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Altered expression of signalling lymphocyte activation molecule (SLAM) family receptors CS1 (CD319) and 2B4 (CD244) in patients with systemic lupus erythematosus

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Summary

CS1 (CRACC, CD319) and 2B4 (CD244), members of the signalling lymphocyte activation molecule (SLAM) family receptors, regulate various immune functions. Genes encoding SLAM family receptors are located at 1q23, implicated in systemic lupus erythematosus (SLE). In this study, we have investigated the expression and alternative splicing of CS1 and 2B4 in immune cells from SLE patients. The surface expression of CS1 and 2B4 on total peripheral blood mononuclear cells (PBMCs), T, B, natural killer (NK) cells and monocytes in 45 patients with SLE and 30 healthy individuals was analysed by flow cytometry. CS1-positive B cell population was increased significantly in SLE patients. Because CS1 is a self-ligand and homophilic interaction of CS1 induces B cell proliferation and autocrine cytokine secretion, this could account for autoreactive B cell proliferation in SLE. The proportion of NK cells and monocytes expressing 2B4 on their surface was significantly lower in patients with SLE compared to healthy controls. Our study demonstrated altered expression of splice variants of CS1 and 2B4 that mediate differential signalling in PBMC from patients with SLE.

Keywords: 2B4, CS1, PBMC, SLAM family receptors, SLE

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease, characterized by the improper regulation of B cells that leads to the production of autoantibodies. The incidence of disease is gender-biased, with a female to male ratio of 9 : 1, and the onset of disease is usually during the child-bearing years [1]. Using lupus model mice such as MRL/lpr, NZB/NZW and NZM2410, which develop SLE spontaneously, mouse chromosome 1 has been shown to contain lupus susceptibility genes [2–5]. Genomic characterization of the Sle1b locus, the most potent member of lupus susceptibility region on murine chromosome 1, identified a highly polymorphic cluster of genes coding for the signalling lymphocyte activation molecule (SLAM) family receptors [6]. Similarly, genome-wide linkage analyses of SLE families have shown a strong association of SLE with the 1q23 region of the human genome, which also includes SLAM family receptors [7–9].

SLAM family receptors are expressed broadly on haematopoietic cells, and play an important role in immune regulation. Members of this family are SLAM (SLAMF1, CD150), CD229 (SLAMF3, Ly-9), 2B4 (SLAMF4, CD244), CD84 (SLAMF5), NTB-A (SLAMF6; Ly108 in mouse) and CS1 (SLAMF7, CRACC, CD319). All these receptors have immunoreceptor tyrosine-based switch motifs (ITSMs) in their intracellular domain, which can be bound by small adaptor proteins such as SLAM-associated protein (SAP, *SH2D1A*), Ewing's sarcoma (EWS)-activated transcript 2 [Ewing's sarcoma-activated transcript-2 (EAT-2), *SH2D1B*] and EAT-2-related transducer (ERT, *SH2D1C*, only in rodents). Mutations in *SH2D1A*, the gene encoding SAP, are responsible for the primary immunodeficiency X-linked lymphoproliferative disease (XLP) in humans [10–12]. Studies using knock-out mice deficient in SAP and SLAM family receptors indicate that SLAM family receptors and SAP play an important role in T cell-mediated help for humoral immunity [13–16].

2B4 (CD244) is expressed on natural killer (NK) cells, some $CD8^+$ T cells, monocytes, basophils and eosinophils. In both mice and humans, CD48 is the ligand for 2B4 [17,18]. We have originally identified, cloned and characterized the 2B4 receptor in the mouse [19,20]. In the mouse two isoforms of 2B4, m2B4-L and m2B4-S, are expressed which are the products of differential splicing of hnRNA [21]. These two isoforms differ only in the cytoplasmic domain, and they send opposing signals to NK cells [22]. Human NK cells also express two isoforms of 2B4, h2B4-A and h2B4-B, which

differ in a small portion of the extracellular domains [23,24]. The important role of 2B4 has been implicated in various infection and clinical settings. For example, a number of studies revealed that an inability to signal via 2B4 due to a genetic defect in SAP may contribute to the pathogenesis of XLP [25–27]. Human 2B4 expression is up-regulated on $CD8^+$ T lymphocytes raised in response to herpes simplex virus (HSV), which lysed infected cells more efficiently [28]. Soluble CD48 (ligand for 2B4) is detected at elevated levels in the plasma of patients with arthritis and lymphoid leukaemia [29]. 2B4 is expressed early in the differentiation of NK cells and in immature NK cells 2B4 acts as an inhibitory receptor [30]. This allows a fail-safe mechanism to prevent killing of normal autologous cells at early stages of NK cell differentiation when there is no other inhibitory receptors expressed. 2B4/CD48 interactions regulate the proliferation of activated/memory T cells [31]. It was shown that 2B4/CD48 interactions provide a co-stimulatory signal among T cells themselves [32]. Our studies indicated that 2B4 acts as a non-major histocompatibility complex (MHC) binding negative regulator of NK cells in mice [33]. The generation and preliminary characterization of 2B4 gene knock-out mice revealed an important role for 2B4 *in vivo* in rejection of tumour metastases [34]. More interestingly, the immune response against B16 melanoma in 2B4-deficient mice revealed a gender-specific role for 2B4 in the immune system [34]. This led us to reason a role for 2B4 in human autoimmune disorders that tend to be predominant among females. Recently, it was suggested that 2B4 has a role in the autoimmune process shared by rheumatoid arthritis and SLE [35].

CS1 is expressed on NK cells, activated T cells, activated B cells and dendritic cells. CS1 is a self-ligand, and homophilic interaction of CS1 activates NK cell cytolytic function [36]. CS1 induces proliferation and production of autocrine cytokines in B lymphocytes [37]. Two isoforms of CS1, CS1-L and CS1-S are expressed in NK cells. These two isoforms differ in their cytoplasmic domain and signal differently [38]. It has been shown that CS1 can mediate both activating and inhibitory functions, depending upon EAT-2 expression [39]. Recently it has been reported that CS1 is overexpressed in multiple myeloma and an anti-CS1 humanized monoclonal antibody inhibited myeloma cell adhesion and induced antibody-mediated cellular cytotoxicity in bone marrow milieu [40,41]. CS1 promotes multiple myeloma cell adhesion, clonogenic growth and tumorigenicity via cmaf-mediated interactions with bone marrow stromal cells [42]. Family-based association studies in UK and Canadian SLE families identified variants in the promoter and coding region of CS1 contributing to SLE disease susceptibility [43].

Based on the recent finding of a genetic association of SLAM family receptors with SLE, we hypothesized that the alterations in expression of 2B4 and CS1 may mediate the immune dysregulation observed in patients with SLE. In this study, we compared expression levels of 2B4 and CS1 on T, B, NK cells and monocytes in SLE patients *versus* those of healthy controls. The 2B4-expressing NK cells and 2B4-expressing monocytes were reduced in patients with SLE compared to healthy controls. The proportion of CS1-expressing B cells in patients with SLE was significantly higher than that from healthy controls. Our study also demonstrated differential expression of CS1 and 2B4 splice variants in total peripheral blood mononuclear cells (PBMC) in patients with SLE compared to healthy controls.

Materials and methods

Patients and healthy control volunteers

Blood samples were obtained from 45 patients diagnosed with SLE (two males, 43 females) at John Peter Smith (JPS) Hospital, Fort Worth, TX and from 30 healthy volunteers at University of North Texas Health Science Center (UNTHSC), Fort Worth, TX with prior approval from Internal Review Board of JPS Health Network and UNTHSC. Written informed consents were obtained from all of the study subjects. Patients with SLE were classified according to the 1997 revised criteria from the American College of Rheumatology [44,45]. Clinical and demographic characteristics of SLE patients, including SLE Disease Activity Index (SLEDAI), treatments, major disease manifestations and serological parameters, are shown in Table 1. Eight patients had active SLE, defined by a SLEDAI score of ≥ 8 [46]. All 45 patients were positive for anti-nuclear antibody (ANA).

Isolation of PBMCs

PBMCs were isolated from ethylenediamine tetraacetic acid (EDTA)-treated whole-blood samples by Histopaque-1077 (Sigma Chemicals, St Louis, MO, USA) density gradient centrifugation using LeucoSep tubes (Greiner, Monroe, NC, USA). The remaining red blood cells were lysed with ACK lysis buffer. Resulting PBMCs were used for immunostaining or reverse transcription–polymerase chain reaction (RT–PCR).

Antibodies and immunostaining for flow cytometry analysis

Before starting immunostaining, PBMCs were incubated with human IgG Fc fragments (Rockland, PA, USA) for prevention of possible Fc receptor-mediated fluorescence. The tricolour staining [fluorescein isothiocyanate–phycoerythrin–allophycocyanin (FITC-PE-APC)] method was applied for immunostaining. Anti-hCD3-FITC (clone UCHT1; BD Biosciences, San Jose, CA, USA)/anti-hCD19-APC (clone J4·119; Beckman Coulter, Miami, FL, USA) or anti-hCD14-FITC (clone M5E2; BD Biosciences)/anti-hCD56-APC (clone N901 (NKH-1); Beckman Coulter) were used in combination with anti-h2B4-PE (clone C1·7; Beckman Coulter) or anti-hCS1-PE (clone 235614; R&D

Table 1. Clinical and demographic characteristics of patients with systemic lupus erythematosus (SLE).

Patient	Race	Age/sex	Disease duration (months)	Family history	ANA	Anti-dsDNA	Anti-Sm	SLEDAI	Disease manifestations	Treatments
1	AA	25/F	48	No	+	n.d.	+	15	R, HL, AR, LP, PU, LCL, IDB	Pred (40), HCQ, AZA
2	AA	37/F	84	Yes	+	+	+	12	R, AR, PU, LCL, IDB	Pred (20), HCQ, MPA
3	AA	45/F	15	No	–	–	–	12	R, AR, PU, LCL, IDB	Pred (20), HCQ, Others
4	AA	43/F	60	No	+	+	+	10	AR, LCL, IDB	Pred (60), MPA, Others
5	AA	41/F	4	No	+	+	n.d.	9	AR, LP, LCL, IDB	Pred (5), HCQ
6	AA	50/F	24	No	+	n.d.	+	8	R, AR, LCL	HCQ, Others
7	H	56/F	24	No	+	–	–	8	AR, PU, LCL	Pred, MPA, Others
8	H	48/F	24	No	+	+	n.d.	8	AR, LCL, IDB	Pred (5), HCQ, MTX
9	AA	44/F	60	No	+	+	n.d.	6	R, HL, IDB	HCQ, MTX
10	H	28/F	84	No	+	+	n.d.	6	R, LCL, IDB	HCQ, Others
11	AA	43/F	48	No	+	n.d.	n.d.	6	R, PU	Pred (10), HCQ
12	A	40/M	24	No	+	–	–	6	AR, LCL	Pred (30), HCQ
13	H	43/F	36	No	+	+	n.d.	5	R, LP	HCQ
14	AA	50/F	216	No	+	+	n.d.	5	LP, LCL, IDB	Pred (5), HCQ
15	A	40/F	96	No	+	+	n.d.	5	R, LP, LCL, IDB	Pred (5), HCQ
16	AA	37/F	84	No	+	–	n.d.	4	R, LCL	Pred (10), HCQ, Others
17	H	21/F	8	No	+	+	n.d.	4	LCL, IDB	Pred (5), HCQ, AZA, Others
18	C	64/F	24	No	+	n.d.	n.d.	4	AR	None
19	H	22/F	120	No	+	+	n.d.	4	LCL, IDB	Pred (10), HCQ, Others
20	C	47/F	252	Yes	+	–	–	4	AR	Others
21	H	44/F	204	No	+	n.d.	n.d.	4	None	Pred (20), HCQ
22	AA	64/F	12	No	+	n.d.	n.d.	4	AR	Pred (5)
23	H	44/F	192	Yes	+	n.d.	+	4	AR	None
24	AA	40/F	60	No	+	n.d.	+	4	R	Pred (15)
25	AA	57/F	108	No	+	n.d.	n.d.	4	AR	HCQ, Others
26	A	42/F	48	No	+	+	+	3	LP, IDB	Pred (10), AZA
27	C	31/F	24	No	+	+	n.d.	2	LCL	HCQ
28	AA	37/F	132	No	+	–	n.d.	2	HL	Pred (10), HCQ
29	AA	26/F	84	No	+	+	n.d.	2	None	Pred (5), Others
30	H	37/F	240	Yes	+	+	n.d.	2	IDB	Pred (7·5), AZA
31	H	63/F	108	No	+	+	n.d.	2	IDB	Pred (2·5), HCQ
32	AA	25/F	36	No	+	+	n.d.	2	IDB	Pred (15), HCQ
33	C	50/F	12	No	+	n.d.	n.d.	1	LP	HCQ
34	AA	52/F	36	No	+	n.d.	n.d.	1	LP	HCQ
35	C	22/F	120	No	+	–	–	0	None	Pred (20), HCQ, Others
36	H	55/F	Unknown	No	+	–	–	0	None	Others
37	AA	52/F	24	No	+	n.d.	n.d.	0	None	Others
38	H	50/F	12	No	+	n.d.	n.d.	0	R	None
39	AA	27/F	36	No	+	+	n.d.	0	None	Pred (5), HCQ
40	AA	46/F	240	No	+	n.d.	n.d.	0	None	Pred (5), HCQ
41	AA	43/F	72	No	+	–	–	0	None	Others
42	AA	60/F	48	No	+	n.d.	+	0	None	HCQ
43	C	24/F	120	No	+	n.d.	n.d.	0	None	Pred (20), HCQ
44	C	35/F	48	Yes	+	+	n.d.	0	None	HCQ, Others
45	C	22/M	120	Yes	+	+	+	0	R	Pred (10), HCQ, MTX

A, Asian; AA, African American; ANA, anti-nuclear antibody; anti-dsDNA, anti-double-stranded DNA; anti-Sm, anti-Smith antigen; AR, arthritis; AZA, azathioprine; C, Caucasian; F, female; H, Hispanic; HCQ, hydroxychloroquine; HL, hair loss; IDB, increased DNA binding; LCL, low complement levels; LP, leukopenia; M, male; MPA, mycophenolic acid; MTX, methotrexate; n.d., not determined; Pred (X), prednisone (mg/day); PU, proteinuria; R, rash; SLEDAI, systemic lupus erythematosus Disease Activity Index.

Systems, Minneapolis, MN, USA). For isotype controls, mouse IgG1-FITC, mouse IgG1-PE, mouse IgG2a-PE and mouse IgG1-APC were used (all from Caltag Laboratories, Burlingame, CA, USA). Samples were run on a Cytomics FC500 Flow Cytometer (Beckman Coulter, Fullerton, CA, USA). Data were analysed using CXP software (Beckman Coulter). Mean fluorescence intensity ratio (MFIR) was calculated by dividing the mean fluorescence intensity of

Table 2. Mean fluorescence intensity ratio (MFIR) of cells stained positively for CS1 or 2B4 from patients with systemic lupus erythematosus (SLE) *versus* healthy controls.

	MFIR of CS1				MFIR of 2B4		
Cells	Healthy controls ($n = 25$)	SLE patients ($n = 34$)	*P*-level	Cells	Healthy controls ($n = 28$)	SLE patients ($n = 42$)	*P*-level
PBMCs	14·38 ± 1·212	11·74 ± 0·9568	0·0890	PBMCs	22·96 ± 2·290	13·20 ± 1·148	**<0·0001**
T cells	5·672 ± 0·7459	8·986 ± 0·7117	**0·0030**	T cells	21·44 ± 2·50	12·17 ± 1·083	**0·0003**
B cells*	3·235 ± 0·3844	3·934 ± 0·8258	0·4959	Monocytes	18·25 ± 1·779	10·82 ± 0·8689	**<0·0001**
NK cells	12·87 ± 1·485	9·013 ± 0·7335	**0·0124**	NK cells	25·51 ± 2·784	17·98 ± 1·688	**0·0168**

*MFIR of CS1 in B cells were calculated by gating total CS1-positive and negative fraction of cells, because most healthy individuals did not have CS1-positive cells. The rest of MFIR was calculated from just the cells in the positive gate. Values are the mean ± standard error of the mean (s.e.m.). Significant *P*-values are indicated in bold type. NK, natural killer; PBMC, peripheral blood mononuclear cells.

samples with the mean fluorescence intensity of isotype controls.

Semi-quantitative RT–PCR and gel electropheresis for splice variants

Some PBMCs were dissolved with RNA STAT-60 in 5 million cells/1 ml and kept at −80°C until RNA extraction. RNA was extracted by chloroform and precipitated by isopropanol. After resuspension with 0·1% diethylpyrocarbonate (DEPC)-water, RNA purity and concentration were determined by measuring optical density at 260, 280 and 230 nm; 2 μg of RNA was used for cDNA synthesis in the presence of primer mixture of random hexamer (New England Biolabs, Ipswich, MA, USA) and oligodeoxythymidylic acid (oligo-dT) (Integrated DNA Technologies, Coralville, IA, USA). After RT reaction, cDNA was diluted to a concentration of 100 ng/μl and 1–3 μl was used for each PCR reaction as a template. PCR cycle conditions were 94°C for 45 s, 50°C for 45 s and 72°C for 60 s, repeated for 35 cycles using *Taq* DNA polymerase (New England Biolabs, Ipswich, MA, USA). We used PCR primers amplifying simultaneously two splice variants of CS1 and 2B4 (Table 2). CS1 PCR products were run on 2% agarose gels. 2B4 PCR products were electrophoresed on 8–12% non-denaturing polyacrylamide gels. Intensity of PCR bands was estimated using the Area Density Tool of LabWorks software (UVP, Upland, CA, USA).

Statistical analysis

A two-tailed Student's *t*-test was performed to determine significant differences between the SLE patients and healthy individuals. If variances were significantly different between the two populations, Welch's correction was applied to calculate the *P*-value. Spearman's rank was employed to study correlations between percentage of cells and SLEDAI index. Linear regression analysis was also performed. *P*-values below 0·05 were considered statistically significant. Data were analysed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA).

Results

Expression of splice variants of CS1 and 2B4 in SLE

A recent family-based association study in UK and Canadian SLE families identified one single nucleotide polymorphism (SNP) (rs489286) in intron 6 of CS1 contributing to SLE disease susceptibility [43]. Also, a similar study in a Japanese population identified five SNPs in the introns of 2B4 associated with rheumatoid arthritis: rs6682654 (intron 3), rs1319651 (intron 4), rs3766379 (intron 5), rs3753389 (intron 5) and rs11265493 (intron 7) [35]. Because mutations in the intron sequence can affect splicing events, we decided to see whether differential expression of splice variants of CS1 and 2B4 is observed in SLE patients.

Our laboratory has identified two splice variants of human CS1, CS1-L and CS1-S [38]. While CS1-L and CS1-S forms have identical extracellular domains, CS1-S lacks the two ITSMs required for intracellular signalling. CS1-L functions as an activating receptor, whereas CS1-S does not show any signalling function in NK cells [38]. We determined the expression ratio of CS1-L over CS1-S mRNA in PBMCs by RT–PCR. Common PCR primers detecting both CS1 isoforms generated PCR products of 228 base pairs (bp) for CS1-L and 125 bp for CS1-S. As seen in Fig. 1a, while all healthy individuals and most of SLE patients expressed three- to sixfold higher levels of CS1-L than CS1-S, some SLE patients expressed higher levels of CS1-S isoform (SLE 19, SLEDAI = 4 and SLE 36, SLEDAI = 0). Notably, one patient showed no expression of CS1-S isoform (patient 17; SLEDAI = 4). The different-sized PCR bands found in patient 4 and patient 41 were cloned and sequenced and found to be non-specific. There was no correlation between differential expression ratio of CS1 isoforms and SLEDAI.

Previously, we also identified two different splice variants of human 2B4, h2B4-A and h2B4-B [24]. While h2B4-A and h2B4-B share the same intracellular domains, h2B4-B has additional five amino acids between the V and C2 regions compared to h2B4-A. Recently, we have shown that these two isoforms have different functional roles in human NK cells [23]. In order to examine whether these isoforms are

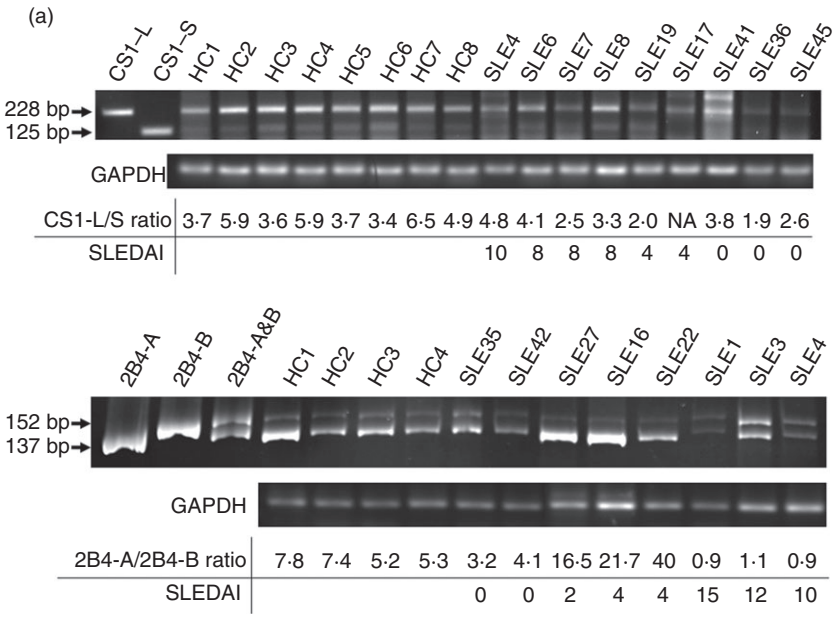

Fig. 1. Altered expression ratio of splice isoforms of human CS1 and 2B4 in systemic lupus erythematosus (SLE). (a) Differential expression of two CS1 transcript variants, CS1-L and CS1-S, was analysed by semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) using total peripheral blood mononuclear cells (PBMCs) from SLE patients and healthy controls. PCR products were run on 2% agarose gels. Lanes 1 and 2 are positive controls amplified from plasmid clones for CS1-L and CS1-S, respectively. Low values of a CS1 : L/S ratio were found in some SLE patients [patient 19, SLE Disease Activity Index (SLEDAI) = 4 and patient 36, SLEDAI = 0]. Notably, one patient showed no expression of CS1-S isoform (patient 17; SLEDAI = 4). (b) Differential expression of two human 2B4 transcript variants, 2B4-A and 2B4-B, in SLE was analysed by semiquantitative RT–PCR. PCR products were electrophoresed on 8–12% non-reducing polyacrylamide gels. Lanes 1, 2 and 3 are positive controls amplified using h2B4-A plasmid clone, h2B4-B plasmid clone and 1 : 1 mixture of h2B4-A and -B as templates, respectively. While some SLE patients (patient 16, SLEDAI = 4, patient 22, SLEDAI = 4 and patient 27, SLEDAI = 2) have dominant expression of h2B4-A, patients with active SLE (patient 1, SLEDAI = 15, patient 3, SLEDAI = 12 and patient 4, SLEDAI = 10) have comparable levels of expression in h2B4-A and h2B4-B. Primers simultaneously amplifying two isoforms of CS1 and 2B4 were used. Thirty SLE patients and 27 healthy controls were used for RT–PCR. Experiments were repeated twice and representative data were shown.

expressed differentially in lupus, we analysed mRNA expression of h2B4-A and h2B4-B in total PBMC from patients with SLE and healthy controls by RT–PCR. We used common PCR primers detecting both h2B4-A and h2B4-B forms, which generate PCR products of 137 bp for h2B4-A and 152 bp for h2B4-B. Because of the small difference in size between h2B4-A and h2B4-B, the PCR products were electrophoresed on 8–12% non-denaturing polyacrylamide gels. As seen in Fig. 1b, healthy individuals expressed five- to eightfold higher levels of 2B4-A than 2B4-B. However, some SLE patients showed more predominance (more than 10-fold) of 2B4-A over 2B4-B than in healthy controls (patient 16, SLEDAI = 4; patient 22, SLEDAI = 4; and patient 27, SLEDAI = 2). Interestingly, some patients with active SLE showed comparable levels of 2B4-A and 2B4-B (patient 1, SLEDAI = 15; patient 3, SLEDAI = 12; and patient 4, SLEDAI = 10). These data indicate clearly that splicing of h2B4 mRNA is regulated differentially in SLE.

Altered surface CS1 expression in SLE

In order to determine whether the surface expression of CS1 is altered in SLE, we examined the proportion of CS1-expressing cells in total PBMCs, $CD3^+$ T cells, $CD19^+$ B cells and $CD56^+$ NK cells in patients with SLE and healthy individuals by flow cytometry. The proportion of CS1-expressing cells in total PBMCs, T cells and NK cells was not significantly different between healthy controls and patients with SLE (Fig. 2a–c). However, we found an increased proportion of CS1-positive B cells in PBMCs of SLE patients *versus* healthy controls (Fig. 2d). When we performed correlation analysis to find the relationship between this population and disease activity, it did not reach statistical significance because the number of patients with active SLE was not great enough (data not shown). However, linear regression analysis showed that the proportion of CS1-positive B cells increases linearly with increased SLEDAI score ($P = 0{\cdot}035$, $R^2 = 11{\cdot}4\%$; Fig. 2e).

Because the proportion of cells can be affected by a relative lymphopenia in patients with SLE, we also determined the mean fluorescence intensity ratio (MFIR), which represents the density of receptors at the single-cell level (Table 2). MFIR of $CS1^+$ cells in total PBMCs was not significantly different between healthy controls and SLE patients. However, $CD3^+$ $CS1^+$ T cells up-regulated CS1 expression significantly at the single-cell level. In contrast, all NK cells down-regulated CS1

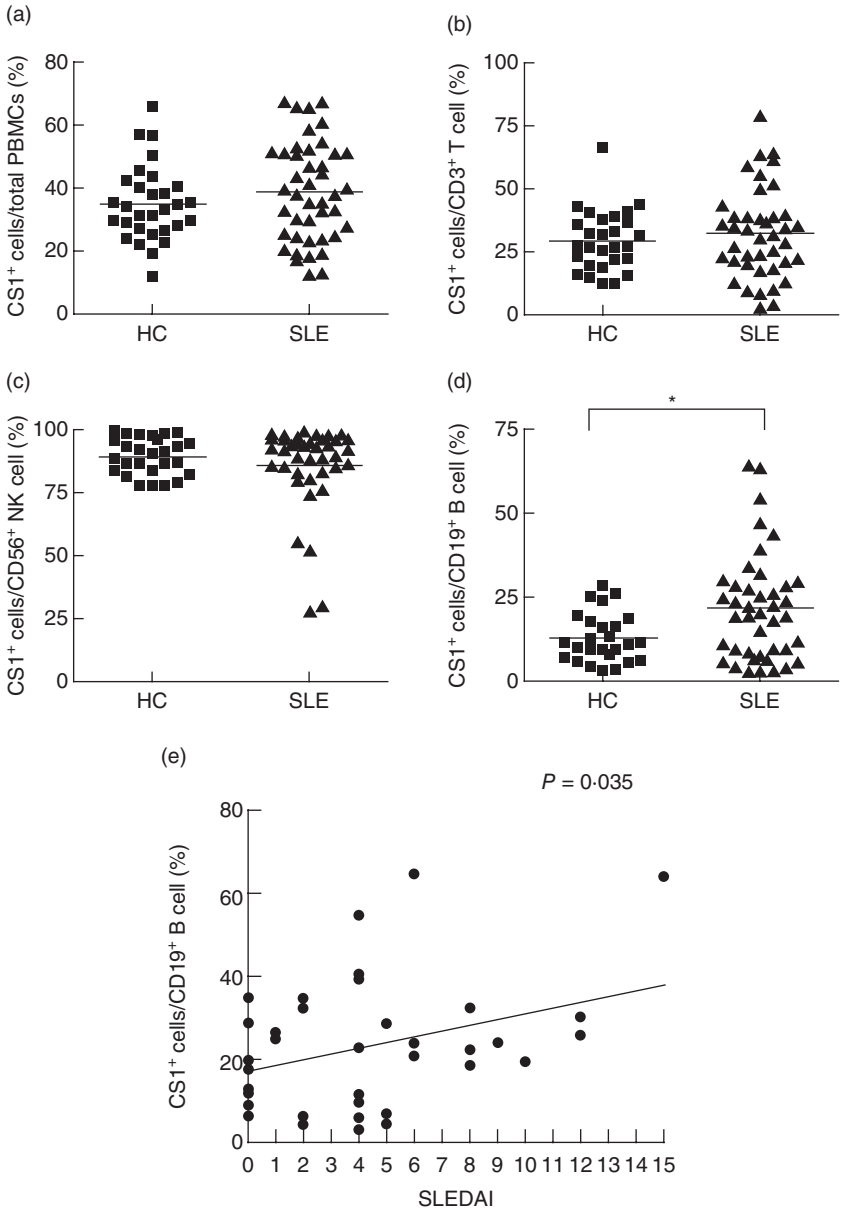

Fig. 2. Increased proportion of CS1-expressing B cells in systemic lupus erythematosus (SLE) patients is observed with increased disease severity. (a–d) Comparison of the proportion of CS1-expressing cells in gated populations from peripheral blood mononuclear cells (PBMCs) between SLE patients (SLE) ($n = 40$) and healthy controls (HC) ($n = 30$). (a) Total lymphocyte population was gated based on forward- and side-scatter. (b) T cells stained with anti-CD3-fluorescein isothiocyanate (FITC) were gated. (c) Natural killer (NK) cells stained with anti-CD56-allophycocyanin (APC) were gated. (d) B cells stained with anti-CD19-APC were gated. For each gate, the proportion of CS1-expressing cells was calculated by subtracting the percentage of phycoerythrin (PE)-conjugated mouse immunoglobulin (Ig)G2a-stained cells from the percentage of PE-conjugated anti-CS1 antibody-stained cells. Asterisk indicates significant differences between HC and SLE groups. (e) Linear regression analysis shows that there is a linear relationship with positive slope between the proportion of CS1-positive B cells and SLEDAI scores ($P = 0{\cdot}035$).

expression significantly compared to healthy controls. For analysis of B cells, we gated total B cells including both CS1-positive and CS1-negative B cells, because percentages of CS1-positive B cells are very low in healthy controls. Despite the significant percentage increase of CS1-positive B cells, MFIR shift in $CS1^+$ cells gated within total B cells did not reach significant levels compared to healthy controls. Collectively, these data suggest that CS1-expression is regulated dynamically at the cellular and molecular levels in SLE.

Analysis of CS1-positive cells in $CD19^+$ B cells in SLE

Recently, a number of different subsets of circulating B cells were reported in SLE, including naive B cells, memory B cells, plasma cells and plasmablasts. These cells can be identified by surface markers such as surface immunoglobulins (IgM and IgD), CD19, CD20, CD21, CD27, CD38, CD95 and human leucocyte antigen D-related (HLA-DR). Interestingly, we found that CS1 expression can also identify different subsets of SLE B cells. Figure 3 shows that co-staining with CD19 and CS1 distinguishes three distinct subsets of B cells: CD19-middle, CS1-negative B cells; CD19-high, CS1-low B cells; and CD19-low, CS1-high B cells (best illustrated by Fig. 3d). As shown in Fig. 3a–c, healthy individuals had CD19-middle, CS1-negative B cells as a major B cell population. In contrast, most SLE patients had all three B cell populations, and all patients exhibiting a high proportion of CS1-positive B cells essentially had CD19-high and CD19-low B cell populations. As shown in Fig. 3e,f, some SLE patients displayed CD19-low, CS1-high B cells as their major B cell populations. Notably, as

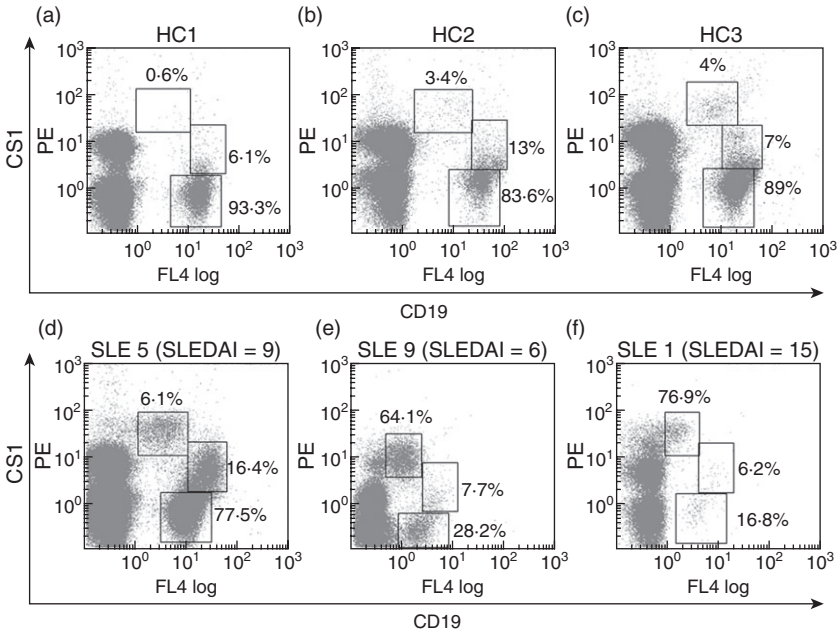

Fig. 3. Co-staining of CD19 and CS1 distinguishes three distinct subsets of B cells in systemic lupus erythematosus (SLE)-CD19-middle, CS1-negative B cells (bottom square); CD19-high, CS1-low B cells (middle square); and CD19-low, CS1-high B cells (upper square). Representative dot plots from three healthy controls (a–c) and three SLE patients (d), patient 5, SLE Disease Activity Index (SLEDAI) = 9; (e), patient 9, SLEDAI = 6; (f), patient 1, SLEDAI = 15) were shown. Anti-CD56-allophycocyanin (APC) and anti-CS1-phycoerythrin (PE) were used to stain B cell populations in healthy control *versus* SLE patient and percentage of each subset in total CD19 B cells was indicated.

seen in Fig. 3f, one patient with active SLE (patient 1, SLEDAI = 15) displaying the highest percentage of CD19-low, CS1-high B cells had a very low number of $CD19^+$ B cells, probably affected by lymphopenia.

Altered surface 2B4 expression in SLE

Next, we analysed the proportion of 2B4-expressing cells in total PBMCs, $CD3^+$ T cells, $CD56^+$ NK cells and $CD14^+$ monocytes in patients with SLE and healthy controls. As shown in Fig. 4, the proportion of 2B4-positive cells in total PBMCs and T cells was not significantly different between healthy controls and SLE patients (Fig. 4a,b). However, the proportion of 2B4-expressing cells was decreased significantly in $CD56^+$ NK cells and $CD14^+$ monocytes from patients with SLE compared to healthy controls (Fig. 4c,d). Although all monocytes are known to express 2B4, monocytes from two patients with SLE (patient 7, SLEDAI = 8 and

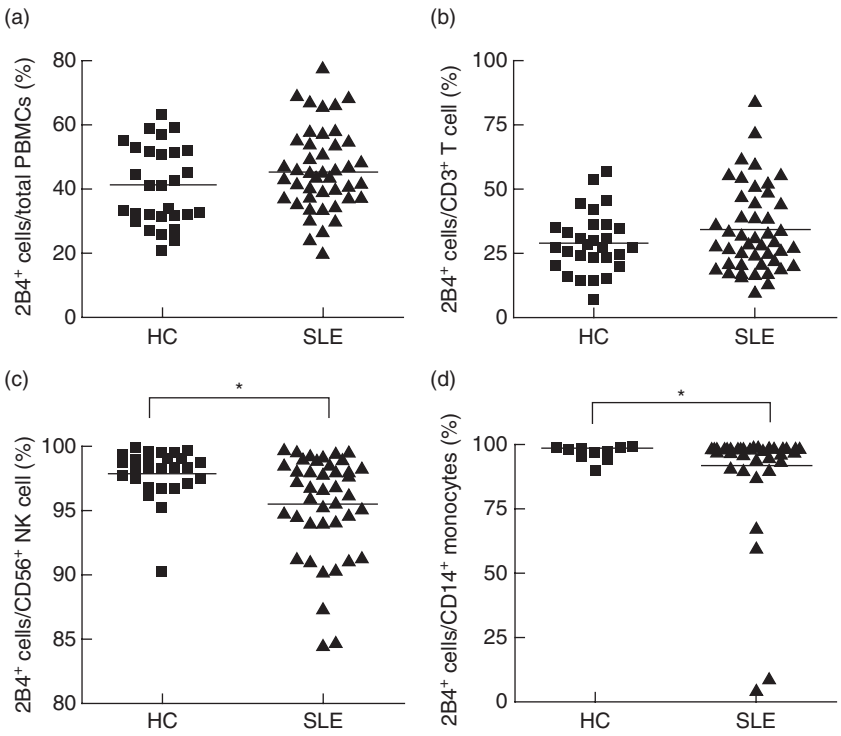

Fig. 4. Comparison of the proportion of 2B4-expressing cells in gated populations from peripheral blood mononuclear cells (PBMCs) between systemic lupus erythematosus (SLE) patients ($n = 45$) and healthy controls (HC) ($n = 30$). (a) Lymphocyte and monocyte populations were gated based on forward- and side-scatter. (b) T cells stained with anti-CD3-fluorescein isothiocyanate (FITC) were gated. (c) Natural killer (NK) cells stained with anti-CD56-allophycocyanin (APC) were gated. (d) Monocytes stained with anti-CD14-FITC were gated. For each gate, the proportion of 2B4-expressing cells was calculated by subtracting the percentage of phycoerythrin (PE)-conjugated mouse IgG1-stained cells from the percentage of PE-conjugated anti-2B4 antibody-stained cells. Asterisk indicates significant differences between HC and SLE groups.

patient 17, SLEDAI = 4) showed almost no expression of 2B4. Interestingly, when we compared the expression of 2B4 at the single-cell level, the MFIR of 2B4 was down-regulated significantly by all 2B4-expressing cells, including total PBMCs, $CD3^+$ T cells, $CD56^+$ NK cells and $CD14^+$ monocytes (Table 2). Consistent with the 2B4 splice variant result, these data indicate clearly that the expression of 2B4 is altered in SLE.

Discussion

In the present study we have analysed the expression and differential splicing of 2B4 and CS1, two members of the SLAM family in PBMCs from patients with SLE. The important roles of SLAM family receptors are recognized increasingly due to their broad expression in immune cells, including haematopoietic stem and progenitor cells [47]. As most SLAM family receptors are self-ligands, one important feature of these receptors is their capability to mediate both homotypic and heterotypic cell-to-cell interactions. For example, CS1-expressing B cells can interact not only with nearby CS1-expressing B cells but also with other immune cells expressing CS1, such as dendritic cells. Unlike other members of the SLAM family, the ligand for 2B4 is CD48. However, 2B4-expressing cells can also interact homotypically with each other because CD48 is expressed on all haematopoietic cells, including 2B4-expressing cells. There is an accumulation of data demonstrating a critical role played by SLAM family receptors in immune regulation [48–50].

SLE is characterized by hyperreactive B cells that produce pathogenic autoantibodies. However, detailed features of B cell abnormalities are largely unknown. Recently, a number of different subsets of circulating B cells were reported in SLE, including naive B cells, memory B cells, plasma cells and plasmablasts [51]. Our flow cytometry study also found distinct subsets of CD19-positive B cells in PBMCs of SLE patients, based on CS1 expression; CS1-negative B cells (CD19-middle), CS1-low B cells (CD19-high) and CS1-high B cells (CD19-low) (Fig. 3). According to a recent study, the majority of $CD19^+$ B cells are IgD^+ and $CD27^-$, indicating naive B cells [52]. They also reported CD19-high B cells as autoreactive memory B cells, and the frequency of this population correlates with disease activity [52,53]. Also, active SLE disease has been shown to correlate with a high frequency of plasma cells, which express high levels of CD27 and low levels of CD19 [54,55]. Based on these studies, we believe that CS1-negative, CD19-middle B cells are naive B cells; CS1-low, CD19-high B cells are memory B cells; and CS1-high, CD19-low B cells are plasma cells. The idea that the CS1-high cells are plasma cells is supported by recent publications [40–42]. The CS1-high, CD19-low B cells expressed high levels of CD27, indicating that they are plasma cells or plasmablasts. It is noteworthy that some patients with active SLE have these CS1-high B cells as their major B cell population (Fig. 3). As HLA-DR staining differentiates CD27-positive cells further into HLA-DR-high plasmablasts or HLA-DR-low plasma cells, it will be interesting to investigate whether CS1-high B cells are plasmablasts or plasma cells [51].

We found that SLE patients have an increased proportion of CS1-positive B cells. In addition, regression analysis showed that there is a linear relationship, with a positive slope between the proportion of CS1-positive B cells and disease activity (Fig. 2e). These data provide the possibility that altered CS1 expression in B cells might be critical in SLE pathogenesis. SLE B cells undergo active proliferation and differentiation [56]. Our previous study showed that CS1 induces B cell proliferation by increasing autocrine cytokine production. This study also showed that the expression of CS1 on B cells is induced upon CD40-mediated B cell activation [37]. Because CS1 is homophilic, it will result in further proliferation of CS1-expressing B cells. Thus, elevated expression of CS1 on B cells in SLE may enhance B cell proliferation. In fact, we observed that B cells isolated from patients with SLE show more proliferation in response to agonist anti-CS1 antibody than those from healthy controls (data not shown). At present, we do not know whether SLE is causing the higher expression of CS1 on B cells, or the elevated CS1 expression seen in B cells from SLE patients is causing the proliferation of B cells. The mechanism of CS1 gene induction is being investigated, which may provide a better understanding of the CS1 function in normal and disease conditions. The critical role of CS1 in controlling B cell proliferation is indicated further by recent multiple myeloma studies. CS1 is overexpressed by multiple myeloma cells and promotes cell adhesion, clonogenic growth and tumorigenicity via interactions with bone marrow stromal cells [40,41]. An anti-CS1 humanized monoclonal antibody has been shown to inhibit multiple myeloma cell adhesion and induce NK cell cytotoxicity against multiple myeloma cells [41]. It will be valuable to find out whether use of anti-CS1 monoclonal antibodies (mAb) could dampen the autoantibody production by B cells in SLE patients.

Our flow cytometry data showed that the proportion of 2B4-expressing NK cells are reduced in SLE patients compared to healthy controls (Fig. 4). In addition, the mean fluorescence intensity ratio (MFIR) of 2B4 was down-regulated significantly by all 2B4-expressing cells, including NK cells (Table 2). A possible mechanism for this 2B4 down-regulation can be suggested from previous studies, showing that 2B4-mediated stimulation of human NK cells leads to the down-regulation of surface 2B4 by reduced promoter activity as well as receptor internalization [57,58]. This implies that 2B4–CD48 interaction might be involved actively in SLE. Furthermore, our study using 2B4-deficient mice showed that 2B4–CD48 interactions play a regulatory role in generating gender-specific immune responses. This gender-specific immune response was mediated by NK cells [34]. Thus, one could speculate that reduced expression of

2B4 on NK cells from SLE patients may be involved in the gender bias seen in SLE.

Analysis of expression of CS1 isoforms indicates differential expression of CS1-L and CS1-S isoform in SLE PBMCs, reminiscent of Ly108 expression in lupus-prone mice [59,60]. The CS1-S isoform does not contain two ITSMs and does not mediate signalling [38]. Healthy individuals express three- to sevenfold higher levels of CS1-L over CS1-S. In SLE patients this expression ratio is altered, affecting signalling via CS1. We have also found that healthy individuals expressed five- to eightfold higher levels of h2B4-A than h2B4-B. However, some patients with SLE showed increased expression of h2B4-B, while some patients with SLE showed more predominance of h2B4-A over h2B4-B than in healthy controls. The structural difference between 2B4 and A and 2B4-B is found in the ligand binding region of the extracellular domain, and our recent study showed that h2B4-A and h2B4-B activate NK cells differentially upon CD48 interaction [23]. At present the ligand for h2B4-B is not known. If h2B4-B interacts with an unidentified ligand, altered expression of h2B4-B in SLE may impact immune signalling in SLE. Further studies on the functional consequences of altered expression of SLAM family receptors will greatly improve our understanding of SLE pathogenesis.

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Disclosure

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. CS1-high expressing B cells are plasma cells. Total peripheral blood mononuclear cells (PBMCs) from healthy individual (a) and systemic lupus erythematosus (SLE) patients with active disease (b) were first analysed by CD19 *versus* CS1. CS1-high B cells (blue dots), CS1-low B cells (red dots) and CS1-negative negative B cells (green dots) were gated and the surface expression of CD27 is shown in histogram. As seen in (b), CS1-high expressing B cells express high levels of CD27 (mean fluorescence intensity: 25), indicating that they are plasma cells.

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