from B6 mice were harvested and then prepared for Affymetrix microarray analysis as described above. Biotinylated antisense cRNA was prepared using two cycles of *in vitro* amplification according to the Affymetrix Small Sample Labeling Protocol II. Fifteen micrograms of biotinylated cRNA was fragmented and hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 arrays at the Takara Bio Genomics Center. Data were analysed using a Bioconductor (version 1.9) and statistical software R and GeneSpring GX version 7.3.1 (Silicon Genetics). All microarray data have been deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-1343. The data set of microarray analysis referred in this article is identical to a data set from our previous study[14].

Quantification of TGF-β family members. Each T-cell subset was plated into anti-CD3- or CD3/CD28-coated wells at 3×10^5 cells per well in serum-free X-Vivo-20 medium (Lonza) for the determination of TGF-β1 and 2, or the RPMI medium described above for TGF-β3, respectively. All cultures were incubated at 37 °C for 72 h, and the supernatants were collected and stored at −80 °C before the measurement of TGF-β family members, unless otherwise mentioned. TGF-β1, 2 and 3 levels in supernatants were determined with the TGF-β1 Emax ImmunoAssay System (Promega), TGF-β2 Quantikine ELISA Kit (R&D Systems) and TGF-β3 ELISA Kit (Mybiosource), respectively, according to the manufacturer's protocol. TGF-β3 levels in the RPMI medium supplemented with 10% FBS were lower than the minimum detectable levels of the TGF-β3 ELISA Kit.

Western blot analysis. MACS-purified B cells from B6 mice were pretreated with 0.75 μM CpG-ODN (ENZO Life Science) for 72 h supplemented with or without rTGF-β3 (20 ng ml^{-1}) during the last 16 h, and subsequently stimulated with 10 μg ml^{-1} anti-CD40 mAb, 10 μg ml^{-1} rIL-4 or 10 μg ml^{-1} anti-IgM F(ab)'$_2$ in RPMI medium for the indicated time. Following the stimulation, cells were prepared in Lysis Buffer (50 mM Tris-HCl, 0.15 M NaCl, 1% Triton X-100, 1 mM EDTA), denatured in 2 × Laemmli Buffer (Bio-Rad) at 95 °C for 5 min and resolved on Mini-PROTEAN TGX precast gels (Bio-Rad). Total protein concentrations in the cell lysates were determined using a BCA Protein Assay kit (Pierce). Following blotting on polyvinylidene fluoride membranes and blocking with 5% BSA, blots were probed with antibodies against phospho- or total STAT6, NF-κB p65, or Syk in 1:1,000 dilution (all purchased from Cell Signaling Technology), as well as with secondary anti-rabbit-IgG-HRP (Invitrogen) in 3:10,000 dilution. Membranes were developed with ECL Prime substrate (GE Healthcare). Images have been cropped for presentation. Full-size images are presented in Supplementary Fig. 10

ChIP sequencing analysis. LAG3$^+$ Treg cells were fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Chromatin was isolated by the addition of lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated and the DNA sheared to an average length of 300–500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantitation of the total chromatin yield. An aliquot of chromatin (30 μg) was precleared with protein A agarose beads (Invitrogen). Genomic DNA regions of interest were isolated using 4 μg of antibody against Krox20 (Covance, PRB-236P). Complexes were washed, eluted from the beads with SDS buffer and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65 °C, and ChIP DNA was purified with phenol–chloroform extraction and ethanol precipitation. Illumina sequencing libraries were prepared from the ChIP and Input DNAs by the standard consecutive enzymatic steps of end-polishing, dA-addition and adaptor ligation. After a final PCR amplification step, the resulting DNA libraries were quantified and sequenced on NextSeq 500. Sequences (75 nt (nucleotides) reads, single end) were aligned to the mouse genome (mm10) using the BWA algorithm (default settings). Duplicate reads were removed and only uniquely mapped reads (mapping quality ≥ 25) were used for further analysis. Alignments were extended *in silico* at their 3′-ends to a length of 200 bp, which is the average genomic fragment length in the size-selected library, and assigned to 32-nt bins along the genome. Krox20 peak locations were determined using the Bioconductor package BayesPeak[51].

***In vitro* helper T-cell differentiation and cytokine analysis.** MACS-sorted CD4$^+$ T cells described above were further purified as CD4$^+$CD25$^-$CD62L$^+$CD44$^-$ naive T cells by FACS, and cells were seeded at a density of 3×10^5 cells per 100 μl of RPMI culture medium described above in 96-well plates coated with 2 μg ml^{-1} anti-CD3 mAb and 2 μg ml^{-1} anti-CD28 mAb. Cytokines for effector cell polarization were as follows: Th0, anti-IFN (interferon)-γ (10 μg ml^{-1}; XMG1.2) and anti-IL-4 (10 μg ml^{-1}; 11B11); Th0, anti-IFN-γ (10 μg ml^{-1}; XMG1.2 (BD Pharmingen)) and anti-IL-4 (10 μg ml^{-1}; 11B11 (BD Pharmingen)); Th1, IL-12 (10 ng ml^{-1} (R&D Systems)), IL-2 (50 μg ml^{-1} (R&D Systems)) and anti-IL-4 (10 μg ml^{-1}; 11B11); Th17, TGF-β1 (1ng ml^{-1}), IL-6 (50 ng ml^{-1} (BioLegend)), IL-23 (50 ng ml^{-1} (R&D Systems)), anti-IFN-γ (10 μg ml^{-1}; XMG1.2) and anti-IL-4 (10 μg ml^{-1}; 11B11); induced Treg, TGF-β1 (5 ng ml^{-1}) and anti-IL-4 (10 μg ml^{-1}; 11B11). The culture supernatants were harvested on day 5, and TGF-β3 levels were analysed using ELISA, as described above.

***In vitro* CD4$^+$Egr2$^+$LAG3$^+$ Treg differentiation by IL-27.** *In vitro* stimulation of MACS-purified naive CD4$^+$ T cells using the CD4$^+$CD62L$^+$ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol was performed in 24- or 96-well plates coated with 2 μg ml^{-1} anti-CD3 mAb and 1 μg ml^{-1} anti-CD28 mAb in RPMI medium described above supplemented with 25 ng ml^{-1} IL-27 for 5 days. IL-27-treated naive T cells were subsequently sorted using flow cytometry for CD4 expression and used for each assay. For determination of TGF-β3 levels in the culture supernatants by ELISA, IL-27-treated T cells were stimulated with phorbol myristate acetate (50 ng ml^{-1}; Sigma) and ionomycin (1 μg ml^{-1}; Sigma) for the last 4 h.

Construction of the TGF-β3 expression plasmid vector. Full-length fragments of murine TGF-β3 were isolated from an OmicsLink Expression-Ready ORF-cloning vector (GeneCopoeia) containing a *Tgfb3* cDNA (NM_009368). *Tgfb3* cDNAs were subcloned into the pCAGGS vector[52], which has the CAG (cytomegalovirus immediately early enhancer/chicken β-actin hybrid) promoter, using EcoRI sites and designated as pCAGGs-*Tgfb3*. Recombinant plasmids were then transformed into competent cells of *Escherichia coli* JM109 and purified using plasmid purification columns using the EndFree Plasmid Maxi Kit (Qiagen) according to the manufacturer's protocol. The purified plasmid DNA was diluted to 1 μg μl^{-1} with sterile PBS (pH 7.4) immediately before use.

Intravenous injection of plasmid DNA. MRL/*lpr* mice were injected i.v. with 100 μg of plasmid DNA (pCAGGs-*Tgfb3* or control pCAGGS) in sterile PBS (pH 7.4) twice at an interval of 4 weeks. Proteinuria was assessed semiquantitatively at weekly intervals, and renal pathology was evaluated as described above 6 weeks after the final administration.

Flow cytometric assessment of human PBMCs. All human samples were obtained under informed consent. The protocol for the human research project has been approved by the Ethics Committee of the University of Tokyo. PBMCs from healthy and SLE patients were isolated by Ficoll-Paque (Amersham Pharmacia Biotech) gradient. After the cells were washed, they were stained with indicated the mAbs for 20 min at 4 °C. To prevent nonspecific binding of mAbs, Human Fc Receptor Binding Inhibitor (eBioscience) was added before staining with labelled mAb. Dead cells were excluded by 7-AAD. The fluorescence-positive cells were analysed by a Moflo XDP cell sorter. The five distinct subpopulations are as follows: (naive T) CD4$^+$CD25$^-$CD45RA$^+$CCR7$^+$ cells; (CD25$^+$ Treg) CD4$^+$CD25$^+$CD127^{dim}CD45RA$^-$ cells; (LAG3$^+$ Treg) CD4$^+$CD25$^-$CD45RA$^-$LAG3$^+$ cells; (Tfh) CD3$^+$CD19$^-$CD4$^+$CD25$^-$LAG3$^-$CXCR5$^+$CD45RA$^-$ cells and (B cells) CD3$^-$CD19$^+$ cells.

Quantitative real-time PCR expression analysis of human T-cell subsets. Total RNA isolated from human naive T and CD25$^+$ Treg, and LAG3$^+$ Treg stimulated with plate-bound 5 μg ml^{-1} anti-CD3 for 72 h were analysed for *EGR2, IL10, IFNG*, and *FOXP3* mRNA expressions, as described above. *TGFB3* mRNA expression was determined using unstimulated cells from each T-cell subset.

Quantification of human IL-10. For cytokine analysis, human naive T, CD25$^+$ Treg and LAG3$^+$ Treg were plated into 5 μg ml^{-1} anti-CD3/anti-CD28-coated 96-well flat-bottomed plates at 2×10^4 cells per well in RPMI-1640 medium supplemented with 10% FBS, 100 μg ml^{-1} L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 50 μM 2-mercaptoethanol. The culture supernatants were harvested on day 3, and IL-10 levels were analysed with ELISA using OptEIA Human IL-10 ELISA Kit II (BD Biosciences) according to the manufacturer's protocol.

Human LAG3$^+$ Treg suppression assays. RPMI-1640 medium as described above was used for co-culture. FACS-purified 1×10^5 human B cells and FACS-purified 5×10^4 human T_{FH} cells were seeded in round-bottom 96-well plates with or without FACS-purified 1×10^5 human CD25$^+$ Treg or LAG3$^+$ Treg in the presence of 2 μg ml^{-1} recombinant staphylococcal enterotoxin B (Toxin Technology). The culture supernatants were harvested on day 12, and total IgG levels were analysed with ELISA using Human IgG Quantitation Set kits (Bethyl Laboratories), according to the manufacturer's protocol.

Statistical analysis. Statistical significance, normal distribution and similar variance between groups were analysed using GraphPad Prism version 5.03 (GraphPad Software Inc.). Quantitative histology and proteinuria progression were analysed with the Mann–Whitney *U*-test. For the comparison of more than three groups, a one-way analysis of variance followed by a Bonferroni multiple comparison test was performed. All other statistical differences were determined using the two-tailed Student's *t*-test. If the variance was unequal, Welch's correction was applied to Student's *t*-test. Differences were considered statistically significant at $P < 0.05$ for all tests. All data in the figures are expressed as mean ± s.d. Sample size was estimated on the basis of numbers typically used in previous studies. No statistical method was used to predetermine sample size. No samples or animals

were excluded from the analyses. We did not perform randomization of animals except for adoptive transfer studies in MRL/*lpr* mice. Animal studies were not performed in a blinded manner, except for histological analyses.

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Author contributions

T.O., K.F., S.S. and K.Y. conceived, designed and analysed the experiments and contributed to writing the manuscript. T.O. carried out all experiments with the exceptions noted below. K.M. and M.I. conducted the *in vitro* B-cell suppression assays. K.F. contributed to the *in vivo* B-cell suppression assays. Y.I. performed the IL-27-treated T-cell experiments. S.S. and S.N. performed experiments using human samples. J.M. contributed to construction of plasmid vectors. T.K. performed systemic treatment of MRL/*lpr* mice using plasmid vectors. H.S. provided transgenic mouse strains and technical assistance.

Additional information

Accession codes: The accession number for the microarray data presented in this study is E-MEXP-1343 [ArrayExpress database].

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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EXTENDED REPORT

Efficacy and safety of subcutaneous tabalumab in patients with systemic lupus erythematosus: results from ILLUMINATE-1, a 52-week, phase III, multicentre, randomised, double-blind, placebo-controlled study

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ABSTRACT

Objectives Evaluate efficacy and safety of tabalumab, a human IgG4 monoclonal antibody that binds and neutralises membrane and soluble B-cell activating factor (BAFF) versus placebo plus standard of care (SoC) in patients with systemic lupus erythematosus (SLE).

Methods This phase III, 52-week study randomised 1164 patients with moderate-to-severe SLE (Safety of Estrogens in Lupus Erythematosus National Assessment—SLE Disease Activity Index ≥6 at baseline). Patients received SoC plus subcutaneous injections of tabalumab or placebo, starting with a loading dose (240 mg) at week 0 and followed by 120 mg every two weeks (120 Q2W, n=387), 120 mg every four weeks (120 Q4W, n=389) or placebo Q2W (n=388). Primary endpoint: proportion of patients achieving SLE Responder Index 5 (SRI-5) response at week 52.

Results Similar proportions of patients in each group achieved SRI-5 response at week 52 (120 Q2W: 31.8%; 120 Q4W: 35.2% and placebo: 29.3%). Key secondary endpoints were not met. In a sensitivity analysis not excluding patients who decreased antimalarials or immunosuppressants, SRI-5 response was achieved with 120 Q4W (37.0% vs 29.8% placebo; p=0.021), but not 120 Q2W (34.1%; p=0.171). Significant reductions in anti-dsDNA antibodies, increases in C3 and C4, and reductions in total B cells and immunoglobulins were observed with tabalumab. No differences were observed between treatment groups in percentage of deaths (120 Q2W: 0.8%; 120 Q4W: 0.5%; placebo: 0.5%), serious adverse events (AEs) (range 11.1–14.4%) or treatment-emergent AEs (range 81.1–82.3%).

Conclusions Tabalumab had biological activity—changes in anti-dsDNA, complement, B cells and immunoglobulins—consistent with BAFF pathway inhibition. Key clinical efficacy endpoints did not achieve statistical significance. Safety profiles were similar with tabalumab and placebo.

Trial registration number NCT01196091.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease with an estimated prevalence of 7–160 cases in 100 000 worldwide.[1 2] Mortality has improved, probably due to faster and more accurate diagnosis, increased disease awareness, more appropriate use of corticosteroids and cytotoxic/immunosuppressive drugs, and a greater focus on treatment of comorbidities. However, few drugs are approved for the treatment of SLE, and a substantial unmet medical need remains.

There is compelling evidence that B cells are involved in the etiopathogenesis of SLE. B cells may be blocked directly by antibodies to CD20 or CD22, or via blockade of vital B-cell survival factors (B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL)).[3–5] BAFF is a tumour necrosis factor family ligand that is important for B-cell development, survival and differentiation.[6–8] BAFF serum is elevated in patients with SLE and correlate with autoantibody production and disease activity.[9 10] Thus, targeting B cells by neutralising BAFF may suppress SLE disease activity. Tabalumab is a fully human IgG4 monoclonal antibody, administered subcutaneously, that binds and neutralises both membrane and soluble BAFF. Two phase III studies, ILLUMINATE-1 and ILLUMINATE-2, evaluated the efficacy and safety of subcutaneous doses of tabalumab plus standard of care (SoC) versus placebo plus SoC in patients with active SLE. We present ILLUMINATE-1 results.

METHODS

Study design

Patients in ILLUMINATE-1, a 52-week, phase III, multicentre, randomised, double-blind, placebo-controlled study, were randomly assigned 1:1:1 (stratified by anti-dsDNA positivity and African descent) to receive subcutaneous tabalumab 120 mg every two weeks (Q2W) plus SoC (120 Q2W), tabalumab 120 mg every four weeks (Q4W) plus SoC (120 Q4W; administered by alternating 120 mg tabalumab and placebo injections Q2W), or placebo Q2W plus SoC. At week 0, the tabalumab groups received a 240 mg loading dose (2×120 mg injection) and the placebo group received two placebo injections. Blinded treatments were administered from week 0 through 50. After week 52, patients discontinued study drug and entered safety follow-up or entered a multiyear, open-label, safety extension study of tabalumab. Data from the double-blind, 52-week period are included in this report.

Patients were allowed to continue SoC for SLE, including non-steroidal anti-inflammatory drugs (unrestricted), corticosteroids, stable doses of antimalarials (such as chloroquine or hydroxychloroquine) and/or stable doses of immunosuppressants (such as azathioprine, methotrexate, mycophenolate, cyclophosphamide or cyclosporine). Increases or decreases in corticosteroid dose were allowed during the first 24 weeks, after which the dose had to be less than or equal to the baseline dose, and only reductions were allowed thereafter. No added, increases in or decreases in antimalarials or immunosuppressants were allowed at any time.

Entry criteria

Eligible patients were 18 years or older and had a diagnosis of SLE (fulfilled 4/11 American College of Rheumatology revised criteria),[11] a positive antinuclear antibody (HEp-2 ANA; titre ≥1:80) test by the central laboratory during screening (patients with negative ANA, but positive anti-dsDNA, were retested before being excluded), and a Safety of Estrogens in Lupus Erythematosus National Assessment—SLE Disease Activity Index (SELENA-SLEDAI) score ≥6[12] (see online supplementary text).

Endpoints

The primary endpoint was the proportion of patients achieving an SLE Responder Index 5 (SRI-5) response at week 52. The SRI-5 is a composite endpoint defined as ≥5 point improvement (reduction) in SELENA-SLEDAI score, no new British Isles Lupus Assessment Group (BILAG) 2004 index score of A or no more than one new BILAG B score, and no worsening (increase ≥0.3 points from baseline) in physician's global assessment (PGA).[13] Patients who did not meet all three clinical criteria, who added, increased or decreased antimalarial or immunosuppressant treatment, who could not adhere to corticosteroid-dosing requirements and/or who discontinued study prior to week 52 were considered non-responders.

Key secondary endpoints included time to first severe SLE flare on the SELENA-SLEDAI Flare Index,[14] proportion of patients with reduction in corticosteroid dose by ≥25% to ≤7.5 mg/day prednisone (or equivalent) for ≥3 consecutive months from weeks 24 through 52, and change from baseline on the Brief Fatigue Inventory at week 52.[15 16]

Other endpoints included change in proportion of SRI-5 responders over time; individual components of SRI-5; the proportion of patients achieving SRI-4 and SRI-6 through SRI-10 at week 52; and mean changes in SELENA-SLEDAI, PGA, Systemic Lupus Erythematosus Disease Activity Index 2000, Systemic Lupus International Collaborating Clinics (SLICC) damage index and disease-related biomarkers (anti-dsDNA, C3 and C4) at week 52. A medical team reviewed efficacy endpoint data for consistency. Serum tabalumab concentrations, changes in pharmacodynamic markers (total B cells, B-cell subsets, mean BAFF levels and immunoglobulin concentrations), on the Columbia-Suicide Severity Rating Scale and on the Quick Inventory of Depressive Symptomatology (QIDS-SR16), and other safety assessments (serious adverse events (SAEs), treatment-emergent AEs (TEAEs), AEs of special interest, vital signs, clinical laboratory data) were analysed (see online supplementary text).

Statistical analysis

All analyses of efficacy, serum tabalumab concentrations and biological activity were conducted on the intent-to-treat population. Safety analyses were applied to the safety population (patients who received ≥1 dose of study drug). For the primary endpoint (SRI-5), a sample size of 380 patients per group was predicted to provide >95% power to detect a statistically significant difference of ≥14% between the tabalumab and placebo groups. Statistical methods used a two-sided, 0.05 significance level and included logistic regression for binary efficacy variables, Cox proportional hazard model for time-to-event variables, Fisher's exact for discrete variables and mixed-effects model for repeated measures or analysis of covariance for continuous variables. A graphical approach for multiple comparisons was used to control the overall type I error in analysis of primary and key secondary endpoints. Additional analyses were performed to identify baseline characteristics associated with responders at week 52 and to evaluate how response to tabalumab relative to placebo varied across different categories within a subgroup. Analyses of efficacy variables were adjusted for randomisation stratification factors (anti-dsDNA status and race) and region. For all non-SRI continuous measures, a last observation carried forward (LOCF) approach was used.

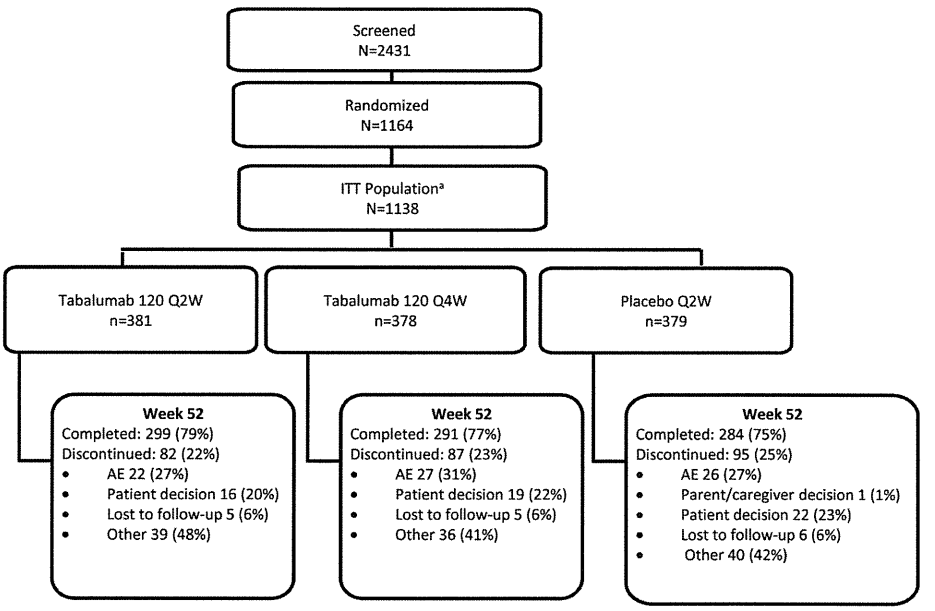

Figure 1 Flow diagram of patient disposition during the study. [a]Patients from two study sites were not included in the intent-to-treat (ITT) population due to violations at the study site. AE, adverse event; Q2W, every two weeks; Q4W, every four weeks.

Table 1 Baseline demographics and clinical characteristics

	120 Q2W n=381	120 Q4W n=378	Placebo n=379
Age, years	40±13	40±11	39±12
Age, range	18–87	19–79	18–72
Women, n (%)	354 (92.9)	352 (93.1)	360 (95.0)
Race, n (%)			
Indigenous American†	65 (17.1)	55 (14.6)	67 (17.7)
Asian	68 (17.8)	61 (16.1)	66 (17.4)
Black/African–American	40 (10.5)	41 (10.8)	39 (10.3)
Native Hawaiian or Pacific Islander	0	1 (0.3)	0
White	204 (53.5)	218 (57.7)	205 (54.1)
Multiple races	4 (1.0)	2 (0.5)	2 (0.5)
Hispanic/Latino origin, n (%)‡	119 (31.2)	116 (30.7)	122 (32.2)
Geographic region, n (%)			
USA/Canada	133 (34.9)	140 (37.0)	129 (34.0)
Mexico/Central America/South America	89 (23.4)	84 (22.2)	92 (24.3)
Asia Pacific	66 (17.3)	58 (15.3)	62 (16.4)
Europe	74 (19.4)	77 (20.4)	75 (19.8)
Africa/Middle East	19 (5.0)	19 (5.0)	21 (5.5)
SLE disease activity			
Time since SLE onset, years	8±7*	8±8*	6±7
SELENA-SLEDAI organ system involvement, n (%)			
CNS	6 (1.6)	3 (0.8)	6 (1.6)
Vascular	28 (7.3)	29 (7.7)	30 (7.9)
Musculoskeletal	318 (83.5)	298 (78.8)	308 (81.3)
Renal	36 (9.4)	40 (10.6)	42 (11.1)
Mucocutaneous	344 (90.3)	348 (92.1)	349 (92.1)
Cardiovascular and respiratory	27 (7.1)	25 (6.6)	26 (6.9)
Immunologic	272 (71.4)	284 (75.1)	273 (72.0)
Constitutional	5 (1.3)	4 (1.1)	5 (1.3)
Haematologic	39 (10.2)	40 (10.6)	46 (12.1)
SELENA-SLEDAI score	10.2±3.5	10.4±3.6	10.7±3.9
SELENA-SLEDAI ≥10, n (%)	222 (58.3)	219 (57.9)	224 (59.1)
SLEDAI-2K score	10.6±3.7	10.7±3.9	10.8±4.0
≥1 A or 2 B BILAG scores, n (%)	228 (59.8)	228 (60.3)	220 (58.2)
PGA score	46.3±15.7	46.1±16.2	47.1±16.1
SLICC damage index	0.5±1.0*	0.6±1.2*	0.4±0.8
Medications			
Prednisone, n (%)	300 (78.7)	296 (78.3)	295 (77.8)
Mean prednisone dose (or equivalent), mg/day	11.9±7.4	12.1±7.8	12.1±7.9
Prednisone >7.5 mg/day, n (%)	197 (51.7)	200 (52.9)	196 (51.7)
Antimalarial, n (%)	241 (63.3)	241 (63.8)	243 (64.1)
Any immunosuppressant, n (%)	158 (41.5)	176 (46.6)	164 (43.3)
MMF, n (%)	24 (6.3)	38 (10.1)	32 (8.4)
AZA, n (%)	66 (17.3)	80 (21.2)	80 (21.1)
MTX, n (%)	50 (13.1)	40 (10.6)	40 (10.6)
Biomarkers and other laboratory measures			
≤45 mg/mmol	330 (86.8)	326 (86.2)	332 (87.6)
>45 mg/mmol	50 (13.2)	52 (13.8)	47 (12.4)
Total B-cell counts, median (range), cells/μL	131.5 (3–1979)	128.0 (6–1727)	129.5 (3–1345)
BAFF concentration, pg/mL	2559±8277	2230±4230	1753±2439
Anti-dsDNA ≥30 IU/mL, n (%)§	228 (59.8)	231 (61.1)	226 (59.6)
Anti-dsDNA, IU	107.2±113.5	110.4±111.6	107.1±112.4
C3 <LLN, n (%)	144 (37.9)	150 (39.8)	163 (43.1)
C3, g/L	1.0±0.3	1.0±0.3	1.0±0.3

Continued

Table 1 Continued

	120 Q2W n=381	120 Q4W n=378	Placebo n=379
C4 <LLN, n (%)	84 (22.1)	85 (22.5)	98 (25.9)
C4, g/L	0.2±0.1	0.2±0.1	0.2±0.1
IgG, g/L	15.6±5.7	15.7±5.9	15.6±5.6
IgG <LLN, n (%)	2 (0.5)	1 (0.3)	2 (0.5)
IgM, g/L	1.1±0.7	1.2±0.8	1.2±1.0
IgM <LLN, n (%)	36 (9.4)	34 (9.0)	35 (9.2)
IgA, g/L	3.1±1.6	3.2±1.6	3.1±1.5
IgA <LLN, n (%)	10 (2.6)	7 (1.9)	11 (2.9)
uPCR, n (%)			

*p<0.05, p value for comparison of treatment groups derived using an analysis of variance model with treatment group and geographic region fitted as explanatory variables.
†American Indian from North, Central and South America and Alaska Native.
‡Ethnicity (Hispanic/Latino) information was only required to be collected in the USA.
§Or >10 IU/mL for patients for whom the original DNA autoantibody assay was performed.
Anti-dsDNA, anti-double-stranded DNA; AZA, azathioprine; BAFF, B-cell activating factor; BILAG, British Isles Lupus Assessment Group index; CNS, central nervous system; Ig, immunoglobulin; LLN, lower limit of normal; MMF, mycophenolate mofetil; MTX, methotrexate; PGA, physician's global assessment; Q2W, every two weeks; Q4W, every four weeks; SELENA-SLEDAI, Safety of Estrogens in Lupus Erythematosus National Assessment—Systemic Lupus Erythematosus Disease Activity Index; SLE, systemic lupus erythematosus; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; SLICC, Systemic Lupus International Collaborating Clinics; uPCR, urine protein to urine creatinine ratio.

RESULTS

Patient disposition and baseline characteristics

Of the 1164 randomised patients, ≥75% in each group completed the 52-week trial (figure 1). Discontinuation rates and reasons for early discontinuation were similar across groups. Baseline demographics, SLE disease activity and medications were generally balanced across groups (table 1).

Efficacy

The primary endpoint, SRI-5 response at week 52, was not achieved in either treatment group (figure 2A, table 2). At week 52, SRI-5 (non-responder imputation (NRI)) response rates were 31.8% in the 120 Q2W and 35.2% in the 120 Q4W group versus 29.3% in the placebo group (p>0.05, each comparison). For all three components of SRI, the percentages of responders were higher in the tabalumab groups versus placebo (table 2). See online supplementary table S1 for specific SELENA-SLEDAI organ improvement.

None of the key secondary endpoints (ie, time to first severe SLE flare, corticosteroid-sparing effects and change from baseline in worst level of fatigue) were significantly different with tabalumab versus placebo (table 2). At week 52, the changes in

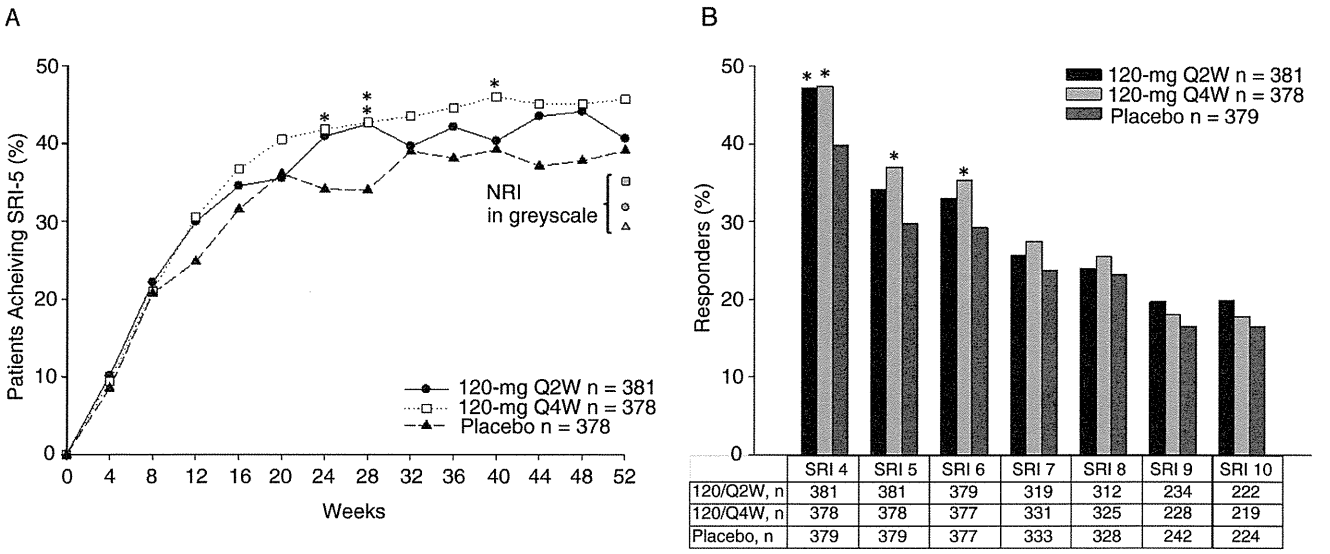

Figure 2 Clinical outcomes. (A) SLE Responder Index 5 (SRI-5) response rates over the 52-week treatment period. Primary endpoint analysis included patients who achieved SRI-5 endpoint without violating the concomitant medication rules. Patients who did not meet all three clinical criteria, who added, increased or decreased antimalarial or immunosuppressant treatment, who could not meet the corticosteroid-dosing requirements and/or who discontinued the study prior to week 52 were considered non-responders. p Value based on odds ratio estimated from logistic regression model. (B) SRI-4 through SRI-10 response rates at week 52. Predefined analyses of SRI endpoints described above used a modified concomitant medication rule where patients who decreased their antimalarials or immunosuppressants during the study were not automatic non-responders. SRI-6 through SRI-10 performed in subset of patients who entered study with Safety of Estrogens in Lupus Erythematosus National Assessment—SLE Disease Activity Index (SELENA-SLEDAI)≥6-SELENA-SLEDAI≥10, respectively. *p≤0.05 vs placebo; **p≤0.001 vs placebo. Symbols in greyscale represent 52-week endpoint data calculated using a non-responder imputation (NRI), circle=120 every two weeks (Q2W), square=120 every four weeks (Q4W) and triangle = placebo.

SELENA-SLEDAI scores were also similar in the 120 Q2W (least-squares (LS) mean: −5.1), 120 Q4W (LS mean: −5.4) and placebo groups (LS mean: −5.2).

In a predefined sensitivity analysis of the primary endpoint, where patients who *decreased* their antimalarials or immunosuppressants during the study were not automatic non-responders[17] SRI-5 (NRI) response rates were 34.1% in the 120 Q2W (p=0.171) and 37.0% in the 120 Q4W group (p=0.021) versus 29.8% in the placebo group at week 52 (figure 2B). In the same predefined analyses of SRI-4 and SRI-6 through SRI-10, both the 120 Q2W (47.2%) and 120 Q4W groups (47.4%) had a significantly higher percentage of SRI-4 responders at week 52 versus placebo (39.8%; p≤0.05, both comparisons), and significantly more 120 Q4W patients achieved SRI-6 response versus placebo (35.3% vs 29.2%, p=0.044) at week 52.

The effect of tabalumab was also evaluated in patients who were anti-dsDNA positive and who had low C3 and C4 levels at baseline. There was no significant difference between tabalumab and placebo in percentage of SRI-5 responders at week 52 (120 Q2W: 34.1%, 120 Q4W: 32.3%, placebo: 35.4%; p>0.05, each comparison).

The anti-dsDNA levels decreased significantly in both tabalumab groups versus placebo as early as week 4 (figure 3) and continued to decrease and remained well below baseline levels through week 52 (LS mean changes, 120 Q2W: −25.60 IU; 120 Q4W: −22.28 IU; placebo: −4.13 IU; p<0.001, each comparison). Of the patients with anti-dsDNA ≥30 IU/mL (positive) at

Table 2 Clinical and biomarker outcomes

	120 Q2W n=381	120 Q4W n=378	Placebo n=379
SRI-5	121 (31.8) p=0.409	133 (35.2) p=0.052	111 (29.3)
Components of SRI-5			
≥5 point improvement in SELENA-SLEDAI score	122 (32.0) p=0.618	134 (35.4) p=0.105	116 (30.6)
No worsening in BILAG*	247 (64.8) p=0.038	234 (61.9) p=0.177	219 (57.8)
No worsening in PGA†	245 (64.3) p=0.062	232 (61.4) p=0.263	220 (58.0)
Other clinical outcomes			
Severe flares on the SELENA-SLEDAI Flare Index			
Median time to first severe flare, days HR for severe flares (95% CI)	169 0.94 (0.67 to 1.32) p=0.724	146 0.79 (0.55 to 1.13) p=0.204	141
Corticosteroid sparing‡	46 (23.4) p=0.280	35 (17.5) p=0.747	37 (18.9)
BFI, LSM change	−1.31 p=0.163	−1.38 p=0.081	−1.01
SELENA-SLEDAI, LSM change	−5.1 p=0.832	−5.4 p=0.492	−5.2
PGA score, LSM change	−23.5 p=0.770	−23.9 p=0.587	−23.1
SLEDAI-2K, LSM change	−5.0 p=0.342	−5.2 p=0.141	−4.8
SLICC, LSM change	0.03 p=0.964	0.03 p=0.921	0.03
Biomarkers			
Anti-dsDNA, IU, LSM change	−25.6 p<0.001	−22.3 p<0.001	−4.1
Anti-dsDNA positive at baseline	n=228	n=231	n=226
Normalisation at week 52	32 (14)	36 (16)	20 (9)
Complement C3, g/L, mean change	0.046 p=0.002	0.047 p=0.007	0.015
Complement C4, g/L, mean change	0.031 p<0.001	0.031 p<0.001	0.005
Low complement (C3 and/or C4) at baseline	n=161	n=168	n=182
Normalised at week 52	47 (29)	61 (36)	40 (22)

All data are n (%) unless indicated otherwise.
*No new BILAG A and no more than one new BILAG B.
†Worsening defined as an increase of ≥0.3 points from baseline.
‡Percent of patients able to decrease corticosteroid dose by at least 25% to ≤7.5 mg/day prednisone for at least three consecutive months from weeks 24 to 52 without an increase in dose of either antimalarials or immunosuppressants at any time during the treatment period.
Anti-dsDNA, anti-double-stranded DNA; BFI, Brief Fatigue Inventory; BILAG, British Isles Lupus Assessment Group index; LSM, least-squares mean change from baseline, PGA, physician's global assessment; Q2W, every two weeks; Q4W, every four weeks; SELENA-SLEDAI, Safety of Estrogens in Lupus Erythematosus National Assessment—Systemic Lupus Erythematosus Disease Activity Index; SLE, systemic lupus erythematosus; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; SLICC, Systemic Lupus International Collaborating Clinics; SRI-5, SLE Responder Index 5.

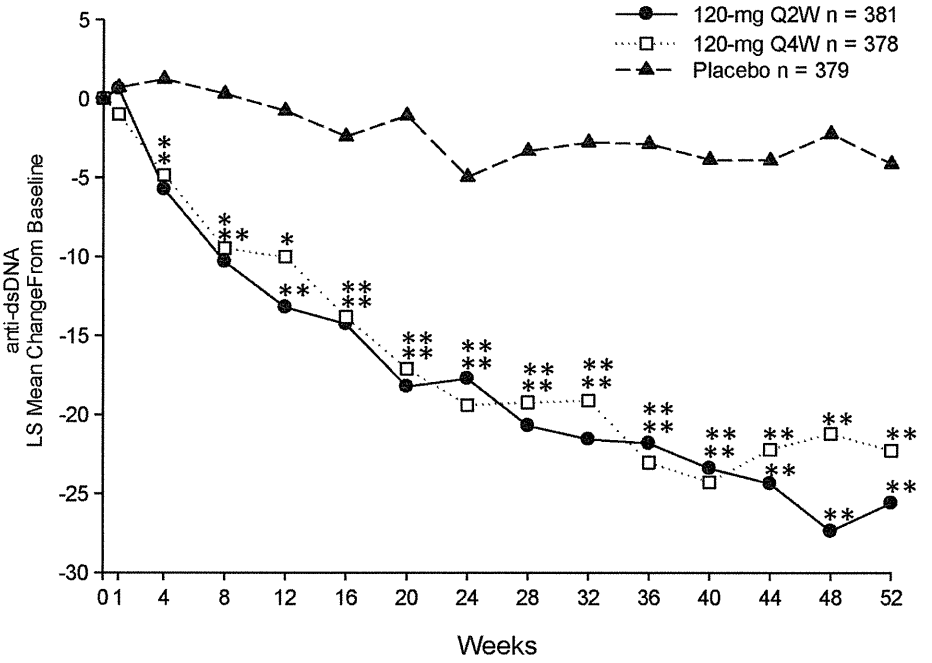

Figure 3 Mean change in anti-dsDNA over the 52-week treatment period. *p≤0.05 vs placebo; **p≤0.001 vs placebo. LS, least squares; Q2W, every two weeks; Q4W, every four weeks.

baseline, 14% in 120 Q2W and 16% in 120 Q4W versus 9% in placebo returned to normal levels (<30 IU/mL) at week 52.

Increases in C3 and C4 were observed in both tabalumab groups versus placebo at week 52 (table 2). Of the patients with low complement C3 and/or C4 at baseline, 29% in 120 Q2W and 36% in the 120 Q4W group versus 22% in the placebo group had normalised at week 52.

Other than sex, there were no baseline variables (ie, age, region, anti-dsDNA status, complement, race, ethnicity, disease severity (SELENA-SLEDAI <10 or ≥10), mucocutaneous or musculoskeletal organ involvement, PGA score, SLICC score, severe seropositivity, concomitant medication use, number of prior treatment failures, comorbidities, time since onset of SLE or BAFF concentration) for which the treatment effect differed across subgroups.

Pharmacodynamics

At week 52, tabalumab treatment was associated with increases in median BAFF concentrations from baseline (120 Q2W: 95349 pg/mL; 120 Q4W: 91332 pg/mL); as expected, these increases were significantly different from changes observed in the placebo group (27 pg/mL, p<0.001, both comparisons) due to presence of active drug binding to BAFF. In the tabalumab groups, median total B-cell counts initially increased from baseline at week 1 (120 Q2W: 64.5 cells/μL; 120 Q4W: 70.0 cells/μL; placebo: −1.0 cells/μL; p<0.001, each comparison) and subsequently decreased below baseline values through week 52 (LOCF) (120 Q2W: −68.0 cells/μL; 120 Q4W: −57.0 cells/μL; placebo: −7.5 cells/μL; p<0.001, each comparison) (see online supplementary figure S1A). B-cell subset changes in both tabalumab groups were significantly different from placebo; however, there was no clear dose separation in either tabalumab group. The largest magnitude of change for B cells was the reduction in the mature naive subset. No significant T-cell changes were observed. Statistically significant decreases in serum immunoglobulins were observed at each time point over the 52-week treatment period (see online supplementary figure S1 B,D). However, the number of patients who dropped below normal range was low in the 120 Q2W, 120 Q4W and placebo groups; IgA: 2 (0.5%), 4 (1.1%) and 2 (0.5%); IgG: 4 (1.1%), 2 (0.5%) and 2 (0.5%); and IgM: 64 (18.6%), 52 (15.2%) and 14 (4.0%), respectively.

Pharmacokinetics

Analysis of the pharmacokinetic data indicated that tabalumab concentrations shifted higher for the tabalumab 120 Q2W group. Pharmacokinetic analysis also showed an overlap between the two dosing regimens, as expected (data not shown).

Safety and tolerability

No clinically meaningful or significant differences in the number of deaths, frequency of SAEs or TEAEs, or number of patients who discontinued due to an AE were observed between groups (table 3). The majority of TEAEs were mild-to-moderate. Ten pregnancies were reported during the treatment period (table 3), five of which were carried to term and there were no fetal abnormalities at birth (one in 120 Q2W, one in placebo, three in 120 Q4W). Two fetal deaths in utero (one placebo patient with preeclampsia and one patient in 120 Q2W group), one spontaneous abortion (placebo) and one terminated pregnancy (placebo) were reported; and one pregnant patient was lost to follow-up (120 Q2W).

The incidences of serious infections and severe infections were similar in the tabalumab and placebo groups (table 3). The incidences of opportunistic infections of interest were herpes zoster, reported in 13 patients (3.4%) in 120 Q2W, 4 (1.0%) in 120 Q4W and 10 (2.6%) in placebo; disseminated tuberculosis, reported in 1 patient (0.3%) in 120 Q2W; and cytomegalovirus, reported in 1 patient (0.3%) in 120 Q4W. Oral candidiasis was reported in six (1.6%) patients in 120 Q2W, three (0.8%) in 120 Q4W and eight (2.1%) in placebo; however, no unexpected fungal opportunistic infections were reported. There were no differences in the percentages of patients reporting an injection-site reaction, in the incidences of adjudicated major adverse cardiovascular events or in reported malignancies. No anaphylactic events were reported;

Table 3 Safety and tolerability

n (%)	120 Q2W n=386	120 Q4W n=389	Placebo n=387
Deaths	3 (0.8)*	2 (0.5)†	2 (0.5)‡
Serious AEs	43 (11.1)	56 (14.4)	50 (12.9)
Discontinued due to an AE	26 (6.7)	29 (7.5)	28 (7.2)
Treatment-emergent AEs	317 (82.1)	320 (82.3)	314 (81.1)
AEs possibly related to study drug	175 (45.3)	159 (40.9)	161 (41.6)
Selected treatment-emergent AEs			
Pregnancies	3 (0.8)	3 (0.8)	4 (1.0)
Infections			
Serious	20 (5.2)	19 (4.9)	17 (4.4)
Severe	10 (2.6)	14 (3.6)	15 (3.9)
Adjudicated MACE	5 (1.3)	4 (1.0)	5 (1.3)
Malignancies	1 (0.3)§	1 (0.3)¶	2 (0.5)**
Injection-site reactions	11 (2.8)	7 (1.8)	6 (1.6)
Allergic/hypersensitivity reactions			
Anaphylaxis	0	0	0
Non-anaphylaxis hypersensitivity	5 (1.3)	1 (0.3)	2 (0.5)
Depression and suicide-related events			
Depression	12 (3.1)	14 (3.6)	13 (3.4)
Suicide attempt	0	1 (0.3)	1 (0.3)
Columbia-Suicide Severity Rating Scale††			
Suicidal ideation	1 (0.4)	3 (1.3)	2 (0.9)
Suicidal behaviour	0	1 (0.4)	2 (0.9)
Change in QIDS-SR16 total score, mean±SD	−1.2± 4.09	−1.4±4.37	−1.4±4.58
Immunogenicity			
TE persistent or transient ADA	2 (0.5)	3 (0.8)	12 (3.1)
Positive neutralising ADA	0	0	0

Data are n (%) unless indicated otherwise.
*Antiphospholipid syndrome, pulmonary embolism, disseminated tuberculosis.
†Pneumonia, SLE.
‡Preferred term=death (verbatim reported by investigator was unknown cause of death), CVA.
§Basal cell carcinoma.
¶Squamous cell carcinoma of the skin.
**Breast cancer, intestinal adenocarcinoma.
††Number of patients with at least one post-baseline assessment.
ADA, antidrug antibody; AE, adverse event; CVA, cerebrovascular accident; MACE, major adverse cardiovascular event; Q2W, every two weeks; Q4W, every four weeks; QIDS-SR16, Quick Inventory of Depressive Symptomatology (16-Item); SLE, systemic lupus erythematosus; TE, treatment-emergent.

hypersensitivity reactions occurred in five patients in 120 Q2W, one patient in 120 Q4W and two patients in placebo.

There were no differences in the percentages of patients in the tabalumab groups versus placebo who reported depression, treatment-emergent suicidal ideation or suicidal behaviour during the treatment period (table 3). The change from baseline in the QIDS-SR16 score at week 52 (LOCF) was similar in the tabalumab and placebo groups.

No clinically meaningful differences in laboratory abnormalities were noted. The frequency of treatment-emergent antidrug antibodies was low, and no positive neutralising antidrug antibodies were identified (table 3).

DISCUSSION

This phase III, placebo-controlled study in patients with active, moderate-to-severe SLE assessed the efficacy of tabalumab 120 Q2W or 120 Q4W versus placebo. The primary endpoint, SRI-5 response, was not met for either dose group, though 120 Q4W showed a trend towards improvement versus placebo at week 52 (p=0.052).

Statistical significance was not achieved on secondary measures of clinical efficacy (ie, time to first severe flare, corticosteroid-sparing effects, fatigue and individual components of the SRI). However, a significant pharmacodynamic effect (ie, anti-dsDNA reductions, C3 and C4 increases) was observed in the tabalumab groups. In a flare prevention trial, patients administered atacicept (a fusion protein that blocks B-lymphocyte stimulator and APRIL) showed marked anti-dsDNA reductions and C3 increases, although only patients receiving the higher dose demonstrated any clinical benefit.[18] The reasons for the difference are not obvious. One could speculate that some form of 'threshold effect' is taking place. In general, biomarkers often perform rather variably in their relationship with clinical disease. Based on clinical observations from this trial and ILLUMINATE-2,[17] a similarly designed phase III, global study of tabalumab where the SRI-5 endpoint was met and a dose-effect was observed, it remains possible that higher doses or more frequent dosing may lead to better efficacy despite maximal effect on pharmacodynamic markers.

Significant increases in BAFF concentrations, reductions in total B cells and reductions in immunoglobulins were also observed in the tabalumab groups. These changes were consistent with tabalumab's mechanism of action and with previous studies.[19–21] Increases in BAFF concentration were expected. In the presence of

tabalumab, BAFF concentration increases because the anti-BAFF Ig molecule (tabalumab) binds to BAFF creating a BAFF-anti-BAFF Ig complex. The measurable level of BAFF accumulated in the presence of tabalumab. Overall, these outcomes were indicative of biological activity consistent with BAFF inhibition.

Baseline BAFF concentrations were not predictive of higher SRI response rates with tabalumab. A study evaluating whether serum BAFF levels correlate with SLE relapse or remission following B-cell-depleting therapy demonstrated that patients were more likely to flare when serum BAFF was rising.[22] However, serum BAFF level did not predict subsequent remission or relapse after treatment. Despite being a strong correlate of flares, baseline BAFF concentrations may not be a strong indicator of response to B-cell-targeting therapies.

In a predefined sensitivity analysis of the primary endpoint, where patients who *decreased* antimalarials or immunosuppressants were not automatically non-responders, SRI-5 response was met with 120 Q4W (37.0% vs 29.8% placebo), but not the more frequent 120 Q2W dose (34.1%). In similar analyses of SRI-4 and SRI-6 through SRI-10, both the 120 Q2W (47.2%) and 120 Q4W groups (47.4%) had a significantly higher SRI-4 response than placebo (39.8%) and the 120 Q4W group also had a higher SRI-6 response (35.3% vs 29.2% placebo). The ILLUMINATE-2 primary endpoint applied the same concomitant medication rules noted above and SRI-5 response was met with the more frequent 120 Q2W dose, but not 120 Q4W.[18] Additionally, 120 Q2W also had a significantly higher percentage of responders than placebo when using other SRI cut-offs (ie, SRI-4, SRI-6, SRI-7, SRI-9 and SRI-10).

Belimumab is a monoclonal antibody that targets soluble BAFF and has been approved for the treatment of SLE in a number of countries.[23 24] Tabalumab is a monoclonal antibody that targets both membrane and soluble BAFF. Beyond the differences in mechanism of action, there are no data that further differentiate the biological activity of these drugs. In two phase III trials of belimumab, 43% and 58% of patients, respectively, receiving belimumab 10 mg/kg plus SoC achieved the primary endpoint of SRI-4 (vs 33% and 44%, placebo).[25 26] Unlike the ILLUMINATE-1 study, the belimumab trials allowed the addition of new antimalarial treatments and increases in immunosuppressants up to week 16, and corticosteroid use was less restrictive. Despite similarities in trial design, it is difficult to determine if any dissimilarities in baseline variables, background medications or mechanism of action had an impact on the different study outcomes.

The safety data show no unexpected events and low levels of antidrug antibody. Although a higher incidence of herpes zoster in the Q2W group was noted, there were no other indications of opportunistic infection. Overall, there was no increased risk of mortality, or of serious, severe adverse effects with tabalumab relative to placebo.

This study enrolled patients that principally had joint and skin manifestations, which may limit applicability of the results to the broader, more heterogeneous features of SLE. In addition, variations in clinical practices worldwide were indicated by a relatively small percentage of patients (~63%) taking an antimalarial at baseline as well as other differences in SoC. Although baseline variables were balanced across groups, it is possible that differences in SoC may also represent differences in degrees of illness or a patient's responsiveness to treatment. Additionally, in the absence of mild-or-moderate flare analyses, the impact of treatment on flares of lesser severity is unknown.

Tabalumab did not meet the primary endpoint, SRI-5 response, or key secondary endpoints, despite pharmacodynamic evidence of biological activity that was consistent with BAFF pathway inhibition. Safety profiles of the tabalumab groups were similar to placebo with no unexpected signals.

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Original article

Amplification of IL-21 signalling pathway through Bruton's tyrosine kinase in human B cell activation

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Abstract

Objective. B cells play an important role in the pathogenesis of autoimmune diseases. The role of Bruton's tyrosine kinase (Btk) in cytokine-induced human B cell differentiation and class-switch recombination remains incompletely defined. This study analysed the effect of Btk on human activated B cells.

Methods. Purified B cells from healthy subjects were stimulated with B cell receptor (BCR) and other stimuli with or without a Btk inhibitor and gene expression was measured. The B cell line BJAB was used to assess Btk-associated signalling cascades. Phosphorylated Btk (p-Btk) in peripheral blood B cells obtained from 10 healthy subjects and 41 patients with RA was measured by flow cytometry and compared with patient backgrounds.

Results. IL-21 signalling, in concert with BCR, CD40 and BAFF signals, led to robust expression of differentiation- and class-switch DNA recombination-related genes and IgG production in human B cells, all of which were significantly suppressed by the Btk inhibitor. Although phosphorylation of STAT1 and STAT3 was induced by co-stimulation with IL-21, BCR and CD40, STAT1 phosphorylation in the nucleus, but not in the cytoplasm, was exclusively impaired by Btk blockade. High levels of p-Btk were noted in B cells of RA patients compared with controls and they correlated significantly with titres of RF among RF-positive patients.

Conclusion. The findings elucidate a model in which Btk not only plays a fundamental role in the regulation of BCR signalling, but may also mediate crosstalk with cytokine signalling pathways through regulation of IL-21-induced phosphorylation of STAT1 in the nuclei of human B cells. Btk appears to have pathological relevance in RA.

Key words: B cells, autoimmune diseases, BCR signalling, rheumatoid arthritis, IL-21, class-switch DNA recombination, STAT1.

Rheumatology key messages

- Btk is a critical molecule for differentiation and class switching of human B cells.
- Btk regulates IL-21-induced phospho-STAT1 in the nucleus of human B cells.
- Phospho-Btk in B cells of RF-positive RA patients is closely related to RF.

Introduction

Activated autoreactive B cells contribute to the pathological process of various autoimmune diseases, including RA. The importance of B cells is underscored by several functions: antigen presentation, secretion of pro-inflammatory cytokines and autoantibodies such as RF. A previous open-label study indicated that depletion of B cells using the anti-CD20 antibody rituximab led to sustained clinical improvements in RA patients [1]. Thus B

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cells are assumed to be a legitimate therapeutic target for RA. Rituximab was approved for RA in 2006 in the USA. Today, rituximab is ranked as the second-line biologic product after TNF inhibitor therapy. Over the last few years, several small-molecule inhibitors of various kinases, such as Janus kinase (JAK) and spleen tyrosine kinase (SYK), have also been developed for the treatment of RA.

Bruton's tyrosine kinase (Btk) is a key intracellular kinase known to regulate B cell function. Btk mutations in humans cause X-linked agammaglobulinaemia, a severe inherited disease associated with peripheral blood B cell and Ig deficiencies [2]. Transgenic mice that overexpress Btk exhibit spontaneous germinal centres and large numbers of plasma B cells, with antinuclear autoantibody production and lupus-like autoimmune diseases [3]. Btk deficiency is associated with B cell development arrest at the pre-B cell stage [4]. Growing interest in Btk stems from its role in the B cell receptor (BCR) and pre-BCR signal transduction pathways [5]. Currently the therapeutic use of Btk inhibitors is being tested in clinical trials in patients with B cell lymphoma [6] and in a series of experiments on autoimmune diseases [7]. However, the role of Btk in the pathogenesis of RA remains to be elucidated.

B cell differentiation and class-switch DNA recombination (CSR) are essential for specific antibody production. Cytokines are key effectors, and IL-21 is one of the most influential in driving B cell fate. IL-21 is produced by activated NK or T cells and has been reported to regulate germinal centre formation, B cell differentiation and CSR [8–10]. IL-21 also plays an important role in B cell development, and experimental animal studies have demonstrated its contribution here in both adjuvant-induced arthritis and CIA models [11]. Ettinger *et al.* [12] confirmed that IL-21 can induce maximal plasma cell differentiation from human cord blood naive B cells following co-stimulation with IL-21 and CD40, but not with IL-2 or IL-10. A clinical study in RA patients demonstrated a significant correlation of IL-21 levels with disease activity [13].

However, little is known about the role and mechanisms of Btk in cytokine-induced human B cell differentiation and CSR. In the present study we described the establishment of a robust system of human peripheral B cell activation and also a Btk-knock-down (KD) B cell line with BCR-, CD40-, BAFF- and IL-21-mediated signalling. Both systems were used, together with Btk-specific inhibitors, to assess the role of Btk in B cell activation. We also assessed phosphorylation of Btk in B cells purified from the peripheral blood of patients with RA and the relevance of Btk phosphorylation to B cell activation.

Materials and methods

B cell isolation from healthy donors and RA patients

Table 1 lists the characteristics of RA patients at baseline. All 41 study subjects satisfied the ACR diagnostic criteria for RA [14]. Control subjects were either staff members of our hospital or healthy subjects who visited our hospital for medical examinations. After withdrawing blood from both healthy donors and RA patients at the same time points, peripheral blood mononuclear cells (PBMCs) were immediately separated using lymphocyte separation medium (ICN, Cappel Pharmaceuticals, Aurora, OH, USA) and incubated with CD19 Dynabeads (Dynal, Oslo, Norway) and/or CD27 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously [15]. B cell purity was >90%. The study, including the collection of peripheral blood samples from healthy adults and patients, was approved by the university's Human Ethics Review Committee. All subjects provided a signed informed consent form.

Table 1 Baseline characteristics of patients

Characteristic	Value
Age, mean (S.D.), years	62.0 (13.9)
Females/males, *n/n*	31/10
Disease duration, mean (S.D.), months	8.8 (10.1)
Stage (I/II/III/IV), %	22/46/17/14
Class (I/II/III/IV), %	2/90/5/2
Prednisolone (or equivalent) treatment	
n (%)	5 (12.1)
Dose, mean (S.D.), mg/day	5.6 (2.3)
MTX treatment	
n (%)	31 (75.6)
Dose, mean (S.D.), mg/week	13.7 (3.0)
CRP, mean (S.D.), mg/dl	2.0 (3.0)
ESR, mean (S.D.), mm/h	48.9 (33.6)
IgG, mean (S.D.), mg/dl	1447 (376)
RF positive, *n* (%)	29 (70.7)
Mean (S.D.), IU/ml	189 (367)
Anti-CCP positive, *n* (%)	34 (82.9)
Mean (S.D.), U/ml	293 (498)
MMP-3, mean (S.D.), ng/ml	206 (270)
DAS28 (CRP), mean (S.D.)	3.7 (1.4)
DAS28 (ESR), mean (S.D.)	5.0 (1.6)
Clinical Disease Activity Index, mean (S.D.)	21.8 (15.0)
Simple Disease Activity Index, mean (S.D.)	23.8 (16.5)
HAQ, mean (S.D.)	1.4 (0.8)
Number of patients treated with biologics, *n* (%)	9 (22.0)

DAS28: 28-joint DAS.

B cell in vitro culture conditions

Purified $CD19^+$ B cells (2×10^5 cells/200 μl) were cultured alone or with 1 μg/ml anti-BCR mAbs (anti-Igλ and anti-Igκ, BD Pharmingen, Franklin Lakes, NJ, USA), 2 μg/ml human sCD40L or 2 μg/ml sBAFF (both from PeproTech, Rocky Hill, NJ, USA) and 100 ng/ml IL-21 (Miltenyi Biotec). To test viability, cells were stained with annexin V/propidium iodide in the presence or absence of BCR stimulation at days 2 and 5. Culture medium was replenished on day 3. In the absence of stimuli, survival rates (annexin V/propidium iodide-negative cells) were 20–30%

at day 5, which was not different from that in BCR stimulation at the same time point (see supplementary Fig. S1, available at *Rheumatology* Online). IgG secretion was measured by ELISA (Bethyl Laboratories, Montgomery, TX, USA). Different doses of pharmacological inhibitors were added to the medium 30 min before stimulation. For immunoblot analysis, 10 ng/ml IFN-γ and 100 ng/ml IL-6 (both from Miltenyi Biotec) were added as positive controls. Human primary B cells and Burkitt's lymphoma cell line BJAB were cultured in RPMI 1640 (Wako Pure Clinical Industries, Osaka, Japan) supplemented with 10% fetal calf serum (Tissue Culture Biologicals, Tulare, CA, USA) and 100 U/ml penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA). Src-family kinase PP1 and PP2 were both from Merck (Darmstadt, Germany) and ONO-A Btk inhibitor was kindly provided by ONO Pharmaceutical (Tokyo, Japan). Btk inhibitor selectivity is Fyn [IC_{50}]/Btk [IC_{50}] = 2500; LynA [IC_{50}]/Btk [IC_{50}] = 1767. IC_{50} values of ONO-A against recombinant human Btk, Lck, Fyn and LynA were determined by the optimized Z′-Lyte kinase assay kit (Invitrogen).

Quantitative PCR

AICDA, BCL-6 and *XBP-1* gene expression levels were detected by quantitative RT-PCR (Applied Biosystems, Foster City, CA, USA). mRNA concentrations were normalized to 100 pg/reaction. All reactions were standardized to the expression of 18S ribosomal RNA. The relative quantity was calculated using the quantification comparative cycle threshold formula-referenced sample (see supplementary Table S1, available at *Rheumatology* Online).

SDS-PAGE and immunoblot analysis

Cells were washed with cold PBS and lysed in a 10% solution of NP-40 buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 8.0) and a mixture of protease/phosphatase inhibitors (both from Roche Applied Science, Indianapolis, IN, USA). Lysates were boiled for 5 min in an equal volume of sample buffer solution (catalogue no. 161-0737, Bio-Rad Laboratories, Hercules, CA, USA) with 1% 2-mercaptoethanol (Wako). Samples were electroporated in 10% Tris–glycine gels (Novex, Grand Island, NY, USA) and transferred onto nitrocellulose membrane (GE Healthcare Biosciences, Uppsala, Sweden), blocked with 5% bovine serum albumin/5% skimmed milk, then incubated overnight with phospho-STAT1 (T701), phospho-STAT3 (T705), STAT1 and STAT3 (all from Cell Signaling Technology, Danvers, MA, USA), anti-β-actin (Sigma, St Louis, MO, USA) or anti-TBP (Abcam, Cambridge, UK) mAbs at 4°C. Supplementary Table S2 (available at *Rheumatology* Online) provides a complete list of the antibodies used in immunoblots. Bands were detected with ECL western blotting detection reagents (GE Healthcare Biosciences, Uppsala, Sweden) and visualized with a light-capture instrument (ATTO, Tokyo, Japan). Nuclear and cytoplasmic extracts of cells were separated using a nuclear extraction kit (Affymetrix, Santa Clara, CA, USA).

Analysis of shRNA

KD of Btk in Burkitt's lymphoma cell line BJAB was established by electroporation with pcDNA 6.2-GW/EmGFP-miR vectors from BLOCK-iT Pol II miR RNAi Expression Vector Kits; Life Technologies, Grand Island, NY, USA) using a pool of four soluble inhibitory RNA duplexes available from the Invitrogen database. miR-lacZ oligo was used as a control. Electroporation was carried out as described previously [16]. Briefly, XL10-Gold Ultra competent cells treated with 2 μl of ligation reaction (Stratagene, La Jolla, CA, USA) were selected with 50 μg/ml spectinomycin (Sigma) at 37°C overnight. Clonal plasmid DNA (15–20 μg/ml) was transfected into 1×10^7 BJAB cells and GFP-positive cells were sorted after 17 h using a FACS-Vantage Sorter (BD Biosciences, Heidelberg, Germany). pcDNA 6.2-GW/Em GFP-miR-negative plasmid and miR-lacZ-positive oligo were used as negative and ligation efficiency controls, respectively. shRNA-expressing cells were selected in blasticidin for 7–10 days. KD efficiency was tested by real-time PCR (Btk mRNA expression) immunoblotting (Btk protein).

Flow cytometric analysis

For investigating phospho-Btk in $CD19^+$ B cells, $0.5–1 \times 10^7$ PBMCs were incubated with phosphatase inhibitor cocktail (Sigma) and fixed with Phosflow Fix Buffer (BD Biosciences) for 30 min at 37°C. After washing, cells were resuspended in Perm/Wash buffer (BD Bioscience) and stained with unconjugated rabbit anti-human phospho-Btk mAb (Novus Biologicals, Littleton, CO, USA), rabbit anti-human Btk mAb (Cell Signaling Technology, Beverly, MA, USA), rabbit IgG isotype control mAb (Cell Signaling) and APC-H7-conjugated mouse anti-human CD19 mAb (BD Pharmingen) for 30 min at room temperature. PE-labelled goat anti-rabbit IgG pAb (BD Pharmingen) was used as the secondary antibody. After washing with saponin–PBS, cells were analysed on a flow cytometer (FACSCalibur, BD Biosciences). Events were collected and analysed with FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis

Data were expressed as percentages of patients or mean (S.D.) as appropriate. The correlation between clinical indices and phosphorylated Btk (p-Btk) expression level was tested by linear regression with the use of Spearman's correlation coefficients. Statistical significance was tested using analysis of variance (ANOVA) and Student's *t*-test. A *P*-value <0.05 denoted the presence of a statistically significant difference. All statistical analyses were conducted using SPSS software (version 18; SPSS, Chicago, IL, USA).

Results

Btk is important in the regulation of human B cell differentiation and CSR

First, we assessed the expression of IL-21 receptor (IL-21R) in naive ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$)

B cells from human peripheral blood at 0 and 72 h after BCR and/or CD40/BAFF stimulation (see supplementary Fig. S2, available at *Rheumatology* Online). In the absence of stimuli, IL-21R was expressed in memory B cells, while it was marginally expressed in naive B cells. BCR stimulation significantly enhanced IL-21R expression in memory B cells compared with naive B cells. Although CD40 and BAFF stimulation had no effect on IL-21R expression, BCR and CD40/BAFF co-stimulation induced comparable levels of IL-21R relative to BCR-stimulated memory B cells. These results suggest that IL-21R is induced during B cell differentiation and activation and that the signalling pathway downstream of IL-21R is vital for the function of human memory B cells, in agreement with previous reports [17].

CSR and differentiation of B cells requires robust stimulation. *AICDA* expression was slightly induced by BCR, with or without CD40/BAFF, or IL-21 stimulation alone (Fig. 1A). Importantly, in concert with co-stimulation by BCR, CD40/BAFF and IL-21 caused robust *AICDA* expression (Fig. 1A). *AICDA* expression was different in naive ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells, with a strong increase in memory B cells compared with marginal expression in naive B cells (Fig. 1B). In response to triple stimulation with BCR, CD40/BAFF and IL-21, IgG induction was similar to *AICDA* expression (Fig. 1C).

Although Btk is widely expressed in the haematopoietic system, its expression in B cells is largely dependent on Btk signalling [18]. The specific Btk inhibitor ONO-A significantly inhibited *AICDA* expression induced by stimulation with BCR, CD40/BAFF and IL-21, and the inhibition was dose dependent (Fig. 1D, right panel). In contrast, two inhibitors of the Src-family kinases (PP1 and PP2) had no effects on *AICDA* expression, even at 1 μM. These results validate the specificity of the Btk inhibitor in our system (Fig. 1D, left panel). Similar results were observed with *BCL6* and *XBP1*, transcription factors involved in B cell differentiation, after stimulation with BCR, CD40/BAFF and IL-21, with or without Btk inhibitor (Fig. 1E). In addition, IgG production induced by the triple stimulation was again markedly reduced by the Btk inhibitor in a dose-dependent manner (Fig. 1F). These results suggest that Btk may function downstream of IL-21R, and BCR and CD40/BAFF co-stimulation, in the regulation of CSR and differentiation of human B cells.

Btk-mediated signal is important for IL-21-mediated STAT1 phosphorylation in human B cell nuclei

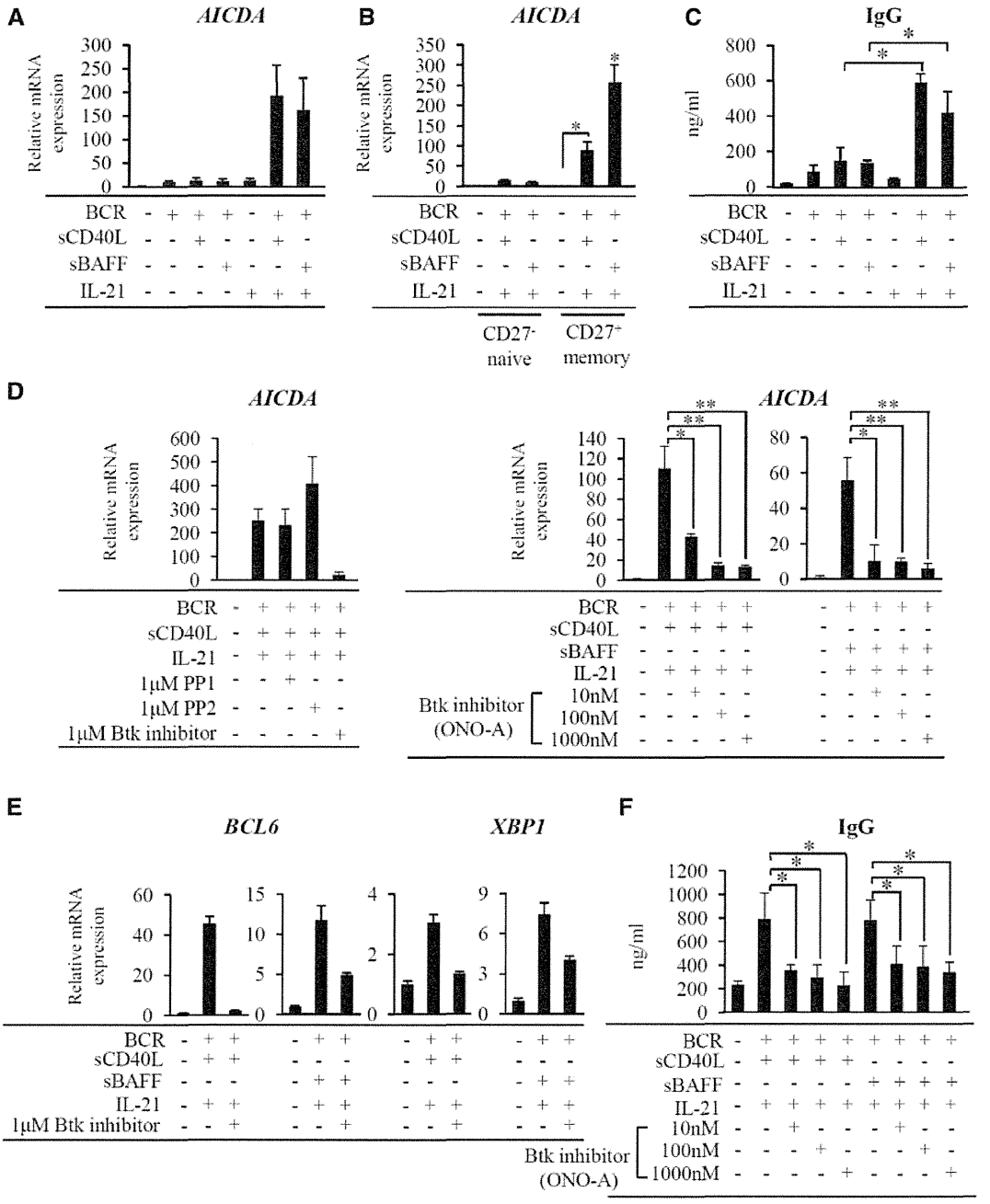
To investigate Btk activities associated with IL-21 and BCR/co-stimulatory signals, we measured p-Btk levels in primary B cells. First we investigated p-Btk levels after IL-21 stimulation alone. However, phosphorylation of Btk was not induced by IL-21 in primary B cells after 5, 10 or 15 min, suggesting that IL-21 predominately activates JAK-STAT signal transduction without directly activating Btk (see supplementary Fig. S3, available at *Rheumatology* Online). Second, we investigated the effects of Btk inhibitor on STAT1 and STAT3 phosphorylation in B cells stimulated with IL-21, BCR and CD40. Phosphorylation of STAT1 and STAT3 in the cytoplasmic and nuclear fractions increased rapidly after triple stimulation in both fractions as predicted. Interestingly, the increase in pSTAT1 nuclear localization in stimulated cells was markedly reduced by the Btk inhibitor (Fig. 2B). On the other hand, there was no difference in STAT3 phosphorylation in both fractions in the presence or absence of Btk inhibitor (Fig. 2A). In these experiments, cells treated with IL-6 and IFN-γ were used as positive controls for pSTAT3 and pSTAT1, respectively.

To rule out possible non-specific effects for the Btk inhibitor on nuclear STAT1 phosphorylation in human B cells, we established a Btk KD system in BJAB cells. Btk-specific shRNA reduced Btk mRNA expression by 80% in two independent B cell lines (KD1 and KD2) compared with negative controls, together with a marked reduction in Btk protein expression in both KD cells (see supplementary Fig. S4, available at *Rheumatology* Online). In KD cells, marked phosphorylation of STAT1 and STAT3 was noted at 20 min after IL-21 stimulation, and the level plateaued over a 60 min period (Fig. 3A). Furthermore, both STAT1 and STAT3 were markedly phosphorylated after IL-21 stimulation in whole lysates of Btk KD BJAB cells (Fig. 3B). Phosphorylation of STAT1 and STAT3 in the cytoplasm after IL-21 stimulation was comparable in control and Btk KD cells (Fig. 3C). It should be noted, however, that phosphorylation of STAT1, but not STAT3, was preferentially decreased in the nuclei of Btk KD cells after IL-21 stimulation, similar to primary B cells (Fig. 3D). These results suggest that Btk-mediated signals directly regulate IL-21-induced phosphorylation of STAT1 in B cell nuclei. Thus B cells seem to require Btk activation to regulate IL-21-induced STAT1 phosphorylation and/or its translocation to the nucleus.

Btk activation in B cells is associated with RF production in RF-positive patients with RA

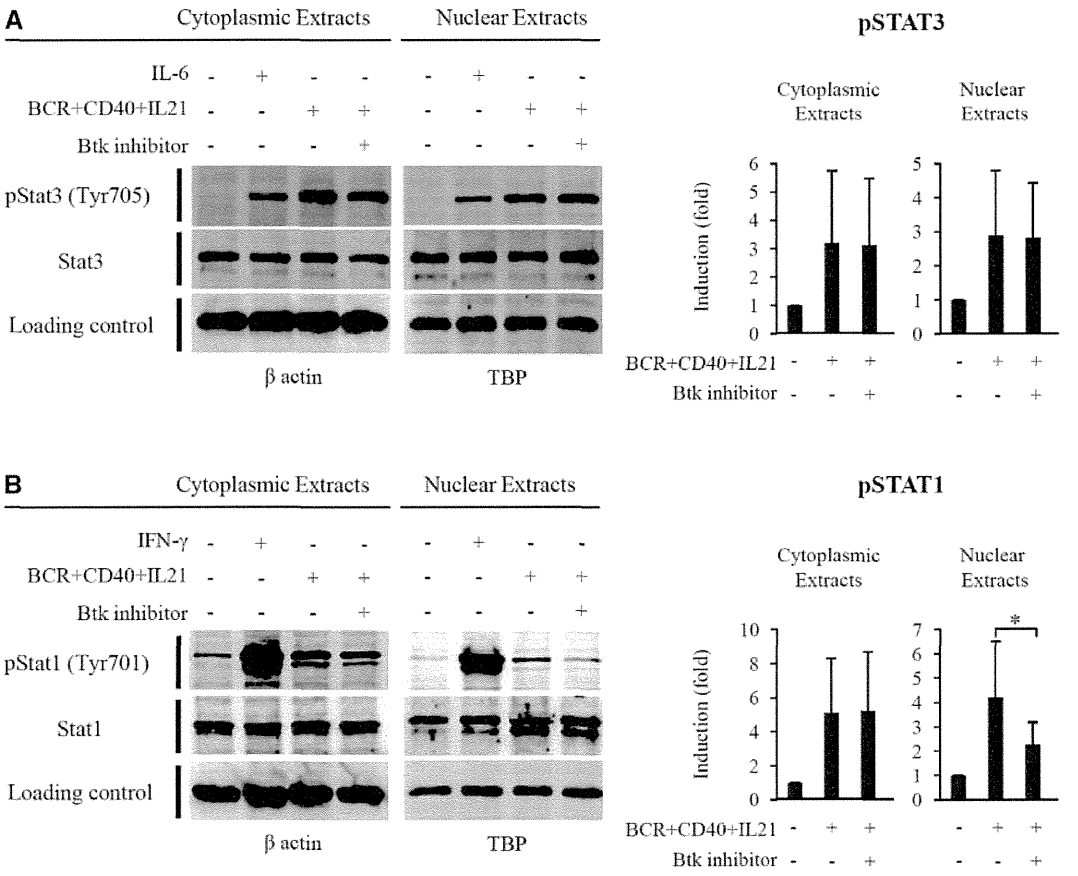
To test our hypothesis we investigated the levels of p-Btk in B cells from patients with RA compared with healthy donors (control) and assessed the relationship between p-Btk levels and clinical features, including disease activity index and autoantibody titre. Peripheral blood $CD19^+$ B cells from 10 control subjects and 41 RA patients were examined. Table 1 summarizes the clinicopathological features of RA patients ($n = 41$). Although the expression level of Btk in B cells was not different between the two groups (data not shown), phosphorylation of Btk in B cells was significantly higher in patients with RA compared with controls [mean fluorescence intensity of p-Btk in $CD19^+$ B cells: control, 144 (S.D. 73.4); RA, 224 (S.D. 171); $P = 0.031$] (Fig. 4A). Even though p-Btk levels were not significantly different between RF-negative and RF-positive RA patients ($P = 0.651$) (Fig. 4B), there was significant correlation in RF titre and phosphorylation of Btk within RF-positive RA patients ($n = 29$; $P = 0.003$, $r = 0.539$) (Fig. 4C). In contrast, there was no correlation in RF-negative RA patients ($n = 12$; $P = 0.265$, $r = -0.350$) (data not shown).

FIG. 1 Bruton's tyrosine kinase (Btk) inhibitor ONO-A inhibits differentiation and class-switch DNA recombination in human primary B cells



(**A** and **C**–**F**) Peripheral blood CD19$^+$ B cells or (**B**) naive (CD19$^+$ CD27$^-$) and memory (CD19$^+$CD27$^+$) B cells were purified from healthy donors and were cultured with 1 μg/ml anti-BCR mAbs, 2 μg/ml human sCD40L or 2 μg/ml human sBAFF and 100 ng/ml of IL-21 for 2 or 5 days in the absence or presence of inhibitors (PP1/PP2/ONO-A Btk inhibitor). *AICDA* (day 2), *BCL6* (day 2) and *XBP1* (day 5) gene expression levels were detected by quantitative PCR, with each sample 100 pg of mRNA template/reaction. (**C** and **F**) Levels of total IgG secretion from cultured B cells were measured by ELISA. Data are mean (S.E.M.) of three independent experiments. $^*P < 0.05$ by one-way analysis of variance with the Games–Howell comparison test.

FIG. 2 Effects of Bruton's tyrosine kinase (Btk) inhibition on phosphorylation of STAT1 in human B cells following stimulation with BCR, CD40 and IL-21



(**A** and **B**) Immunoblot analyses of cytoplasmic and nuclear extracts of $CD19^+$ peripheral blood B cells from healthy donors were cultured with and without 10 μM Btk inhibitor for 30 min before stimulation with 1 μg/ml anti-BCR mAbs, 2 μg/ml human sCD40L and 100 ng/ml of IL-21 for 5 min. Cells were stimulated with 100 ng/ml IL-6 or 10 ng/ml IFN-γ as control for pSTAT3 and pSTAT1, respectively. (**A**) pSTAT3 and (**B**) pSTAT1 band intensity relative to that of cells cultured in medium alone, and normalized to loading control. Data are representative of one of three independent experiments. $^*P < 0.05$ by one-way analysis of variance with the Games–Howell comparison test.

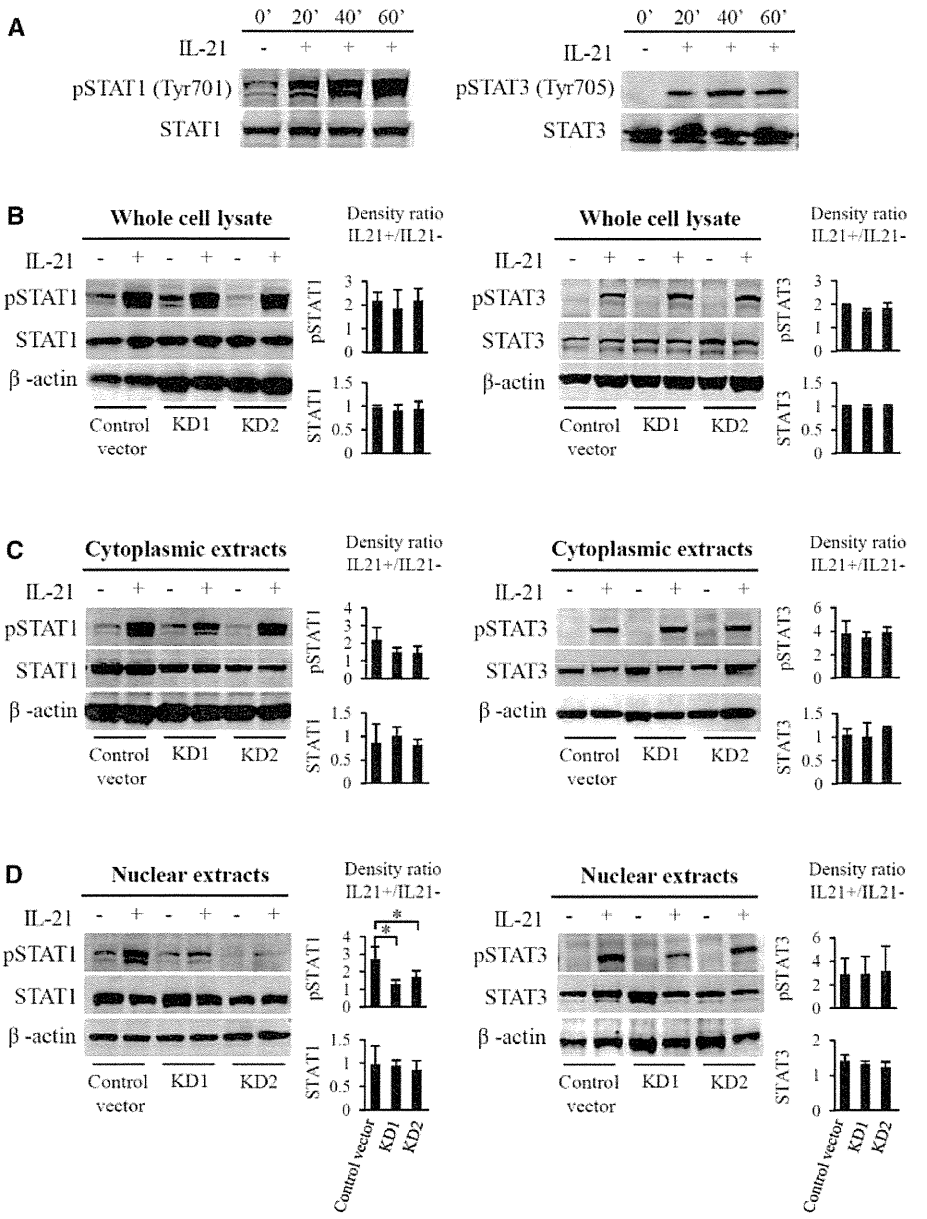
Next, the level of Btk phosphorylation in B cells was analysed according to clinical features among RF-positive RA patients (Table 2). The level of p-Btk in B cells did not correlate with indicators of RA disease activity such as CRP, ESR, MMP-3 and the Simple Disease Activity Index. There was also no correlation between p-Btk levels in B cells and age, sex, steroids or dosage of oral MTX. However, the level of p-Btk correlated significantly with RF titre ($P = 0.003$, $r = 0.539$), suggesting that p-Btk levels were constitutively higher in B cells of patients with RA and might be correlated with RF production within RF-positive patients.

Discussion

Initially we established that IL-21 signalling, in concert with BCR and CD40/BAFF co-stimulation, resulted in robust expression of differentiation- and CSR-related genes, as well as IgG production in human B cells. This was accompanied by JAK-STAT pathway signalling activities, as has been described in detail by Ettinger *et al.* [12]. Other observations also highlight the important role of IL-21 with BAFF in B cell plasma cell differentiation [19]. Building on this, we demonstrated here that efficient BCR-CD40/BAFF-IL-21 signalling requires Btk activity. Specifically, Btk was required for normal IL-21 induction of nuclear phosphorylated STAT1 levels. We demonstrated this using both the Btk inhibitor ONO-A and a Btk-KD model. The important role of Btk was further supported by our observation that p-Btk levels of B cells were increased in RA patients. Specifically, p-Btk levels correlated with RF titre among RF-positive patients.

The induction of *AICDA* and Ig class switching in human B cells, as stimulated with BCR, sCD40/sBAFF and IL-21,

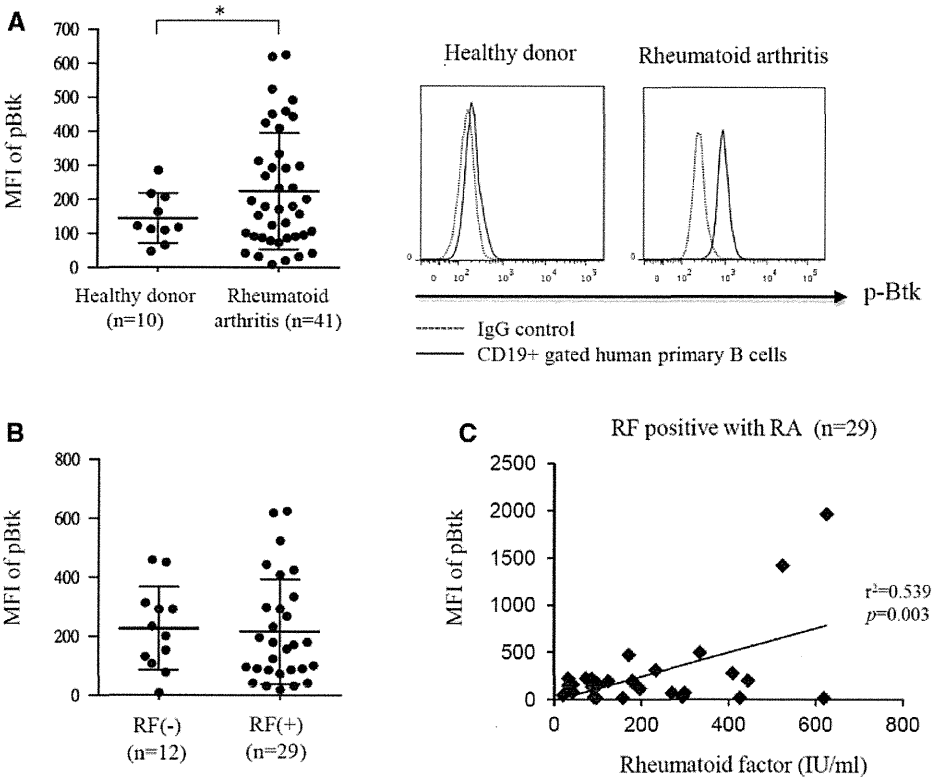
Fig. 3 STAT-1 phosphorylation in cell nuclei is exclusively decreased in Bruton's tyrosine kinase (Btk) knock-down (KD) BJAB cells after stimulation with IL-21



(**A**) Immunoblot analysis of phospho-STAT1/-STAT3 and total STAT1/STAT3 in BJAB cells was carried out after stimulation of cells with IL-21, as indicated. (**B**–**D**) The BJAB and Btk KD cell lines were cultured in the absence or presence of IL-21 for 30 min in RPMI medium supplemented with 10% fetal calf serum. The whole cell lysates (**B**), cytoplasmic extracts (**C**) and nuclear extracts (**D**) of Btk KD cells were prepared and then subjected to SDS-PAGE and transferred to nitrocellulose membranes, as indicated. The band intensity of the immunoblots was quantified using CS Analyzer (ATTO, Tokyo, Japan). (**A** and **B**) β-actin and (**C**) thioredoxin binding protein served as loading controls. The data represent one of four independent experiments. (**B**–**D**) Lanes 1 and 2: miR-negative control; lanes 3 and 4: KD1 (monoclonal line 6–17); lanes 5 and 6: KD2 (monoclonal line 6–21).

was significantly suppressed by Btk inhibitors such as ONO-A. Ample evidence indicates that cytokines in B cells activate mainly through the JAK-STAT pathway [20]. Our data showed that ONO-A Btk inhibitor selectively blocked nuclear phospho-STAT1 accumulation after BCR, CD40/BAFF and IL-21 ligation. Consistent with this effect, KD of Btk in BJAB cells also resulted in impaired phosphorylation levels of nuclear STAT1. These results suggest

FIG. 4 High levels of Bruton's tyrosine kinase (Btk) phosphorylation in primary B cells from RA patients



(**A**–**C**) Peripheral blood CD19$^+$ B cells were collected from healthy control subjects and RA patients. (**A**) Subjects were tested for phosphorylated Btk (p-Btk) expression levels (mean fluorescence intensity units, ordinate) on the CD19$^+$ pan B cell subset by intracellular staining for FACS analysis. (**B**) p-Btk levels in CD19$^+$ B cells from RF-negative/-positive RA patients. (**C**) Correlation between p-Btk level and RF titre from RF-positive RA patients. *$P < 0.05$.

a novel role for Btk in the efficient propagation of IL-21 signalling. Btk appears to regulate STAT1 phosphorylation and/or nuclear localization, which is critical for B cell differentiation and CSR in human B cells.

Avery *et al.* [21] reported a lack of synergistic effects of IL-21 and IL-4 on IgE production by splenic B cells in patients with STAT3 mutations, but not those with STAT1 mutations in *ex vivo* experiments. They reported that although both STAT1 and STAT3 were markedly phosphorylated in response to IL-21 in human peripheral B cells, STAT3 was functionally more important for the differentiation of B cells [16]. It should be noted, however, that STAT3 mutant B cells fail to up-regulate BLIMP-1 in response to IL-21 and CD40L, while up-regulation of *AICDA* expression and CSR were unaffected by this mutation. The present results show that IL-21-induced nuclear phosphorylation of STAT1, but not STAT3, was preferentially abrogated by Btk blockade. This finding suggests that like STAT3, STAT1 may be crucial in IL-21-mediated CSR and differentiation in B cells. In addition, our findings suggest a novel action of Btk in the transport of phosphorylated STAT1 from the cytoplasm to the nucleus. Further work is needed to elucidate the exact mechanism underlying this process.

RA pathogenesis is associated with systemic dysregulation of T and B cell responses, which involves various pro-inflammatory pathways. Rituximab-induced B cell depletion therapy is widely used in chronic lymphocytic leukaemia, non-Hodgkin's lymphoma and autoimmune diseases, including RA. Although rituximab-induced B cell depletion therapy is often successful, some patients are either non-responsive or develop early relapse. The precise contribution of B cells to RA is not fully understood; however, we demonstrated the presence of high levels of p-Btk in B cells in RA patients, which correlated with RF titres in RF-positive patients. These findings suggest that Btk phosphorylation in B cells may have an important role in RF production in the immunopathogenesis of RA. These results emphasize the importance of RF-positive B cells in the immunopathogenesis of RA, as those B cells can bind to immune complexes and exert the ability of antigen presentation to T cells [22]. Although further assessment will be needed, evaluation of Btk phosphorylation may serve to predict the efficacy of B cell-targeted treatment based on our finding. In addition,