Regulation effects of fusion protein IgD-Fc-Ig targeting T cells via IgD-IgDR-Lck-ZAP70-PI3K-NF-κB signaling in rheumatoid arthritis

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Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Abstract

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by synovitis and the destruction of small joints. Emerging evidence had shown that the stimulation of immunoglobulin D (IgD) induced T cell activation which may contribute to diseases pathogenesis in RA. In this study we demonstrated that IgD could induce the activation of T cells through affecting IgDR-Lck-ZAP70-PI3K-NF-κB signaling, IgD-induced $CD4^+$T cells promoted the proliferation of $CD19^+$B cells in RA patients. IgD-Fc-Ig fusion protein (composed of human IgD Fc domain and IgG_1 Fc domain, specifically blocks the IgD-IgDR pathway) inhibited the co-expression of IgDR and p-Lck and the expressions of p-Lck, p-ZAP70, p-PI3K on $CD4^+$T cells, and decreased NF-κB nuclear translocation in Jurkat cells. Meanwhile, IgD-Fc-Ig down-regulated the protein expressions of CD40L on $CD4^+$T cells and CD40, CD86 on $CD19^+$B cells in RA patients and healthy controls. It also decreased the protein expressions of CD40L on $CD4^+$T cells and CD40 on $CD19^+$B cells from spleens of CIA mice and reduced IL-17A level in mouse serum. Moreover, *in vivo*, IgD-Fc-Ig administration dose-dependently down-regulated the protein expressions of CD40, CD40L and IgD in spleens from CIA mice. IgD-Fc-Ig restrains the activations of T cells through inhibiting IgD-IgDR-Lck-ZAP70-PI3K-NF-κB signaling, thus inhibiting the activation of B cells. Our data provides experimental evidence for application prospect of IgD-Fc-Ig as a highly selective targeting T cell treatment for RA.

Key words: rheumatoid arthritis; immunoglobulin D; IgD-Fc-Ig; immunoglobulin D

receptor; $CD4^+$T cells; $CD19^+$B cells

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease manifested by a persistent inflammation of synovial joints, bone destruction, loss of cartilage and increased risk of cardiovascular and other inter-current diseases [1]. The etiopathogenesis of RA has not been thoroughly clarified, so the appropriate drug for RA treatment is still in development. The strategy currently in use mainly including non-steroidal anti-inflammatory drugs (NSAIDs), conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), biological DMARDs (bDMARDs), and targeted synthetic DMARDs (tsDMARDs) [2]. However, some of the drugs are not suitable in some patients with contraindications or early intolerance during clinical use, or accompanied by serious adverse drug reactions including severe bone marrow suppression, infection and even tumors. Till now the latest recommendations published in 2020 will allow most RA patients reach the treatment target. However, about 20%–30% patients remain refractory to current therapies [3]. For these, new treatment options, but also better insights into the pathogenesis of RA will be needed.

Immunoglobulin D (IgD) was discovered in 1965 and consists of two identical light chains and two heavy chains, including variable (V) and constant (C) [4]. IgD acts as B cell surface receptors (BCR) in the form of membrane-bound IgD (mIgD) or in small amounts as secreted IgD (sIgD) in human and mice [5]. Meanwhile, IgD is an important immunomodulatory molecule and promotes immune defense by inducing the activation and infiltration of IgD-interacting cells into tissues and their production of immune-activating factors, and over-activation of this pathway can cause inflammation and tissue damage [6, 7]. SIgD was found abnormally high expressed in

serum of patients with multiple diseases such as immunodeficiency, chronic infection, autoimmune disease, etc [8]. Newly reports showed that sIgD appears to enhance mucosal homeostasis and immune surveillance by "arming" myeloid effector cells [9]. IgD exerts biological functions by binding to a specific membrane IgD Fc receptor (IgDR) which was first reported expressed on human T cells in 1980 [10]. Our group has reported abnormal serum levels of IgD and IgDR in RA patients [11]. Meanwhile, the sIgD concentrations were positively associated with disease activity score in 28 joints (DAS28) and anti-cyclic citrullinated peptide (Anti-CCP) in RA [12]. Moreover, IgD can induce the abnormal activation of T cells and imbalance in the proportion Th1/Th2/Th17/Treg subsets by promoting phosphorylation of lymphocyte-specific tyrosine kinases (Lck) [13], suggested protein tyrosine kinase (PTK) is involved in the process of IgD-induced IgDR expression.

With a particular emphasis on the novel T cell-targeted therapeutic strategies, our work highlights the immunologic features of IgD/IgDR and a novel direction of discovery and development of new drugs for the treatment of T cell related diseases. Based on these, our group successfully linked the human IgD-Fc segment to the human IgG_1-Fc segment to form a fusion protein (IgD-Fc-Ig) targeting IgD/IgDR, and a patent license was acquired in China (No: 201510600762X). Recently, IgD-Fc-Ig was demonstrated to alleviate the arthritis manifestation in CIA rodent models *in vivo*, and could inhibit the activation and proliferation of T cells in healthy controls and PBMCs in RA patients stimulated by IgD *in vitro* [12][14]. In addition, the abnormal activations and proliferations of T cells and B cells could promote the occurrence and

development of RA. T cells behave more like as a hub, in that B cells, DCs, and FLSs can interact with T cells to inhibit their activation and interfere with the process of RA [15]. We have observed that in RA patients, excessive IgD enhances the proliferation of PBMCs and increased the secretion of inflammatory cytokines which may be caused by the activation of T and B cells, while further elucidation of molecular mechanisms that regulate T and B cell activation is needed.

We now investigated whether IgD induced T cell activation through affecting IgDR-Lck-ZAP70-PI3K-NF-κB signaling, thus IgD activated T cells could cause the activation of B cells both in human and mice. IgD-Fc-Ig plays a role in the regulation IgD activated T cells, as this may represent an important application prospect of targeting T cell treatment for RA.

Materials and Methods

Drugs and Reagents

IgD-Fc-Ig fusion protein and IgG_1-Fc protein were supplied by the Institute of Clinical Pharmacology, Anhui Medical University (Hefei, Anhui Province, China), IgD-Fc-Ig can be applied for study with a purity of more than 90% [12]. Lck inhibitor A770041was purchased from Axon Medchem (Groningen, Netherlands). rhTNFR:Fc fusion protein was purchased from Guojian Pharmaceutical Company (Shanghai, China). Anti-mouse IgD antibodies were purchased from eBioscience (San Diego, CA, USA).

Samples collection, Jurkat cell line and animals

The study protocol was carried out in accordance with the Declaration of Helsinkiand approved by the Ethics Committee of Anhui Medical University (No. 20160119, 20160095). Peripheral blood samples of healthy donorsand RA patients were collected from the First Affiliated Hospital, Anhui Medical University. Written informed consent was obtained from all participants before enrollment in the study in accordance with the Declaration of Helsinki, and all donor samples were made anonymous to maintain health information confidentiality. Jurkat, Clone E6-1 cells (RRID: CVCL_0367) were obtained from *Procell Life Science & Technology Co, Ltd.* and cultured according to their guidelines. Seven-week old male DBA/1 mice (18±2g) (Certificate No: 11400700370327) were obtained from *Beijing Wei Tong Li Hua Laboratory Animal Technology Co., Ltd.* and maintained in a specific pathogen-free animal laboratory at Anhui Medical University.

Isolation of human $CD4^+$T cells and $CD19^+$B cells

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of healthy controls and RA patients by ficoll gradient centrifugation. PBMCs were stained with fluorescently labeled mAbs against surface molecules as FITC anti-human CD4 (BD; Cat. No. 555346) and PE-cy5 anti-human CD19 (BD; Cat. No. 555414). $CD4^+$T cells and $CD19^+$B cells were sorted and collected by flow cytometry sorter (BD FACSAria^{TM}II, BD, US). Preparations were typically > 95% pure.

Co-culture of human $CD4^+$T cells and $CD19^+$B cells

$CD4^+$T cells and $CD19^+$B cells from PBMCs of healthy controls and RA patients were co-cultured in RMPI 1640 with 10% fetal bovine serum (FBS; HyClone, Carlsbad, CA, USA) in transwell chambers (0.4um pore size/24well plates), $CD4^+$T cells (1×10^6 cells/well) were seeded in the lower chambers, $CD19^+$B cells (1×10^6 cells/well) were seeded in the upper chambers. $CD4^+$T cells were cultured within creased concentrations of IgD (1, 3 and 10μg/mL) (abcam; Cat. No. ab91022) or IgD (3μg/mL) treated with different concentrations of IgD-Fc-Ig fusion protein (3, 10μg/mL), Lck inhibitor A770041 (0.5μM) and IgG_1-Fc (10μg/mL). B cells in the absence of T cells was set as control group, T cells and B cells without any treatment was set as T cells + B cells group. Each treated group was co-cultured with $CD19^+$B cells simultaneously for 24h or 48h at 37°C.

Biotin labeling of IgD protein

The primary antibody for biotin labeled IgD was prepared, the human IgD protein was labeled with biotin according to the biotin labeling Kit-NH2 kit (DOJINDO; Cat. No. LK03) instructions and the labeled protein were stored at -20°C.

Immunofluorescence confocal microscopy analysis

Pre-treated $CD4^+$T cells were fixed with 4% paraformaldehyde for 15min and rinsed three times with PBS for 10 min each. The cells were permeabilized with 0.1% Triton X-100 solution at room temperature and then rinsed with PBS three times. The cells were blocked for 1h in a 37°C incubator with 10% sheep occluded serum. The

serum was discarded and then the primary antibody to p-Lck（tyr 394）(rabbit; Gene Tex; Cat. No. GTX133876)at a 1:1000 ratio and primary antibody for biotin labeled IgD at a 1:25 ratio were stored at 4°C overnight. The primary antibody was then discarded and rinsed three times with PBS. Fluorescent secondary antibody conjugated Alexa Fluor 488 goat anti-mouse IgG (Fcmacs; Cat. No. FMS-Msaf48801) and PE-cy7 streptavidin (Biolegend; Cat. No. 405206) of a 1:100 ratio were incubated in a 37°C incubator for 1h in the dark. The fluorescent secondary antibody was then discarded and rinsed three times with PBS. Nuclei were stained with DAPI staining solution for 10min and rinsed four times with PBS. Images were acquired on a Leica SP8 TCS STED 3X microscope system (Leica, Germany) with an HC PL APO 100×/ 1.4 NASTED objective either in confocal (IgDR and p-Lck).

Quantification of co-localization

The images were analyzed by “Colocalization Module” of Leica SP8 TCS STED 3X microscope system. The colocalization index of IgDR and p-Lck was determined through Colocalization rate, Pearson’s correlation and Overlap coefficient. Background correction in manual mode was determined and kept consistent for all images that were compared to confirm co-localization of IgDR and p-Lck on $CD4^+$ T cells.

Co-immunoprecipitation (Co-IP) analysis

Pre-treated $CD4^+$T cells plated in 6-well plate (5×10^6/well) were centrifuged at

2,000 rpm/min for 10 min for collecting to 1.5 mL EP tubes, then added 200μL of lysate to each tube. Suspended cells to lyse the cells, and shook them on ice for 30min. The cells were centrifuged at 12,000 rpm/min for 15 min at 4°Cand then extracted the supernatant on ice and added antibody homologous IgG (Rabbit; Santa Cruz Blotechnology; Cat. No. sc-2025) at a ratio of 1:500 along with A/G agarose beads (1:50) (Santa Cruz Blotechnology; Cat. No. sc-2003). This was followed by centrifugation at 4°C for 15min. The supernatant was collected at 14,000 rpm/min. Then, A/G agarose beads were added once again (1:100), and the p-Lck（tyr 394）primary antibody (1:1000) was added at 4°C overnight. The agarose beads were then rinsed 3 times with 1 × IP buffer. Each time, the agarose beads were collected by centrifugation at 3000g for 5min in a 4°C centrifuge, 50μL of 1 × IP buffer was added to the agarose bead binding protein, and the loading buffer was added at a ratio of 1:4 and then boiled for 8min after 10% SDS-page analysis. The membrane was scanned on a GS-700 Imaging Densitometer. The image was analyzed with Image J software (1.4.3.67).

Activation and immunofluorescent detection of NF-κB nuclear translocation

Jurkat cells (2×10^6 cells/mL) in exponential growth phase were treated with 3μg/ml IgD. IgD-induced Jurkat cells incubated with 10μg/ml IgD-Fc-Ig and 0.5μM Lck inhibitor A770041 for 4h in 34.8 mm diameter dishes. Cells in diameter dishes were

centrifuged at 2000rpm/min for 10 min for collecting, followed by cytoperm/cytofix buffer for 30 min, washed with PBS. After washing, the cells were incubated for 30 min with PE anti-human phospho-transcription factor nuclear factor-κB (NF-κB) p65 (ser 529) (invitrogen-Thermo Fisher Scientific; Cat. No. 2038939), washed with PBS. After washing, followed by DNA-binding dye DAPI (Beyotime; Cat. No. C1005) for 30 min, then washed and suspended in PBS. The samples were run directly on the Image Stream system (ImageStream^X Mark Ⅱ, Germany) and all fixation and staining reactions were carried on ice in the dark. All washes were performed with cold PBS.

Image Stream data acquisition and analysis

The fluorescence image-based method for quantifying nuclear translocation described here relies on the differential spectral isolation of NF-κB and DAPI images of nuclear images by the Image Stream technology [16]. Determination of NF-κB translocation from a similarity score [16]: The assessment of nuclear translocation was determined in a qualitative manner by comparing a cell's nuclear fluorescence (DAPI fluorescence) image to the pattern of fluorescence produced by the NF-κB label (PE). Nuclear translocation is judged to have occurred if the NF-κB and nuclear fluorescence signals overlap with similar shape. Conversely, if the NF-κB signal surrounds the nucleus, it is judged not to exhibit significant translocation. The similarity score is a method of quantitatively performing this assessment. The similarity score has a high positive value if the NF-κB and nuclear images are alike.

In contrast, if the NF-κB and image is dim where the nuclear image is bright, the score has a large negative value. The data pairs are the pixel intensities at the same location in each image of the two different fluorescence intensities of each pixel. Plotting the pixel intensities of the nuclear image against the transcription factor image reveals an inverse correlation for un-translocated protein and a positive correlation for trans-located protein in a cell.

Cell Counting Kit-8 analysis

$CD4^+$T cells and $CD19^+$ B cells from RA patients were treated and co-cultured as describe previously. $CD4^+$T cells were cultured in the presence or absence of increased concentrations of IgD (1, 3 and 10μg/mL). The control and T cells + B cells groups were set as previously described. To assess proliferative responses of cultured cells, a cell counting kit-8 (CCK-8; DOJINDO; Cat. No. CK04) was used to explore the viability of $CD19^+$B cells after co-culture for 24h and 48h. 100μL per well of $CD19^+$B cells in the upper chamber was collected and seeded in triplicate in 96-well round-bottom microtiter wells. 10μL CCK-8 solution was added per well. The cells were incubated for 4h at 37°C in an atmosphere containing 5% CO_2, and the absorbance at 450 nm was measured on a microplate reader (Infinite M1000 PRO, TECAN, Switzerland).

Flow cytometry analysis

$CD19^+$B cells were harvested from chambers at the end of the incubation period

and stained for 30 min on ice with PE anti-human CD86 (Biolegend; Cat. No. 305405). Each sample was collected for 20,000 cells, and as a result the density of the dot plots reveals relative cell numbers. The Median Fluorescence Intensity (MFI) of CD86 expression were collected by using flow cytometry (Beckman Coulter; Beckman Coulter Biotechnology; USA) and analyzed with CytExpert (2.1.0.92).

Induction and treatment of CIA mice

Type II collagen sample was dissolved in acetic acid (0.1mol/L) at 2 mg/mL and incubated overnight at 4 °C. The sample was then emulsified with an equal volume of complete Freund's adjuvant. Arthritis was induced in seven-week-old male DBA/1 mice by intradermal injection of 0.15 mL CII emulsion into the base of the tail followed by a booster injection of 0.1 mL emulsion on day 21. Mice were maintained in a specific pathogen-free animal laboratory at Anhui Medical University. Mice were divided into nine treatment groups: Normal, CIA, IgD-Fc-Ig (1.625 mg/kg, 3.25 mg/kg, 6.5 mg/kg and 13 mg/kg), IgG_1-Fc (13 mg/kg), rhTNFR:Fc (4.5 mg/kg) and anti-mouse IgD antibody (2 mg/kg). After the onset of arthritis at day 30, mice were treated with IgD-Fc-Ig, IgG_1-Fc or rhTNFR:Fc through tail intravenous injection (twice weekly, for 4 weeks), and anti-IgD antibody (once daily, for 3 days). rhTNFR:Fc and anti-IgD antibody treatment groups were used as positive controls. The normal control group mice were treated with equal volume of saline by intravenous injection. Mice were sacrificed on day 54, and spleens collected for

immunohistochemistry analysis.

Mice splenocyte isolation and co-culture

Mice spleen were pressed and washed through a 200-gauge mesh. Splenocytes were isolated by ficoll gradient centrifugation. Splenocytes were stained with fluorescently labeled mAbs against surface molecules as FITC anti-mouse CD4 (BD; Cat. No. 553047) and PE-cy5 anti-mouse CD19 (BD; Cat. No. 552854). $CD4^+$T cells and $CD19^+$B cells were sorted and co-cultured as described previously.

Western blot analysis

For the *in vitro* study, $CD4^+$T cells and $CD19^+$B cells from PBMCs of healthy controls and RA patients were collected after T-B cells co-culture. Cells were lysed in lysis buffer supplemented with protease inhibitors and phosphatase inhibitors for 30 min on ice. In animal study, $CD4^+$T cells and $CD19^+$B cells from splenocytes of CIA mice were collected and lysed after T-B cells co-culture. Primary antibodies: Lck (rabbit, 1:1000; Gene Tex; Cat. No. GTX107785), p-Lck (tyr394) (rabbit, 1:1000; Gene Tex; Cat. No. GTX133876), ZAP-70 (rabbit, 1:500; Affinity; Cat. No. AF6312), p-ZAP70 (rabbit, 1:500; Affinity; Cat. No. AF3312), PI3K (rabbit, 1:1000; Cell Signaling; Cat.No. 4257T), p-PI3K (rabbit, 1:1000; Cell Signaling; Cat.No. 4228T), CD40L (rabbit, 1:500; Affinity; Cat. No. DF2301), CD40 (rabbit, 1:500; Affinity; Cat. No. AF5336) and β-actin (mouse, 1:1000; protein tech; Cat. No. 66009-I-Ig) were then incubated at 4 °C overnight, and the goat anti-rabbit (1:1000; Cell Signaling; Cat.

No. 7074P2)/mouse conjugated secondary antibodies (1:1000; proteintech; Cat. No. SA00001-1) were incubated for 2h at 37°C. The membrane was scanned on a GS-700 Imaging Densitometer. The image was analyzed with Image J software (1.4.3.67).

Immunohistochemistry (IHC)

The spleens of each mice group were dehydrated (2 × 70% EtOH, 2 × 90% EtOH, 2 × 100% EtOH, 3 × 100% Xylene, 3 × Paraffin; each 90min), embedded in paraffin in plastic holders, followed by preparation 5μm paraffin microtome slices. After blocking with 2% BSA/PBS, sections were labeled with primary antibodies including CD40, CD40L and IgD. Detection of primary anti-bodies was performed using goat anti-rabbit conjugated secondary antibodies and peroxidase-based detection systems using the ABC complex and DAB as substrate. Images were acquired on a Olympus BX53F microscope system (Olympus, Japan) with an 40x objective.

ELISA analysis

The levels of IL-17A were determined in serum samples and cellular supernatant samples using the ELISA method (abcam, USA; FcMACS, China) according to the manufacturer's instructions. ODs of each sample were detected at 450nm with the Infinite M1000 PRO Micro-plate Reader.

Statistical analysis and quantification

In this study, SPSS 16.0 was used for the statistical analysis, and the two groups

were compared using the t-test. Data were presented as means ± standard error (SEM) and calculated using Prism 6 software with 2-way ANOVA multiple comparison tests. $P < 0.05$ was considered statistically significant.

Results

Effects of IgD-Fc-Ig on co-expression of IgDR and Lck in human CD4$^+$T cells

We used immunofluorescence confocal microscopy and co-immunoprecipitation methods to examine the co-expression of IgDR and p-Lck proteins on CD4$^+$T cells in healthy controls. Confocal microscopy results clearly show that IgDR fluorescence (red) on CD4$^+$T cells occurred in the same area as p-Lck fluorescence (green), proving the combination of the two molecules (**Fig.1A**). Colocalization rate, Pearson's correlation and Overlap coefficient were used to quantify colocalization. Co-localization rate (%) showed that red-green pair implies that all red pixels co-localized with green. Pearson's correlation described the correlation of the intensity distribution between channels (ranges from -1.0 to 1.0; 0 indicates no significant correlation and -1.0 indicates complete negative correlation). Overlap coefficient splits the value of colocalization into the two separate parameters, allows determining the contribution of each antigen to the areas with co-localization. Our results showed that in these co-localization coefficient graphs, IgD (3μg/mL) significantly promoted the co-expression of IgDR and p-Lck on CD4$^+$T cells (**Fig.1B-D**) compared to control group (P<0.01). IgD-Fc-Ig (10μg/mL) and Lck inhibitor A770041 (0.5μM) could significantly decrease the co-expression of IgDR and p-Lck (**Fig.1B-D**) compared to

IgD (3μg/mL) stimulation group (P<0.01).

The results of co-immunoprecipitation are consistent with those of laser confocal. IgD (3μg/mL) could significantly promote the co-expression of IgDR and p-Lck on $CD4^+$T cells (**Fig.1E**) compared to control group (P<0.05). IgD-Fc-Ig (10μg/mL) and Lck inhibitor A770041 (0.5μM) significantly decreased the co-expression of IgDR and p-Lck (**Fig.1E**) compared to IgD (3μg/mL) stimulation group (P<0.05).

Effects of IgD-Fc-Ig on protein expressions of p-Lck, ZAP-70, p-ZAP70, PI3K, p-PI3K in $CD4^+$T cells of healthy controls

The expression levels of p-Lck, ZAP70, p-ZAP70, PI3K and p-PI3K were analyzed by Western blotting. No significant differences were observed in the total protein expressions of ZAP70 and PI3K between treatment groups (**Fig.2A**). The expression levels of p-Lck, p-ZAP70 and p-PI3K were obviously increased in $CD4^+$T cells stimulated by IgD (3, 10μg/mL) compared to control group (P<0.01). Both IgD-Fc-Ig (10μg/mL) and Lck inhibitor A770041 (0.5μM) treatment significantly reduced the expression of p-Lck, p-ZAP70 and p-PI3K (**Fig.2B-D**) compared to IgD (3μg/mL) stimulation group (P<0.01).

Effects of IgD-Fc-Ig on NF-κB nuclear translocation stimulated by IgD in Jurkat cells

NF-κB nuclear translocation was monitored and quantitated using the similarity score by Image Stream technology described in the Material and Methods (**Fig.3A,**

3B). We have observed that the optimal stimulate time point is 4h (**Supplementary data**). **Figure 3B$_1$** illustrates Image Stream images of four control cells, **Figure 3B$_2$** illustrates Image Stream images of four IgD-induced cells, **Figure 3B$_3$** illustrates Image Stream images of four IgD-induced cells incubated with IgD-Fc-Ig, **Figure3B$_4$** illustrates Image Stream images of four IgD-induced cells incubated with Lck inhibitor A770041. Stimulation of Jurkat cells with 3μg/mL of IgD clearly resulted in nuclear translocation of NF-κB within 4h which as expected as measured by the G-mean (**Fig.3C**) compared to control group (P<0.01). Meanwhile, IgD-induced cells incubated with IgD-Fc-Ig (10μg/mL) and Lck inhibitor A770041 (0.5μM) revealed a decrease in NF-κB nuclear translocation (**Fig.3C**) compared to IgD (3μg/mL) stimulation group (P<0.01).

Effects of IgD activated CD4$^+$ T cells on CD19$^+$ B cells in healthy controls

To confirm the effect of IgD-induced CD4$^+$T cells on CD19$^+$B cell proliferation and activation in healthy controls, CD19$^+$B cells were co-cultured with IgD (3μg/mL) treated CD4$^+$T cells or CD19$^+$B cells were treated with IgD (3μg/mL) *in vitro* for 24h and 48h, and the proliferation of CD19$^+$B cells was measured. IgD activated CD4$^+$ T cells could cause the proliferation of CD19$^+$B cells (P<0.01), while CD19$^+$ B cells directly stimulated by IgD could not cause B cell proliferation (**Fig.4A, 4B**). The expressions of CD40L on CD4$^+$T cells and CD40 on CD19$^+$B cells which were used to indicate the activation of T and B cells were analyzed by Western blotting after co-culture. The expression of CD40L on CD4$^+$T cells was up-regulated after IgD (3,

10μg/mL) treatment (**Fig.4C**) compared to control group (P<0.01). In addition the expression of CD40 on $CD19^+$B cells (**Fig.4D**) was up-regulated after co-culture with IgD activated $CD4^+$T cells (P<0.01).

Effects of IgD-Fc-Ig on IgD activated $CD4^+$T cells in RA patients

Human $CD4^+$T cells activated by IgD have been shown to induce B cell activation from peripheral blood in healthy controls. Then we extend this result to demonstrate the effect of IgD-Fc-Ig on the co-culture of IgD activated $CD4^+$T cells and $CD19^+$B cells in RA patients. To confirm the finding in healthy controls, we observed the effect of IgD activated $CD4^+$T cells on $CD19^+$B cell proliferation and activation. $CD4^+$T cells were incubated with different concentrations of IgD (1, 3, 10μg/mL) and co-cultured with $CD19^+$ B cells *in vitro* for 24h and 48h, and the proliferation of $CD19^+$B cells was measured. As expected, IgD (1, 3, 10μg/mL) activated $CD4^+$T cells could increase the proliferation of $CD19^+$ B cells in a concentration-dependent manner (P<0.05) (**Fig.5A, 5B**). The expressions of CD40L on $CD4^+$T cells and CD40 on $CD19^+$B cells were analyzed, separately. Consistent with the finding in healthy controls, the expressions of CD40Lon $CD4^+$T cells were up-regulated after IgD (3, 10μg/mL) treatment (**Fig.5C)** compared to control group (P<0.01). In addition the expression of CD40 on $CD19^+$B cells (**Fig.5D**) were up-regulated after co-culture with IgD activated $CD4^+$T cells (P<0.01). The up-regulated expression of CD40L on $CD4^+$ T cells by IgD (3μg/mL) stimulation could be down-regulated by IgD-Fc-Ig (3, 10μg/mL) treatment (P<0.01). Meanwhile, the expression

of CD40 on $CD19^+$ B cells was down-regulated after T-B cell co-culture (**Fig.5E, 5F**). The effect of IgD-Fc-Ig on CD86 expression on $CD19^+$ B cells was analyzed by FCM. The expression of CD86 was up-regulated after IgD (10μg/mL) treatment (**Fig.5G)** compared to T cells + B cells group (P<0.05). The up-regulated expression of CD86 on $CD19^+$ B cells by IgD (10μg/mL) stimulation could be down-regulated by IgD-Fc-Ig (3, 10μg/mL) treatment (P<0.01) (**Fig.5G**).

Effects of IgD-Fc-Ig on the expressions of CD40, CD40L and IgD in CIA mice

We established the CIA model and mice were treated with IgD-Fc-Ig, IgG1-Fc, rhTNFR:Fc or anti-IgD antibody through tail intravenous injection. To observe the effect of IgD-Fc-Ig on the overall mice model, the expressions of CD40, CD40L and IgD in mice spleens were analyzed by immunohistochemistry. The expressions of CD40, CD40L and IgD in spleens from CIA mice were higher than those in normal mice (P<0.01). Etanercept (4.5mg/kg) and anti-mouse IgD antibody (2mg/kg) administration could down-regulate the expressions of CD40, CD40L and IgD in CIA mice spleens (P<0.01). IgD-Fc-Ig (3.25, 6.5, 13mg/kg) administration dose-dependently down-regulated the expressions of CD40, CD40L and IgD in spleens (P<0.05). In contrast, the expressions of CD40, CD40L and IgD in IgG_1-Fc (13mg/kg) administration group did not undergo efficient changes compared to CIA mice group (**Fig.6A-C**).

Effects of IgD-Fc-Ig on IgD activated $CD4^+$ T cells in CIA mice *in vitro*

To identify the effect of IgD-Fc-Ig on the co-culture of IgD activated $CD4^+$ T cells and $CD19^+$ B cells in CIA mice, the expressions of CD40L on $CD4^+$T cells and CD40 on $CD19^+$ B cells from spleens of CIA mice were analyzed by Western blotting. Consistent with the finding in human samples, the expressions of CD40Lon $CD4^+$T cells were up-regulated after IgD (3μg/mL) treatment (**Fig.7A**) compared to control group (P<0.01). In addition the expression of CD40 on $CD19^+$B cells (**Fig.7B**) were up-regulated after co-culture with IgD activated $CD4^+$T cells (P<0.01). The up-regulated expression of CD40L on $CD4^+$ T cells by IgD (3μg/mL) stimulation could be down-regulated by IgD-Fc-Ig (10μg/mL) treatment or Lck inhibitor A770041 treatment (0.5μM) (P<0.01). Meanwhile, the expression of CD40 on $CD19^+$ B cells was down-regulated after T-B cell co-culture (**Fig.7A, 7B**). IgG_1-Fc (10μg/mL) treatment did not undergo efficient changes on the expressions of CD40L and CD40 (**Fig.7A, 7B**).

To determine the cytokine profile of the IgD activated T cells, the levels of IL-17A from $CD4^+$T cells were detected by ELISA from culture supernatant following 48h co-culture treatment. Substantial amounts of IL-17 were produced by IgD activated T cells (**Fig.7C**) compared to control group (P<0.01). The up-regulated levels of IL-17A by IgD (3μg/mL) stimulation could be down-regulated by IgD-Fc-Ig (3, 10μg/mL) treatment or Lck inhibitor A770041 treatment (0.5μM) (**Fig.7C**) (P<0.01). IgG_1-Fc had no significant effect on IL-17A secretion (**Fig.7C**).

Discussion

RA is an autoimmune disease characterized by chronic inflammation, synovium hyperplasia, joint destruction of bone and cartilage interface, and T cell hyper-activation [1]. The global prevalence of all species is 1%~2%, the ratio of male to female is 1:2.5, with 30~50 years of age as the peak, the RA prevalence in China is about 0.2%~0.4% [17]. The exact etiology and pathogenesis of RA are unclear. Studies have shown that sIgD plays a significant role in multiple diseases and serum IgD levels were found abnormally high expressed in serum of patients with multiple diseases such as immunodeficiency, chronic infection, allergic disease, autoimmune disease, etc [8]. Meanwhile, In an IgD transgenic mouse model, skin ulcers and abnormal swelling of the liver, spleen and kidney were found, which may be related to the high expression of sIgD in serum [18]. The results of mice experiments have shown that anti-IgD antibody alleviated arthritis indices in mice with CIA [19]. Our group has reported that the levels of plasma sIgD in RA patients and CIA mice were observed higher than that in healthy controls and normal mice. Moreover, the sIgD concentrations were positively associated with disease activity score in 28 joints (DAS28) and anti-cyclic citrullinated peptide (Anti-CCP) in RA [12]. Till now, IgD has been known as a very important mediator, which can work via its effector IgDR. In 1980, IgDR was first discovered on T cells in human peripheral blood [10]. Our group further demonstrated the dissociation constant (KD) of IgD binding to IgDR was 8.10×10^{-10}mol/L on human $CD4^+$ T cells [20]. IgD could stimulate the expression of IgDR on T cells *in vitro*. The above suggest the abnormal high expression of IgD

plays an important role in pathological mechanism of RA.

Our group found that IgD induced IgDR on T cells more obviously, so we focused on the effect and function of IgD-IgDR on T cells [21]. $CD4^+$T cells play a vital role in coordinating protective immune responses to various pathogens and also contribute to the development of allergies, inflammation, and immune responses in autoimmune diseases [22, 23]. Previous studies suggested that IgD could activate the Lck signaling pathway of mice and our group also found that IgD could induce phosphorylation of human Lck [13]. The activation of Lck kinase induces the production of the SH2 domain, which binds to ZAP70 kinase, thereby activating ZAP70 kinase. The activation of ZAP70 kinase further stimulates multiple signaling networks downstream, including calcium mobilization, Ras/MAPK and PI3K pathways [24]. Moreover, ZAP-70 has a critical role in the initiation of T-cell signaling and initiates a sequence of events leading to the activation of the transcription factor nuclear factor-κB (NF-κB), which, in a step-wise procedure, results in the transcription of genes coding for proteins exerting T-cell functions [25].We have found thatthe expression levels of p-Lck (tyr394), p-ZAP70 and p-NF-κB65 were obviously increased in CIA mice [12] and rats [14]. However, the specific mechanism by which IgD activates T cells through the Lck signaling pathway and the interaction between IgDR and Lck remains unclear. To prove our hypothesis, we observed IgDR and p-Lck were co-expressed on $CD4^+$T in healthy controls and IgD up-regulated the co-expression of IgDR and p-Lck. The results indicated that IgD binding receptors, IgDR could up-regulate Lck activity through binding Lck and phosphorylating Lck site (tyr394).

Phosphorylated Lck could induce abnormal activation of T cells by direct interaction with IgDR. The results of Western blot showed that IgD up-regulated the expressions of p-Lck、 p-ZAP70、 p-PI3K on $CD4^{+}$T cells in healthy controls. We further observed NF-κB translocation in Jurkat cell line. Stimulation of Jurkat cells with IgD clearly resulted in nuclear translocation of NF-κB. Our data suggested IgD could induce the abnormal activation of T cells through affecting IgDR-Lck-ZAP70-PI3K-NF-κB signaling.

During the RA immune response, B cells can identify and present antigens to T cells. B cells provide co-stimulatory signals to aid in T cell activation, secrete inflammatory factors, produce rheumatoid factors and anti-cyclic citrullinated peptide antibody, and participate in the formation of germinal centers (GCs) [15]. Co-stimulatory molecules CD40-CD40L play a crucial role in the interaction between T and B cells, which involved in RA occurrence and development. The interactions of CD40 and CD40L can induce expressions of TNF-α and co-stimulatory molecules CD80-CD86, promote differentiation, proliferation and maturation of B cells, and also promote excitation and differentiation of T cells [26, 27], thus further promoting the interaction between T and B cells. The trans-activation of CD40L is tightly regulated by several transcription factors (NFAT, CD28RE, NF-κB, TFE3/TFEB, EGR, AKNA, and AP1) that bind in the promoter region [28]. Although NFAT is the key transcription factor found in the minimal CD40L promoter [29], several reports demonstrated significant involvement of NF-κB in the up-regulation of CD40L

expression in both activated mouse and human T cells [30]. Previous studies in our lab showed that IgD could increase the proportion of $CD154^+$ (CD40L) cells. Moreover, the proportion of $CD154^+$ cells in CIA mice was significantly increased compared with normal mice [12]. In this study, IgD could up-regulate the expressions of CD40L on $CD4^+$T cells and CD40 on $CD19^+$B cells in healthy controls and RA patients. As expected, consistent results were observed in parallel experiments in CIA mice. As we known, the up-regulated expression of CD86 is a sign of B cell activation [31]. We demonstrated that IgD-induced $CD4^+$ T cells up-regulated the expression of CD86 on $CD19^+$B cells in RA patients, IgD-induced $CD4^+$ T cells could induce the proliferation of $CD19^+$B cells. These results suggested that the mechanism of T cell activation induced by IgD involved IgDR-Lck-ZAP70-PI3K-NF-κB signaling and CD40-40L co-stimulatory molecules promotion. The previous studies shift our focus to IgD-IgDR interactions on T cells, which may offer a potential strategy to neutralize over-expressed IgD in RA.

Our lab synthesized and purified a novel biological agent, IgD-Fc-Ig fusion protein, by combining an IgG_1-Fc domain to IgD-Fc. IgD-Fc-Ig binds to IgDR thereby preventing excessive IgD activation. We have confirmed that IgD-Fc-Ig exhibited a similar binding affinity as IgD for IgDR, and competitively inhibited IgD binding to IgDR. In CIA rodent model, disease activity, manifestation, T cells activation and the expressions of p-Lck, p-ZAP70 and p-NF-κB were significantly reduced after IgD-Fc-Ig administration [12, 14]. This study further confirmed that IgD-Fc-Ig could down-regulate the interaction of IgDR and p-Lck, decrease the NF-κB

nuclear translocation stimulated by IgD. Moreover, in healthy controls, RA patients and CIA mice, IgD-Fc-Ig could also down-regulate the expressions of CD40L on $CD4^+$ T cells and CD40 on $CD19^+$ B cells stimulated by IgD. However, IgDR gene has not yet been cloned, the exact molecular mechanism of IgD participated in abnormal activations of T and B cells through IgDR are needed. In summary, IgD-Fc-Ig could affect the activation of Lck and its downstream signaling pathways by inhibiting the binding of IgD to IgDR, which also could restrain the activations of T cells through inhibiting IgD-IgDR-Lck-ZAP70-PI3K-NF-κB signaling, thus inhibiting the activation of B cells.

Conclusion

In conclusion, IgD can induce the abnormal activation of T cells through affecting IgDR-Lck-ZAP70-PI3K-NF-κB signaling on T cells. The co-culture of IgD-induced T cells and B cells can up-regulate the expressions of co-stimulatory molecules CD40-CD40L, and then induce the activation of B cells. IgD-Fc-Ig restrains the activations of T cells through inhibiting IgD-IgDR-Lck-ZAP70-PI3K-NF-κB signaling, thus inhibiting the activation of B cells. These findings provide a theoretical basis for further elucidating the occurrence and development of RA and provide experimental evidence for the development of IgD-Fc-Ig as a new targeted the therapeutic drug for highly selective therapy of RA.

Abbreviations: CCK-8, Cell Counting Kit-8; CIA, Collagen-induced arthritis;

DMARDs, disease-modifying antirheumatic drugs; FcR, Fc region; FLS, fibroblast-like synoviocytes; GCs, germinal centers; IgD, immunoglobulin D; IgDR, IgD receptor; IL, interleukin; KD, dissociation constant; Lck, lymphocyte-specific protein tyrosine kinase; mIgD, membrane IgD; NSAIDs, non-steroidal anti-inflammatory drugs; PBMCs, peripheral blood mononuclear cells; PI3K, phosphatidylinositol 3-kinase; PTK, protein tyrosine kinase; RA, rheumatiod arthritis; RT, room temperature; SI, stimulation index; sIgD, secreted IgD; TCR, T cell antigen receptor; TNF-α, tumor necrosis factor α; Tregs, regulatory T cells; ZAP70, zeta-associated protein 70.

Authors' contributions

XXH performed the experiments, and wrote the manuscript. AJZ and WWP participated the experiments, collected the samples and did immunohistochemistry experiments. JYC helped guide the cytometry sorter operation. LLZ and YC helped to revise the manuscript. YJW designed the study, participated the experiments and revised the manuscript. WW conceived of the study and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Fig 1. Effects of IgD-Fc-Ig (DG) on the IgDR-p-Lck interaction occurs on $CD4^+$ T

cells in healthy controls induced by IgD. Obtained $CD4^+$ T cells from PBMCs of healthy controls, $CD4^+$ T cells were incubated with IgD (3μg/mL), IgD-Fc-Ig (10μg/mL) and 0.5μM Lck inhibitor A770041 for 24h. **(A)** Confocal microscopy of $CD4^+$ T cells stained for IgDR and p-Lck: red, staining of $CD4^+$ T cells with labeled IgDR protein; green, staining of $CD4^+$ T cells with labeled p-Lck protein; blue, staining of nuclei with the DNA-binding dye DAPI. **(B)** Colocalization rate of IgDR and p-Lck proteins (%). **(C)** Pearson's correlation of IgDR and p-Lck proteins. **(D)** Overlap coefficient of IgDR and p-Lck proteins. Data were analyzed by "Colocalization Module" of LeicaSP8 TCS STED 3X microscope system and were expressed as mean ± SEM (n=5). $^{**}P<0.01$ *vs.* control (T cells), $^{\#\#}P<0.01$ *vs.* IgD (3μg/mL) stimulation group. **(E)** Co-immunoprecipitation analysis of IgDR-p-Lck interaction occurs on $CD4^+$ T cells in healthy controls. Data were expressed as mean ± SEM (n=5). $^{**}P<0.01$ *vs.* control (T cells), $^{\#\#}P<0.01$ *vs.* IgD (3μg/mL) stimulation group.

Fig 2. Effects of IgD-Fc-Ig (DG) on the protein expressions of p-Lck, ZAP70, p-ZAP70, PI3K and p-PI3K. Western blot analysis of p-Lck **(A)**, ZAP70, p-ZAP70 **(B)**, PI3K and p-PI3K **(C)** expressions on $CD4^+$ T cells from PBMCs of healthy controls, $CD4^+$T cells were incubated with IgD (3, 10μg/mL), DG (10μg/mL) and 0.5μM Lck inhibitor A770041 for 24h and lysed. Data were expressed as mean ± SEM (n=3). $^{**}P<0.01$ *vs.* control, $^{\#\#}P<0.01$ *vs.* IgD (3μg/mL) stimulation group.

Fig 3. Image Stream fluorescence imaging of IgD induced and incubated with IgD-Fc-Ig (DG) and Lck inhibitor A770041 NF-κB nuclear translocation in Jurkat cells. Quantification of nuclear translocation using the similarity algorithm. **(A)** The region High Sim drawn on the dark field/DAPI similarity plot represents the region for positive image correlation for the similarity algorithm. This region is applied to the NF-κB/DAPI similarity plot. The percentages of cells that fall within the High Sim region are displayed on the right of each histogram. **(B)** Control un-stimulated Jurkat cells **(B1)**, Jurkat cells treated with 3μg/mL IgD for 4h **(B2)**, and IgD-induced Jurkat cells incubated with 10μg/mL DG **(B3)** and 0.5μM Lck inhibitor A770041**(B4)** for 4h were probed for NF-κB expression and DAPI as described in the Materials and Methods and run on the Image Stream. The multispectral imaging system acquires up to 6 images per cell in three different imaging modes: bright field (morphology) (1), and fluorescence; Ch07-DAPI (Purple) (2), Ch03-NF-κB (yellow-PE) (3), and NF-κB/DAPI composite images (4) for five representative (of 10000 images) cells and are shown for each treatment group. Pixel intensities from the NF-κB images are plotted against the corresponding pixel intensities from the DAPI or dark-field images. **(C)** NF-κB nuclear translocation in Jurkat cells. The G-means represent the geometric means of the similarity of nuclear/cytoplasmic NF-κB intensity ratio to DAPI intensity. Jurkat cells were either left un-stimulated or were stimulated with IgD3μg/mL, IgD-induced Jurkat cells incubated with 10μg/mL DG and 0.5μM Lck inhibitor A770041. The results show the mean ± SEM of 5 independent experiments in which the similarity ratio was calculated from 10000 cells in each independent

experiment. $^{**}P<0.01$ *vs.* control, $^{\#\#}P<0.01$ *vs.* IgD (3μg/mL) stimulation group.

Fig 4. Effects of IgD on $CD4^+$ T cells and $CD19^+$B cells actions in healthy controls. Obtained $CD4^+$T cells and $CD19^+$B cells from PBMCs of healthy controls. $CD4^+$ T cells were incubated with IgD (10μg/mL) co-cultured with $CD19^+$ B cells *in vitro* for 24h and 48h, $CD19^+$ B cells were incubated with IgD (10μg/mL) *in vitro* for 24h and 48h. **(A, B)** After co-cultured, $CD19^+$ B cells were obtained, CCK-8 analysis of effect of IgD-induced $CD4^+$ T cells on proliferation of $CD19^+$ B cells. Western blot analysis of CD40L expressions on $CD4^+$ T cells **(C)** and CD40 expressions on $CD19^+$B cells **(D)** from PBMCs of healthy controls, $CD4^+$ T cells were incubated with IgD (3, 10μg/mL) co-cultured with $CD19^+$ B cells *in vitro* for 48h and lysed. Data were expressed as mean ± SEM (n=3). $^{**}P<0.01$ *vs.* Control, $^{\#\#}P<0.01$ *vs.* T cells + B cells.

Fig 5. Effects of IgD-Fc-Ig (DG)on the $CD4^+$ T cells and $CD19^+$ B cells actions in RA patients. Obtained $CD4^+$ T cells and $CD19^+$ B cells from PBMCs of RA patients, $CD4^+$ T cells were incubated with different concentrations of IgD (1, 3, 10μg/mL) and DG(3, 10μg/mL) co-cultured with $CD19^+$ B cells *in vitro* for 24h **(A)** and 48h **(B)**. After co-cultured, $CD19^+$B cells were obtained, CCK-8 analysis of effect of IgD-induced $CD4^+$T cells on proliferation of $CD19^+$B cells. After co-cultured, $CD4^+$ T cells and $CD19^+$ B cells were obtained and lysed. Western blot analysis of CD40L **(C, E)** and CD40 **(D, F)** expressions on $CD4^+$T cells and $CD19^+$B cells from PBMCs of RA patients. Representative flow cytometry dot plot from each group. The MFI of CD86

expression on $CD19^+$B cells stimulated by IgD **(G)**. Data were expressed as mean ± SEM (n=4). $^{**}P < 0.01$ *vs.* Control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ *vs.* T-B cells and IgD (3μg/mL) group and IgD (10μg/mL) stimulation group.

Fig 6. Effects of IgD-Fc-Ig (DG) on CD40, CD40L and IgD expressions on spleens of CIA mice *in vivo*. CIA mice were treated with IgD-Fc-Ig, IgG_1-Fc, rhTNFR:Fc and anti-mouse IgD antibody by tail intravenous administration. Immunohistochemistry analysis of CD40 **(A)**, CD40L **(B)** and IgD **(C)** expressions on spleens of CIA mice. Data were expressed as mean ± SEM (For each group 5 mice). $^{**}P<0.01$ *vs.* Normal, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ *vs.* Model.

Fig 7. Effects of IgD-Fc-Ig (DG) on the $CD4^+$T cells and $CD19^+$B cells actions in CIA mice *in vitro*. Obtained $CD4^+$T cells and $CD19^+$B cells from spleens of CIA mice, $CD4^+$T cells were incubated with IgD(3μg/mL), DG (3, 10μg/mL), 0.5μM Lck inhibitor A770041 and IgG_1-Fc (10μg/mL) co-cultured with $CD19^+$ B cells *in vitro* for 48h. Western blot analysis of CD40L **(A)** and CD40 **(B)** expressions on $CD4^+$T cells and $CD19^+$B cells from spleens of CIA mice. After co-cultured, obtained $CD4^+$T cells and $CD19^+$B cells and lysed. **(C)** ELISA analysis of IL-17A expression in $CD4^+$ T cellular supernatant of CIA mice. Data were expressed as mean ± SEM (Both normal group and CIA group3-5 mice). $^{**}P<0.01$ *vs.* Control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ *vs.* IgD (3μg/mL) stimulation group.

Fig 8. The hypothetical schematic diagram of IgD-Fc-Ig mechanism. IgD induces the abnormal activation of T cells through affecting IgDR-Lck-ZAP70-PI3K-NF-κB signaling on T cells, which can up-regulate the expression of CD40L on $CD4^+$T cells. The co-cultivation of IgD-activated T cells can up-regulate the expressions of co-stimulatory molecules CD40-CD40L, then induce the activation of B cells. IgD-Fc-Ig could down-regulate CD86 expression and IL-17A level, decrease CD40 and CD40L expression. IgD-Fc-Ig restrains the activations of T cells through inhibiting IgD-IgDR-Lck-ZAP70-PI3K-NF-κB signaling, thus inhibiting the activation of B cells.