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Copy number of the X-linked genes TLR7 and CD40L influences innate and adaptive immune responses

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Abstract

The number of the X chromosome–linked genes has been previously suggested to influence immune responses and the development of autoimmune diseases. In the present study, we aimed at evaluating the level of expression of CD40L (an X-linked gene involved in adaptive immunity) and TLR7 (an X-linked gene involved in innate immunity) in a variety of different karyotypes. Those included males, females and patients with X chromosome aneuploidy. Healthy females (46, XX; n = 10) and healthy males (46, XY; n = 10) were compared to females with Turner syndrome (TS) (45, X; n = 11) and males with Klinefelter syndrome (KS) (47, XXY; n = 5). Stimulation of peripheral blood mononuclear cells (PBMCs) with PMA and ionomycin resulted in higher percentage of CD3 + CD40L+ T cells ($P < 0.001$) and higher level expression of CD40L in T cell ($P < 0.001$) in female and KS patients compared with male and TS patients. TLR7-mediated IFN-alpha production by HLADR + CD3− CD19− cells was significantly upregulated in healthy women compared with healthy males, TS and KS patients ($P < 0.001$). TLR7 agonist-stimulated PBMCs from healthy females and KS patients expressed significantly higher levels of TLR7 mRNA than those from male and TS patients ($P < 0.05$). The increased expression of the X-linked genes TLR7 and CD40L in healthy females and KS patients suggests that the presence of two X chromosomes plays a major role in enhancing both innate and adaptive immune responses. These results may contribute to the explanation of sex-based differences in immune biology and the sex bias in predisposition to autoimmune diseases.

1 | INTRODUCTION

Strong evidence shows that females are generally characterized by a greater immunoreactivity and a more vigorous humoral and cell-mediated immune response to microbial infection and vaccination when compared to men.[1,2] Such a female "advantage" in response to infection may however bring about an enhanced autoreactivity to self-antigens and thereby contribute to the induction of autoimmunity.[2] A conservative estimate indicates that nearly 80% of individuals with autoimmune diseases are women.[3] However, the reason for the female preponderance to autoimmune diseases remains largely unknown.[4] Sex-related differences in immune response are often ascribed to the dissimilar hormonal balance between men and women, as well as the dissimilar genetic makeup involving the sex chromosomes karyotype.[5] Indeed, unlike males, who have only one X chromosome, female cells carry two of them: one maternally and the other paternally inherited. Most of the genes located on the X chromosome are not sex-specific, so that the equivalency in X-related genes

Sarmiento and Svensson contributed equally to this work.

products between males and females is guaranteed by the random inactivation of one of the two X chromosomes in each female cell.[6] The inactivation randomly affects the paternal or maternal X chromosome during the early development of female embryos, so that the inactive state is thereafter stably inherited by daughter cells upon successive cell divisions. As a result, each female develops as a mosaic of two mixed cell populations, in which either the maternally or paternally derived X chromosome is silenced.[7] The overall process is controlled by the X-linked non-coding Xist RNA, which is expressed asymmetrically from one of the two X chromosomes in females.[8,9] Eventually, most of genes on the inactive X chromosome are silenced. However, some of the genes encoded by the inactive X chromosome may "escape" from inactivation, resulting in the expression of X-related genes from both the active and inactive X chromosomes in female cells.[10]

The X chromosome contains several paramount immune-related genes, including CD40 ligand (CD40L) and Toll-like receptor 7 (TLR7).[11] The gene encoding CD40L has been mapped to Xq26.3–27.1 and is a key co-stimulatory molecule capable of modulating the adaptive immune response by regulating B cell activation/differentiation and T cell survival.[12] On the other hand, the TLR7 gene (mapped to Xp22.3) plays a central role in pathogen identification. TLR7 is an innate immune receptor recognizing endosomal single-stranded RNA and triggering innate and inflammatory immune responses through NF-kB activation and IFN-alpha secretion.[13] In this study, we hypothesized that the different dosage of these two aforementioned immune-related genes in women respect to men may influence their innate and adaptive immunological responses. The aim of this study was to evaluate the level of CD40L and TLR7 expression in patients with an abnormal number of X chromosomes in comparison with male and female individuals with a normal karyotype.

2 | MATERIALS AND METHODS

2.1 | Study population

Eleven patients with Turner syndrome (TS, 45,X; mean age ± SD: 24 ± 8.5 years) and five patients with Klinefelter syndrome (KS, 47,XXY; mean age ± SD: 26.4 ± 9.0 years) were recruited among those referring to the Pediatric and Adult Endocrinology outpatient clinics of Skåne University Hospital, Malmö, Sweden. Ten healthy women (46,XX; mean age ± SD: 31.6 ± 6.0 years) and 10 men (46,XY; mean age ± SD: 29.5 ± 6.4 years) without history of autoimmunity were also enrolled from the same hospital. None of the study participants received treatment with immunosuppressive or immunostimulant therapies, nor they had any established diagnosis/clinical suspect of infectious diseases at the time of the enrolment. The study protocol was reviewed and approved by the local Ethics Committee in Malmö-Lund under the permit number Dnr 194/2004 and conducted in conformance with the Helsinki Declaration. Written consent was obtained from all patients before the entering the study.

2.2 | Expression of CD40L on activated T lymphocytes

Human peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Lymphoprep (Axis-Shield PoC, AS) from heparinized blood according to the manufacturer's protocol. The isolated lymphocytes were washed with 1X PBS (Applichem) and frozen in freezing media containing 90% human serum (Invitrogen) and 10% DMSO (Sigma-Aldrich), which was added dropwise to the cells before they were frozen and stored in liquid nitrogen. Aliquots of 1.5×10^6 PBMC (viability approximately 90% or more for each population) were diluted in culture medium containing RPMI 1640 (Gibco BRL, Life Technologies), 10% human serum pooled (Invitrogen), 2 mmol/L L-glutamine (Sigma-Aldrich), 50 μg/L streptomycin sulphate (Sigma-Aldrich) and 10 μg/L gentamicin sulphate (Sigma-Aldrich). PBMCs were incubated in 1 mL culture medium alone or with 5 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 500 ng/mL ionomycin (IO, Sigma-Aldrich) for 3 hours at 37°C in 5% CO_2. Unstimulated and stimulated cells were harvested and stained with CD40L-FITC, 7-AAD and CD3-APC (all from BD Pharmingen™). Data were acquired on a FACS Calibur (Becton Dickinson and Company) and analysed by FlowJo software (Tree Star, Inc). CD40L expression was calculated as the percentage of CD3 + CD40L+ T cells among the total CD3+ cells. Mean fluorescence intensity (MFI) was calculated for each histogram.

2.3 | Stimulation of TLR7

In order to evaluate IFN-alpha expression on peripheral blood antigen-presenting cells (APCs), PBMCs were stimulated in vitro with the imidazoquinoline derivative CLO97 (Invitrogen), a TLR7 agonists capable of inducing the IFN-alpha production in a TLR7-specific manner.[14] IFN-alpha expression was evaluated by flow cytometry on $HLADR^+CD3^-CD19^-$ cells, representing most of the peripheral blood APCs. Cells were plated with 0.5 and 10 μg/mL CLO97. After 2 hours incubation, Brefeldin A (Sigma-Aldrich) was added to a final concentration of 10 μg/mL. Cells were then stained for HLADR-APC (BD Biosciences, AS) and CD3/CD19-PerCP (BD Biosciences, AS), and red blood cell lysis was obtained using the BD FACS lysing solution (BD Biosciences, AS). The intracellular staining for IFN-alpha was performed by BD's fix/perm solutions according to the protocol (BD Biosciences, AS). Samples were acquired on the FACS Calibur machine and analysed in

FlowJo (Tree Star, Inc). IFN-alpha expression was assessed on $HLADR^{+}CD3^{-}CD19^{-}$ cells.

2.4 | Quantitative polymerase chain reaction

Total RNA from unstimulated and stimulated cells was extracted using RNeasy mini kit (Qiagen). cDNA was obtained by reverse transcription (RT) with Maxima™ First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas, Thermo Scientific). Gene expression level was quantified using a Maxima™ Probe/ROX quantitative polymerase chain reaction (qPCR) Master Mix (Thermo Scientific) on an ABI PRISM 7900 (Applied Biosystems ViiA™ Real-Time PCR System, Life Technologies) using oligonucleotide primers (all from Thermo Scientific) for amplification of human CD40L (sense, 5′-CCAGGTGCTTCGGTGTTTGT-3′; antisense, 5′-ATGGCTCACTTGGCTTGGAT-3′) and TLR7 (sense, 5′-AAAATGGTGTTTCCAATGTGG-3′; antisense, 5′-GGCAGAGTT TTAGGAAACCATC-3′) The relative fold change in gene expression was normalized to RPLP0 (ribosomal protein, large, P0, [Thermo Scientific]) and compared with the expression in non-stimulated control cells, used as a calibrator. The relative gene expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method.

2.5 | Statistical analysis

All the analyses were performed by using SPSS version 23 statistical package. Comparisons among multiple groups were evaluated by one-way ANOVA test. Mann-Whitney *U* test was used for comparisons between two groups. Experimental results are reported as mean value ± SD or percentage, as appropriate. A two-tailed *P*-value < 0.05 was considered statistically significant with a confidence interval of 95%.

3 | RESULTS

3.1 | Expression of X-linked gene CD40L in healthy males and females and in patients with abnormal X chromosome number

In line with the notion that CD40L expression is induced in T cells only post-activation, none of the study participants had detectable CD40L expression on CD3+ cells before

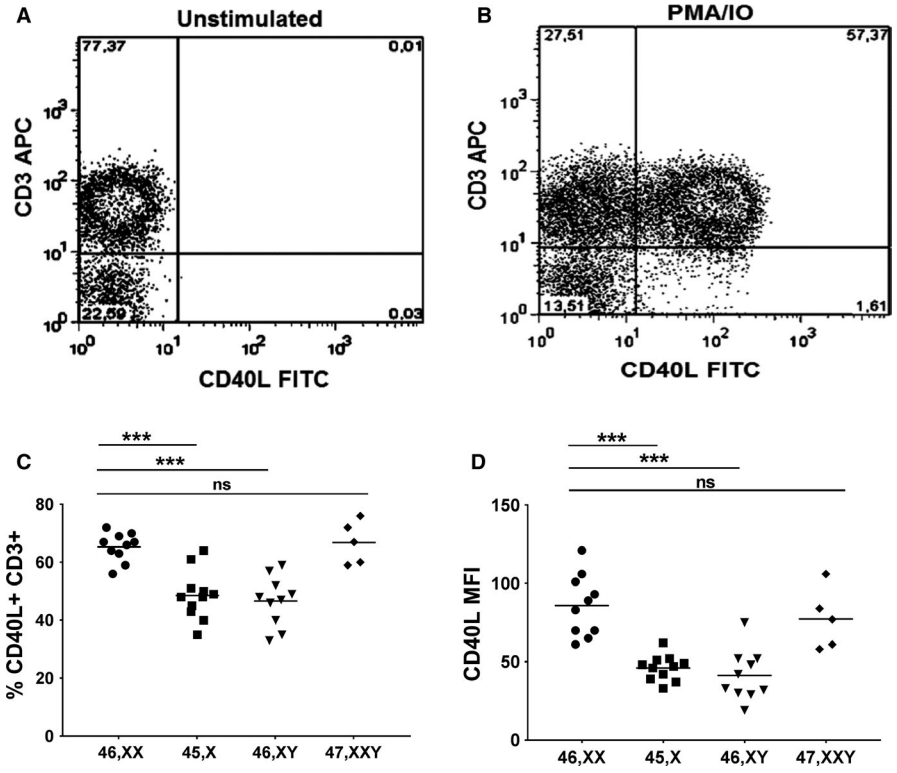

FIGURE 1 Expression of CD40L on PBMCs from healthy females (46,XX; n = 10), males (46,XY; n = 10), Turner syndrome (45,X; n = 11) and Klinefelter syndrome (47,XXY; n = 5). (A) Representative dotplots show the CD40L expression on CD3 + T cells in PBMC before stimulation. (B) Representative dotplots show the CD40L expression on CD3+ T cells in PMA/IO-stimulated PBMCs. (C) Percentage of CD3+ T cells expressing CD40L after in vitro stimulation (ns, not significant; ***$P < 0.001$, Mann-Whitney, two-tailed *t* test: $P = 0.0009$ 45X vs 47XXY and $P = 0.0006$ 46XY vs 47XXY). (D) CD40L expression (gating on CD3+ T cells) demonstrating the intensity of CD40L antigen expression on CD3+ T cells (ns, not significant; ***$P < 0.001$, Mann-Whitney, two-tailed *t* test: $P = 0.0004$ 45X vs 47XXY and $P = 0.0003$ 46XY vs 47XXY). All data are cumulative from three different experiments

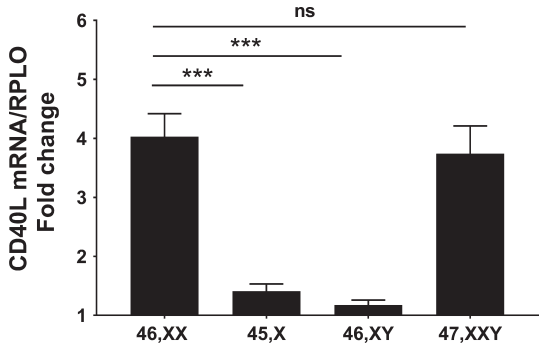

FIGURE 2 CD40L mRNA expression by qPCR on PMA/IO-stimulated PBMCs from healthy females (46,XX; n = 10), males (46,XY; n = 10), Turner syndrome (45,X; n = 11) and Klinefelter syndrome (47,XXY; n = 5). The expression of CD40L gene was normalized to the expression of the reference genes RPLP0 and is displayed as fold up change compared to the mRNA level of the corresponding unstimulated PBMC, which was set to 1. Data are presented as means ± SD and were based on observations from at least three experiments (ns, not significant; ***$P < 0.001$, Mann-Whitney, two-tailed *t* test: $P = 0.0005$ 45X vs 47XXY and $P = 0.0002$ 46XY vs 47XXY)

stimulation (Figure 1A). However, optimal expression could be achieved after in vitro stimulation with PMA/IO (Figure 1B). Striking, in vitro stimulation induced significantly higher percentage of CD40L-expressing CD3+ T cells in 46,XX women than both 46,XY men and 45,X TS patients (both $P < 0.001$), whereas KS patients with two X chromosomes did not significantly differ from females (Figure 1C). Also, CD40L protein density (Figure 1D) and mRNA expression (Figure 2) were significantly increased in 46,XX women ($P < 0.05$) and KS men ($P < 0.05$) but not in 45,X TS and 46,XY normal male karyotypes.

3.2 | Expression of X-linked gene TLR7 in healthy males and females and in patients with abnormal X chromosome number

In order to investigate if a similar pattern was true for the innate immunity gene TLR7, we stimulated PBMCs with the imidazoquinoline derivative CLO97, which is a TLR7 agonist. Healthy females showed a significantly higher percentage of $HLADR^{+}CD3^{-}CD19^{-}$ cells expressing intracellular IFN-alpha post-stimulation than healthy males, TS women and KS patients (all $P < 0.001$; Figure 3). In contrast, only healthy females and KS patients—but not 46,XY men and TS subjects—showed significantly higher TLR7 mRNA levels ($P < 0.05$) after stimulation with CLO97 (Figure 4).

4 | DISCUSSION

The first finding of this study is the striking difference of CD40L expression on T cells after activation between female and KS patients compared with male and TS patients. Interestingly, although KS subjects carry the Y chromosome, conferring them a male hormonal pattern, they also carry two X chromosomes and showed high CD40L protein expression and mRNA level after stimulation, as females did. Therefore, these data suggest that the presence of two X chromosomes plays a major role in enhancing adaptive immune responses. A previous study has shown that several

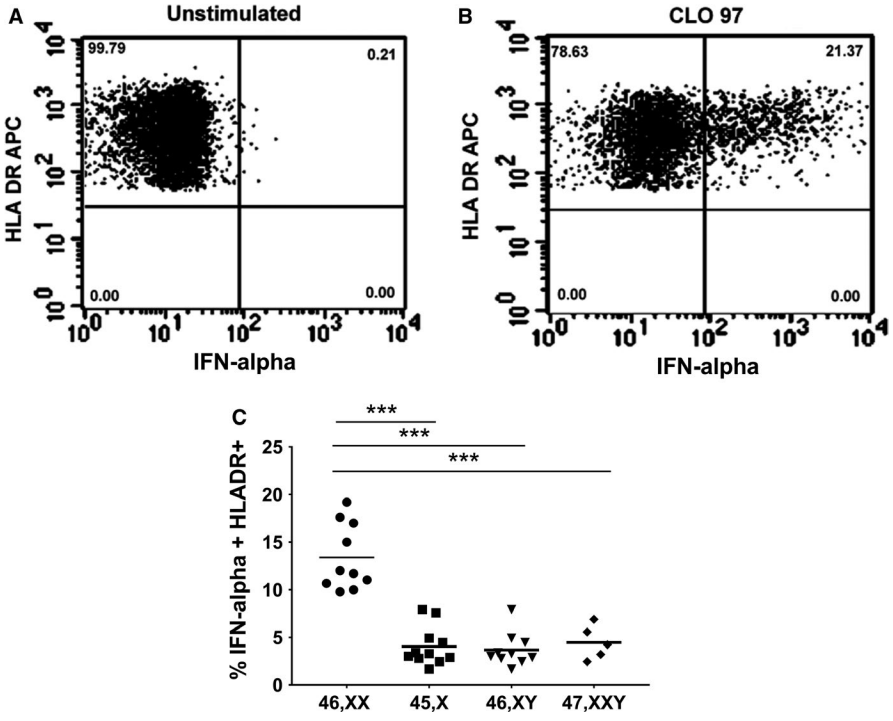

FIGURE 3 IFN-alpha production through TLR7 stimulation on PBMC from healthy females (46, XX; n = 10), males (46,XY; n = 10), Turner syndrome (45,X; n = 11) and Klinefelter syndrome (47,XXY; n = 5). (A) Representative dotplots showing the expression of IFN-alpha in HLADR + CD3-CD19 cells before stimulation with TLR7 agonist CLO97. (B) Representative dotplots showing the expression of IFN-alpha in HLADR + CD3-CD19 cells after stimulation with TLR7 agonist CLO97. (C) TLR7 protein expression levels presented as percentage of IFN-alpha–positive cells on total HLADR+ cells. All data are cumulative from three different experiments. ***$P < 0.001$, one-way ANOVA test

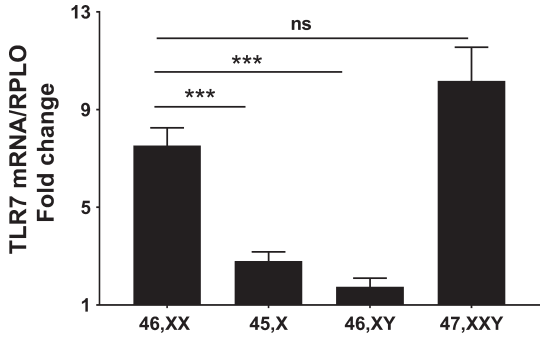

FIGURE 4 TLR 7 mRNA expression by qPCR on CLO97-treated PBMCs from healthy females (46,XX; n = 10) males (46, XY; n = 10), Turner syndrome (45,X; n = 11) and Klinefelter syndrome (47,XXY; n = 5). The expression of TLR7 gene was normalized to the expression of the reference genes, RPLP0, and is displayed as fold up change compared to the mRNA level of the corresponding unstimulated PBMC, which was set to 1. All data are cumulative from three different experiments. (ns, not significant; ***$P < 0.001$, Mann-Whitney, two-tailed t test: $P = 0.0005$ 45X vs 47XXY and $P = 0.0007$ 46XY vs 47XXY)

immune-related X-linked genes (ie CD40L, CXCR3, OGT) are upregulated in patients with systemic lupus erythematosus (SLE).[15] Importantly, an increased level of expression of CD40L in CD4+ and CD8+ T cells has been demonstrated in female patients with SLE.[16-18] On the other hand, KS patients share the same risk of women of developing SLE[19] and other female-predominant autoimmune diseases such as acquired hypothyroidism, Addison's disease and multiple sclerosis.[20] These data support the hypothesis that expression of immune-related genes that escape from X chromosome inactivation may expose women to greater risk of certain autoimmune diseases.

In a previous study, Lu Q et al evaluated differences in CD40L expression between healthy females and males after a first cycle of T cell activation through phytohemagglutinin (PHA) and further stimulation with PMA/IO.[18] The authors did not find any significant differences in $CD4^+CD40L^+$ cells between men and women, although there was a small increase in $CD4^+CD40L^+$ cells in women. We can speculate that differences in methodological procedure and the intensity of activation through PHA and PMA/IO could account for the different results observed. We chose to stimulate T cells using only PMA/IO in order to evaluate CD40L expression without depending on TCR stimulation, which could introduce additional variables. To the best of our knowledge, this is the first study demonstrating differential CD40L expression between males and females. Another major strength of this study is the genetic comparison among several cohorts with different X chromosome number.

Previous research has demonstrated that female peripheral blood lymphocytes (PBLs) produce significantly higher levels of IFN-alpha in response to TLR7 stimulation compared with male PBLs.[21] In the present study, we demonstrated that TLR7 expression in cells followed the same pattern observed for CD40L among the study subgroups; however, KS patients showed functional outcomes similar to healthy males. Thus, male-related determinants (ie, hormones levels) may still play a central role in defining the innate immune response, although many other mechanisms, like epigenetic changes, might additionally contribute to this complex process. For example, estrogens have been shown to control a number of key negative regulators of protein synthesis**,** such as microRNAs.[22] As suggested by Berghöfer et al,[21] the epigenetic sex-dependent regulation of TLR7 signalling pathways may explain the higher IFN-alpha production that is observed in females. A distinct sex-specific pattern of microRNA expression has been observed in neonatal rodent brain[23]; interestingly, this difference almost disappeared after blocking the testosterone to estradiol conversion in males, suggesting that estrogens may play a role in microRNAs regulation.[23] Thus, a hormone-mediated epigenetic modulation of TLR7 gene could justify the lack of IFN-alpha response reported in KS subjects, despite increased TLR7 mRNA levels after stimulation.

In humans, the greater TLR7 transcriptional expression in a large proportion of plasmacytoid dendritic cells (pDCs), B cells and monocytes from normal women and KS males has been linked to the development of SLE and other autoimmune diseases.[24] These findings are supported by animal studies, demonstrating that TLR7 gene dosage is both necessary and sufficient to promote autoantibody production and pathological disease associated with systemic autoimmune diseases.[25,26]

In conclusion, our data demonstrate a direct role of X chromosome genes dosage expression in the development of innate and adaptive immune responses, which may underlie sex differences in immune biology and the sex bias in predisposition to autoimmune diseases.

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CONFLICT OF INTEREST

No potential conflicts of interest relevant to this article were reported.

AUTHOR CONTRIBUTIONS

CC and JS conceived and designed the study. LS, IB and CC wrote and edited the manuscript. JS, LS, AG and CC acquired data, revised and edited the intellectual content of the article. All authors reviewed and approved the final version

of the manuscript. CC is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the work.

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