Polymorphic estrogen receptor binding site causes Cd2-dependent sex bias in the susceptibility to autoimmune diseases

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Complex autoimmune diseases are sexually dimorphic. An interplay between predisposing genetics and sex-related factors probably controls the sex discrepancy in the immune response, but the underlying mechanisms are unclear. Here we positionally identify a polymorphic estrogen receptor binding site that regulates Cd2 expression, leading to female-specific differences in T cell-dependent mouse models of autoimmunity. Female mice with reduced Cd2 expression have impaired autoreactive T cell responses. T cells lacking Cd2 costimulation upregulate inhibitory Lag-3. These findings help explain sexual dimorphism in human autoimmunity, as we find that CD2 polymorphisms are associated with rheumatoid arthritis and 17-β-estradiol-regulation of CD2 is conserved in human T cells. Hormonal regulation of CD2 might have implications for CD2-targeted therapy, as anti-Cd2 treatment more potently affects T cells in female mice. These results demonstrate the relevance of sex-genotype interactions, providing strong evidence for CD2 as a sex-sensitive predisposing factor in autoimmunity.

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Women generally mount a more vigorous immune response than men, and are more susceptible to most autoimmune diseases\(^1,2\). These diseases have a strong but complex genetic component, and it has been difficult to identify the underlying polymorphisms\(^3-5\). The female predominance in autoimmunity is controlled by sex hormones\(^6\) and genetics\(^7\), not only through sex chromosomes but also through sex hormone-mediated regulation of autosomal genes. However, conclusive evidence is lacking, as it is difficult to positionally identify the underlying polymorphisms controlling complex traits in a sex-dependent manner.

Analysis of genetically segregated inbred animal strains dramatically enhances the power to isolate polymorphisms underlying complex diseases. Compared with association studies of human cohorts, studies in mice reduce environmental variability and allow for proof-of-concept experiments in biologically relevant systems, making it possible to conclusively identify genes underlying complex traits. In the context of previous such work to identify genetic loci that regulate autoimmune arthritis\(^8-10\) our research group identified a locus on mouse chromosome 3 (Cia21) that affects expression of the T cell activation marker Cd2 and regulates arthritis severity in females, but not in males\(^9\).

Here we report the causative mechanism to be a single polymorphism in an oestrogen receptor binding site (ERBS) within Cia21. This polymorphic ERBS orchestrates the expression of its surrounding genes in a sex-specific manner, including Cd2. We isolate this polymorphic ERBS in a congenic mouse line (D3-31) and use these mice to study the consequences of oestrogen-mediated regulation of Cd2 for T cell-dependent autoimmunity. Importantly, we find oestrogen regulation of Cd2 expression to be a conserved mechanism in humans likely contributing to the sexual dimorphism in T cell-mediated autoimmune diseases.

**Results** We have set out to identify major genetic polymorphisms underlying the development of autoimmune arthritis, using animal models. As part of these efforts a major quantitative trait locus (QTL) was identified on chromosome 3 qF2.2, which was termed Cia21\(^9\). Cia21 was identified from an intercross between the collagen-induced arthritis (CIA)-susceptible C57BL/10.

\textit{R}III (BR) and the CIA-resistant RIIIS/J (R3) mouse strains\(^11\). Cia21 contains several differentially expressed genes, including Cd2 and Ptpn22\(^9\). Both Cd2 and Ptpn22 play a key role in T cell activation and were proposed as strong candidate genes. The aim of the present study is to identify the polymorphisms underlying the Cia21 QTL.

A minimal non-coding genetic interval proximal to Cd2 explains Cia21. To dissect the Cia21 QTL, we bred heterozygous Cia21 mice and evaluated the resulting recombinant mice (shown in Fig. 1a) using CIA (Fig. 1b–g). Out of all the evaluated recombinants, only two, numbers 1 and 5, recapitulated the protective phenotype previously observed in Cia21 mice\(^9\). Thus, the Cia21 QTL results from individual contributions of these two sub-QTLs. Importantly, the phenotype driving recombinant regions 1 and 5 mapped to the previously proposed candidate genes Cd2 and Ptpn22, respectively. Recombinant fragment 1 (proximal to Cd2), however, was significantly smaller than fragment 5 providing better conditions for the positional identification of underlying polymorphisms. Therefore, we focused our efforts on the former.

Recombinant fragment 1 stretched from markers D3KV1 to MF31 (Fig. 1a, ca. 0.2 Mbp), but could be further redefined to the significantly smaller D3KV1-MF96 interval (ca. 0.02 Mbp) through a recombination assisted breeding strategy. Although recombinant fragments 1, 2 and 3 overlapped significantly, only fragment 1 regulated arthritis. Thus, we concluded that the causative polymorphisms must be positioned between markers D3KV1 and MF96 (Fig. 1a, highlighted yellow). D3KV1-MF96 is a non-coding 0.02 Mbp region proximal to Cd2, located in between the genes Atp1a1 and Igf3 (Fig. 1h).

We isolated the D3KV1-MF31 recombinant fragment (termed D3-31) in a congenic mouse line for further investigations. D3-31 congenic mice carry the parental R3 allele of D3-31 on an otherwise BR background. For simplicity, we hereon refer to the congenic line as D3-31 and to wild-type littermates as BR.

**Female D3-31 mice are protected from T-cell-dependent models of autoimmunity.** In accordance with previous data on Cia21, the R3 allele of D3-31 protected congenic mice in T-cell-dependent\(^12-14\) autoimmune inflammatory models, including collagen-induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE) and delayed type hypersensitivity (DTH) (Fig. 2a–f). We also investigated the T-cell-independent collagen antibody-induced arthritis (CAIA) model, but observed no phenotypic differences (Supplementary fig. 1). As the DTH model does not depend on B cells\(^15\), these results indicated a critical role for T cells. Interestingly, and as previously described for Cia21\(^9\), only female D3-31 mice were protected from T-cell-mediated autoimmunity (Fig. 2a–f). Thus, we concluded that D3-31 regulates T-cell-dependent autoimmune phenotypes, and likely T cells, in a sex-specific manner.

**Female sex hormones are required for the protective phenotype in D3-31 mice.** To discriminate between influence of sex chromosomes versus hormones, we performed CIA and EAE experiments in castrated female mice (Fig. 3a–c). Castration of female mice depletes gonadal production of 17-β-estradiol (E2)\(^16\), which constitutes the major circulating oestrogenic compound in females. Castration reverted the protective effect of the D3-31 fragment both in CIA and EAE (Fig. 3a–c), which demonstrated the crucial contribution of female sex hormones, most likely E2, to the protective phenotype in female D3-31 mice. We next set out to define the genetic mechanisms underlying this sexually dimorphic immune phenotype by sequencing the D3-31 fragment.

**Polymorphic ERBS in D3-31 interval affects E2-mediated transcriptional activity.** DNA sequencing of the D3-31 BR and R3 alleles revealed four single-nucleotide polymorphisms (SNPs) in the critical D3KV1-MF96 interval (Fig. 4a, b). None of the variants affected the coding region of known genes, indicating distal (cis) regulation of gene expression, likely by interfering with regulatory elements. Given our previous observations, we speculated that the identified polymorphisms could be located within an ERBS, interfering with sex-dependent regulation of gene expression.

Oestrogen receptors (ER\(\alpha\) and ER\(\beta\)) are nuclear hormone receptors that translate E2-mediated signalling. Both ER\(\alpha\) and ER\(\beta\) are expressed in immune cells\(^17\), and act as transcription factors regulating the expression of proximal and distant genes\(^18,19\). To test our hypothesis, we screened publicly available ChiP-seq data for Era binding sites overlapping with one or more of the sequenced SNPs within D3KV1-MF96 interval. Indeed, one of the SNPs, AC > GG on chr3:101310478-479 (termed SNP478), clearly overlapped with an Era binding site (Fig. 4c). In fact, bioinformatic analysis also revealed an oestrogen response element (i.e. an ER core binding motif) in close proximity to SNP478. We sought to verify this finding and could confirm binding of Era to SNP478 in mouse spleen cells using ChiP-qPCR (Fig. 4d). Comparison of
SNP478 between mouse inbred strains revealed that this SNP is in fact part of a highly polymorphic AC/GT simple repeat (Supplementary fig. 2, extracted from Kent et al.20).

To address whether SNP478 had functional consequences for E2-mediated transcriptional activity (i.e. interfered with the binding of Erα to the DNA), we cloned the candidate D3KV1 ERBS (±100 bp) in its two variant forms (AC and GG) into luciferase reporter constructs. Leveraging the fact that human and mouse Erα are highly conserved21, we assessed transcriptional activity of these constructs in the Erα expressing MCF-7 human cell line, treating them with increasing concentrations of E2 (Fig.4e). In the context of the reporter construct, an increased occupancy of the candidate ERBS by Erα (as a function of increasing E2) resulted in dose-dependent suppression of transcriptional activity. Although counterintuitive, similar observations have been reported elsewhere22. Given the stronger transcriptional inhibition when using the BR derived construct, we concluded that Erα/Erα has a higher affinity for the BR allele than for the D3-31 allele. Importantly, these data demonstrate that SNP478 has functional consequences for E2-mediated transcriptional activity.

Polymorphic ERBS in D3-31 interval leads to female-specific changes in Cd2 expression. Next, we sought to test the biological relevance of our findings by comparing the gene expression profile in lymph node cells from male and female D3-31 and BR mice. We observed female-specific changes in the expression of three genes adjacent to the polymorphic ERBS, namely Cd2, Igsf3 and Mab21l3 (Fig. 5a, b). We also investigated the expression of Atp1a1 as well as more distal genes (Cd101 and Slc22a15) previously implicated in the non-obese diabetic (NOD) mouse model of type 1 diabetes23, but found no changes in their expression level. Notably, the female-specific reduction of Cd2 expression in D3-31 mice was also evident at protein level (Fig.5c), correlating with our gene expression results and those previously reported in Johanesson et al.9.

Out of the differentially expressed genes, Cd2 was the only gene predominantly expressed in lymphoid tissue (Fig. 5d), particularly in activated Cd4+ T cells (Fig. 5e). Igsf3 and Mab21l3 regulate neural18 and ocular25 development, whereas Cd2 has been involved in immune function26 and associated with human autoimmune conditions4,27. Indeed, treatment of lymph node cells with anti-Cd2 mAb inhibited T cell activation as demonstrated by reduced secretion of pro-inflammatory cytokines (Fig. 5f). Considering these data and normal development of D3-31 mice, we concluded that Cd2 is driving the T-cell-dependent immune phenotype observed in D3-31 mice.
Given the sex-specific differences in gene expression, we next investigated the relation between E2 and Cd2 expression. T cells cultured in the presence of E2 upregulated Cd2 in a dose-dependent manner (Fig. 5g). Conversely, use of E2 depleted medium (achieved by using charcoal-stripped serum) reduced the expression of Cd2, and, more importantly, neutralised the observed differences in Cd2 expression between BR and D3-31 mice. Additionally, differences in Cd2 expression could be re-established by reintroducing E2 to the medium (Fig. 5h). This not only demonstrates direct regulation of E2 on Cd2 expression, but also proves that the identified polymorphisms interfere with this regulation. Consequently, we speculated that E2-mediated regulation of Cd2 was contributing to sex-specific differences in the T cell responses. A sex-dependent reduction of Cd2 expression in female D3-31 mice could likely limit the T cell responses.

E2-dependent regulation of Cd2 leads to sex-specific differences in T cell activation. To investigate the impact of sex hormone-dependent alterations in Cd2 expression on the T cell

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**Fig. 2** D3-31 mice are protected from T cell-dependent autoimmunity in a sex-specific manner. Collagen-induced arthritis (CIA) in a female and b male BR and D3-31 mice. Data was pooled from two independent experiments. Delayed-type hypersensitivity (DTH) reaction in c female and d male BR and D3-31 mice. Box upper and lower limits indicate interquartile range (25th/75th percentiles), the middle line indicates the median. Whiskers indicate 10th and 90th percentiles. Min. and max. values are plotted as individual dots. Data is representative of three independent experiments with similar results. MBP89-101-induced experimental autoimmune encephalomyelitis (EAE) in e female and f male BR and D3-31 mice. Data was pooled from two independent experiments. For all graphs, incidence and total number of mice used are indicated in parenthesis. Data are summarised as mean (SEM). Statistical significance was evaluated using a two-tailed non-parametric Mann-Whitney U test.
responses, we compared the activation of T cells between BR and D3-31 female mice. In a first set of in vitro experiments, we found an impaired response in D3-31 T cells to Tcr stimulation, as evidenced by reduced proliferation and IL-2 production (Fig. 6a, b). Importantly, the difference in T cell proliferation between BR and D3-31 mice could be enhanced in a dose-dependent manner by E2 (Fig. 6c), much like the E2-dependent expression differences observed for Cd2 (Fig. 5h).

A diminished T cell response in D3-31 mice was also evident in vivo. Compared to BR mice, D3-31 mice showed a lower level of antigen-specific T cells responses 10 days after immunisation with CIA antigen bovine collagen type II, as demonstrated by reduced secretion of pro-inflammatory cytokines in lymph node cell cultures recalled with antigen (Fig. 6d). Flow cytometry analysis of D3-31 draining lymph node cells revealed lower numbers of antigen experienced Cd40l^+ Cd4^+ T cells (Fig. 6e), which expressed reduced levels of Cd2 (Fig. 6f) and IL-17a after ex vivo restimulation with PMA (Fig. 6g, h). Differences in T cell activation status were also evident given lower numbers of induced regulatory T cells after immunisation (Fig. 6i, j).

Importantly, the observed differences in T cell activation were strictly sex-specific (Fig. 6e–j), mirroring sex-specific differences in Cd2 expression. Treatment with anti-Cd2 strongly reduced the expression of IL-17a in autoreactive Cd4^+ T cells (Fig. 6h), as well as expression of IL-17a and Foxp3 in naïve T cells (Fig. 6k), demonstrating the importance of Cd2 costimulation for the differentiation of Th17 and Treg type cells. Consequently, we concluded that reduced Cd2 expression in female D3-31 mice limits T cell activation in a sex-specific manner.

To further characterise how impaired Cd2 signalling affected T cells, we compared the proteomic landscape of anti-Cd3-stimulated Cd4^+ T cells in the presence or absence of anti-Cd2 mAb. Blocking of Cd2 signalling by means of anti-Cd2 mAb resulted in the selective upregulation of the immune inhibitory marker Lag-3 (Fig. 6l–n). Thus, we concluded that Cd2 costimulation is required for T cell activation and that impaired Cd2 signalling results in the upregulation of the inhibitory marker Lag-3.

**CD2 is associated with rheumatoid arthritis and is regulated by E2 in humans.** Our results in mice suggested a regulatory role for CD2 on T-cell-dependent autoimmunity, which is genetically determined in a sex-linked manner. We therefore went on to explore the relevance of our findings in humans, specifically in the context of rheumatoid arthritis (RA). In a genetic association study, we found a significant association between CD2 polymorphisms and RA (Fig. 7a and Supplementary Fig. 8). While this association was more often found in females than in males, this was likely due to higher prevalence of RA in females (female to male ratio 3:1). Interestingly, several of the SNPs associated with RA can enhance expression of CD2 (Fig. 7b), as we determined from the GTEx database. Further analysis of available microarray datasets revealed a mild yet significant correlation between CD2 expression in RA synovia and disease activity (Fig. 7c). Moreover, CD2 is strongly upregulated in the synovial tissue from RA patients when compared to osteoarthritis or healthy synovium (Fig. 7d). Thus, it is likely that CD2 is involved in joint inflammation, and that CD2 polymorphisms affecting its expression contribute to the development or perpetuation of joint autoimmunity.

Importantly, women expressed higher levels of CD2 than men, both in RA synovium and healthy PBMCs (Fig. 7c, e,
respectively), suggesting the E2-mediated regulation of CD2 observed in mice is conserved in humans as well. To corroborate our findings, we stimulated CD4+ T cells from healthy human donors with increasing amounts of E2. Firstly, we noticed a strong upregulation of CD2 in antigen experienced CD45RO+ T cells compared to their naïve CD45RA+ counterparts (Fig. 7f, g). But more importantly, expression of CD2 could be enhanced in CD45RO+ T cells by incubation with E2 in a concentration-dependent manner (Fig. 7h). Indeed, analysis of available ChIP-seq data30 revealed that ERα robustly binds the human CD2 gene locus (Supplementary Fig. 8). Thus, these data demonstrate the evolutionary conserved nature of E2-mediated regulation of CD2.

Anti-Cd2 mAb treatment affects T cells in female mice more than in male mice. We reasoned that hormonal regulation of CD2 expression could have implications for anti-CD2-mediated therapy, as previous research suggests that anti-CD2 (Alefacept) preferentially targets CD2hi T cells31. To test this, we compared the in vivo effects of anti-Cd2 mAb administration on circulating T cells from male and female mice (Fig. 8). Anti-Cd2 mAb treatment partially depleted circulating T cells and resulted in a relative expansion of effector CD44+ T cells in the remaining T cell pool, skewing the naïve CD62L−/effector CD44+ T cell ratio (Fig. 8c–h). This effect was significantly more pronounced in females, which, like in humans, expressed higher levels of CD2 in circulating T cells. Taken together, these data demonstrate that sex-dependent differences in Cd2 expression determine the response to anti-Cd2 mAb.

Discussion

Using forward genetics, we have positionally identified a polymorphic ERBS regulating T-cell-dependent autoimmunity. This site orchestrates expression of surrounding genes in a sex-specific manner, including expression of the T cell co-stimulatory molecule, Cd2. We find that E2-mediated regulation of Cd2 is a conserved mechanism that influences T cell activation in a sex-specific manner, contributing to the sexual dimorphism in autoimmune diseases.

Understanding the sexual dimorphic immune responses is fundamental for personalised medicine but is methodologically challenging. Common approaches to study this phenomenon rely on intricate manipulation of gonadal or hormonal systems32, which has yielded valuable insights but with limited physiological relevance. Our study provides a more physiological perspective by the identification of a naturally occurring polymorphism in an ERBS, which enables studies on sex-associated differences in T-cell-mediated autoimmunity. Since we used a hypothesis-free approach, our findings strongly suggest E2-mediated regulation of CD2 as a key physiological mechanism contributing to sex
differences in T cell responses and the susceptibility to autoimmunity.

Our results also highlight the importance of genotype-sex interactions for the sexual dimorphism in autoimmune diseases. While much attention has been devoted to the contribution of sex chromosomes, epigenetic mechanisms or direct actions of hormones to the sexually dimorphic immune responses, the interactions between sex and predisposing autosomal polymorphisms have remained elusive. Isolated studies have demonstrated sex-dependency of expression (e) QTLs and sex differences in the genetic associations to inflammatory diseases, but evidence is limited. Using a hypothesis-free approach, we conclusively identify a sex biased QTL with direct consequences for the development of autoimmunity. Polymorphisms in an ERBS within this QTL modulate E2-driven Cd2 expression, leading to sex-specific differences in T cell autoimmunity. Our results demonstrate not only that genetic polymorphisms influence hormonal regulation of gene expression, but also that genotype-sex interactions shape the sexually dimorphic immune response.

Independent of our sex-related findings, this study provides valuable insights into CD2 immunobiology. Polymorphisms in the CD2 locus have been previously associated with several autoimmune diseases, but not much attention was given to the mechanism of action of these polymorphisms. Similarly, CD2 has been explored as a therapeutic target, but its mechanism of action beyond its ability to stabilise the immune synapse, as blocking CD2 resulted in selective upregulation of the exhaustion marker, Lag-3. This finding is supported by studies showing an inverse correlation between CD2 expression and T cell exhaustion, and others showing upregulation of LAG-3 in CD8+ T cells after treatment with anti-CD25. Taken together, the data suggest that costimulation through CD2 is important for proper T cell activation, and that impaired CD2 signalling results in upregulation of inhibitory or anergy markers such as LAG-3.

Thus, there is a need to study this therapeutically promising pathway in a physiologically relevant context. The D3-31 mice used in this study exhibit discrete changes in Cd2 expression mediated by E2, thus enabling us to study the effect of Cd2 on T-cell-mediated autoimmunity in a physiological setting.

We show for the first time that changes in Cd2 expression, caused by natural polymorphisms, affect the T cell responses. Reduced Cd2 expression protected mice from T-cell-dependent inflammation and autoimmunity by reducing the activation and proliferation of antigen-specific T cells. This is consistent with studies demonstrating that CD2 membrane density is proportional to TCR signalling strength, and that peptide-based blocking of Cd2 signalling reduces CIA severity. Our results also implicate CD2 in the generation of Th17 and Treg-type responses. Mice with reduced Cd2 expression had a diminished T cell response characterised by a reduced expansion of Th17 and Treg cells. Accordingly, blocking Cd2 resulted in the suppression of both cell types in vitro. Indeed, Cd2 has been linked to Treg and Th17 phenotypes, and targeting CD2 is effective in the treatment of Th17-mediated inflammatory diseases like psoriatic arthritis. In summary, these data suggest a key role for CD2-mediated activation in the induction of Th17 and Treg cells.

Mechanistically, CD2 seems to play a role in T cell activation beyond its ability to stabilise the immune synapse, as blocking Cd2 resulted in selective upregulation of the exhaustion marker, Lag-3. This finding is supported by studies showing an inverse correlation between CD2 expression and T cell exhaustion, and others showing upregulation of LAG-3 in CD8+ T cells after treatment with anti-CD25. Taken together, the data suggest that costimulation through CD2 is important for proper T cell activation, and that impaired CD2 signalling results in upregulation of inhibitory or anergy markers such as LAG-3.
Our findings in mice are likely relevant to the sexual dimorphism observed in human autoimmune conditions. CD2 associates with RA and E2 regulation of CD2 expression is highly conserved in human T cells. Women, who are generally more prone to autoimmunity, express higher levels of CD2 than men. In mice, we demonstrate that these type of discrete and sex-specific differences in Cd2 expression result in sexually dimorphic T cell responses that affect autoimmune phenotypes. Thus, subtle, physiological changes in CD2 expression caused by natural polymorphisms likely modify the risk of T cell-dependent autoimmunity in humans. E2-mediated regulation of CD2 probably contributes to sex differences in the

**Fig. 6** Sex-specific differences in Cd2 expression limit the T cell responses in female D3-31 mice. a, b Proliferation (a) and IL-2 secretion (b) of Cd4+ lymph node T cells after stimulation with anti-Cd3/anti-Cd28 mAbs. c Proliferation of BR and D3-31 Cd4+ T cells as in a in the presence of increasing concentrations of E2 (10–100 nM). d Antigen recall assay showing pro-inflammatory cytokine secretion by lymph node cell cultures from CIA mice after recall with bovine collagen type II (bCII). Lymph nodes were harvested 10 days after immunization with bCII (day 10). e, f Quantitation of antigen-experienced Cd40I+Cd4+ T cells in lymph nodes from CIA mice (day 10) (e), and expression of Cd2 in these cells (f). Representations of gating (g) and quantification (h) of IL-17a+ Cd40I+ lymph node T cells from CIA mice (day 10) after ex vivo restimulation with PMA in the presence or absence of soluble anti-Cd2 mAb. i, j Representative gating (i) and quantification (j) of Cd25+Foxp3+ Tregs in lymph nodes from CIA mice (day 10). k Expression of IL-17a and Foxp3 in Cd4+ naïve T cells stimulated with PMA in the presence or absence of anti-Cd2 mAb. The data in a–k are representative of two independent experiments with similar results. l, m Flow cytometry data showing Lag-3 expression in Cd4+ T cells stimulated with immobilised anti-Cd3 mAb in the presence or absence of immobilised anti-Cd2 mAb. This experiment was performed once with n = 8 independent mouse biological replicates per group. n, o Flow cytometry data showing Lag-3 expression in Cd4+ T cells after culture with anti-Cd2 mAb. Representative gating in n and quantification in o. This experiment was performed three independent times with similar results. In all figures, each dot represents one independent mouse biological replicate. Data are summarised as mean (SEM). Statistical significance was evaluated using a parametric two-tailed t-test in d, and non-parametric two-tailed Mann-Whitney U test in all other experiments. Sequential flow cytometry gating strategies for a, e–j, m, n, as well as cell purity for l are provided in Supplementary figs. 10–15.
immune response, both in homeostasis and in autoimmune conditions.

Sex-dependent differences in CD2 expression have implications for several sexually dimorphic immune processes involving T cells or other CD2 expressing cells. Hormonal regulation of CD2 could contribute to more vigorous humoral immune responses in women, helping to protect their offspring from infections and viral infections, thus providing a possible explanation for the observed differences in the expression of CD2 in women. Indeed, we demonstrate that CD2 expression in women is preferentially higher than in men, and this is consistent with previous findings in mice.

Methods

Mice. The BR.Ca21.D3-31 congenic founder mice were obtained from a partial advanced intercross (PAI) described elsewhere and they were subsequently backcrossed for four additional generations. In order to ensure strain purity, BR.Ca21.D3-31 mice were screened with a custom designed 8k illumina chip at genome wide level and the mice were found to be devoid of any contaminating RIJS/J alleles. No SNPs were present between the congenic and the B10.RIII S/J alleles. No SNPs were present between the congenic and the B10.RIII S/J alleles. No SNPs were present between the congenic and the B10.RIII S/J alleles.

Preparation of mouse single cell suspensions. Briefly, spleen or lymph nodes were harvested and mechanically dissociated on a 40 µM cell strainer (Falcon) followed by $\text{CD}^2$+ T cells from blood of a representative human biological replicate. The experiment was done twice with similar results. Data shown is pooled from two independent experiments. In all figures, each dot indicates one independent human biological replicate. Data are summarised as mean (SEM). In d, e, and g, statistical significance was evaluated using a two-tailed non-parametric Mann-Whitney U test. Sequential flow cytometry gating strategies for f-h are provided in Supplementary fig. 16.

that has implications for the sexual dimorphism in the susceptibility to and treatment of autoimmune diseases like RA.
density gradient medium (Sigma) according to the manufacturer. Ethical approval by the Swedish ethical Review authority Etikprövningsmyndigheten, Uppsala, Sweden. Ethical permit number: Dnr 2020-05001. Informed consent was received from all participants.

**Cell culture.** $10^6$ splenocytes, $5 \times 10^5$ lymph node cells, or $10^5$ PBMCs were cultured in 200 µl of complete RPMI per well in Nunclon U-shaped bottom 96-well plates (Thermo Scientific). Cells were incubated at 37 °C and 5% CO2. Complete RPMI: RPMI 1640 with GlutaMAX™ (Thermo Scientific); 10% heat inactivated FBS (Thermo Scientific); 10 µM HEPES (Sigma); 50 µg/ml streptomycin sulfate (Sigma); 60 µg/ml penicillin G (Sigma); and 50 µM β-Mercaptoethanol (Thermo Scientific). FBS was heat-inactivated for 30 min at 56 °C. To assess the effect of 17-β-estradiol (Sigma) on CD2 expression, FBS was replaced with charcoal-stripped FBS (Thermo Scientific), and RPMI was replaced with RPMI without phenol red (Thermo Scientific). 17-β-estradiol was dissolved in ethanol.

**ELISA.** $10^6$ lymph node cells from CIA mice were plated per well and stimulated with 100 µg/ml bovine collagen type II (bCII) in complete RPMI for 48 h as described in cell culture. Supernatants were used for cytokine analysis. Flat 96-well plates (Maxisorp, Nunc) were coated overnight at 4 °C with the capture antibody (Ab, listed below) in PBS. After removing the coating solution, supernatant from cell cultures were added. Plates were incubated for 3 h at RT before washing (0.05% Tween PBS) and adding the biotinylated detection Ab (listed below) in PBS (1 h at RT). Plates were washed and incubated 30 min at RT with Eu-labelled streptavidin (PerkinElmer, 1:1000) in 50 mM Tris-HCl, 0.9% (w/v) NaCl, 0.5% (w/v) BSA and 0.1% Tween 20, 20 µM EDTA. After washing, DELFIA Enhancement Solution (PerkinElmer) was added, and fluorescence read at 620 nm (Synergy 2, BioTek). Monoclonal antibodies (mAbs) to Il-2 (capture Ab 5 µg/ml JES6-IA12; detection Ab 2 µg/ml biotinylated-JES6-5H4, Mabtech), Il-17a (capture Ab 5 µg/ml TC11-18H10.1; detection Ab 2,5 µg/ml TC11-8H4, BD), Ifn-γ (capture Ab 5 µg/ml AN18; detection Ab 2,5 µg/ml biotinylated R46A2, Mabtech).

**Analysis of mRNA expression.** $10^6$ lymph node cells per well were stimulated for 24 h using mAb LEAF hamster anti-mouse Cd3 (1 µg/ml, 500A2, BD Pharmingen) and LEAF hamster anti-mouse Cd28 (1 µg/ml, 37.51, BD Pharmingen) as described in cell culture. Cells were washed in PBS and RNA was extracted using Qiagen RNeasy columns according to the manufacturer without DNAse digestion. RNA concentration was determined using a NanoDrop 2000 (Thermo Scientific). Sample concentrations were normalised before proceeding with reverse transcription. Samples were stored at −20 °C for short-term storage. cDNA synthesis was carried out using the 2xScript cDNA synthesis kit (Bio-Rad) according to the manufacturer. qRT-PCR primers covered an exon–exon junction to minimise amplification of genomic DNA and were used at a final concentration of 300 nM.
The qPCR reaction was carried out using the iQSYBR Green Mix (Bio-Rad) in 100 µl of a 1:1 emulsion with CFA (BD) and PBS. Collagen-induced arthritis

Proliferation parameters were analysed and calculated using FlowJo 8.8.7.

Proliferation assay

2,10); TCRb (IP26); CD4 (OKT4); CD45RA (Hl100); and CD45RO (UCHL1).

Buffer. For intracellular cytokine staining, cells were stimulated in vitro with (H57-597); Cd4 (RM4-5); Cd8 (53-6.7); Cd19 (1D3, 6D5); Cd11b (M1/70); Cd11c

vortex. Beads were pelleted and incubating beads in 100 µl elution buffer56 at 65 °C for 30 min with occasional

Flow cytometry

10 µg/ml RNAse A (Thermo Scientific) to baselines

5×1 05 naïve lymph node cells were cultured per well in U 96-well plates as

1% formaldehyde

Ficoll/Transcription Factor Staining

Preparation of enriched Cd4

Luciferase reporter assay

2 × 104 MCI-7 cells were seeded into flat 96-well flat bottom plates (Thermo Scientific) and left to adhere overnight. Then cells were transfected with pGL4.17 (Promega) luciferase reporter construct containing the (H57-597) and pGL4.17.R3, respectively. ERBS cloning primers 5′-3′: Fw: AGATCTCGAGGGGAAAAAGCTCTGATT GGG: Rv: GTCAAGCTTTGAAAGAATTGTGATTGATGG. Cells were transfected in OPTIMEM medium (Thermo Scientific) using lipofectamine 3000 (Thermo Scientific) according to the manufacturer. The transfection mix (per well) contained 400 ng plasmid, 0.3 µl lipofectamine and 0.2 µl P3000 reagent. Respective stimuli (20 µg/ml PMA, 10-100 nM E2) were added after 24 h, and cells were further incubated overnight before lysis. Luciferase activity was measured using Pierce Firefly Luc One-Step Glow Assay Kit (Thermo Scientific) in a Synergy-2 plate reader (BioTek).

SNP sequencing and sequence comparison. SNP sequencing was performed on a Qiagen PSQ HS 96 Pyrosequencing using PyroMark Gold reagents (Qiagen) according to the manufacturer. Sequences were compared using Clustal Omega v1.2.4.9.

Genetic association study. Data for genetic variations within CD2-CD58 locus was extracted from previous Immunochip data published elsewhere (PMID: 23145596). After filtering these data correspond to 263 SNPs in 1940 healthy controls (M/F 524/1416) and 2762 RA patients (M/F 817/1945) from the Swedish EIRA study. Association was analysed by PLINK separately for female and male individuals. Data from IEU Open GWAS database used in Supplementary fig, 8 was analysed using l夙gwas in R v4.1.8.

Analysis of public microarray expression data. Microarray data was extracted from NCBI GEO Database and analysed using GEO2R (R v3.2.3; Bioconductor v2.14.16) and 2762 RA patients (M/F 817/1945) from the Swedish EIRA study. Association was analysed by PLINK separately for female and male individuals. Data from IEU Open GWAS database used in Supplementary fig, 8 was analysed using l夙gwas in R v4.1.8.

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Statistical analysis. Statistical analysis was performed using GraphPad Prism v6.0 or higher. Statistical significance was evaluated using non-parametric two-tailed Mann–Whitney U test unless stated otherwise in the figure legends. P values under 0.05 were considered statistically significant.

Proteomic analysis of enriched Cd4

T cells were enriched from naïve spleens using untouched Cd4 T cell mouse kit (Dynabeads, Life Technologies, purity 92%). 96-well U bottom plates were pre-coated with 1 µg/ml of anti-Cd3 and 1 µg/ml of anti-Cd2 in PBS for 3 h at 37°C. 2.5 × 104 CD4+ T cells were plated on the pre-coated plates and cultured for 48 h. Cell pellets were lysed in a buffer consisting of 1% SDS, 8 M urea and 20 mM EPPS pH 8.5 and sonicated using a Branson probe sonicator (3 s on, 3 s off pulses, 45, 30, 50 amplitude). Protein concentration was measured using BCA assay and subsequently 50 µg of protein from each sample were reduced with 5 mM DTT at RT for 30 min followed by alkylation with 15 mM IAA in the dark at RT for 45 min. The reaction was quenched by adding 10 mM DTT and the samples were precipitated using methanol-chloroform mixture. Dried protein pellets were dissolved into 8 M urea, 20 mM EPPS pH 8.5. EPPS (20 mM, pH 8.5) was added to lower the urea concentration to 4 M and LysC digestion was done at a 1:100 ratio (1 µg of protein, 30 µg of LysC). Then urea concentration was lowered to 1 M and trypsin digestion was conducted at a 1:100 ratio (Trypsin/protein, w/w) at RT for 5 h. TMTpro plex (Thermo Fisher Scientific reagents) were dissolved into dry

Delayed type hypersensitivity. A DTH reaction was elicited in the ear by immunising mice with 100 µg bCII (as described for CIA), and challenging 10 days later with 10 µg bCII intradermally in the dorsal part of the ear. The right ear was challenged with bCII, and the left one with vehicle (10 mM acetic acid). Ear swelling was measured at baseline, 48 h, and 72 h after challenge using a calliper. Plots show ear swelling as difference (Δ) to baseline.

Ovariectomy. In brief, ovaries of female mice were removed after a single incision through the back skin and bilateral flank incision through the peritoneum. Sham animals underwent the same procedure (i.e. incision in the back and peritoneum) without removing the ovaries. After the operation, mice were rested for a minimum of 14 days prior to immunisation for EAE or CIA as described elsewhere.
The dependent manner with automatic switching between MS and MS/MS modes using Fischer Scientiﬁc with 4% B for 5 min and increased to 26% B in 91 min, 95% B in 9 min, stayed at 63% B in 2 min and stayed at 4% B for 5 min and finally back to 1% B and stayed at 1% B for 7 min. This resulted in 96 fractions that were concatenated into 24 fractions. Samples were then dried using Speedvac and re-suspended into 2% ACN and 0.1% FA. The gradient was started with buffer A (98% milliQ water, 2% ACN and 0.1% FA) and buffer B (98% ACN, 2% milliQ water and 0.1% FA). The gradient was started with 4% B for 5 min and increased to 26% B in 91 min, 95% B in 9 min, stayed at 95% B for 4 min and finally decreased to 4% B in 3 min and stayed at 4% B for 8 more min. The injection was set to 5 µl corresponding to approximately 1 µg of peptides.

Mass spectra were acquired on a Q Exactive High Mass spectrometer (Thermo Fisher Scientiﬁc). The Q Exactive High MS frequency was performed in a data dependent manner with automatic switching between MS and MS/MS modes using a top-17 method. MS spectra were acquired at a resolution of 120,000 with a target value of 3 × 10^6 or maximum integration time of 100 ms. The m/z range was from 375 to 1500. Peptide fragmentation was performed using higher-energy collision dissociation (HCD), and the normalised collision energy was set at 33. The MS/MS spectra were acquired at a resolution of 60,000, with the target value of 2 × 10^5 ions and a maximum integration time of 120 ms. The isolation window and ﬁrst ﬁxed mass were set at 1.6 m/z units and m/z < 100, respectively.

TMT-10 labelling quantiﬁcation. Protein identiﬁcation and quantiﬁcation were performed with MaxQuant software (version 1.6.2.3). MS2 was selected as the quantiﬁcation mode and masses of TMTpro labels were added manually and selected as peptide modiﬁcation. Acetylation of N-terminal, oxidation of methionine and deamidation of asparagine and glutamine were selected as variable modiﬁcations while carbamidomethylation of the cysteine was selected as ﬁxed modiﬁcation. The Andromeda search engine was used with the UP00000589 Mus musculus database (22129 entries) with the precursor mass tolerance for the ﬁrst searches and the main search set to 20 and 45 ppm, respectively. Trypsin was selected as the enzyme, with up to two missed cleavages allowed; the peptide minimum length was set to seven amino acids. Default parameters were used for the instrument settings. The FDR was set to 0.01 for peptides and proteins. “Match between runs” option was selected with a time window of 0.7 min and an alignment time window of 20 min.

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Author contributions

G.F.L wrote the manuscript with the help of M.F. and R.H.; G.F.L. designed, performed, analysed and interpreted most experiments. M.F. and M.J. designed, performed and analysed all the experiments shown in Figs. 1b, 2 and 3. R.Z. and P.S. performed and analysed experiments requiring mass spectrometry (Fig. 6h). K.S.N. helped secure funding and reviewed the manuscript. E.L., M.A. and Y.H. helped with data collection, analysis and interpretation. All authors revised and approved the manuscript. R.H. initiated, designed and supervised the project and takes overall responsibility for the data.

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Competing interests

The authors declare no competing interests.

Additional information

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