Letter to the Editor

CD40LG duplication-associated autoimmune disease is silenced by nonrandom X-chromosome inactivation

To the Editor:

Gene duplication expands genome size and is the initial step in paralog formation. Not all gene duplication events are immediately beneficial; some result in functional disomy and convey a survival disadvantage until silenced.[1] On an autosome, gene silencing requires the random accumulation of loss-of-function mutations, but on a sex chromosome, females can rapidly silence disadvantageous alleles via nonrandom X-chromosome inactivation (XCI).[2] It has been proposed that reactivation of sex-linked, immune-related genes may contribute to the observed female bias of autoimmune diseases.[3,4]

The X-linked gene *CD40LG* encodes CD40L, a T-cell coactivation receptor expressed under transcriptional control of nuclear factor of activated T cells.[5] CD40L-deficient patients display impaired immunoglobulin class-switching and defective cellular immunity, whereas CD40L-overexpressing mice develop high-titer autoantibodies and chronic inflammation.[5,6] Here, we report the first 2 subjects, a boy (III.1) and his mother (II.2), with *CD40LG* duplication-associated autoimmune diseases (Fig 1, *A*).

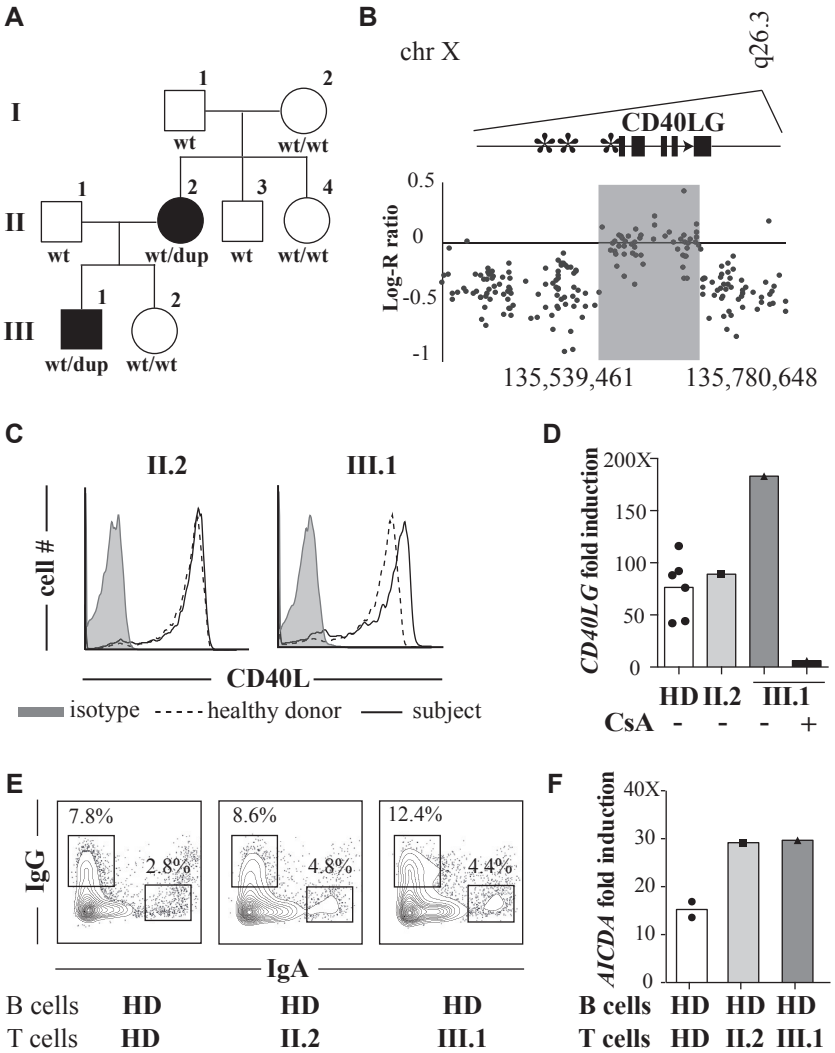

FIG 1. *CD40LG* duplication results in functional disomy for subject III.1. **A,** X-chromosome duplication (dup) status of affected (filled) and unaffected pedigree members. **B,** *CD40LG* with its promoters (asterisks) within log-R ratio–indicated duplication boundaries are depicted. **(C)** Increased CD40L expression and **(D)** *CD40LG* transcripts on subject III.1's, not II.2's, $CD4^+$ T cells after PMA/ionomycin activation *(right)*. Cyclosporine (CsA) therapy is indicated. **(E)** Increased IgG and IgA expression (day 7) and **(F)** activation-induced cytidine deaminase (*AICDA*) transcripts (48 hours) from healthy donor (HD) naive B cells heterologously cocultured with $CD4^+$ T cells from indicated subjects and HDs. *chr*, Chromosome; *PMA*, phorbol 12-myristate 13-acetate; *wt*, wild-type.

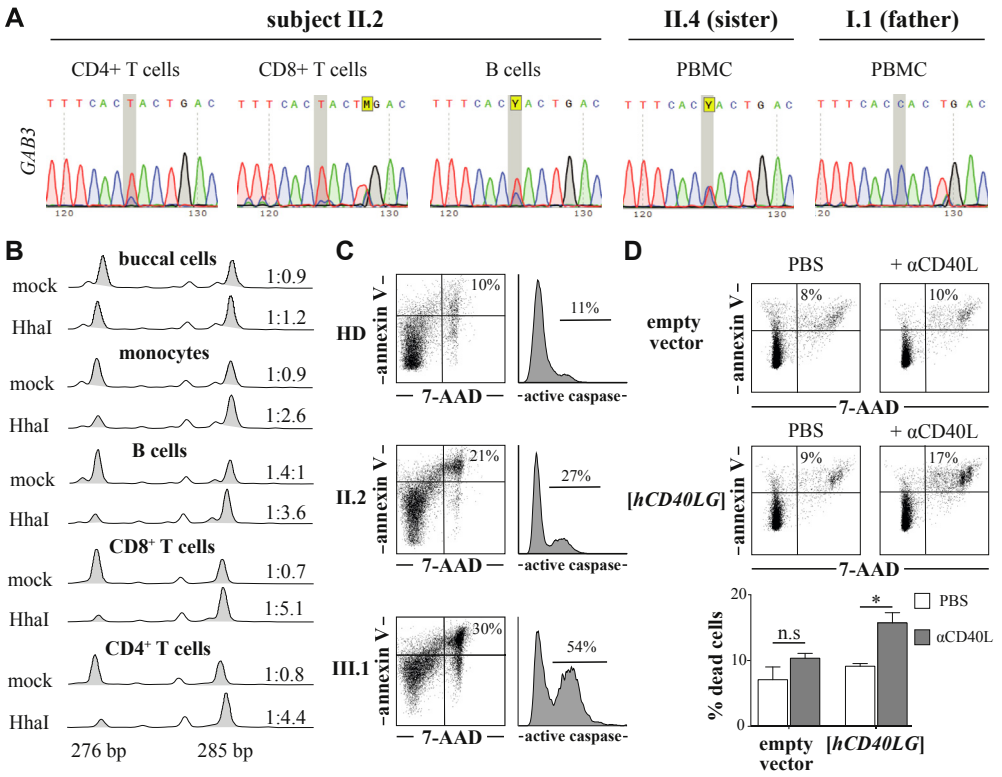

FIG 2. CD40-mediated cell death generates nonrandom XCI in II.2 lymphocytes. **A,** Chromatograms depict single nucleotide polymorphisms within *GAB3* transcripts. **B,** Electropherograms display *HUMARA* amplicons from II.2 DNA after mock or HhaI digestion. Areas under the peak ratios of the duplicated X-chromosome *HUMARA* amplicon (276 base pairs [bp]) to the nonduplicated X-chromosome amplicon (285 bp) are indicated. **C,** 7-Aminoactinomycin D (7-AAD) and annexin V (dead, *left*) and Z-VAD-FMK active caspase-stained (dying, *right*) $CD4^+$ T cells are displayed after 72 hours in CD3/CD28-stimulated PMBC cultures. **D,** Dead cell frequencies in CD40L overexpressing and control Jurkat lines cultured for 7 days with 100 μg/mL of anti-CD40L antibody, or not, are displayed *(upper panels)*. Mean, SDs, and differences between technical triplicates are displayed (*P = .02, n.s., not significant; paired Student t test).

The index subject, III.1, presented as a 6-month-old boy with coombs-positive hemolytic anemia, which evolved into multilineage autoimmune cytopenias associated with massive splenomegaly. Peripheral blood flow cytometric analysis revealed moderate T-cell lymphopenia and mild B-cell lymphopenia with increased frequencies of $CD45RO^+$ memory T cells, $CD27^+IgD^-$ class-switched memory B cells, and plasmablasts (see Table E1 and Fig E1 in this article's Online Repository at www.jacionline.org). Although initial immunoglobulin A, G, and M serum concentrations exceeded age-matched control ranges, IgM progressively declined until subject III.1 became selectively IgM deficient at age 5 years (Table E1). His autoimmune cytopenias were refractory to glucocorticoids, rapamycin, intravenous inmmunoglobulin, and rituximab.

Subject II.2 presented as a 25-year-old woman with Raynaud phenomenon and arthralgia. Serologic testing revealed high-titer antinuclear (1:320), antiribonucleoprotein (>8 Avidity Index [AI]), and antithyroid antibodies. She was diagnosed with mixed connective tissue disease and autoimmune thyroiditis. After 8 years, her symptoms resolved spontaneously during pregnancy. Recent serological testing demonstrated lower antinuclear antibody and ribonucleoprotein titers (1:160 and 0.4 AI, respectively).

To identify a genetic basis for our subjects' autoimmune diseases, we performed whole- exome sequencing. Pathologic mutations, including autoimmune lymphoproliferative syndrome-associated gene mutations, were not identified, but increased chromosome Xq26.3 copy number was detected in both subjects. Confirmatory testing with single nucleotide polymorphism arrays revealed a 240-kb microduplication encompassing *CD40LG*, its regulatory elements, and 3 other protein-coding genes, none immunologically significant (Fig 1, *B*; see Table E2 in this article's Online Repository at www.jacionline.org). Similarly discrete microduplications were not identified in unaffected family members (Fig 1, *A*), nor in publicly available or institutionally held human genetic variation databases.

To determine whether *CD40LG* duplication resulted in functional disomy, we measured CD40L induction on $CD4^+$ T cells. After phorbol 12-myristate 13-acetate/ionomycin stimulation, III.1 cell CD40L expression and *CD40LG* transcripts were twice that of unaffected relatives (Fig 1, *C*, right, and *D*). Furthermore, in heterologous cocultures, anti-CD3/anti-CD28–stimulated III.1's $CD4^+$ T cells compelled control naive B cells to express more activation-induced cytidine deaminase transcripts (Fig 1, *E*) and undergo more class-switching than when cocultured with stimulated heterologous control $CD4^+$ T cells (Fig 1, *F*).

Because other immunomodulatory treatments were ineffective, we sought to control III.1's CD40L expression by blocking nuclear factor of activated T cells nuclear translocation with cyclosporine A[7] and found low concentrations (50-100 ng/mL) of normalized CD40L expression *in vitro* (see Fig E2 in this article's Online Repository at www.jacionline.org) and *ex vivo* (Fig 1, *D*). During 14 months of daily cyclosporine A monotherapy (4 mg/kg/d), the spleen size significantly decreased, and he required no blood product transfusions. Thus, in a male, *CD40LG* duplication produces pathologic, functional disomy that is amenable to pharmacologic modulation.

In contrast to her son's cells, CD40L induction on II.2's $CD4^+$ T cells was indistinguishable from that on unaffected relatives, suggesting nonrandom XCI (Fig 1, *C*, left, and *D*). To investigate this possibility at the transcriptional level, we analyzed single nucleotide polymorphisms within 3 nonduplicated X-linked genes in $CD4^+$ T-cell, $CD8^+$ T-cell, and B-cell transcripts from the mother and her unaffected sister (II.4). One gene, *ZFX*, is known to escape XCI; *GAB3* and *MMGT1* do not.[8] As expected, II.4's *ZFX*, *GAB3*, and *MMGT1* transcripts were expressed by her X-chromosomes in equal proportions, whereas most *GAB3* and *MMGT1* (but not *ZFX*) transcripts originated from II.2's nonduplicated X-chromosome (Fig 2, *A*; see Fig E3 in this article's Online Repository at www.jacionline.org). A distinguishing feature of the inactive X-chromosome is hypermethylation. To analyze X-chromosome methylation patterns in different II.2 tissues, we digested buccal or PBMC-derived maternal DNA with the methyl-sensitive restriction enzyme HhaI and amplified the X-linked human androgen receptor (*HUMARA*) gene with primers spanning an HhaI restriction site. *HUMARA* harbors a polymorphic trinucleotide repeat, permitting discrimination of allele-specific amplicons by size. We found that buccal-derived, HhaI-digested II.2 DNA yielded 276 base pair (bp) *HUMARA* amplicons from the duplicated X-chromosome and 285-bp amplicons from the nonduplicated X-chromosome in near-equal quantities (276:285 ratio = 1:1.2; Fig 2, *B*). In contrast, 276-bp amplicons were significantly underrepresented in $CD4^+$ T-cell–derived (1:4.4), $CD8^+$ T-cell–derived (1:5.1), and EBV-transformed B-cell–derived HhaI-digested II.2 DNA (1:3.6; Fig 2, *B*). Monocytes, which unlike other PBMCs do not express detectable *CD40LG* transcripts (see Fig E4 in this article's Online Repository at www.jacionline.org), exhibited the least skewed 276:285 ratio (1:2.6; Fig 2, *B*).

Nonrandom XCI may reflect differential cell survival. To determine whether CD40L overexpression affects cell viability, we cultured III.1's and II.2's PBMCs for 3 days and analyzed $CD4^+$ gated T cells for apoptosis markers. Compared with a modest frequency of dead (7-Aminoactinomycin D [7-AAD] and annexin V positive, 10%) and dying (active caspase-positive, 11%) healthy control cells, we identified modestly increased frequencies of dead and dying II.2 cells (21% and 27%, respectively) and dramatically increased frequencies of dead and dying III.3 cells (30% and 54%, respectively; Fig 2, *C*). Furthermore, II.2's PBMCs surviving after 7 days in culture (7-AAD negative cells) exhibited a more skewed 276:285 *HUMARA* amplicon ratio (1:4; see Fig E5 in this article's Online Repository at www.jacionline.org) than did cells analyzed immediately *ex vivo* (1:3.4), suggesting that an active duplicated X-chromosome conferred a relative survival disadvantage. To confirm that *CD40LG* duplicated–associated cell death was dependent on CD40/CD40L, we measured apoptosis markers in Jurkat lines transfected with either *CD40LG* or an empty control vector. After 7 days in culture, CD40L-overexpressing cells did not die more than control cells (9% vs 10%, respectively) unless treated with hu5c8, a CD40 mimetic anti-CD40L antibody, which significantly increased dead cell frequency (9% vs 17%; $P < .02$). Hence, CD40-mediated apoptosis preferentially affects CD40L-overexpressing cells, which, in a female, produces nonrandom XCI.

Herein, we describe, for the first time, the deleterious immunologic consequences of *CD40LG* duplication in humans, but ours is not the first autoimmune disorder associated with CD40L overexpression. Quantitative increases in CD40L on T cells were previously reported to correlate with disease activity and lymphopenia in patients with lupus.[3,9] Our observation of high-titer autoantibodies and accelerated lymphocyte apoptosis in *CD40LG*-duplicated subjects reinforces this gene's role in autoimmune pathogenesis and suggests that CD40/CD40L interactions contribute to lupus-associated lymphopenia. Previously, we reported that *CD40LG* is susceptible to X-chromosome reactivation in female donor T cells stimulated for several days with anti-CD3/anti-CD28 antibodies.[4] Similarly, we demonstrate here that despite epigenetic silencing of their duplicated X-chromosome, II.2's T cells stimulated with anti-CD3/anti-CD28 antibodies induce B-cell class-switching and activation-induced cytidine deaminase expression with a capacity intermediate between cells from subject III.1 and healthy controls (Fig 1, *E* and *F*). On this basis, we speculate that the temporary reactivation of the *CD40LG*-duplicated X-chromosome, with resultant functional trisomy, may have contributed to the development of subject II.2's historical autoimmune disorders and the resumption of epigenetic control, to resolution of her disease. Because XCI is unavailable to males, subject III.1 has been continuously susceptible to the deleterious effects of his *CD40LG* duplication until his CD40L expression was normalized through pharmacological intervention.

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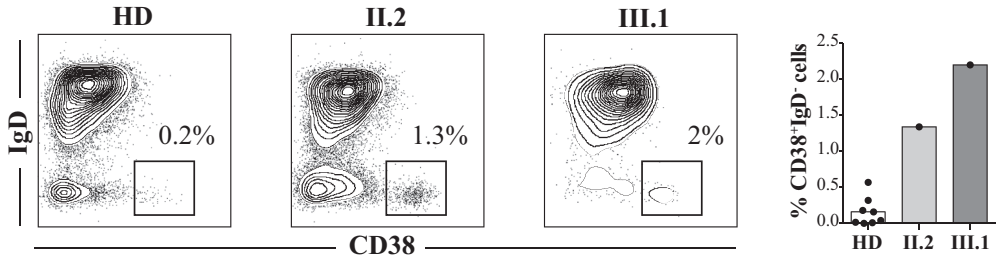

FIG E1. An increased frequency of circulating plasmablasts in subjects III.1 and II.2 compared with sex/age-matched and related controls. Plasmablasts were identified as $CD19^{+}IgD^{-}CD38^{+}$ cells via flow cytometry.

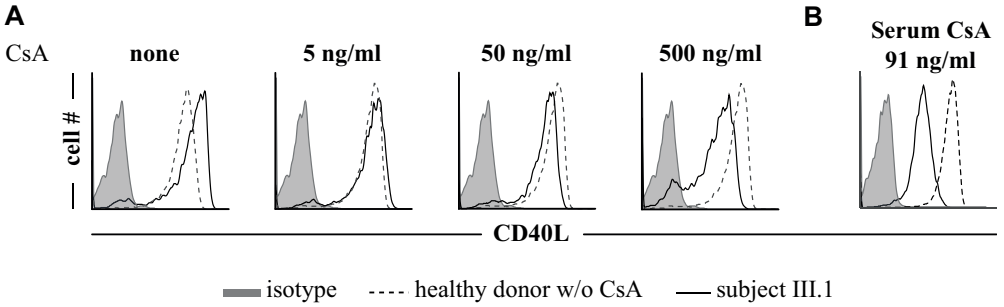

FIG E2. CD40L expression on subject III.1's $CD4^+$ T cells is reduced by cyclosporine A (CsA) in a dose-dependent manner. **A,** Subject III.1's $CD4^+$ T cells were cultured overnight at the indicated CsA concentration and activated for 4 hours with PMA/ionomycin before measuring CD40L expression by flow cytometry. **B,** After determining the ideal CsA dose range, the subject III.1 received low-dose CsA monotherapy (4 mg/kg/d). CD40L expression on subject III.1's $CD4^+$ T cells was determined by flow cytometry when his serum CsA concentration was 91 ng/mL. *PMA*, Phorbol 12-myristate 13-acetate; *w/o*, without.

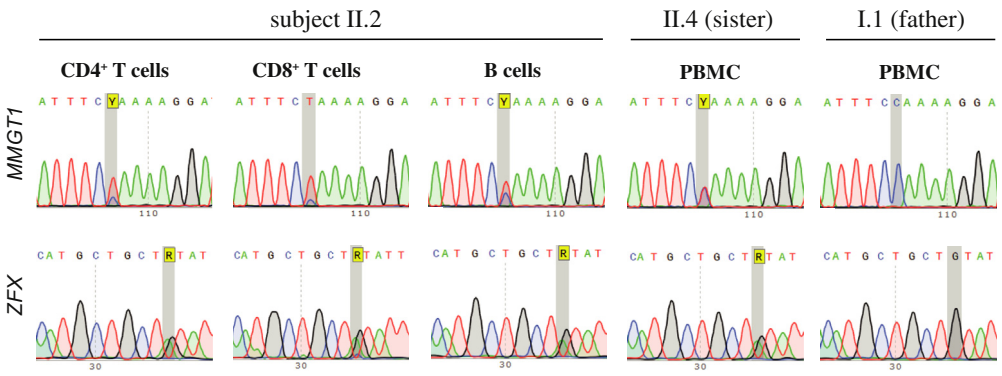

FIG E3. Subject II.2's MMGT1, but not ZXF, transcripts are primarily generated by the nonduplicated X-chromosome. Chromatograms depict single nucleotide polymorphisms in MMGT1 (sensitive to XCI) and ZXF (escapes XCI) transcripts from subject II.2, her sister (II.4), and her father (I.1).

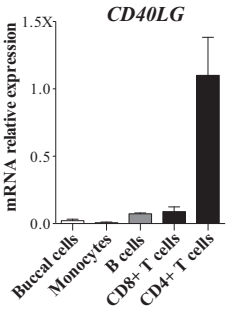

FIG E4. CD40LG expression in immune and nonimmune cells from healthy donor controls (n = 3). Total RNA was isolated from unstimulated, FACS-sorted PBMC subsets or buccal cells, and expression of CD40LG transcripts was determined by quantitative PCR. Results are expressed as the fold induction of gene transcription relative to median transcript values for $CD4^+$ T cells.

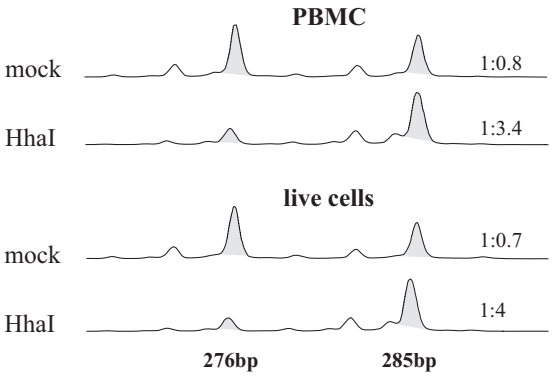

FIG E5. Preferential inactivation of the duplicated X-chromosome among surviving subject II.2's PBMCs. Electropherograms display HUMARA amplicons from II.2's PBMC DNA generated either immediately *ex vivo* or from live, 7-Aminoactinomycin D (7-AAD)-negative cells sorted after 7 days in culture without stimulation. Both mock and HhaI digestion conditions are depicted. Area under the peak ratios of the duplicated X-chromosome HUMARA amplicon (276 base pairs [bp]) to the nonduplicated X-chromosome amplicon (285 bp) are indicated.

TABLE E1. Peripheral blood lymphocyte and serum immunoglobulin concentrations of index patient III.1 and patient II.2

Laboratory assessment category	Laboratory determinate	Index patient III.1				Patient II.2
Age (y) at assessment		2	3	5	6*	39
Cell concentrations (cells/μL) and frequencies	$CD3^+$	829 (L)	923	665 (L)	455 (L)	561 (L)
	$CD3^+CD4^+$	475 (L)	473 (L)	366 (L)	214 (L)	329
	$CD45RO^+$ (%)	64.9 (H)	65.9 (H)	19	51.7 (H)	41.4
	$CD3^+CD8^+$	295 (L)	372	267 (L)	181 (L)	178 (L)
	$CD45RO^+$ (%)	37.6 (H)	28.8 (H)	—	34.3	31.6
	$CD16^+CD56^+$	305	217	128	91	52
	$CD19^+$	76 (L)	153 (L)	66 (L)	0	203
	$CD27^+IgD^-$ (%)	37 (H)	7 (L)	14.2	0	19
Immunoglobulin isotype concentration (mg/dL)	IgG	1130 (H)	1212 (H)	1110	953	—
	IgA	215 (H)	190 (H)	173 (H)	60	—
	IgM	296 (H)	194	47 (L)	12 (L)	—

H, Above normal range for age; *L*, below normal range for age; —, number not determined.
*Patient status post rituximab and receiving antibody replacement therapy.

TABLE E2. Protein-coding genes within chrX: 135,539,461-135,780,648

Gene	Tissue with elevated expression*	Function
BRS3	Ovary, testis	Metabolic regulator
HTATSF1	Pituitary gland	Transcriptional elongation
VGLL1	Bladder	Unknown
CD40LG	Lymphoid organ, blood	Immune response

Chr, Chromosome.
*Data obtained from the Genome-Tissue Expression Project on June 7, 2017.