Published in final edited form as:

Ann Rheum Dis. 2012 August ; 71(8): 1418–1422. doi:10.1136/annrheumdis-2011-201246.

The XX Sex Chromosome Complement in Mice is Associated with Increased Spontaneous Lupus as compared to XY

Manda V. Sasidhar^a, Noriko Itoh^a, Stefan M. Gold^{a,b}, Gregory W. Lawson^c, and Rhonda R. Voskuhl^a

^aDepartment of Neurology, David Geffen School of Medicine University of California Los Angeles, Charles Young Dr S, Los Angeles, CA 90095

^bCenter for Molecular Neurobiology, University Hospital Hamburg-Eppendorf, Falkenried 94, 20251 Hamburg, Germany

^cDivision of Laboratory Animal Medicine, David Geffen School of Medicine University of California Los Angeles, 10833 LeConte Avenue, Los Angeles, CA 90095

Abstract

Objectives—Many autoimmune diseases are characterized by a female predominance. This may be caused by sex hormones, sex chromosomes or both. Here, we use a transgenic mouse model to investigate how sex chromosome complement, not confounded by differences in gonadal type, might contribute to lupus pathogenesis.

Methods—Transgenic NZM2328 mice were created by deletion of the *Sry* gene from the Y chromosome, thereby separating genetic from gonadal sex. We compared survival, renal histopathology, and markers of immune activation in mice carrying the XX versus the XY⁻ sex chromosome complement, with each genotype being ovary bearing.

Results—Mice with XX sex chromosome complement as compared with XY⁻ exhibited poorer survival rates and increased kidney pathology. Splenic T lymphocytes from XX mice demonstrated upregulated X-linked CD40Ligand expression and higher levels of activation markers *ex vivo*. We found increased MMPs, TGF β and IL13 production, while IL2 was lower in XX mice. Finally, we observed an accumulation of splenic follicular B cells and peritoneal marginal zone B cells, coupled with upregulated costimulatory marker expression on B cells in the XX mice.

Conclusion—Together, these data show that the XX sex chromosome complement, as compared to XY⁻, is with associated accelerated spontaneous lupus.

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Corresponding author: Rhonda R. Voskuhl, M.D., Department of Neurology, University of California, Los Angeles, Neuroscience Research Building 1, Room 475D, 635 Charles Young Drive South, Los Angeles, CA 90095, Phone: 310-206-4636, Fax: 310-206-7282, rvoskuhl@ucla.edu.

Competing Interests: Competing Interest: None declared.

Keywords

Sex chromosome complement; gender bias; autoimmune disease; X chromosome and CD40Ligand

Introduction

Most autoimmune diseases including systemic lupus erythematosus (SLE) are characterized by a female gender bias¹. Increased susceptibility in females has also been observed in many autoimmune animal models such as spontaneous lupus in NZM2328 mice². Sex differences in these diseases may be caused by sex hormones, sex chromosomes or both. Generally, the XX genotype is linked with the development of ovaries and a female hormonal environment, while the XY genotype is linked with the development of testes and a male hormonal environment. Thus, previous studies have not been able to distinguish between the contributions of sex hormones versus sex chromosomes.

Novel transgenic mouse models now allow for investigation of the independent contribution of sex chromosome complement to pathogenesis³. To this end, an informative system has been created where the *Sry* gene, which encodes for testicular development, has been deleted from the Y chromosome. Mice which have a deletion of the *Sry* gene on the Y chromosome are phenotypic females (ovary bearing). They are denoted XY⁻ since they have other Y genes remaining, with only the *Sry* gene deleted. A comparison between XX and XY⁻ thus reveals the impact of sex chromosomes on a given outcome measure (such as disease susceptibility) in a setting of identical gonadal sex (both female). Here, we have backcrossed XY⁻ mice derived from the outbred MF1 strain⁴ onto the NZM.2328 lupus prone strain to determine whether there is an effect of sex chromosome complement on spontaneous lupus.

Methods

Detailed methods are available as Supplementary Material.

Assessment of survival

Mice were monitored daily for signs of lupus (lethargy, ruffled fur, and distended abdomen) and survival rates were attained.

Kidney pathology

Kidneys were fixed in formalin and sections underwent Trichrome and H&E staining using standard conditions for evaluation of fibrosis and inflammation.

Flow Cytometry

Splenocytes stained with T cell and B cell specific antibodies were acquired on a FACSCalibur cytometer and analyzed with FlowJo software.

Cytokine analysis

Splenocytes were stimulated with anti-CD3/CD28 for 48 hours and cell supernatants were assessed for cytokines and MMPs using commercially available multiplexing arrays (Aushon Bio Systems, Billerica, MA).

Statistics

Survival curves between groups were compared using Mantel-COX log-rank test (GraphPad Prism, San Diego, CA). Pathology scores, frequencies of lymphocyte subpopulations as well as cytokine levels were analyzed using independent samples t tests or Mann-Whitney U tests as appropriate. All statistical tests were two-tailed and a value of $p < 0.05$ was considered statistically significant. Data are given as means+standard error of mean (s.e.m.).

Results

The XX sex chromosome complement, as compared to the XY⁻ complement, is associated with accelerated spontaneous lupus in NZM.2328 mice

Survival curves revealed significantly more severe disease in XX, as compared to XY⁻, mice (Fig. 1A). Kidney pathology was then assessed by histological analysis. Glomerular fibrosis as indicated by collagen deposition was minimal in kidneys from XY⁻ mice, while more severe fibrosis was evident in XX (Fig. 1 B-F). Interstitial nephritis, perivascular lymphoid hyperplasia and tubular atrophy were also observed, but levels did not differ significantly between groups (not shown). Together these data demonstrate that mice carrying the XX sex chromosome complement have accelerated spontaneous lupus as compared to the XY⁻ complement.

Could there be differences between XX and XY⁻ mice in levels of ovarian hormones mice which might underlie the accelerated disease in XX mice? We found no difference in serum estradiol levels in XX versus XY⁻ mice, when tested at either age 4-6 or age 7-9 months (Supplemental Figure 1). A lack of a difference in hormone levels at any given timepoint cannot however rule out differences in hormone levels at all other time points. Since it is not practical to assess hormone levels daily from the perinatal period to adulthood, others have assessed whether differences exist in regions known to be hormone responsive. Such differences in sexually dimorphic neural structures and behavioral traits have not been found, thereby providing very strong evidence that indeed such comparisons in this mouse model do indeed reveal differences in sex chromosome complement, not differences in hormonal status⁵. Further, we also assessed spontaneous lupus in mice which had the *Sry* gene inserted at an autosomal location. Both XX*Sry* and XY⁻*Sry* mice are gonadal males. As shown in Supplemental Figure 2, survival curves and kidney pathology both revealed accelerated disease in XX*Sry* as compared to XY⁻*Sry* mice, thereby confirming the disease promoting effect of the XX genotype, which in this case would not be due to differences in ovarian hormones. We also determined testosterone levels in XX*Sry* versus XY⁻*Sry* mice and found no differences (Supplemental Figure 1).

Mice carrying the XX sex chromosome complement, as compared to the XY⁻ complement, show increased T cell markers of activation and migration

To explore the effect of sex chromosome complement on lupus immunopathogenesis, splenic lymphocyte subpopulations were examined *ex vivo*. There were no group differences in total T cells or CD4 and CD8 populations in the spleen (Fig. 2A and 2B). Nevertheless, within the CD4⁺ T cell pool, higher expression of the X-linked costimulatory molecule CD40 ligand (CD40L) was observed in XX mice (Fig. 2 C, D). This is consistent with accelerated disease in XX mice since CD40L is thought to be immunostimulatory in mice with lupus⁶. CD69 and CD147 expression was also increased in XX as compared to XY⁻ mice (Fig. 2 E-H), indicating an activated, disease-promoting T cell phenotype⁷. Since CD147 has been associated with increased activity levels of matrix metalloproteinases in lupus⁸, we then assessed MMPs in supernatants of anti-CD3/CD28-stimulated splenocytes. MMP9 and MMP2 levels were higher in XX (Fig. 2 I, J). This is important since MMP9 is known to promote inflammation in a variety of autoimmune diseases. In lupus specifically, the activity level of secreted MMP9 is higher in SLE compared to healthy PBMCs⁸, and MMP9 was shown to be hypomethylated in lupus T cells⁹ with DNA methylation known to suppress gene expression.¹⁰ Our data of accelerated spontaneous lupus and increased MMP9 levels in XX mice are consistent with these previous observations and suggest that sex chromosome complement may be a contributing factor. In addition, levels of TGFβ, a cytokine involved in the late fibrotic stage of lupus¹¹, were increased in XX mice (Fig. 2 K), as was the cytokine IL13 (Fig. 2L). Conversely, IL2, which may play a protective role in lupus¹², was decreased in supernatants from XX splenocytes as compared to XY⁻ (Fig. 2 M). Other cytokines falling within the Th1, Th2, or Th17 framework, namely IFNγ (Fig. 2), TNFα, IL4, IL5, IL6, IL10, and IL17, were no different between groups (not shown). Together these data indicate a pro-inflammatory effect of sex chromosome complement on T lymphocytes during spontaneous lupus.

Mice carrying the XX sex chromosome complement, as compared to the XY⁻ complement, had increased B cell markers of activation

Given the effect of sex chromosome complement on T lymphocytes and the known role of T cell dependent B cells in lupus pathogenesis¹³, we next ascertained sex chromosome complement effects on B lymphocytes. Follicular B cells, which are thought to play a pathogenic role in lupus¹⁴, were increased in the spleens of XX as compared to the XY⁻ mice (Fig. 3 A-C). Peritoneal marginal zone B cells were also elevated in XX as compared to the XY⁻ mice (Fig. 3 D-F). Since CD40L on T cells is thought to drive B cell activation via CD40 on B cells, we assessed CD40 on B cells. We found CD40 on B cells to be no different (Fig. 3G) indicating that the XX complement effect was on the T cell ligand (CD40L) not the B cell receptor (CD40). Consistent with T cell dependent B cell activation, splenic B cells from XX compared to XY⁻ were shown to be more activated as indicated by increased costimulatory molecule (CD80, CD86) expression (Fig. 3 H-J).

Discussion

Relative to other autoimmune diseases, SLE in humans has one of the largest female to male ratios of 9:1¹. Since it is impossible to ascertain sex chromosome effects that are not

confounded by differences in sex hormone levels in humans with SLE, one must turn to the animal model. Sex hormones have previously been shown to play a role in susceptibility to spontaneous lupus in mice. Specifically, castration of males accelerates disease, and testosterone treatment has been shown to be protective, while ovariectomy generally reduces disease, and estrogen treatment is thought to be disease accelerating¹⁵⁻¹⁷. A role for sex chromosome effects, not confounded by differences in sex hormones, has not been previously studied in spontaneous lupus.

In an informative transgenic system, we demonstrate for the first time a role for sex chromosome complement in spontaneous lupus, while controlling for the effect of gonadal type. Previously we demonstrated a role of sex chromosome complement in pristane induced lupus in SJL mice¹⁸. Ovariectomized female XX, as compared to ovariectomized female XY⁻ mice, had more severe lupus. In addition, ovariectomized female XX, as compared to ovariectomized female XY⁻ mice, had more severe experimental autoimmune encephalomyelitis (EAE) in SJL mice. The sex chromosome effect in spontaneous lupus described herein is clinically significant since it was shown in the absence of chemical disease induction and was found in gonadally intact mice, each more closely aligned with the human condition in SLE. Further, the finding of an effect of sex chromosome complement in another strain, the NZM2328, shows that the disease promoting effect of the XX chromosome complement is not limited to one strain. This has implications for how ubiquitous sex chromosome effects might be in the genetically heterogeneous human population.

It has been previously shown that men with Klinefelter's syndrome (XXY) are more susceptible to lupus¹⁹ than those with the normal complement of sex chromosomes. This might suggest a role for sex chromosome complement in disease. However, since XXY men have low testosterone levels, one cannot rule out that low levels of potentially protective testosterone may be responsible. Our finding that ovary bearing mice of the XX complement have more severe lupus as compared to ovary bearing mice of the XY⁻ complement is consistent with a sex chromosome effect.

The precise mechanism of how the XX genotype promotes lupus remains to be determined. Nonmutually exclusive possibilities include: 1) XX has a higher dose of disease promoting X genes which escape X-inactivation since approximately 3% of genes in mice (and up to 15% in humans) demonstrate some degree of escape from X-inactivation²⁰, 2) differential imprinting of X-inactivated genes, since XY⁻ mice always carry the maternal X, while XX mice can express either the maternal or paternal X, or 3) the presence of disease protective genes on the Y chromosome. The latter is less likely since most genes on the Y chromosome have been lost during evolution with the exception of those that are directly related to reproductive function, although there are some data showing that the Y chromosome may contain polymorphic genes that can confer protection in EAE.²¹ Of the above possibilities, the preponderance of recent data in lupus supports a disease promoting role of the XX genotype. X-chromosome demethylation has been described in humans with lupus, as well as in other lupus mouse models, whereby both X chromosomes are available for transcription (reviewed in Sawalha et al¹⁰). Such a methylation defect may take place in our mouse model as well and would be consistent with our observation of increased expression

of X-linked CD40L on T lymphocytes from XX, as compared to XY⁻ mice. This is consistent with previous findings that in CD4⁺ T cells derived from female patients with either active lupus or systemic sclerosis, both CD40L alleles are demethylated and CD40L is expressed at two fold higher levels than those from male patients or healthy females.^{22 23} Future studies of autoimmune diseases that compare XX and XY⁻ mice with those that are XO or XXY⁻ will help to further distinguish between these possibilities.²⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank Dr. Paul Burgoyne and Dr. Arthur Arnold for providing the original MF1 XY⁻ mice as well as for their insights. We thank Son Pham, Ph.D. for technical assistance.

Funding: Funding for this project was provided by NIH grant R21 NS071210 (RRV), an NIH sponsored training grant through the UCLA Laboratory of Neuroendocrinology, and the Jack H. Skirball Foundation. Dr. Manda V Sasidhar (FG-1857) and Dr. Stefan Gold (FG-1702-A1) were supported by fellowships from the National Multiple Sclerosis Society.

Reference List

1. Whitacre CC, Reingold SC, O'Looney PA. A gender gap in autoimmunity. *Science*. 1999; 283(5406):1277–8. [PubMed: 10084932]
2. Waters ST, Fu SM, Gaskin F, Deshmukh US, Sung SS, Kannapell CC, et al. NZM2328: a new mouse model of systemic lupus erythematosus with unique genetic susceptibility loci. *Clin Immunol*. 2001; 100(3):372–83. [PubMed: 11513551]
3. Arnold AP. Sex chromosomes and brain gender. *Nat Rev Neurosci*. 2004; 5(9):701–8. [PubMed: 15322528]
4. Mahadevaiah SK, Odorisio T, Elliott DJ, Rattigan A, Szot M, Laval SH, et al. Mouse homologues of the human AZF candidate gene RBM are expressed in spermatogonia and spermatids, and map to a Y chromosome deletion interval associated with a high incidence of sperm abnormalities. *Hum Mol Genet*. 1998; 7(4):715–27. [PubMed: 9499427]
5. De Vries GJ, Rissman EF, Simerly RB, Yang LY, Scordalakes EM, Auger CJ, et al. A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits. *J Neurosci*. 2002; 22(20):9005–14. [PubMed: 12388607]
6. Lettesjo H, Burd GP, Mageed RA. CD4⁺ T lymphocytes with constitutive CD40 ligand in preautoimmune (NZB × NZW)F1 lupus-prone mice: phenotype and possible role in autoreactivity. *J Immunol*. 2000; 165(7):4095–104. [PubMed: 11034421]
7. Ishikawa S, Akakura S, Abe M, Terashima K, Chijiwa K, Nishimura H, et al. A subset of CD4⁺ T cells expressing early activation antigen CD69 in murine lupus: possible abnormal regulatory role for cytokine imbalance. *J Immunol*. 1998; 161(3):1267–73. [PubMed: 9686587]
8. Pistol G, Matache C, Calugaru A, Stavaru C, Tanaseanu S, Ionescu R, et al. Roles of CD147 on T lymphocytes activation and MMP-9 secretion in systemic lupus erythematosus. *J Cell Mol Med*. 2007; 11(2):339–48. [PubMed: 17488482]
9. Jeffries MA, Dozmorov M, Tang Y, Merrill JT, Wren JD, Sawalha AH. Genome-wide DNA methylation patterns in CD4⁺ T cells from patients with systemic lupus erythematosus. *Epigenetics*. 2011; 6(5):593–601. [PubMed: 21436623]
10. Sawalha AH, Harley JB, Scofield RH. Autoimmunity and Klinefelter's syndrome: when men have two X chromosomes. *J Autoimmun*. 2009; 33(1):31–4. [PubMed: 19464849]

11. Okuda S, Languino LR, Ruoslahti E, Border WA. Elevated expression of transforming growth factor-beta and proteoglycan production in experimental glomerulonephritis. Possible role in expansion of the mesangial extracellular matrix *J Clin Invest.* 1990; 86(2):453–62.
12. Humrich JY, Morbach H, Undeutsch R, Enghard P, Rosenberger S, Weigert O, et al. Homeostatic imbalance of regulatory and effector T cells due to IL-2 deprivation amplifies murine lupus. *Proc Natl Acad Sci U S A.* 2010; 107(1):204–9. [PubMed: 20018660]
13. Kotzin BL. Systemic lupus erythematosus. *Cell.* 1996; 85(3):303–6. [PubMed: 8616885]
14. Cappione A 3rd, Anolik JH, Pugh-Bernard A, Barnard J, Dutcher P, Silverman G, et al. Germinal center exclusion of autoreactive B cells is defective in human systemic lupus erythematosus. *J Clin Invest.* 2005; 115(11):3205–16. [PubMed: 16211091]
15. Carlsten H, Tarkowski A, Holmdahl R, Nilsson LA. Oestrogen is a potent disease accelerator in SLE-prone MRL lpr/lpr mice. *Clin Exp Immunol.* 1990; 80(3):467–73. [PubMed: 2372993]
16. Dumont F, Monier JC. Sex-dependent systemic lupus erythematosus-like syndrome in (NZB × SJL)F1 mice. *Clin Immunol Immunopathol.* 1983; 29(2):306–17. [PubMed: 6605226]
17. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice. *Journal of Experimental Medicine.* 1978; 147(6):1568–83. [PubMed: 308087]
18. Smith-Bouvier DL, Divekar AA, Sasidhar M, Du S, Tiwari-Woodruff SK, King JK, et al. A role for sex chromosome complement in the female bias in autoimmune disease. *J Exp Med.* 2008; 205(5):1099–108. [PubMed: 18443225]
19. Scofield RH, Bruner GR, Namjou B, Kimberly RP, Ramsey-Goldman R, Petri M, et al. Klinefelter's syndrome (47,XXY) in male systemic lupus erythematosus patients: support for the notion of a gene-dose effect from the X chromosome. *Arthritis Rheum.* 2008; 58(8):2511–7. [PubMed: 18668569]
20. Berletch JB, Yang F, Xu J, Carrel L, Disteche CM. Genes that escape from X inactivation. *Human Genetics.* 2011; 130(2):237–45. [PubMed: 21614513]
21. Teuscher C, Noubade R, Spach K, McElvany B, Bunn JY, Fillmore PD, et al. Evidence that the Y chromosome influences autoimmune disease in male and female mice. *Proc Natl Acad Sci U S A.* 2006; 103(21):8024–9. [PubMed: 16702550]
22. Lian X, Xiao R, Hu X, Kanekura T, Jiang H, Li Y, et al. DNA demethylation of CD40L in CD4(+) T-cells from women with systemic sclerosis - A possible explanation for female susceptibility. *Arthritis Rheum.* 2012
23. Lu Q, Wu A, Tesmer L, Ray D, Yousif N, Richardson B. Demethylation of CD40LG on the inactive X in T cells from women with lupus. *J Immunol.* 2007; 179(9):6352–8. [PubMed: 17947713]
24. Chen X, Watkins R, Delot E, Reliene R, Schiestl RH, Burgoyne PS, et al. Sex difference in neural tube defects in p53-null mice is caused by differences in the complement of X not Y genes. *Dev Neurobiol.* 2008; 68(2):265–73. [PubMed: 18004765]

Figure 1. The XX sex chromosome complement is associated with greater lupus disease severity compared to the XY⁻

Intact NZM2328 mice were monitored daily for clinical signs of lupus from week 16 – 40 weeks. Decreased survival was observed in XX genotype (n=27) as compared to XY⁻ (n=22) (Log-rank (Mantel-COX) p=0.0082) (A). Further, kidney pathology was increased in XX mice (B) compared to XY⁻ mice (D) at 36 weeks of age as indicated by fibrosis collagen deposition (original magnification 20×). Panels C and E show magnified view of the rectangles shown in panels B and D. Quantitative fibrosis scores were significantly higher in XX mice compared to XY⁻ (independent samples t tests p=0.0063) (F). Fibrotic scoring was performed according to a scale where 0 indicates no fibrosis, 1 minimal, 2 mild, 3 moderate and 4 severe fibrosis. Fibrosis scores were acquired from twelve kidneys from a total of 6 mice at 36 weeks of age. All data are shown as mean+ standard error of mean (SEM).

Figure 2. T lymphocytes have a more pro-inflammatory phenotype in the XX sex chromosome complement as compared to the XY⁻ complement

Splenocytes from XX and XY⁻ NZM2328 mice were isolated and phenotyped by flow cytometry for the expression of activation and inflammation markers. XX and XY⁻ mice did not differ in CD3, CD4⁺ and CD8⁺ T cell percentages (A and B). CD4⁺ T cells from XX expressed significantly more CD40ligand compared to XY⁻ ($p < 0.0001$), (representative plots in C, quantification in D). CD4⁺ T cells derived from XX mice also showed increased expression of CD69 compared to XY⁻ animals, ($p = 0.0079$), (E-F). CD3⁺ T cells from XX mice showed increased expression of CD147 compared to XY⁻ mice, ($p = 0.0002$), (G-H). Finally, splenocytes from XX and XY⁻ mice were stimulated *in vitro* with anti CD3/CD28 antibody (both 1μg/ml) for 48 hours and supernatants were analyzed for cytokine/MMP levels. MMP-9, (I), ($p = 0.0052$), MMP-2, (J), ($p = 0.0027$), TGFβ, (K), ($p = 0.0052$) and IL-13, (L) ($p = 0.018$) were significantly higher in splenocyte cultures from XX mice compared to XY⁻ mice. In contrast, IL-2 levels were decreased in XX compared to XY⁻ (M) ($p = 0.0022$). No group differences were observed for IFNγ (N). Results are shown from animals at 36 weeks of age combined from 2 independent experiments (each with 2-3 mice in each group). Group differences in lymphocyte subpopulations, activation markers, MMPs and cytokines levels were analyzed using independent samples t tests or Mann-Whitney U tests, as appropriate. All data are shown as mean+standard error of mean (s.e.m).

Figure 3. B lymphocytes have a more activated phenotype in the XX sex chromosome complement as compared to the XY⁻ complement

Splenocytes and peritoneal lymphocytes from XX and XY⁻ mice were isolated and phenotyped by flow cytometry. CD23⁺CD21⁺ follicular B cells and CD23⁻CD21⁺ marginal zone B cells (MZBs) were identified after gating on CD19⁺B cells. There was a significant increase in percentage of splenic CD23⁺CD21⁺ B cells in XX genotype (A) compared to XY⁻ (B) (quantification in C, p=0.0309). In the peritoneal pool, a significant accumulation of marginal zone B cells, MZBs, (CD23⁻CD21⁺) was observed in XX genotype (D) as compared to the XY⁻ (E) (quantification in F, p=0.041). Finally, increased expression of costimulation markers was observed for CD86 (H) (p<0.0001) and CD80 (I) (p=0.0022) in XX compared to XY⁻, while no significant differences were observed for CD40 (G) expression on B cells. Quantification is shown in (J). Results are shown from animals at 36 weeks of age combined from 2 independent experiments (each with 2-3 mice in each group). Group differences in B lymphocyte subpopulations and costimulation markers were analyzed using independent samples t tests or Mann-Whitney U tests, as appropriate. All data are shown as mean+standard error of mean (s.e.m).