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A Yin-Yang Effect between Sex Chromosome Complement and Sex Hormones on the Immune Response

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Sex chromosome complement, by determining whether an ovary or testis develops, exerts indirect hormone-mediated effects on the development of sex-specific traits. However, this does not preclude more direct effects that are independent of gonadal hormones. To look for gonadal hormone-independent effects in sexually dimorphic immune responses, we used mice in which the testis determinant *Sry* has been moved from the Y chromosome to an autosome, thus allowing the production of mice that differ in sex chromosome complement while having the same gonadal type. This model permits comparison of XX and XY mice with ovaries or testes. These mice were immunized with an autoantigen, and draining lymph node cells were assessed for autoantigen-specific proliferative responses and cytokine production. Surprisingly, we found that the male complement of sex chromosomes (XY) was relatively stimulatory, whereas male sex hormones were inhibitory, for this immune response. This is the first experimental evidence of a compensatory yin-yang effect of sex chromosome complement and sex hormones on a biologic process. (*Endocrinology* 146: 3280–3285, 2005)

IN MALE MAMMALS, THE Y-linked gene *Sry* is expressed in cells of the undifferentiated gonadal ridges to cause them to differentiate into Sertoli cells, which begins the differentiation of the testes (1). Once the testes have formed, they secrete hormones distinct from those of the ovary, and these hormonal differences generate sex differences in many nongonadal tissues such as the external genitalia, immune system, brain, cardiovascular system, and skeletal system. Indeed, the effects of these hormones account for the majority of sex differences in nongonadal tissues identified to date. However, there are other genetic differences between males and females arising from the difference in sex chromosome complement that could also contribute to sex differences in phenotype (2, 3). There are other Y genes whose role in nongonadal tissues has been little studied, and there are also differences in X gene expression arising from the difference in X chromosome complement. Because it has been much easier to study the effects of gonadal hormones than these possible direct effects of X and Y genes, the sexually differentiating effects of hormones are much better understood. A major question addressed here is whether direct actions of X or Y genes on tissues are also important, aside from *Sry*'s effect on the gonad.

To begin to approach this question in our system of interest (the immune system), we used mice in which the *Sry* gene has been moved from the Y chromosome to an autosome so that gonadal sex (ovaries *vs.* testes) no longer correlates with sex chromosome complement (XX *vs.* XY). This model allows comparison of XX and XY mice within the same gonadal type (either ovaries or testes) to identify any separate (gonadal hormone independent) sex chromosome effects. These mice have been previously described in studies of gonadal and brain development (3–8).

Sex differences in immune responses as well as susceptibility to autoimmune diseases have been recognized for decades. Autoimmune diseases characterized by a female preponderance are numerous, including multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus. A variety of sex-related differences in immune responses have been described, with females generally having increased cellular and humoral responses, compared with males (9). Extensive research has been devoted to the role of sex hormones on immune responses and autoimmunity, and numerous effects of sex hormones have indeed been shown (9). Here we ask whether there are also gonadal hormone-independent (sex chromosome complement) effects on an autoantigen-specific immune response.

Materials and Methods

Experimental animals

The mouse model system used here has previously been described (6). Briefly, a 129 strain Y chromosome (129/SvEi-*Gpi1*^{c}) deleted for the testis determinant *Sry* (10) here designated Y$^-$, has been complemented by an *Sry* transgene at a single autosomal location. The variant Y$^-$ together with the transgene is maintained on an outbred MF1 background at the Medical Research Council National Institute for Medical Research (London, UK) by breeding from fertile XY$^-$*Sry* transgenic males (11). MF1 XY$^-$*Sry* males were bred with wild-type SJL females purchased from The Jackson Laboratory (Bar Harbor, ME). Male F1

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Abbreviations: IFN, Interferon; LNC, lymph node cell; MBP, myelin basic protein.

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progeny of the XY$^-$*Sry* genotype were then bred with wild-type SJL females to create the F2 generation. This process continued to F11. All progeny (XX females, XY$^-$ females, XX*Sry* males, and XY$^-$*Sry* males) of the F11 generation were used in experiments. All experiments were done with the approval of the University of California, Los Angeles, Animal Research Committee with regard to meeting established standards of humane animal care.

Genotyping

Tail samples were obtained from mice and genomic DNA isolated using the DNeasy tissue kit (QIAGEN, Valencia, CA). The *Sstyl* multicopy gene on the Y chromosome (12) was amplified (primers: YMTFP1, CTG GAG CTC TAC AGT GAT GA; YMTRP1, CAG TTA CCA ATC AAC ACA TCA C) to test for the presence of the Y$^-$ chromosome; the autosomal gene *Myogenin* [primers MYOF (forward): TTA CGT CCA TCG TGG ACA GCA T; MYOR (reverse): TGG GCT GGG TGT TAG TCT TAT] served as an amplification control (4, 13). Amplification of DNA from XY$^-$ females and XY$^-$*Sry* males mice yielded the 245-bp *myogenin* product and the 342-bp *Sstyl* product, whereas amplification of DNA from XX females and XX*Sry* males yielded only the 245-bp *myogenin* product.

Gonadectomies and hormone treatment

Ovariectomies were carried out at 11 or 10 wk of age and castrations at 11, 10, or 4 wk of age. Methods for ovariectomy and castration have been previously described (14). Testosterone (5 mg, 90 d sustained release) or placebo pellets were implanted at the time of ovariectomy, as described (14).

Hormone levels

Blood was obtained by intracardiac puncture at age 8 wk in a subset of gonadally intact XX*Sry* and XY$^-$*Sry* phenotypic male mice. Serum testosterone levels were determined by RIA by Diagnostic Systems Laboratories (Webster, TX) as described (14).

Immunization and immune responses

Immunizations took place at 12 wk of age in gonadally intact as well as gonadectomized mice. A subset of females had been ovariectomized at 11 or 10 wk of age (1 or 2 wk before immunization, respectively). A subset of males had been castrated at 11, 10, or 4 wk of age (1, 2, or 8 wk before immunization, respectively). Myelin basic protein (MBP) emulsified in complete Freund's adjuvant was used during immunization. After 10 d, draining lymph node cells (LNCs) were cultured with MBP and assayed for proliferation by thymidine incorporation. Cytokine production [TNF; interferon (IFN)-γ; and IL-10] was determined using the mouse inflammation cytokine cytometric bead array kit (BD Biosciences, San Diego, CA) according to manufacturer's instructions as previously described (15).

Results and Discussion

MBP and proteolipid protein are autoantigens in experimental autoimmune encephalomyelitis in animals and candidate autoantigens in humans with multiple sclerosis. It has previously been demonstrated that after MBP or proteolipid protein immunization of SJL mice, there is a greater increase in proinflammatory cytokine production in draining LNCs of gonadally intact females than males (16–20). To begin to dissect the role of sex chromosome complement *vs.* sex hormones in this sexually dimorphic immune response, we used intact mice, ovariectomized females, and castrated males. Proliferative responses as well as levels of TNFα, IFNγ, and IL-10 production were significantly higher in LNCs of MBP immunized, intact females, compared with intact males, and immune responses were also different between ovariectomized females and castrated males but less so, compared with intact mice (Fig. 1). The decreased difference between gonadectomized females *vs.* males, compared with intact females *vs.* males, demonstrated a role for circulating adult sex hormones in the sex difference in the immune response of intact mice. The residual difference between ovariectomized females and castrated males could be due to either direct effects of sex chromosome complement or effects of sex hormones acting before gonadectomy.

In the same experiment, we then made a second comparison. We ascertained whether circulating adult levels of fe-

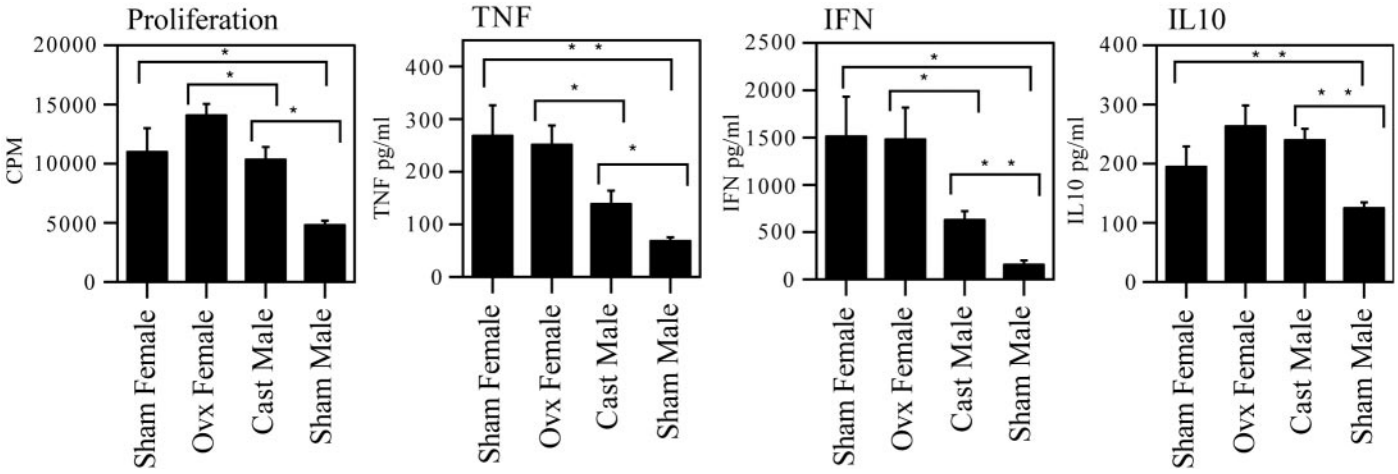

FIG. 1. Effect of adult sex hormones on MBP-specific immune responses. SJL mice were immunized with MBP, and then draining LNCs were assayed for MBP-specific proliferative responses and cytokine production. Sham females had significantly increased proliferative responses as well as TNFα, IFNγ, and IL-10 production, compared with sham males. Ovariectomized females had increased proliferative responses, TNFα, and IFNγ, compared with castrated males, which was significant but less different from comparisons between gonadally intact females *vs.* males. Castrated males had increased immune responses, compared with those of sham males, whereas ovariectomized females did not have significantly different immune responses, compared with sham females. Ovariectomies and castrations were performed at 10 wk of age, as described (14). Immunization took place 2 wk after surgery (gonadectomized or sham operated), at 12 wk of age. Ovx, Ovariectomized; cast, castrated. Sham females, n = 6; ovariectomized females, n = 7; castrated males, n = 4; sham males, n = 4. Histograms show means and SEM of mice in each group. *, $P < 0.05$; **, $P < 0.01$, Student's *t* test. Data are representative of three repeated experiments.

male *vs.* male gonadal hormones, or both, were contributing to the sex difference in the immune response in intact mice. We found that castration increased immune responses, compared with sham surgery in male mice, whereas ovariectomy had no significant effect in female mice (Fig. 1). These data indicated that the hormonal effect on sex differences in this immune response in intact mice was primarily due to an immunosuppressive effect of testes. These results were consistent with previous descriptions by our group and others of an immunosuppressive effect of testosterone treatment in a variety of autoimmune diseases (14, 21–26).

We next evaluated the separate role of sex chromosome complement by comparing groups of mice that had the same gonadal type but differed in sex chromosome complement. For this we backcrossed the *Sry*-negative Y^- chromosome and the *Sry* transgene onto the SJL strain, the strain with a known gender dimorphism in this immune response. We assessed immune responses in LNCs from MBP-immunized mice, making comparisons between XX and XY^- females (with ovaries) and between XX*Sry* and XY^-*Sry* males (with testes). Whereas significantly greater proliferative responses and TNF production were observed in gonadal females (both XX and XY^-), compared with gonadal males (XX*Sry* and XY^-*Sry*), there were no significant differences between XX and XY^- chromosome complements within each gonadal type in these gonadally intact mice (Fig. 2). Regarding IFN and IL-10 production, there were again significantly increased responses in female XY^-, compared with male XY^-*Sry* mice, and trends for an increase in female XX, compared with male XX*Sry* mice, but again there were no significant differences between XX and XY^- chromosome complements within each gonadal type for these responses.

We then ascertained whether an effect of sex chromosome complement might become evident if adult circulating sex hormones were removed. Gonadal females (XX and XY^-) were ovariectomized 1 wk before immunization to remove an effect of adult circulating female hormones on immune responses. Interestingly, immune responses were significantly higher in ovariectomized XY^- mice, compared with ovariectomized XX mice (Fig. 3). An immunostimulatory effect of the XY^- chromosome complement was quite surprising for the following reason. Intact, wild-type males have decreased immune responses, compared with females, and testosterone is known to be inhibitory (9, 14, 19, 21–26). Therefore, one would have predicted that the effect of the male sex chromosome complement would be synergistic with, not opposed to, effects of male hormones, together combining to give the relatively low level of immune responsiveness of SJL males, compared with females (9, 16–18, 21). Instead, the male sex chromosome complement was relatively stimulatory.

Consistent with a significant role for testosterone in inhibiting the immunostimulatory effect of the XY^- genotype in intact male mice, we were not able to detect an effect of sex chromosome complement on the immune response in intact gonadal males (XX*Sry vs.* XY^-*Sry*) (Fig. 2) or gonadal males that were castrated 1 wk (age 11 wk) before immunization at 12 wk of age (not shown). However, when gonadal male mice were castrated much earlier, 8 wk (age 4 wk) before immunization at 12 wk of age, immune responses were significantly higher in castrated XY^-*Sry* mice, compared with castrated XX*Sry* mice (Fig. 4). These data are consistent with the conclusion that male sex hormones play a significant role in opposing, or masking, the immunostimulatory effect of the male genotype in intact mice.

Finally, to directly demonstrate the opposing effect of male genotype (XY^-) and male sex hormone phenotype, testosterone, or placebo was administered to ovariectomized, female XY^- mice that had the significantly increased immune response. Indeed, testosterone treatment suppressed the otherwise elevated immune response in XY^- mice (Fig. 5).

To our knowledge, this is the first demonstration of an

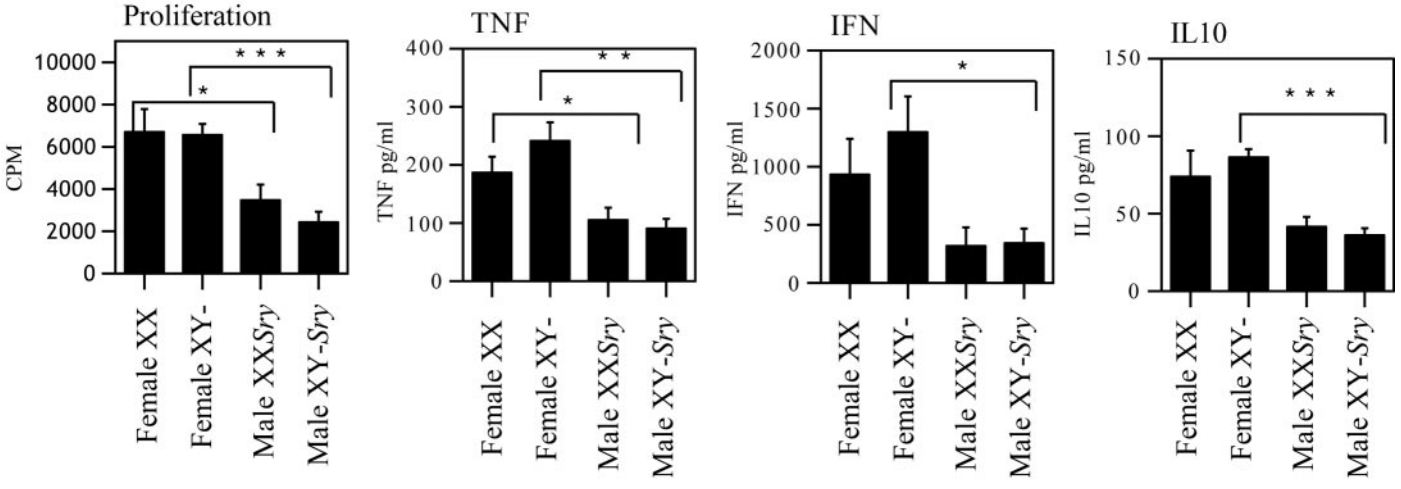

FIG. 2. Effect of sex chromosome complement on MBP-specific immune responses in gonadally intact SJL XX, XY^-, XX*Sry*, and XY^-*Sry* transgenic mice. MF1 XY^-*Sry* gonadal males were backcrossed with female wild-type SJL females for 11 generations. Gonadal female (XX and XY^-) and gonadal male (XX*Sry* and XY^-*Sry*) progeny were immunized with MBP and immune responses assessed as in Fig. 1. Greater immune responses were observed in gonadal females (XX and XY^-), compared with gonadal males (XX*Sry* and XY^-*Sry*), but there were no significant differences between XX and XY^- genotypes within each gonadal type. XX females, n = 6; XY^- females, n = 8; XX*Sry* males, n = 8; XY^-*Sry* males, n = 7. Histograms show means and SEM for mice in each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Student's *t* test. Data are representative of three independent experiments on different sets of mice.

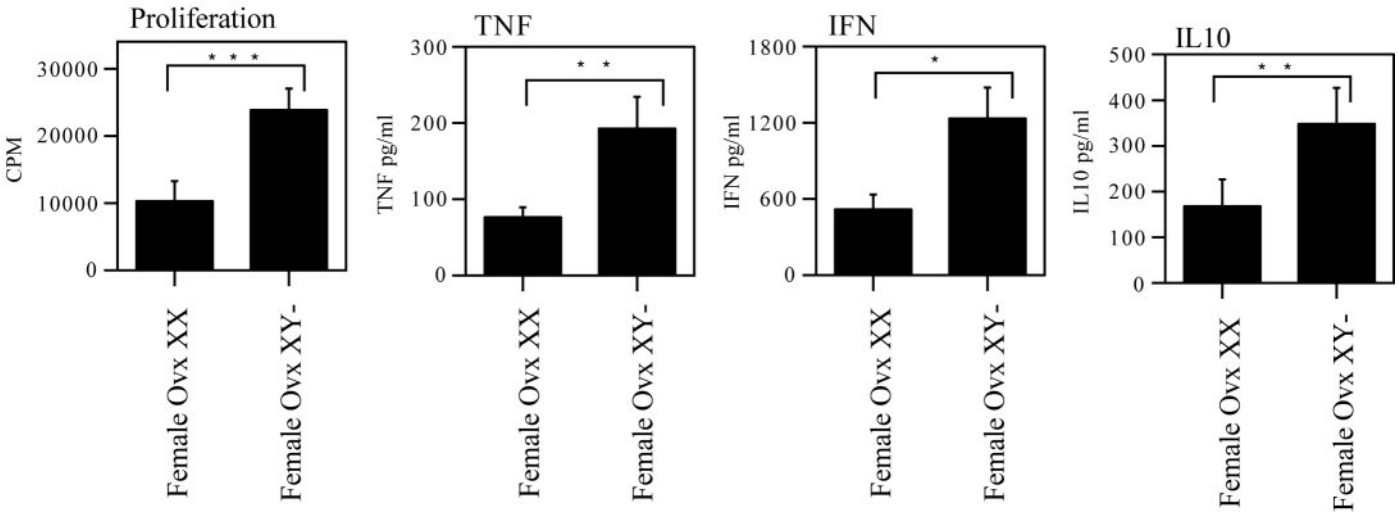

FIG. 3. Effect of sex chromosome complement on MBP-specific immune responses in ovariectomized female SJL XX and XY$^-$ mice. Immune responses were assessed in F11 SJL XX and XY$^-$ female mice as described in Fig. 2. Ovariectomized (Ovx) XY$^-$ females had increased proliferative responses and cytokine production, compared with Ovx XX females. Ovariectomy was done at 11 wk of age, 1 wk before immunization at 12 wk of age. Ovx XY$^-$ females, n = 8; Ovx XX females, n = 7. Histograms show means and SEM for mice in each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Student's *t* test. Data are representative of five independent experiments on different sets of mice.

effect of sex chromosome complement on immune responses in mice that have the same gonadal type. Because we compared gonadectomized mice, the group differences in immune responses cannot be attributed to differences in levels of gonadal hormones in the weeks before harvesting the cells. When testosterone levels were measured in intact XX*Sry* and XY$^-$*Sry* gonadal males, at age 8 wk, levels were no different (XX*Sry* = 0.8 ± 0.1 ng/ml, n = 11; XY$^-$*Sry* = 0.8 ± 0.1 ng/ml; n = 11, $P = 0.908$). However, might the putative sex chromosome effect have been mediated by long-lasting effects of different levels of neonatal gonadal secretions in XX *vs.* XY$^-$ mice? This is a remote possibility, at best, due to the following reasons. First, it is very unlikely that the same difference in neonatal levels of hormones would occur in mice with both ovaries (XX *vs.* XY$^-$) and testes (XX*Sry vs.* XY$^-$*Sry*). Second, if the increase in immune responses were caused by differences in neonatal steroid levels, one would suspect that XY$^-$ mice would have lower neonatal testosterone levels because it is known that exposure to testosterone early in development decreases immune responses, as has been shown in diabetes in nonobese, diabetic (NOD) mice (27), lupus in [New Zealand black (NZB) × New Zealand White (NZW)] F1 mice (28), and thyroiditis in rats (29). Importantly, XY$^-$ gonadal females were not less masculine than XX gonadal females, nor were XY$^-$*Sry* gonadal males less masculine than XX*Sry* gonadal males, on any trait that has been measured to

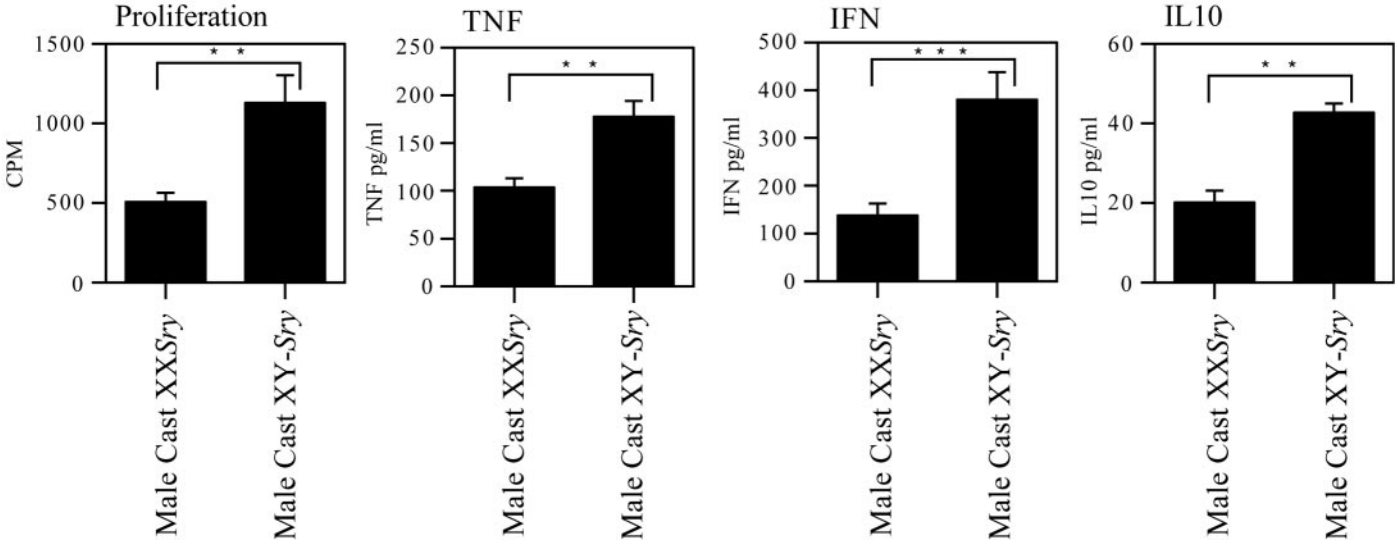

FIG. 4. Effect of sex chromosome complement on MBP-specific immune responses in castrated male SJL XX*Sry* and XY$^-$*Sry* mice. Immune responses were assessed in F11 SJL XX*Sry* and XY$^-$*Sry* male mice as described in Fig. 2. Castrated XY$^-$*Sry* males had increased proliferative responses and cytokine production, compared with castrated XX*Sry* males. Castration was done at 4 wk of age, 8 wk before immunization at 12 wk of age. Castrated XY$^-$*Sry* males, n = 7; castrated XX*Sry* males, n = 7. Histograms show means and SEM for mice in each group. **, $P < 0.01$; ***, $P < 0.001$, Student's *t* test.

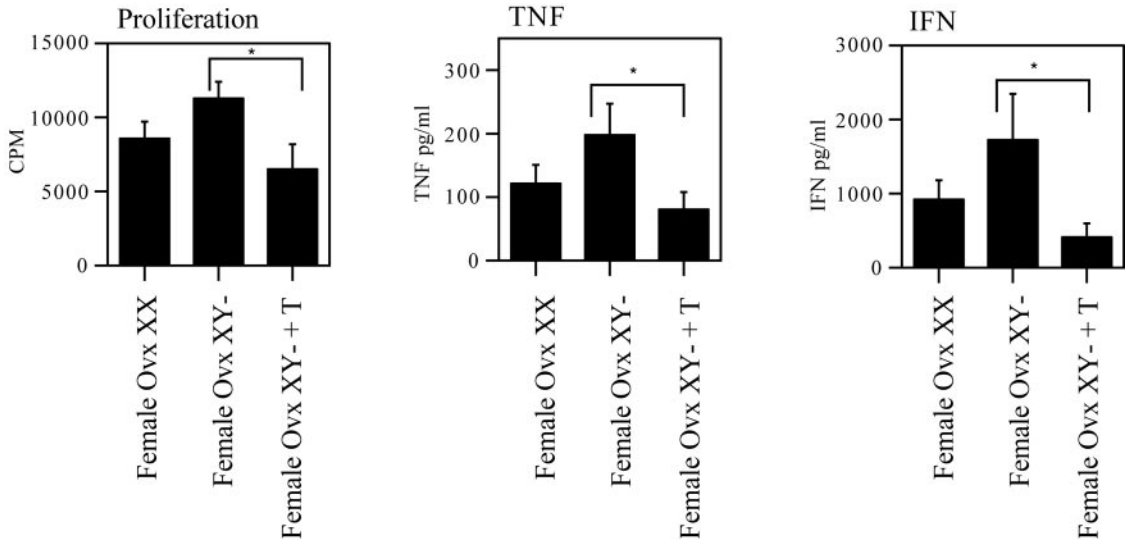

FIG. 5. Testosterone (T) and the XY^- genotype have compensatory effects on immune responses. Ovariectomized (Ovx) XY^- females had increased immune responses, compared with Ovx XX females, replicating the effect shown in Fig. 3, whereas testosterone treatment decreased immune responses in Ovx XY^- females. Ovariectomy was done at 11 wk of age, 1 wk before immunization at 12 wk of age. Testosterone (5 mg) or placebo pellets were implanted at the time of ovariectomy. Ovx XX females treated with placebo, Ovx XX, n = 5; Ovx XY^- females treated with placebo, Ovx XY, n = 5; Ovx XY^- females treated with testosterone, Ovx XY + T, n = 5. Histograms show means and SEM for mice in each group. *, $P < 0.05$, Student's t test. Data are representative of three independent experiments on different sets of mice.

date (3–8). Together, these observations argue strongly against the possibility that neonatal levels of testosterone were lower; hence, immune responses were higher in XY^- mice, compared with XX mice. Whereas we have shown a direct effect of sex chromosome complement on an autoantigen-specific immune response, it remains to be determined whether this effect is due to: (1) gene(s) unique to the Y chromosome, (2) a higher dose of X genes that escape X inactivation in XX mice, or (3) paternal imprinting of X genes, which is present in XX, but not XY^-, mice (3). Regarding clinical significance, study of a direct effect of sex chromosome complement on a variety of autoimmune diseases is now warranted.

Surprisingly, we have discovered that the XY^- genotype is relatively immunostimulatory, compared with the XX genotype, whereas confirming that the male hormone phenotype is indeed inhibitory. Together these data reveal a compensatory or yin-yang effect between sex chromosome complement and sex hormone status. This compensatory effect of sex chromosome complement and sex hormones has been recently proposed in a hypothetical fashion in brain development as part of a review article by De Vries (30). Our data are the first experimental evidence that sex chromosomes and sex hormones may indeed compensate for each other and that this may occur not only in brain but also in other tissues. The opposing action of male hormone and male genome suggests the evolution of a compensatory relationship between the two, serving to decrease immune response differences between females and males in situations in which extreme differences might be maladaptive. In the case of the MBP-specific immune response in the SJL mouse, testosterone has indeed overcompensated, such that intact, wild-type males have a less robust immune response, compared with females. It is possible that in other strains of mice, in which there is not a gender difference in immune responses, these two forces may more precisely offset each other. This theory has broad implications for the study of the effects of sex hormones and sex chromosome complement even on biologic events that are not characterized by a gender dimorphism. In essence, the two genders may reach a common and equal biologic end point (phenotypic sexual equality) through opposing contributions of sex-specific (sexually unequal) hormonal or genetic signals. Whether a yin-yang (compensatory) effect or an additive (complementary) effect of sex hormones and sex chromosome complement exists in other organ systems remains to be determined. Evolutionary pressure may favor an antagonistic effect in some organ systems, whereas it may favor a synergistic effect in other organ systems, depending on pressures on each gender.

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