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

Carole Le Coz, PhD[a], **Melissa Trofa, BA**[a], **Camille M. Syrett, BS**[b], **Anna Martin, BS**[b], **Harumi Jyonouchi, MD**[d], **Soma Jyonouchi, MD**[a,c], **Montserrat C. Anguera, PhD**[b], and **Neil Romberg, MD**[a,c]

[a]Division of Immunology and Allergy, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA

[b]Department of Biomedical Sciences, School of Veterinary Medicine, Philadelphia, Pennsylvania, USA

[c]Department of Pediatrics, The Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania, USA

[d]Pediatric Allergy/Immunology, Department of Pediatrics, Children's Hospital at Saint Peter's University Hospital, New Brunswick, New Jersey, USA

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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 non-random 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]

Correspondence to: Neil Romberg M.D., Leonard and Madlyn Abramson Pediatric Research Center, 3615 Civic Center Blvd., Philadelphia, PA 19104, rombergn@email.chop.edu.

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The X-linked gene *CD40LG* encodes CD40L, a T-cell coactivation receptor expressed under transcriptional control of nuclear factor of activated T-cells (NFAT).[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 two subjects, a boy (III.1) and his mother (II.2), with *CD40LG* duplication-associated autoimmune diseases (Fig 1A).

The index subject, III.1, presented as a six-month-old male with coombs-positive hemolytic anemia that evolved into multi-lineage 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 (Table E1 and Fig E1). While 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 five years old (Table E1). His autoimmune cytopenias were refractory to glucocorticoids, rapamycin, IVIG and rituximab.

Subject II.2 presented as a 25-year-old female with Raynaud's phenomenon and arthralgia. Serologic testing revealed high-titer anti-nuclear (ANA, 1:320), anti-ribonucleoprotein (RNP, >8 AI) and anti-thyroid antibodies. She was diagnosed with mixed connective tissue disease and autoimmune thyroiditis. After eight years, her symptoms resolved spontaneously during pregnancy. Recent serological testing demonstrated lower ANA and RNP titers (1:160 and 0.4 AI, respectively).

To identify a genetic basis for our subjects' autoimmune diseases, we performed whole exome sequencing (WES). 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 (SNP) arrays revealed a 240 kb microduplication encompassing *CD40LG*, its regulatory elements and three other protein-coding genes, none immunologically significant (Fig 1B and Table E2). Similarly discrete microduplications were not identified in unaffected family members (Fig 1A), nor in publicly available or institutionally held human genetic variation databases.

To determine if *CD40LG* duplication resulted in functional disomy, we measured CD40L induction on $CD4^+$ T cells. After phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation, III.1 cell CD40L expression and *CD40LG* transcripts were twice that of unaffected relatives (Fig 1C, right and D). Further, in heterologous co-cultures, anti-CD3/anti-CD28 stimulated III.1 $CD4^+$ T cells compelled control naïve B cells to express more activation-induced cytidine deaminase (*AICDA*) transcripts (Fig 1E) and undergo more class-switching than when co-cultured with stimulated heterologous control $CD4^+$ T cells (Fig 1F).

As other immunomodulatory treatments were ineffective, we sought to control III.1's CD40L expression by blocking NFAT nuclear translocation with cyclosporine A (CsA)[7] and found low concentrations (50–100 ng/ml) normalized CD40L expression *in vitro* (Fig E2)

and *en vivo* (Fig 1D). During 14 months of daily CsA monotherapy (4mg/kg day), 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 $CD4^+$ T cells was indistinguishable from unaffected relatives suggesting non-random XCI (Fig 1C, left and D). To investigate this possibility at the transcriptional level, we analyzed SNPs within three non-duplicated 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 the majority of *GAB3* and *MMGT1* (but not *ZFX*) transcripts originated from II.2's non-duplicated X-chromosome (Fig 2A and Fig E3). 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 peripheral blood mononuclear cell (PBMC)-derived maternal DNA with the methyl-sensitive restriction enzyme HhaI and amplified the X-linked human androgen receptor (*HUMARA*) gene with primers spanning a 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 non-duplicated X-chromosome in near-equal quantities (276:285 ratio=1:1.2; Fig 2B). 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; Figure 2B). Monocytes, which unlike other PBMCs do not express detectable *CD40LG* transcripts (Fig E4), exhibited the least skewed 276:285 ratio (1:2.6; Fig 2B).

Non-random XCI may reflect differential cell survival. To determine if CD40L overexpression affects cell viability, we cultured III.1 and II.2 PBMCs for three days and analyzed $CD4^+$ gated T cells for apoptosis markers. Compared to a modest frequency of dead (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 2C). Furthermore, II.2 PBMCs surviving after seven days in culture (7-AAD negative cells) exhibited a more skewed 276:285 *HUMARA* amplicon ratio (1:4; Fig E5) than cells analyzed immediately *ex-vivo* (1:3.4), suggesting an active duplicated X-chromosome conferred a relative survival disadvantage. To confirm that *CD40LG* duplicated-associated cell death was CD40/CD40L dependent, we measured apoptosis markers in Jurkat lines transfected with either *CD40L*G or an empty control vector. After seven 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%, $p<0.02$). Hence, CD40-mediated apoptosis preferentially affects CD40L overexpressing cells, which, in a female, produces non-random 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 of CD40L on T cells were previously reported to correlate with disease activity and lymphopenia in lupus patients.[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 re-activation 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 T cells stimulated with anti-CD3/anti-CD28 antibodies induce B cell class-switching and *AIDCA* expression with a capacity intermediate between cells from subject III.1 and healthy controls (Fig 1E 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. Since 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AICDA	activation-induced cytidine deaminase
ANA	anti-nuclear antibody
CsA	cyclosporine
HUMARA	human androgen receptor
NFAT	nuclear factor of activated T-cells
PMA	phorbol 12-myristate 13-acetate
RNP	ribonucleoprotein
SNP	single nucleotide polymorphism
WES	whole exome sequencing

XCI X-chromosome inactivation

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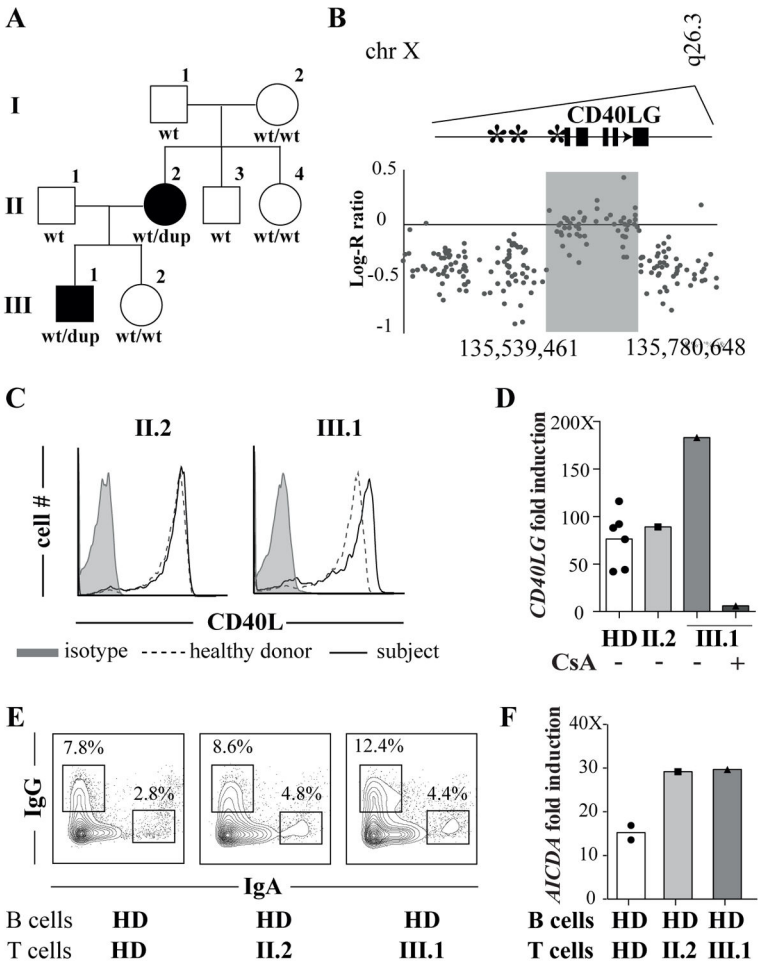

FIG. 1.

CD40LG duplication results in functional disomy for subject III.1. **A,** X-chromosome duplication (dup) status of affected (filled) and unaffected pedigree members and **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, not II.2, $CD4^+$ 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) naïve B cells heterologously co-cultured with $CD4^+$ T cells from indicated subjects and HDs.

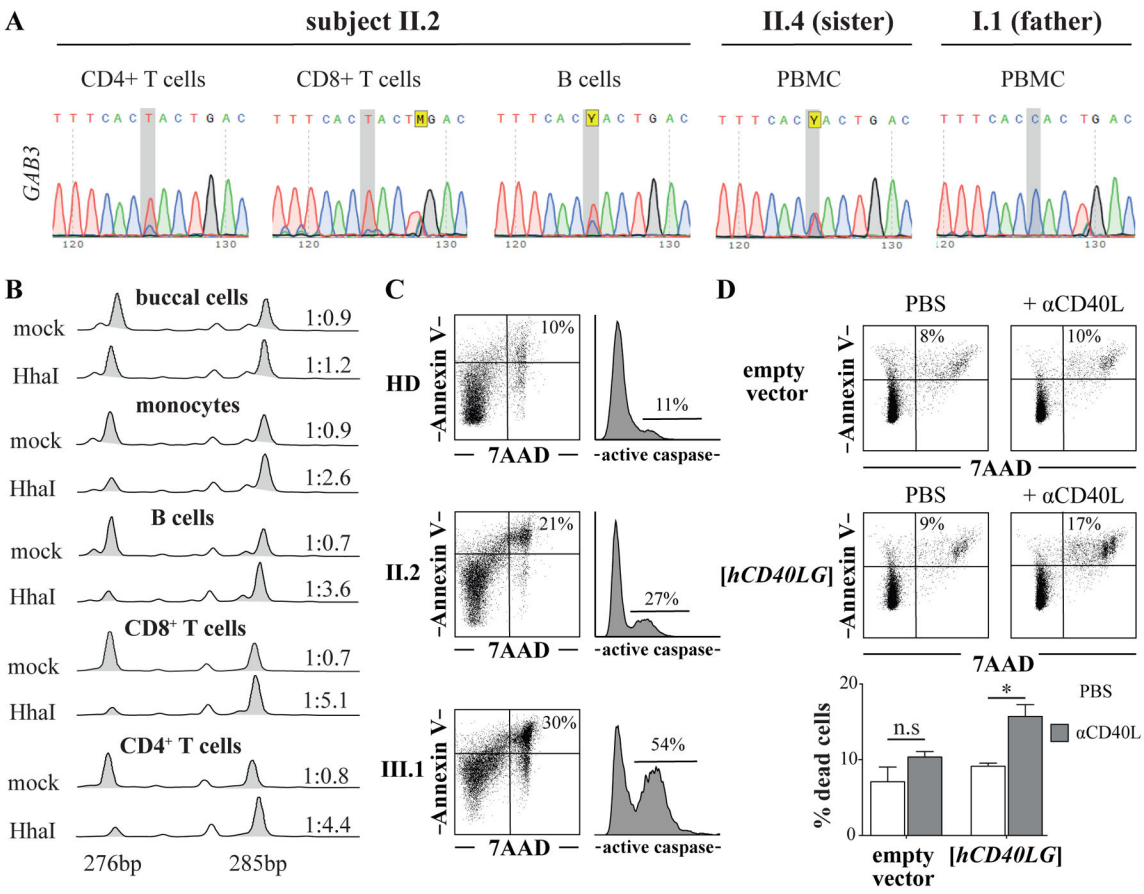

FIG. 2.

CD40-mediated cell death generates non-random X-chromosome inactivation 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 Hhal digestion. Area under the peak ratios of the duplicated X-chromosome *HUMARA* amplicon (276 base pairs (bp)) to the non-duplicated X-chromosome amplicon (285 bp) are indicated. **C,** Annexin V/7-AAD (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 100μg/ml of anti-CD40L antibody, or not, are displayed (upper panels). Mean, standard deviations and differences between technical triplicates are displayed (*, p=0.02, n.s., not significant; paired student t-test).