.

2.8. Statistical analysis

All statistical analyses were performed using GraphPad (version 7.0a). Soluble OX40L were non-normally distributed and were presented as median with interquartile range (IQR). Student's *t*-test or a nonparametric Mann-Whitney *U* test was used for independent samples. For multiple comparisons, one-way ANOVA or the Kruskal-Wallis test was performed. A p-value > 0.05 was considered to denote a significant difference.

3. Results

3.1. Identification of three novel anti-human OX40L mAbs

To identify OX40L monoclonal antibodies, we screened a large panel of mAbs (> 1000) produced from BALB/c mice immunized with OX40L, and > 200 clones showed positive binding by ELISA. Three specific anti-human OX40L mAbs (clone 3D2, 3F7 and 2H3) were characterized further and used for the following experiments. The isotype of 3D2, 3F7 and 2H3 were all IgG1,κ (Supplement Fig. 1). First, the specificity of the mAbs for human OX40L was identified by flow cytometry. The mAbs could specifically bind to transfected cells L929/OX40L but not to L929/Mock (Fig. 1A). ELISA assay was also used to identify the OX40L mAbs. The results indicated that 3D2, 3F7 and 2H3 can bind to OX40L-Ig protein (Fig. 1B).

With recombinant OX40LIg protein and L929/OX40L transfected cells as antigens, Western Blot showed that 3D2 and 2H3 could recognize the target protein bands but not IgG protein or L929/Mock (Fig. 1C). To identify the epitopes on OX40L antibodies, competitive binding assays were performed. The results showed that 3D2 mAb could not inhibit the binding of biotin-conjugated 3F7. Meanwhile, 3F7 mAb also could not inhibit the binding of biotin-conjugated 3D2 (Fig. 1D). These data indicated that 3D2 and 3F7mAbs recognized different epitopes of OX40L. The immunofluorescence analysis showed that 3D2 specifically recognized L929/OX40L transfected cells but not L929/Mock transfected cells (Fig. 1E).

3.2. 3D2, 3F7 and 2H3 blocked the OX40/OX40L interaction

To assess the impact of the mAbs on the OX40/OX40L interaction, competition assay was performed between the recombinant OX40 protein and anti-OX40L mAbs. The results showed that 3D2, 3F7 and 2H3 mAbs could partially block OX40/OX40L binding, suggesting that 3D2, 3F7 and 2H3 mAbs may close to OX40 binding site (Fig. 2A).

3.3. 3F7 inhibited the proliferation of PBMC and cytokines production

To further characterize the effect of OX40L mAbs, we analyzed whether OX40L mAbs affected PBMC differentiation. Compared with the IgG mAb groups, 3F7 mAbs inhibit PBMC proliferation in a dose-dependent manner (Fig. 2B). Meanwhile, co-culture of 3F7 also inhibited IL-2, IL-6, IFN-γ and TNF-α production, but not IL-4, IL-10 or IL-17a (Fig. 2C). Therefore, 3F7 mAb is a functional antibody that can inhibit OX40/OX40L costimulatory signals on T cells and cytokines release.

3.4. Establishment of the novel sandwich sOX40L ELISA

Competition assay suggested that 3F7 and 3D2 mAbs recognized two different epitopes respectively. After optimizing working concentrations of coating and detection mAbs, a sensitive ELISA kit was developed using 5 μg/ml 3F7 mAb as the capture antibody and 0.5 μg/ml biotin-labeled 3D2 mAb for detection. The detectable limitation of OX40L-Fc was 0.78125–50 ng/ml, with R^2 is 0.9995 (Fig. 3A). For more extensive studies on the potential application of this sOX40L ELISA, it is essential to know how precise and stable this system is. First, the intra-assay and inter-assay precision were assessed. The data indicated that the ELISA was very robust with little well-to-well and plate-to-plate variation (CV% < 5%) (Table 1). The stability assay indicated that no significant loss in signal intensity of different time was detected (CV % < 10.5%). Thus, a long time storage of the precoated ELISA plates does not affect the quality of sOX40L (Table 2). Meanwhile, the kit did not cross-react with other soluble molecules such as sOX40, sPD-L1 or sB7-H4 recombinant protein (Fig. 3B, C and D). The results showed that a high sensitivity sOX40L sandwich ELISA system was established. Therefore, we selected this system to study the production of sOX40L. Using this Kit, we detected L929/OX40L but not L929/B7H3, L929/OX40 and L929/mock cells produced high levels of sOX40L in the supernatants in a time-dependent manner (Supplement Fig. 2).

3.5. sOX40L exists in the sera of healthy donors and showed increased levels in the sera of autoimmune diseases

Using the sOX40L ELISA kit, all the healthy volunteers showed detectable levels of sera sOX40L (Fig. 4). Meanwhile, we found that the level of sOX40L in < 10-year-old children was significantly lower than the other groups but over 60-year-old group had the highest level of sOX40L (2.025 ± 0.04891 ng/ml) among the groups (Fig. 4A). Additionally, the levels of sOX40L in human sera were significantly different between the sexes (Fig. 4B). The male group was significantly higher than the female (1.623 ± 0.0496 ng/ml vs 1.382 ± 0.0489 ng/ml; $p = 0.023$). The result showed that the sOX40L expression levels in human sera exhibits age dependency and sexes difference.

3.6. Increased levels of sOX40L in the serum of autoimmune diseases

We detected the levels of sOX40L in autoimmune diseases patients using ELISA assays. As shown in Fig. 4C, compared with the HC (1.627 ± 0.2617 ng/ml), the concentration of sOX40L was increased in SS (5.175 ± 1.109 ng/ml; $p = 0.054$), SLE (3.978 ± 0.563 ng/ml; $p = 0.059$) patients and significantly increased in RA patients (14.36 ± 2.156 ng/ml; $p < 0.001$). Regarding the PBMC expression of OX40L detected by 3D2 or commercial mAb ab89896, sOX40L were significantly higher in RA patients than those in healthy controls (Fig. 4D).

4. Discussion

Costimulatory molecules played an important role in the regulation of the immune response in autoimmune diseases [20]. Therefore, the costimulatory molecules were always used as early diagnosis and

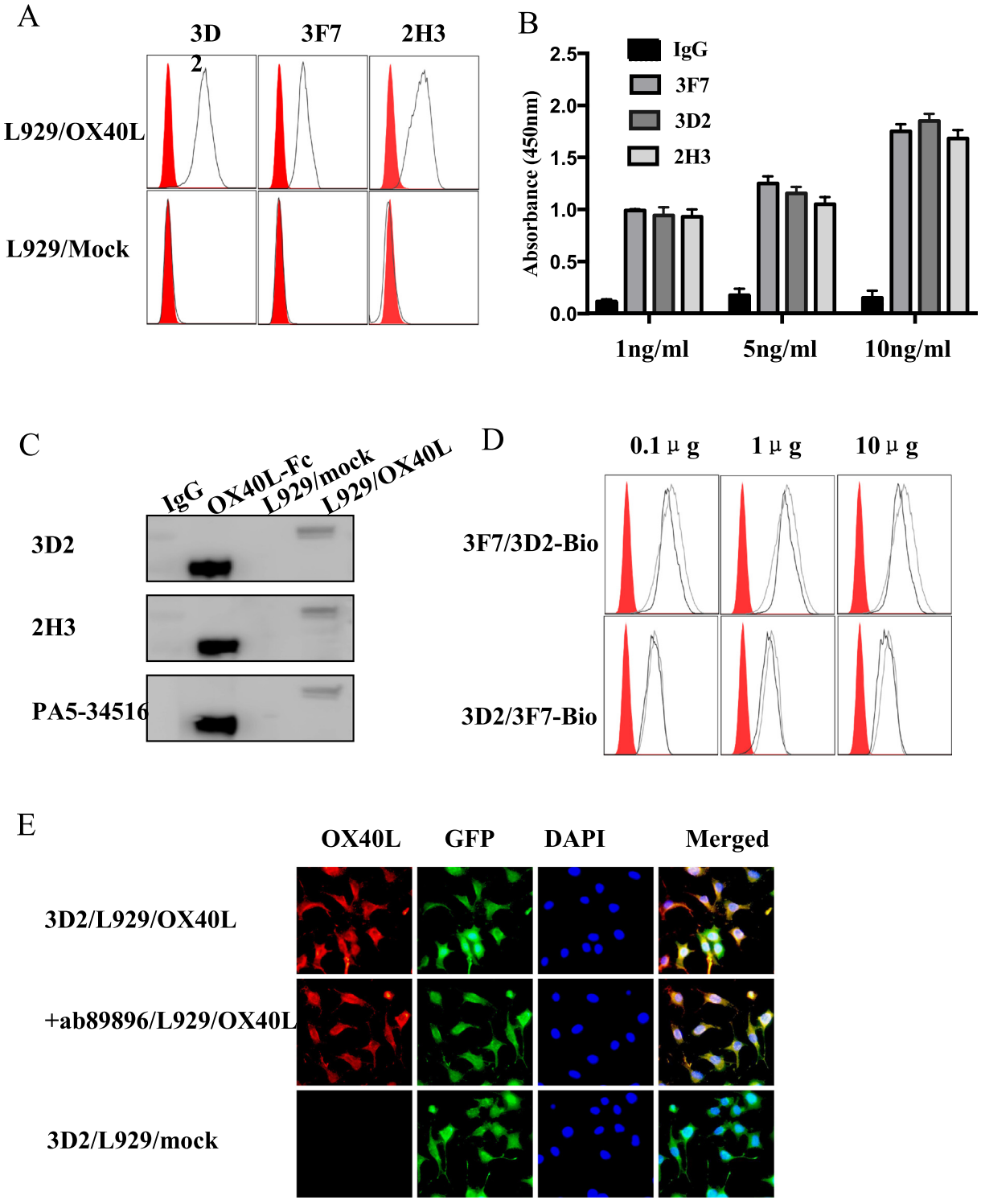

Fig. 1. Three novel monoclonal antibodies specifically recognized human OX40L. (A) L929/mock and L929/OX40L cell lines were stained with the anti-OX40L mAbs (3D2, 3F7 and 2H3) for 30 min followed by PE-goat anti-mouse IgG. Then the cells were analyzed by flow cytometry. (B) Determination of the binding ability of the obtained mAbs to OX40LIg antigen by indirect ELISA. The recombinant OX40LIg protein with different concentrations was coated on the ELISA plate. Then, the indirect ELISA was performed with the obtained mAbs 3D2, 3F7 and 2H3. The absorbance was measured at 450 nm by a microplate reader. (C) Western blot analyzed the specific mouse anti-human OX40L mAbs. The commercial OX40LIg protein or L929/OX40L cells were loaded to electrophoresis and transferred onto nitrocellulose membranes. The membranes were incubated with 3D2 and 2H3 mAbs followed by goat anti-mouse IgG-HRP. The protein bands were visualized using chemical reagents. PA5-34516 mAb was used as a positive control. (D) For the mutual competition assay of the mAbs, L929/OX40L cells were incubated with 3F7 mAb for 30 min at 4 °C, then the cells were washed and stained with biotinylated 3D2 mAb followed by streptavidin-PE for 30 min at 4 °C. The fluorescence intensity of the stained cells was analyzed by flow cytometry. The red solid histograms are negative control which L929/OX40L cells were stained directly with biotinylated mouse IgG followed by streptavidin-PE. Biotinylated anti-OX40L mAbs as positive control (grey lines). (E) The expression of anti-OX40L mAb was detected by immunofluorescence. 3D2 expressed on L929/OX40L but not L929/Mock. The anti-OX40L commercial mAb ab89896 was used as a positive control in this experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

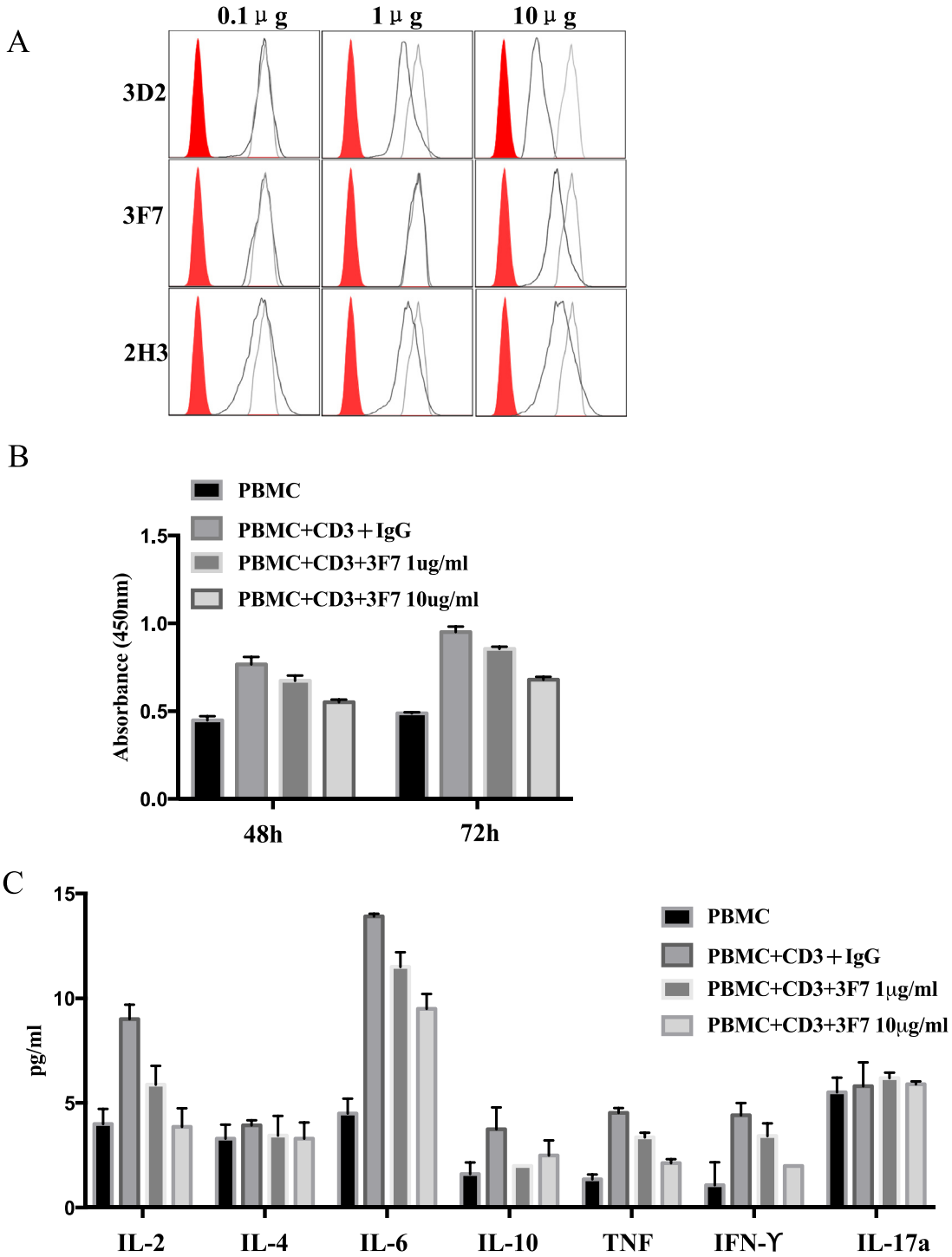

Fig. 2. Effect of the anti-OX40L mAbs on OX40/OX40L interaction and the inhibition role of 3F7 on PBMC proliferation and cytokines production. (A) The fluorescence intensity of the stained cells was analyzed by flow cytometry. The red solid histograms are human IgG which as negative control. The grey lines as positive control. From the blacks we found that 3D2, 3F7 and 2H3 mAbs could partially block OX40 binding to OX40L. (B) The proliferation of PBMC was decreased after stimulating with 3F7 mAb in vitro. PBMC cells were incubated with CD3 antibody in the presence or absence of 3F7 mAb. After 48 or 72 h, the proliferation was analyzed by the Elisa assay. (C) The supernatants from the 3F7 mAb group or PBMC mixture after 48 or 72 h were collected, and the production of IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α and IL-17a in the presence of 3F7 compared with the control IgG group was measured by the CBA assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treatment indicators of autoimmune disease [21,22]. The most well studied examples for this clinical function are CTLA-4 and PD-L1 [23,24]. OX40 expressed on cell surface of activated $CD4^+$ T cells [25,26] and OX40 signaling increase the permanence of T cells, cytokine production and the population of memory T-cell [27]. OX40L signaling enhanced B-cell proliferation and differentiation [28]. The OX40/OX40L interaction recruited a series of immune reactions, such as increasing $CD4^+$ T cells proliferation and cytokine secretion [3,29–31].

Therefore, inhibition of OX40/OX40L especially using a blocking

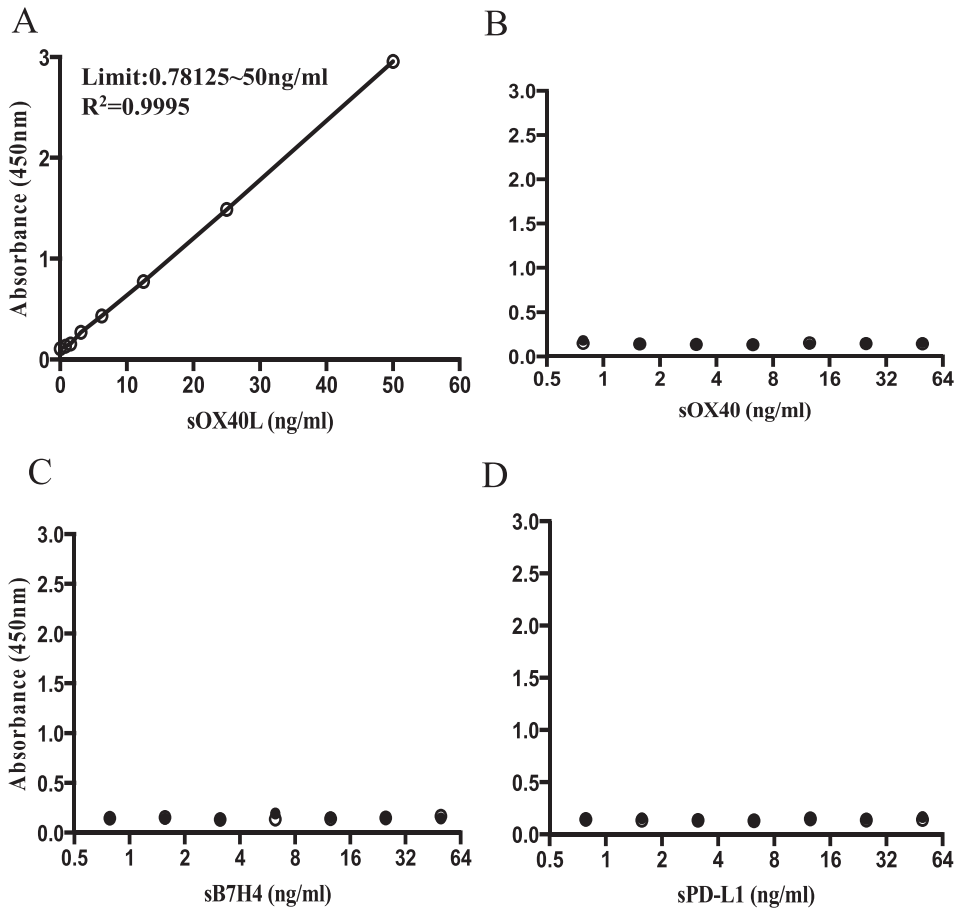

Fig. 3. Establishment of the sOX40L ELISA. (A) The capture anti-OX40L mAb (3F7) was at 5 μg/ml, the biotinylated anti-OX40L mAb (bio-3D2) was at 0.5 μg/ml. The two-fold serial dilutions of the soluble OX40L Ig protein starting from 50 ng/ml were detected by the ELISA. The absorbance was measured at 450 nm using a microplate reader. R^2 represents the correlation coefficient. (B), (C) and (D) The specificity of sOX40L ELISA. The proteins of sOX40, sB7H4 and sPD-L1, starting from 50 ng/ml, with two-fold serial dilutions were detected by the ELISA. The absorbance was measured at 450 nm using a microplate reader.

Table 1
The precision of human sOX40L ELISA system.

	Intra-assay precision					Inter-assay precision				
Sample	1	2	3	4	5	1	2	3	4	5
n	15	15	15	15	15	10	10	10	10	10
$\bar{x}$	1.12	6.18	12.65	24.8	50.76	1.18	6.21	12.84	25.3	51.02
SD	0.08	0.31	0.37	0.81	1.81	0.04	0.29	0.5	0.97	1.67
CV%	7.14	5.01	2.92	3.26	3.56	3.38	4.67	3.8	3.83	3.27

Five samples of known concentration (1, 6.25, 12.5, 25 and 50 ng/ml) were tested 15 times on one plate to assess intra-assay precision. In addition, the same five samples were tested in 10 separate assays to assess inter-assay precision.

anti-OX40L mAb represents a better therapeutic way for autoimmune disease [32]. Recently, many research and clinical trials showed their therapeutic value by blocking the costimulatory pathway. Usage of OX40L antibody for therapy has been widely studied. Huang et al. observed that blockade of OX40L with anti-OX40L mAb could inhibit the differentiation of helper T cells in chronic asthma [33]. To date, a phase II clinical trial used a humanized anti-OX40L mAb (oxelumab), developed by Roche and Genentech has been finalized for preventing allergen-induced airway inflammation and mild asthma disease in adults. If oxelumab was beneficial to patients, then anti-OX40L mAb would be developed for other autoimmune diseases.

In our current work, we have screened and characterized three novel mAbs against the OX40L molecule. Importantly, 3F7 and 3D2 bound to different antigenic epitopes. 3F7, 2H3 and 3D2 have the ability to prevent the binding of OX40L to OX40. 3D2 can be used in the immunofluorescence assay. The 3F7 mAb effectively inhibits the proliferation of PBMC and the production of IL-2, IL-6, IFN-γ and TNF-α, suggesting that the 3F7 mAb may be useful for blocking the OX40/OX40L positive signal.

A variety of costimulatory molecules exist both membrane-bound and soluble forms, such as B7-H3, B7-H4, CD28, CTLA-4, CD80, CD86 and ICOSL. Both membrane-bound and soluble forms of costimulatory molecules played important roles in immune-regulatory networks [34].

Table 2
The stability of human sOX40L ELISA system.

Time (day)	1 ng/ml		6.25 ng/ml		12.5 ng/ml		25 ng/ml		50 ng/ml	
	$\bar{x} \pm s$	CV%	$\bar{x} \pm s$	CV%	$\bar{x} \pm s$	CV%	$\bar{x} \pm s$	CV%	$\bar{x} \pm s$	CV%
0	1.08 ± 0.10	9.26	6.28 ± 0.34	5.41	12.44 ± 0.59	4.74	25.12 ± 1.93	7.68	50.73 ± 2.61	5.14
10	1.14 ± 0.11	9.65	6.23 ± 0.56	8.99	12.51 ± 0.65	5.19	25.95 ± 1.20	4.62	51.23 ± 3.93	7.67
20	1.12 ± 0.08	7.14	6.15 ± 0.43	6.99	12.26 ± 0.71	5.79	24.82 ± 2.44	9.83	49.52 ± 4.14	8.36
30	1.18 ± 0.12	10.17	6.28 ± 0.56	8.92	12.41 ± 0.78	6.28	24.54 ± 2.45	9.98	48.91 ± 4.68	9.57

After the coated plates were preserved at 4 °C for 0, 10, 20 or 30 days, 5 concentrations (1, 6.25, 12.5,25 and 50 ng/ml)) were tested to evaluate the variation.

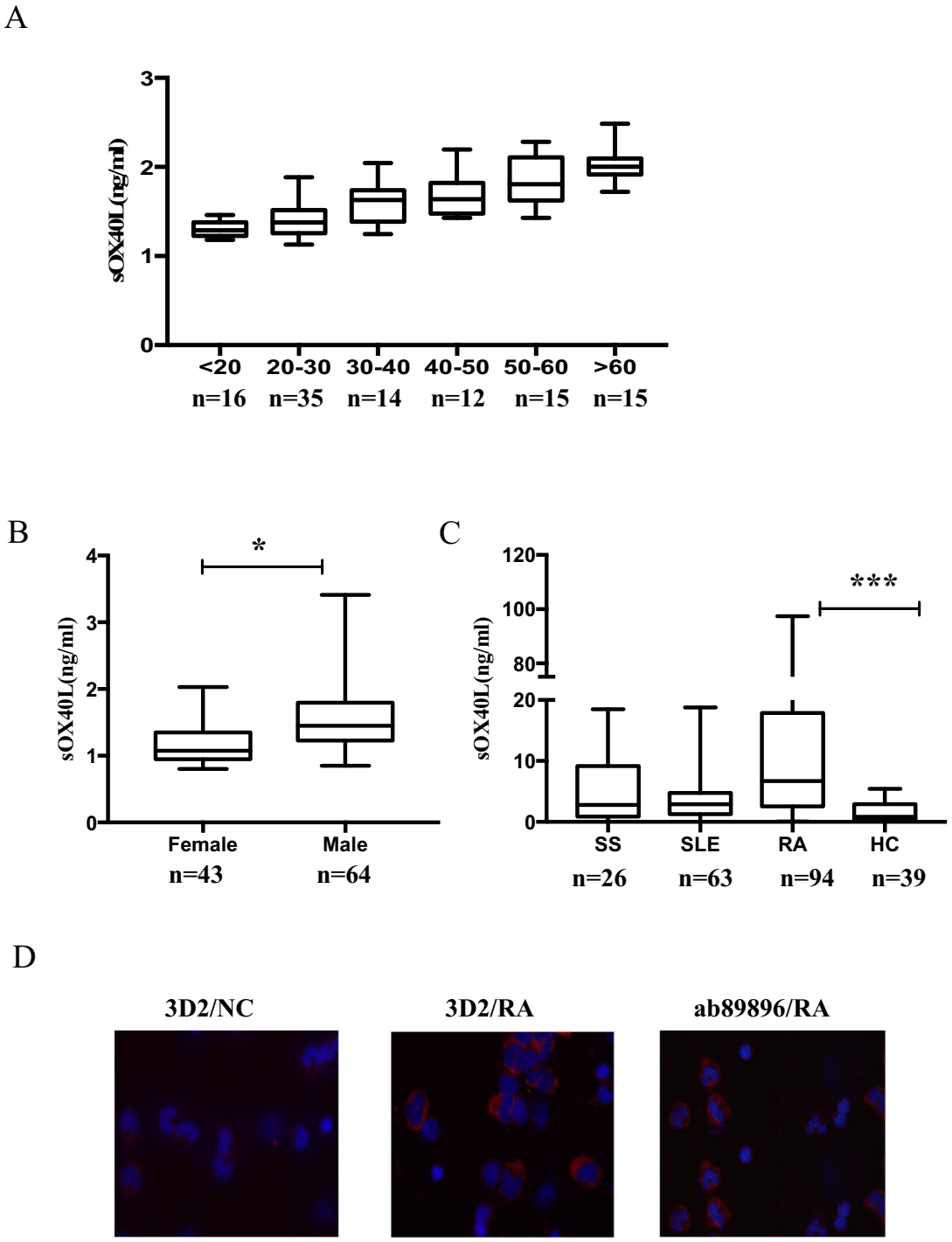

Fig. 4. Expression levels of sOX40L in human sera from healthy donors and autoimmune diseases. Sera from healthy donors were collected. sOX40L was detected by the ELISA system. (A) The level of sOX40L in the serum from 0 to 10 year old children was the lowest among the six groups, whereas the more than 60-year-old donors had the highest concentration of sOX40L. The sOX40L expression level in healthy donors has a distinct age-dependent manner. (B) A higher level of sOX40L in males was found than in females. (C) Significantly increased sOX40L levels were observed in sera from patients with autoimmune diseases especially in RA patients compared with those healthy controls group. (D) Immunofluorescence analysis of OX40L levels in the PBMCs of the patients with RA and NC was detected by anti-OX40L mAb 3D2 and commercial mAb ab89896. The results shown are from one experiment representative of three. The bar represents the mean value of every group, and the upper number is the mean ± SD. *$p < 0.05$, **$p < 0.01$.

Soluble forms of some costimulatory molecules, like sOX40L, have been found specially contribute to autoimmune disease [35,36]. As reported, sOX40L increased in asthma patient sera compared with healthy controls [33]. The plasma levels of sOX40L also increased with disease activity and the production of IgM rheumatoid factor (IgM-RF) and anti-citrullinated peptide antibody (ACPA) in RA disease [37]. Sera levels of OX40L were also significantly higher in systemic lupus erythematosus (SLE) patients than controls [30,38]. Previously, we found sOX40L is detectable in the sera of patients with Graves' disease [39]. In summary, OX40L has been identified in different pathological conditions and may become a promising target for disease treatment. However, a larger sample is necessary to validate the importance of sOX40L.

In this report, we established a sensitive and precise sandwich ELISA kit by two anti-hOX40L mAbs with the working range 0.78125–50 ng/ml to detect sOX40L. The establishment of the kit has made a solid foundation for soluble OX40L research. Using this kit, we detected sOX40L in the sera of healthy donors and found that sOX40L expression levels in human sera exhibit an age-dependent manner. The sera levels of sOX40L in samples from men were higher than in those from women. Meanwhile, the elevated sOX40L levels were also found in autoimmune disease especially in RA. Therefore, this ELISA kit might distinguish the RA disease from other autoimmune diseases.

In conclusion, we developed three novel mAbs against OX40L. The antibodies were valuable for study biological function of OX40L such as flow cytometry, immunoblotting, immunofluorescence and ELISA assay. Additionally, we established the sandwich ELISA kit to identify sOX40L, and detected the levels of sOX40L in the sera of healthy individuals and autoimmunity disease patients. From this point of view, above-mentioned findings have future application for the use of sOX40L antibodies in age-dependent and autoimmunity diseases.

Abbreviations

ACPA	anti-citrullinated peptide antibody
APC	antigen-presenting cells
DCs	dendritic cells
ELISA	enzyme-linked immunosorbent assay
HAT	hypoxanthine-aminopterin-thymidine
HC	healthy controls
HT	hypoxanthine-thymidine
IgM-RF	IgM rheumatoid factor
mAbs	monoclonal antibodies
OX40Ig	OX40 immunoglobulin
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
SS	Sjögren's syndrome
TNF	tumor necrosis factor

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The study was approved by the Ethics Committee of first affiliated hospital of Soochow University. All participants of this study had been informed and signed the consent for participation in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.11.053.

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