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A tetrameric form of CD40 ligand with potent biological activities in both mouse and human primary B cells

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ABSTRACT

CD40 ligand (CD40 L) expressed by activated T cells interacts with CD40 on B cells and triggers B cell survival, proliferation and differentiation. Deficiency in CD40 L or CD40 in humans causes hyper IgM syndrome due to a defect in T-B interaction that is essential for Ig gene class switch recombination (CSR). CD40 L belongs to the tumor necrosis factor family and normally forms a homotrimer on the cell surface, which is important for its biological activity. To generate a multimeric CD40 L that can be used to stimulate both mouse and human B cells, we fused the extracellular domain of mouse CD40 L, which is known to also bind human CD40, with streptavidin (SA) that forms a stable tetramer under physiological conditions. As expected, 293 T cells transiently transfected with an SA-CD40 L expression vector secreted tetrameric SA-CD40 L in the culture supernatant. The secreted SA-CD40 L exhibited > 25-fold stronger activities in inducing the survival, activation and proliferation of both mouse and human primary B cells than did an agonistic anti-mouse or anti-human CD40 antibody. In the presence of IL-4, SA-CD40 L also induced efficient CSR and plasma cell differentiation in both mouse and human B cells. Moreover, administration of SA-CD40 L in mice induced activation and proliferation of spleen B cells *in vivo*. These results demonstrate that the SA-CD40 L fusion protein generated in the present study recapitulates the function of membrane-bound trimeric CD40 L and has potent biological activities in both mouse and human primary B cells.

1. Introduction

CD40 ligand (CD40 L), also known as CD154, gp39, TRAP and TNFSF5, is a type II transmembrane protein that belongs to the tumor necrosis factor (TNF) superfamily. CD40 L was first identified on activated T cells (Armitage et al., 1992) and is also expressed by NK cells, basophils, eosinophils, monocytes and platelets (Andre et al., 2002; Carbone et al., 1997; Pinchuk et al., 1996). The murine CD40 L cDNA predicts a polypeptide of 260 amino acids (aa) consisting of a 22-aa cytoplasmic domain, a 24-aa transmembrane domain, and a 214-aa extracellular domain with four cysteines. The extracellular domain of mouse CD40 L shares 75% sequence identity with human CD40 L (Spriggs et al., 1992). Previous studies have revealed that mouse CD40 L can bind human CD40 and activate human cells expressing CD40 (Lane et al., 1993). Like other TNF family members, CD40 L normally forms a homotrimer on the cell surface. The membrane bound homotrimeric CD40 L can be cleaved by the TNFα-converting enzyme (TACE) and secreted as a homotrimeric soluble CD40 L (Black et al., 1997; Contin et al., 2003). CD40 L expressed by activated T cells interacts with CD40 on B cells and mediates B cell survival, activation, proliferation and differentiation (Arpin et al., 1995; Banchereau and Rousset, 1991; Majlessi and Bordenave, 2001). Deficiency in CD40 L or CD40 in humans and mice causes X-linked hyper-IgM immunodeficiency syndrome and common variable immunodeficiency, respectively, manifesting as an inability to produce IgG, IgA, and IgE immunoglobulin isotypes that are needed for an effective humoral response (Aruffo et al., 1993; By Blair et al., 1994; van Kooten and Banchereau, 2000).

Previous studies have shown that monomeric CD40 L is biologically less active than membrane-bound CD40 L, and its biological activity increases with a higher multimeric organization (Mazzei et al., 1995; Morris et al., 1999). Hence various approaches have been used to develop different forms of recombinant multimeric CD40 L, including incorporation of an isoleucine zipper motif, hexameric formation by

Abbreviations: CD40L, CD40 ligand; CSR, class switch recombination; SA, streptavidin; SP, signal peptide; CBB, coomassie brilliant blue; GC, germinal center

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linking CD40 L to collagen domain of ACRP30/adiponectin, or dodecameric production with the lung surfactant protein-D (SP-D) (Gupta et al., 2015; Haswell et al., 2001; Holler et al., 2003; Morris et al., 1999). The isoleucine zipper or the natural stalk domain did increase CD40 L bioactivity by stabilizing the multimer structure (Morris et al., 1999). At present, anti-CD40 antibody (Ab), recombinant monomeric CD40 L, with or without a crosslinking Ab, and CD40L-expressing feeder cells are commonly used in immunological experiments for triggering CD40 on B cells (Ivanov et al., 2005; Kofod-Olsen et al., 2016; Liu et al., 2017; Naito et al., 2013). However, the commercially available anti-CD40 Ab and monomeric recombinant CD40 L are biologically less active than CD40 L trimers (Fischer et al., 2001). While CD40L-expressing feeder cells can effectively support B cell long-term culture, these cells need to be precultured to form a monolayer and irradiated to inhibit their overgrowth before use. A soluble, multimeric CD40 L with strong biological activities will greatly facilitate the analysis of T cell dependent B cell function.

Streptavidin (SA) is a homotetrameric protein expressed in *streptomyces avidinii* and has remarkably high binding affinity for a small water-soluble vitamin, biotin. SA-biotin system is a powerful tool in biological and immunological researches. Using its homotetrameric characteristics, a partner protein could be fused to SA to form a tetramer. A few of SA-conjugated tetramers have been reported, such as SA–Ag and SA-IL-4 fusion proteins (Stanek et al., 2012; Zhang et al., 2012), and have stronger biological activity than their natural, monomeric forms. In the present study, we have generated a soluble SA-CD40 L tetramer fusion protein and evaluated its *in vitro* and *in vivo* biological activities in both mouse and human primary B cells. Our results demonstrate that SA-CD40 L could form a stable tetramer and has a much stronger activity in inducing B cell survival, activation, proliferation and Ig gene class switch recombination (CSR) in both mouse and human B cells than did agonistic anti-mouse or anti–human CD40 Ab. Our SA-CD40 L can be widely used in studying T cell-dependent B cell function in both mice and humans.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from Shanghai Laboratory Animal Corporation and maintained in specific pathogen free conditions. All animal experiments were approved by Fudan University Animal Committee. In addition, all methods were performed in accordance with the guidelines and regulations of Fudan University. Mice of 8–10 weeks old were used for all the experiments.

2.2. Human blood samples

Adult peripheral blood samples were obtained from healthy volunteers after obtaining informed consent, and all the experiments were approved by the ethics committee of Fudan University (approval number 103).

2.3. Construction of SA-CD40L expression vector

The extracellular domain of murine CD40 L was amplified using CD40L-specific primers and the first strand cDNA generated from activated mouse T cells as a template. The gene encoding core SA was amplified using the genomic DNA of *S. avidinii* as a template. The signal peptide (SP) of human IL-4 was amplified using the first strand cDNA of human peripheral blood lymphocytes. The high fidelity KOD-plus DNA polymerase (Toyobo, Japan) was used in all PCR reactions. The following forward and reverse primers were used in PCR: mouse CD40 L, 5′-CGGGATCCCAAATTGCAGCACACGTTG-3′, which included a BamHI site, and 5′-GCTCGAGTCAGAGTTTGAGTAAGCCAAA-3′, which included a stop codon and a SacI site; SA, 5′-CGGGATCCGACCCCTCCA AGGACTCGAAG-3′ and 5′-GCCCTAGGCTGCTGAACGGCGTCGAG CGG-3′, both of them included a BamHI site; SP, 5′−CCAAGCTTATG GGTCTCACCTCCCAAC-3′, which included a HindIII site, and 5′-GCCC TAGTTCCGTGGACAAAGTTGCC-3′, which included a BamHI site. The amplification was carried out for 30 cycles at 94 °C for 15 s, annealing temperature for 30 s, and 68 °C for 30 s. The annealing temperature was 65 °C for CD40 L and SP, and 68 °C for SA. Each PCR product was subcloned into a T vector for sequencing. CD40 L, SP and SA were then sequentially cloned into the eukaryotic expression vector pcDNA3.0 using restriction sites mentioned above. The plasmid DNA was purified with an endotoxin-free plasmid purification kit (Gene Mark) according to the manufacturer's instructions.

2.4. Expression of SA-CD40L fusion protein

293 T cells in a 10 cm dish were transfected with pcDNA3.0-SA-CD40 L plasmid (8 μg) using Lipofectamine 2000 transfection kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Empty pcDNA3.0 vector was used as a negative control. Transfected 293 T cells were cultured in serum-free medium and the supernatant was collected 3 days after the transfection. The expression of SA-CD40 L fusion protein in culture supernatants was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by coomassie brilliant blue (CBB) staining or immunoblotting.

2.5. CBB staining and immunoblot

The culture supernatant was mixed with 1/5 vol of non-reducing sample loading buffer (6x) and the proteins were resolved by SDS-PAGE (10% separating gel). An anti-CD40 monoclonal Ab (eBioscience) was used as a standard for estimating the amount of SA-CD40 L fusion protein by CBB staining. For immunoblot analysis, proteins in the supernatants were resolved by SDS-PAGE under non-reducing conditions and transferred onto a polyvinylidene difluoride (PVDF) membrane in a transfer apparatus. The membrane was first blocked in TBS containing 0.5% Tween-20 (TBST) and 5% nonfat milk at 37 ℃ for one hour and then incubated in biotinylated anti-human Ab (PeproTech) at 1:2000 dilution in the blocking buffer at 4 ℃ overnight. The membrane was then washed with TBST three times (10 min each) and incubated with HRP-conjugated anti-rabbit IgG (SouthernBiotech) at 1:5000 dilution at 37 °C for 1 h. After washing three times (10 min each) in TBST, the proteins were detected using the enhanced chemiluminescence detection system and Luminescent Image Analyzer LAS-4000 mini (Fujifilm).

2.6. B cell enrichment

Mouse primary B cells were isolated from the spleen of C57BL/6 mice using mouse B lymphocyte enrichment set (BD Biosciences) according to the protocol of the manufacturer. The purity of B cells was > 95% as judged by their B220 expression. Naïve human B cells were purified from human peripheral blood mononuclear cells (PBMC), which were isolated with the use of ficoll (TBD science) density gradient centrifugation. Briefly, PBMC were incubated with a biotin-conjugated anti-human IgG Ab (BD Biosciences) and biotinylated Ab cocktail of human naïve B cell isolation kit (Biolegend) for 15 min, and the cells were washed and incubated with streptavidin nanobeads. Finally, purified cells were analyzed for B cell purity by flow cytometry and usually > 95% of the cells were $CD19^+CD27^-IgG^-$ naïve B cells.

2.7. Culture of mouse and human B cells

Purified B cells were cultured in RPMI 1640 containing 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin, supplemented with 10% heat-inactivated fetal bovine serum (all from Thermo Fisher Scientific). SA-CD40 L (0.1 μg/ml), anti-human (2.5 μg/ml; BD Biosciences) or -mouse CD40

(2.5 μg/ml; BD Biosciences) Ab (Joo et al., 2017; Meednu et al., 2016; Zhang et al., 2016), and recombinant human (25 ng/ml; R&D) or mouse (20 ng/ml; R&D) IL-4 were freshly added. Mouse B cells were seeded at 5×10^5 cells/ml density in 24-well plates in a final volume of 0.5 ml while human naïve B cells were seeded at 4×10^5 cells/ml density in 96-well plates in a final volume of 0.2 ml. All the cells were cultured at 37°C in humidified air and 5% CO_2. On day 3, half of the medium was removed and replaced with medium supplemented with fresh SA-CD40 L, anti-human or -mouse CD40 Ab, and IL-4. As a negative control of SA-CD40 L, cells were stimulated with culture supernatants of 293 T cells transfected with pcDNA3.0 empty vector. B cells were cultured for two days for survival and activation assays, three days for proliferation assay, four or six days for CSR and antibody secretion assays. B cell viability was determined by 7-amino-actinomycin D (7-AAD) staining followed by FACS analysis.

2.8. Antibodies and flow cytometry

Anti-mouse B220-APC (clone RA3-6B2), anti-mouse CD86-APC (clone GL1), anti-mouse CD40-APC (clone 3/23), anti-mouse IcosL-PE (clone HK5.3), anti-mouse IgG_1-FITC (clone A58-1), anti-mouse GL7-FITC (clone GL7), anti-human HLA-DR-PE (clone G46-6), anti-human CD80-FITC (clone L307.4), anti-human CD86-PE (clone 2331 FUN-1), anti-human CD40-PE (clone 5C3) and anti-human IgG-PE-Cy7 (clone G18-145) were purchased from BD Biosciences; anti-mouse MHC class II-FITC (clone AF6-120.1), anti-mouse Fas-PE (clone 15 A7) and anti-mouse CD80-PE (clone 16-10A1) were purchased from eBioscience; anti-human CD19-APC (clone HIB19); anti-human CD20-FITC (clone 2H7); anti-human CD38-PE-Cy7 (clone HIT2) and 7AAD were purchased from Biolegend. Cultured B cells were washed with cold PBS containing 2% FBS and incubated with diluted Abs at 4°C for 15 min. The cells were then washed twice with cold PBS containing 2% FBS, resuspended in 0.3 ml of PBS containing 2% FBS and analyzed with a FACSVerse flow cytometer using the FACSuite software.

2.9. Analysis of B cell proliferation

To examine cell division induced by SA-CD40 L, B cells were labeled with the fluorescent proliferation marker carboxyfluorescein succinimidyl ester (CFSE). Briefly, B cells (1×10^6 cells/ml) were stained in serum-free RPMI 1640 containing 1 μM of CFSE for 10 min in the dark. The reaction was stopped by addition of 8 ml of RPMI 1640 containing 10% FBS. The labeled B cells were washed and then cultured under various conditions. B cell proliferation was evaluated 3 days later by analyzing CFSE intensity.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The amount of mouse and human IgG in the culture supernatants were measured by standard ELISA. Briefly, 96-well ELISA plates (Costar) were coated with goat anti-human or -mouse Ig (Southern Biotech) in PBS at 4°C overnight, and then blocked with 1% BSA blocking buffer at room temperature for 1 h. The culture supernatants and serially diluted standards were then added and incubated at room temperature for 2 h. After washing, biotin-conjugated anti-mouse IgG_1 (Southern Biotech) or anti-human IgG (Southern Biotech) Ab was added and incubated at room temperature for 2 h. After washing, avidin-HRP (Biolegend) (1:1000 dilution) was added to the plates, incubated for 30 min and visualized with TMB substrate reagents (Invitrogen). OD_{450}-OD_{570} was measured by a spectrophotometer (Bio-Rad). The amount of Ig in duplicate wells was calculated based on the standard.

2.11. Effects of SA-CD40 L in vivo

C57BL/6 mice (8–10 weeks old) were injected *i.p.* with culture supernatants containing 1 μg of SA-CD40 L every three days for two weeks. As a control, mice were injected with the same volume of the culture supernatants of 293 T cells transfected with pcDNA3.0 empty vector. Mice were then euthanized and the spleens were analyzed for their weight, cellularity, the expression of co-stimulatory molecules in B cells and the proportion of the germinal center (GC) B cells.

2.12. Statistical analysis

Data obtained from flow cytometry were analyzed with FlowJo 7.6 Software, exported to Excel spreadsheets and subsequently analyzed with GraphPad Prism 6. Results are presented as mean ± standard deviation (SD). Statistical analysis of the data was performed with Student *t* test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Generation of SA-CD40L fusion protein

To generate soluble tetrameric SA-CD40 L fusion protein, we used the core SA, which forms a tetramer under physiological conditions, as a fusion partner of CD40 L. We chose human IL-4 SP to direct SA-CD40 L fusion protein secretion. To prevent proteolytic digestion of CD40 L by metalloproteases, we used the extracellular domain of murine CD40 L but did not include its natural cleavage site. The molecular structure of the SA-CD40 L is illustrated in Fig. 1A.

293 T cells were transfected with pcDNA3.0-SA-CD40 L and cultured in serum-free medium for 3 days. A non-reducing SDS-PAGE analysis revealed an expected 136 kDa band, which corresponded to the tetrameric SA-CD40 L, in the supernatants of 293 T cells transfected with SA-CD40 L (lane 2, Fig. 1B) but not the empty vector (lane 1, Fig. 1B). Immunoblot analysis confirmed the production of the tetrameric SA-CD40 L only in 293 T cells transfected with SA-CD40 L (Fig. 1C). Monomeric band (34 kDa) was not observed both by CBB staining and by immunoblot. To estimate the amount of SA-CD40 L in the culture supernatants, we stained the gel with CBB and used a commercial anti-CD40 Ab as a standard (Fig. 1D). By comparing the band intensities of the anti-CD40 Ab and SA-CD40 L, the concentration of SA-CD40 L in the supernatants was estimated to be 0.91 μg/ml (Fig. 1E). To quickly evaluate the biological activity of the SA-CD40 L, we stimulated mouse B cells with increasing doses of the SA-CD40 L. As shown in Fig. 1F, SA-CD40 L at 0.09, 0.18 and 0.36 μg/ml all induced a significant increase in B cell viability. These results indicate that the SA-CD40 L fusion protein formed a tetramer and was able to enhance the survival of mouse B cells.

3.2. Biological activities of SA-CD40L

CD40 L induces B cell survival, activation and proliferation. To analyze the biological activities of the tetrameric SA-CD40 L, we first cultured purified mouse spleen B cells and human peripheral blood naïve B cells in the presence of 0.1 μg/ml SA-CD40 L, or 2.5 μg/ml of the agonistic anti-mouse or anti-human CD40 Ab which were considered to be an optimal concentration in previous studies (Joo et al., 2017; Meednu et al., 2016; Zhang et al., 2016). Two days later, we analyzed B cell survival and activation. The viability of both mouse and human B cells was significantly enhanced in the presence of SA-CD40 L as compared with cells cultured in the presence of anti-mouse or anti-human CD40 Ab (Fig. 2A–B). In addition, SA-CD40 L was much more effective than the anti-mouse or anti-human CD40 Ab in inducing B cells to form clusters, an indicator for B cell activation (Fig. 3A). The size of the clusters (Fig. 3B, left panels) was significantly increased in both mouse and human B cells cultured in the presence of SA-CD40 L relative to those cultured with the anti-mouse or anti-human CD40 Ab. Consistently, the forward scatter (FSC), which reflects the cell size, was also significantly increased in mouse and human B cells cultured in the

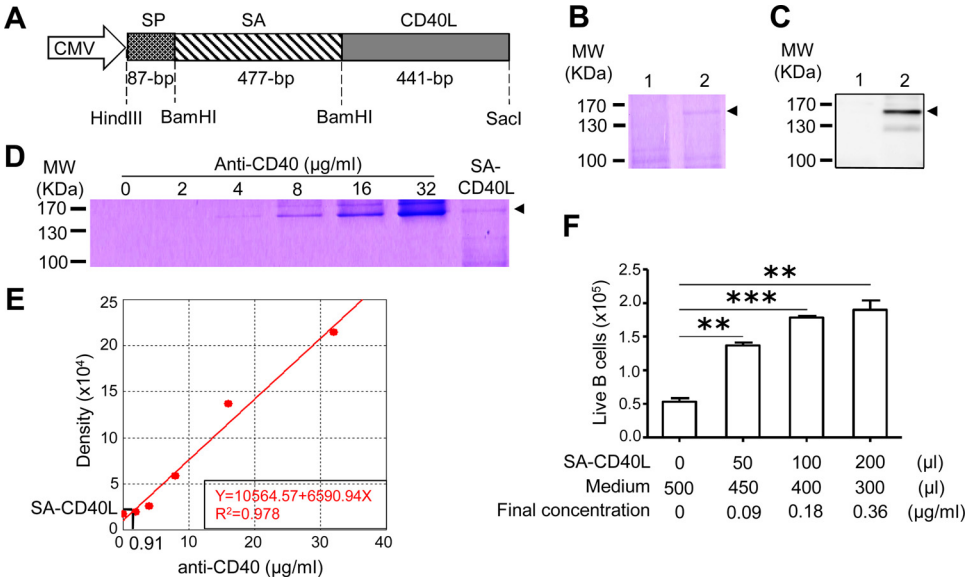

Fig. 1. Generation of a soluble SA-CD40L tetrameric molecule. (A) Schematic structure of SA-CD40 L. SP, signal peptide derived from human IL-4; SA, streptavidin core domain; CD40 L, mouse CD40 L extracellular domain with the proteolytic site (EM) being deleted. **(B–C)** Culture supernatants of 293 T cells transfected with pcDNA3.0 empty vector or pcDNA3.0-SA-CD40 L were analyzed by CBB staining **(B)** and immunoblot **(C)** under non-denaturing conditions. Lane 1, empty vector; lane 2, SA-CD40 L. Arrows indicate tetrameric SA-CD40 L protein band. **(D)** Serially diluted commercial anti-human CD40 Ab and SA-CD40 L culture supernatants were analyzed *via* CBB staining. **(E)** Estimation of the amount of SA-CD40 L protein in the culture supernatant using an anti-CD40 Ab as a standard. **(F)** Purified mouse B cells were stimulated with different doses of SA-CD40 L for 2 days and analyzed for cell viability. Live B cells were enumerated by trypan blue exclusion. The error bars represent mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$ (unpaired *t*-test).

presence of SA-CD40 L (Fig. 3B, right panels). An additional indicator for B cell activation is the upregulation of costimulatory molecules (Oberwalleney et al., 2000). As shown in Fig. 4, SA-CD40 L together with IL-4, but not control supernatant + IL-4, induced upregulation of MHC class II, CD80, CD86 and CD40 expression in both mouse and human B cells.

We further analyzed the effect of SA-CD40 L on B cell proliferation. SA-CD40 L alone induced a significant (Fig. 5A) and moderate (Fig. 5C) reduction in CFSE intensity in mouse and human B cells, respectively, than did anti-mouse or anti-human CD40 Ab. In the presence of IL-4, SA-CD40 L induced a more dramatic reduction in CFSE intensity in both mouse (Fig. 5B) and human (Fig. 5D) B cells than did anti-CD40 Abs. Taking into consideration that the concentration of SA-CD40 L (0.1 μg/ml) was much lower than that of anti-CD40 Abs (2.5 μg/ml), these results collectively demonstrate that tetrameric SA-CD40 L exhibited > 25-fold stronger activities than the anti-mouse or anti-human CD40 Ab in inducing B cell survival, activation and proliferation.

3.3. SA-CD40L induces efficient CSR in both mouse and human B cells

CD40L-CD40 interaction has been shown to be essential for the induction of Ig gene CSR (Fluckiger et al., 1998; Fuleihan et al., 1993). In the presence of IL-4, CD40 L induces naïve B cells to switch from IgM to IgG_1 in mice and IgM to IgG in humans. As shown in Fig. 6, SA-CD40L + mIL-4 induced an increased proportion of the IgG_1^+ population (Fig. 6 A–B), concomitant with the induction of the $B220^{dull}CD138^+$ plasma cells (Fig. 6C) and the secretion of the IgG_1 Ab in the culture supernatants (Fig. 6D). These observations suggest that some of the IgG_1 class-switched cells further differentiated into antibody-secreting plasma cells. Similarly, SA-CD40 L induced the switching to IgG in human B cells (Fig. 6E) and increased proportion of the $CD20^-CD38^+$ plasma cells (Fig. 6F), and the secretion of IgG Abs in the culture supernatant (Fig. 6G). These results indicate that SA-CD40 L in the presence of IL-4 is able to induce Ig gene CSR and the differentiation into plasma cells in both mouse and human B cells.

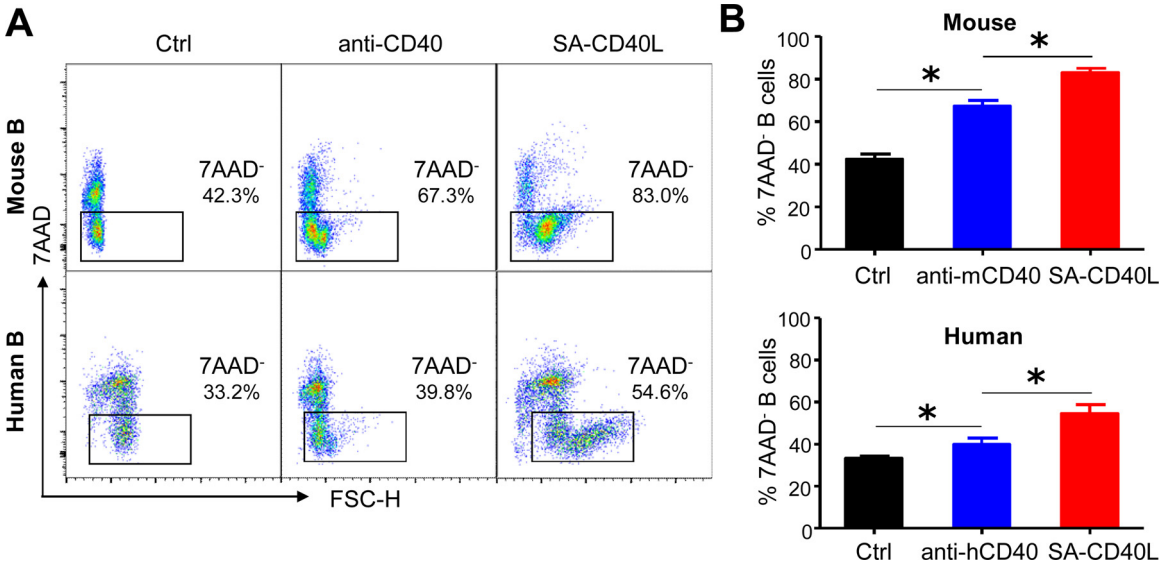

Fig. 2. SA-CD40L induces the survival of both mouse and human primary B cells. (A) Mouse and human B cells were cultured in the presence of control supernatants (Ctrl), anti-mouse CD40 (anti-mCD40) or anti-human CD40 Ab (anti-hCD40), or SA-CD40L for 2 days, stained with 7AAD and analyzed for $7AAD^-$ B cells by flow cytometry. **(B)** Mean ± SD of three independent experiments. $^*p < 0.05$ (unpaired *t*-test).

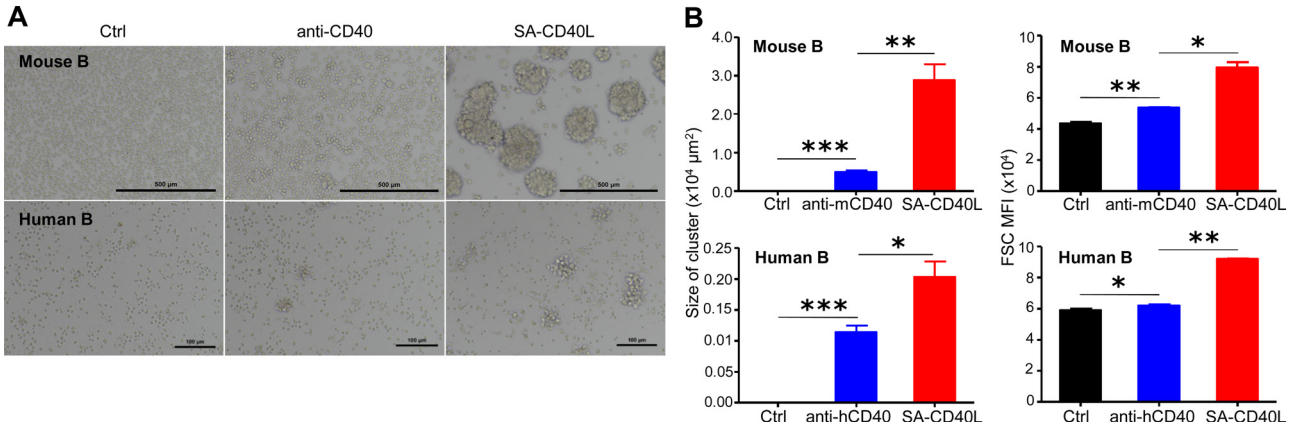

Fig. 3. SA-CD40 L induces the activation of both mouse and human primary B cells. Purified B cells were cultured for 2 days and analyzed for their cluster formation and cell sizes. **(A)** Representative images showing clustering of B cells. **(B)** Average size of B cell clusters (left panels) and the size of single B cells (right panels). Mean ± SD of 3 independent experiments are shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (unpaired *t*-test).

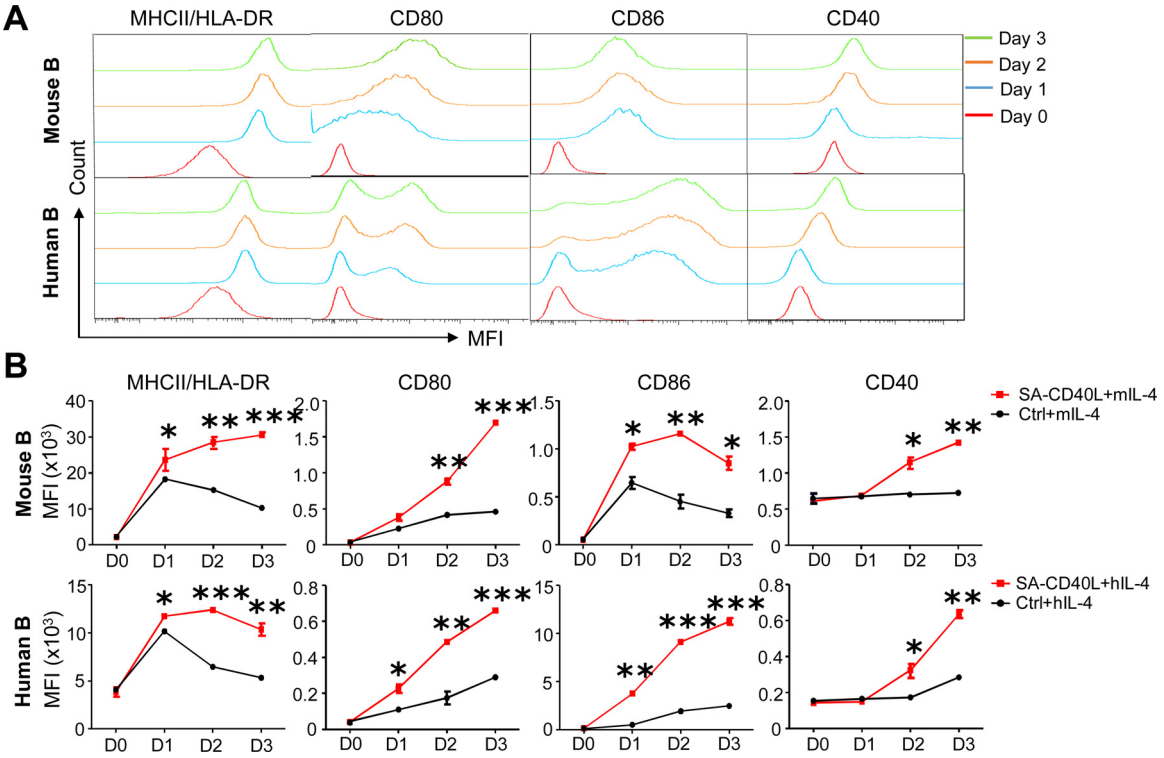

Fig. 4. SA-CD40 L induces upregulation of co-stimulatory molecules in both mouse and human primary B cells. Mouse and human B cells were stimulated with SA-CD40 L + IL-4 or control supernatants + IL-4 for 1–3 days. **(A)** Representative FACS profiles showing the expression of MHCII/HLA-DR, CD80, CD86 and CD40. **(B)** Mean ± SD of three independent experiments. *$p < 0.05$; **$P < 0.01$; ***$P < 0.001$ (unpaired *t*-test).

3.4. SA-CD40L is functional in vivo

Having shown that SA-CD40 L has potent biological activities under *in vitro* culture conditions, we next examined whether the SA-CD40 L is functional *in vivo*. We injected 1 μg of SA-CD40 L *i.p.* into C57BL/6 mouse every three days for two weeks and then analyzed the phenotypes of spleen B cells. We first noticed that the weight of the spleen was moderately but statistically significantly increased in mice received SA-CD40 L as compared to those received control supernatant (Fig. 7A–B). In addition, the total number of B cells in the spleen was also significantly increased (Fig. 7C). Furthermore, B cells from SA-CD40L-treated mice had increased expression of several costimulatory molecules, including CD80, CD86, MHC class II and IcosL (Fig. 7D). In contrast, the proportion of the GC B cells was not significantly affected by administration of SA-CD40 L (Fig. 7E-F). Taken together, these results demonstrate that SA-CD40 L induced B cell activation (as evidenced by increased levels of costimulatory molecules) and proliferation *in vivo* but did not trigger GC formation.

4. Discussion

CD40L plays critical roles in the survival, activation, proliferation and differentiation of B cells (D'Orlando et al., 2007; Kehry, 1996). CD40 L forms a trimer on the cell surface naturally, and CD40 ligand clustering is required to induce clustering of CD40 on B lymphocytes, which triggers efficient CD40 signaling (Grassme et al., 2002). Many of the earlier studies employed monoclonal anti-CD40 IgG to mimic the function of CD40 L (Bishop and Hostager, 2001). While agonistic anti-CD40 Ab has potent activity, the commercially available low endotoxin and azide-free anti-CD40 Abs are quite expensive. Monomeric soluble

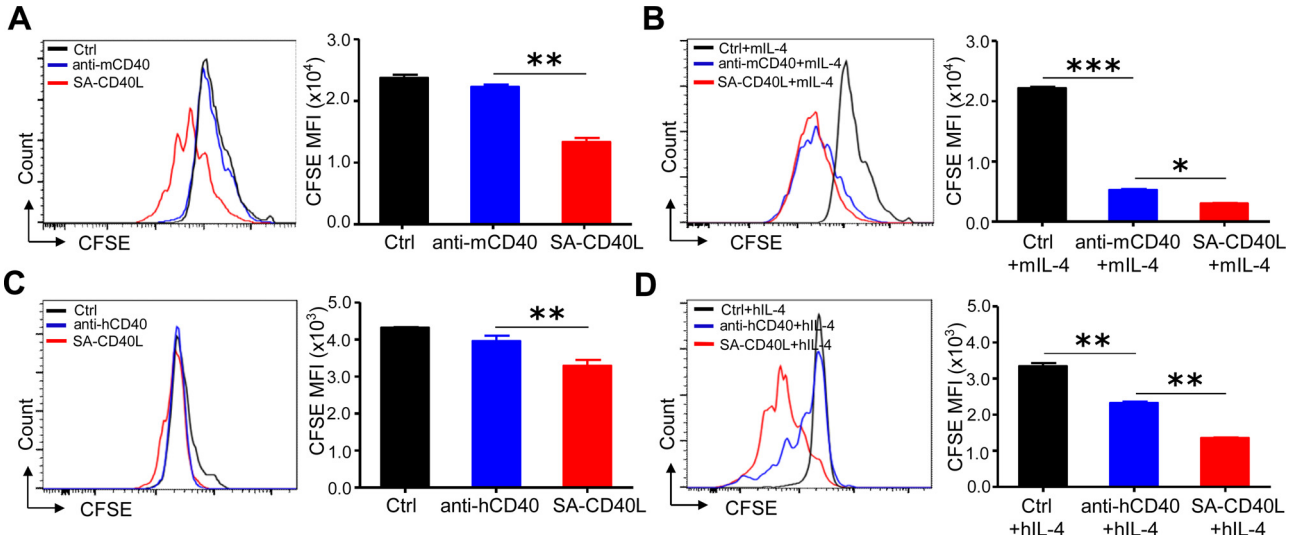

Fig. 5. SA-CD40 L induces vigorous B cell proliferation. Mouse **(A–B)** and human **(C–D)** primary B cells were stimulated with control supernatants (Ctrl), anti-CD40 Abs or SA-CD40 L in the absence **(A and C)** or presence **(B and D)** of IL-4 and analyzed for CFSE dilution. For each panel, left, representative CFSE profile; right, Mean ± SD of 3 independent experiments. $*p < 0.05$; $**P < 0.01$; $***P < 0.001$ (unpaired *t*-test).

CD40L-CD8 chimeric molecule has also been used but needs to be crosslinked by anti-CD8 Ab to increase its activity (Garcia-Marquez et al., 2014; Haswell et al., 2001). To solve these problems, intense efforts have been made to produce CD40 L molecules with higher order of structures. Several higher-ordered CD40 L have been generated that include trimeric recombinant human CD40 L with a leucine zipper (He et al., 2006; Pullen et al., 1999) and hexameric and dodecameric soluble forms of CD40 L (Haswell et al., 2001).

SA is a homo-tetrameric protein and expected not only to facilitate the oligomerization of protein molecules, but also to provide structural durability under native conditions, both of which are required for improving biological activity of chimeric proteins. In the present study, we engineered an SA-CD40 L recombinant expression vector, which contained the tetramer-forming domains of SA. We also removed the natural cleavage site in the extracellular domain of CD40 L to prevent its proteolytic degradation by metalloproteases. Our results demonstrate that the vast majority of the fusion proteins formed tetramers, which exhibited much stronger biological activities than did anti-mouse and

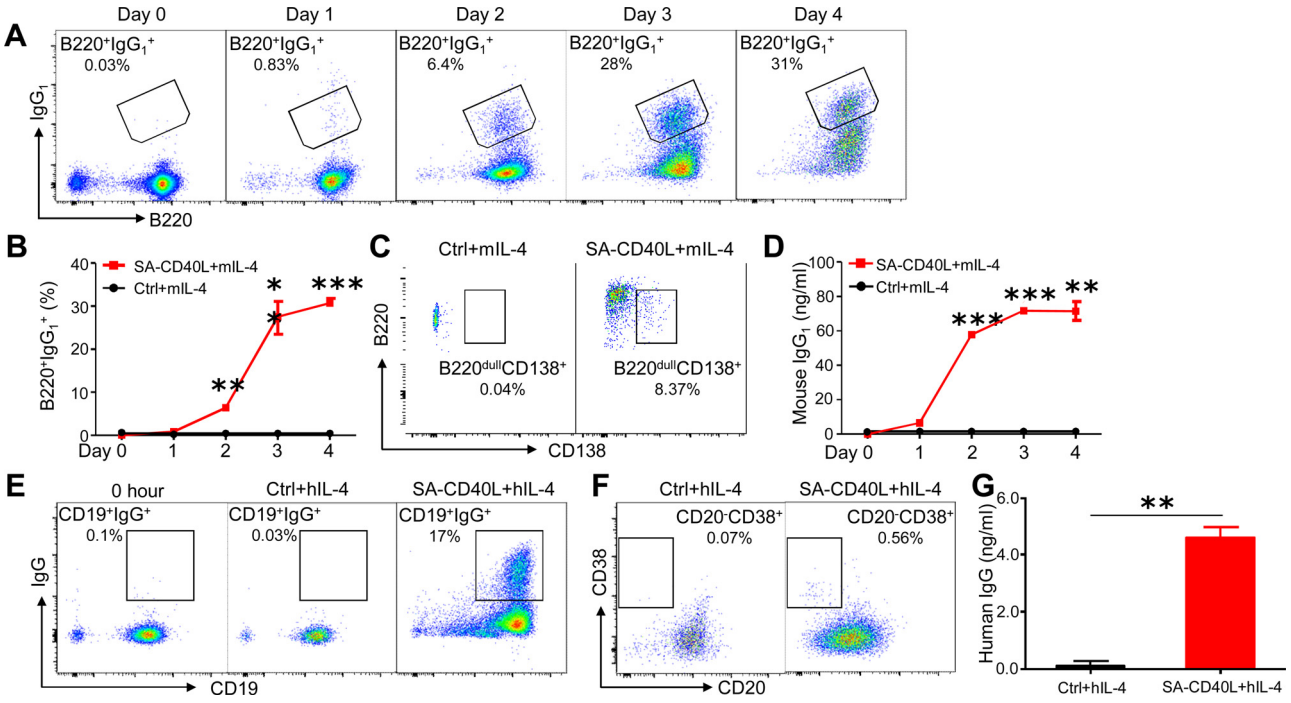

Fig. 6. SA-CD40 L + IL-4 induces CSR and plasma cell differentiation in both mouse and human primary B cells. (A) Mouse B cells were cultured for 4 days in the presence of SA-CD40 L + mIL-4 and analyzed for IgG_1^+ cells each day. Representative FACS profiles are shown. **(B)** Summary of 3 independent experiments shown in **(A)**. **(C)** Proportion of the $B220^{dull}CD138^+$ plasma cells in mouse B cells cultured for 4 days in the presence of SA-CD40 L + mIL-4. **(D)** The concentration of mouse IgG_1 in the culture supernatants was determined by ELISA. **(E)** FACS profiles of human naïve B cells before culture (left panel), or cultured for 6 days in the presence of control supernatants + hIL-4 (middle panel) or SA-CD40 L + hIL-4 (right panel). **(F)** Proportion of the $CD20^-CD38^+$ plasma cells in human B cells cultured for 6 days in the presence of control supernatants + hIL-4 (left) or SA-CD40 L + hIL-4 (right). **(G)** The concentration of human IgG in the culture supernatants was determined by ELISA on day six. Mean ± SD of 2 independent experiments are shown. $**p < 0.01$; $***P < 0.001$ (unpaired *t*-test).

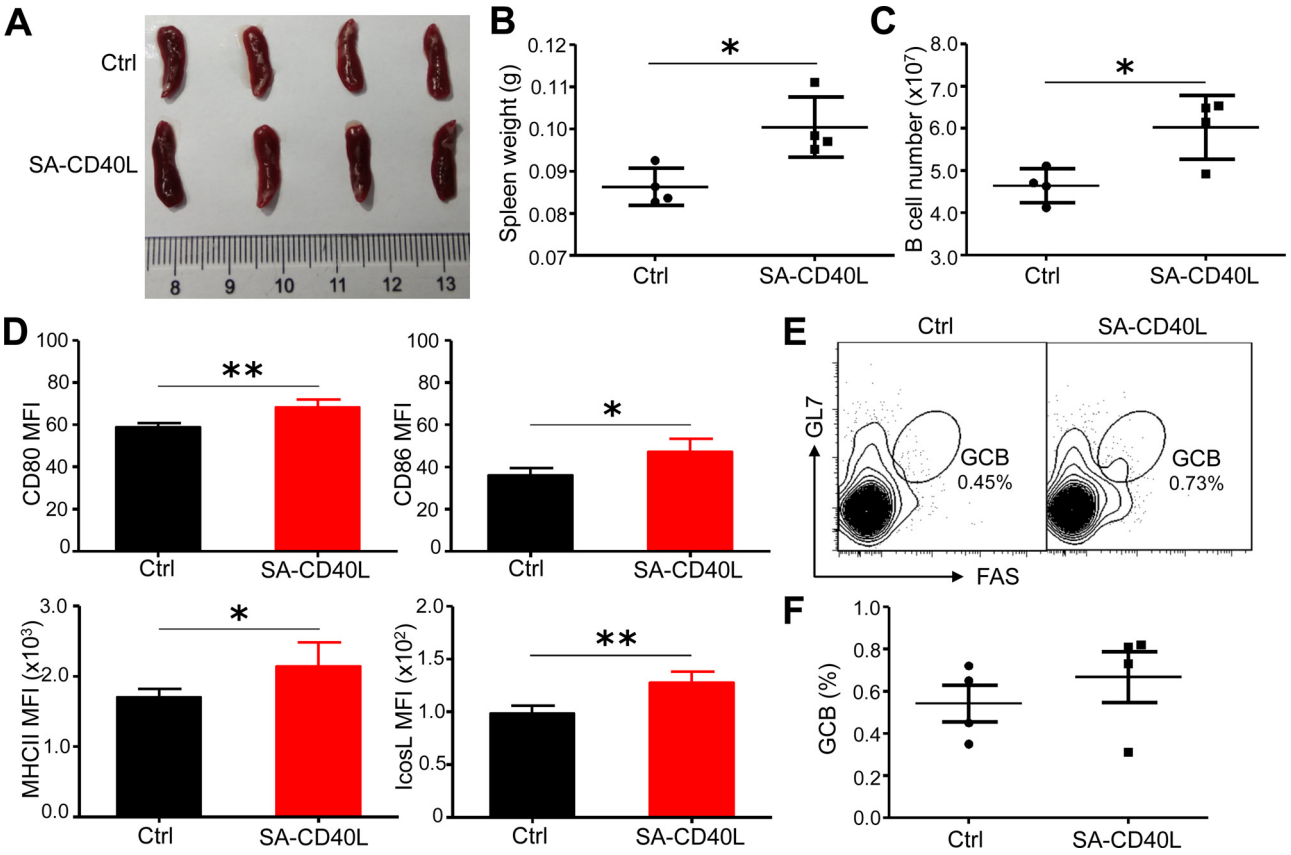

Fig. 7. SA-CD40 L induces B cell activation and expansion *in vivo*. Mice were injected *i.p.* with 1 μg of SA-CD40 L every three days for two weeks and their spleens were analyzed. **(A)** Spleens from mice received control (upper) or SA-CD40 L (lower) supernatants. **(B)** Weight of spleen from control and SA-CD40L-injected group ($n = 4$). **(C)** Total B cell numbers in spleen from control and SA-CD40L-injected group ($n = 4$). **(D)** The levels of CD80, CD86, MHC class II and IcosL in spleen B cells from mice received control or SA-CD40 L. **(E)** and **(F)** Proportion of the GC B cells ($GL7^+FAS^+$) among $B220^+$ cells. **(E)** Representative FACS profiles. **(F)** Mean ± SD of the percentages of GC B. $^*p < 0.05$; $^{**}P < 0.01$ (unpaired *t*-test).

anti-CD40 Ab in terms of inducing B cell survival, activation, proliferation and differentiation.

The aa sequence indicates that mouse CD40 L exhibits 75% amino acid identity with human counterpart in the extracellular region (Spriggs et al., 1992). A previous study demonstrated that a soluble recombinant human CD40 L was effective in inducing co-stimulatory molecule expression on human B cells without concomitant increase on mouse B cells (Garcia-Marquez et al., 2014). Unlike the human CD40 L that shows species specificity, the mouse CD40 L has the cross-species activity and can interact with both mouse and human CD40 to trigger effective signaling (Hostager et al., 1996). The CD40 L of SA-CD40 L was of mouse origin and indeed the SA-CD40 L produced in the present study exhibited strong activity for both mouse and human primary B cells. Furthermore, the SA-CD40 L was active *in vivo* in inducing B cell activation and proliferation even only a very small amount (1 μg) was administered into C57BL/6 mouse for 5 times.

Humans lacking CD40 L suffers from hyper-IgM immunodeficiency syndrome due to an inability to produce IgG, IgA, and IgE immunoglobulins. CD40 L combined with specific cytokines, such as IL-4 and IL-10, can induce B cells to switch from IgM/IgD- to IgG_{1-4}, IgA_{1-2} or IgE (Hummelshoj et al., 2006). The tetrameric SA-CD40 L produced in the current study could induce efficient CSR in B cells in the presence of IL-4. Moreover, SA-CD40 L combined with IL-4 also promoted B cell differentiation into plasma cells, as judged by the increased amount of mouse IgG_1 and human IgG Abs in the culture supernatants of mouse and human B cells, respectively. Collectively, the SA-CD40 L generated in the present study has four important characteristics and advantages: 1) it has much stronger biological activities than anti-CD40 Abs; 2) it is active for both mouse and human B cells; 3) it is functional *in vivo*; 4) it recapitulates the known biological activities of membrane-type CD40 L. The SA-CD40 L might also have potential therapeutic application. Considering the significance of CD40/CD40 L in T-B interaction and B cell function, we expect that the SA-CD40 L generated in the present study will be widely used in both mouse and human studies.

Author contributions

J.Y.W designed the study, N.L. performed the most of the experiments, Q.M., E.X, J.L., L.Z. and S.Y. analyzed the data, and N.L. and J.Y.W. wrote the paper. All authors read and approved the final manuscript.

Conflict of interest

The authors have no financial conflicts of interest to declare.

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