Parent-of-origin differences in DNA methylation of X chromosome genes in T lymphocytes

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Many autoimmune diseases are more frequent in females than in males in humans and their mouse models, and sex differences in immune responses have been shown. Despite extensive studies of sex hormones, mechanisms underlying these sex differences remain unclear. Here, we focused on sex chromosomes using the "four core genotypes" model in C57BL/6 mice and discovered that the transcriptomes of both autoantigen and anti-CD3/CD28 stimulated CD4^{+} T lymphocytes showed higher expression of a cluster of 5 X genes when derived from XY as compared to XX mice. We next determined if higher expression of an X gene in XY compared to XX could be due to parent-of-origin differences in DNA methylation of the X chromosome. We found a global increase in DNA methylation on the X chromosome of paternal as compared to maternal origin. Since DNA methylation usually suppresses gene expression, this result was consistent with higher expression of X genes in XY cells because XY cells always express from the maternal X chromosome. In addition, gene expression analysis of F1 hybrid mice from CAST × FVB reciprocal crosses showed preferential gene expression from the maternal X compared to paternal X chromosome, revealing that these parent-of-origin effects are not strain-specific. SJL mice also showed a parent-of-origin effect on DNA methylation and X gene expression; however, which X genes were affected differed from those in C57BL/6. Together, this demonstrates how parent-of-origin differences in DNA methylation of the X chromosome can lead to sex differences in gene expression during immune responses.

global DNA methylation | parental imprinting | sex differences | X chromosome | autoimmunity

Women have more robust immune responses to self and foreign antigens compared to men. This robust immune response is consistent with a higher incidence of autoimmune diseases in women (1, 2). In the autoimmune disease multiple sclerosis (MS), women are more susceptible than men by a 3:1 ratio, and in systemic lupus erythematosus (SLE) the female bias is 9:1. This female preponderance across distinct autoimmune diseases suggests a fundamental mechanism underlying its etiology. Female-predominate immune responses are also observed across species (1, 3), consistent with the importance of sex as a biological variable (4, 5). Sex differences can be due to sex hormones, sex chromosomes, or both (6). While the role of sex hormones has been well studied in pre-clinical models of autoimmune diseases and in clinical trials, the role of sex chromosomes in autoimmunity remains unclear (6, 7).

Sex chromosomes can cause differences in gene expression between males (XY) and females (XX) due to the expression of Y chromosome genes, X gene dosage effects, or parent-of-origin differences in DNA methylation of X genes (6). The Y chromosome has evolved from an autosomal ancestor to primarily include genes involved in male reproduction, with only a few nonreproduction-related genes remaining (8, 9). That said, consomic mice previously showed a strain-specific Y chromosome effect on autoimmune disease susceptibility, suggesting that allelic variants of Y genes in a given strain may confer increased disease risk (10, 11).

The X chromosome has many immune-related genes and has been widely implicated in sex differences in autoimmunity (3, 12–14). Females have 2 X chromosomes while males have 1. To compensate for double expression of X genes, females randomly silence gene expression from one of their X chromosomes by a dosage compensation mechanism called X inactivation. X inactivation is initiated by the expression of *Xist* on the inactive X chromosome. *Xist* RNA transcripts associate with the inactive X chromosome to induce gene silencing (15). While the vast majority of genes on the inactive X are silenced by random X inactivation, 3% of X genes in mice (15% in humans) escape inactivation (16–18). That said, the expression level from the inactive X chromosome is typically less than that from the active X (16, 17). Together, this can lead to X dosage effects with higher expression of X genes in females (XX) as compared to males (XY).

The third possible mechanism underlying differences in gene expression between XY and XX involves parent-of-origin differences

Significance

Sex differences are naturally occurring disease modifiers that, if understood, could lead to novel targets for drug development. Autoimmune diseases are more prevalent in women than in men, and sex differences in immune responses have been shown in humans and mice. Here, we discover a global parent-of-origin difference in DNA methylation on the X chromosome that affects gene expression in activated CD4^{+} T lymphocytes. The paternal X has more methylation than the maternal X, with higher expression of X genes in XY cells since they only express from the maternal X. Thus, parent-of-origin differences in DNA methylation of X genes can play a role in sex differences in immune responses.

in DNA methylation of X genes. Using the "four core genotypes" (FCG) model, we previously showed higher expression of the X gene *toll-like receptor 7* (*Tlr7*) in XY cortical neurons compared to XX in experimental autoimmune encephalomyelitis (EAE), a classic CD4$^+$ T lymphocyte-mediated model of MS (19). Higher expression of *Tlr7* in XY as compared to XX cannot be explained by an X dosage effect, since X dosage effects lead to higher expression in XX. It could, however, be due to differences in DNA methylation of X chromosome genes. Inherited differences in DNA methylation that depend on parent of origin are often due to epigenetic modifications in the parental germ line, namely parental imprinting. Males and females differ in X chromosome origin in that females (XX) inherit both an X chromosome of maternal origin (X$_m$) and of paternal origin (X$_p$), while males (XY) inherit only X$_m$. Random X inactivation in females inactivates X$_m$ in half of the cells, and X$_p$ in the other half. Thus, females are a mosaic of cells expressing genes from either X$_m$ or X$_p$ (20, 21), whereas males always express genes from X$_m$. Since DNA methylation typically silences gene expression, parental imprinting of X genes can induce gene expression differences in XX versus XY (22, 23).

The DNA of the inactive X is highly methylated due to X inactivation (24). This creates a major confound in investigating possible parent-of-origin differences in DNA methylation patterns when comparing XX and XY. Studies to date addressing differential DNA methylation based on parent of origin have only been done at the transcription level and have not shown direct DNA methylation differences of the X chromosome (25, 26). Here, in order to study parent-of-origin effects on DNA methylation of the X chromosome without the confound of DNA methylation from X inactivation, we used a model in which there is only one X chromosome, and therefore no X inactivation. The one X chromosome was of either maternal (X$_m$) or paternal (X$_p$) origin. We used this model to show a direct parent-of-origin difference in DNA methylation of the X chromosome in autoantigen-stimulated CD4$^+$ T lymphocytes.

Results

Several X Genes Have Higher Expression in CD4$^+$ T Lymphocytes from XY Compared to XX. We previously identified a role for sex chromosomes in modulating the immune response in EAE (12, 27), but whether this was due to Y gene expression, X dosage effects, or parent-of-origin differences in DNA methylation of X genes remained unknown. Here, to investigate transcriptional differences arising from different sex chromosome genotypes in autoimmunity, we analyzed genome-wide transcriptomes of autoantigen-stimulated CD4$^+$ T lymphocytes from the FCG mouse model using high-throughput RNA sequencing (RNA-Seq). The FCG model utilizes the Y$^-$ chromosome, a Y chromosome with a deletion of the gene responsible for testicular development, namely *sex determining region of the Y* (*Sry*). Thus, XY$^-$ mice are gonadal females. Comparison between XX and XY$^-$ gonadal females permits the study of sex chromosome genes without confounding effects of differences in sex hormones (28, 29). Transcriptomes of autoantigen-stimulated CD4$^+$ T lymphocytes from XX and XY$^-$ mice 10 d after immunization with autoantigen showed separation using principal component analysis (PCA) (Fig. 1*A*), indicating that sex chromosomes altered the transcriptome. The transcriptome data were then used to generate a volcano plot to display differentially expressed genes between XX and XY$^-$. Two X genes known to escape X inactivation, *Kdm6a* and *Kdm5c* (16, 30), had higher expression in XX compared to XY$^-$, thereby validating sequencing results. Interestingly, we found a cluster of 5 X genes, *Msl3*, *Prps2*, *Hccs*, *Tmsb4x*, and *Tlr7*, which had higher expression in XY$^-$ than in XX (Fig. 1*B*). This result could not be an effect of X gene dosage, which would result in an opposite effect, namely higher expression in XX. Instead, higher expression of an X gene in an XY genotype could be explained by differential DNA methylation of X genes, with more
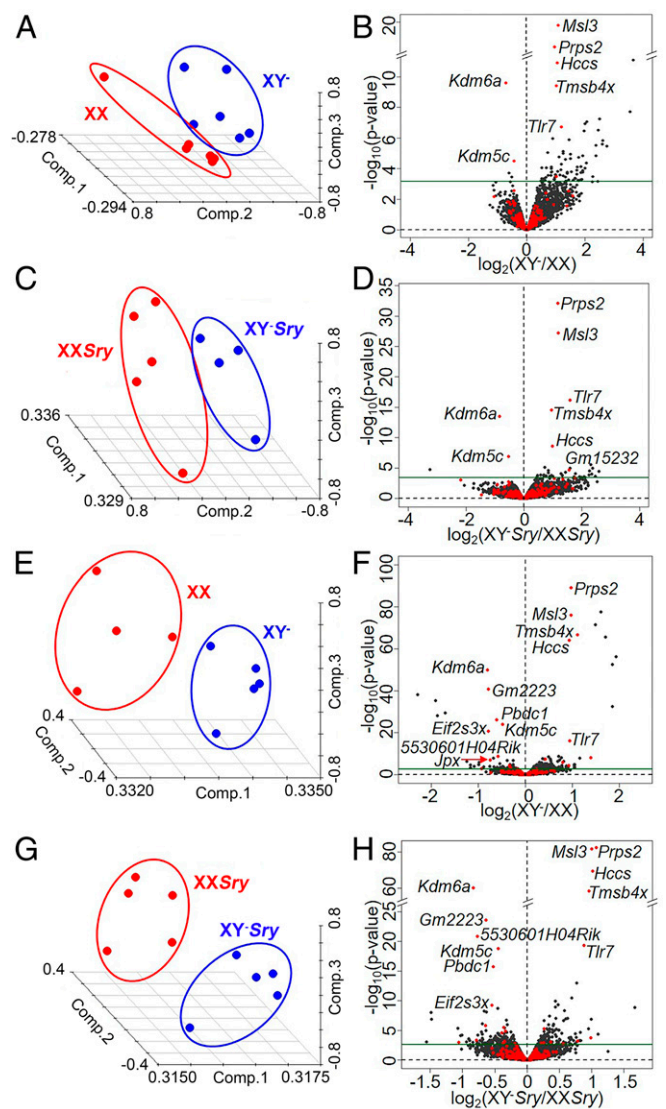

Fig. 1. CD4$^+$ T lymphocytes from XX and XY$^-$ mice have distinct transcriptomes. RNA from autoantigen- (*A–D*) and anti–CD3/CD28-stimulated (*E–H*) CD4$^+$ T lymphocytes from C57BL/6 FCG mice was analyzed by RNA-Seq. (*A*) PCA of autoantigen-stimulated CD4$^+$ T lymphocyte transcriptomes showed separation of XX ($n = 6$) and XY$^-$ ($n = 6$). (*B*) Volcano plots showed the distribution of differentially expressed autosomal (black) and X (red) genes between XX and XY$^-$. A cluster of 5 X genes had higher expression in XY$^-$ than XX. (*C*) PCA of autoantigen-stimulated CD4$^+$ T lymphocyte transcriptomes showed separation of XX*Sry* ($n = 5$) and XY$^-$*Sry* ($n = 5$). (*D*) Volcano plot showed higher expression of the 5 X gene cluster in XY$^-$*Sry* compared to XX*Sry*. (*E*) PCA of anti–CD3/CD28-stimulated CD4$^+$ T lymphocyte transcriptomes showed separation between XX ($n = 4$) versus XY$^-$ ($n = 5$) (*F*) Volcano plot showed higher expression of the 5 X gene cluster in XY$^-$ than XX. (*G*) PCA of anti–CD3/CD28-stimulated CD4$^+$ T lymphocyte transcriptomes showed separation of XX*Sry* ($n = 5$) and XY$^-$*Sry* ($n = 5$). (*H*) Volcano plot showed higher expression of the 5 X gene cluster in XY$^-$*Sry* compared to XX*Sry*. False discovery rate (FDR) < 0.1 was used as the threshold for significance (green line); any gene above this line was considered significantly different (FDR was calculated using R package edgeR).

methylation on X$_p$ compared to X$_m$. A complete list of differentially expressed genes is shown in Dataset S1.

We repeated this experiment in gonadal males of the FCG which have the *Sry* transgene inserted at chromosome 3 (31, 32), so XX*Sry* and XY$^-$*Sry* mice both have testes (28, 29). Comparison between XX*Sry* versus XY$^-$*Sry* gonadal males also permits the

study of sex chromosome genes without confounding effects of differences in sex hormones. Higher expression of the same cluster of 5 X genes was observed in XY⁻Sry compared to XXSry (Fig. 1 *C* and *D*). Also, higher expression of *Kdm6a* and *Kdm5c* in XXSry compared to XY⁻Sry was again observed.

To determine if the observed pattern of X gene expression was related to autoimmune activation, we analyzed the transcriptomes of CD4$^+$ T lymphocytes from nonimmunized, healthy XX and XY⁻ mice of both gonadal types that were stimulated with anti-CD3 and anti-CD28 antibodies. Consistent with results in CD4$^+$ T lymphocytes from immunized mice, the transcriptomes from CD4$^+$ T lymphocytes from nonimmunized, healthy XX and XY⁻ mice separated by PCA in both gonadal types (Fig. 1 *E* and *G*) and showed higher expression of the cluster of 5 X genes in XY⁻ compared to XX in both sexes (Fig. 1 *F* and *G*). These findings in nonimmunized, healthy mice suggested parent-of-origin imprinting effects in CD4$^+$ T lymphocytes. Notably, higher expression of *Kdm6a* and *Kdm5c* in XX compared to XY⁻ was again observed in anti–CD3/CD28-stimulated CD4$^+$ T lymphocytes from nonimmunized, healthy mice.

Expression of a Cluster of 5 X Genes Is Higher in CD4$^+$ T Lymphocytes from XY Compared to XX.

To complement our genome-wide, unbiased approach, we validated the expression of the 5 X genes with higher expression in XY⁻ from our RNA-Seq experiments (*Msl3*, *Prps2*, *Hccs*, *Tmsb4x*, and *Tlr7*). Quantitative RT-PCR analysis of RNA from autoantigen-stimulated CD4$^+$ T lymphocytes from 2 separate sets of immunized mice validated higher expression of all 5 genes in XY⁻ compared to XX (Fig. 2 *A–E*). *Tlr7* is of particular interest due to its known complex role in immunity (33–40); therefore, we further investigated TLR7 expression at the protein level. Indeed, autoantigen-stimulated CD4$^+$ T lymphocytes from XY⁻ mice had higher TLR7 protein expression than those from XX (Fig. 2 *F–H*).

In addition to XX and XY⁻ gonadal females, we analyzed the expression of the same 5 X genes in gonadal males of the FCG. We found higher expression of all 5 X genes in autoantigen-stimulated CD4$^+$ T lymphocytes from XY⁻Sry compared to XXSry (Fig. 2 *I–M*). TLR7 protein expression was also found to be higher in XY⁻Sry compared to XXSry (Fig. 2 *N–P*).

We also analyzed TLR7 protein expression in B lymphocytes to determine if differential expression was limited to CD4$^+$ T lymphocytes. We found higher expression of TLR7 in CD19$^+$ B lymphocytes from XY⁻ and XY⁻Sry compared to XX and XXSry, respectively (*SI Appendix*, Fig. S1), consistent with findings in CD4$^+$ T lymphocytes (Fig. 2).

A Model to Directly Study Parent-of-Origin Differences in DNA Methylation.

To directly investigate potential parent-of-origin differences in DNA methylation of X chromosome genes, we used CD4$^+$ T lymphocytes from X-monosomic mice from the XY* mouse model (32, 41), permitting the comparison of DNA methylation of X_m and X_p without the confound of methylation due to X inactivation (Fig. 3). The Y* chromosome is a rearranged Y chromosome with a translocated X chromosome centromere and a modified X pseudoautosomal region (PAR) (Fig. 3*A* and ref. 32). When breeding XY* male mice with XX females, the Y* chromosome undergoes recombination with the X chromosome to produce gonadally female XY*ˣ offspring (Fig. 3*D* and ref. 32). The Y*ˣ chromosome is an X chromosome with a major deletion of about 99% of genes, leaving only the PAR and about 8 non-PAR X genes (Fig. 3 *A* and *B* and ref. 32). Since XY*ˣ mice are almost completely monosomal for the X chromosome, we refer to them here as "XO" for simplicity. In this genotype, there is no *Xist* expression (Fig. 3*E*) and therefore no DNA methylation related to X inactivation, permitting the direct study of parent-of-origin differences in DNA methylation of X genes.

To determine if differential DNA methylation could be playing a role in differential expression of *Tlr7*, we used CD4$^+$ T lymphocytes from this model to analyze the region upstream of the *Tlr7* transcriptional start site (TSS) using targeted bisulfite sequencing. We identified more methylation on X_p than X_m at all 4 CpG sites analyzed (Fig. 3*F*). This result was consistent with higher *Tlr7* RNA expression from X_mY^- versus X_mX_p (Figs. 1 and 2).
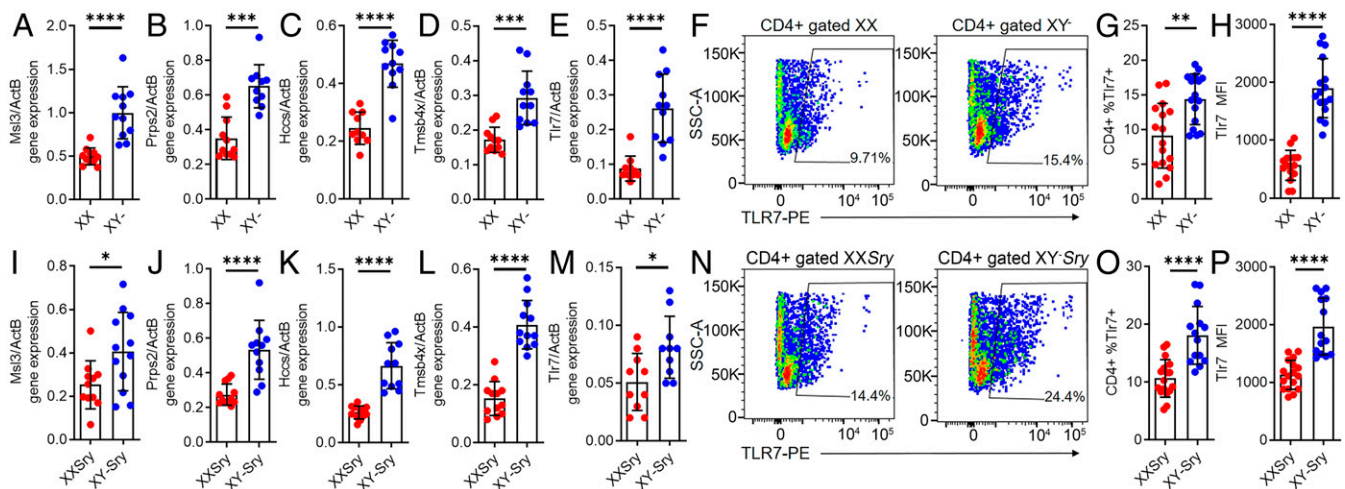
Fig. 2. XY⁻ has higher expression of a cluster of 5 X genes compared to XX. RNA and protein expression were measured in autoantigen-stimulated CD4$^+$ T lymphocytes from XX and XY⁻ mice immunized with autoantigen. (*A–E*) RNA expression of (*A*) *Msl3*, (*B*) *Prps2*, (*C*) *Hccs*, (*D*) *Tmsb4x*, and (*E*) *Tlr7* was measured by quantitative RT-PCR. XY⁻ (n = 10 or 11) had higher RNA expression than XX (n = 10 or 11) for all 5 genes (****P < 0.0001, ***P < 0.0002). (*F–H*) CD4$^+$ T lymphocytes were analyzed for TLR7 protein expression by flow cytometry. See *SI Appendix*, Fig. S2 for gating strategy. (*F*) Representative flow plots for TLR7 expression in XX versus XY⁻. (*G*) XY⁻ mice (n = 16) had a higher percentage of TLR7 expressing CD4$^+$ T lymphocytes than XX (n = 16) (**P = 0.0017). (*H*) XY⁻ mice (n = 16) had higher mean fluorescence intensity (MFI) of TLR7 expression in CD4$^+$ T lymphocytes than XX (n = 16) (****P < 0.0001). (*I–P*) The same analyses in *A–H* were performed in XXSry and XY⁻Sry gonadal males. (*I*) *Msl3*, (*J*) *Prps2*, (*K*) *Hccs*, (*L*) *Tmsb4x*, and (*M*) *Tlr7* RNA expression; XXSry n = 10 to 12, XY⁻Sry n = 10 to 12 (*P < 0.045, ****P < 0.0001). (*N*) Representative flow plots for TLR7 expression in XXSry versus XY⁻Sry. (*O*) TLR7% expression; XXSry n = 17, XY⁻Sry n = 14 (****P < 0.0001). (*P*) TLR7 MFI; XXSry n = 17, XY⁻Sry n = 14 (****P < 0.0001). All data are representative of 2 replicate experiments. Error bars represent SD. *P* values were calculated by Mann–Whitney *U* test.
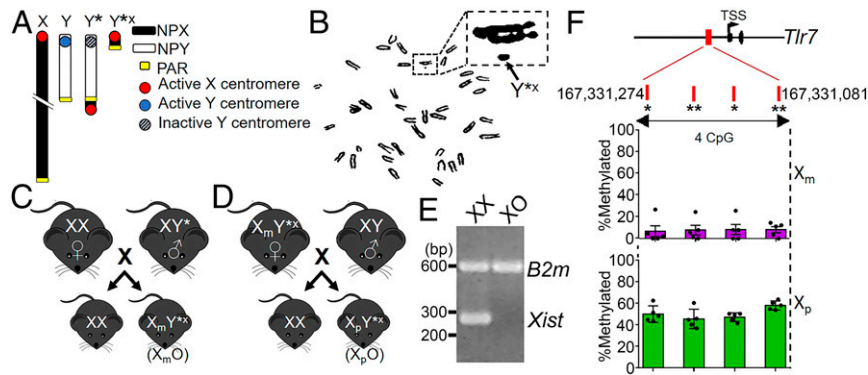
Fig. 3. A monosomic X mouse model to directly study parent-of-origin differences in DNA methylation. (A) Diagrams of the sex chromosomes used in the breeding of XY*^{x} (XO) mice. The Y* chromosome is a rearranged Y chromosome with a translocated X chromosome centromere and a modified X PAR (see ref. 32 for details). The Y*^{x} chromosome is produced from recombination of the X and Y* chromosomes. It is an X chromosome with a massive deletion of about 99% of genes, leaving the PAR and about 8 non-PAR genes (32). (B) Representative metaphase spread of XY*^{x} (XO) chromosomes. An XY*^{x} animal has 39 normal chromosomes, plus 1 small "spec" representing the Y*^{x} chromosome (Inset). (C) Female X_mO mice were generated by crossing wild-type XX females and XY* males. (D) Female X_pO mice were generated by crossing X_mO females with wild-type XY males. (E) Xist expresses in XX, but not in XO. RT-PCR was performed on RNA from ear tissue. The expression of B2m was used as the internal control. (F) DNA methylation of Tlr7 was analyzed using targeted bisulfite sequencing in CD4$^+$ T lymphocytes of X_mO (n = 5) and X_pO (n = 5) SJL mice 12 d after immunization with autoantigen. The DNA methylation on X_m and X_p is shown as the percentages of methylation at CpG sites of a CpG island upstream of the Tlr7 TSS. X_p had more methylation than X_m at each site analyzed (*P < 0.0120, **P < 0.0008, Mann–Whitney U test). Error bars represent SEM.

The X_p Chromosome Has Significantly More DNA Methylation in CpG Islands than X_m. Next, we asked if differential DNA methylation could be occurring on other X genes as it did for Tlr7. Thus, we directly analyzed the DNA methylome of CD4$^+$ T lymphocytes from X_mO and X_pO mice 12 d after immunization with auto-antigen. The X_p chromosome displayed a strikingly higher number of methylated CpG islands in comparison to X_m (Fig. 4 A and B and SI Appendix, Fig. S3 and Table S1; X_p = 45.2%, X_m = 1.1%, $P < 2.2 \times 10^{-16}$), while no significant difference was observed between X_pO and X_mO autosomes (Fig. 4 A and B and SI Appendix, Fig. S4 and Table S1; P = 0.4258). A complete list of differentially methylated genes is shown in Dataset S2. The relationship between RNA expression and DNA methyl-

ation was examined for the genes which had higher expression in XY$^-$ compared to XX in Fig. 1 (Hccs, Msl3, Prps2, Tlr7, and Tmsb4x). All 5 X genes showed higher DNA methylation on X_p, consistent with the role of DNA methylation in X gene expression (Table 1).

Parent-of-Origin Differences on DNA Methylation Are Not Strain-Specific. To determine whether the effects of DNA methylation are strain-specific, we analyzed allele-specific gene expression patterns from F1 hybrid mice. By using the F1 generation from CAST/EiJ × FVB/NJ reciprocal crosses, the sequences from X_m or X_p chromosomes can be identified based on single-nucleotide polymorphism differences between these 2 strains. This permits
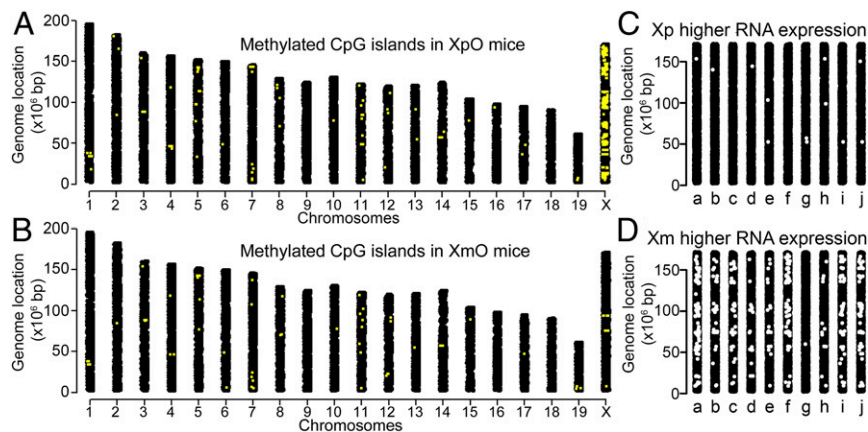

Fig. 4. Genes on the X_p have more DNA methylation in CpG islands than X_m. X_mO and X_pO female mice were immunized with autoantigen for 12 d. DNA from CD4$^+$ T lymphocytes isolated from lymph nodes was analyzed by bisulfite sequencing to generate the whole methylome of X_pO (n = 4) and X_mO (n = 5) SJL mice. Yellow spots indicate CpG sites in CpG islands that had DNA methylation in >50% of reads on average. Black dots were mapped based on all gene locations from the Ensemble genome browser (uswest.ensembl.org/index.html) and form the chromosome shapes. (A) The X_p chromosome showed greater accumulation of CpG island DNA methylation compared to autosomes ($P < 2.2 \times 10^{-16}$, Fisher's exact test). (B) The X_m chromosome did not have accumulation of DNA methylation compared to autosomes (P = 1, Fisher's exact test). Comparing X_p (A) and X_m (B), the number of methylated CpG islands was significantly higher on X_p than on X_m ($P < 2.2 \times 10^{-16}$, Fisher's exact test). No difference in DNA methylation was observed for autosomal genes between X_pO and X_mO (P = 0.4258, Fisher's exact test). (C and D) Gene expression data from F1 hybrid mice derived from CAST/EiJ × FVB/NJ and FVB/NJ × CAST/EiJ reciprocal crosses was analyzed to show parent-of-origin differences in X gene expression in multiple tissues (a: embryonic day [E] 16.5 liver, b: E16.5 brain, c: E16.5 heart, d: day-3 tongue, e: day-3 brain, f: adult brain, g: adult liver, h: adult heart, i: adult lung, j: adult spleen). White dots represent genes on the X chromosome with higher expression from X_p (C) or from X_m (D). There were many more X genes with higher expression from X_m compared to X_p across tissues.

Table 1.   X genes with higher expression in XY have more DNA methylation on X_p compared to X_m

	RNA expression			DNA methylation				
	log₂FC (XY/XX)	*P* value	FDR	methDiff ($X_m - X_p$)	SD	DM CpGs/all CpG	q-value (minimum)	q-value (maximum)
Hccs	1.06	1.18E-11	1.19E-08	−25.6	4.4	11/14	0.0001	0.1590
Msl3	1.09	2.44E-20	3.11E-17	−30.3	2.7	6/7	0.0024	0.1230
Prps2	0.97	5.44E-18	6.36E-15	−41.4	8.5	5/7	0.0263	0.1410
*Tlr7**	1.19	1.87E-07	1.25E-04	−38.0	4.9	4/4	0.0079**	0.0119**
Tmsb4x	1.02	3.80E-10	3.33E-07	−46.1	9.4	4/6	0.0166	0.1890

The difference in DNA methylation (methDiff) between X_m and X_p was analyzed for the cluster of 5 X genes that had higher RNA expression in XY⁻ compared to XX in autoantigen-stimulated CD4⁺ T lymphocyte transcriptome data from C57BL/6 mice (*Hccs*, *Msl3*, *Prps2*, *Tlr7*, and *Tmsb4x*; Fig. 1) to investigate the relationship between RNA expression and DNA methylation. All 5 genes had more DNA methylation on X_p. FDR < 0.1 was used as the threshold for significance for differential RNA expression (R package edgeR). DNA methylation differences for all CpG sites in each gene were averaged to generate one value of differential methylation (DM). q-value < 0.1 was used as a threshold for significance of differentially methylated CpG sties (R package methylSig). The q-values for the most significant (minimum) and least significant (maximum) CpG site are listed. *Tlr7* DNA methylation data were obtained from targeted DNA methylation analysis and *P* value < 0.05 (Mann–Whitney *U* test) was used as a threshold for significance of differential methylation.
*Measured by targeted DNA methylation analysis.
**P values.

detection of gene expression differences between X_m and X_p. There was a preference for gene expression from the X_m compared to X_p across several tissues, while very few X genes had higher expression in X_p (Fig. 4 *C* and *D*). Further, we observed that many genes which had higher expression from the X_m in F1 mice were the same genes with more DNA methylation on the X_p (*SI Appendix*, Fig. S5). Together, this is consistent with parental imprinting as a mechanism for increased expression of X genes in XY mice in more than one strain.

Which Genes Show Differential Expression due to Parent-of-Origin DNA Methylation Depends on Genetic Background. Our data indicated that differential parent-of-origin DNA methylation of the X chromosome can occur in several strains, but whether gene expression changes are the same across different genetic backgrounds was not known. To investigate this, we analyzed the transcriptomes of both autoantigen and anti–CD3/CD28-stimulated CD4⁺ T lymphocytes from XX and XY⁻ SJL mice of both gonadal sexes (*SI Appendix*, Fig. S6). The SJL is known to have sex differences in immune responses (42–45). While SJL mice did not show differential expression of the same 5 X genes observed in C57BL/6 mice, CD4⁺ T lymphocytes from XY⁻ as compared to XX SJL mice had higher expression of *Xlr3b*, an X gene previously reported to be imprinted (46), and this was observed in both immunized and nonimmunized, healthy SJL mice. Thus, which genes show differential expression due to parent-of-origin DNA methylation are not the same across strains. Additionally, there was an *Sry* effect in the SJL, whereby the *Xlr3b* gene did not have higher expression in XY⁻*Sry* compared to XX*Sry* gonadal males. A difference in gonadal females versus males in the FCG model is consistent with previous reports showing an effect of endogenous testosterone in the SJL strain, but not in the C57BL/6 strain in EAE (47). Notably, *Kdm6a* and *Kdm5c* again had higher expression in XX compared to XY⁻ regardless of immunization, gonadal type, or strain.

Discussion

In this study, we performed genome-wide transcriptome analyses in the FCG mouse model to determine differences in gene expression due to sex chromosome complement. RNA-Seq analyses of autoantigen-stimulated CD4⁺ T lymphocytes from autoantigen-immunized C57BL/6 mice showed a cluster of 5 X genes with higher expression in XY⁻ as compared to XX. Quantitative RT-PCR in a separate set of mice confirmed higher expression of *Msl3*, *Prps2*, *Hccs*, *Tmsb4x*, and *Tlr7* in XY⁻ compared to XX. These results were also observed in CD4⁺ T lymphocytes from nonimmunized, healthy FCG mice stimulated with anti-CD3/CD28 antibodies. Higher expression of an X gene in the XY

compared to the XX genotype is in the opposite direction from an X dosage effect but is consistent with a parent-of-origin effect on DNA methylation (23). Examination of the whole methylome in CD4⁺ T lymphocytes from X-monosomic mice (X_mO and X_pO) showed that the X_p chromosome had a global increase in DNA methylation at CpG islands compared to X_m. CpG islands are regions of DNA with high CG content found near gene promoters and TSSs that repress gene expression when methylated. The accumulation of DNA methylation at CpG islands throughout the X_p indicated specific silencing of several genes on the paternal X chromosome. Indeed, DNA methylation analysis for differentially expressed genes in CD4⁺ T lymphocyte transcriptome data showed that X genes with higher expression in XY⁻ compared to XX had more DNA methylation on X_p.

Our methylome data showing more DNA methylation on X_p than X_m in adult CD4⁺ T lymphocytes is consistent with known paternally biased DNA methylation during development, including the preferential inactivation of X_p prior to embryo implantation, thought to be due to imprinting (48–52), and meiotic sex chromosome inactivation during spermatogenesis (53), leading to a more methylated X_p in the germ line. Since DNA methylation patterns from parental imprinting are inherited by all progeny cells, canonically imprinted genes are not tissue-specific (54), consistent with our finding of preferential gene expression from X_m across tissues (Fig. 4 *C* and *D*). Our DNA methylation results are also consistent with previous reports in neonatal brains that showed preferential gene expression from X_m over X_p (25, 26). This study directly demonstrates parent-of-origin differences in DNA methylation between X_m and X_p without the confound of X inactivation as a chromosome-wide, rather than a localized (25), effect on the X chromosome. Moreover, these differences in DNA methylation aligned with differential expression of X genes.

Other mechanisms affecting X chromosome gene expression include skewed X inactivation, whereby X inactivation is not random but instead biased toward either the X_m or X_p, and this has previously been suggested as a possible factor in female susceptibility to autoimmune disorders (55, 56), albeit this remains unproven (57). Skewed X inactivation is not infrequent in healthy females (58). Notably, parent-of-origin differences in DNA methylation which drive differences in gene expression from X_m versus X_p are a means by which skewed X inactivation could alter gene expression. Thus, our findings here of parent-of-origin effects on DNA methylation of the X chromosome support a mechanism of action regarding how skewed X inactivation could alter gene expression.

Determining why most autoimmune diseases have a higher prevalence in women compared to men is challenging in humans (1, 2). Since the X chromosome contains many immune-related genes, it has been implicated in sex differences in patients with autoimmune diseases (59, 60). SLE is more prevalent in women than men by 9:1, and men with Klinefelter's syndrome (XXY) have increased susceptibility to SLE as compared to XY men (61, 62). There have only been 5 reports of Turner syndrome patients (XO) with SLE, suggesting a lower risk of SLE in XO as compared to XX women (63, 64). Together, the association of these chromosomal abnormalities with SLE has suggested a possible role of X dosage in autoimmunity; however, studies in humans are confounded by differences in sex hormones in XXY and XO genotypes. In mouse models of SLE, a role of sex chromosomes was shown without the confound of differences in sex hormones. More severe disease and immune dysregulation was demonstrated in XX compared to XY$^-$ mice when the spontaneous lupus susceptible strain (NZM2328) was back-crossed onto the FCG model (13) as well as in pristane-induced lupus when SJL mice were back-crossed onto the FCG model (12).

In MS, the ratio of women to men is 3:1 (6), and autoantigen-specific immune responses were shown to be more robust in MS women (65, 66). Both sex hormones and sex chromosomes have been shown to play a role in sex differences in immune responses during EAE. Previously, draining lymph node cells (LNCs) from myelin basic protein (MBP)-immunized SJL mice were stimulated in vitro with MBP, then cytokines (tumor necrosis factor-α [TNFα], interferon-γ [IFNγ], and interleukin 10 [IL-10]) were assessed in supernatants (27). Wild-type females had higher levels of cytokines than males, and gonadectomy (GDX) suggested a role for both sex hormones and sex chromosomes. An activational effect of adult testosterone to reduce cytokines was shown by an increase in cytokines when males were castrated. A sex chromosome effect was suggested by a difference in castrated males versus ovariectomized females, but this could also be due to an organizational effect of sex hormones prior to GDX. Experiments in the FCG model disentangled these possibilities. There were lower levels of cytokines in gonadally intact males (XXSry and XY$^-$Sry) as compared to females (XX and XY$^-$), revealing a role for testosterone in decreasing cytokines, either organizational during development or activational during adulthood. The activational effect of testosterone in decreasing cytokines in adults was confirmed by exogenous treatment of adult females with testosterone. Given the major effect of sex hormones, experiments were next done in FCG that were GDX to focus on sex chromosome effects that might be masked by sex hormone effects. Cytokines were higher in XY$^-$ ovariectomized females (XY$^-$ > XX) and castrated males (XY$^-$Sry > XXSry). Together, this was consistent with male sex hormones (testosterone) and male sex chromosome complement (XY) coevolving to achieve balance (6, 27, 67), with testosterone decreasing cytokines and the XY$^-$ complement increasing cytokines.

Since cytokine changes can have different effects on EAE (TNFα proinflammatory, IFNγ variable, and IL-10 antiinflammatory), the role of sex chromosomes on disease was determined by inducing EAE in SJL FCG mice that were GDX (12). Adoptive transfer of proteolipid protein (PLP)$_{139-151}$-specific LNCs from XX as compared to XY$^-$ mice showed that XX cells were more encephalitogenic than XY$^-$. When SJL FCG mice were immunized and draining LNCs were restimulated in vitro with PLP$_{139-151}$, Th2 cytokines IL-13 and IL-5 were higher in XY$^-$ compared to XX, with similar trends for TNFα, IFNγ, and IL-10. In the current study, we examined genome-wide effects, as opposed to hypothesis-driven cytokines of interest, in both immunized and nonimmunized, healthy FCG mice on the C57BL/6 and SJL genetic backgrounds. In C57BL/6 mice, XY$^-$ as compared to XX had numerous genes expressed higher in both immunized and nonimmunized mice. This included a cluster of 5 X chromosome genes which could not be due to X dosage. Investigation of the methylome provided direct evidence for differences in DNA methylation that aligned with transcriptome data. Notably, in the SJL strain, the same 5 X chromosome genes were not increased in XY$^-$ mice, but instead there was increased expression of another X gene, one known to undergo parental imprinting (Xlr3b) (46). Another difference between genetic backgrounds (SJL versus C57BL/6) was the role of endogenous testosterone. XX versus XY$^-$ results were the same as XXSry versus XY$^-$Sry in the C57BL/6 but differed in the SJL, consistent with a role of endogenous testosterone in EAE in SJL mice (42–45), but not in C57BL/6 mice (47).

A finding shared by the C57BL/6 and SJL strains was higher expression of Kdm6a and Kdm5c in XX compared to XY$^-$ (and in XXSry compared to XY$^-$Sry). These are genes known to escape X inactivation and are capable of inducing X dosage effects (16, 30). Kdm6a is a histone demethylase that regulates expression of other genes, and it showed the greatest increase in XX as compared to XY$^-$. Previously, when Kdm6a was selectively deleted in CD4$^+$ T lymphocytes, EAE was ameliorated and the transcriptome showed a decrease in the neuroinflammatory signaling pathway (68). Thus, an X dosage effect of Kdm6a in females is proinflammatory. While females are more susceptible to EAE in the SJL strain, this is not the case in C57BL/6. We speculate that strain differences in the C57BL/6 and the SJL may be related to the degree of imbalance between higher expression of Kdm6a in the XX genotype on the one hand and the 5 X genes with higher expression in the XY$^-$ genotype on the other. If the 5 X chromosome genes that are increased in XY$^-$ due to parental imprinting in the C57BL/6 strain have a net proinflammatory effect, then this would balance the proinflammatory effect in XX due to an X dosage effect of Kdm6a. In contrast, the SJL does not have an increase in the 5 X chromosome genes in XY$^-$, thereby not balancing the proinflammatory effects in XX due to Kdm6a. Clearly the assumption that the net effect of the 5 X chromosome genes increased in XY$^-$ in the C57BL/6 is proinflammatory is highly speculative since Tmsb4x and Tlr7 have complex roles in the immune response (34–40, 69–71), and the remaining genes either involve general functions in apoptosis, differentiation, and proliferation (72, 73) or their role is not yet defined. Regardless of whether this imbalance is ultimately someday shown to contribute to the difference in the sex bias in EAE susceptibility in SJL versus C57BL/6 mice, findings here in the context of existing literature reveal that several factors are involved in sex differences in immune responses. Our overarching hypothesis is that sex differences in the immune system are due to the balance between parental imprinting of X genes that do not escape X inactivation and X dosage effects of X genes that do escape X inactivation, which can be modulated by an effect of sex hormones, as illustrated in Fig. 5.

While our focus here was to study CD4$^+$ T lymphocytes, it is notable that our TLR7 protein expression data in B lymphocytes derived from LNC cultures restimulated with autoantigen was inconsistent with what has been previously described in B lymphocytes from healthy human and mouse cells (74, 75) and gonadally intact female versus male mice during vaccination (76). This is likely due to 2 methodological differences. First, differences in sex hormones in gonadally intact females versus males presents a major confound when studying sex chromosome effects, a confound not present in our studies which used GDX mice of the FCG model. Indeed, many studies have established a role of estrogen on B cells in SLE (humans and mice) (77), and TLR7 function is affected by interactions between estrogen receptor-α and estrogen response elements (78). Second, previously observed biallelic expression of Tlr7 in B lymphocytes was found in cells that were not in vitro-stimulated (74–76, 79). Indeed, within 1 to 2 d of in vitro stimulation, most cells had monoallelic expression (70 to 80% in mice) (74). Thus, our LNC cultures stimulated for 36 h in vitro would not be expected to
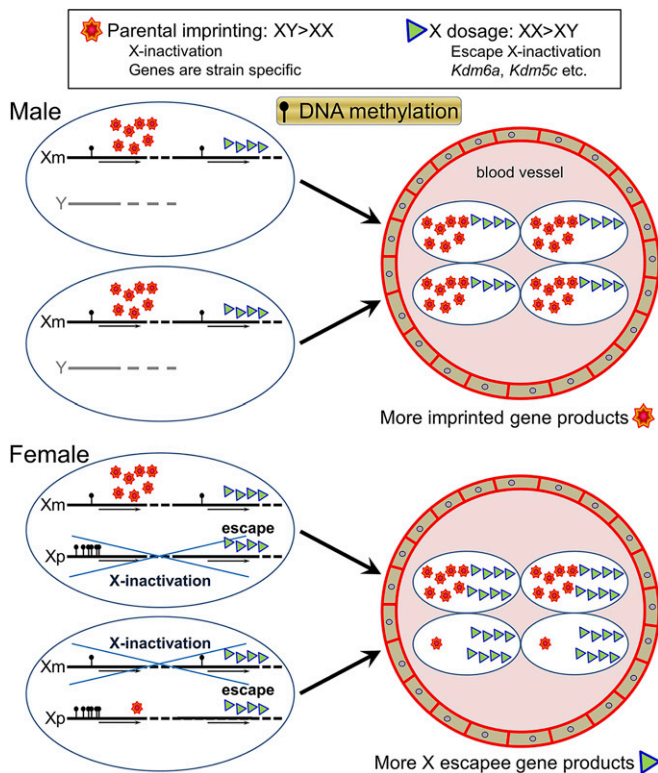
Fig. 5. Overarching hypothesis that sex differences in the immune system are due to the balance between parental imprinting of X genes that do not escape X inactivation (red stars) and X dosage effects of X genes that do escape X inactivation (blue triangles), which can be modulated by an effect of sex hormones. Males (XY) have 1 X chromosome with maternal imprinting (higher expression). On the other hand, females (XX) have 2 X chromosomes, 1 with maternal and 1 with paternal imprinting. Due to random X inactivation in females, the X_m is inactivated in half of the cells, while X_p is inactivated in the other half, creating a mosaic of cells expressing genes from either X_m (higher expression) or X_p (lower expression). Additionally, some genes escape X inactivation (*Kdm6a* for example), expressing from both X chromosomes and creating an X dosage effect with higher expression in XX versus XY. Together, this results in higher expression of parentally imprinted genes in XY and higher expression of X escapee genes in XX. These sex chromosome complement effects are not mutually exclusive of sex hormone effects, both organizational effects during development and activational effects during adulthood.

show significant biallelic expression of X genes, thereby permitting detection of higher expression of TLR7 in XY^- compared to XX due to parental imprinting.

In summary, our discovery of chromosome-wide hypermethylation of CpG islands on the paternal X chromosome aligns with differential X chromosome gene expression in X_mX_p females compared to X_mY males and provides evidence supporting a mechanism involved in sex differences in immune responses.

Materials and Methods

Mice. SJL/J, C57BL/6J, and C57BL/6J XY* mice were obtained from The Jackson Laboratory. MF1 XY^-Sry males were back-crossed to C57BL/6J and SJL/J to generate the FCG mice. C57BL/6J XY* males were back-crossed onto the SJL. X_mY^{*x} mice were generated by crossing wild-type females with XY* males. X_pY^{*x} mice were generated by crossing X_mY^{*x} females with wild-type XY

males. All procedures were reviewed and approved by the Chancellor's Animal Research Committee of the University of California, Los Angeles Office for the Protection of Research Subjects.

GDX Surgery. All mice were gonadectomized (females: ovariectomy, males: castration) between 4 and 6 wk of age.

Immunization. C57BL/6J mice were immunized with MOG_{35-55} in complete Freund's adjuvant for 10 d. SJL/J mice were immunized with $PLP_{139-151}$ in complete Freund's adjuvant for 10 to 12 d.

Lymphoid Tissue Collection and Lymphocyte Stimulation. For RNA and flow cytometry studies, LNCs from immunized C57BL/6J and SJL/J mice were cultured with MOG_{35-55} or $PLP_{139-151}$ autoantigen, respectively, in the presence of IL-12 for 36 h, followed by isolation of $CD4^+$ T lymphocytes by negative selection or flow cytometric analyses. $CD4^+$ T lymphocytes were isolated by negative selection from LNCs from nonimmunized, healthy C57BL/6J and SJL/J mice and cultured with anti-CD3/CD28 antibodies for 36 h.

High-Throughput Sequencing and Analysis. Standard procedures were used to isolate RNA. $CD4^+$ transcriptome analyses were performed using a high-throughput sequencing approach.

Quantitative RT-PCR. Standard procedures were used to quantify gene expression (see *SI Appendix, Supplementary Materials and Methods* for primer sequences).

Flow Cytometry. Lymphocytes were analyzed for CD4, CD19, and TLR7 expression using standard methods. All flow cytometry was performed at the University of California, Los Angeles (UCLA) Jonsson Comprehensive Cancer Center and Center for AIDS Research Flow Cytometry Core Facility.

DNA Methylation. DNA was isolated from $CD4^+$ T lymphocytes using standard procedures and analyzed by enhanced reduced representation bisulfite sequencing or targeted bisulfite sequencing as previously described (80–84).

Further details of methods and statistics are provided in *SI Appendix, Supplementary Materials and Methods*.

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