CD40LG triplication associates with immune dysregulation and exhaustion

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To the Editor,

CD40LG gene in the X chromosome encodes the T cell coactivation receptor CD40 ligand (CD40L) primarily expressed on activated $CD4^+$ T lymphocytes [1]. The CD40L-CD40 interaction is required, for example, for somatic hypermutation in B cells, class switch recombination and IgG1/IgG3 responses. Loss of function mutations in the *CD40LG* causes Hyper-IgM (HIGM) syndrome with defective immunoglobulin class-switching, elevated IgM and low IgG/IgA concentrations [2]. CD40L overexpression in mice is associated with chronic inflammation, autoantibodies and high IgG1 serum concentration [3].

Sun *et al* described successful hematopoietic stem cell transplantation in a child with *CD40LG* duplication [4]. The patient suffered from severe immune dysregulation with autoimmune cytopenia, splenomegaly and IgM deficiency caused by 240 kb X chromosomal microduplication covering *CD40LG, BRS3, HTATSF1* and *VGLL1* genes [5]. In this case report, we describe natural course and immunological findings in a 52-year-old male with early onset infection susceptibility and autoimmunity. This male has X chromosomal triplication rearrangement including *ARHGEF6, CD40LG* and *RBMX* genes (Figure 1A) (Primary Immunodeficiency and Primary Ciliary Dyskinesia Panel Plus and Whole Exome Sequencing, Blueprint Genetics, Finland). Molecular karyotyping confirmed this 381 kb Xq26.3 (GRCh37, ChrX:135659127-136040115) triplication rearrangement. No other genetic variants were reported (Supplemental methods). Mother of the patient was negative for the variant suggestive of de novo mutation. The index does not have any children. Copy number variation of *CD40LG* is shared by the patient of the current study and the previously reported pediatric case. Similar genetic rearrangements including the *CD40LG* have been reported in X-linked acrogigantism (X-LAG) syndrome. Interestingly, the X-LAG patients do not present with obvious immune dysfunction.

Our 52-year-old patient has suffered repeated episodes of otitis media, sinus infections and tonsillitis from early childhood. He underwent tonsillectomy and antrostomy at the age of 8 without obvious benefit. He has experienced limited exercise capacity and recurrent episodes of rales

and wheezing. His first pneumonia was diagnosed at the age of 9 and he continues to suffer from chronic bronchitis and recurrent episodes of otitis media. At the age of 12, he started receiving respiratory physiotherapy. At the age of 15, bronchiectasis was suspected; although alpha-1-antitrypsin and serum IgG (10.0 g/L) concentrations were normal, low serum IgA and IgG2 concentrations at that time were recognized. At the age of 17, he developed synovitis in multiple joints. At early twenties he experienced progression of synovitis with chronic swelling of his hands and feet suggesting diagnosis of remitting seronegative symmetrical synovitis with pitting edema (RS_3PE) syndrome. Response to prednisolone, sulfasalazine and hydroxychloroquine was at best partial. At the age of 24, he required hospitalization and intravenous acyclovir due to generalized skin varicella zoster infection. At the age of 28, he was confirmed to have extensive bronchiectasis after experiencing more than 20 pneumonia episodes. At the age of 30, he developed widespread verrucosis on his hands. He also suffered from chronic diarrhea; although colonoscopy appeared unremarkable, mucosal biopsies were positive for lymphatic hyperplasia. The patient has also suffered from chronic prostatitis, repeated urinary tract infections, acne vulgaris, folliculitis and episodes of erysipelas. IgG substitution (0.4 g/kg, 3-week intervals) improved the airway infection susceptibility. Alternative disease-modifying antirheumatic drugs had not been given because of recurrent infections and highly variable clinical presentations.

Peripheral white blood cell counts were normal (Supplemental data, Table 1). Naïve $CD27^-IgD^+IgM^+$ B-cells (87.1%, normal range 43.2-82.4%) and activated $CD21^{low}CD38^{low}$ B-cells (45.4%, normal range 0.8-7.7%) were high (Figure 1B). The switched memory ($CD27^+IgD^-IgM^-$) B cells (1.6%, normal range 6.5-29.2%), marginal zone ($CD27^+IgD^+IgM^+$) and $CD27^+$ memory B (8.4%) cells were low. $CD19^+CD27^-$CD10-$CD21^{low}$ B cell population was very high (patient 46,7%, controls 2,66 - 3,48%) (Figure 1C). Expression of BAFF-R was slightly reduced (Figure 1D), and CD19 expression was elevated (Figure 1E). CD20 expression was found to be elevated as well (data not shown). These findings are suggestive of B cell exhaustion. Immunoglobulin

concentrations were analyzed after a six month break in IgG substitution at the age of 34. Serum IgG (19.6 g/L), IgG1 (17.6 g/L) and IgG3 (1.6 g/L) were high while IgG2 (0.29 g/L), IgG4 (<0.1 g/L) and IgA (<0.13 g/L) concentrations were low (Figure 1B). Serum IgM (0.37 g/L) was within the normal range (Figure 1B). Although the patient was positive for serotype specific pneumococcal polysaccharide antibodies, no response to vaccination was seen (Supplemental data, Table 2).

Blood T cells and their subpopulations were normal (Supplemental data, Table 1). Importantly, $CD4^+$ T cells expressed high amounts of CD40L (Figure 1G) when compared to controls before stimulation (MFI: patient 235, control 107) with phorbol 12-myristate 13-acetate and ionomycin (PMA/ionomycin). However, the stimulation of the $CD4^+$ T cells with PMA/ionomycin caused a rapid and nearly complete loss of $CD4^+CD40L^+$ among the activated $CD69^{high}$ T cell populations (Figure 1H). The activated $CD69^{high}$ T cell populations were reduced in the patient's blood sample compared to control (Figure 1F). Upon PMA/ionomycin stimulation, the number of apoptotic cells in patient's PBMCs was significantly increased compared to controls measured with Annexin V/propidium iodide staining (AV-PI) (Figure 1F, Supplemental data, Figure 2). The largest difference in AV-PI negative cells was observed after 4 hours of stimulation in the patient (35%) compared to controls (59-61%). Same difference was not found in unstimulated PBMCs (80% AV-PI negative). Most likely, the rapid T cell apoptosis after stimulation leads to lack of proper CD40L-CD40 interaction. This may explain the incomplete B cell maturation, inadequate peripheral blood $CD27^+$ memory B cell development and inefficient class-switch recombination. Evidence of T cell exhaustion, however, was not observed although some differences in the expression of PD-1, CTLA-4 and CD160 were seen between the patient and control T cells (Supplemental data, Figure 1).

Proinflammatory cytokines were measured because of continuous inflammatory symptoms. Interleukin-1β (*in vitro*) or serum interleukin-6 and tumor necrosis factor alpha concentrations

were not elevated (Supplemental data, Table 3). Despite the multiple copies of *CD40LG*, soluble CD40L in serum (1944 pg/mL) was comparable to that seen in healthy controls (Supplemental data, Table 3). The results do not, for example, support a significant role for canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediated inflammation.

In conclusion, the adult patient with X chromosomal rearrangement including the *CD40LG,* has suffered from life-long symptoms of autoimmunity and infection susceptibility. These severe clinical and immunological findings are in line with previous observations on the child who received allogeneic transplantation [5][4]. Our report and the pediatric patient contribute to the understanding of biological and clinical consequences of *CD40LG* copy number variation. Although serum IgM in the patient reported here is normal, IgG1 is elevated, the B cell development is affected, class switching is disturbed, and B cells are exhausted. We can only speculate that similar mechanisms may explain other cases of IgG subclass deficiencies. It is also possible that *CD40LG* copy number variation may at least partly explain immunological presentations in additional cases with elevated IgG and IgG1 levels.

Statements and declarations

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Competing interests

The authors have no relevant financial or non-financial interests to disclose

Author contributions

WS, NP, VG, PÅ: laboratory analysis, writing

OK: Genetic analysis, writing

TH: patient care, study design, writing

Data availability

Data obtained during the study are available from the corresponding author on reasonable request

Ethics approval

This study has been completed according to principles of the Helsinki Declaration. The study has been approved by Oulu University Hospital Ethics committee.

Consent to participate

Informed consent to participate was obtained from the patient.

Consent to publish

Informed consent to publish was obtained from the patient.

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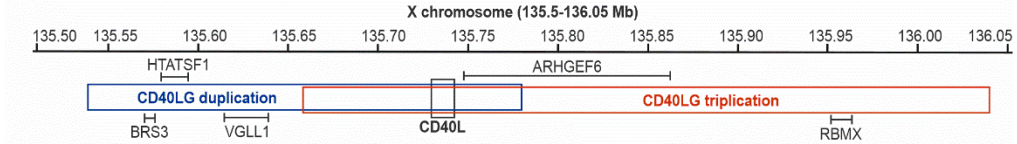
Figure legend

Figure 1. (A) Gene map comparing the locations and affected genes on previously published CD40L duplication (blue) and our patient with novel CD40L triplication (red). **(B)** A table of absolute counts or percentages of circulating B lymphocyte subsets and serum immunoglobulin concentrations in the patient. **(C)** The patient has a very high percentage of B cells with exhausted phenotype (CD19+CD27-CD10-CD21low) compared to the controls (46.7% vs 2.66-3.48%)**. (D)** BAFF-R expression is lower in patient’s CD19+ cells compared to the controls. **(E)** The CD19 expression in patient B cells is elevated compared to the controls. (**F**) Apoptosis rate was analyzed after 4, 6 and 8 hours of PMA/Ionomycin stimulation with Annexin V-PI staining kit. The apoptosis rate was higher in the patient cells compared to control cells (delta apoptotic cells (%) / delta time) from 0h to 4h; 16.4 vs. 9.9 correspondingly. The apoptosis rates are shown in figure as red (patient) and blue (control average) typing. The percentages of non-apoptotic patient PBMCs were lower in all four time points compared to the PBMCs of two controls. **(G)** CD40L expression in CD4+ T cells is high in unstimulated patient cells (P PMA/Iono-) compared to control (C PMA/Iono-) (58.4% vs 13.0%). After 18h PMA/Ionomycin stimulation, the number of CD4+CD40L+ cells is very low in the patient (P PMA/Iono+) compared to control (C PMA/Iono+). The low number of surviving patient cells were positive for CD40L expression (patient 57.9% vs control 39.6%). **(H)** CD69 expression in the patient’s CD4+ T cells (P PMA/Iono+) is low compared to control (C PMA/Iono-) after PMA/ionomycin stimulation. The Proportion of apoptotic cells is increased in patient PBMCs

A



B

B lymphocytes	Patient	Normal range
CD19^{+} B cells	310	80-616×10^{6}/L
CD38^{hi} IgM^{hi}	1.8%	0.6-3.5%
CD27^{-} IgD^{+}	87.1%↑	43.2-82.4%
CD27^{+}	8.4%	
CD27^{+} IgD^{+} IgM^{+}	4.9%↓	7.2-30.8%
CD27^{-} IgD^{-} IgM^{-}	1.6%↓	6.5-29.2%
CD38^{++} IgM^{-}	<0.2%	0.4-3.6%
CD38^{low} CD21^{low}	45.4%↑	0.8-7.7%

Immunoglobulins	Patient	Normal range
IgG	19.6 ↑	6.77-15.0 g/L
IgG1	17.6 ↑	4.9-11.4 g/L
IgG2	0.29 ↓	1.5-6.4 g/L
IgG3	1.6 ↑	0.2-1.1 g/L
IgG4	<0.1 ↓	0.1-1.4 g/L
IgA	<0.13 ↓	0.88-4.84 g/L
IgM	0.37	0.36-2.59 g/L
IgE	<5	<110 U/L
IgD	0.14	<0.12 g/L