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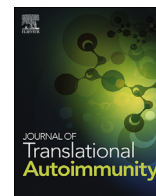
Citation of this paper:

Cairns, Ewa; Saunders, Sheri; Bell, David A.; Blackler, Garth; Lac, Patrick; and Barra, Lillian, "The effect of sex on immune responses to a homocitrullinated peptide in the DR4-transgenic mouse model of Rheumatoid Arthritis" (2020). *Bone and Joint Institute*. 194.

<https://ir.lib.uwo.ca/boneandjointpub/194>

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The effect of sex on immune responses to a homocitrullinated peptide in the DR4-transgenic mouse model of Rheumatoid Arthritis



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ARTICLE INFO

Keywords:

Rheumatoid arthritis
Anti-citrullinated protein/peptide antibodies
Anti-homocitrullinated (carbamylation) protein/peptide antibodies
Sex
Mouse model
Shared epitope
Cytokines

ABSTRACT

Rheumatoid Arthritis (RA) is more common and severe in women compared to men. Both women and men with RA express autoantibodies to post-translationally modified antigens, including citrullinated and homocitrullinated proteins or peptides. These autoantibodies are strongly linked with the HLA-DR4 gene. The objective of this study was to determine sex differences in immune responses to homocitrullinated antigens. We used a humanized animal model of RA, DR4-transgenic mice and immunized them with a homocitrullinated peptide called HomoCitJED. Immune responses in these mice were measured for splenocyte proliferation by tritiated thymidine incorporation, serum autoantibody production by ELISA and cytokine levels by multiplex. We found that T cell and antibody responses to homocitrullinated antigens were similar in male and female mice. However, we found sex differences in serum cytokine profiles with female mice having higher ratio of IL-1 α to IL-5, suggesting imbalances in immune regulation. This is the first study to report that immune responses to homocitrullinated antigens can be differentiated by sex.

1. Introduction

Rheumatoid Arthritis (RA) is a systemic inflammatory autoimmune disease predominantly affecting the synovial joints. It is 2–3 times more common in women who are less likely to respond to therapies and achieve remission than men [1–5]. The reasons for sex and/or gender differences in RA are incompletely understood: in addition to sociodemographic factors, sex differences in immune responses are likely to contribute. The X-chromosome contains many immune-related genes and sex hormones are known to affect immune cell function [6]. In RA, the disease improves with pregnancy and testosterone-deficient men are more likely to develop RA [7,8]. In some studies, T cell subtypes and autoantibody expression were shown to differ amongst men and women with RA [9–11].

RA is characterized by the production of autoantibodies: rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA)

were the first to be described [12,13]. More recently, anti-homocitrullinated protein/peptide antibodies (AHCPA), also known as anti-carbamylated protein antibodies (anti-CarP), were shown to be specifically expressed in a large proportion of patients with RA, including those negative for ACPA [14–16]. Like ACPA, AHCPA is associated with more severe erosive disease [16,17]. There is conflicting evidence whether women with RA are more likely to be positive for ACPA [10,11].

ACPA is highly associated with the strongest genetic risk factor for RA, HLA-DR4 alleles encoding major histocompatibility complex (MHC) II containing a consensus sequence known as the Shared Epitope [18]. The Shared Epitope binds to citrullinated peptides with high affinity leading to T cell activation and the production of ACPA [19,20]. Homocitrulline and citrulline are structurally similar and *in silico*, a homocitrullinated collagen II peptide was found to bind the Shared Epitope [14]. Mice transgenic for the Shared Epitope (DR4tg) immunized with a homocitrullinated peptide (HomoCitJED) developed T and B cell

Abbreviations: ACPA, anti-citrullinated protein/peptide antibodies; AHCPA, anti-homocitrullinated protein/peptide antibodies; anti-CarP, anti-carbamylated protein antibodies; ConA, Concanavalin A; HomoCitFib, homocitrullinated fibrinogen; RA, rheumatoid arthritis; RF, rheumatoid factor; sc, subcutaneous; SL, stimulation index.

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<https://doi.org/10.1016/j.jtauto.2020.100053>

Received 8 March 2020; Accepted 2 April 2020

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responses to homocitrullinated antigens, but not to peptides containing lysine instead of homocitrulline on the same amino acid backbone [21]. HomoCitJED is a synthetic peptide that captures immune reactivities to multiple naturally-occurring homocitrullinated and citrullinated protein/peptides known to be present in the joints of RA patients [14,15]. The objective of the present study was to determine sex differences in immune responses to homocitrullinated antigens.

2. Materials and methods

2.1. Antigens

The following antigens were employed in this study: a) Homocitrullinated JED (HomoCitJED), which is an 18 amino acid long cyclic peptide with 9 homocitrullines [14,15], synthesized by Creative Peptides (Shirley, NY, USA); b) human fibrinogen (Fib) from VWR and c) homocitrullinated human fibrinogen (HomoCitFib). Homocitrullination was performed as previously described [14,22].

2.2. Mice and immunizations

DR4-IE transgenic mice on C57Bl/6 background (referred to as DR4tg) lacking endogenous MHC class II molecules were bred in-house [22]. They were compared to wild type C57Bl/6 (B6) (The Jackson Laboratories, Maine, USA). Mice were housed at the Animal Care and Veterinary Services pathogen-free facility at the University of Western Ontario according to the guidelines of the Canadian Council on Animal Care. The study was approved by the Animal Care and Use Committee (The University of Western Ontario, London, ON, Canada). Female and male mice received primary and booster immunization of HomoJED peptide (100 µg each time) or PBS control via subcutaneous (sc) route as described by Lac et al. [21].

2.3. Splenocyte proliferation

At various times post-immunizations, mouse spleens were harvested and splenocytes were cultured in media containing 100 µg/mL of HomoCitJED or 1 µg/mL Concanavalin A (ConA) (Sigma) or media alone. After 54 h of culture, half of the supernatant was replaced by media containing ³H-thymidine (1 µCi/well). After an additional 18 h, radioactivity was measured. Proliferation is reported as a Stimulation Index (SI; cpm of samples with HomoCitJED or ConA/cpm of samples with media) +/− standard deviation. SI > 2 was considered as a positive proliferative response. Samples were tested at least in quadruplicate (coefficient of variation <20%). Additional methodological details can be found in Lac et al. [21].

2.4. Antibody assays

Each mouse serum sample was tested at least in duplicate for the presence of IgG antibodies to HomoCitJED, unmodified and homocitrullinated human fibrinogens using ELISA [21]. Antibody concentrations to each antigen are expressed as relative units (RU)/mL which were determined from a standard curve generated from a pooled reference mouse serum. The cut-off for positive values was the lower detection limit of the ELISA (0.1 OD, equivalent to 20.3 RU/mL and 2.9 RU/mL for anti-HomoCitJED and anti-HomoCitFib antibody, respectively). For each mouse sample, the coefficient of variation across replicates was <20%.

2.5. Cytokines and chemokines assay

Twenty-four cytokines and chemokines were measured in the sera of HomoCitJED immunized DR4tg and B6 mice on days 10 and 70 post-immunization using a commercial multiplex immunoassay (ThermoFisher). Samples were tested in duplicate.

2.6. Statistical analyses

Comparisons were performed for four groups of mice: female DR4tg, male DR4tg, female B6 and male B6. T cell proliferation and antibody responses at each timepoint were compared using 2-way ANOVA with Bonferroni correction ($p < 0.001$ considered statistically significant). Frequency of positive proliferative and detectable antibody responses over the course of the study were compared using Chi-square test ($p < 0.01$). Discriminant analysis of the log-transformed cytokine data was performed to determine whether the cytokine profiles for the four mouse groups were distinguishable ($p < 0.05$). Stepwise discriminant analysis was also performed to identify the cytokines that contribute most to the group separation. Individual log-transformed cytokine levels were compared using the Mann-Whitney test and corrected for multiple comparisons ($p < 0.01$). Cytokine and chemokine levels at day 10 and day 70 were similar and these were therefore combined. Statistical analyses were performed using GraphPad Prism 6.0 and SAS 9.4 software.

3. Results

3.1. Splenocyte proliferative responses

Splenocytes from HomoCitJED immunized DR4tg and B6 mice were isolated at various time points and examined for T cell proliferative responses to HomoCitJED and ConA (Fig. 1, Supplementary Fig. 1). Over the time course of the study, there were no significant differences in T cell responses to HomoCitJED or ConA in female versus male DR4tg and B6 mice. Responses to HomoCitJED were more frequent in DR4tg compared to B6 mice: 24/116 (21%) versus 8/98 (8%); $p = 0.0150$. For DR4tg mice the responses were stable over time; whereas, for B6 mice, the responses were higher at later timepoints ($p < 0.0001$). There were no significant differences in responses to ConA between mouse strains and over time. PBS immunized mice did not have detectable splenocyte proliferative responses to HomoCitJED but responded to ConA as expected with no significant differences by strain or sex (data available from corresponding author).

3.2. Antibody responses to homocitrullinated antigens

After immunization with HomoCitJED, serum IgG antibodies to this peptide and to HomoCitFib were mostly detected from day 30 to day 100 in DR4tg and B6 mice (Fig. 2 and Tables 1 and 2). For DR4tg mice, the levels peaked at day 70 and for B6, day 50; changes over time were significantly different for DR4tg ($p < 0.0001$), but not B6 mice. There were no significant differences in the levels or frequency of antibodies in female versus male mice for both strains over the course of the study. The number of mice positive for anti-HomoCitJED antibodies was higher in DR4tg versus B6 mice: 90/161 (56%) and 39/137 (28%), respectively; $p < 0.0001$. High positive AHCPA (>than the 75th percentile) was uncommon in B6 mice: 8/137 (6%) vs. 39/161 (24%) in DR4tg mice for anti-HomoCitJED antibodies ($p < 0.0001$) and 13/144 (9%) vs. 35/157 (22%) in DR4tg for anti-HomoCitFib antibodies ($p = 0.0017$). DR4tg males had higher mean antibody levels ($p < 0.0001$) than B6 males; results were similar for females but did not reach statistical significance. PBS immunized mice did not have any detectable AHCPA and none of the mice had antibody responses to unmodified human fibrinogen (data available from corresponding author).

3.3. Cytokine and chemokine responses

Twenty of twenty-four cytokines/chemokines were detected in the sera of HomoCitJED immunized DR4tg and B6 mice. The following were not detected in any of the animals: IL-4, IL-9, IL-13 and TGFβ2. Four mice had detectable IL-1β, three had IL-2, two had IL-25 and nine had IL-17; however, the mean values for these cytokines were below the detection limit and they were excluded from further analyses. The included

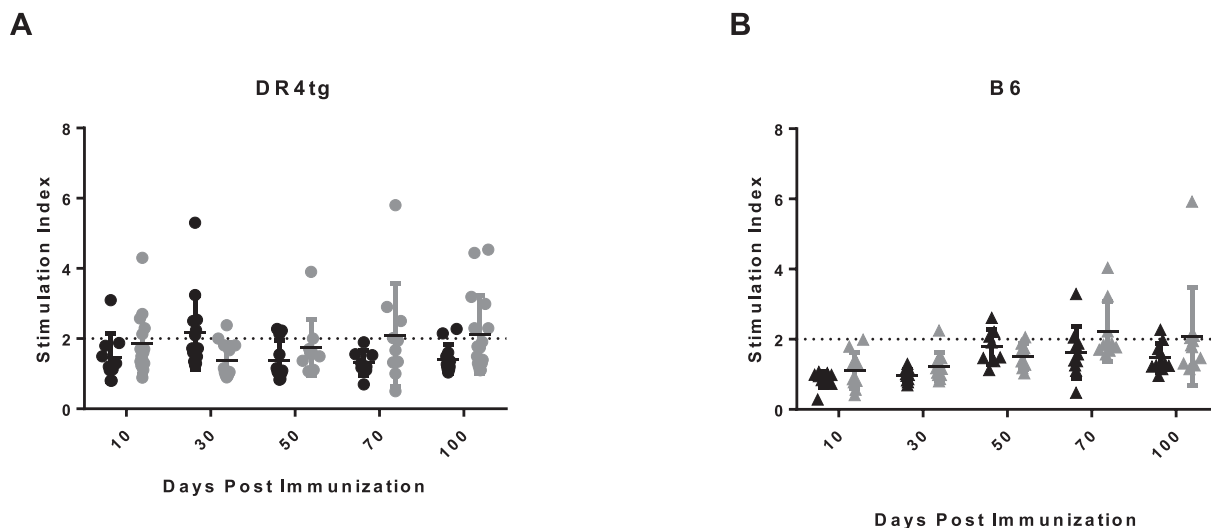


Fig. 1. Splenocyte proliferative responses in DR4tg (●Females; ●Males) and B6 (▲Females; ▲Males) mice. Splenocytes isolated from HomoCitJED-immunized DR4tg (A) and B6 (B) mice were cultured in the presence of 100 $\mu\text{g}/\text{mL}$ of HomoCitJED. Splenocyte proliferation is shown as a mean stimulation index (SD). Positive proliferative responses were defined as stimulation indices ≥ 2.0 . Each symbol represents one mouse; N = 8–16.

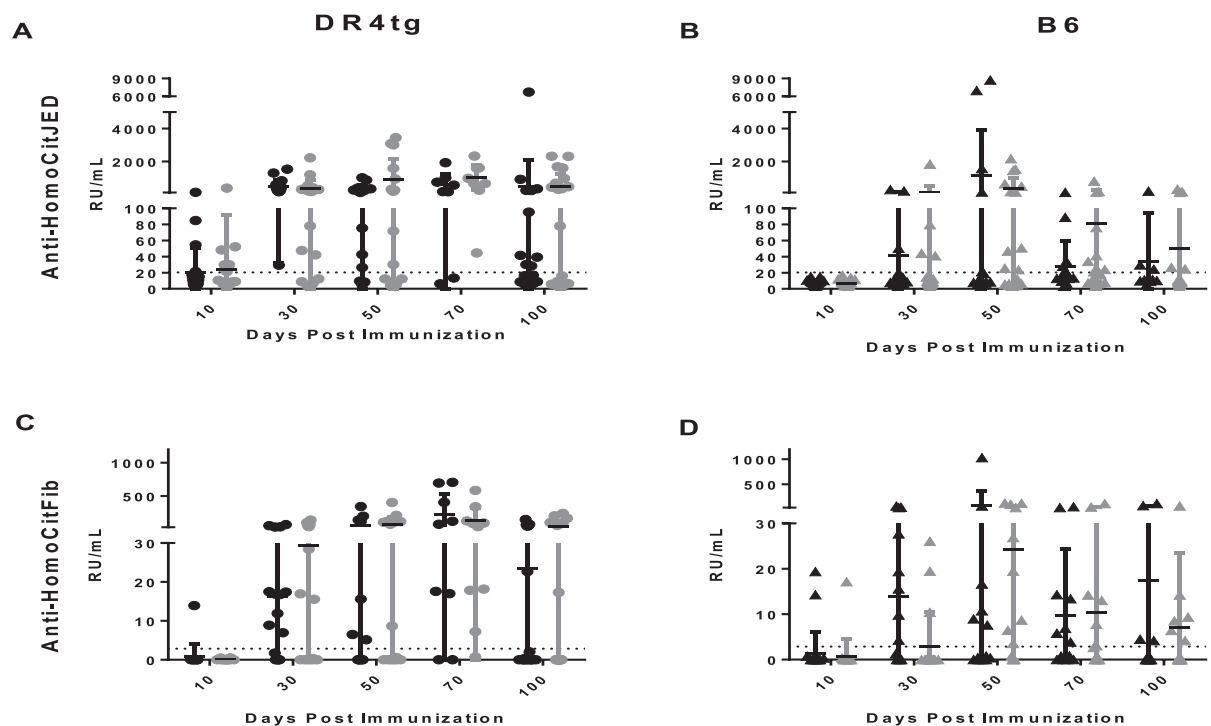


Fig. 2. Sera antibody responses in DR4tg (●Females; ●Males) and B6 (▲Females; ▲Males) mice. Sera at various time points were tested for IgG anti-HomoCitJED antibodies in DR4tg (A) and B6 (B) mice and for IgG anti-HomoCitFib antibodies in DR4tg (C) and B6 (D) mice. Values are in RU/mL (SD). Each symbol represents one mouse; N = 8–29.

cytokines/chemokines (N = 16) are shown in Table 3. In DR4tg mice, IL-5 was significantly higher in females compared to males ($p = 0.0004$). Both male and female B6 mice had higher levels of IL-12p40/IL-23 than DR4tg mice ($p < 0.0001$). Analyses of the levels for the other individual cytokines and chemokines did not reveal any other statistically significant differences.

Discriminant analysis was performed to determine whether a profile consisting of all the detectable cytokines and chemokines (N = 16) could differentiate mice by sex and strain. This analysis generated 3 functions (Can1, Can2, Can3), accounting for 86% of the variance seen in the dataset. Can1 primarily differentiated the cytokine profile of DR4tg vs.

B6 mice ($p < 0.0001$) and Can2, female vs. male DR4tg mice ($p = 0.0118$) (Fig. 3). Group differentiation by Can3 was not significant ($p = 0.4311$) (data available from corresponding author). The accuracy of the group differentiation by the discriminant analysis model was 92%. The cytokines that contributed most to the differentiation between mouse groups were: IL-12p40/IL-23 ($R^2 = 0.7346$, $p < 0.0001$), IL-1 α ($R^2 = 0.3891$, $p = 0.0009$) and IL-5 ($R^2 = 0.2193$, $p = 0.0404$).

4. Discussion

In this study we found that there were no sex differences in T cell

Table 1
Sera IgG anti-homocitrullinated JED antibody responses.

DR4tg Mice	Females					Males				
	10	30	50	70	100	10	30	50	70	100
Days	10	30	50	70	100	10	30	50	70	100
N	19	17	13	8	18	29	19	15	8	23
Mean (SD), RU/ mL	20.5 (30.9)	312.6 (146.4)	255.5 (328.5)	567.0 (653.9)	484.3 (1571)	23.8 (68.1)	215.7 (170.8)	874.6 (1268)	1007 (772.0)	486.9 (755.1)
Positive, N (%)	4 (25)	15 (100)	9 (75)	6 (75)	10 (56)	3 (10)	13 (76)	11 (73)	8 (100)	11 (48)
High Positive, N (%)	0 (0)	8 (53)	2 (17)	4 (50)	2 (11)	1 (4)	3 (18)	5 (33)	6 (75)	8 (35)

B6 Mice	Females					Males				
	10	30	50	70	100	10	30	50	70	100
Days	10	30	50	70	100	10	30	50	70	100
N	27	15	15	15	9	25	20	19	17	14
Mean (SD), RU/ mL	8.2 (3.3)	45.1 (84.2)	1170 (2770)	27.5 (32.5)	34.0 (8.4)	6.9 (3.6)	105.2 (406.3)	355.2 (641.3)	81.2 (190.0)	50.2 (88.4)
Positive, N (%)	0 (0)	3 (25)	4 (33)	5 (39)	3 (33)	0 (0)	4 (25)	7 (54)	8 (57)	5 (42)
High Positive, N (%)	0 (0)	0 (0)	3 (25)	0 (0)	0 (0)	0 (0)	1 (6)	4 (31)	0 (0)	0 (0)

Days are post-HomCitJED primary immunization.

RU/mL = relative units per millilitre.

Positive values were defined as higher than the detection limit of the assay (>20.3 RU/mL).

High positive values were defined as > than the 75th percentile = 340 RU/mL.

Table 2
Sera IgG anti-homocitrullinated fibrinogen antibody responses.

DR4tg Mice	Females					Males				
	10	30	50	70	100	10	30	50	70	100
Days	10	30	50	70	100	10	30	50	70	100
N	15	18	14	9	18	20	16	16	9	22
Mean (SD), RU/mL	0.8 (3.3)	16.3 (20.5)	58.8 (104.1)	226.5 (296.8)	23.4 (40.8)	0.1 (0.2)	29.4 (42.4)	71.5 (109.7)	131.6 (188.3)	48.3 (73.9)
Positive, N (%)	1 (7)	11 (61)	5 (36)	5 (56)	6 (33)	0 (0)	8 (50)	7 (44)	8 (89)	9 (41)
High Positive, N (%)	0 (0)	2 (11)	2 (14)	3 (33)	5 (28)	0 (0)	4 (25)	7 (44)	4 (44)	8 (36)

B6 Mice	Females					Males				
	10	30	50	70	100	10	30	50	70	100
Days	10	30	50	70	100	10	30	50	70	100
N	21	13	11	11	11	14	16	16	16	15
Mean (SD), RU/mL	1.4 (4.7)	14.0 (18.9)	81.6 (274.3)	9.6 (14.7)	17.3 (37.6)	0.8 (3.7)	2.9 (7.5)	24.4 (42.6)	10.4 (27.2)	7.1 (16.3)
Positive, N (%)	2 (10)	6 (46)	5 (45)	8 (72)	4 (36)	1 (7)	3 (19)	8 (50)	5 (31)	6 (40)
High Positive, N (%)	0 (0)	1 (8)	2 (18)	2 (18)	2 (18)	0 (0)	0 (0)	3 (19)	2 (13)	1 (7)

Days are post-HomCitJED primary immunization.

RU/mL = relative units per millilitre.

Positive values were defined as higher than the detection limit of the assay (>2.9 RU/mL).

High positive values were defined as > than the 75th percentile = 40 RU/mL.

Table 3
Serum Log-Transformed Cytokine and Chemokine levels.

Cytokine	DR4tg			B6			DR4tg vs. B6 Females	Dr4tg vs. B6 Males
	Female	Male	p-value	Female	Male	p-value		
TGFβ1b	10.968 (0.702)	11.246 (0.591)	0.3507	10.969 (0.812)	11.528 (0.444)	0.0722	0.9972	0.2429
TGFβ2b	7.552 (0.473)	7.520 (0.503)	0.8876	7.501 (0.703)	7.833 (0.559)	0.2703	0.8532	0.2054
IFNγ	1.845 (0.903)	1.675 (0.739)	0.6501	2.375 (1.686)	1.813 (1.011)	0.3776	0.3923	0.7318
IL-1α	1.309 (0.826)	0.665 (0.731)	0.0814	1.277 (1.158)	0.431 (0.337)	0.0398	0.9437	0.3704
IL-5	3.134 (0.245)	2.402 (0.475)	0.0004	2.773 (0.554)	2.776 (0.688)	0.9912	0.0756	0.1738
IL-6	1.431 (1.948)	1.688 (2.140)	0.7825	1.660 (2.253)	1.835 (1.625)	0.8441	0.8109	0.8640
IL-10	0.855 (0.471)	0.291 (0.635)	0.0369	1.269 (0.853)	0.783 (1.048)	0.2703	0.1951	0.2202
IL-12p40/IL-23	2.550 (0.511)	2.117 (0.514)	0.0751	3.899 (0.653)	3.723 (0.403)	0.4784	<.0001	<.0001
IL-18	6.214 (0.385)	6.036 (0.462)	0.3619	5.748 (1.049)	5.171 (1.454)	0.3227	0.2036	0.0900
MCP-1	2.773 (1.532)	2.343 (1.359)	0.5151	2.185 (2.017)	2.938 (0.520)	0.2697	0.4744	0.2123
MIP-1α	0.514 (0.395)	0.447 (0.171)	0.6315	0.600 (0.203)	0.594 (0.386)	0.9628	0.5448	0.2863
MIP-1β	0.909 (0.491)	0.694 (0.427)	0.3096	0.943 (0.555)	0.893 (0.409)	0.8225	0.8856	0.2998
MIP-2	1.887 (0.478)	1.910 (0.503)	0.9193	1.674 (0.493)	2.161 (0.224)	0.0107	0.3393	0.1737
Eotaxin	3.454 (2.775)	3.981 (2.142)	0.6405	3.067 (2.850)	5.080 (1.348)	0.0586	0.7618	0.1864
RANTES	2.125 (1.378)	2.479 (1.468)	0.5847	3.178 (2.525)	4.2835 (1.252)	0.2306	0.2622	0.0084
VEGF	1.538 (0.956)	0.896 (0.958)	0.1510	1.674 (1.180)	1.898 (0.918)	0.6430	0.7804	0.0283

Values are mean levels in pg/mL (SD), unless otherwise indicated.

proliferative responses and antibody responses to homocitrullinated antigens in DR4tg mice immunized with a homocitrullinated peptide.

However, using discriminant analysis, the cytokine profile of female mice was significantly different from male mice. To our knowledge, this is the

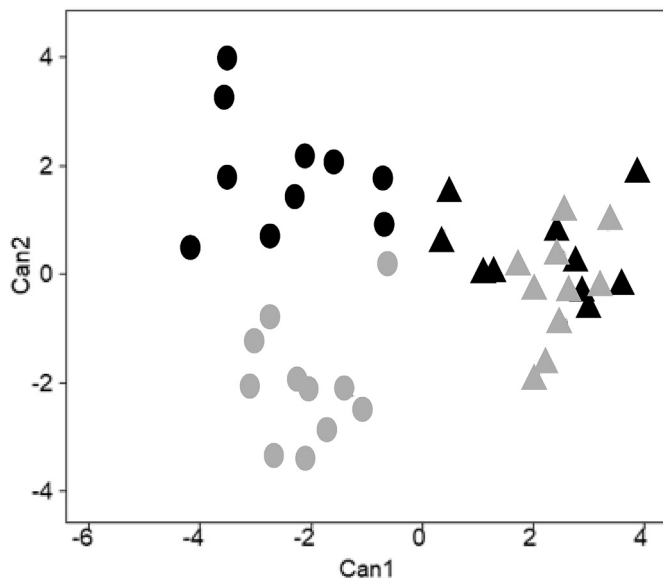


Fig. 3. Discriminant analysis based on serum cytokine profile for differentiating the mouse groups: DR4tg females (●), DR4tg males (◐), B6 females (▲) and B6 males (◑); $N = 10$ mice/group. Canonical discriminant functions (Can1 and Can2) were generated using the standardized coefficients derived from the levels of all individual detectable serum log-transformed cytokine levels. The plot shows each subject's calculated value for Can1 (X axis) and Can2 (Y axis). Can3 did not differentiate the mouse groups and is not shown.

first published study to investigate sex differences in anti-homocitrullinated immune responses in mouse models of RA.

The DR4tg mouse model used in this study closely resembles human RA in that it expresses human Shared Epitope [20]. We and others have previously shown that DR4tg mice have immune responses to antigens known to be involved in the pathogenesis of RA and can develop RA-like inflammatory arthritis [22–24]. In humans, RA is more common in females [3]; in DR4tg mice, arthritis was also more common in females when immunized with collagen II [24]. The mechanistic role of immune responses in the observed sex differences in RA are unclear. Although evidence supports arthritogenicity of ACPA *ib25* [19,25] and some studies show that ACPA are more common in women with RA [10,11], most studies either did not report or did not find sex differences in the expression of the autoantibodies. Similarly, we found that the frequency and levels of AHCPA (anti-HomoCitJED antibodies) were not significantly different in women compared to men in a cohort of patients with established RA that we previously published [15]: 70.1 (SD 138.0) RU/mL, (47/100) positive and 85.9 (135.7) RU/mL (22/37 positive), respectively. Therefore, our findings of no sex differences in antibody expression in the DR4tg mouse model are consistent with prior human studies.

There are a paucity of studies investigating sex differences in T cell function and frequency of T cell subtypes in RA and the results are conflicting: Aldridge et al. did not find significant sex differences in the number or proportion of peripheral T cell subsets [9]; whereas, Mateen et al. found that men had a higher proportion of Th17 cells than women [26]. Both studies investigated patients with early, untreated RA and the reason for the discrepancy of their results is unknown but could be due to other differences in the populations, such as age, genetics and environmental exposures. In these studies, which had small sample sizes, it can be difficult to account for possible confounders; therefore, animal models with controlled settings can provide insight into the sex differences. In our study of HomoCitJED immunized DR4tg mice, specific T cell proliferative responses to homocitrullinated antigens and responses to a general T cell mitogen (ConA) did not reveal any sex differences. Behrens et al. found that sex effects in T cell proliferative responses to collagen II peptides in collagen II-immunized DR4tg mice depended upon the type of

antigen presenting cell; females had higher responses to peptides presented by B cells and males by dendritic cells [27]. In our experiments, we studied splenocytes and did not examine separately the T cell responses to HomoCitJED presented by different antigen presenting cells.

Another limitation of our study is that we did not analyze multiple immune cell types, including T cell subtypes. However, we measured various serum cytokines and chemokines, which can be markers of immune cell phenotype. We found that the cytokine/chemokine profile differentiated HomoCitJED immunized female from male mice by discriminant analysis. The differences in the profile were mostly driven by IL-1 α and IL-5 levels, which were higher in female mice. IL-1 α is a pro-inflammatory cytokine involved in the pathogenesis of RA; it activates immune cells in the joint to release proteoglycans and proteases, leading to cartilage destruction and bone erosions [28]. Studies have shown that women with RA are more likely to have joint erosions [5] and that an antagonist of the IL-1 receptor effectively treats RA [29]. Less is known about the role of IL-5 in RA. It primarily acts upon eosinophils and appears to be involved in the regulatory mechanisms leading to the resolution of inflammation in autoimmunity [30]. In a serum transfer mouse model of RA, over-expression of IL-5 resulted in decreased arthritis severity [31]. In our DR4tg model, females had a higher ratio of IL-1 α /IL-5 compared to males (0.42 and 0.28, respectively), suggesting that in females the regulation of pro-inflammatory immune responses could be dysfunctional and lead to a higher incidence and severity of arthritis. Our HomoCitJED immunized DR4tg mice of either sex did not develop arthritis, likely due to the absence of the homocitrullinated or citrullinated targets in the joint.

The main focus of our study was DR4tg mice as a model for RA, but we also investigated wild-type B6 mice. We found that the effect of sex on immune responses to HomoCitJED were similar for B6 and DR4tg mice. Consistent with our prior study where the Shared Epitope influenced immune responses to homocitrullinated peptides, B6 mice were less likely to develop T cell and antibody responses to homocitrullinated antigens [21]. Surprisingly, B6 mice had higher levels of IL-12p40/IL-23 than DR4tg mice. These cytokines activate Th1 and Th17 cells, respectively that are critical in RA disease mechanisms [32]. However, IL-12 has been shown to be a regulator of autoantibody production [33,34] and may contribute to the lower AHCPA levels we detected in B6 versus DR4tg mice.

In conclusion, HomoCitJED induces higher levels of IL-1 α and IL-5 in female compared to male DR4tg mice, which may provide insight into mechanisms of sex differences in RA. Although T cell proliferative responses to HomoCitJED and levels of IgG AHCPA did not vary by sex, we did not investigate specific T cell subtypes nor different IgG isotypes. Given the sex differences we found in the cytokine profile, HomoCitJED immunized female mice may have an imbalance towards a more pro-inflammatory state. Future studies in mouse models and humans are required to further understand the mechanisms of sex differences in RA pathogenesis to identify sex-specific biomarkers and treatments.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Gagandeep Singh and Yifeng Song for their assistance with ELISAs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtauto.2020.100053>.

Author contributions

Study design: L.B., E.C., D.A.B. Data Acquisition: S.S., G.B., P.L. Data analysis and interpretation: L.B., E.C., D.A.B., S.S. Manuscript drafting: L.B., E.C. All authors critically reviewed and approved the manuscript.

Funding

This study was funded by a grant (#133685) from the Canadian Institutes of Health Research to E.C., D.A.B. and L.B. E.C. is supported by an award from the Calder Foundation.

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