Blockade of OX40/OX40L signaling using anti-OX40L delays disease progression in murine lupus

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Research Article

Keywords: OX40L, Systemic lupus erythematosus (SLE), B cell, Treatment

Posted Date: July 11th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3139603/v1

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Abstract

Objectives

OX40 ligand (OX40L) locus genetic variants have relationships with the risk for systemic lupus erythematosus (SLE), OX40L blockade has been shown to ameliorate renal damage and suppress autoantibody production in NZB/W F1 mice. However, it is unclear how OX40L blockade delays lupus phenotype.

Methods

In present study, we examined the impact of blocking OX40L using anti-OX40L in the MRL/lpr murine model of lupus. Mice were sorted into 3 groups with 9 ~ 11 mice per group as follows: IgG treatment, Cyclophosphamide (CTX) treatment, and anti-OX40L treatment. Treated mice were harvested, and samples of serum, kidney, and spleen were collected for outcome evaluation. Next, we investigated the impact of anti-OX40L on immunosuppression in KLH-immunized C57BL/6J mice aged 8 weeks through the detection of serum immunoglobulins (Igs) and splenocyte flow cytometry. In vitro treatment of anti-OX40L in CD4+ T and CD19+ B cells were used for exploring the roles of OX40L in SLE pathogenesis.

Results

Anti-OX40L delayed disease progression in MRL/lpr mice, accompanied by decreased production of anti-dsDNA, proteinuria, and Ig deposition in kidney, as well as lower frequencies of Th1 and Tfh cells in the spleen. Compared to the IgG group, anti-OX40L was found to up-regulate polyclonal CD4+ T cell differentiation into Tregs in vitro. In KLH-immunized mice, decreased levels of Igs, and plasmablast cells were observed in anti-OX40L group. Blocking OX40/OX40L signaling also inhibited TLR7-mediated differentiation of antibody secreting cells (ASCs) and production of antibody through the up-regulation of SPI-B, IRF8, and PAX5, and down-regulation of Xbp-1 in B cells in vitro.

Conclusion

Together, these results propose OX40L as a promising therapeutic target for SLE.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that arises from the loss of tolerance to nuclear antigens [1]. Genome-wide association studies demonstrated CD252, also known as OX40 ligand (OX40L), as a susceptibility gene for SLE [2, 3]. OX40L is expressed mainly by antigen-presenting cells (APCs) [4], activated T cells [5, 6] and B cells [7], while its receptor, OX40 (CD134), is
mainly present on activated T cells and delivers co-stimulatory signals that enhance T cell survival and activation [8, 9], promote plasma cell development and increase immunoglobulin (Ig) secretion [10, 11]. Up-regulated OX40/OX40L signaling is linked to abnormal T follicular helper (Tfh) cell activity, which results in the development of autoimmune diseases including rheumatoid arthritis (RA) [12], Grave's disease [13], and SLE [14]. Furthermore, activation of OX40/OX40L pathway increased T helper cell 1 (Th1) [15, 16], Th2 [17, 18], Th17 [17, 19] polarization, and antagonized the Treg generation [20]. Recent reports have underlined the crucial role of the OX40/OX40L signaling pathway in SLE pathogenesis. Elevated OX40L expression has been observed in the kidneys of lupus-prone mice, and blocking OX40L signaling using OX40L-Fc fusion protein has been shown to improve renal function and reduce autoantibody production in NZB/W F1 mice [21]. OX40L-expressing B cells aids Tfh cell development and results in SLE pathogenesis in murine models [14]. Furthermore, the OX40/OX40L signaling impairs Treg-mediated immunosuppressive function in human SLE [10].

These findings propose OX40L as a novel therapeutic target for SLE. Whereas, further research is warranted to comprehend the function of OX40L in SLE pathogenesis and evaluate the prognosis of OX40L-targeted therapies in lupus. In this study, we explored the impact of OX40L blockade in the MRL/lpr mice, investigated the mechanism of autoantibody reduction caused by blockade of OX40L signaling.

Methods

Animals

At eight weeks of age, female MRL/lpr mice were acquired from SPF Biotechnology Co. Ltd, while C57BL/6J mice were obtained from Jiangsu GemPharmatech Co. LTD. Prior to use, all necessary sterilization measures were taken, including autoclaving of cages, bedding, nestlets, food, and water. The experimental protocols adhered to the animal ethical and care guidelines of the Institute of Dermatology, Chinese Academy of Medical Sciences (no. 2022-DW-004) and were approved by local government authorities to ensure compliance.

Experimental design

MRL/lpr mice

At 11 weeks of age, 30–33 female mice were randomly assigned to one of three groups (n = 10 ~ 11 per group) and received intraperitoneal injections until they reached 21 weeks of age. The treatment groups included a vehicle control (IgG isotype control, 100 µg/mouse) obtained from Bio X cell (BE0093), Cyclophosphamide (CTX) (Sigma, C0768) at a weekly dose of 30 mg/kg, or Anti-OX40L (Bio X cell, BE0033-1) at a dose of 100 µg/mouse, twice a week (Fig. 1. a). Body weight was monitored weekly throughout the study period. At 21 weeks of age, all mice were euthanized, and serum, spleen, and kidney were isolated for further analysis. Lymph nodes and spleen were also collected and weighed, with the spleen or lymph node index being calculated as the weight of the organ divided by body weight.
**KLH immunization**

We aimed to evaluate the impact of Anti-OX40L on immunosuppression in female C57BL/6J mice aged 8 weeks. 17 female mice were randomly assigned to one of three groups (Blank group: n = 5, no-KLH immunized mice; negative control: n = 7, KLH + IgG treated group; anti-OX40L group: n = 6, KLH + anti-OX40L treated group). The mice were subcutaneously injected with KLH (0.5 mg/ml, Sigma) emulsified in CFA on the tail, once a week for two weeks. To explore the role of Anti-OX40L, we administered anti-OX40L (100 µg) or control IgG systemically twice a week, starting at 8 weeks of age. After 2 weeks, we collected serum and spleen samples from the mice for further analysis (Fig. 4. a).

**Evaluation of systemic lupus and glomerulonephritis**

Serum samples were collected from MRL/lpr mice via orbital vein blood after anesthesia every 3 ~ 4 weeks. We used an ELISA kit (CUSABIO, CSB-E11194m) to detect the levels of anti-dsDNA IgG in the serum. Blood urea nitrogen levels were measured using a colorimetric method (Sangon Biotech, D799850-0100) at the end of the experiment. To quantitatively assess proteinuria, we used a Bradford assay kit (Nanjing Jiancheng Bioengineering Institute, C035-2-1).

Renal histopathology in mice was assessed by embedding the left kidneys in paraffin and cutting them into 5-µm sections. We used a standard technique to stain the sections with hematoxylin and eosin (HE) to visualize tissue morphology. The pathological scores of glomerulonephritis were determined by the average of the results from 50 glomeruli. The scoring system for glomerulonephritis was as follows: Normal (0), cell proliferation or infiltration (1), membrane proliferation, hyaline deposition, or lobulation (2), global hyalinosis or crescent formation (3). To detect IgG depositions, direct immunofluorescence (IF) was performed on paraffin-embedded kidney section via Goat Anti-Mouse IgG2a heavy chain (FITC) (Abcam, ab97244, 1:500). C3 deposits were detected using C3/C3b/C3c Polyclonal antibody (Proteintech, 21337-1-AP, 1:500) and Goat Anti-Rabbit IgG (FITC) (Proteintech, SA00003-2, 1:200) by IIF. The HE slides and immune-complex (IC) depositions in the mouse kidneys were analyzed and evaluated using a digital fluorescence microscope (Olympus, BX53F2). The fluorescent staining was recorded and analyzed using OLYMPUS cellSens Standard Imaging Software for presentation.

**RNA isolation and qPCR**

Total RNA of kidney was extracted using Total RNA Extraction Reagent (Vazyme, R401-01-AA). RNA quality and concentration were assessed using a NanoDrop spectrophotometer (Thermo, ND-2000). mRNA was reverse-transcribed using the HiScript III RT SuperMix for qPCR (Vazyme, R323-01) following the manufacturer's protocol. qRT-PCR was conducted with ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711-02) using a thermocycler (Roche, LightCycler 480 II). Normalized to the control group, the gene mRNA level was calculated as $2^{\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct$ of experiment group - $\Delta Ct$ of control group). mRNA primers for cytokines, chemokines, immune cell markers, and β-actin were obtained from Tsingke Biotechnology Co. (Supplemental Table 1).
Flow cytometry

Spleen tissues were mechanically disrupted and filtered to obtain cell suspensions. About \(1.5 \times 10^6\) cells were incubated with mouse Fc-R block (BD Pharmingen, 553141) for 15 minutes (min) at 20–25°C, following with the surface staining using antibodies against specific markers for 45 min at 4°C, away from dark. Intracellular staining was performed via fixing and permeabilizing the cells with a Transcription Factor Buffer Set (BD Pharmingen, 562574) and staining with fluorescent antibodies for 1.5 h at 4°C. To assess cytokine production by lymphocytes in spleens, \(2 \times 10^6\) cells/well were stimulated with the Leukocyte Activation Cocktail (BD GolgiPlug™ antibody, BD 550583) in 6-well plates for 6–8 hours at 37°C with 5% CO\(_2\). Flow cytometry results were downloaded analyzed using FlowJo software 10.8.1. The following antibodies were used: FITC anti-mouse CD3 Antibody (BioLegend, 100204), Alexa Fluor® 700 anti-mouse CD4 Antibody (BioLegend, 100430), CD25 Monoclonal Antibody (PC61.5), Super Bright™ 780 (eBioscience, 78-0251-82), Zombie Aqua™ Fixable Viability Kit (BioLegend, 423101), PE-CF594 Rat Anti-Mouse IFN-γ (BD Pharmingen, 562303), IL-17A Monoclonal Antibody (eBio17B7), eFluor™ 450 (eBioscience, 48-7177-82), PE/Cyanine7 anti-mouse IL-4 Antibody (BioLegend, 504118), FOXP3 Monoclonal Antibody (FJK-16s), PE (eBioscience, 12-5773-82), PerCP/Cyanine5.5 anti-mouse/human CD45R/B220 Antibody (BioLegend, 103236), PE anti-mouse CD19 Antibody (BioLegend, 152408), IgD Monoclonal Antibody (11-26c (11–26)), Super Bright™ 600 (eBioscience, 12-5773-82), Brilliant Violet 421™ anti-mouse CD138 (Syndecan-1) Antibody (BioLegend, 142523), Alexa Fluor® 647 anti-mouse/human GL7 Antigen (T and B cell Activation Marker) Antibody (BioLegend, 144606), PE-CF594 Hamster Anti-Mouse CD95 (BD Pharmingen, 562499), CD8a Monoclonal Antibody (53 – 6.7), APC-eFluor™ 780 (eBioscience, 47-0081-82), PE/Cyanine7 anti-mouse CD62L Antibody (BioLegend, 104418), Brilliant Violet 785™ anti-mouse/human CD44 Antibody (BioLegend, 103059), Biotin Rat Anti-Mouse CD185 (CXCR5) (BD Pharmingen, 551960), APC Streptavidin (BioLegend, 405207), BB700 Hamster Anti-Mouse CD279 (PD-1) (BD Pharmingen, 566514). Flow cytometry was performed using a Cytek NL-CLC V16B14R8 instrument.

Cytometric bead array

Serum samples from KLH-immunized mice and supernatants from cultured splenic B cells were collected and analyzed using a LEGENDplex™ Mouse Immunoglobulin Isotyping Panel (6-plex) (BioLegend, 740492), following the manufacturer's protocols. The concentrations of mouse Igs (IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3) were calculated through LEGENDplex software after flow cytometry analysis on a BD FACSVerse™ Flow Cytometer instrument.

Isolation and stimulation of CD4\(^+\) T cells

Spleen cells of 8-week-old C57BL/6J mice were isolated and purified using the MojoSort™ Mouse CD4 T Cell Isolation Kit (BioLegend, 480006) and added at an intensity of \(5 \times 10^5\) cells/well in a 24-well plate. The cells were activated using plate-bound anti-CD3 (Invitrogen, 16-0031-86, 3 µg/ml) and anti-CD28 (Invitrogen, 16-0281-85, 2 µg/ml) antibodies for 3 days with the addition of isotype IgG or anti-OX40L
(100 µg/ml), furthermore, blank group without CD3/CD28 stimulation was also established. Subsequently, Th1, Th2, Th17, Tfh, and Treg cells were detected by flow cytometry.

**Culture of splenic B cell**

To obtain Mouse Pan B cells, splenocytes were isolated and purified using the MojoSort™ Mouse Pan B Cell Isolation Kit II (BioLegend, 480088). The purified B cells were then cultured in RPMI 1640 (Gibco, 21870076) supplemented with IL-4 (PeproTech, 214-14-20, 30 ng/ml) and R848 (Sigma, SML0196-10MG, 100 ng/ml) to promote activation as described previously [22]. Cultures were set up in 24-well plates with 5×10^5 cells/well in 500 µl of medium at 5% CO_2 and 37°C. To block OX40/OX40L signaling, anti-OX40L (100 µg/ml) or IgG (100 µg/ml) was added to the culture. Blank control was set up without the addition of IL-4 and R848 in the medium. After 72 h, all cells were harvested for the evaluation of B cell differentiation and the supernatant was collected to detect antibody production using flow cytometry. Next, we investigated the effect on transcription factors related to B-cell proliferation and differentiation using western blot assay, after 48 h culture.

**Western blotting**

Mouse splenic B cells were isolated after 2 days of culture, and lysed using RIPA buffer (Beyotime, P0013K), and the protein was quantified via a BCA Protein Assay Kit (Beyotime, P0011). A 10% SDS-page was loaded with the protein and then transferred to polyvinylidene difluoride membranes. Subsequently, western blotting was performed using antibodies that were specific to various proteins such as Blimp-1 (CST, 9115S, 1:1000), BCL6 (CST, 5650S, 1:1000), XBP1 (Affinity, AF5110, 1:1000), IRF8 (Proteintech, 18977-1-AP, 1:500), PAX5 (Affinity, AF5110, 1:500), SPI-B (Santa Cruz, sc-517204, 1:1000) and GAPDH (CST, 5174S, 1:2000). The blots were then visualized using a Tanon 5200 imaging system.

**Statistical analysis**

The statistical analysis was performed using GraphPad Prism 9.5.0. The results are displayed as mean ± SEM, and the data were analyzed for normal distribution and equal variance between groups. Two-tailed unpaired Student's t-tests were used to compare two groups. For multigroup comparisons, we used and one-way analysis of variance (ANOVA) with appropriate post hoc tests. The 2-tailed Mann-Whitney U test was performed when the data had unequal variances or were not normally distributed between two groups. The sample sizes were not predetermined using a statistical method, and mice were randomly assigned to different groups.

**Results**

**The impact of systemic administration of anti-OX40L on the body weight, as well as the weight of lymph nodes and spleen in MRL/lpr mice**

We investigated the potential therapeutic effect of systemic administration of anti-OX40L treatment on the lupus phenotype in MRL/lpr mice. Although there was a trend towards a decrease in body weight in
MRL/lpr mice following anti-OX40L treatment, compared with the IgG group \((P>0.05)\). However, anti-OX40L led to a significant decline in both spleen and lymph node weight and index (spleen or lymph node weight divided by body weight) in comparison to the IgG group \((P<0.05)\) (Fig. 1.b).

**Anti-OX40L efficiently controls autoantibodies and lupus nephritis activity**

Compared with the negative control (NC) group, the administration of anti-OX40L and CTX significantly reduced the anti-double stranded DNA antibody (anti-dsDNA) levels in serum \((P<0.05)\) at 17 weeks of age in MRL/lpr mice, and blood urea nitrogen levels \((P<0.05)\) at the end of experiment (Fig. 1.b). At 21 weeks of age, MRL/lpr mice displayed kidney damage, including high proteinuria levels \((P<0.05)\) (Fig. 1.b), severe glomerular expansion, and increased HE scores (Fig. 2.a). Notably, treatment with anti-OX40L and CTX resulted in a reduction in glomerular damage with less HE scores when compared to IgG-treated mice (Fig. 2.a) \((P<0.05)\).

**Anti-OX40L suppresses glomerular immune complex deposits in the kidney of MRL/lpr mice**

Immunofluorescence analysis revealed substantial deposition of C3 (green) and IgG (green) in the mesangium and capillary loops within glomeruli of IgG-treated mice. However, treatment with anti-OX40L significantly reduced the fluorescence intensities of IgG \((P<0.05)\), which are indicators of the severity of immune-complex deposition within glomeruli (Fig. 2.a).

**Anti-OX40L inhibits inflammatory cytokines and chemokines in the kidney of MRL/lpr mice**

To investigate the potential anti-inflammatory effects of anti-OX40L treatment in MRL/lpr mice, we performed *in vitro* RNA experiments to identify cytokines, chemokines and markers of immune cells in kidneys. Among the 15 cytokines and 3 chemokines identified, four mRNAs (IL-21, CCL8, IL-5, and IFN-\(\gamma\)) were greatly down-regulated \((P<0.05)\) after treatment with anti-OX40L mAb (Fig. 2.b). While there was a trend towards a decrease in the expression of more than six mRNAs, including CD3, IFN-\(\beta\), IL-4, IL-13, CCL2, and CCL7 after anti-OX40L treatment, these trends were not statistically significant (Fig. 2.b). Other qPCR results of genes were shown in Supplementary Fig. 2. Supplementary Table 1 provides a complete list of the mRNA expressions analyzed.

**Anti-OX40L reduces splenic Th1 and Tfh proportion in MRL/lpr mice**

To study the effect of anti-OX40L treatment on specific cell subsets, we conducted flow cytometry analysis on splenocytes obtained from MRL/lpr mice. After administering anti-OX40L therapy for 10 weeks, we found a notable decrease in proportion of Th1 \((P<0.05)\) and Tfh cells \((P<0.001)\) compared
with the NC group (Fig. 3. a). Conversely, we observed only a minimal effect on central memory T cell (TCM), effector memory T Cell (TEM), terminally differentiated effector memory CD45RA\(^+\) T cells (TEMRA), and B cell subsets in anti-OX40L group in comparison to the NC group (Supplementary Fig. 1).

**Reduced KLH-response and plasmablast cell proportion in the C57 mice treated with anti-OX40L**

We evaluated the effects of anti-OX40L treatment on antigen-specific B-cell responses in C57BL/6J mice after KLH immunization. On day 14 following immunization, we conducted flow cytometry analysis of immune cells and serum Ig levels. Our findings revealed that KLH-immunized mice treated with anti-OX40L exhibited a significant decrease in plasmablast cell frequencies \((P<0.05)\) (Fig. 4. b), but no significant differences were noticed in other B-cell subsets. Anti-OX40L treatment had a minimal impact on Tfh, TCM, TEM, and TEMRA (Supplementary Fig. 3). Of note, mice treated with anti-OX40L demonstrated lower serum concentrations of total IgG1 \((P<0.05)\), IgG2a \((P<0.05)\), IgG2b \((P>0.05)\), IgG3 \((P>0.05)\), and IgA \((P>0.05)\) than IgG-treated mice (Fig. 4. c). Our findings suggest that OX40L blockade may lead to down-regulated production of antibody and antigen-specific responses.

**Up-regulated Treg differentiation after anti-OX40L treatment in activated CD4\(^+\) T cells**

In the presence of anti-OX40L, the functions of OX40L in the adjustment of monoclonal CD4\(^+\) T cell differentiation activated with anti-CD3/CD28 antibodies were investigated. After activation, all Th cells, Treg, Tfh greatly increased compared with the bank group with no addition of anti-CD3/CD28 \((P<0.05)\).

Anti-OX40L (100 µg/ml) promoted CD4\(^+\) T cell differentiation into Treg cells \((P<0.001)\), while no significant polarization of other Th and Tfh cells were observed in CD4\(^+\) T cells treated with anti-OX40L in comparison to the NC group \((P>0.05)\) (Fig. 5).

**Blocking OX40L inhibits TLR7-mediated differentiation of ASCs and antibody production**

The findings unveiled a crucial role of OX40L in Ig production in MRL/lpr and KLH-immunized mice. Next, we cultured spleen B cells from C57 mice and incubated with R848 (Toll-like receptor 7 agonist) and IL-4. R848 and IL-4 treatment up-regulated the proportion of ASCs \((CD19^+ \text{ CD44}^{hi} \text{ CD138}^+\)) , plasmablast cells, memory cells, and plasma cells \((P<0.05)\), suggesting the induction and differentiation of B cells. OX40L blockade significantly inhibited the differentiation of ASCs and plasma cells (PC) (Fig. 6. a) \((P<0.05)\), and reduced supernatant levels of IgM \((P<0.0001)\) and IgG2b \((P<0.001)\), had a decreasing tendency against IgA and IgG3 \((P>0.05)\) (Fig. 6. b).

**Anti-OX40L up-regulates SPI-B, IRF8, and PAX5, and down-regulates Xbp-1 in mouse splenic B cells**
Blimp-1, BCL6, XBP1, SPIB, IRF8, and PAX5 are transcription factors that have been proved to be key regulators of B-cell proliferation and differentiation, of which SPIB, IRF8, and PAX5 mainly inhibit plasma induction and Ig production. Anti-OX40L up-regulated protein levels of SPIB, IRF, and PAX5, and down-regulated Xbp-1 in mouse splenic B cells after 48 h culture of activated-splenic B cells, which had no significant effect on Blimp-1 and BCL6 (Fig. 6. c).

Discussion

OX40L is involved in activating and maintaining T cell-mediated immune responses, and has association with several autoimmune diseases, including SLE. The loss of OX40L in B cells ameliorated the lupus phenotype [14]. In NZB/W F1 mice, agonistic anti-OX40 monoclonal antibody exacerbated renal disease. Conversely, when the same pathway was blocked using an Fc-fusion protein, significant benefits were observed in terms of disease progression and mortality [21]. While the therapeutic potential of anti-OX40L in this lupus model has been suggested, it is important to evaluate the response to OX40/OX40L blockade in other lupus models, such as the MRL/lpr mouse. Our team was the first to report that blockade of OX40/OX40L signaling using anti-OX40L delayed disease progression in MRL/lpr mice. This was characterized by decreased production of anti-dsDNA, proteinuria, and Ig deposition in the kidney. A recent report discovered the lack of OX40-OX40L signaling contributed to the amelioration of disease symptoms in lupus models [14]. Moreover, it was discovered that genetically predisposed high OX40L expression increases B cell expression, which in turn promotes Tfh growth and predisposes individuals to SLE. In vitro stimulation of human T cells with OX40 resulted in a Tfh cell-like transcriptional program and phenotype [23]. Our study in the MRL/lpr lupus model similarly demonstrated a reduction in Tfh cells in the spleen following anti-OX40L administration. Interestingly, we also observed a down-regulation of Th1 cells after OX40L blockade, which is in line with a recent report showing that OX40 ligation by OX40L accelerated Th1 differentiation [24]. Furthermore, a down-regulation in Th1-type cytokines with OX40/OX40L blockade has been confirmed in an inflammatory bowel disease model [15]. Activation of the OX40/OX40L signaling pathway was found to result in the down-regulation of Foxp3 in Treg cells [20], and counts in SLE Tregs, had associations with the frequency of circulating OX40L-expressing myeloid dendritic cells [10]. Although no significant up-regulation of Tregs was observed after anti-OX40L administration compared to the IgG group, anti-OX40L was found to up-regulate mouse splenic CD4+ T cell differentiation into Tregs in vitro. OX40 appears to connect the TCR signal's intensity with its sensitivity to IL-2 [25], and IL-2 treatment could selectively modulate the abundance of Tregs in patients with SLE [26], which may account for the phenomenon of the promotion of Treg differentiation mediated by OX40L blockade.

An extra follicular response produces short-lived plasma cells and low-affinity antibody production, while a germinal center (GC) response develops memory B or long-lived plasma cells, accompanied with high-affinity antibodies. To probe the roles of OX40L in antigen-specific B-cell responses after KLH immunization, we evaluated the proportion of B subtypes and serum concentrations of Igs in C57 mice treated with anti-OX40L in comparison to IgG-treated mice. The results demonstrated a greatly lower
frequency of plasmablast cells and lower serum concentrations of total IgG2a and IgG1 in anti-OX40L-treated mice. However, in anti-OX40L-treated MRL/lpr mice, no significant difference was found in B cell subsets, the heterogeneity of the of lupus progression between NZB/W F1 and MRL/lpr mice may impact the therapeutic effects of OX40L as a target, whereas, OX40L blockade may play a role in B cell function, and contributing to the reduce of autoantibodies. These findings suggest an important role for OX40L signaling in Ig production in both MRL/lpr and KLH-immunized mice, next, we cultured murine spleen B cells from C57 mice and treated them with R848 and IL-4, following treatment with anti-OX40L. We observed the inhibition of ASCs differentiation and reduced levels of IgG2b, IgM, IgG3, and IgA in the supernatant of murine splenic B cells co-cultured with anti-OX40L, which supports the view that OX40L signaling is required for the formation of high-affinity Ig-producing plasma cells [27], and plasmablast cells [10, 21], as well as the production of antibody by ASCs [11]. To further explore the mechanism of autoantibody reduction caused by blockade of OX40L signaling, we conducted western blotting on key regulators of B-cell differentiation and found up-regulation of SPI-B, IRF8, and PAX5, and down-regulation of Xbp-1 in mouse splenic B cells after 48 hours of co-culture with anti-OX40L and activated splenic B cells. These transcription factors are key regulators of B-cell pro differentiation and mainly inhibit plasma induction and Ig production [28–32]. Our findings suggested that anti-OX40L treatment may suppress the production of antibody by ASCs, via the up-regulation of SPI-B, IRF8, and PAX5, and down-regulation of Xbp-1 which revealed the potential mechanism of autoantibody reduction caused by blockade of OX40L signaling in B cells. Whereas, anti-OX40L has a minimal impact on Blimp-1 and BCL6, positive and negative feedback from a complex regulatory network of transcription factors may contribute to this outcome.

Our study demonstrated that anti-OX40L therapy effectively ameliorated the lupus disease phenotype. We found a suppress in the production of anti-dsDNA, proteinuria, blood urea nitrogen, and Ig deposition in the kidneys of treated MRL/lpr mice. We also noted reduced frequencies of Tfh and Th1 cells in MRL/lpr splenocytes, and decreased plasmablast cells and Ig levels in KLH-immunized mice following treatment with anti-OX40L. In addition, we identified decreased mRNA expressions of CCL8, IFN-γ, IL-5, and IL-21 from the kidney of anti-OX40L-treated MRL/lpr mice. These cytokines affect B cell function and result in the development of autoantibodies in SLE [33–35]. Recently, a study suggested that CCL8 from classical monocytes and its receptor network could exacerbate SLE autoimmune phenotype [36]. Additionally, in vitro treatment with anti-OX40L promoted Treg differentiation in murine CD4+ T cells and decreased ASC frequencies and Ig levels in B cell cultivation. Whereas, minimal impact on Th1 and Tfh in vitro treatment with anti-OX40L during CD4+ T activation by CD3/CD28 was observed, which may be resulted from the different expression of OX40L in different Th subsets. Together, our findings indicate OX40L blockade may act as a novel target for immunotherapy in autoimmune diseases, including SLE.

Monoclonal antibodies targeting OX40 are currently being investigated in early-stage clinical trials for various human cancers [37–39]. However, recent study has raised concerns about the potential for OX40 stimulation to trigger autoimmune or inflammatory responses in humans [21]. Our anti-OX40L therapy demonstrates promising results in delaying renal disease, sheds light on the mechanism of autoantibody
reduction through the up-regulation of SPI-B, IRF8, and PAX5, and down-regulation of Xbp-1 in B cells. Although our findings provide insight into the possible therapeutic use of anti-OX40L therapy in lupus, more preclinical and animal researches are required to comprehend the potential adverse effects of modulating the OX40/OX40L pathway, particularly with regards to when and how it can be safely and effectively targeted in autoimmune diseases, especially in SLE.

Conclusion

We firstly demonstrated ameliorated lupus phenotype in MRL/lpr mice after anti-OX40L treatment, and found that blocking OX40/OX40L signaling inhibited TLR7-mediated differentiation of ASCs and production of antibody through the up-regulation of SPI-B, IRF8, and PAX5, and down-regulation of Xbp-1 in B cells in vitro. These findings provide insight into the possible therapeutic use of anti-OX40L therapy in lupus.

Abbreviations

SLE  Systemic lupus erythematosus
CTX  Cyclophosphamide
Ig  Immunoglobulin
ASC  Antibody secreting cell
Th1  T helper cell 1
HE  Hematoxylin and eosin
TCM  Central memory T cell
TEM  Effector memory T Cell
TLR7  Toll-like receptor 7
GC  Germinal center

Declarations

Acknowledgements

This work was supported by the National Key R&D Program of China (No. 2022YFC3601800), the CAMS Innovation Fund for Medical Sciences (No. 2021-I2M-1-059), Non-profit Central Research Institute Fund of Chinese Academy of Medical Sciences (No. 2021-RC320-001, No. 2020-RC320-003), National Natural
Science Foundation of China (No. 81830097), and the Special Program of National Natural Science Foundation of China (No. 32141004).

Authors’ contributions

QL, HY, MZ and JZ had the idea for the article and drafted the manuscript. JZ, LL and HY wrote the methods and conducted the experiments. All the other authors critically revised the work.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

We adhered to the animal ethical and care guidelines of the Institute of Dermatology, Chinese Academy of Medical Sciences (no. 2022-DW-004) and were approved by local government authorities to ensure compliance.

Consent for publication

Not applicable.

Competing Interest

The authors declare no conflicts of interest.

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References


Figures
Figure 1

Anti-OX40L ameliorates the lupus progression. (a) A schematic of the treatment of anti-OX40L in MRL/Lpr mice. (b) Body weight, urine protein, anti-dsDNA (IgG), blood urea nitrogen, spleen and lymph node weigh, as well as spleen and lymph node index were shown. Horizontal bars represent the mean ± SEM. n = 11 in MRL/Lpr group + IgG isotype control (NC) (4 mice died); n = 10 in MRL/Lpr + CTX treated group (1 mouse died); n = 11 in MRL/Lpr + Anti-OX40L treated group (2 mice died). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Anti-OX40L improves histological damage, reduces immune-complex deposition, and controls inflammatory response in MRL/Lpr mice. (a) H&E staining, IgG and C3 deposition in the of kidney MRL/lpr mice. (b) Anti-OX40L treatment reduced renal mRNA levels of pro-inflammatory cytokines (INF-γ, IL-5, IL-21), chemokine (CCL8), and marker of inflammatory cell (CD3) in MRL/Lpr mice. Horizontal bars represent the mean ± SEM. n = 11 in MRL/Lpr group+ IgG isotype control (NC) (4 mice died); n = 10 in
MRL/Lpr + CTX treated group (1 mouse died); n = 11 in MRL/Lpr + Anti-OX40L treated group (2 mice died). *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 3

Blocking OX40L results in down-regulation of Th1 and Tfh cells in CD4⁺ T cells. Representative flow cytometry diagrams and statistical analysis of the frequencies of Th1, Th2, Th17, Tfh, and Treg cells in the spleens of the MRL/Lpr mice treated by IgG (NC group), CTX, and anti-OX40L respectively. All Th subtype results are presented as proportions of CD4⁺ T cells. Horizontal bars represent the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
The KLH-induced response in anti-OX40L treated mice. (a) A schematic of the treatment of anti-OX40L in KLH-immunized C57 mice. (b) Representative flow cytometry diagrams and statistical analysis of the percentages of naïve B cells, memory B cells, GC B cells, plasmablast cells, plasma cells in the spleens of the C57 mice. (c) The serum levels of total IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM from the KLH-immunized mice on days 14. Horizontal bars represent the mean ± SEM. n = 5 in C57 blank group (no
KLH immunization); n = 7 in C57 + KLH treated group; n = 6 in C57 + KLH + anti-OX40L treated group. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5

OX40L blockade promotes polyclonal CD4⁺ T cell to differentiate into Tregs. Representative flow cytometry diagrams and statistical analysis of the percentages of Th1, Th2, Th17, Tfh, and Treg cells in the polyclonal CD4⁺ T cells activated with anti-CD3/CD28 Abs. All Th subtype results are presented as proportions of CD4⁺ T cells. Horizontal bars represent the mean ± SEM. n = 4 in Blank group: CD4⁺ T + no CD3/CD28 stimulation; n = 4 in NC group: CD4⁺ T + IgG isotype control; n = 4 in anti-OX40L group: CD4⁺ T + anti-OX40L. *p < 0.05, **p < 0.01, ***p < 0.001.
Anti-OX40L inhibits TLR7-mediated differentiation of ASCs and antibody production through up-regulation of SPI-B, IRF8, and PAX5, and down-regulation of Xbp-1. (a) Representative flow cytometry diagrams and statistical analysis of the percentages of naïve B cells, memory B cells, plasmablast cells, plasma cells, and ASCs (CD44^hi CD138^+) in the splenic B cells stimulated by R848 and IL-4. (b) The levels of IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM from the splenic B cell supernatant after 72 h of culture. (c)
Regulation of key transcription factors related to B-cell proliferation and differentiation after anti-OX40L treatment for 48 h. Horizontal bars represent the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**Supplementary Files**

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