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Original Article

Androgen receptor signaling protects male mice from the development of immune response to peanut

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Abstract: Objectives: Peanut (PN) allergy is a major public health concern. Recent research has brought clarity about how individuals become sensitized to PN allergen with routes known through the skin, as well as the airway. Still unclear, however, is the role of sex hormones on the development of allergic immune responses to PN. This study examines the role of androgen receptor (AR) signaling in regulating PN-specific immune responses. Methods: We utilized a 4-week inhalation mouse model of PN allergy that is known to drive the production of PN-specific antibodies and elicit systemic anaphylaxis following PN challenge. Wildtype (WT) male, female, and androgen receptor-deficient testicular feminization mutant (AR^{Tfm}) male mice were examined using this model to document sex differences in PN allergy. To determine if sex differences also existed in the cellular immune response, this study utilized a 3-day inhalation mouse model of PN to examine the response of group 2 innate lymphoid cells (ILC2s). WT male and female mice were examined using this model to document sex differences in ILC2 response within the lungs. Results: AR use is critical in regulating PN-specific antibody levels. We found that AR^{Tfm} males have a higher antibody response and significantly worse anaphylactic response following PN challenge relative to WT males. WT males also exhibit a less severe anaphylactic response compared to AR^{Tfm} male and female mice. Lastly, we discovered that lung ILC2s from female mice respond more robustly to PN compared to ILC2s within WT male mice. Conclusions: Taken together, this study suggests that male sex hormones, namely androgens, negatively regulate allergic immune responses to PN.

Keywords: Peanut (PN) allergy, androgen receptor, AR^{Tfm} (androgen receptor-deficient) male, PN-specific antibodies, systemic anaphylaxis, group 2 innate lymphoid cells (ILC2s)

Introduction

Peanut (PN) allergy is a major medical problem as it remains one of the most common, severe, and persistent food allergies, and its prevalence is increasing rapidly [1-6]. Although progress has been made in the last decade to better understand PN allergy, our knowledge of the immunological mechanisms involved in the initial development of the disease remains incomplete. Specifically, how sex hormones regulate the immune pathways associated with the development of PN allergy is unknown. An examination of food allergies revealed that under the age of 18, males are almost twice as likely to have a food allergy compared to females [7]. In adulthood, however, the ratio dramatically shifts as females are more likely to have a food allergy (female to male ratio of 1:0.53) [8]. Post-menopause, males and females appear evenly impacted [7]. The sex reversals during puberty and following menopause suggest that sex hormones impact the development of food allergy [7, 8]. PN allergy also displays a clinical sex bias. An analysis of US adults allergic to PN showed that female adults were twice as likely to develop PN allergy during their childhoods than males [9]. The twofold difference favoring females with PN allergy was maintained into adulthood in the US and Mexico [9, 10]. Collectively, these data strongly suggest that allergic reactions to foods like PN are sensitive to sex hormones and that studying the immune mechanisms impacted by sex hormones are clinically relevant.

Recent clinical trials have provided strong evidence that eating PN early in life allows the development of an oral tolerance that protects children from developing allergic responses to

PN [11, 12]. Studies using mouse models and human cell-based systems have shown that sensitization to PN via the airways is a likely route of sensitization [13]. PN is commonly found in household dust and is biologically active [14-16]. We were the first to show that PN exposure through the airways elicited PN sensitization in mice, and upon PN challenge, anaphylaxis occurs [17]. While we discuss in greater detail the known mechanisms of peanut allergy in a recent review [18], we and others showed that following inhalation of PN, IL-1α stimulates type 2 innate lymphoid cells (ILC2s) to secrete IL-13 to activate dendritic cells (DCs) that work to trigger a T follicular helper (Tfh) cell-mediated, PN-specific IgE antibody response that sensitizes the mice [17, 19]. Of note, a recent study showed that sensitization to PN via the airways can be inhibited by oral exposure to PN prior to inhalation [20]. The significance of these discoveries is that for the first time in an animal model, sensitization via inhalation of PN was reported and this sensitization can be blocked by oral tolerance, mirroring what was observed in the Learning Early About Peanut Allergy study [11, 12].

Sex hormones have been shown to modulate airway inflammation. Estrogen has been shown in multiple studies to drive airway inflammation (reviewed in 22), while testosterone was shown to reduce airway inflammation in murine models of airway inflammation. Interestingly, this decrease was linked to the ability of testosterone to negatively regulate group 2 innate lymphoid cells (ILC2s), as well as stabilizing the suppressive function of T regulatory cells [21-25]. Knowledge about how sex hormones influence the development of allergic immune responses to PN initiated within the airways remains unclear. The goal of this study was twofold. First, we wanted to document whether androgens impact the development of PN allergy. Second, we examined how androgens impact allergic immune responses to PN.

To accomplish this, we exposed WT male, WT female, and androgen receptor-deficient testicular feminization mutant (AR^{Tfm}) male mice, to PN using established inhalation models [17, 19]. AR^{Tfm} male mice lack both functional ARs and the ability to produce endogenous testosterone [26-28]. To assess whether differences existed in the ability of the mice to become sensitized to PN, plasma was examined for the presence of PN-specific antibodies and mice were challenged with PN to induce systemic anaphylaxis. Using these methods, we identified that WT female and AR^{Tfm} male mice developed worse PN-induced anaphylactic reactions and that AR^{Tfm} males developed higher PN-specific antibody responses than WT mice. We also investigated the response of ILC2s in the lungs of mice exposed to PN. We found that the ILC2 response in female mice is more robust than their male counterparts. These data suggest that androgens play a role in negatively regulating allergic responses to PN, likely through an ILC2-mediated mechanism.

Material and methods

Mice

Male and female BALB/c or C57BL/6 mice were obtained from Taconic Farms (Germantown, NY). Cynthia Jordan, Adam Moeser, and S. Marc Breedlove at Michigan State University generously donated breeder female mice that were used to start an AR^{Tfm} mouse colony at the University of Nebraska at Kearney (Kearney, NE). Briefly, the breeder females were generated using the cre-lox system using mice supplied by The Jackson Laboratory (stock 003724) and mice containing a “floxed” AR gene from De Gendt and colleagues at K.U. Leuven in Belgium [29]. Mice were bred at the University of Nebraska at Kearney under pathogen-free conditions. All protocols and procedures for handing the mice were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Nebraska at Kearney (IACUC protocol #210823). Mice used in the study ranged from 6-20 weeks of age. Mice used in each experiment were aged-matched and placed in one of the following respective groups: PBS WT male, PBS WT female, PBS AR^{Tfm} male, Peanut (PN) WT male, PN WT female, and PN AR^{Tfm} male. Upon completion of the experiment, the mice were euthanized via inhalation of carbon dioxide (flow rate: 3 L/min).

Allergens

Peanut flour (14.4% protein) was donated from the Golden Peanut Company (Alpharetta, GA) as a bulk raw material; endotoxin was undetectable (<0.5 EU/mg flour) in the product by

Limulus Amebocyte Lysate assay (Lonza, Walkersville, MD) [17]. Crude peanut extract (CPE) (20.0% protein) was purchased from Stallergenes Greer (Lenoir, NC).

Sensitization PN allergy models

Using established inhalation models [17, 19], groups of WT male, WT female, and AR^{Tfm} mice were exposed to the solution of their respective group, consisting of either 100 µg peanut flour suspended in 50 µl of 1X PBS or 50 µl 1X PBS alone. Isoflurane, delivered via a vaporizer, was used to anesthetize the mice prior to delivery of either PN or PBS solution. For exposure, mice were held upright and 50 µl of solution was placed on the tip of nose. Mice were held upright for 30 seconds to facilitate the airway aspiration. After administration, mice were placed into their respective cages until they recovered from anesthesia. Once recovered, they were transferred back into the housing room until the next date of exposure. Mice are deemed to successfully undergo sensitization to PN if they displayed PN-specific antibodies. Mice that undergo anaphylaxis upon challenge with PN at day 28 were marked as having successfully developed PN allergy. Additional details about how this study measured levels of PN-specific antibodies and monitored the development of anaphylaxis are described below. The inhalation models are as follows:

3-consecutive day inhalation model: For experiments examining lung ILC2s, mice were exposed to either PN or PBS solution on days 0, 1, and 2. Twenty-four hours after last exposure (on day 3), tissue was harvested for analysis (please see tissue harvest section for more information).

28-day inhalation model: Mice were exposed to either PN or PBS solution twice a week for four weeks (d0, d3, d7, d10, d14, d17, d21, and d24). Plasma was taken via retroorbital bleed on day 27 for analysis of PN-specific antibodies. On day 28, mice were challenged via intraperitoneal injection with 2.5 mg CPE resuspended in 500 µl 1X PBS to induce systemic anaphylaxis. Following injection, rectal temperature was measured every 10 minutes for 60 minutes to quantitatively analyze the degree to which anaphylaxis was occurring in the peanut-challenged mice. Change in (Δ) temperature was calculated by taking the baseline temperature (temperature recorded prior to CPE injection) and subtracting the lowest temperature value recorded in the 60-minute period following injection. Retroorbital blood was collected immediately after 60 minutes to harvest serum and examine MCPT-1 levels post-anaphylaxis.

ELISA for PN-specific antibodies and MCPT-1

Blood was taken retroorbitally on day 27, treated with 0.05% EDTA and centrifuged at 4000 RPM for 5 minutes at 4°C to harvest plasma. Plasma was analyzed via ELISA to identify levels of PN-specific IgE, IgG1, and IgG2a antibodies as described [17, 30]. ELISA plates were read at 450 nm using a Synergy H1 microplate reader (BioTek, Winooski, VT). Blood taken on day 28 was collected into a serum separator tube (BD Microtainer Tube SST Gel, Franklin Lakes, NJ) and centrifuged at 4000 RPM for 10 minutes at 4°C to obtain serum. Total protein levels of MCPT-1 were determined using a commercial mouse MCPT-1 ELISA Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions using a 1:20 dilution in assay diluent of serum samples.

Lung tissue harvest and processing

Lung tissue was harvested for analysis of ILC2 on day 3 after completion of the 3-day inhalation model. Following harvest, lungs were processed by cutting them into small pieces and physically dissociating each lung into FACS buffer (PBS; 0.5% BSA; 1% 0.5 M EDTA) with the back of a 3 mL syringe plunger through a 70 µm cell strainer in a six well plate to dissociate the tissue into a single cell suspension able to be further analyzed using flow cytometry.

Flow cytometry

Following tissue processing, lung single cell suspensions were treated with red blood cell lysis buffer to eliminate erythrocyte contamination. The cells were washed and suspended in FACS buffer followed by obtaining the cell count using a hemocytometer. Cells were then plated on a 96-well plate to perform antibody staining. All antibodies were purchased from BioLegend (San Diego, CA). The plated cells were incubated with TruStain FcX PLUS (anti-mouse CD16/32) antibody on ice for 10 minutes to block Fc receptors. Following the Fc block, cells were stained for 30 minutes on ice in the dark with

the following combinations of primary mAb: KLRG1 (Brilliant Violet 421; clone: 2F1), CD45.2 (FITC; clone: 104), and a biotin-conjugated lineage cocktail to select for lineage negative (Lin-) cells composed of 11 antibodies: CD3ε; clone: 145-2C11, CD4; clone: GK1.5, CD8; clone: 53-6.7, CD19; clone: 6D5, B220; clone: RA3-6B2, CD11b; clone: M1/70, Ter119; clone: TER-119, Gr-1; clone: RB6-8C5, CD16/32; clone: 93, CD49b; clone: DX5, and CD11c; clone: N418. After primary staining was complete, cells were washed and stained for 15 minutes on ice in the dark with streptavidin-Brilliant Violet 711 to visualize the biotinylated antibodies. Surface marker expression was visualized with the Sony SH800S flow sorter (Sony Biotechnology, San Jose, CA). Data were analyzed using FlowJo software (Becton Dickinson, Ashland, OR).

Statistical analysis

PN-specific antibody, anaphylaxis, and correlation data analysis: Statistical analysis was done using GraphPad Prism 9. Statistical significance for effects of various treatments were calculated using a Welch's ANOVA with multiple comparisons adjusted with post-hoc Games-Howell Test to define any significant differences. Correlations were analyzed using a Pearson correlation coefficient and simple linear regression to understand slope significance from zero. All numerical data are represented as the mean ± SEM. Results were considered significant at $\alpha = 0.05$.

ILC2 data analysis: Statistical analysis was done using GraphPad Prism 9.5. Statistical significance for the treatments were calculated using a Two-Way ANOVA. The data did not meet the normality assumption, therefore the data was transformed using $\log_{10}$ equation. Results were considered significant at $P \leq 0.05$.

Results

AR^{Tfm} male and female mice sensitized to peanut (PN) develop worse anaphylactic reactions when challenged with PN

We first sought to investigate whether sex impacted the ability of sensitized WT mice to undergo an allergic response to PN. Male and female mice were sensitized using our established four-week PN inhalation model and challenged on day 28 with crude PN extract to induce systemic anaphylaxis [17]. The severity of anaphylaxis was quantitatively analyzed by monitoring reductions in rectal temperature and measuring the amount of mast-cell protease tryptase-1 (MCPT-1) in post-anaphylactic serum. MCPT-1 is a type of protease that is released by mast cells during degranulation caused by allergen challenge [31]. PN-sensitized WT female mice underwent more severe anaphylactic reactions compared to their WT male counterparts (females: -5.63°C±0.7°C; males: -3.31°C±0.54°C) (**Figure 1A**). MCPT-1 levels in post-anaphylactic serum were also higher in female versus male mice (**Figure 1B**). Taken together, these data suggest that female mice sensitized to PN develop stronger symptoms of systemic anaphylaxis (e.g., reduction in body temperature and mast cell activation) than male mice following challenge with PN.

Given WT male mice exhibited milder anaphylaxis, we wondered whether androgen receptor (AR) signaling played a role in protecting male mice against developing allergic reactions against PN. To investigate, we sensitized AR^{Tfm} male mice to PN alongside the WT mice as described above. AR^{Tfm} male mice have a mutation in the AR gene that makes AR^{Tfm} male mice unresponsive to androgens, including testosterone and testosterone derivatives [32]. Following PN challenge, AR^{Tfm} male mice developed systemic anaphylactic reactions similar to female mice, and much worse than male mice (AR^{Tfm} male: -6.16°C±1.6°C) (**Figure 1A**). MCPT-1 levels were also female-like (**Figure 1B**). These data suggest that AR signaling plays a role in inhibiting the allergic immune response following PN exposure.

AR^{Tfm} mice develop greater PN-specific antibody responses than WT mice

To further investigate whether AR signaling plays a role in modulating PN-specific immune responses, we examined levels of PN-specific IgE, IgG1, and IgG2a antibodies in the plasma of WT male, female, and AR^{Tfm} male mice on day 27 of our inhalation model. Mice sensitized to PN developed PN-specific antibody responses, while these responses were not observed in mice exposed to PBS as control (**Figure 2**). Because PBS mice displayed negligible PN-specific antibody responses, we grouped all PBS mice together in the analysis of the data. WT male and female mice sensitized to PN

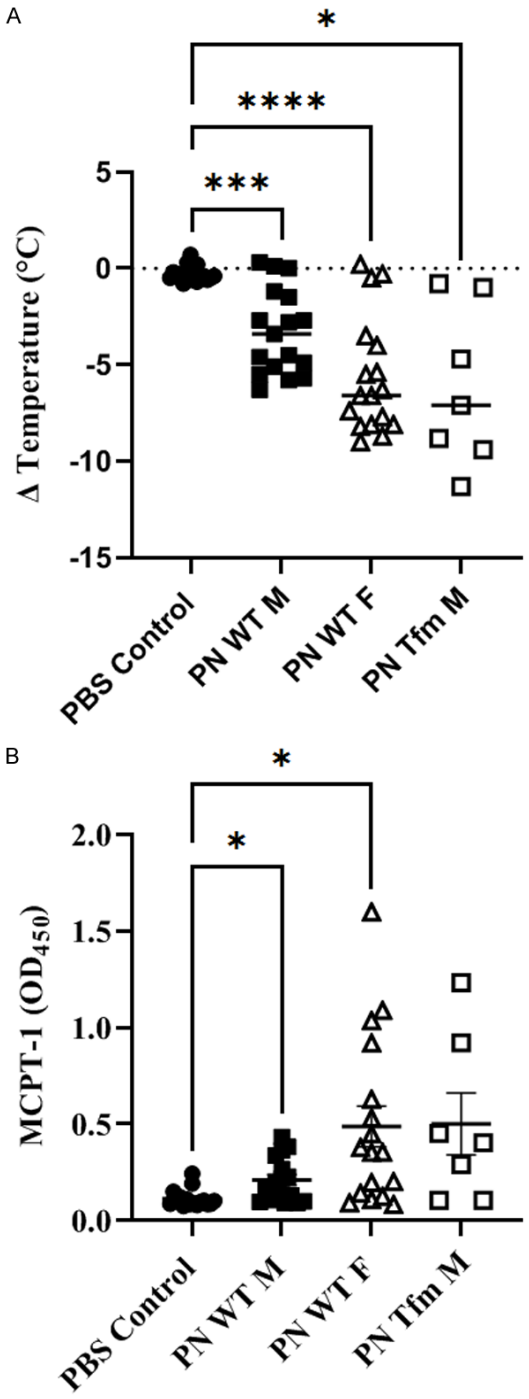

Figure 1. AR^{Tfm} male and female mice sensitized to peanut (PN) develop worse anaphylactic reactions when challenged with PN. On day 28, post sensitization each mouse underwent PN challenge via intraperitoneal injection of crude peanut extract (CPE) to induce an anaphylactic reaction. (A) Baseline temperatures were taken pre-challenge and then every 10 minutes for 60 minutes post-challenge to measure anaphylactic response. The change in (Δ) temperature was calculated using the baseline temperature and the lowest temperature reading during the 60-minute period. (B) MCPT-1 levels in serum taken on day 28 post-anaphylaxis were analyzed via ELISA. Data represents 16 PBS controls (male, female, and AR^{Tfm} male mice were grouped together for this analysis because PBS mice do not react to the CPE challenge), 16 WT PN-sensitized males, 17 WT PN-sensitized females, and 7 AR^{Tfm} PN-sensitized males. *, ***, and **** denote statistical significance by the Welch's ANOVA with multiple comparisons adjusted with post-hoc Games-Howell Test as described in *Statistical Analysis*. For both (A and B), * reveals significance at P<0.05, *** depicts significance at P<0.001, and **** indicates significance at P<0.0001.

exhibited similar levels of PN-specific IgE antibody responses. PN-sensitized AR^{Tfm} male mice developed much higher levels of PN-specific IgE antibodies (**Figure 2A**). Similar results were observed for PN-specific IgG1 and IgG2a. AR^{Tfm} male mice displayed significantly higher levels of PN-specific IgG1 and IgG2a in comparison to WT male and female mice (**Figure 2B**, **2C**). Overall, these data suggest that AR signaling regulates the development of PN-specific antibody responses and taken together with the systemic anaphylactic data, that a lack of AR signaling leads to a heightened allergic response to PN.

Correlation between PN-specific IgE and severity of anaphylactic reactions are higher in females than male mice

Next, we investigated the relationship between levels of PN-specific IgE and the severity of the anaphylactic reaction caused by PN challenge. For this correlation analysis, AR^{Tfm} male mice were grouped with female mice due to phenotypic similarities in mounting allergic responses to PN. While no correlation was found between PN-specific IgE and change in temperature (Δ temperature) in male mice (R^2 value = 0) (**Figure 3A**), a significant correlation was observed between these two metrics in WT female/AR^{Tfm} male mice (R^2 value = 0.1860) as depicted by the significant slope for Δ temperature (P<0.001) (**Figure 3B**). This comparison reveals that in females, higher levels of PN-specific IgE correlated with more severe anaphylactic reactions (as measured by reductions in body temperature), whereas males showed no such correlation.

ILC2s in lungs are more abundant in female mice exposed to PN via inhalation

Currently, it is unknown why females are more likely to develop PN allergy than males begin-

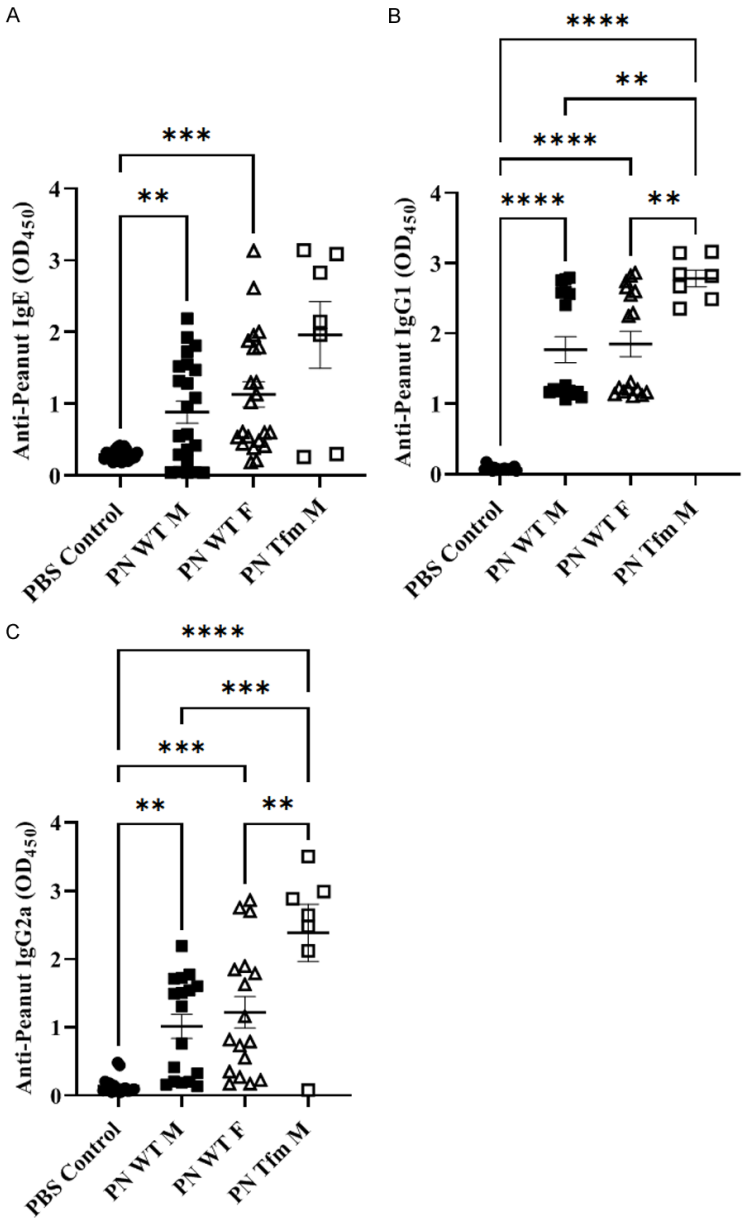

Figure 2. Mice deficient in androgen receptor (AR^{Tfm}) develop greater PN-specific antibody responses than wildtype mice. Blood was collected on day 27 retroorbitally and plasma was examined by ELISA for presence of PN-specific IgE (A), IgG1 (B), and IgG2a (C) from mice that were sensitized to PBS or PN via inhalation in a four-week inhalation model. Data is representative of 22 control mice (male, female, and AR^{Tfm} male mice were grouped together for this analysis because PBS mice do not react to inhalation of PN), 17-22 WT PN-sensitized females, 17-22 WT PN-sensitized males, and 7 AR^{Tfm} PN-sensitized males. **, ***, and **** denote statistical significance by the Welch's ANOVA with multiple comparisons adjusted with post-hoc Games-Howell Test as described in *Statistical Analysis*. For each (A-C), ** reveals significance at P<0.01, *** depicts significance at P<0.001, and **** indicates significance at P<0.0001.

ning in childhood. In this study, we have shown that female mice develop more severe PN allergy than males and this response is likely regulated by androgens. Next, we aimed to elucidate whether the mechanism that drives the development of PN-specific immune responses was impacted by sex differences. To investigate, we exposed WT male and female mice in an established three-day PN inhalation model to examine whether ILC2s known to be activated against PN are sensitive to sex differences [19]. Using flow cytometric analysis, we defined ILC2 population in the harvested lung tissue as lineage negative (Lin-) CD45.2+ KLGR1+ (**Figure 4**). Female mice exposed to PN displayed a more robust ILC2 response within the lungs than their male-exposed counterparts in terms of both cellular frequencies and numbers (**Figure 4**). Females sensitized to PN developed significantly different ILC2 responses in comparison to females exposed to PBS (**Figure 4**). In sharp contrast, PN failed to induce a significantly different ILC2 response in WT males exposed to PN verses PBS (**Figure 4**). These data suggest the development of the innate immune response activated by PN is more intense in females than males. In addition, these data provide support to the concept that AR signaling is regulating allergic immune responses to PN.

Discussion

Within allergic disease, sex hormones are best known to regulate immune responses in allergic asthma [33-41]. However, it remains unclear how sex hormones may influence the development of peanut (PN) allergy. Recent epidemiological data suggests that females are two times as likely to develop PN allergy than males [9]. These data support that sex differences exist in PN allergy

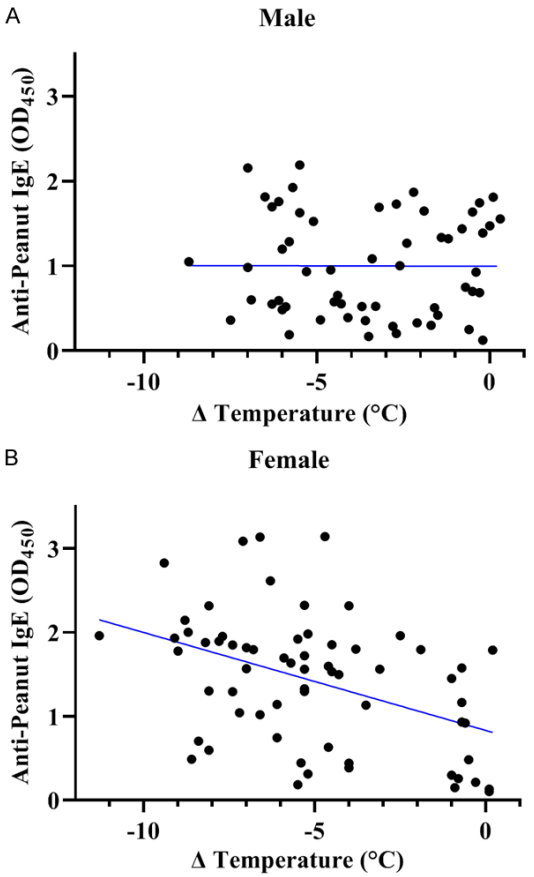

Figure 3. Correlation between PN-specific IgE and severity of anaphylactic reactions is higher in female than male mice. Pearson correlation analysis between PN-specific IgE levels compared to change in temperature. Females have a stronger negative correlation between PN-specific IgE than males (A. R^2 = 0; B. R^2 = 0.1860) with no significant difference in slope from zero in males, but a significantly different slope in females ($P<0.001$) determined by a simple linear regression. AR^{Tfm} males are included in the female analysis.

and support further investigation of this phenomenon. In this study, we compared WT male, WT female, and AR^{Tfm} male mice to examine how androgen hormones influenced the allergic immune response to PN. PN-specific antibodies were measured to examine if sex impacted the development of sensitization to PN. Additionally, reductions in body temperature following PN challenge were documented to elucidate how WT male, WT female, and AR^{Tfm} male mice differed in their ability to undergo systemic anaphylaxis. MCPT-1 levels were measured in these mice to better understand whether levels of mast cell degranulation caused by PN challenge was sensitive to sex differences. We discovered that while WT male and female mice developed similar levels, AR^{Tfm} male mice developed higher levels of PN-specific antibodies. However, WT female and AR^{Tfm} male mice developed worse anaphylactic reactions and had greater mast cell degranulation compared to WT male mice. Using these data, we ran a correlation analysis and found a significant correlation between levels of PN-specific IgE and the severity of anaphylaxis in WT female/AR^{Tfm} male mice, whereas this correlation was absent in the WT male mice. Taken together, this data strongly suggests that AR signaling regulates the allergic immune response to PN.

One finding we found particularly interesting is that WT male and female mice developed similar levels of PN-specific antibodies yet had different reactions upon PN challenge. While total PN-specific antibodies were measured, we did not measure the epitope specificity of the PN-specific antibody response. *Arachis hypogaea* (Ara h), the scientific name for PN, defines the nomenclature of epitopes within PN. Ara h epitopes function as seed storage proteins or plant defenses for the PN plant. Ara h 1, 2, 3, and 8 are most associated with PN allergy with Ara h 2 being associated with the most severe reactions [42-44]. Each mouse is sensitized to PN nonspecifically in this experiment, and we only measured total PN-specific IgE, IgG1, and IgG2a. Understanding the epitope specificity of the IgE response (e.g., Ara h 1-specific IgE, Ara h 2-specific IgE, etc.) would be useful in understanding why some mice reacted with weak to mild anaphylactic reactions even though they had high levels of IgE. It would also provide additional insight as to why male and female mice appear to generate the same amount of PN-specific antibodies yet react differently following PN challenge. For instance, a sensitized mouse expressing a mixture of antibodies specific for both non-allergy associated epitopes, such as Ara h 5, with allergy-associated epitopes, would result in a lowered anaphylactic response than a mouse with a high level of only Ara h 2-specific antibodies [44]. From the correlation data, one could hypothesize that females are likely creating greater amounts of antibody against PN epitopes associated with severe PN allergy due to the stronger reactions they are developing than their male counterparts. It would be of interest to know how lack

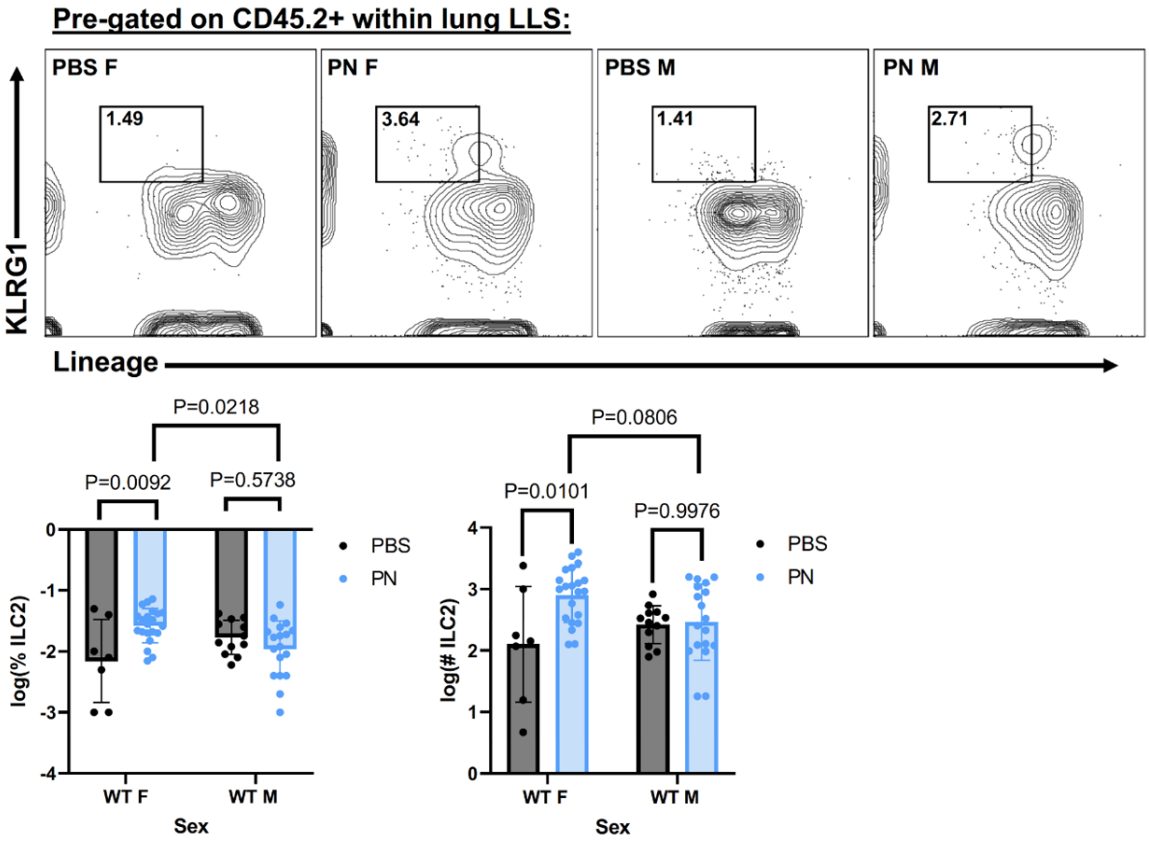

Figure 4. ILC2s in lungs are more abundant in female mice exposed to PN via inhalation. Flow cytometric analysis of ILC2 cells defined as CD45.2+ Lineage- KLRG1+ within the lungs after exposure to PN in 3-day inhalation model. ILC2s were stained according to the procedures outlined in the *Methods* section. Bar graph depicting the log transformed data represents the percentage of ILC2 population of 7 PBS-sensitized female, 12-PBS sensitized male, 21-PN sensitized female and 18-PN sensitized male mice. Note: the smaller the negative value the higher the percentage of ILC2. Bar graphs depicting the log transformed data represent the number of ILC2 population. Statistics were performed using two-way ANOVA with statistically significant differences measured at P≤0.05.

of androgen receptor in AR^{Tfm} mice impacts the ability of the immune system to mount PN epitope-specific antibody responses.

Further, the notion that AR signaling plays, at least in part, a role in protecting male mice from developing PN allergy is supported by our observation that AR^{Tfm} male mice had higher levels of PN-specific antibodies, as well as more severe anaphylactic reactions, than male mice. Considering that PN-specific IgE-bound mast cells are activated to degranulate upon PN challenge, we correlated the production of PN-specific antibodies with the anaphylactic response. The correlation analysis showed that female mice combined with AR^{Tfm} males (who reacted very similarly to females verses male) had a negative correlation between PN-specific IgE production and anaphylactic response. This data indicates a significantly greater increase in PN-specific IgE levels corresponding with a greater decrease in body temperature in female versus male mice that showed no correlation between these metrics. The absence of a relationship between the levels of PN-specific antibodies, namely PN-specific IgE, and the development of severe systemic anaphylactic reactions in males suggests that testosterone, via AR signaling, is suppressing the development of immune responses against PN. The role of AR signaling is further supported by the negative correlation between PN-specific IgE and systemic anaphylactic reactions in females. Females are known to produce much less androgens than males [45, 46]. Therefore, the milder reactions we observe in WT male mice likely indicates that the presence of more androgens drives stronger AR signaling that

functions to negatively regulate allergic immune responses to PN. The strong reactions to PN in PN-sensitized WT females, as well as the even more severe reactions observed in the PN-sensitized androgen receptor-deficient AR^{Tfm} male mice support this notion. This is the first time a study represents such a correlation being found in a food allergy mouse model.

While the data suggest that AR signaling is negatively regulating allergic immune responses to PN, an understanding of how this could be occurring mechanistically at the cellular level is unclear. To address this, we harvested lung tissue from PN-sensitized WT male and female mice to study how ILC2s may be impacted by sex differences. ILC2s have been shown to be activated against PN following inhalation of the allergen [19]. We found that female ILC2s responded stronger when exposed to PN than their male counterparts. This study marks the initial report where sex differences have been shown to impact the cellular response to PN. We and others have detailed the ILC2-dependent dendritic cell process that is required to activate PN-specific T follicular helper cells [17, 19]. Additional studies will need to examine whether sex differences impact activation of other innate and adaptive cells following PN inhalation. Future studies pinpointing how PN activates innate and adaptive cells in mice that lack androgen receptor signaling will be necessary to further elucidate the role male sex hormones have on the development of PN-specific allergic responses.

In this study, we document that while PN-specific IgE is similar between WT males and females, female mice underwent more severe systemic anaphylaxis when challenged with PN than their male counterparts. Additionally, the robust response of female ILC2s to PN suggests that mast cells (MCs) are being regulated by sex hormones. Best known for their proinflammatory roles in allergic disease and anaphylaxis [47], MCs express the high affinity FceRI that binds PN-specific IgE generated following initial exposure to PN. Upon subsequent exposure, PN crosslinks the IgE bound to FceR causing the MCs to degranulate, and this causes the release of preformed proinflammatory mediators such as histamine and heparin. Excess activation of MCs can lead to life-threatening anaphylaxis [47], and in the case of PN allergy, this reaction is more frequent and more severe than other food allergies [48]. In addition to these classic activities, MCs also orchestrate innate and adaptive immune responses to allergens [49]. Studies have shown that MC-associated diseases exhibit a female sex bias [50-56]. Furthermore, female MCs have been shown to have increased levels of proinflammatory mediators, a greater ability to drive anaphylaxis, and these differences between male and females are established before puberty by androgens [47, 57]. It will be interesting to examine how sex hormones modulate the MC response to PN in future experiments. A greater understanding about how sex hormones modulate the MC response to PN will provide clarity into why anaphylactic reactions following PN challenge were sensitive to sex differences.

Understanding how sex differences impact the development of PN allergy is critical in explaining the female bias in PN allergy. As novel therapies to mitigate PN allergy continue to be developed, it becomes important to document how male and females may respond differently to these therapies because of the way hormones have regulated how the immune system mounts the PN-specific response. Lastly, these studies will likely guide our understanding of other food allergies.

Conclusion

Peanut (PN) allergy continues to be a growing public health issue, and while females have been shown to develop PN allergy twice as frequently as males in recent clinical data, little is known about how sex differences impact allergic immune responses to PN. This study documents for the first time a sex difference in a mouse model of PN allergy. Female mice develop stronger anaphylactic reactions than their male counterparts. Furthermore, male mice deficient in androgen receptor signaling develop higher levels of PN-specific antibodies and severe anaphylaxis upon PN challenge, suggesting that androgen receptor signaling plays a role in inhibiting the allergic immune response following PN exposure. Finally, we show that ILC2s in the lungs of female mice respond more robustly to inhaled PN than their male counterparts. Taken together, this study supports further investigation about how sex hormones impact the development of PN allergy. Such

work is critical to obtain a greater understanding about how females and males react differently to exposure to PN.

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Disclosure of conflict of interest

None.

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