TLR7 escapes X chromosome inactivation in immune cells

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Toll-like receptor 7 (TLR7) is critical to the induction of antiviral immunity, but TLR7 dosage is also a key pathogenic factor in systemic lupus erythematosus (SLE), an autoimmune disease with strong female bias. SLE prevalence is also elevated in individuals with Klinefelter syndrome, who carry one or more supernumerary X chromosomes, suggesting that the X chromosome complement contributes to SLE susceptibility. TLR7 is encoded by an X chromosome locus, and we examined here whether the TLR7 gene evades silencing by X chromosome inactivation in immune cells from women and Klinefelter syndrome males. Single-cell analyses of TLR7 allelic expression demonstrated that substantial fractions of primary B lymphocytes, monocytes, and plasmacytoid dendritic cells not only in women but also in Klinefelter syndrome males express TLR7 on both X chromosomes. Biallelic B lymphocytes from women displayed greater TLR7 transcriptional expression than the monoallelic cells, correlated with higher TLR7 protein expression in female than in male leukocyte populations. Biallelic B cells were preferentially enriched during the TLR7-driven proliferation of CD27+ plasma cells. In addition, biallelic B cells showed a greater than twofold increase over monoallelic cells in the propensity to immunoglobulin G class switch during the TLR7-driven, T cell–dependent differentiation of naive B lymphocytes into immunoglobulin-secretting cells. TLR7 escape from X inactivation endows the B cell compartment with added responsiveness to TLR7 ligands. This finding supports the hypothesis that enhanced TLR7 expression owing to biallelism contributes to the higher risk of developing SLE and other autoimmune disorders in women and in men with Klinefelter syndrome.

INTRODUCTION

Toll-like receptor 7 (TLR7), together with TLRs 3, 8, and 9, forms a family of intracellular nucleic acid sensors maintained under strong purifying selection, which attests to their essential role in host survival to viral infection (1). TLR7 localizes to the endosomal compartment, where it binds microbial or self-derived single-stranded RNA ligands (2). In plasmacytoid dendritic cells (pDCs), TLR7 engagement elicits strong type I interferon (IFN) production and is critical to the induction of antiviral immune responses (3). TLR7 is also an essential component of antibody-mediated immunosurveillance against the reactivation of endogenous retroviruses (4).

RNA-containing immune complexes associated with severe systemic lupus erythematosus (SLE) (5) are recognized by TLR7, and disease severity in lupus-prone mice depends on the Tlr7 gene dose (6–8). TLR7 deficiency protects mice against lupus by decreasing the production of autoantibodies to ribonucleoproteins and mitigates lupus-associated kidney disease (9). In contrast, Tlr7 overexpression induces systemic autoimmunity even in a non–lupus-prone genetic background (6). B cell–intrinsic TLR7 signaling is essential to the development of spontaneous germinal centers, autoantibody production, and systemic inflammation (10, 11), which supports the notion that RNA sensing through TLR7 represents a central mechanism of chronic B cell activation in SLE.

Like other autoimmune disorders, SLE is markedly sex-biased, and female-to-male incidence peaks at 9:1 in adulthood (12). SLE susceptibility correlates with the number of X chromosomes in the karyotype and is thus increased in men with Klinefelter syndrome (47,XXY; KS) (13), whereas a lower risk has been suggested for women affected with Turner syndrome (45,X) (12). These observations support a gene-dose effect of X chromosome loci in SLE predisposition (13, 14).

The gene encoding TLR7 maps to the short arm of the X chromosome. In placental mammals, dosage compensation for X-linked gene products between XX females and XY males is achieved by the random silencing of one of the two X chromosomes during the early development of female embryos (15, 16). This essential mechanism, called X chromosome inactivation (XCI), was first thought to be extremely stable in adult somatic cells, with only a minority of genes, notably those in the pseudoautosomal regions shared with the Y chromosome, endowed with biallelic expression (17–19). However, studies of global X-linked gene expression in rodent-human somatic cell hybrids, using limited numbers of available exonic polymorphisms, found that up to 30% of X-linked genes, in and outside the pseudoautosomal regions, exhibited some variable degree of escape from XCI (17–19). Recently, advances in high-throughput and single-cell technologies enabled a systematic RNA sequencing analysis of up to 29 tissues in hundreds of individuals, which confirmed that about 23% of X-linked genes show incomplete XCI, with variability between tissues and individuals (19, 20). Because TLR7 is a non-pseudoautosomal locus, we investigated whether this gene escaped from XCI in women and KS males. Here, we demonstrate that TLR7 is transcribed on both X chromosomes in a large proportion of the pDCs, B cells, and monocytes from normal women and KS males. Focusing on B lymphocytes, we show that biallelism leads to increased TLR7 gene products and imparts a selective advantage at key TLR-dependent development checkpoints of effector B cells.

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RESULTS

TLR7 evades XCI in female primary immune cells

We developed an assay to measure the relative abundance of TLR7 transcripts derived from either X chromosome in female cells at single-cell resolution (Fig. 1A). The assay used the allele-specific KASP (KBiosciences) polymerase chain reaction (PCR) chemistry (21) to discriminate a diallelic (G or C) single-nucleotide polymorphism (SNP) of TLR7 in the 3′ untranslated region of the mRNA (rs3853839; Fig. 1B). We were able to analyze, in this way, immune cells from women who carried allele C on one X chromosome and allele G on the other. We calculated the relative proportions of the two alleles from the ratio of the respective endpoint fluorescence signals in the KASP PCR amplification; for this, we built a standard curve based on genomic DNA samples from men of G/0 and C/0 genotypes in different mix ratios (Fig. 1, C and D). B lymphocytes, monocytes, and pDCs were individually sorted by the gating strategy in fig. S1A, and each cell was classified as monoallelic or biallelic for TLR7 expression when the relative abundance of the minor allele (i.e., the less represented allele among total TLR7 transcripts in each cell) fell below or above 15%, respectively. This ex vivo approach categorized primary B lymphocytes into the two monoallelic groups predicted by the paradigm of stochastic XCI (15, 16) plus a substantial stratum of biallelic cells where TLR7 escaped from XCI (Fig. 1E; green or blue, monoallelic; red, biallelic). pDCs split up in a similar way, as did monocytes (Fig. 1, F and G). The accuracy of this allele-of-origin classification was verified by sequencing the TLR7 complementary DNA (cDNA) amplimers from random monoallelic and biallelic B cells (Fig. 1H), and we confirmed single-cell resolution by assaying mixed male B cells of G/0 and C/0 genotypes (1:1), which generated only correct monoallelic calls (Fig. 1I). Last, similar biallelic cell frequencies were found in a comparison of KASP results for SNP rs3853839 and for the alternative TLR7 exonic polymorphism rs179008 (A to T substitution near the 5′ terminus of exon 3; Fig. 1B), ruling out an SNP-dependent bias in the allele-specific PCR (fig. S1B). The allelic expression pattern of TLR7 among B cells, monocytes, and pDCs was consistent across a cohort of eight heterozygous women (Fig. 1J): 30% of cells were biallelic, on average, with some interindividual variability but similar monoallelic/biallelic ratios between same-donor

**Fig. 1. TLR7 escape from X inactivation in immune cells.** (A) Workflow of the allele-of-origin analysis using a genetic marker observable in mature TLR7 transcripts. (B) Map of TLR7 and the useful exon 3 SNPs, rs179008 (A/T) and rs3853839 (C/G). (C) KASP assay of mixed genomic DNA samples from men genotyped G/0 (green) and C/0 (blue) for SNP rs3853839, showing the variation of fluorescence intensities according to the mix ratio. (D) Standard curve of the KASP assay. (E to G) Representative allele-of-origin profile of individual B cells (E), monocytes (F), and pDCs (G) from a woman of G/C heterozygous genotype, displaying a proportion of biallelic cells; each dot in the graphs represents one cell with monoallelic (blue or green) or biallelic (red) expression of TLR7. (H) Confirmation by Sanger sequencing (right) of the KASP monoallelic (green or blue) and biallelic (red) expression calls (left) in primary female B cells. (I) Single-cell resolution of the assay. Mixed male B cells of G/0 and C/0 genotypes correctly generate no biallelic events. (J) Frequency of single-cell biallelic TLR7 expression among pDCs (BDCA4+ CD123+), monocytes (CD14+), and B cells (CD19+) from eight heterozygous women (HD1 to HD8). The dotted line denotes the global average.
cell types. Among steady-state CD19+ B lymphocytes, biallelism levels were similar between CD27− IgM− IgD+ (naive) and CD27+ IgM+ IgD− (memory) cells in a cohort of 13 heterozygous women (fig. S1C).

In addition, we observed simultaneous TLR7 transcription on both X chromosomes using RNA fluorescence in situ hybridization (RNA FISH; Fig. 2) (22). A fraction of female CD27+ B cells, pDCs, and monocytes exhibited two nuclear foci of nascent TLR7 RNAs at two sites of transcription, one of which colocalized with the inactive X chromosome (Fig. 2 and fig. S2). The inactive X territory was detected by using probes against the XIST (X-inactive specific transcript) non-coding transcripts that coat the chromosome to initiate XCI and form the so-called XIST cloud (23). Further hybridizations on B cells showed the expected double signals from SLC25A6, a pseudoautosomal gene on the tip of Xp constitutively expressed from the active and the inactive X (17), paired with signals from TLR7 nascent transcripts on both chromosomes (movie S1). These experiments confirmed that TLR7 escapes from XCI in a proportion of immune cells.

**Escape from X inactivation increases TLR7 gene products**

To measure the effect of escape from X inactivation on TLR7 mRNA levels, we quantified total TLR7 transcripts by real-time PCR in single CD19+ CD27+ IgD+ naive B cells from three women and determined in parallel the relative abundance of allelic TLR7 transcripts in each cell (Fig. 3A). The relative abundance of TLR7 transcripts from the minor allele in each cell ranged from ≈0% (apparent TLR7 mono-allelism) to nearly 50% (apparent balanced biallelism). Because gene transcription is stochastic and may occur in nonsynchronous bursts on either allele, single-cell analysis may count stably and transiently monoallelic cells together (24). We considered that cells where the minor allele accounted for less than 10% of TLR7 transcripts [as per (17)] represented the stratum with the highest likelihood of stable monoallelism and defined a reciprocal biallelic stratum of cells where the minor allele accounted for 35 to 50% of total TLR7 transcripts (Fig. 3A). The two strata were sharply different as regards the proportions of low (below the first-quartile threshold) and high TLR7 expression cells (P < 10−4, two-sided Fisher’s exact test). Low-expression cells were depleted in the biallelic stratum (Fig. 3B), and median TLR7 expression increased 1.8-fold relative to the monoallelic stratum (1.27 versus 0.70 times the population mean; Fig. 3B, white dots), consistent with a shift from one to two contributing alleles in the burst-like stochastic transcription model (24).

In parallel to TLR7, we quantitated by single-cell real-time PCR the transcripts from 15 well-expressed housekeeping and TLR signaling–related genes, but these were not significantly elevated in TLR7 biallelic cells (fig. S3A). We calculated the relative expression of TLR7 with reference to 12 autosomal and 3 X chromosome genes by the ΔCt method on a per-cell basis. As expected, we observed a significant difference between monoallelic and biallelic cells when TLR7 expression was normalized to nine autosomal genes and to the three X-linked genes (IL2RG, IRAK1, and TMSB4X; Fig. 3C and fig. S3, B to E). This indicated that greater TLR7 expression in biallelic cells was specific (i.e., did not mirror a hypothetical increase across the entire gene panel) and suggested that the inactive X chromosome was not globally reactivated (although our sample of X-linked genes was small). Principal components analysis of the real-time PCR data set showed a groupwise difference between the monoallelic and biallelic cells in the first and second principal components (Fig. 3D). We could not correlate increased TLR7 transcripts in biallelic naive B cells with any significant changes in other assayed genes. TMSB4X lies only 85 kb from TLR7 and reportedly evades XCI (25); elevated TLR7 transcripts over the TMSB4X internal control in biallelic cells (~0.534 ΔCt, units on average) suggest that the escape from XCI of TLR7 and TMSB4X is uncoupled despite the close physical linkage. Consistent with biallelic gene expression in a large cell subset, Western blot analysis (using a newly validated monoclonal antibody to the C terminus of human TLR7; see Materials and Methods and fig. S4) showed the full-length and proteolytically mature forms of TLR7 to be increased 1.38- and 1.31-fold, respectively, in resting peripheral blood mononuclear cells (PBMCs) from women relative to the male cells (Fig. 4).

**TLR7 escape from X inactivation enhances the B cell response to TLR7 engagement**

We investigated the B cell response to TLR7 engagement attending to sex and to the allelic pattern of TLR7 expression. In the presence of type 1 IFN, nucleic acid sensor (TLR7 or TLR9) engagement elicits proliferation and immunoglobulin secretion by B cells (Fig. 5A) (26). We stimulated CD19+ B cells in vitro for 6 days with IFN-β, an up-regulator of TLR7 expression (27), and with either TLR7 or TLR9 ligands. The frequencies of proliferating plasma cell (PC)–committed (CD27hi) and naive (CD27−) B cells showed that the CD27hi PC lineage population (28) expand more vigorously in female than in male cells (Fig. 5B), but only when TLR7-stimulated. One hypothesis was that this bias could arise from TLR7 biallelic expression in women’s cells. TLR7 biallelism was enriched among CD27hi PCs that proliferated in response to TLR7 engagement specifically (P = 0.016, Wilcoxon’s paired test; Fig. 5C). Proliferating CD27− (naive) B cells trended in the same direction, but the difference was not conclusive. Biallelic CD27hi PC lineage B cells were counterselected upon TLR9 engagement (Fig. 5C), consistent with the notion that TLR7 and TLR9 compete for binding to UNC93B1, the chaperone protein that regulates the trafficking of both receptors to the endosomal compartment (29, 30). This predicts that, at least under IFN-β
stimulation, greater TLR7 expression in biallelic B cells should result in lower levels of functional TLR9, and the converse in monoallelic cells.

Activation of human naïve B cells requires a combination of T cell help and TLR engagement (31, 32). Figure 6A illustrates this dependency using an in vitro model (32, 33) that measured proliferation and immunoglobulin class switch in response to T cell–dependent signals and ligands for TLR7 (CL097) or, alternatively, TLR9 (class B CpG oligodeoxynucleotides). On CD19+CD27−IgG+ naïve B cells from five women, this model showed the frequency of TLR7 biallelic B cells to be significantly enriched among immunoglobulin G (IgG)–switched proliferative cells after TLR7 but not TLR9 engagement (Fig. 6B). We further analyzed these data to quantify the effect of biallelism on class switch. In each instance of B cell donor and TLR ligand used to promote class switch, we wrote the biallelic and monoallelic cell counts for the IgG+ and IgG− populations as a 2 × 2 contingency table (table S6), by analogy with a case-control study of epidemiological association (34). We next pooled all the 2 × 2 tables for the same TLR ligand in a statistical meta-analysis to extract a summary odds ratio (OR). The summary OR measured the strength of the association between biallelism and IgG class switch and is formally the estimated ratio of the odds of class switch in biallelic cells to the odds of this outcome in monoallelic cells (Fig. 6C). By this approach, we observed non-association overall between biallelism and IgG expression in response to the

TLR7 ligand (summary ORTLR7 ≈ 1; Fig. 6C). In contrast, upon TLR7 engagement, biallelic cells were more likely to undergo IgG class switch than monoallelic cells, with summary ORTLR7 = 2.4, 95% confidence interval (CI) [1.6 to 3.7] (Fig. 6C).
Because immunoglobulin class switch represented <10% of outcomes in this model (e.g., 4.45% for TLR7 and 7.03% for TLR9; Fig. 6A), the OR makes a good approximation to the relative risk (35). With OR_{TLR7} = 2.4, the propensity of biallelic cells to differentiate into isotype-switched cells in the presence of T cell help and TLR7 agonist ligands was thus more than twofold greater than that of monoallelic cells. In addition, OR_{TLR7} enabled the percentage of IgG^+ cells due to biallelism to be estimated as the population attributable fraction (PAF) = 29% (34), 95% CI [14 to 45%] (assuming 30% biallelism as observed earlier under IFN-β stimulation; Fig. 1J and Materials and Methods). This experiment confirmed that TLR7 escape from XCI may endow the B cell compartment with added responsiveness to TLR7 ligands and contribute a large proportion of immunoglobulin class switch events.

**The TLR7 allelic expression profile is unstable in EBV-transformed B cells**

TLR7 escape from XCI was reported recently in human Epstein-Barr virus (EBV)-transformed lymphoblastoid B cells (36, 37). We investigated whether monoallelic or biallelic expression was stable in the progeny of lymphoblastoid cell clones. Similar to the primary B cells of the female donor, EBV-transformed cells comprised three subsets with monoallelic or biallelic TLR7 expression (fig. S5A). We characterized the TLR7 allele of origin in clonal subcultures derived by limiting dilution (fig. S5, B and C) and selected a predominantly monoallelic subline, F2, for further subcloning. Follow-up at single-cell resolution of three clonal sublines (F2-2G7 and F2-3E10 plus G9 derived from the initial lymphoblastoid population) evidenced a striking relaxation toward biallelism in one-half of the cells (fig. S5, D and E).

**TLR7 evades X inactivation in immune cells from KS males**

The carriage of a supernumerary X chromosome in KS multiplies the risk of developing SLE and other female-biased autoimmune pathologies (13, 14). We examined whether higher TLR7 dosage arising from XCI escape also operates in 47,XXY males. Similar to female single cells, we observed biallelic TLR7 expression in 19.3 to 39% of primary pDCs, monocytes, and B lymphocytes from two KS males (Fig. 7, A to D) using either rs3853839 as above or, alternatively, rs179008 as a TLR7 allelic marker. Improving the IFN-β stimulation step in the RNA FISH protocol (see Materials and Methods) again showed biallelic TLR7 expression at high frequencies (49 and 59%; Fig. 7, E and F) in CD19^+ B cells from two other KS males. These results demonstrate that the female pattern of biallelic TLR7 expression also occurs in 47,XXY males and suggest TLR7 escape from XCI as a mechanism for the elevated risk of SLE in KS men.

DISCUSSION

X disomy and polysomy are thought to contribute to the greater risk of autoimmunity in women and also in KS men, who carry one or more supernumerary X chromosomes (13, 14). We demonstrate here that TLR7 gene products are increased in women owing to consistent escape from XCI in a large proportion of B lymphocytes, monocytes, and pDCs. Previous studies have shown that TLR7 is required for the production of autoantibodies to ribonucleoproteins in SLE and that TLR7-dependent B cell responses are TLR7 dose-sensitive (7–9, 38). In agreement with this, we observed enhanced TLR7-driven responses of biallelic female B cells during key stages of effector B cell development. In addition, we found a significant TLR7 biallelism among immune cells of KS males. Together, our findings strongly support the hypothesis that TLR7 overexpression through biallelism is a candidate contributor to the risk of SLE not only in women but also in 47,XXY men (13, 14).

Our data show TLR7-expressing B cells as a mosaic population of individual cells expressing transcripts from either one or two alleles at a given time. The current model of stochastic burst–like gene expression (24) predicts a similar pattern for autosomal genes expressed at low-to-medium levels. In this model, transcription occurs not continuously but in pulses of RNA molecules from one allele or the other. The timing of these pulses is stochastic, and they alternate with periods of transcriptional inactivity. This model may explain that B cells with low levels of TLR7 transcripts were almost absent among the biallelic population (Fig. 3B), given that transcription from one allele can occur during the inactivity periods of the other if both are expressed. However, as we note below, our functional experiments argue not for complete biallelism but for distinct monoallelic and biallelic cell subsets with differential responsiveness to TLR7 ligands.

TLR engagement is necessary for immunoglobulin class switch in the generation of antigen-specific antibody responses (31, 32). We analyzed in vitro the impact of TLR7 biallelism on class switch through the response of naive B cells from women to a TLR7 agonist and found that biallelic cells display more than twofold greater propensity to class switch toward IgG than monoallelic cells. This is a conservative estimate of overall class switch propensity because we only analyzed for the IgG isotype. We ascribed this effect to the higher dose of the protein product of TLR7 specifically, in light of the neutral outcome (summary OR_{TLR9} ≈ 1) of TLR9 engagement. These results show that the observed increase in TLR7 biallelism among class-switched IgG+ cells did not result from TLR stimulation at large. The effects on the immunoglobulin repertoire of overexpressing TLR7 in murine models of lupus have been addressed by several studies (38–40), which suggest that increased TLR7 dose in women owing to biallelism could contribute to higher SLE risk by fostering a larger pool of PC lineage cells producing high-affinity IgG autoantibodies; these have been recognized as a factor in the pathogenesis of human SLE (41, 42).

TLR7 evaded XCI in all the female and KS male patients we have studied thus far, with a wide range of biallelic cell frequencies but similar within-donor values in B lymphocytes, monocytes, and pDCs. Although this suggests intracellular stability, variation in respect of physiological processes, age, genetics, and the environment remains to be examined. Longitudinal investigation of biallelism during B cell differentiation, activation, and proliferation is challenging because single-cell longitudinal studies would require nondestructive sample analysis. In addition, our study does not directly demonstrate that an increase in TLR7 expression drives autoimmunity in individuals carrying two X chromosomes. Because we are dealing with human primary cells, the participants of study were not directly amenable to...
experimentation. Dissection of the mechanisms involved should be aided by cellular and animal models, although we note that XCI in mice appears to be more complete than in humans (19). To assess the maintenance of XCI in the progeny of monoclonal B cells, we generated EBV-transformed B cell clones, because escape from XCI has been reported for TLR7 in lymphoblastoid cells (36, 37). However, marked degradation of monoallelicism in these lines over time in culture suggested loose control of XCI as a consequence of EBV immortalization, which is known to cause large-scale epigenetic remodelling of the host cells (43).

In conclusion, our study provides proof that TLR7 evades XCI in human B cells, pDCs, and monocytes, endowing women and KS males with subpopulations of immune cells where TLR7 expression is enhanced. In B lymphocytes, we found evidence for a causal relationship between biallelism and TLR7-dependent cell responsiveness. TLR7 function has been also well documented in pDCs (3) and other specialized cell types such as CD4+ T cells (44), monocytes (45), and neutrophils (46), implying a potential impact of sex-dependent TLR7 dosage on multiple immune processes. This could be particularly relevant to the TLR7-driven production of type I IFNs by pDCs, which exhibits a strong female bias (47). Although the female sex hormone, estrogen, appears to be an important regulator of TLR responsiveness in these cells, an added nonredundant effect of X chromosome dosage has also been suggested (48). The tissue distribution of biallelism and its longitudinal within-subject stability, variability between individuals, and functional consequences in different immune cell lineages will be important topics of study in the context of the TLR7-dependent autoimmune and infectious pathologies of women and KS men.

**MATERIALS AND METHODS**

**Donors and ethical compliance**

This study complied with the ethical principles of the Declaration of Helsinki and with applicable French regulations. PBMCs of anonymous, healthy blood donors at the Toulouse blood transfusion center (Etablissement Français du Sang) were from a biobank authorized under dossier number 2-15-36 by the competent ethics board (Comité de Protection des Personnes Sud-Ouest et Outre-Mer II, Toulouse).
The study of KS donors was approved by this board under dossier number 1-16-28. Patients with 47,XXY karyotypes [aged 11 (KS3), 13 (KS1), 16 (KS4), and 21 (KS2)] were recruited at the Toulouse University Hospital, and informed consent was obtained in writing from the patient or the patient’s parents or guardians as appropriate.

**Genotyping and single-cell analysis of TLR7 allelic expression**

Typing for the frequent diallelic polymorphisms of TLR7, rs3853839 (NM_016562.3:c.*881C>G) and rs179008 (NM_016562.3:c.32A>T), at the genomic and single-cell cDNA levels relied on custom KASP competitive allele-specific PCR assays (21) from LGC. rs3853839 localizes to the 3′ untranslated region in exon 3, 881 base pairs downstream from the stop codon; rs179008 is a coding-sequence polymorphism near the 5′ end of exon 3. The workflow for single-cell cDNA analysis is summarized in Fig. 1A. Immune cells from men or heterozygous women were sorted with a FACSaria II cell sorter (BD Biosciences) at one cell per well of a 96-well plate preloaded with a medium containing 2% Triton X-100, RNaseOUT recombinant ribonuclease inhibitor (1 U/μl; Thermo Fisher Scientific), 940 μM deoxynucleoside triphosphates, and random hexamer primers (12.5 ng/μl). For RNA reverse transcription, single-cell lysates were supplemented with Maxima H Minus reverse transcriptase (50 U per well) and the supplied reaction buffer (Thermo Fisher Scientific). Before KASP typing, target molecules were PCR-amplified using TLR7 cDNA-specific primer pairs, and negative wells were screened out by quantitative real-time PCR with nested primers (table S1), using LightCycler 480 SYBR Green I Master reagents from Roche. The quantitative real-time PCR and KASP fluorescence-based assays were performed in a LightCycler 480 instrument (Roche). Relative allelic expression was calculated from the ratio of the 6-carboxyfluorescein (FAM) (alleles C and T) and chloromito-6-carboxyfluorescein (HEX) (alleles G) fluorescence signals from the respective KASP probes; a four-parameter standard curve was generated monthly for each LightCycler 480 unit, using an R script based on package drc (Fig. 1, C and D) (49). Biallelic TLR7 expression in a cell was inferred when the relative expression values of TLR7 transcripts bearing the rs3853839 G allele were comprised between 15 and 85%. Further information on the single-cell assay can be found in the Supplementary Materials.

**Immunostaining for flow cytometry**

Human immune cells were stained with the following fluorescent mouse antibodies conjugates against specific surface markers: R-phycocerythrin (PE) anti-CD14 for monocytes; allophtochocyanin (APC) anti-CD14, isothiocyanate (FITC) anti-CD19 (CD14/CD16/CD19/CD56/CD235a), and PE anti-CD123 for pDCs; and PE-Cy5 anti-CD19, APC anti-CD27, BV421 anti-IgA, BV510 anti-IgG, PE anti-IgM, and FITC anti-IgD for B cell subsets (table S2). Flow cytometry analysis was performed on a BD Biosciences LSRII or FACSaria II cell sorter, and the data were processed with the FlowJo software (FlowJo LLC).

**RNA FISH**

RNA FISH was performed as described by Chaumeil and colleagues (22). Briefly, spreads of primary nonadherent cells on poly-l-lysine-coated coverslips were fixed for 10 min with 3% paraformaldehyde at room temperature and permeabilized for 7 min in ice-cold cytosal buffer containing 0.5% Triton X-100 and 2 mM vanadyl-ribonucleoside complex (New England Biolabs). The cells were dehydrated through successive ethanol baths, air-dried briefly, and incubated with the labeled probes (described in the Supplementary Materials) overnight at 42°C. The coverslips were rinsed twice with 50% formamide in 2× SSC buffer and thrice with 2× SSC alone, and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in phosphate-buffered saline. The coverslips were mounted using Dako fluorescence mounting medium before microscopy on a Leica TCS SP8 or Zeiss LSM 710 microscope using a 63× oil immersion objective. Image data were processed with the Fiji software (50). Improved cell culture conditions were applied to CD19+ B cells from KS patients, which boosted hybridization-detectable TLR7 transcripts. The cells were grown for 5 days in the presence of the imidazoquinoline CL097 (250 ng/ml; InvivoGen) and human IFN-β (1 ng/ml; PeproTech), starved of IFN-β for 24 hours, and last, IFN-β–stimulated for 2 hours immediately before harvesting for RNA FISH.

**High-throughput quantitative real-time PCR**

Cells from specific immune compartments were flow-cytometrically fractionated and then individually sorted using a C1 Single-Cell Auto Prep system (Fluidigm) at the Genomic and Transcriptionic (GeT) core laboratory, Toulouse. Single-cell RNA was reverse-transcribed, and the cDNAs from genes of interest were amplified by PCR before quantitation by real-time PCR using the Fluidigm BioMark technology.

**TLR activation assay**

CD19+ B cells were isolated to >93% purity by negative selection (Human B cell enrichment kit, STEMCELL Technologies), stained with a tracing dye (CellTrace Violet, Thermo Fisher Scientific), and seeded in 96-well U-bottom plates at 5 × 10^4 cells per well in RPMI 1640 medium with 10% fetal bovine serum (FBS). The cells were then stimulated with human IFN-β (1 ng/ml), class B CpG oligodeoxynucleotides (300 ng/ml; InvivoGen), or CL097 (250 ng/ml). After 6 days, the cells were labeled for CD19 and CD27 expression, and the proliferative response was analyzed by flow cytometry.

**T cell–dependent activation assay**

The T cell– and TLR-dependent model of naive B cell activation was implemented as described by Ruprecht and Lanzavecchia (32). Naive B lymphocytes were enriched by negative selection (EasySep human B cell isolation kit, STEMCELL Technologies) on the PBMCs of healthy donors, and CD16– CD3+ CD14– CD19+ CD27– IgG– IgA– cells were sorted on a FACSaria II cell sorter. CD4+ T cells were isolated from the same donor (CD4+ CD45+ T cell isolation kit, human, Miltenyi Biotec) and irradiated at 20 grays. Naive B cells (1 × 10^4 per well) were loaded with CellTrace Violet and cocultured with 2 × 10^4 irradiated T cells in 96-well round bottom culture plates, using RPMI 1640 medium supplemented with 10% FBS; CD40L + enhancer (1 μg/ml; Enzo Life Sciences); interleukin-6 (IL-6), IL-10, and IL-12 (10 ng/ml each); F(ab')2 anti-human IgM-IgG-IgA (2.5 μg/ml); and the following superantigens at 0.5 ng/ml each: toxic shock syndrome toxin 1 and Staphylococcus aureus SeA, SeB, SeE, and SeC. TLR signaling was elicited by CL097 (250 ng/ml; TLR7) or CpG-B (300 ng/ml; TLR9). After 7 days in culture, cells were stained for CD19, IgA, IgG, IgM, and IgD and sorted by differentiation status on a FACSaria II instrument.

**EBV transformation of B cells**

Lymphoblastoid cell lines were generated by a standard protocol. Briefly, 10^6 PBMCs were incubated for 3 hours at 37°C with 1 ml of EBV viral supernatant from B95-8 cells. The cells were washed, resuspended in 1 ml of RPMI 1640 medium with 20% FBS in the presence of
phytohemagglutinin (2 μg/ml), and grown in 1 well of a 24-well plate until the appearance of clumps of transformed B cells.

### Statistical analysis

Paired analysis of variance (ANOVA) and two-sided Welch’s t test, Mann-Whitney, Student’s t test, paired t test, and Wilcoxon’s paired test were performed using Prism 4 (GraphPad Software). All data subjected to a parametric test satisfied the Shapiro-Wilk test of normality at α = 0.05. The normality test and two-sided Fisher’s exact test were performed with R. A summary OR and 95% CI were extracted from sets of $2 \times 2$ contingency tables by the exact meta-analysis method of Liu and colleagues (31) implemented in R package gmeta. The PAF and its 95% CI were calculated from the summary OR using the following formula (34):

$$\text{PAF} = \frac{P_e(\text{OR} - 1)}{P_e(\text{OR} - 1) + 1} \times 100$$

where $P_e$ is the prevalence of exposure to the risk factor. Here, $P_e = 0.3$, corresponding to 30% TLR7 biallelism in naive B cells (Fig. 1).

### SUPPLEMENTARY MATERIALS

immunology.sciencemag.org/cgi/content/full/3/19/eaap8855/DC1

Materials and Methods

Fig. S1. Determination of TLR7 monoallelic or biallelic expression at the single-cell level.

Fig. S2. In situ visualization of monoallelic and biallelic TLR7 transcripts at the single-cell level.

Fig. S3. Relationship between transcript quantitation and level.

Fig. S4. PCR primer pairs used in the preparation of the

Fig. S5. Unstable


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**TLR7 escapes X chromosome inactivation in immune cells**

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**The X chromosome link to lupus**

Nine of 10 individuals who develop systemic lupus erythematosus (SLE) are women. Furthermore, individuals with Klinefelter syndrome (47,XXY) also have increased incidence of SLE, suggesting that X chromosome dosage could be an important risk factor in SLE. Using sensitive quantification methods, Souyris *et al.* demonstrate that Toll-like receptor 7 (TLR7) that is encoded from the X chromosome escapes X inactivation in B cells and myeloid cells in females and Klinefelter individuals. TLR7 binds single-stranded RNA and activates type I interferon signaling, a pathway that is also activated in SLE patients. On the basis of this, the authors propose that biallelic expression of TLR7 contributes to greater SLE risk in individuals with two X chromosomes.