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c-kit signaling differentially regulates ILC2 accumulation and susceptibility to CNS demyelination in male and female SJL mice

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Abstract

Multiple sclerosis (MS) preferentially affects women and this sex dimorphism is recapitulated in the SJL mouse model of MS, experimental autoimmune encephalomyelitis (EAE). Here we demonstrate that signaling through c-kit exerts distinct effects on EAE susceptibility in male and female SJL mice. Previous studies in females show that *Kit* mutant (W/W^v) mice are less susceptible to EAE than wildtype mice. However, male W/W^v mice exhibit exacerbated disease, a phenotype independent of mast cells and corresponding to a shift from a Th2 to a Th17 dominated T cell response. We demonstrate a previously undescribed deficit in c-kit^+ type 2 innate lymphoid cells (ILC2s) in W/W^v mice. ILC2s are also significantly reduced in EAE-susceptible WT females indicating that both c-kit signals and undefined male-specific factors are required for ILC2 function. We propose that deficiencies in Th2-promoting ILC2s removes an attenuating influence on the encephalitogenic T cell response and therefore increases disease susceptibility.

Introduction

There is abundant evidence that females are more susceptible than males to most autoimmune diseases. Multiple sclerosis (MS), a T cell-mediated demyelinating inflammatory disease of the central nervous system (CNS), is no exception to this as there are sex-biased differences in the incidence, age of onset, and clinical course of MS (1). Two clinically predominant variants of MS are recognized. Relapsing-remitting (RR) MS is defined by transient neurological symptoms; while primary progressive (PP) MS is characterized by steadily decreasing neurological function. Women are at least three times more likely than men to develop MS (1). Women are also more likely to present at a younger age and follow a RR course. In contrast, men are diagnosed later and more readily exhibit a PP course. The reason for this bias is not fully understood; however, X-chromosome dosage, differences in commensal microbiota, and most convincingly, the effects of sex hormones likely all contribute to the sex differences (2).

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The SJL mouse model of MS, experimental autoimmune encephalomyelitis (EAE), is an attractive model for studying the sexual dimorphism in MS susceptibility. Female SJL mice exhibit a higher incidence, more severe disease, and a more consistent relapsing pattern than their male counterparts (3). Previous studies in our laboratory using female SJL-*Kit* mutant mice (SJL-W/W^v) revealed a contribution of c-kit, the stem cell factor (SCF) receptor, to EAE pathogenesis. c-kit is expressed by most hematopoietic precursors and plays a role in the early development and survival of several lineages. W/W^v mice retain 10–20% of c-kit signaling, which permits the normal development of most hematopoietic cells (4). However, these mice have a profound mast cell deficiency and thus have been used extensively to study the contribution of mast cells to disease (4). Similar to what was first observed in female (WB × C57BL/6)F1-W/W^v mice (5), female SJL-W/W^v mice are mast cell deficient and exhibit decreased EAE severity, a phenotype that is reversed by selective mast cell reconstitution (6). Activated early in disease, mast cells regulate blood brain barrier (BBB) integrity and inflammatory cell influx into the CNS (7, 8).

The current studies are based on the surprising observation that unlike their WT male counterparts, male SJL-W/W^v mice are not protected from EAE nor do they exhibit the Th2-dominated T cell response associated with protection. Rather, they demonstrate exacerbated disease, corresponding to a more robust Th17 peripheral T cell response. Reconstitution of mast cells is not sufficient to restore protection indicating other c-kit regulated cell(s) mediate disease protection in WT male mice. Here we provide evidence of a previously undescribed developmental deficit in type 2 innate lymphoid cells (ILC2s) in male W/W^v mice. Best studied in allergic airway models, ILC2s are c-kit$^+$ and are essential for inducing Th2 immunity through production of IL-13 (7). We propose that an ILC2 deficiency in W/W^v male mice removes an attenuating influence on the autoreactive T cell response and therefore, increases disease susceptibility. Notably, the disease-induced accumulation of ILC2s to the CNS is also significantly reduced in WT female mice, corresponding to a reduced Th2 response and increased susceptibility to EAE. These data indicate that both c-kit signals and male-specific influences are required for ILC2 function in EAE.

Material and Methods

Mice

WT and W/W^v SJL mice were bred and genotyped as previously described (6). All mice were housed under specific pathogen free conditions in the Association for Assessment of Accreditation of Laboratory Animal Care approved facility at Northwestern University.

Active EAE Induction

Age-matched (6–10 weeks of age) littermates were immunized with 100 μg $PLP_{139–151}$ (Genemed Biotechnologies Inc.) emulsified in 500 μg CFA (4). One subcutaneous injection of 100 μl was administered to each posterior hind flank. Disease was scored as previously described (8).

Passive EAE Induction

Homogenized cells from isolated lymph nodes (inguinal, axillary, and brachial) of immunized donor mice were harvested 10 dpi and cultured for four days with 50 μg/mL $PLP_{139–151}$ and 10 ng/mL rmIL-23 (R & D Systems). 15×10^6 cells were transferred intravenously to recipient mice.

Isolation of CNS Leukocytes

Leukocytes were isolated from the combined digested spinal cord and brain of individual perfused mice using a Percoll gradient as previously described (9).

Flow Cytometry

Cells were stained with the indicated antibodies (eBioscience and Biolegend). Lineage stain included antibodies to CD3, CD19, Gr-1, and FcεRIα. For intracellular cytokine staining, the Fixation & Permeabilization Kit (eBioscience) was used and cells were stained after a 5 hour restimulation period with 50 μg/mL $PLP_{139–151}$.

Serum Testosterone Assay

Serum testosterone levels in naïve male weanlings (5–7 weeks old) caged in isolation were assessed using Parameter testosterone immunoassay (R&D).

Bone Marrow Mast Cell (BMMC) Reconstitutions

BMMCs were generated as previously described (8). For reconstitution, W/W^v mice were reconstituted intravenously with 4×10^6 or intracranially with 1×10^6 BMMCs.

Isolation of Peritoneal Cells

Anesthetized mice were injected intraperitoneally with 5–7mLs of PBS. Fluid was then drained from the peritoneum, centrifuged, and isolated cells were analyzed.

Toluidine Blue Staining of Meninges

Dural mast cells were identified using toluidine blue as previously described (8).

Quantitative Real-time PCR Analysis

RNA was isolated from spleens using the SV Total RNA Isolation System (Promega) after a 5-hour stimulation period with 50 μg/mL of $PLP_{139–151}$. cDNA was generated using SuperScript III Reverse Transcriptase (Life Technologies) and gene expression quantification was performed as previously described (6).

T cell differentiation

$CD4^+$ isolated T cells (Miltenyi L3T4 Microbeads) were cultured under polarizing conditions in wells coated with 1μg/mL anti-CD3 and 1 μg/mL anti-CD28 for 40 hours as previously reported (9, 10). On day 2, the cells were removed from co-stimulation and cultured with rmIL-2 (10 ng/mL) and polarizing cytokines for 6 days with media and cytokine replacement every two days.

Statistics

All statistics were performed using Prism 6 software (GraphPad Software, Inc.).

Results and Discussion

Male SJL-W/W^v mice exhibit mast cell-independent, exacerbated EAE compared to their WT counterparts

In contrast to the disease course in female W/W^v mice (6), male W/W^v mice exhibit significantly more severe EAE than their non-susceptible wild type (WT) male littermates (Figure 1A). Consistent with more severe clinical disease, the percentage and number of CD45^{hi} infiltrating leukocytes, predominantly T cells, is significantly increased in the spinal cords of W/W^v male mice at peak disease (17–19 days post-immunization [dpi]) (Figures 1B–D).

Testosterone is protective in EAE as evidenced by the increased susceptibility of castrated males and the reduced susceptibility of testosterone-treated females (11). As shown in Figure 1E, c-kit does not affect systemic testosterone levels, although we cannot eliminate the possibility that testosterone requires intact c-kit signaling to exert its protective effects.

In female W/W^v mice, mast cell reconstitution restores WT-like EAE susceptibility, indicating mast cells are pathogenic (6). We initially hypothesized that there are male-dependent differences in mast cell responses and that while mast cell activation in females elicits a pro-inflammatory response, mast cells promote an anti-inflammatory response in males. To test this hypothesis, mast cells (c-kit$^+$ FcεRIα$^+$) from male WT donors were transferred to male W/W^v mice and reconstitution was confirmed in the peritoneum and meninges (Supplemental Figure 1). Surprisingly, mast cell reconstitution had no effect on the disease course of the W/W^v males (Figure 1F). These results demonstrate that c-kit signals exert disparate effects on disease susceptibility in males and females. In females, c-kit$^+$ mast cells are pathogenic and promote inflammatory cell influx into the CNS by compromising the integrity of the blood-brain barrier (6–8). However in males, c-kit signals are protective and this protection is not evoked by restoration of mast cells alone.

Male SJL-W/W^v mice exhibit an increased encephalitogenic T cell cytokine response in preclinical EAE

We next asked if differences in autoreactive T cell responses could account for disparities in disease susceptibility. We first evaluated mRNA transcripts that encode the pro-inflammatory mediators IL-17, GM-CSF, and IFNγ in the spleen 10 dpi (12). As shown in Figures 2A and B, IL-17 and GM-CSF transcripts were increased in male W/W^v mice compared to WT controls; while no significant differences were observed in IFNγ transcripts (data not shown).

The PLP$_{139–151}$-specific T cell response of male WT and W/W^v mice was next compared directly *ex vivo* 8–10 dpi. W/W^v mice have increased numbers of CD3$^+$CD4$^+$ T cells in their draining lymph nodes during preclinical EAE, mirroring the expansion observed in immunized female WT mice (Figure 2C). Importantly, the PLP$_{139–151}$-specific T cell

response in male W/W^v mice is shifted from the Th2 response observed in WT males to a Th17 skewed response. A higher proportion of CD4$^+$ T cells in the draining lymph nodes produce IL-17 and GM-CSF in W/W^v mice (Figures 2D–E); whereas, IL-4 and IL-10 production dominate in WT male mice Figures 2D–E.

The enhanced pro-inflammatory PLP$_{139–151}$-specific T cell response in male SJL-W/W^v mice is not due to c-kit regulated T cell-intrinsic differences

The increased pro-inflammatory cytokine response of T cells in W/W^v males suggested that c-kit might influence intrinsic T cell function. T cells express c-kit during early maturation, but expression is lost during the double-negative stage of thymic development (13). Although W/W^v females have normal peripheral T cell responses (9), the long-term consequences of c-kit deficiency on T cell development and intrinsic function in male mice have not been determined. However, equivalent percentages and numbers of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells are present in the thymus, spleen, lymph nodes (cervical and inguinal), and blood of naïve male WT and W/W^v mice (Figures 2C, 4A–B and data not shown). Furthermore, *in vitro* differentiation of naive T cells from male WT and W/W^v donors yields similar frequencies of Th1, Th2, Th17 and Treg cells as defined by lineage-specific cytokine production and Foxp3 expression (Figures 3A and Supplemental Figure 2). Finally, encephalitogenic T cells from either male WT or W/W^v mice transfer equivalent disease to WT recipients (Figure 3B). Taken together, these data indicate that there are no intrinsic differences in T cell responsiveness between WT and W/W^v mice.

Male SJL-W/W^v mice have decreased numbers of type 2 innate lymphoid cell precursors (ILC2Ps)

We hypothesized that a c-kit$^+$ accessory cell population acts extrinsically to attenuate the autoreactive T cell response in male WT mice and that this population is deficient in W/W^v mice. While we have eliminated mast cells as sole contributors to protection in males (Figure 1F), subsets of both dendritic cells and innate lymphoid cells (ILCs) also retain c-kit expression as mature cells. Type 2 ILCs (ILC2s) are particularly attractive candidates for providing such an attenuating influence. Defined as CD45$^+$ Lineage$^-$ IL-7Rα$^+$ ST2$^+$ c-kit$^+$, ILC2s produce IL-5, -9 and -13 and thus exhibit functional parallels to Th2 cells (14). Despite very small numbers, ILC2s can have a profound impact on T cell priming (15–17). Their impact has been best studied in allergic airway models where they elicit a Th2-dominated response and suppress the expression of pro-inflammatory cytokines, such as IL-1β, TNF, and IL-23 (18).

Of note, while analyzing the thymic profiles of male WT and W/W^v mice, we observed differences in populations reported to be precursors of ILC2s (19). Despite comparable frequencies of CD8$^+$ and CD4$^+$ single and double positive cells (Figures 4A and B), there is a significantly reduced frequency of Lineage$^-$ CD44$^+$ CD25$^+$ c-kit$^+$ cells in W/W^v mice (Figures 4C and D). W/W^v mice also have deficits in the proportion of CD45$^+$ Lineage$^-$ $\alpha_4\beta_7^+$ CD25$^+$ cells in the bone marrow (Figures 4E–F). These cells express high levels of IL-7Rα, ST2, and c-kit (data not shown) and thus are similar to the previously defined bone marrow ILC2 precursors (20).

Mature ILC2s are reduced in EAE-susceptible SJL-W/W^v males and WT females compared to EAE-resistant WT male mice

We next quantified the mature ILC2 population in the draining lymph nodes and CNS of WT and W/W^v male mice during preclinical EAE using the gating strategy shown in Figure 5A. Ten dpi, ILC2 (CD45$^+$ Lin$^-$ IL-7Rα$^+$ ST2$^+$ c-kit$^+$) numbers significantly increase in the draining lymph nodes of WT, but not W/W^v, male mice (Figure 5B). A similar and more striking increase is observed in the CNS. Again, ILC2s accumulate in EAE-resistant WT but not EAE-susceptible W/W^v males (Figures 5C–D). Surprisingly, ILC2s also fail to significantly accumulate in the CNS of EAE-susceptible WT females (Figures 5C–D).

Furthermore, in immunized WT male mice, the increases in ILC2 numbers in the CNS correspond with a robust Th2 response as measured by IL-4 and IL-10 production by T cells (Figures 5E–F). In contrast, a relatively low percentage of T cells in the CNS of EAE-susceptible female and male W/W^v mice produce these Th2 cytokines.

Based on the previously described activities of ILC2s, we speculate that these cells mediate disease protection by limiting the encephalitogenic T cell response in male WT mice. Several independent studies provide support for this hypothesis. ILC2-deficient mice (ST2$^{-/-}$) exhibit increased production of IL-17 and GM-CSF by T cells and are more susceptible to EAE than their WT counterparts (21, 22). Transfer of WT encephalitogenic T cells to ST2$^{-/-}$ mice does not rescue WT-like EAE susceptibility, demonstrating that the protective effect of ST2 is T cell extrinsic, mirroring our observations (21). Finally, treatment with IL-33, which expands ILC2s (17), results in a Th2-dominated response and ameliorates EAE (23, 24).

We show that in the setting of diminished c-kit signals, ILC2s fail to develop. However, given the inability of ILC2s to protect in WT females, the functionality of these cells also depends on male-specific influences (hormones, microbiota, chromosomes, etc.). Although not yet well documented in humans, it is notable that these differences in ILC2 populations are also observed in the umbilical cord blood of newborns, where males have an increased frequency of ILC2s (25). Taken together with our novel observations, these data provide new avenues for investigation into sex-determined disease susceptibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMMCs	bone marrow derived mast cells
CNS	central nervous system
Dpi	days post immunization
EAE	experimental autoimmune encephalitomyelitis
ILC2	type 2 innate lymphoid cells
ILC2P	type 2 innate lymphoid cell precursor
MS	Multiple sclerosis
WT	wildtype

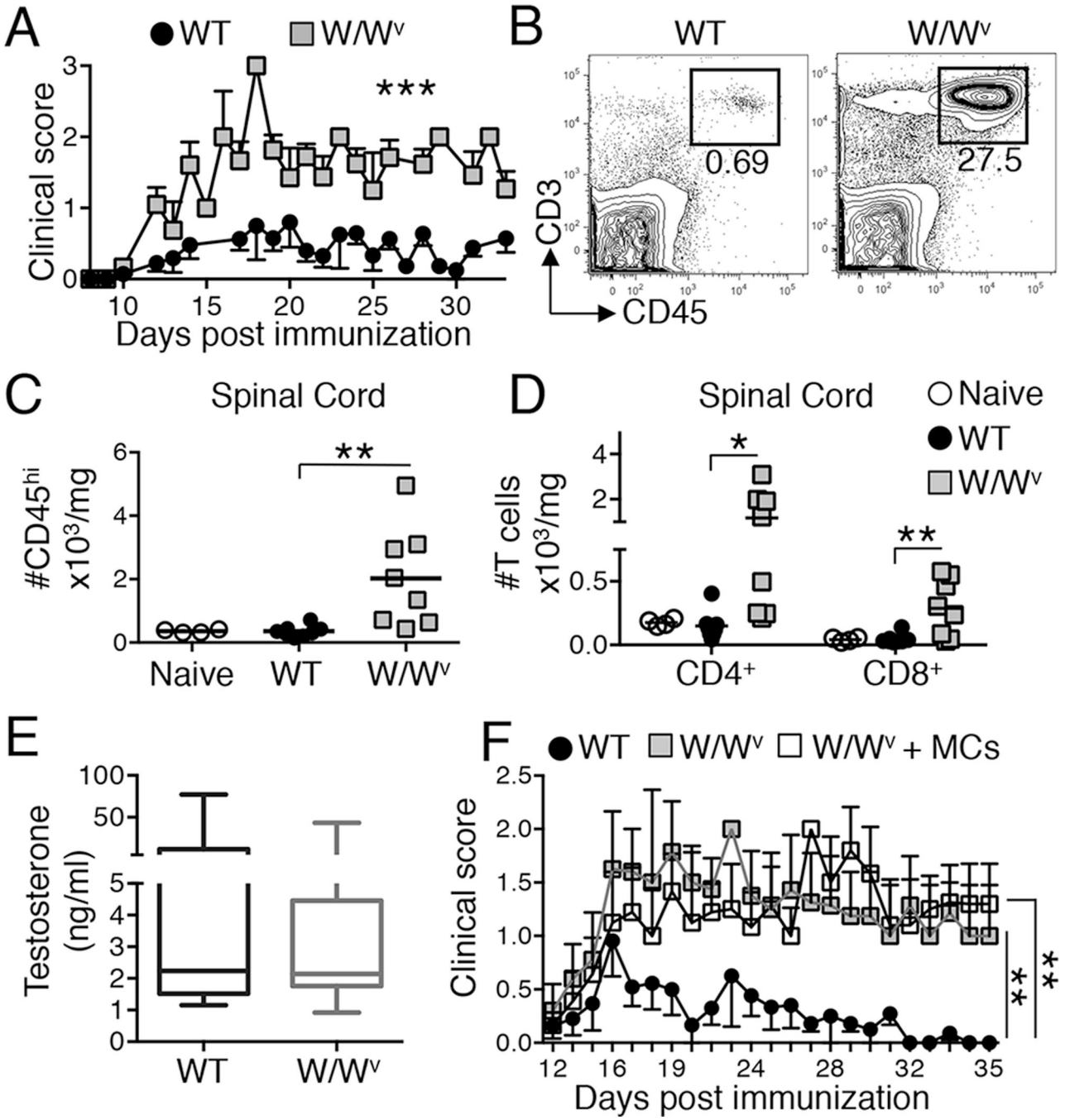

Figure 1. SJL-W/W^v male mice exhibit more severe EAE than their WT counterparts

A: Comparison of mean clinical score (A, *** p<0.001 by two way ANOVA) between male W/W^v (n=20) and WT (n=31) mice. 5 experiments. **B–D:** Comparison of infiltrating CD45^{hi} leukocytes (B, gate shows percentage of CD45^{hi}CD3$^+$ cells of total cells) in the spinal cords of male WT (n=8) and W/W^v (n=8) mice 17–19 dpi. The number of CD45^{hi} (C), CD45^{hi}CD3$^+$CD4$^+$ or CD8$^+$ T cells (D) in the spinal cord of male WT (n=8) and W/W^v (n=8) mice 17–19 dpi. N= naïve WT male mice (n=4). *p<0.05 and **p<0.01 by Student's *t* test. 2 experiments. **E:** Serum testosterone concentrations from naïve male W/W^v (n=13)

and WT (n=25) littermates assessed by ELISA. p>0.25 by Student's *t* test with Welch's correction. 3 experiments. **F:** W/W^v recipients were reconstituted with mast cells (MCs) and disease was induced 8 weeks later. Comparison of mean clinical score (F, ** p<0.01 by two way ANOVA) between reconstituted W/W^v (n=9), non-reconstituted W/W^v (n=7), and WT (n=17) mice. NS= not significant. 5 experiments.

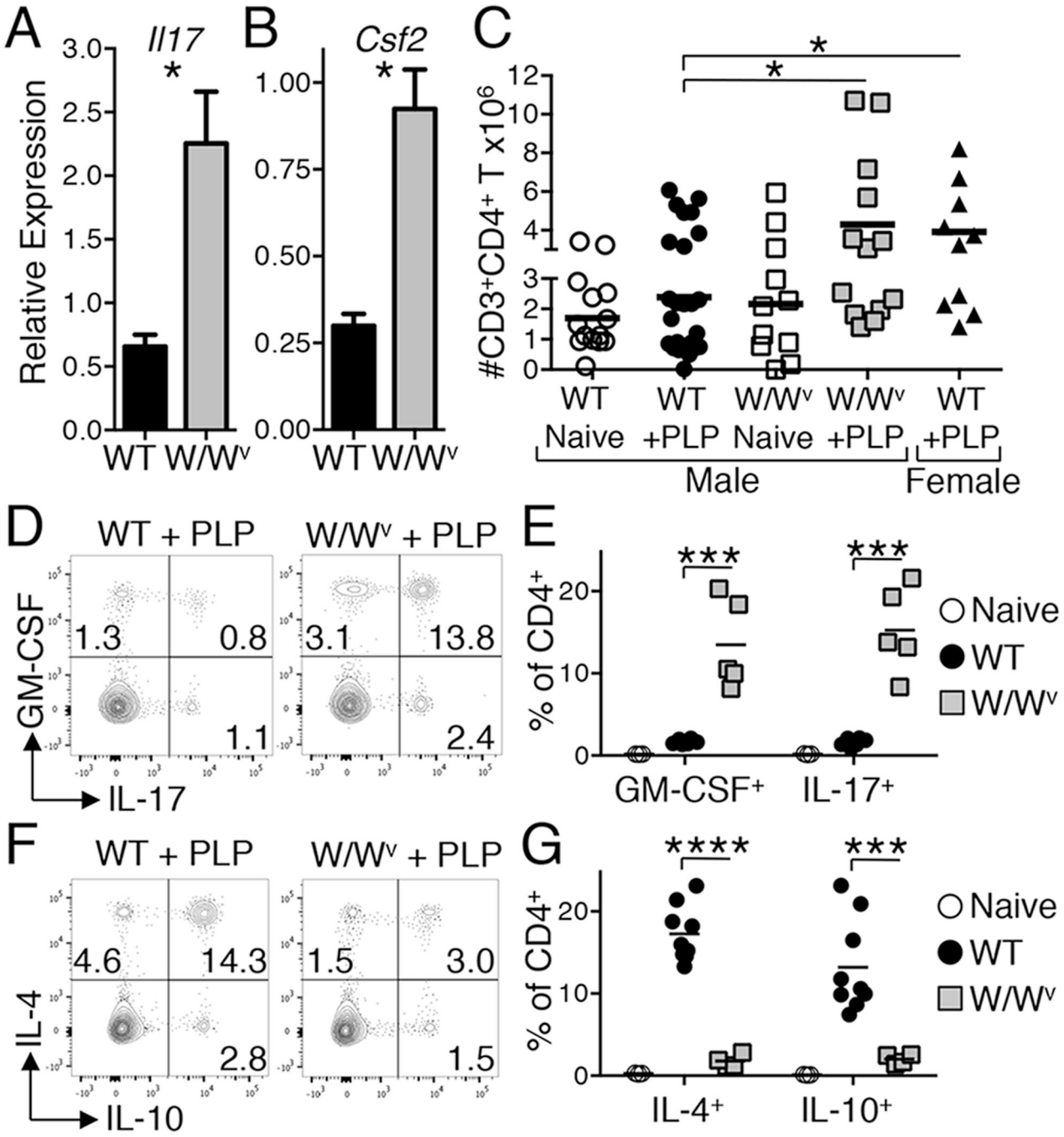

Figure 2. SJL-W/W^{v} male mice exhibit a more robust pro-inflammatory peripheral T cell response

A–B: Quantitative real-time PCR analysis of *Il17* (A) and *Csf2* (B) gene expression in the spleen of WT (n=4) and W/W^{v} (n=3) male mice 10 dpi. 2 experiments. **C**: Number of CD3^{+}CD4^{+} T cells in the draining lymph nodes of male WT (n= 26) and W/W^{v} (n= 16) mice 8–10 dpi. 5 experiments. **D–G:** *Ex vivo* IL-17/GM-CSF (D–E) and IL-4/IL-10 (F–G) expression by CD3^{+}CD4^{+} T cells in the draining lymph nodes of male WT naïve (n=3), WT immunized (n>6), and W/W^{v} immunized (n>5) mice 8–10 dpi after a 5-hour peptide

restimulation. Gates show percentage of cytokine producing cells of $CD3^+CD4^+$ cells. ***p<0.001 and ****p<0.0001 by Student's *t* Test. 3 experiments.

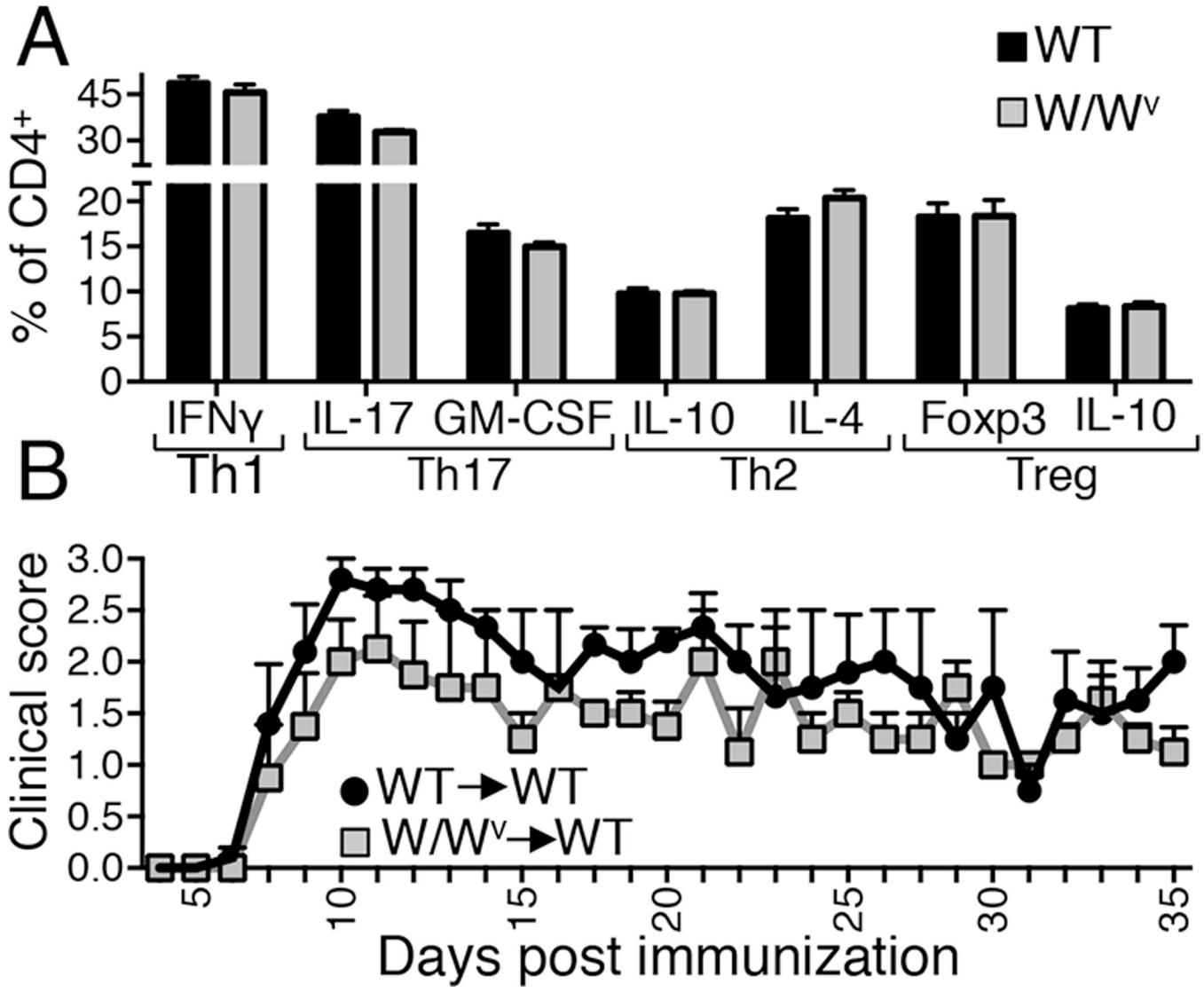

Figure 3. T cells from SJL-W/W^v male mice are not intrinsically more encephalitogenic

A: Percentage of cytokine-producing or Foxp3-expressing splenic CD4$^+$ T cells under Th1, Th2, Th17, and Treg differentiating conditions (A). Not significant by Student's *t* Test. n=3. 3 experiments. **B:** Encephalitogenic T cells from WT or W/W^v donors were transferred to naïve WT recipients (B). 2 experiments, n=4 for W/W^v and n=5 for WT T cell recipients. Not significant by 2-way ANOVA.

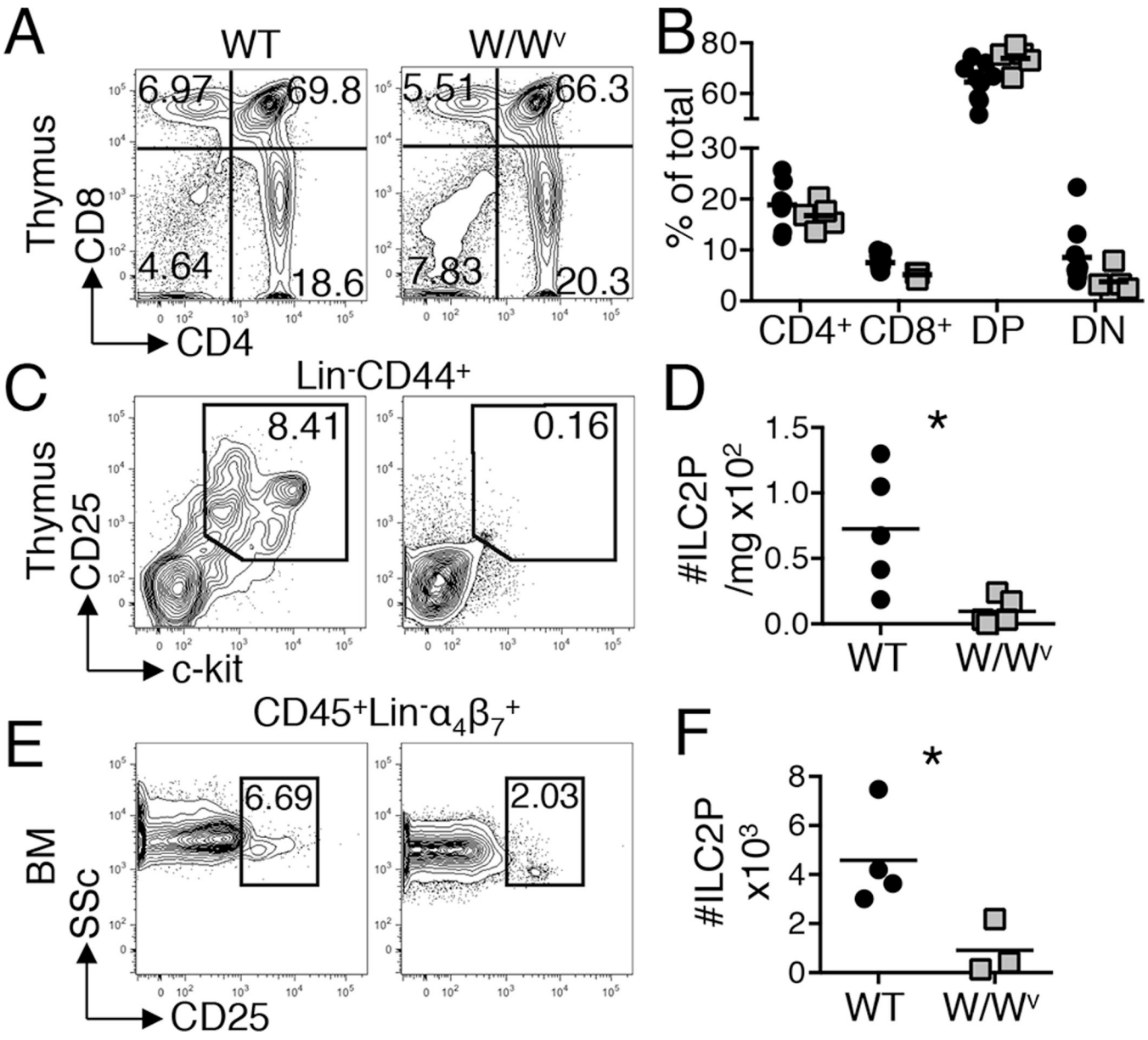

Figure 4. SJL-W/W^v males are deficient in type 2 innate lymphoid cell precursors (ILC2Ps)

A–B: Percentage of CD4$^+$ or CD8$^+$ single positive, double positive (DP), and double negative (DN) thymic T cells of WT (n=9) and W/W^v (n=5) mice. **C–D:** Percentage and number of thymic ILC2 precursors (ILC2P, Lineage$^-$ CD4$^-$ CD8$^-$ CD44$^+$ CD25$^+$ c-kit$^+$) of total thymic cells. 3 experiments. **E–F:** Percentage and number of bone marrow ILC2 precursors (CD45$^+$ Lineage$^-$ $\alpha_4\beta_7^+$ CD25$^+$). 2 experiments. *p<0.05 by Student's *t* Test.

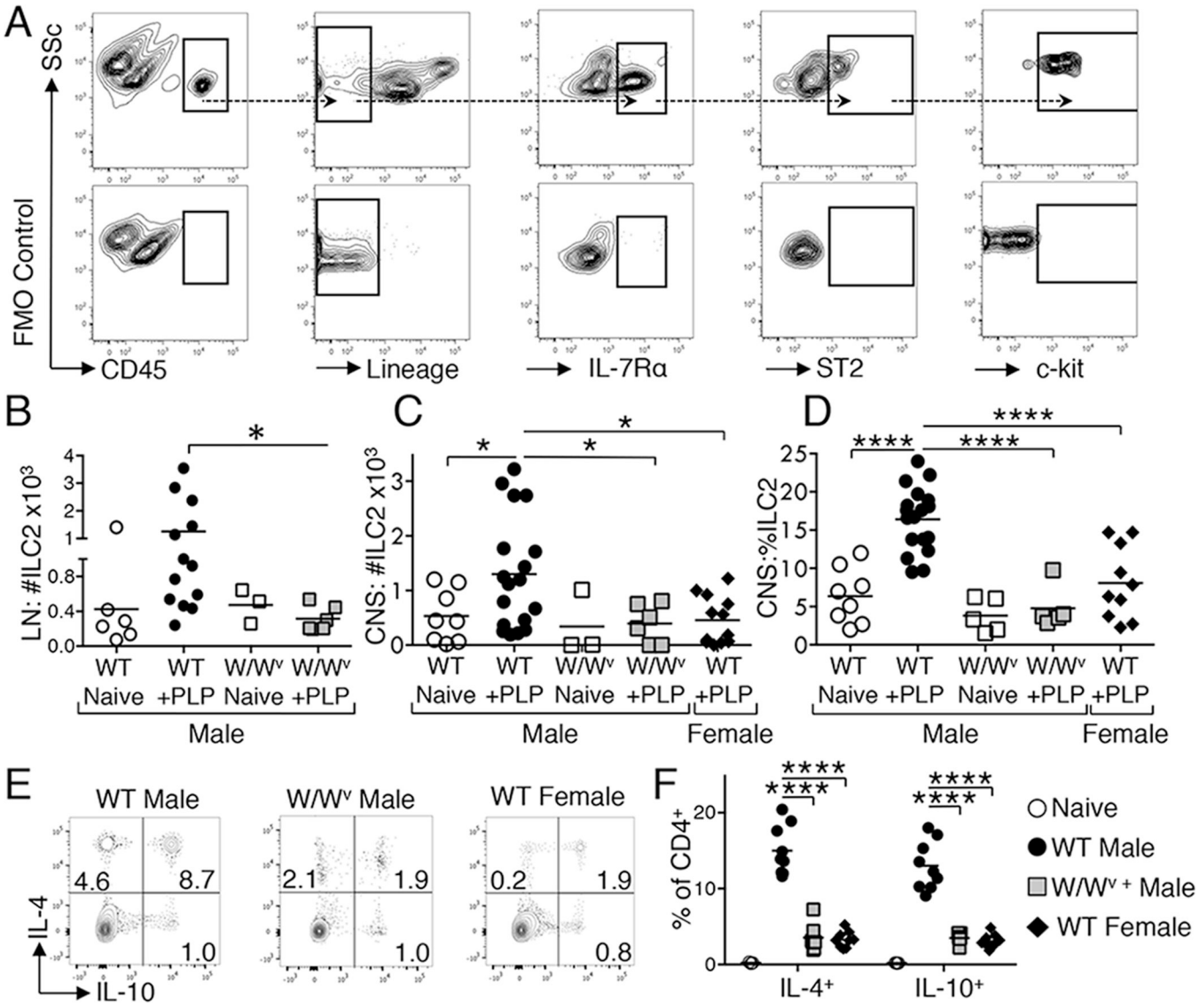

Figure 5. ILC2s accumulate and correspond to a Th2 dominated response in EAE-resistant WT male mice but not in susceptible W/W^v male or WT female mice

A: Gating scheme to identify mature ILC2s (top panels) using FMO negative controls (bottom panels). **B–C:** Number of mature ILC2s ($CD45^+Lineage^-IL\text{-}7R\alpha^+ST2^+ckit^+$) in the inguinal LNs (B) and CNS (C) 10 dpi. **D:** The percentage of ILC2s of the total ILC population ($CD45^+Lineage^-IL\text{-}7R\alpha^+$) in the CNS 10 dpi. 3 experiments. **E–F:** IL-4 and IL-10 production by infiltrating $CD45^{hi}$ $CD3^+$ $CD4^+$ $CD4^+$ T cells after a 5- hour restimulation period with $PLP_{139-151}$. (E). Percentage of $CD4^+$ cells producing IL-4 and IL-10 (F, n=3 for naïve and n>6 for immunized groups). 3 experiments. *p<0.05 and ****p<0.001 by Student's *t* Test.