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T cell *CD40LG* gene expression and the production of IgG by autologous B cells in systemic lupus erythematosus

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Abstract

CD40 ligand (CD40LG), encoded on the X chromosome, has been reported to be overexpressed on lupus Tcells. Herein, we investigated the effect of DNA demethylation on Tcell CD40LG expression and the production of IgG by autologous B cells in lupus. We found normal human T cells transfected with CD40LG induced autologous B cell activation and plasma cell differentiation. Both female lupus CD4+ T cells and demethylating agents treated CD4+ T cells overexpressed CD40LG mRNA. Further, lupus T cells from both genders or demethylated CD4+ T cells from healthy women overstimulated autologous B cells, and this could be reversed with anti-CD40LG Ab in only females. We demonstrated that female lupus CD4+ T cells and demethylated CD4+ T cells express high level of CD40LG and overstimulate B cells to produce IgG. This is due to DNA demethylation and thereby reactivation of the inactive X chromosome in female.

Keywords

CD40 ligand; DNA methylation; Immunoglobulin G; Systemic lupus; erythematosus

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by antibody production to a variety of nuclear autoantigens [1]. The disease has a strong gender bias with a female:male ratio of 9:1 [2,3]. Pathogenic autoantibodies synthesized by B lymphocytes are due to the activation of both B and T lymphocytes. One of the important T–B collaboration is the interaction of costimulatory receptor/ligand pairs, CD40–CD40LG.

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The interaction between CD40LG and CD40 is important for T cell-dependent immune response. CD40LG, encoded on X chromosome, is a type II membrane protein which belongs to the tumor necrosis factor family [4,5]. CD40LG is mainly expressed on the surface of activated CD4+ T cells and binds to CD40 on B cells to provide a necessary signal for initiation of immune response, including B cell activation, differentiation and production of pathogenic autoantibodies [6]. Elevated soluble CD40LG in sera and overexpressed CD40LG on T lymphocytes were observed in human and murine lupus [7–9]. Protein overexpression or gene activation may be caused by gene-specific DNA demethylation or hypomethylation [10]. In vitro, gene demethylation could be induced by DNA methyltransferase inhibitors such as 5-azaC and procainamide, or hydralazine and PD98059 which decrease DNA methyltransferase expression by inhibiting ERK pathway signaling [11–13]. Recently, we showed that CD40LG was demethylated on CD4+ T cells from women with lupus indicating the reactivation of the inactive X chromosome in female lupus [14]. Therefore, DNA demethylation reactivates *CD40LG* gene in the silenced X chromosome on T cells and overstimulates B cells to produce a large amount of autoantibodies. We hypothesize that the expression of CD40LG on CD4+ T cells may differ in SLE patients and normal subjects, in demethylating agents treated T cells and untreated normal cells, and overexpression of CD40LG may cause a high level of IgG production by T–B interaction. Furthermore, those differences may be different between women and men if the inactive X chromatin reactivation happened in CD4+ T cells from female lupus.

In the present study, we transfected normal T cells with CD40LG to induce autologous B cell activation and plasma cell differentiation in vitro. Expression of CD40LG mRNA on CD4+ and CD8+ T cells from patients with lupus were detected. We also measured IgG production by coculturing autologous B cells with T cells from lupus patients with or without CD40LG blockage by anti-CD40LG antibody. Similar experiments were performed on T cells from healthy subjects with or without treatment of DNA methylation inhibitors. The differences between the two genders were compared respectively.

Materials and methods

Subjects

The subjects recruited in this study include 15 lupus patients (6 women, 9 men; 23.2±5.27 years) and 11 age-, sex-matched healthy controls (6 women, 5 men; 26.9±3.83 years). All patients satisfied at least 4 criteria for lupus classification from the American College of Rheumatology and was assessed by disease activity based on SLE Disease Activity Index (SLEDAI) [15]. There was no difference in SLEDAI scores between female patients and male patients ($P>0.05$). Relevant clinical information is shown in Table 1. Another 6 healthy subjects (3 women, 3 men; ages 28.8±3.60 years) were enrolled for drug-treated studies, and normal healthy subjects were enrolled for T cell transfection experiments. All patients and controls enrolled in this study signed informed consent. This study was approved by the Human Ethics Committee of the Central South University Xiangya Medical College and the Institutional Review Boards at the University of Oklahoma Health Sciences Center and the Oklahoma Medical Research Foundation.

Cells preparation, purification and transfection

PBMC were obtained by density-gradient centrifugation. In lupus case, CD4+, CD8+ and B cell subsets were isolated by positive selection using magnetic beads (Miltenyi Biotec, Germany), the cells were then stimulated with 5 ng/ml PMA and 500 ng/ml ionomycin for 6 h. In drug-treated case, PBMC were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin and stimulated with 1 μg/ml of PHA (Sigma, USA) in 37 °C/5% CO2 for 16–24 h, then cultured in the presence of DNA methylation inhibitors

including 5-azaC (Aldrich, MO), procainamide (Aldrich, MO), hydralazine (Aldrich, MO), or PD98059 (Promega, USA) for 66 h and received the restimulation of 5 ng/ml PMA and 500 ng/ml ionomycin for additional 6 h. CD4+ and CD8+ subsets were purified and autologous B cells from original donors were separated by positive selection with magnetic beads (Miltenyi, Auburn Germany). For T cell transfection experiments, CD3+ T cells were isolated from normal healthy donors. PBMCs by negative selection (Miltenyi Biotec, CA) and cell purity checked by flow cytometry using conjugated anti-CD3 antibody. Autologous B cells were isolated by positive selection from each donor using the same blood sample. Human CD40LG cDNA (Mammalian Gene Collection, ATCC number 79815) was subcloned into the expression vector pEGFP-C1. T cells from a normal healthy female donor were transfected with pEGFP-C1/CD40LG using the nucleofection approach (Amaxa, Germany). Transfection efficiency was confirmed by flow cytometry for the GFP protein. Controls included T cells transfected with the empty vector pEGFP-C1.

Cell culture

T cell subsets (1×10^5, 100 μl) or drug-treated T cell subsets were cultured with autologous B cells (4×10^5, 100 μl) in U-bottom 96 well plates (5×10^5, 200 μl volume/well) where 0.625 μg/ml of PWM (Aldrich) were added. 1 μg/ml of anti-CD40LG monoclonal antibody (eBioscience, USA) or Mouse isotype control IgG1 (eBioscience, USA) was used to observed the CD40–CD40LG blocking role when cells were incubating. B cells alone, B cells plus lipopolysaccharide (LPS) (Sigma, USA), and B cells plus LPS and anti-CD40LG were cultured as controls. After incubating the cells in RPMI 1640/10% FBS/penicillin/streptomycin at 37 °C/5% CO2 for 8 days, supplementing 50 μl fresh media to each well at day 4, supernatants (200 μl) were harvested and stored at 4 °C. To determine if T cells transfected with CD40LG become are capable of stimulating B cells, a T cell–B cell costimulation assay was also performed. 5×10^6 T cells were transfected with CD40LG or empty vector. 24 h later, cells were washed and 1×10^6 T cells added to autologous B cells in a T cell: B cell ratio of 1:1. The cells were cultured in RPMI 1640/10% FBS/penicillin/streptomycin for 8 days. Flow cytometry was performed to determine the expression of B cell activation markers CD25 and CD69, and plasma cell differentiation marker CD138. Flourochrome-conjugated antibodies to CD3, CD19, CD25, CD69, and CD138 were purchased from BD Pharmingen (San Diego, CA).

IgG enzyme-linked immunosorbent assays (ELISAs)

IgG in the supernatants of the T cell–B cell cultures was measured by total IgG ELISA per kit (Bethyl, USA) following the manufacturer's protocol.

RNA extraction and real-time RT-PCR

Total RNA was extracted from $CD4^+$ and $CD8^+$ T cells using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using the RevertAidTM First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA). Random hexamer primers were used to initiate cDNA synthesis of 0.5 μg total RNA per 20 μl reaction.

CD40LG and β-actin transcripts were measured in magnetic bead purified CD4+ and CD8+ T cells using a Rotor-Gene (Corbett, Sydney, Australia) thermocycler. The CD40LG and β-actin primers and amplification were as published [14,16].

Statistical analysis

Statistical difference between means was tested using Student's *t*-test or Mann–Whitney test based on SPSS13.0 software. Correlation was determined by Pearson's rank order correlation. *P* value below 0.05 was considered significant.

Results

CD40LG overexpression in human T cells results in autologous B cell activation and plasma cell differentiation in vitro

To determine if CD40LG overexpression is sufficient to induce B cell activation and plasma cell differentiation, we transfected primary T cells from normal human donors with CD40LG cDNA cloned into the expression vector pEGFP-C1 (pEGFP-C1/CD40LG). Controls included cells transfected with the empty vector. Transfection efficiency (≥40%) was confirmed by flow cytometry for the GFP protein. CD40LG overexpression was confirmed in transfected T cells by flow cytometry using a fluorochrome conjugated anti-CD40LG antibody (Fig. 1A). T cells transfected with either pEGFP-C1 or pEGFP-C1/CD40LG were washed and added to autologous B cells in a T:B cell ratio of 1:1. Flow cytometry was used to determine the expression of the activation markers CD25 and CD69 and the plasma cell differentiation marker CD138 on B cells (CD19+ cells). We found that B cells expressing the activation marker CD25 increased from 35.70%±3.57% to 63.16%±5.68% (t = 7.86, P=0.0002, n=7) when cocultured with pEGFP-C1/CD40LG transfected T cells compared to B cells cocultured with empty vector transfected T cells (Fig. 1B). Similar results were obtained by looking at CD69 expression. B cells expressing the activation marker CD69 increased from 31.20%±4.60% to 54.05%±7.90% (t = 5.01, P=0.0041, n=6) when cocultured with pEGFP-C1/CD40LG transfected T cells compared to B cells cocultured with empty vector transfected T cells (Fig. 1C). Further, the percentage of B cells expressing the plasma cell differentiation marker CD138 increased from 15.59%±3.06% to 30.71%±6.49% (t = 3.74, P=0.0096, n=7) in transfected T cell cocultures, indicating that increased plasma cell differentiation results from CD40L overexpression (Fig. 1D).

CD40LG mRNA overexpression in women lupus CD4+ T cells and DNA methylation inhibitors treated CD4+ T cells from women

To investigate whether the expression of CD40LG on T lymphocytes is different between women and men, we measured CD40LG mRNA level in CD4+ and CD8+ T cells from both patients with lupus and normal controls by real-time RT-PCR. Women lupus patients had a significantly elevated amount of CD40LG mRNA on CD4+ T cells (8.48±2.13 versus 1.29 ±0.28; P=0.020) and CD8+ T cells (5.16±2.19 versus 1.22 ±0.64; P = 0.045) compared with female healthy donors. While in men, CD40LG mRNA in both CD4+ (4.27± 1.84 versus 1.83 ±1.23; P=0.283) and CD8+ T cells (1.78±0.68 versus 1.71 ±0.77; P=0.957) from lupus showed no significant differences from that of healthy controls. However, CD40LG mRNA expression in CD4+ T cells from women lupus was increased by nearly 2-fold compared with men lupus (8.48±2.13 versus 5.16±2.19) (Fig. 2A).

Previous studies showed that T cells from patients with active lupus have decreased levels of genomic deoxymethylcytosine (dmC) content, similar to that in T cells treated with demethylating agents [17]. In order to test whether CD40LG expression was also increased in demethylating agents treated T cells as in SLE patients, CD4+ and CD8+ T cells were collected from normal subjects and then treated with DNA demethylating agents 5-AzaC, procainamide, hydralazine and PD98059, and CD40LG expression was measured by mRNA level using real-time RT-PCR. As shown in Figure 2B, drug-treated women CD4+ T cells expressed a significantly higher amount of CD40LG than untreated women cells (5-AzaC, P=0.002, procainamide, P=0.047, hydralazine, P=0.039, PD98059, P=0.048) and CD4+ T cells from men treated with these same drugs (5-AzaC, P=0.009, procainamide, P=0.046, hydralazine, P=0.032, PD98059, P=0.025). While in the male group, there were no differences between drug-treated CD4+ T cells and untreated cells (P>0.05). CD40LG expression levels in CD4+ T cells from healthy women and men were similar. After treating with DNA methylation inhibitors, the level of CD40LG mRNA in CD4+ T cells from women increased nearly 2-fold,

but remained the same as in the untreated cells in men, confirming findings in our previous study [14]. CD8+ T cells treated with these DNA methylation inhibitors expressed a slightly increased CD40LG mRNA but did not reach statistical significance (data not shown).

Overstimulated autologous B cells secreting IgG in lupus patients in vitro

The interaction of T–B cell via CD40LG–CD40 is important for the immune response. To assess the IgG synthesis in lupus patients in vitro, B cells from SLE patients and healthy subjects were cocultured with autologous CD4+ or CD8+ T cells in media for 8 days respectively. The levels of IgG in the supernatants were detected by ELISA as described. IgG production of CD4+ or CD8+ T cocultured with autologous B cells between female and male lupus patients was not significantly different ($P>0.05$). Figure 3 showed that total IgG production was significantly higher when B cells were cultured with autologous CD4+ or CD8 + T cell from both female lupus ($P=0.000$, $P=0.000$, respectively. Fig. 3A) and male lupus ($P=0.000$, $P=0.000$, respectively. Fig. 3B) compared with normal subjects. This finding is consistent with the previous reports that lupus T cells overstimulate autologous B cells to yield much more autoantibodies [18].

Furthermore, the correlation between total IgG production and SLE disease activity were assessed. We compared SLEDAI of 15 lupus patients who had different degrees of disease activity with their IgG production detected in the supematants of coculturing CD4+ T cells and autologous B cells. A positive linear correlation was found between SLEDAI scores and IgG levels ($R = 0.668$, $P=0.007$) (Fig. 4A).

To further investigate if increased level of CD40LG on lupus CD4+ T cell correlates with autoantibody secretion, we compared the relationship of CD40LG mRNA on CD4+ T cells and the total IgG produced in coculturing CD4+ T cell with autologous B cells in vitro. IgG production was positively correlated with the amount of CD40LG mRNA expressed on lupus CD4+ T cells ($R = 0.605$, $P=0.017$) (Fig. 4B).

Anti-CD40 LG inhibition of IgG synthesis induced by women lupus T cells

T cell dependent B cell proliferation and activation require both cytokines and physical cell-cell contact [19]. The present study shows that T/B cell-cell interaction involved CD40–CD40LG playing different roles in women lupus or men lupus patients. In women lupus, the IgG level yielded by B cells alone was significantly lower than by adding CD4+ T cells ($P=0.011$) or CD8+ T cells ($P=0.012$) to the media (Fig. 3A). While in male lupus, there were also significant differences between culturing B cells alone and coculturing B cells with autologous CD4+ T cell ($P=0.000$) or CD8+ T cells ($P=0.000$) (Fig. 3B).

To assess whether the effect of CD40LG–CD40 interaction on IgG production is different in women and men lupus patients, we used a blocking anti-CD40LG mAb when coculturing CD4 + or CD8+ T cells with autologous B cells. To evaluate the role of anti-CD40L mAb, we compared it with its isotype control IgG (both were bought from eBioscience, USA). The result showed that the IgG level was decreased significantly by anti-CD40LG mAb but not its isotype control IgG. In women lupus patients, shown in Figure 3A, cocultured CD4+ T cells or CD8 + T cells with autologous B cells produced much more IgG than cultured B cells alone ($P=0.011$, $P=0.012$, respectively). Anti-CD40LG mAb significantly diminished IgG level produced by CD4+ T plus B cells ($P=0.034$) and CD8+ T plus B cells ($P=0.047$). However, as showed in Figure 3B, the influence of anti-CD40LG mAb on IgG production is weak in men lupus patients. Neither IgG levels produced by CD4+ T cells plus B cells or by CD8+ T cells plus B cells were affected by adding anti-CD40LG mAb ($P=0.238$, $P=0.607$, respectively).

Demethylated CD4+ T cells from women overstimulate B cells to produce IgG and be blocked by anti-CD40LG mAb

Since CD40LG expression enhancement is different in demethylated CD4+ T cells between women and men, we sought to determine whether IgG level secreted by the collaboration of B cell and demethylated T cell is different between women and men. We pretreated T cells with a panel of DNA methylation inhibitors. PBMCs isolated from normal subjects were stimulated with PHA and treated with 5-AzaC, procainamide, hydralazine and PD98059 respectively as described in Materials and methods. After the second stimulation with PMA and ionomycin, CD4+ and CD8+ T cells were isolated from PBMCs using magnetic beads respectively and then cultured for 8 days with autologous B cells at a ratio of 1:4, with or without anti-CD40LG mAb. IgG in the supernatants was measured by ELISA. Figure 5A shows the situation in women. B cells cultured with autologous CD4+ T cells pretreated with 5-AzaC (P=0.02), procainamide (P=0.026), hydralazine (P=0.013) and PD98059 (P=0.008) secreted significantly greater amount of IgG than did B cells cocultured with untreated normal CD4+ T cells. Adding anti-CD40LG mAb with these cells significantly reduce IgG production (P<0.05). While the enhancement of IgG yielded by coculturing autologous B cells with demethylated CD8+ T cells versus normal CD8+ T cells didn't reach a statistically significant level. Anti-CD40LG mAb had no effect either. Figure 5B shows that males had no statistically significant differences (P>0.05) in IgG production produced by untreated or demethylating treated CD4+ or CD8+ T cells cultured with autologous B cells, with or without anti-CD40LG mAb present.

Comparison of IgG production between female and male groups showed that CD4+ T cells from women pretreated by demethylating agents could produce a significantly higher amount of IgG than T cells from men. Similar to non-pretreated normal CD4+ T cells, there was no significant differences between women and men (P>0.05) (Fig. 5C).

We proceeded to analyze the relationship between increased CD40LG mRNA expression on women CD4+ T cells treated with DNA methylation inhibitors and IgG produced by coculturing these drug-treated cells with autologous B cells. Figure 4C shows the positive linear association between IgG secreted in the supernatant and CD40LG mRNA expressed on CD4 + T cells treated with the demethylating drugs 5-AzaC, procainamide, hydralazine and PD98059 (R = 0.641, P=0.01).

Discussion

Systemic lupus erythematosus (SLE) is a female predominant autoimmune disease with overproduction of various autoantibodies. The CD40LG–CD40 interaction between T and B lymphocytes plays a key role in this pathological process. CD40LG, encoded on the X chromosome, is overexpressed on T cells in lupus patients. Whether CD40LG overexpression on T cells is sufficient to induce T cell autoreactivity and whether CD40LG overexpression is associated with women susceptibility to SLE are not clear. The present study demonstrates that CD40LG overexpression is solely capable of inducing T cell autoreactivity, in the absence of a foreign antigen. This is indicated by the ability of CD40LG over-expressing T cells to induce activation of autologous B cells and plasma cell differentiation in the absence of any T cell stimulation and antigen presentation. We further studied the effect of DNA demethylation on T cell CD40LG expression and the production of IgG by autologous B cells via CD40LG–CD40 in lupus. The differences between women and men were focused upon.

Increasing evidence shows DNA hypomethylation of SLE T cells and induction of lupus-like autoimmunity in vivo and in vitro by demethylating agents [20–21]. T cells from active SLE patients have genome-wide reduction in deoxymethylcytosine and gene-specific hypomethylation [22]. *CD11a, perforin, CD70* and *CD40LG* genes on T cells have been proved

relevant to lupus [14,16,23,24]. In the present study, we used 5-azaC and procainamide which directly inhibit DNA methytransferase, and PD98059 and hydralazine which affect ERK signaling pathway to decrease DNA methytransferase expression, to induce a hypomethylation status on T cells imitating lupus-like status [13]. We demonstrated that although CD4+ T cells from healthy women and men expressed equivalent levels of CD40LG mRNA, female lupus patients expressed 2-fold higher levels than male lupus (8.48±2.12 versus 4.27 ± 1.84) (Fig. 2A). Drug-treated CD4+ T cells from females increased almost 2-fold than untreated female CD4+ T cells and treated male cells which did not changed significantly by demethylating treatment (Fig. 2B). These results are in accordance with the previous study [14]. CD40LG is encoded on the X chromosome [25]. Men have a single X chromosome and women have two, but the dosage imbalance is solved by transcriptionally silencing one X chromosome in women during early development [26,27]. Xist RNA, histone modification and DNA methylation are involved in the X chromosome inactivation [28,29]. In a previous study, we used bisulfite DNA sequencing to show that CD40LG promoter and enhancer sequence was half methylated and half unmethylated in women while the same sequence was completely unmethylated in men. Further, CD40LG promoter and enhancer methylation in CD4+T cells from women decreased after 5-azaC treatment [14]. In the present study, CD40LG mRNA expressed on CD4+T cells from men is almost half of that from women after treating with DNA methylation inhibitor, supporting that DNA demethylation reactivates the inactive X chromosome and leads to the doubling of the CD40LG expression. It could be deduced that in females with lupus, DNA hypomethylation which happened on CD4+ T cells causes the silenced X chromosome to be activated and then increases CD40LG expression.

Oversecreting pathogenic autoantibodies is a major character of B cells from active SLE. The role of the CD40LG–CD40 interaction contributing to this process has been proved in human and animal lupus [8,9,30,31]. However, the difference of this contribution between two genders is rarely reported. Our results suggested that CD4+ or CD8+ T cells from both female and male lupus patients overstimulated autologous B cells secreting IgG (Fig. 3), and that IgG production is significantly correlated with SLE disease activity (Fig. 4A). In female lupus patients, IgG produced by coculturing T and B cells in vitro could be reversed with anti-CD40LG (Fig. 3A). In accord with this, demethylated CD4+ T cells from women but not men stimulated autologous B cells oversecreting IgG, again, and anti-CD40LG antibody inhibited the overexpression of IgG production induced by demethylated CD4+ T cells from women but not men (Fig. 3). We also showed a positive correlation between IgG production and the increasing expression of CD40LG mRNA in both lupus CD4+ T cells and drug-treated CD4+ T cells (Figs. 4B, C). Although CD40LG mRNA expression in male lupus T cells is lower than that in female lupus T cells, yet the production of total IgG from B cells cocultured with T cells looked similar between the male and female patients. We suppose that the role of CD40LG–CD40 in IgG production is much weaker in male than in female lupus patients. It seems that CD40LG–CD40 mediated stimulation has a major role in the B cell activation and enhanced autoantibody production in female lupus. Blocking the CD40LG–CD40 pathway is now an established therapeutic approach to alleviate IgG secretion and disease activity [32,33]. In our study, we observed that in men, anti-CD40LG had poor effects in reducing IgG production in coculturing of lupus T cells and autologous B cells or in demethylating drug-treated T cells and autologous B cells. In male lupus, it is possible that T cells interact with B cells via different pathways. Figure 3 shows that LPS could irritate B cells to yield IgG in healthy subjects but not in active SLE patients, and the blocking effect of anti-CD40LG is also limited in culturing B cells with LPS in active SLE patients. This could be interpreted that in active SLE patients, B cells were already in an activated status and could not been affected much by LPS externally.

In conclusion, CD40LG overexpression is sufficient to induce autologous B cell activation and plasma cell differentiation in vitro. Moreover, CD40L overexpression on women lupus CD4+ T cells which may be a fruit of the reactivation of the silenced X chromosome caused by

demethylation of *CD40LG* gene plays a rival role in women lupus pathogenesis. In women lupus, high level of CD40LG may overstimulate B cell to produce various autoantibodies via the T–B cell interaction. Thus, increased expression of *CD40LG* on CD4+ T Cells caused by DNA demethylation may lower the threshold for B cell stimulation and production of autoantibodies thereby contributing the pathogenesis of lupus.

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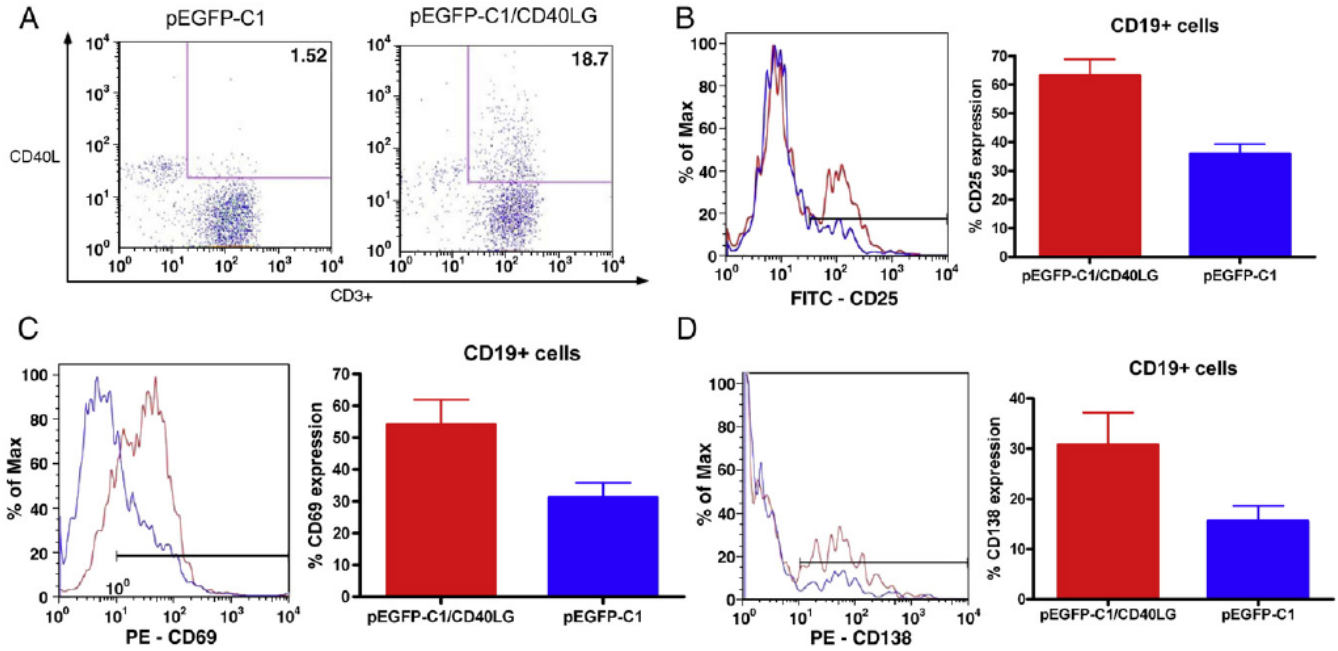

Figure 1.

(A) Increased surface expression of CD40LG on human T cells transfected with pEGFP-C1/CD40LG compared to empty vector transfected controls. (B) Increased percentage of CD25 expression (P=0.0002, n=7), (C) CD69 expression (P=0.0041, n=6), and (D) CD138 expression (P=0.0096, n=7) on CD19+ gated cells that were cocultured with CD40LG overexpressing T cells as compared to T cells transfected with empty vector. Representative histograms are shown on the right.

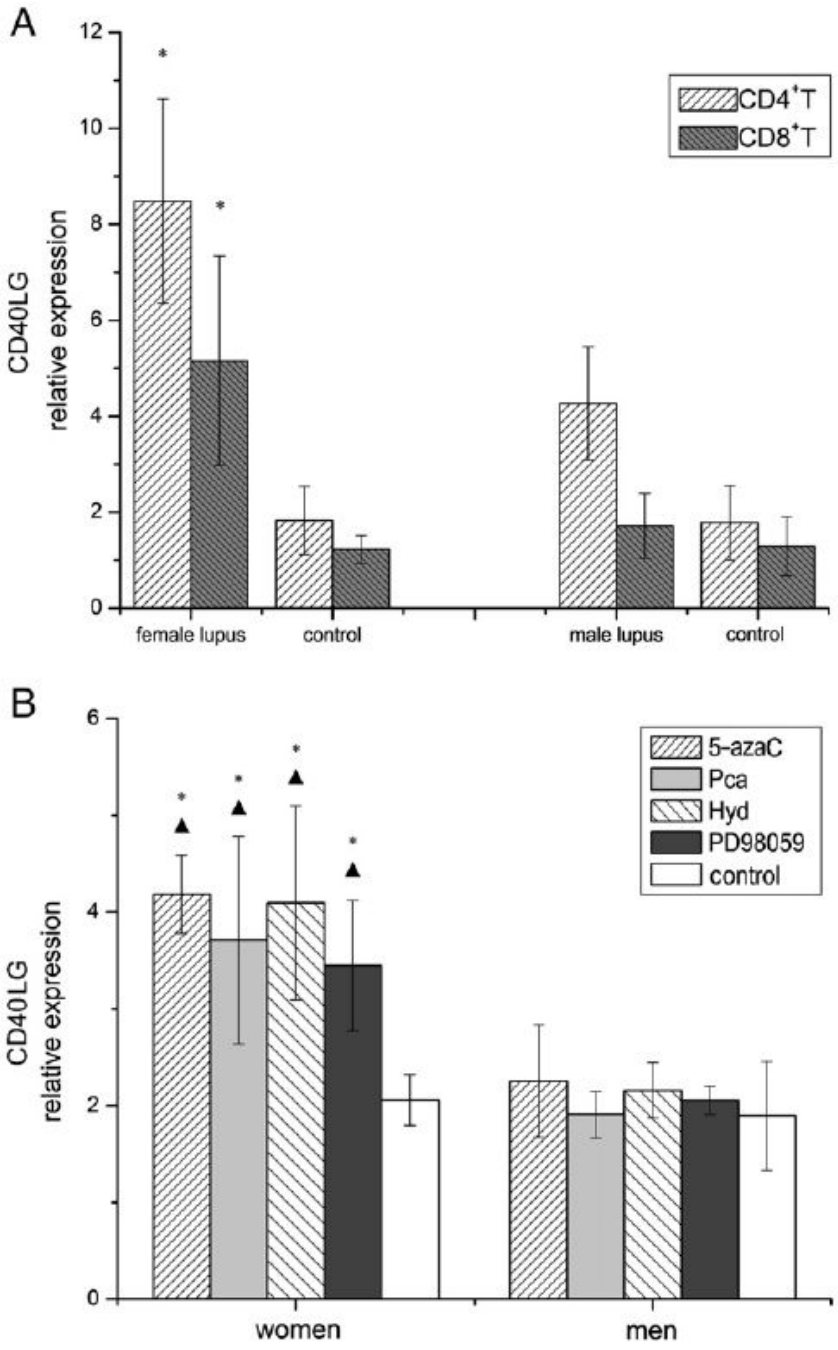

Figure 2.

CD40LG mRNA expression on CD4+ and CD8+ T cells. CD40LG transcripts relative β-actin was measured using realtime RT-PCR. Results represent the mean±SEM of the indicated numbers of subjects. (A) in women (*n*=6), T cells from lupus patients expressed significantly higher amount of CD40LG mRNA than normal controls (*n*=6), *=*P*<0.05 versus normal controls (CD4+, *P*=0.020, CD8+, *P*=0.045). As in men, CD40LG expression on CD4+ and CD8+ T cells was not significantly different between patients with lupus (*n*=9) and normal controls (*n*=5) (*P*>0.05). (B) in DNA methylation inhibitors treated or untreated T cells. Treated CD4+ T cells from women expressed significantly increased CD40LG mRNA compared with untreated CD4+ T cells from women (*n*=3), *=*P*<0.05 versus untreated CD4+ T cells (5-AzaC,

P=0.002; procainamide, *P*=0.047; hydralazine, *P*=0.039 and PD98059, *P*=0.048). The difference was also significant between values of treated CD4+ T cells from women and treated CD4+ T cells from men(*n* = 3), ▲=*P*<0.05 versus CD4+ T cells from men(5-AzaC, *P*=0.009; hydralazine, *P*=0.046; procainamide, *P*=0.032 and PD98059 *P*=0.025).

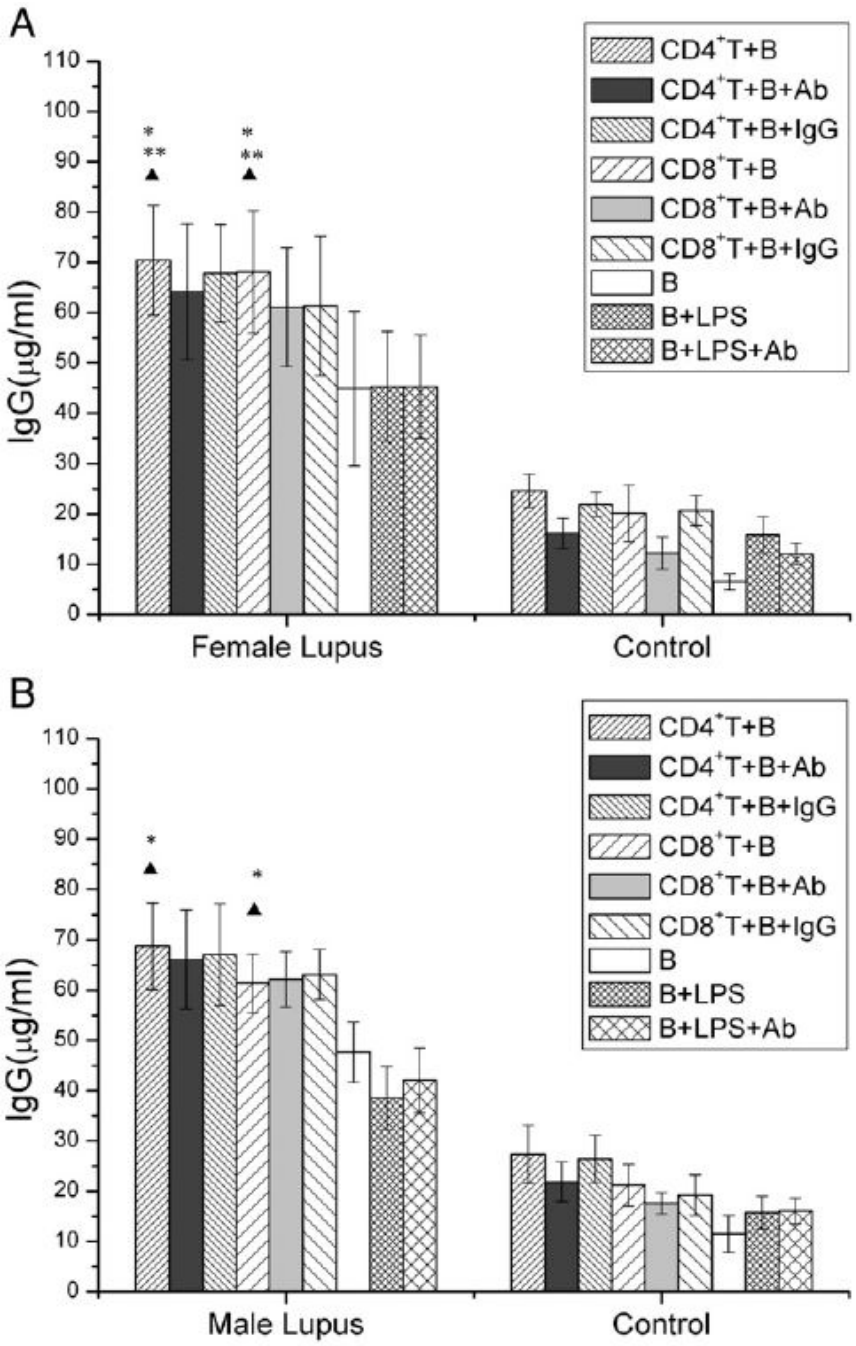

Figure 3.

IgG production induced by T–B cells culture in vitro. Controls included B cells cultured alone, B cells plus lipopolysaccharide (LPS), and B cells plus LPS and anti-CD40LG. (A) women group: IgG production of B cell cultured with autologous CD4+ or CD8+ T cell from female lupus was significantly higher in lupus patients. Lupus patients (n=6) versus normal controls (n=6) (P=0.000, P=0.000, respectively); * = P<0.05 versus normal controls. CD4+ T plus B cells with versus without anti-CD40LG: P=0.034; CD8+ T plus B cells with versus without mAb: P=0.047; **=P<0.05 versus cultures without pretreatment of T cells with anti-CD40LG. CD4+ T plus B cells versus B cells alone: P=0.011, CD8+ T plus B cells versus B cells alone: P=0.012; ▲=P<0.05 versus B cells cultured alone. (B) men group: IgG level of B cell cultured

with autologous CD4+ or CD8+ T cell from female lupus was also significantly higher in lupus patients (n=9) than that in normal controls (n=5) (P=0.000, P=0.000, respectively) normal controls, *=P<0.05 versus normal controls. CD4+ T plus B cells plus with versus without anti-CD40LG: P=0.238; CD8+ T plus B cells with versus without anti-CD40LG: P=0.607. CD4+ T plus B cells versus B cells alone: P=0.000, CD8+ T plus B cells versus B cells: P=0.000.

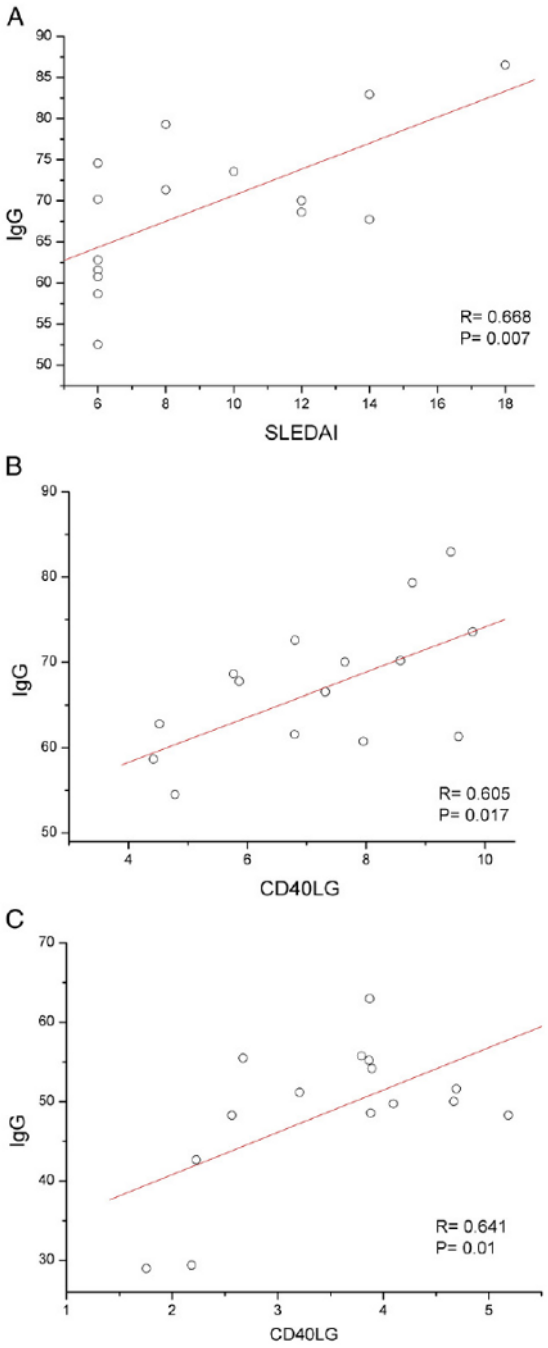

Figure 4.

(A) Correlation between the IgG production and disease activity as measured by SLE disease index score analyses for all patients (R=0.668, P=0.007). (B) IgG secreted by coculturing CD4 + T cells and autologous B cells in vitro is plotted against the expression of CD40LG mRNA on CD4+ T cells from 15 lupus patients. Where indicated, IgG was measured by ELISA as described in the text. CD40LG mRNA was detected by real-time-PCR. (R=0.605, P=0.017). (C) Correlation of CD40LG mRNA on 5-AzaC, procainamide, hydralazine and PD98059 treated[Float1] CD4+ T cells from women and IgG secreted by coculturing 5-AzaC, procainamide, hydralazine and PD98059 treated CD4+ T cells and autologous B cells (R=0.641, P=0.01).

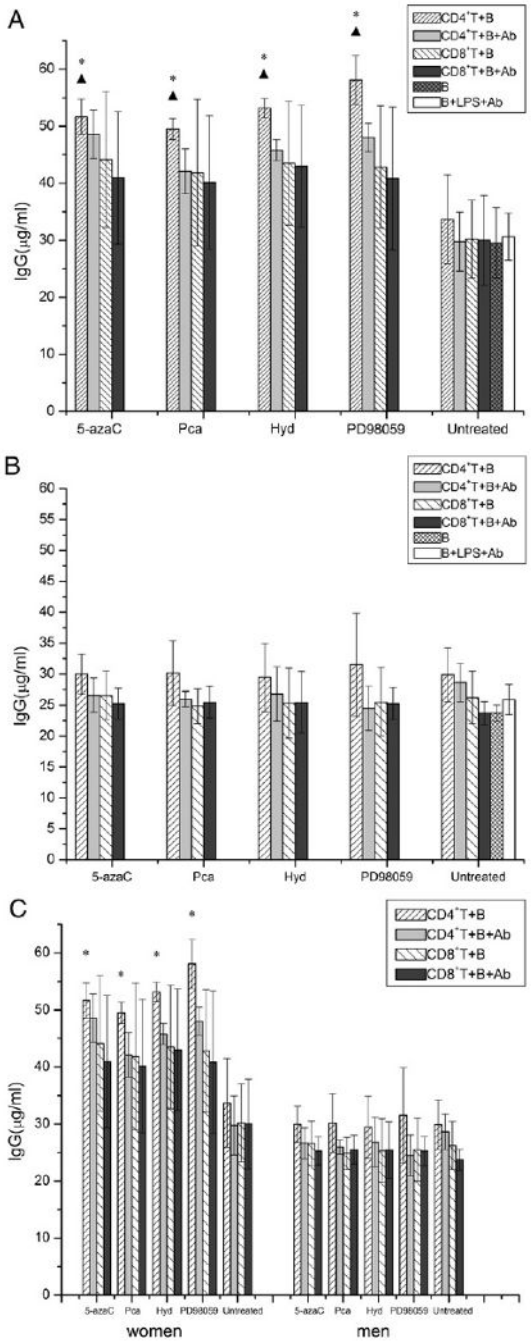

Figure 5.

IgG synthesis induced by B cell cocultured with autologous demethylated T cell in vitro. CD4 + and CD8+ T cells from normal controls were pretreated with DNA methylation inhibitors and cultured with autologous B cells, controls included B cells cultured alone, B cells plus LPS, and B cells plus LPS and anti-CD40LG. (A) IgG was significantly promoted by CD4+ T cells pretreated with 5-AzaC (P=0.02), procainamide (P=0.026), hydralazine (P=0.013) and PD98059 (P=0.008) compared with untreated CD4+ T cells, ▲ =P<0.05 versus untreated CD4 + T cells. Anti-CD40LG inhibited IgG synthesis induced by CD4+ T treated with 5-AzaC (P=0.038), procainamide (P=0.042), hydralazine (P=0.024) and PD98059 (P=0.003), *=P<0.05 versus cultures with and without pretreatment of T cells with anti-CD40LG.

Demethylated CD8+ T cells did not promote IgG production to a statistically significant level compared with untreated CD8+ T cell ($P>0.05$). Anti-CD40LG mAb inhibition of IgG was slightly on CD8+ T cells. (P=0.775) (B) drug-treated CD4+ or CD8+ T cells from men cultured with autologous B cells with or without anti-CD40LG mAb did not significantly affect IgG production. (C) CD4+ T cells from women pretreated with 5-AzaC ($P=0.025$), procainamide ($P=0.041$), hydralazine ($P=0.022$) and PD98059 ($P=0.02$) promoted IgG production compared with CD4+ T cells from men. * = $P<0.05$ versus IgG production of drug-treated CD4+ T cocultured with B cell form men.

Table 1

Summary of study subjects.

Subjects	Sex	Age	SLEDAI score	Medications
A. SLE patients				
1	F	30	6	Pred,10 mg/day
2	F	32	18	CTX.Pred,25 mg/day
3	F	17	12	HCQ.Pred,25 mg/day
4	F	14	6	None
5	F	28	6	HCQ.Pred,20 mg/day
6	F	18	12	HCQ.Pred,30 mg/day
7	M	18	14	HCQ.Pred,40 mg/day
8	M	19	6	Pred,10 mg/day
9	M	24	8	Pred,20 mg/day
10	M	26	8	CTX.Pred,25 mg/day
11	M	22	14	None
12	M	23	6	CTX
13	M	23	6	Pred, 25 mg/day
14	M	25	10	Pred, 12.5 mg/day
15	M	29	6	None
Healthy controls				
1	F	25	0	None
2	F	26	0	None
3	F	30	0	None
4	F	25	0	None
5	F	24	0	None
6	F	24	0	None
7	M	36	0	None
8	M	28	0	None
9	M	30	0	None
10	M	25	0	None
11	M	23	0	None
Drug-treated ones				
1	F	26	0	None
2	F	27	0	None
3	F	28	0	None
4	M	36	0	None
5	M	28	0	None
6	M	28	0	None

SLEDAI = Systemic Erythematosus Disease Activity Index; SLEDAI score > 5 is described as active disease, ≤5 inactive disease, 0 no disease; HCQ = hydroxychloroquine; Pred = prednisone; CTX=cyclophosphamide.