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The XX sex chromosome complement is required in male and female mice for enhancement of immunity induced by exposure to 3,4-dichloropropionanilide

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Abstract

Problem—The chemical propanil enhances antibody responses to a heat killed-*Streptococcus pneumoniae* (HKSP) vaccine. The enhanced response is dependent on gonads in females, but independent of gonads in males. The sex differences in the immune response may be due to sexual differentiation of the immune system or sex chromosome complement.

Method of Study—To test the hypothesis that the immune system is sexually differentiated, newborn C57BL/6 pups were treated with testosterone propionate (TP) or placebo. The role of sex chromosome complement was investigated using the 4-core genotypes (FCG) model of XXF and XYF gonadal females (ovaries), and XXM and XYM gonadal males (testes). For some experiments mice were gonadectomized or sham gonadectomized. All mice were vaccinated with HKSP, treated with propanil, and the antibody response determined at day seven. Results. Neonatal TP did not alter the response to HKSP. In FCG mice propanil significantly enhanced the immune response in XXF females and XXM males, but not in XYF females or XYM males.

Conclusion—The immune system of females was not masculinized by neonatal TP treatment. Sex chromosome complement significantly contributes to the sexually dimorphic immune response after propanil exposure.

Keywords

sex differences; propanil; sex chromosome

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INTRODUCTION

Sexually dimorphic immune responses are well characterized with females, in general, exhibiting stronger immune responses and a greater probability of developing autoimmune disease than males. Although autoimmune diseases are dependent on a number of factors, including genetics and environmental influences; the predominance of autoimmunity in females has largely been attributed to hormonal differences in males and females [1–3]. However, there is increasing evidence that developmental processes due either to genetic differences or the organizational actions of testosterone in males may contribute to sexual dimorphism. Immune responses to infectious diseases can also differ significantly between males and females [4, 5]. Hormonal differences have been thought to be the predominant determinant in the response to infections, with estrogen enhancing the immune response and testosterone suppressing immunity. The disparity between male and female responses is probably the result of complex interactions that depend on the specific pathogen, genetic factors, and innate and adaptive immune responses to infection [4, 5].

Sexual differentiation of the reproductive system, central nervous system and reproductive behavior in response to the organizational effects of hormones are well established, however, there are few studies to determine if sexual differentiation occurs in the developing immune system. Sexual differentiation occurs in response to testosterone at a critical period of development resulting in sexually dimorphic anatomy and function in adults. A common method to study sexual differentiation is to administer testosterone to pregnant animals during the prenatal period of development or to newborns during the neonatal period of development. Early studies using prenatal or neonatal exposure to testosterone propionate (TP) demonstrated increased aggression and fighting in females when exposed to exogenous testosterone as adults [6, 7]. Mice, prenatally androgenized by treatment with dihydrotestosterone during late gestation, have irregular estrous cycles, altered reproductive hormone levels, increased fasting glucose and impaired glucose tolerance demonstrating effects of prenatal androgen exposure on reproductive and metabolic function [8, 9]. Hawkins et al. (1993) examined the effect of neonatal TP treatment on the immune system. They demonstrated that neonatal treatment with TP reduced the incidence of diabetes in the female offspring in a mouse model of autoimmune Type 1 diabetes [10]. This suggests that manipulation of the neonatal hormone environment alters the subsequent immune response in female and male mice in the development of autoimmune mediated diabetes.

Recently it has begun to be appreciated that sex chromosome complement, XX versus XY, can influence sexually dimorphic phenotypes independent of gonadal hormones [11]. Features unique to the X chromosome, including X-chromosome inactivation, escape or skewed inactivation, and X chromosome dosage, may impact responses that contribute to autoimmune diseases [12–14]. The X chromosome also encodes the highest number of immune-related genes identified to date in the human genome [12, 14]. Studies into the role of sex chromosome complement have become feasible with development of the 4-core genotypes (FCG) model, which consists of XX and XY gonadal females with ovaries (XXF and XYF) and XY and XX gonadal males with testes (XYM and XXM) [15, 16]. This provides a model to determine the contribution of XX or XY sex chromosome complement, gonadal secretions, and interactions between the two factors in a given response. Sex chromosome

complement affects sexually dimorphic patterns in the brain and behavior, adiposity and metabolism, and the response to alcohol [17–22]. Sex chromosome complement also contributes to sex differences in the immune response to viral infection and autoimmune disease [23–25].

Previous studies demonstrated that exposure to the chemical 3, 4-dichloropropionanilide (propanil) enhances the immune response to a heat-killed *Streptococcus pneumoniae* (HKSP) vaccine [26]. HKSP elicits a robust antibody response to the immunodominant T-independent type 2 polysaccharide phosphorylcholine (PC) [27]. When propanil is administered to mice simultaneously with HKSP, the number of splenic antibody secreting cells (ASC) specific for PC is increased significantly over controls [28]. Interestingly, the increased response in females is primarily dependent on the ovaries, while the testes are not required for the enhanced response in males. Although sexually dimorphic responses are often the result of differential effects of male and female hormones, additional studies demonstrated that the major gonadal hormones, estrogen, progesterone, and testosterone were not necessary for the increased response in gonadally-intact females or males [28]. It is hypothesized that two additional sex-biasing factors may contribute to the sex difference: long-lasting ("organizational") effects of androgens acting to masculinize males perinatally, or differences in the direct effects of sex chromosome complement (XX vs. XY) acting in tissues other than the gonads, for example, directly on immune system cells [15, 29]. In the present study, due to the effect of propanil on the immune response to HKSP vaccination, propanil was used as a unique chemical tool to test for sex-biasing effects of these two factors. Experiments were performed to determine: (1) if sexual differentiation of the immune system is responsible for the sexual dimorphism in the response to propanil using neonatal exposure to TP; and (2) if sex chromosome complement contributes to the differential response to propanil in male and female mice using the FCG model.

MATERIALS AND METHODS

Mice

Mice were housed in microisolator cages in specific pathogen free conditions on a 12 hr light-dark cycle with food and water provided *ad libitum*. Studies were conducted in accordance with all federal and institutional guidelines for animal use and were approved by the WVU Institutional Animal Care and Use Committee.

Four core genotypes model

Six to eight week old C57BL/6J female and B6.Cg-Tg(Sry)2Ei *Sry*^{dl1Rlb}/ArnoJ (XY^{-}*Sry*) male mice (The Jackson Laboratory, Bar Harbor, Maine) were allowed to acclimate for one week before use. In the XY^{-}*Sry* male mice the sex determining region of the Y chromosome, the *Sry* gene, has been deleted from the Y chromosome and *Sry* is inserted as a transgene onto an autosome. C57BL/6 female mice were bred to XY^{-}*Sry* male mice to produce FCG mice: XX or XY gonadal females (XXF and XYF) and XY or XX gonadal males (XYM and XXM). Mice were weaned at 21 days of age. The genotype of offspring was confirmed by PCR of DNA isolated from tail samples obtained at weaning [19].

Gonadectomy

Adults underwent castration or ovariectomy by standard procedure. Briefly, mice were anesthetized with isoflurane. Incisions were made through the skin and the underlying abdominal wall. The testes or ovaries were isolated and forceps used to cauterize the vas deferens and the blood vessel or transect the tip of the uterine horn and cauterize the blood vessels. The abdominal wall was closed with a suture and skin incisions closed with wound clips. Sham operated mice (Sham) underwent the same procedure, but the testes or ovaries were left intact. Gonadectomized mice (GDX) were housed 5–6 weeks following surgery before used in experiments.

Bacterial preparation and immunization

S. pneumoniae strain R36A, an avirulent, nonencapsulated strain, was grown to mid-log phase in Todd-Hewitt broth + .05% yeast extract (Becton Dickinson, Sparks, MD) and stored at −80°C. For immunization, stock was cultured in a candle jar for 18 hrs at 37°C on blood agar plates (Becton Dickinson). Colonies were selected and suspended in 200 ml broth, grown at 37°C to an absorbance reading at 650nm of 0.4 and heat killed for 4 hours in a 60°C water bath. A final concentration of 10^9 CFU/ml was established in PBS based on colony counts. Sterility was confirmed by culture and heat-killed *S. pneumoniae* (HKSP) stored at −20°C. Mice were immunized intraperitoneally with 2×10^8 CFU HKSP which elicits an optimal PC-specific antibody response 7 days post-vaccination [27, 30].

Propanil treatment

Propanil (99% pure, Chem Service, West Chester, PA) was diluted in peanut oil for administration to mice at a dose of 200 mg propanil/kg of body weight (mg/kg). Vehicle treated animals received peanut oil alone. Mice were treated intraperitoneally with propanil, 200 mg/kg, or vehicle on the day of vaccination with HKSP. The dose of propanil was based on previous studies that demonstrate an increase in the splenic antibody response to PC in vaccinated mice [26].

Neonatal androgen treatment

Eight week old C57BL/6Hla untimed mid-gestation pregnant mice were purchased from Hilltop Lab Animals (Scottsdale, PA). Pups were treated subcutaneously with 400 μg testosterone propionate (TP, Steraloids, Newport, RI) dissolved in 10 μl corn oil (CO) or CO alone within 24 hours of birth. Pups were returned to their mothers and weaned at 21 days of age. To confirm the efficacy of TP treatment the estrous cycle of adult female mice was determined by examination of vaginal smears taken daily for ten days at 8:00 a.m. Females were then sham operated or gonadectomized at 6 weeks of age (Figure 1). Males and females were immunized with HKSP and treated with propanil or the vehicle at 10 weeks, and the response to HKSP determined one week later. Uterine weights were determined at the time of sacrifice.

Serum and organs

Mice were euthanized with 100 μl Euthasol (50 mg/ml, TW Medical) 7 days following propanil exposure and vaccination. Serum was collected by cardiac puncture. Spleens were

dissociated through nylon mesh (Spectrum Labs, Rancho Dominguez, CA) in RPMI-1640 (BioWhitaker, Walkersville, MD), 10% heat inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc, Logan, UT), 10 mM HEPES (Sigma), 1 mM L- glutamine (Gibco, Rockville, MD), 5×10^{-5} M 2-mercaptoethanol (Sigma), 100 U/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco). Red blood cells were lysed with Tris-buffered ammonium chloride. Cell suspensions were washed and counted using a hemacytometer. Viability was determined using Trypan blue dye exclusion.

Measurement of antibody secreting B cells (ASC)

Acrowell™ 96 well filter plates (Pall Life Sciences, Ann Arbor, MI) were coated with 50 μl PC-BSA (Biosearch Technologies, Novato, CA) (10 μg/ml) overnight at 4°C. In subsequent steps, plates were washed with PBS + 0.01% Tween-20. Plates were blocked with 200 μl/well RPMI medium + 25% FBS for 2 hours at 37°C. Plates were washed and cells (100 μl/well) added at 5×10^6 cells/ml or 1×10^6 cells/ml. Samples were plated in triplicate. Plates were incubated for 4–6 hours at 37°C 5% CO_2. After washing, goat anti-mouse alkaline phosphatase (AP) conjugated IgM or IgG antibodies (Southern Biotechnology Associates, Birmingham, AL), diluted 1/2000 in PBS + 1%BSA + 0.05% Tween-20, were added to the appropriate wells (100 μl/well). Plates were incubated overnight at 4°C and washed. SIGMAFAST 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma-Aldrich) were dissolved in water and 100 μl added to each well. Color development was stopped by washing with water. The number of spots/well was counted using a dissection microscope (Olympus Optical Co., Melville, NY). The number of ASC was calculated using the mean number of spots from triplicate wells. The number of ASC was normalized to 1×10^6 splenic B cells which were determined by flow cytometric analysis. Comparable fold increases were noted when normalized to whole spleen [26].

Measurement of PC-specific titers

Immulon 2 plates (ThermoLabsystems, Franklin, MA) were coated overnight at 4°C with 5 μg/ml PC-BSA (50 μl/well). Plates were washed, blocked with 3% BSA + PBS at 37°C for 2 hours, washed, and 100μl/well of eight twofold dilutions of sera in PBS +1% BSA were added starting at 1/400 for the IgG and IgM ELISAs and 1/50 for the IgG subclasses. Plates were incubated for 1 hour at 37°C and washed. Goat anti-mouse AP conjugated antibodies (100μl/well) were added for 1 hour at 37°C. Plates were washed, phosphatase substrate tablets (Sigma-Aldrich) dissolved in p-Nitrophenyl Phosphate, Disodium Salt (PNPP) buffer and added to wells. Absorbance was read at 405nm on a μQuant spectrophotometer using KCJunior software (Bio-Tek Instruments, Winooski, VT).

Flow cytometry

Cells were stained with rat anti-mouse B220-APC (RA3-6B2), rat anti-mouse CD4-FITC (GK1.5), or rat anti-mouse CD8α-PE (53–6.7) (BD PharMingen, San Diego, CA). Staining was performed in PBS with 1% FBS and 0.04% sodium azide. 1×10^6 cells were stained with antibodies for 25 minutes on ice in the dark. After incubation, cells were washed and fixed in 0.04% paraformaldehyde (Fisher Scientific, Pittsburgh, PA). For each sample, 10,000 cells were collected for analysis (CellQuest software) on a FACSCalibur (Becton Dickinson Immunocytometry Systems, Mansfield, MA). Population percentages were used

to calculate the absolute cell number by multiplying the percentage of cells in a population by the total number of cells harvested per organ.

Serum testosterone

Serum testosterone levels were determined using the Testosterone Assay (R&D Systems, Inc., Minneapolis, MN), according to the instructions (level of detection is 0.041 ng/ml). There was no significant difference in serum testosterone levels between XYM and XXM mice (data not shown and [23]).

Statistics

For each of the dependent variables (ASC and antibody titers), data were initially analyzed by ANOVA specific to each experimental design as described below. Following significant main effects ($P<0.05$) or interactions, Tukey-Kramer least-square means - multiple comparison tests with adjusted p-value to protect the error rate were used to estimate specific individual group differences. The number of ASC were not normally distributed, therefore they were transformed before ANOVA using Log_{10} transformation. All analyses were performed using the Statistical Analysis System (vs. 9.3; SAS Institute, Inc., Cary, NC).

Split-split plot ANOVA with main effect in randomized blocks was used for the neonatal TP exposure experiments. This design was chosen due to the randomization restriction that all of the pups of a given litter were treated either with TP or CO and the treatment was not randomized within a litter. The TP or CO treatment which constitutes the main plot was assigned to litters. A pair of litters, one treated with TP, one with CO, constituted a ‘block’ in experimental design, and we used 8 such blocks of litters with a total of 77 female pups. Within litters, female pups were assigned to GDX or Sham treatment constituting the ‘subplot’, and further to ‘sub-sub plot’ which were propanil or vehicle treatment. Gonadectomy randomization was not required for males (N=47, only testes intact males were used) and data were analyzed using split-plot ANOVA with main ‘plot’ of TP or CO and ‘subplot’ of propanil or vehicle.

All data on four core mice (N=108) were analyzed by 4-way ANOVA in 2×2×2×2 factorial arrangement of treatments, using the following factors and their respective levels: Gonadal sex (Ovaries =F, Testes=M), Sex chromosome complement (XX, XY), Gonadectomy (GDX, Sham), and treatment (propanil, vehicle).

RESULTS

Neonatal testosterone propionate treatment does not alter the adult immune response

Earlier studies demonstrated a sexually-dimorphic effect of propanil on the immune response of gonadectomized adult male and female mice. This led to the hypothesis that the immune system undergoes sexual differentiation. To test this hypothesis, neonatal mice were treated with CO or TP within 24 hours of birth. This treatment has been used to induce sexual dimorphism in a number of systems [6, 7]. To confirm the efficacy of TP treatment the estrous cycle was determined by vaginal smears and the uterine weight measured at the time

of the assay. Constant estrus is one indicator of neonatal androgen treatment in female mice [31]. An enlarged uterus (hydrometrocolpos) after androgen- induced- masculinization has been reported in female offspring of prenatally TP-treated female rats [31, 32]. Androgen has proliferative [33] and anti-apoptotic [34] effects on the mouse uterine epithelium. In addition, androgen aromatization increases estrogen levels which has a proliferative effects on the uterus. Vaginal smears confirmed that TP treatment abrogated the estrous cycle (data not shown) and TP-treated females had a significant increase in uterine weight (CO 0.056 g + 0.012/20g body weight vs. TP 0.176 + 0.014 g/20g body weight).

Propanil exposure significantly enhanced the PC-specific antibody response in TP and CO treated male mice compared to the vehicle controls (Figure 2A, * P <0.02, Tukey-Kramer). There was no statistically significant main effect of TP treatment as neonatal treatment of male mice with TP did not alter the PC-specific IgM or IgG antibody response in the spleen after vaccination with HKSP compared to the vehicle controls (Figure 2A). As previously demonstrated (25), males have a lower HKSP-specific ASC response than females (Figure 2A versus 2B and 2C; note difference in y-axes).

In female mice, propanil exposure significantly enhanced the IgM and IgG antibody response to HKSP in the CO and TP treated mice compared to the vehicle controls (Figure 2B and 2C, * P <0.02, Tukey-Kramer). Interestingly, there was a significant main effect of TP treatment demonstrated by the increased antibody response in TP Sham and GDX vehicle treated mice compared to the CO Sham and GDX vehicle controls (Table 1, Figure 2B and 2C, # P <0.01, ANOVA). There were also significant main effects of GDX and propanil exposure (Table 1). The most important test for our hypothesis was the three-way interaction to determine if the interaction between GDX and propanil was abrogated by TP treatment. Specifically, did neonatal TP treatment masculinize the response in females such that the ovaries were no longer necessary for the enhanced antibody response? There was a statistically significant two-way interaction of GDX and propanil as demonstrated by the significantly decreased antibody response after GDX in CO and TP treated mice confirming that the response to propanil was largely dependent on the ovaries (Figure 2B and 2C, ** P <0.01, ANOVA), however, the three-way interaction was not statistically significant (Table 1). These initial studies did not support a role for sexual differentiation of the immune system when TP was given during the neonatal period on the adult response to HKSP vaccination and propanil exposure.

Sex chromosome complement significantly influences the immune response to HKSP after exposure to propanil

To determine if sex chromosome complement contributes to the sexually-dimorphic immune response after propanil exposure, the FCG model of gonadal females (XXF and XYF) and gonadal males (XYM and XXM) was utilized. To examine the role of both gonadal hormones and the role of sex chromosome complement independent of hormones, the immune response to HKSP after exposure to propanil was determined in female and male Sham and GDX mice.

XXF Sham females exposed to propanil had significantly higher PC-specific IgM and IgG ASC compared to the XXF Sham vehicle group (Figure 3A, IgM, and 3B, IgG, * P <0.01,

Tukey-Kramer). The ovaries were not required for propanil exposure to enhance the antibody response as XXF GDX mice had a significant increase in IgM and IgG ASC after propanil exposure compared to the XXF GDX vehicle group (Figure 3A and 3B, * P <0.01, Tukey-Kramer). Although a slightly lower response in the XXF GDX mice exposed to propanil was noted, it was not significantly different from the XXF Sham propanil-exposed group. Surprisingly, propanil exposure did not cause an increase in PC-specific IgM or IgG ASC in XYF female mice. Neither XYF Sham nor XYF GDX mice exposed to propanil had a significant increase in ASC compared to the vehicle controls (Figure 3A and 3B).

Previous studies demonstrated that wild type C57BL/6 males had an enhanced response to HKSP vaccination after exposure to propanil that did not require the testes, suggesting that testosterone was not integral to the response to propanil. To determine the role of the XY sex chromosome complement in gonadal males, the PC-specific IgM and IgG responses in the spleen were determined in Sham and GDX XYM and XXM males. XYM Sham males had a slightly increased response after exposure to propanil compared to XYM Sham vehicle controls (Figure 3A, IgM, and 3B, IgG). The XYM GDX males did not have an increased response after propanil exposure compared to the XYM GDX vehicle controls (Figure 3A and 3B). In contrast, both XXM Sham and XXM GDX males had a significantly increased response to vaccination after exposure to propanil compared to the XXM Sham and GDX vehicle controls (Figure 3A and 3B, * P <0.01, Tukey-Kramer). These data demonstrate a significant role for the XX sex chromosome complement in increased immune responses after exposure to propanil that is independent of ovarian or testicular hormones.

The statistically significant Main Effects and two-way interactions from the 2×2×2×2 factorial design ANOVA are summarized in Figure 4 and Table 2. The results demonstrated that all mice with an XX sex chromosome complement (XXF and XXM) have a higher response to HKSP vaccination than mice with XY sex chromosome complement (XYF and XYM, P <0.001, Figure 4A and Table 2). There was also a Main Effect of gonadal sex demonstrating that gonadal female mice (ovary) have a higher response to HKSP vaccination than gonadal male mice (testes) (P <0.001, Figure 4B and Table 2). These results support a critical role for the XX sex chromosome complement in the antibody response in males and females and potential organizational effects of gonadal hormones. There was not a Main Effect of gonads (Sham vs GDX) (Table 2) suggesting there is not an activational effect of gonadal hormones in the immune response.

There was a statistically significant two-way interaction between sex chromosome complement and propanil exposure. There was a significant increase in IgM and IgG ASC in FCG XX mice (XXF and XXM) after exposure to propanil compared to FCG XY mice (XYF and XYM, P <0.05, Figure 4C and Table 2). These data further demonstrate a significant role for the XX sex chromosome complement in the immune response after propanil exposure.

Serum antibody titers after HKSP vaccination are not affected by exposure to propanil

PC-specific IgM, IgG3, and IgG2b serum antibody titers were determined for the FCG mice. IgM titers for XXF, XYF, XYM, and XXM mice, vaccinated with HKSP and exposed to propanil or the vehicle, were comparable among all groups (data not shown). Similar results

were determined for IgG3 and IgG2b (data not shown). Several earlier reports have established the bone marrow as the primary source of serum antibodies [35]. These results suggest that propanil exposure does not alter the production of antibody in the bone marrow in FCG mice as had been previously demonstrated for wild type C57BL/6 mice [26].

DISCUSSION

Previous studies demonstrated that propanil acts as a novel endocrine disrupter to enhance the immune response to HKSP vaccination in C57BL/6 female and male mice and that the ovaries were required for the enhanced response in females, but the testes were not required in males [28]. Although gonadal hormones are known to influence immune responses, the effect of propanil was not influenced by treatment of females with estrogen or progesterone agonists, or by manipulations of levels of androgens by castration of males [28]. The sexually dimorphic immune response to HKSP vaccination after exposure to propanil provided a model system to study the potential for sexual differentiation of the immune system utilizing neonatal testosterone exposure and to investigate a role for sex chromosomes in immune responses.

TP administration during the prenatal or neonatal period has been used to examine the organizational effect of hormones on the development of the brain, the reproductive system, and aggressive behavior in adults [6, 7, 9]. Manipulation of the neonatal hormone environment alters the immune response in the development of Type I autoimmune diabetes in female NOD mice[10]. The current studies used neonatal exposure to TP to determine if the female immune response was masculinized by early exposure to TP. The response after treatment with propanil and vaccination with HKSP in females was not altered by exposure to TP and did not support a role for permanent masculinization by testosterone of the immune system. There are limitations to the model of neonatal exposure to TP that may influence the outcome. Neonatal administration of TP was chosen instead of prenatal treatment since exposure of females to TP during pregnancy can result in fetal death [7]. The timing of exposure to TP during development is a significant factor and neonatal exposure to TP may have been after a critical period of development of the immune system during gestation. During the prenatal period migration of stem cells and expansion of progenitor cells followed by colonization of the bone marrow and thymus occur but the murine immune system is still relatively immature at birth and continues to develop during the neonatal period [36]. Therefore, although the results do not definitively rule out a role for testosterone, due to temporal considerations, data from the FCG model argues strongly against it.

It is also important to determine the role of X and Y chromosomes, the complement of sex chromosomes, XX or XY, and the role of the genes encoded on X and Y chromosomes. Using the FCG model we demonstrated a significant role for the XX sex chromosome complement in the enhanced immune response to HKSP vaccination after propanil exposure (Figure 3). XXF and XXM mice exposed to propanil had a significant increase in the immune response in the spleen compared to XXF and XXM controls. Propanil exposure in XYF and XYM mice, in contrast, did not enhance the response to HKSP. The HKSP-specific response in the XYF and XYM mice was significantly lower than the response of XXF and XXM mice. This demonstrates a phenotypic effect mediated by sex chromosomes

in mice that are the same gonadal sex. Smith-Bouvier et al. [25] examined the role of sex chromosome complement in two autoimmune models in GDX SJL mice, experimental autoimmune encephalomyelitis (EAE) and lupus. Similar to our results, they demonstrated that mice with the XX sex chromosome complement, XXF and XXM, had increased autoimmune responses and increased pathology compared to those with an XY sex chromosome complement, XYF and XYM. This indicates that regardless of gonadal structures and hormones, the XX sex chromosome complement enhanced responses in both EAE and lupus, and after exposure to propanil.

GDX of XXF and XXM mice did not alter the response to HKSP vaccination suggesting that there was not an activational effect of gonadal hormones as is usually seen in female C57BL/6 mice. Comparison of XYF gonadal females (ovary) and XYM gonadal males (testes), which have the same chromosomal sex but different gonadal sex, suggest that organizational effects of gonadal hormones are nevertheless important in immunity. XYF GDX mice had a significantly higher response to HKSP vaccination compared to XYM GDX mice (Figure 3). It is also possible that in the FCG model there may be direct effects of the *Sry* gene outside of the gonad on the immune system that could account for differences between males and females.

There were two unexpected outcomes from the experiments in the FCG model. The first was that XXF females, in contrast to previous studies using wild type C57BL/6 XX females [28], did not require the ovaries for the enhanced response induced by exposure to propanil. The second was that exposure to propanil did not enhance the immune response in either the XYF or XYM mice, in contrast to wild type C57BL/6 XY males ([28] and Figure 2). Interestingly, a study on juvenile social behavior also demonstrated that the differences in behavior were different in FCG mice compared to C57BL/6 mice [37]. The primary difference between C57BL/6 mice and the FCG mice is the deletion of the *Sry* gene from the Y chromosome of the XY^{-}*Sry* father and the insertion of the *Sry* transgene on an autosome. One potential explanation is that there are transgenerational genetic effects that result in phenotypic differences in offspring whose fathers have a different Y chromosome. Nelson et al. [38] compared forty-one physiological and behavioral multigenic traits in genetically identical female mice from fathers' who only differed in their Y chromosome. There were significant differences between the two daughter populations demonstrating a role for transgenerational genetic effects from the paternal Y chromosome [38]. Transgenerational genetic effects have been described in a number of systems and the mechanisms may include epigenetic changes in DNA methylation, histone modifications, RNA functions, or regulation by microRNAs (miRNAs) [39]. It was recently demonstrated using chromosome Y consomic mouse strains that the Y chromosome contains regulatory elements that altered gene expression in $CD4^{+}$ T cells and macrophages and susceptibility to autoimmune disease [40]. Together these studies suggest an important role for genes encoded on the Y chromosome in addition to its function in sexual differentiation and development. Another possible contributing factor to the results obtained in earlier studies [28] and the current results is the substrain of C57BL/6 mice. Previous studies were performed using C57BL/6Hla from Hilltop Labs which has maintained a closed colony since 1991. The FCG are on the C57BL/6J background from The Jackson Laboratories which has had an established colony

since 1947. A preliminary experiment demonstrated that propanil exposure in XY male C57BL/6J mice did not enhance the immune response to HKSP, similar to the results with the XYM FCG mice. XX female C57BL/6J mice had an enhanced immune response after exposure to propanil that was comparable to previous results in Hilltop female mice that was dependent on the ovaries (data not shown and reference 28). Additional studies are necessary to determine if C57BL/6 substrains from different colonies have developed subtle differences in the Y chromosome that affect their responses.

A number of factors may contribute to the differential immune response of animals with an XX sex chromosome complement versus an XY sex chromosome complement. Among these are Y chromosome encoded genes that are not expressed in XX mice including Y genes that have male-specific effects and the gene regulatory elements described above [40]. Potential factors associated with the X chromosome include X gene dosage effects in XX versus XY mice, the X gene parental imprint, and X-chromosome inactivation [12–14, 41]. It has been reported that approximately 3% of X-linked genes escape inactivation in mice [42]. Interestingly, the X chromosome contains the highest number of immune-related genes in the human genome that influence the immune response to infections, X-linked primary immunodeficiencies, and autoimmune disorders [12, 14]. Recently, it was reported that the X chromosome is enriched for miRNAs that act as key regulators of gene expression [43, 44]. In contrast, there are few miRNAs on the Y chromosome. Several of the miRNAs on the X chromosome are involved in immunity and could contribute to sexually dimorphic immune responses [12, 44, 45].

These results demonstrate that exposure to propanil enhances the immune response to HKSP in a XX sex chromosome complement dependent manner in the FCG model. Moreover, moving Sry to an autosomal chromosome abolished the ability of the immune system of XYM and XYF mice to respond to propanil. Future studies will investigate the mechanism by which exposure to propanil results in a sexually dimorphic immune response and examine the role of X chromosome encoded genes and miRNAs, and the regulation of their expression.

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References

1. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. Nat Rev Immunol. 2008; 8:737–744. [PubMed: 18728636]
2. Pennell LM, Galligan CL, Fish EN. Sex affects immunity. J Autoimmun. 2012; 38:J282–J291. [PubMed: 22225601]
3. Quintero OL, Amador-Patarroyo MJ, Montoya-Ortiz G, Rojas-Villarraga A, Anaya JM. Autoimmune disease and gender: plausible mechanisms for the female predominance of autoimmunity. J Autoimmun. 2012; 38:J109–J119. [PubMed: 22079680]

4. Klein SL. The effects of hormones on sex differences in infection: from genes to behavior. Neurosci Biobehav Rev. 2000; 24:627–638. [PubMed: 10940438]
5. McClelland EE, Smith JM. Gender specific differences in the immune response to infection. Arch Immunol Ther Exp (Warsz). 2011; 59:203–213. [PubMed: 21442309]
6. Edwards DA. Mice: fighting by neonatally androgenized females. Science. 1968; 161:1027–1028. [PubMed: 5691460]
7. vom Saal FS. Prenatal exposure to androgen influences morphology and aggressive behavior of male and female mice. Horm Behav. 1979; 12:1–11. [PubMed: 573234]
8. Roland AV, Nunemaker CS, Keller SR, Moenter SM. Prenatal androgen exposure programs metabolic dysfunction in female mice. Journal of Endocrinology. 2010; 207:213–223. [PubMed: 20713501]
9. Sullivan SD, Moenter SM. Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: implications for a common fertility disorder. Proc Natl Acad Sci USA. 2004; 101:7129–7134. [PubMed: 15096602]
10. Hawkins T, Gala RR, Dunbar JC. The effect of neonatal sex hormone manipulation on the incidence of diabetes in nonobese diabetic mice. Proc Soc Exp Biol Med. 1993; 202:201–205. [PubMed: 8424110]
11. Arnold AP. Sex chromosomes and brain gender. Nat Rev Neurosci. 2004; 5:701–708. [PubMed: 15322528]
12. Bianchi I, Lleo A, Gershwin ME, Invernizzi P. The X chromosome and immune associated genes. J Autoimmun. 2012; 38:J187–J192. [PubMed: 22178198]
13. Brown CJ, Greally JM. A stain upon the silence: genes escaping X inactivation. Trends Genet. 2003; 19:432–438. [PubMed: 12902161]
14. Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: when a chromosome makes the difference. Nat Rev Immunol. 2010; 10:594–604. [PubMed: 20651746]
15. Arnold AP, Chen X. What does the “four core genotypes” mouse model tell us about sex differences in the brain and other tissues? Front Neuroendocrinol. 2009; 30:1–9. [PubMed: 19028515]
16. Cox KH, Bonthuis PJ, Rissman EF. Mouse model systems to study sex chromosome genes and behavior: Relevance to humans. Frontiers in neuroendocrinology. 2014
17. Chen X, McClusky R, Chen J, Beaven SW, Tontonoz P, Arnold AP, Reue K. The number of x chromosomes causes sex differences in adiposity in mice. PLoS Genet. 2012; 8:e1002709. [PubMed: 22589744]
18. Chen X, McClusky R, Itoh Y, Reue K, Arnold AP. X and Y chromosome complement influence adiposity and metabolism in mice. Endocrinology. 2013; 154:1092–1104. [PubMed: 23397033]
19. De Vries GJ, Rissman EF, Simerly RB, Yang LY, Scordalakes EM, Auger CJ, Swain A, Lovell-Badge R, Burgoyne PS, Arnold AP. A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits. J Neurosci. 2002; 22:9005–9014. [PubMed: 12388607]
20. Barker JM, Torregrossa MM, Arnold AP, Taylor JR. Dissociation of genetic and hormonal influences on sex differences in alcoholism-related behaviors. J Neurosci. 2010; 30:9140–9144. [PubMed: 20610747]
21. Bonthuis PJ, Cox KH, Rissman EF. X-chromosome dosage affects male sexual behavior. Hormones and behavior. 2012; 61:565–572. [PubMed: 22349083]
22. Gatewood JD, Wills A, Shetty S, Xu J, Arnold AP, Burgoyne PS, Rissman EF. Sex chromosome complement and gonadal sex influence aggressive and parental behaviors in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006; 26:2335–2342. [PubMed: 16495461]
23. Palaszynski KM, Smith DL, Kamrava S, Burgoyne PS, Arnold AP, Voskuhl RR. A yin-yang effect between sex chromosome complement and sex hormones on the immune response. Endocrinology. 2005; 146:3280–3285. [PubMed: 15905317]
24. Robinson DP, Huber SA, Moussawi M, Roberts B, Teuscher C, Watkins R, Arnold AP, Klein SL. Sex chromosome complement contributes to sex differences in coxsackievirus B3 but not influenza A virus pathogenesis. Biol Sex Differ. 2011; 2:8. [PubMed: 21806829]

25. Smith-Bouvier DL, Divekar AA, Sasidhar M, Du S, Tiwari-Woodruff SK, King JK, Arnold AP, Singh RR, Voskuhl RR. A role for sex chromosome complement in the female bias in autoimmune disease. J Exp Med. 2008; 205:1099–1108. [PubMed: 18443225]
26. Salazar KD, de la RP, Barnett JB, Schafer R. The polysaccharide antibody response after Streptococcus pneumoniae vaccination is differentially enhanced or suppressed by 3,4-dichloropropionanilide and 2,4-dichlorophenoxyacetic acid. Toxicol Sci. 2005; 87:123–133. [PubMed: 15976183]
27. Wu ZQ, Vos Q, Shen Y, Lees A, Wilson SR, Briles DE, Gause WC, Mond JJ, Snapper CM. In vivo polysaccharide-specific IgG isotype responses to intact Streptococcus pneumoniae are T cell dependent and require CD40- and B7-ligand interactions. J Immunol. 1999; 163:659–667. [PubMed: 10395655]
28. Salazar KD, Miller MR, Barnett JB, Schafer R. Evidence for a novel endocrine disruptor: the pesticide propanil requires the ovaries and steroid synthesis to enhance humoral immunity. Toxicol Sci. 2006; 93:62–74. [PubMed: 16788000]
29. Becker JB, Arnold AP, Berkley KJ, Blaustein JD, Eckel LA, Hampson E, Herman JP, Marts S, Sadee W, Steiner M, Taylor J, Young E. Strategies and methods for research on sex differences in brain and behavior. Endocrinology. 2005; 146:1650–1673. [PubMed: 15618360]
30. Wu ZQ, Khan AQ, Shen Y, Schartman J, Peach R, Lees A, Mond JJ, Gause WC, Snapper CM. B7 requirements for primary and secondary protein- and polysaccharide-specific Ig isotype responses to Streptococcus pneumoniae. Journal Immunology. 2000; 165:6840–6848.
31. Mori T, Mills KT, Bern HA. Sensitivity of the vagina and uterus of mice neonatally exposed to estrogen or androgen to postnatal treatment with estrogen or androgen. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine. 1992; 199:466–469.
32. Wolf CJ, Hotchkiss A, Ostby JS, LeBlanc GA, Gray LE Jr. Effects of prenatal testosterone propionate on the sexual development of male and female rats: a dose-response study. Toxicol Sci. 2002; 65:71–86. [PubMed: 11752687]
33. Zhang J, Sun Y, Liu Y, Sun Y, Liao DJ. Synergistic effects of androgen and estrogen on the mouse uterus and mammary gland. Oncology reports. 2004; 12:709–716. [PubMed: 15375489]
34. Terada N, Yamamoto R, Takada T, Taniguchi H, Terakawa N, Li W, Kitamura Y, Matsumoto K. Inhibitory effect of androgen on cell death of mouse uterine epithelium. Journal of steroid biochemistry. 1990; 36:305–310. [PubMed: 2391960]
35. Benner R, Hijmans W, Haaijman JJ. The bone marrow: the major source of serum immunoglobulins, but still a neglected site of antibody formation. Clin Exp Immunol. 1981; 46:1–8. [PubMed: 7039877]
36. Landreth KS. Critical windows in development of the rodent immune system. Hum Exp Toxicol. 2002; 21:493–498. [PubMed: 12458906]
37. Cox KH, Rissman EF. Sex differences in juvenile mouse social behavior are influenced by sex chromosomes and social context. Genes, brain, and behavior. 2011; 10:465–472.
38. Nelson VR, Spiezio SH, Nadeau JH. Transgenerational genetic effects of the paternal Y chromosome on daughters' phenotypes. Epigenomics. 2010; 2:513–521. [PubMed: 22121971]
39. Nadeau JH. Transgenerational genetic effects on phenotypic variation and disease risk. Hum Mol Genet. 2009; 18:R202–R210. [PubMed: 19808797]
40. Case LK, Wall EH, Dragon JA, Saligrama N, Krementsov DN, Moussawi M, Zachary JF, Huber SA, Blankenhorn EP, Teuscher C. The Y chromosome as a regulatory element shaping immune cell transcriptomes and susceptibility to autoimmune disease. Genome Research. 2013; 23:1474–1485. [PubMed: 23800453]
41. Arnold AP. The end of gonad-centric sex determination in mammals. Trends in Genetics. 2012; 28:55–61. [PubMed: 22078126]
42. Yang F, Babak T, Shendure J, Disteche CM. Global survey of escape from X inactivation by RNA-sequencing in mouse. Genome Research. 2010; 20:614–622. [PubMed: 20363980]
43. Guo X, Su B, Zhou Z, Sha J. Rapid evolution of mammalian X-linked testis microRNAs. BMC Genomics. 2009; 10:97. [PubMed: 19257908]

44. Pinheiro I, Dejager L, Libert C. X-chromosome-located microRNAs in immunity: might they explain male/female differences? The X chromosome-genomic context may affect X-located miRNAs and downstream signaling, thereby contributing to the enhanced immune response of females. Bioessays. 2011; 33:791–802. [PubMed: 21953569]

45. Dai R, Ahmed SA. MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. Transl Res. 2011; 157:163–179. [PubMed: 21420027]

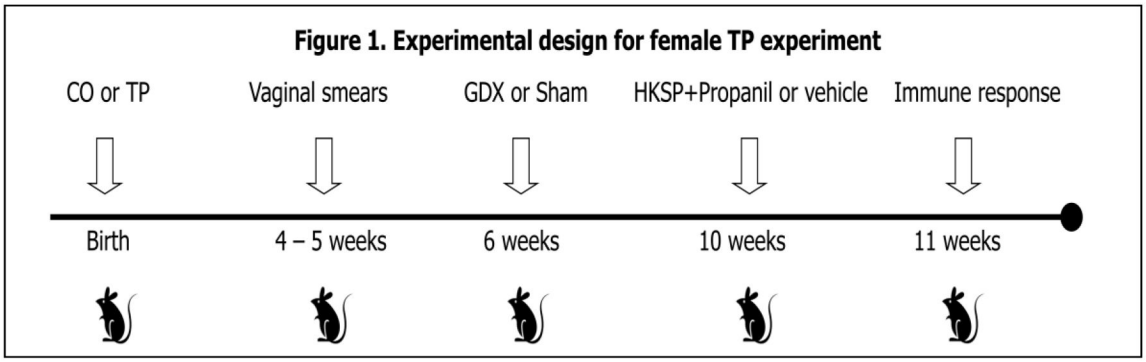

Figure 1.

Neonatal TP treatment and immune response in female mice. All mice (males and females) were treated within 24 hours of birth with TP or the vehicle corn oil (CO). Vaginal smears were performed on the female offspring at 4 – 5 weeks of age and subsequently either sham-ovariectomized (Sham) or ovariectomized (GDX) at 6 weeks of age. At 10 weeks of age mice were either exposed i.p. to propanil (200 mg/kg) or the vehicle peanut oil and all mice vaccinated i.p. with 2×10^8 CFU HKSP. One week later the splenic PC-specific IgM and IgG antibody responses were determined.

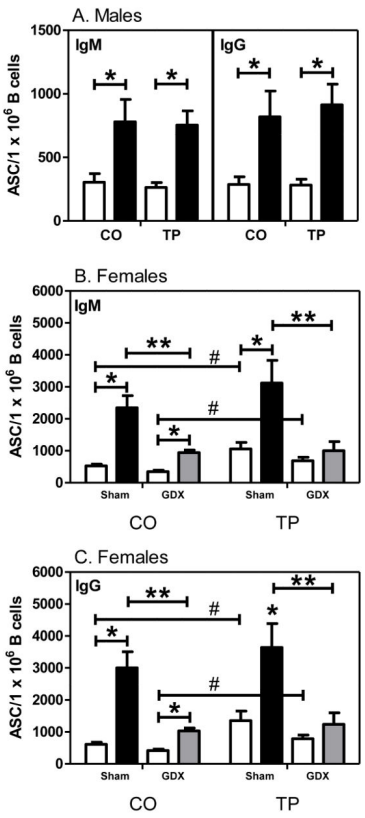

Figure 2.

Neonatal TP treatment does not alter the response to HKSP after propanil exposure. (A) Males. Vehicle (open bars), propanil (black bars) * P <0.02 (Tukey-Kramer); CO vs.CO propanil, TP vs. TP propanil. (B and C) Females. Vehicle (open bars), sham + propanil (black bars), GDX + propanil (gray bars). * P <0.05 (Tukey-Kramer); CO Sham vs CO Sham propanil, CO GDX vs. CO GDX propanil, TP Sham vs. TP Sham propanil. ** P <0.05 (ANOVA) CO Sham propanil vs. CO GDX propanil, TP Sham propanil vs. TP GDX propanil. # P < 0.05 (ANOVA) CO Sham vs. TP Sham, CO GDX vs. TP GDX.

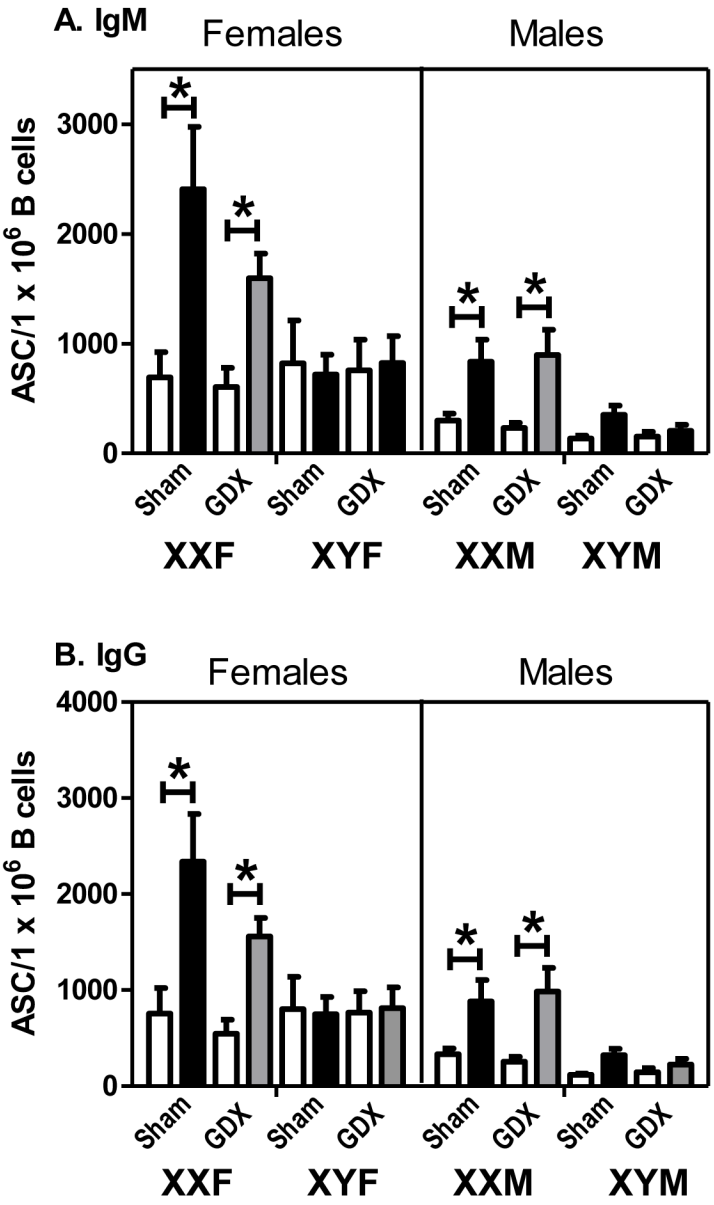

Figure 3.

XX sex chromosome complement is required for enhancement of the immune response to HKSP vaccination after exposure to propanil in females and males. XXF and XYF sham operated (Sham) or GDX (GDX) female mice and XYM and XXM sham operated (Sham) or GDX (GDX) male mice were treated as described in Figure 1. Values represent the mean ± SEM of ASC/1×10^6 B cells. Treatments: vehicle (open bars), sham + propanil (black bars), GDX + propanil (gray bars). (A) IgM and (B) IgG. * $P < 0.05$ (Tukey-Kramer) propanil exposed groups significantly different from matched vehicle treated group.

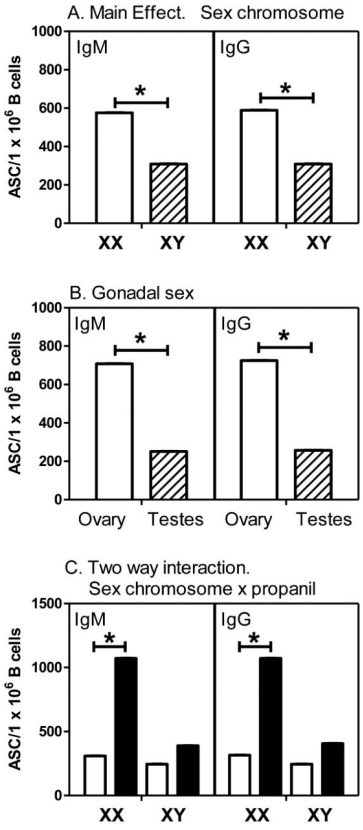

Figure 4.

Statistically significant Main Effects (A) sex chromosomes (B) gonadal sex. (A) XX mice (open bars) significantly higher response than XY mice (hatched bars). *P <0.05. (B) XXF and XYF mice (open bars) significantly higher response than XXM and XYM males (hatched bars). (C) Significant two-way interaction of sex chromosomes and propanil in FCG mice. XX mice significantly higher response after exposure to propanil (black bars) compared to vehicle control (open bars), * P <0.05. (A, B, C) All values represent the least square means SEM of ASC/1×10^6 B cells. The SEM was < 5 for all groups.

Table 1

Summary of the split-plot ANOVA results of the main effects of testosterone, ovariectomy (gonads) and propanil treatment and their interactions on the PC-specific ASC response in female mice.

	IgM		IgG	
Effect	**F-value**	**Probability >F**	**F-value**	**Probability >F**
Main Effects (levels)				
Testosterone [corn oil, testosterone]	**6.2**	**0.04**	**6.46**	**0.039**
Gonads [sham, gonadectomized (GDX)]	**33.96**	**<0.0001**	**35.48**	**<0.0001**
Treatment [vehicle, propanil]	**58.29**	**<0.0001**	**51.99**	**<0.0001**
Two-way interactions:				
Gonads*Treatment	**7.11**	**0.0109**	**7.69**	**0.0083**
Three-way interaction:				
Testosterone*Gonads*Treatment	1.17	0.2865	0.31	0.58

Table 2

Summary of the 2×2×2×2 factorial design ANOVA results of main effects of gonadal sex, sex chromosome complement, gonads, propanil treatment and their interactions on the PC-specific antibody response shown in Figure 4.

	IgM		IgG	
Effect	**F-value**	**Probability >F**	**F-value**	**Probability >F**
Main Effects (levels)				
Gonadal Sex (Ovary, Testes)	**38.74**	**<.0001**	**38.12**	**<.0001**
Sex Chromosome (XX, XY)	**14.15**	**0.0003**	**14.04**	**0.0003**
Gonads (GDX, Sham)	1.20	0.2766	1.05	0.3089
Treatment (Propanil, Vehicle)	**26.55**	**<.0001**	**27.16**	**<.0001**
Two-way interactions:				
Gonadal Sex*Sex Chromosome	1.23	0.2712	2.33	0.1302
Gonadal Sex*Gonads	0.02	0.8976	0.00^	0.9486
Sex Chromosome*Gonads	0.01	0.9137	0.09	0.7706
Gonadal Sex *Treatment	0.00	0.9772	0.08	0.7774
Sex Chromosome*Treatment	**5.44**	**0.0219**	**4.73**	**0.0322**
Gonads*Treatment	0.58	0.4470	0.13	0.7185

^ approaching 0