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Cutting Edge: c-Kit Signaling Differentially Regulates Type 2 Innate Lymphoid Cell Accumulation and Susceptibility to Central Nervous System Demyelination in Male and Female SJL Mice

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Melissa A. Brown*

Multiple sclerosis preferentially affects women, and this sexual dimorphism is recapitulated in the SJL mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). In this study, 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 are wild-type 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 wild-type 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 remove an attenuating influence on the encephalitogenic T cell response and therefore increases disease susceptibility. *The Journal of Immunology*, 2015, 194: 000–000.

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 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 MS is defined by transient neurologic symptoms, whereas primary progressive MS is characterized by steadily decreasing neurologic 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 relapsing-remitting course. In contrast, men are diagnosed later and more readily exhibit a primary progressive 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-linked differences (2).

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 do 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 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 \times C57BL/6) F_1 - 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 integrity and inflammatory cell influx into the CNS (7, 8).

The present studies are based on the surprising observation that unlike their wild-type (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 that other c-Kit-regulated cells mediate disease protection in WT male mice. In this study, we provide

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Abbreviations used in this article: dpi, d postimmunization; EAE, experimental autoimmune encephalomyelitis; ILC2, type 2 innate lymphoid cell; ILC2P, precursor of ILC2; MS, multiple sclerosis; PLP, proteolipid protein; WT, wild-type.

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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.

Materials and Methods

Mice

WT and SJL- W/W^v 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 wk of age) littermates were immunized with 100 μ g proteolipid protein (PLP)_{139–151} (Genemed Biotechnologies) emulsified in 500 μ g CFA (4). One s.c. injection of 100 μ l 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 d postimmunization (dpi) and cultured for 4 d with 50 μ g/ml PLP_{139–151} and 10 ng/ml recombinant murine IL-23 (R&D Systems). Cells (15×10^6) were transferred i.v. 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 Abs (eBioscience and BioLegend). Lineage stain included Abs to CD3, CD19, Gr-1, and Fc ϵ RI α . For intracellular cytokine staining, a fixation and permeabilization kit (eBioscience) was used and cells were stained after a 5-h restimulation period with 50 μ g/ml PLP_{139–151}.

Serum testosterone assay

Serum testosterone levels in naive male weanlings (5–7 wk old) caged in isolation were assessed using a parameter testosterone immunoassay (R&D Systems).

Bone marrow-derived mast cell reconstitutions

Bone marrow-derived mast cells were generated as previously described (8). For reconstitution, W/W^v mice were reconstituted i.v. with 4×10^6 or intracranially with 1×10^6 bone marrow-derived mast cells.

Isolation of peritoneal cells

Anesthetized mice were injected i.p. with 5–7 ml 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 a SV Total RNA Isolation System (Promega) after a 5-h stimulation period with 50 μ g/ml 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 Biotec L3T4 Microbeads) were cultured under polarizing conditions in wells coated with 1 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28 for 40 h as previously reported (9, 10). On day 2, the cells were removed from costimulation and cultured with recombinant murine IL-2 (10 ng/ml) and polarizing cytokines for 6 d with media and cytokine replacement every 2 d.

Statistical analysis

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

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 do their nonsusceptible WT male littermates (Fig. 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 dpi) (Fig. 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 Fig. 1E, c-Kit does not affect systemic testosterone levels,

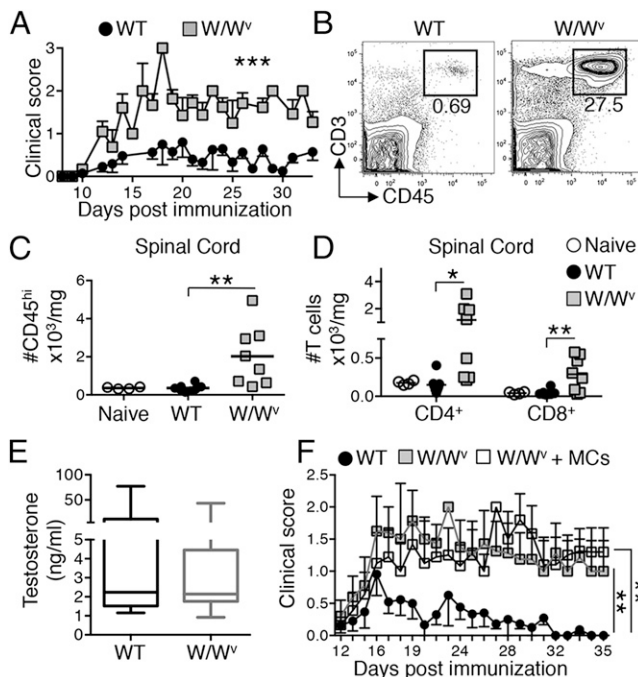


FIGURE 1. SJL- W/W^v male mice exhibit more severe EAE than do their WT counterparts. (A) Comparison of mean clinical score (***) by two-way ANOVA between male W/W^v ($n = 20$) and WT ($n = 31$) mice (five experiments). (B–D) Comparison of infiltrating CD45^{hi} leukocytes (B) (gate shows percentage of CD45^{hi}CD3⁺ cells) in the spinal cords of male WT ($n = 8$) and W/W^v ($n = 8$) mice 17–19 dpi. The numbers 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 are shown (for naive WT male mice, $n = 4$). * $p < 0.05$, ** $p < 0.01$ by Student t test (two experiments). (E) Serum testosterone concentrations from naive male W/W^v ($n = 13$) and WT ($n = 25$) littermates assessed by ELISA; $p > 0.25$ by Student t test with a Welch correction (three experiments). (F) W/W^v recipients were reconstituted with mast cells (MCs) and disease was induced 8 wk later. Comparison of mean clinical score (F) (** $p < 0.01$ by two-way ANOVA) between reconstituted W/W^v ($n = 9$), nonreconstituted W/W^v ($n = 7$), and WT ($n = 17$) mice is shown (five experiments).

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 that mast cells are pathogenic (6). We initially hypothesized that there are male-dependent differences in mast cell responses and that whereas mast cell activation in females elicits a proinflammatory 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 Fig. 1). Surprisingly, mast cell reconstitution had no effect on the disease course of the W/W^v males (Fig. 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 whether differences in autoreactive T cell responses could account for disparities in disease susceptibility. We first evaluated mRNA transcripts that encode the

proinflammatory mediators IL-17, GM-CSF, and IFN-γ in the spleen 10 dpi (12). As shown in Fig. 2A and 2B, IL-17 and GM-CSF transcripts were increased in male W/W^v mice compared with WT controls, whereas no significant differences were observed in IFN-γ 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 (Fig. 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. Higher proportions of CD4⁺ T cells in the draining lymph nodes produce IL-17 and GM-CSF in W/W^v mice (Fig. 2D, 2E), whereas production of IL-4 and IL-10 dominates in WT male mice (Fig. 2D, 2E).

The enhanced proinflammatory 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 proinflammatory 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 naive male WT and W/W^v mice (Fig. 2C 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 regulatory T cells as defined by lineage-specific cytokine production and Foxp3 expression (Fig. 3A, Supplemental Fig. 2). Finally, encephalitogenic T cells from either male WT or W/W^v mice transfer equivalent disease to WT recipients (Fig. 3B). Taken together, these data indicate

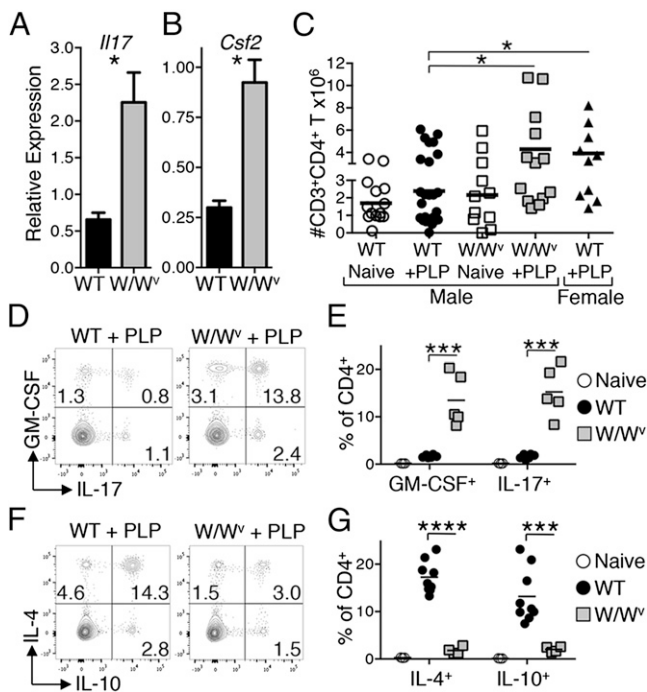


FIGURE 2. SJL-W/W^v male mice exhibit a more robust proinflammatory peripheral T cell response. (A and B) Quantitative real-time PCR analysis of *Il17* (A) and *Csf2* (B) gene expression in the spleen of WT ($n = 4$) and W/W^v ($n = 3$) male mice 10 dpi (two experiments). (C) Number of CD3⁺CD4⁺ T cells in the draining lymph nodes of male WT ($n = 26$) and W/W^v ($n = 16$) mice 8–10 dpi (five experiments). * $p < 0.05$ by Student t test. (D–G) Ex vivo IL-17/GM-CSF (D and E) and IL-4/IL-10 (F and G) expression by CD3⁺CD4⁺ T cells in the draining lymph nodes of male WT naive ($n = 3$), WT immunized ($n > 6$), and W/W^v immunized ($n > 5$) mice 8–10 dpi after a 5-h peptide restimulation. Gates show percentage of cytokine-producing cells of CD3⁺CD4⁺ cells. *** $p < 0.001$, **** $p < 0.0001$ by Student t test (three experiments).

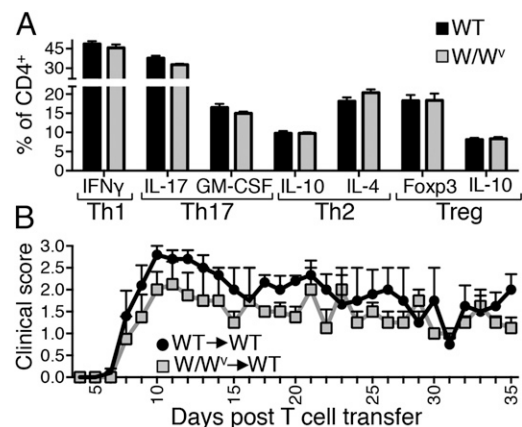


FIGURE 3. T cells from SJL-W/W^v male mice are not intrinsically more encephalitogenic. (A) Percentage of cytokine-producing or Foxp3-expressing splenic CD4⁺ T cells under Th1, Th2, Th17, and regulatory T cell differentiating conditions. Not significant by Student t test ($n = 3$; three experiments). (B) Encephalitogenic T cells from WT or W/W^v donors were transferred to naive WT recipients (two experiments; $n = 4$ for W/W^v and $n = 5$ for WT T cell recipients). Not significant by a two-way ANOVA.

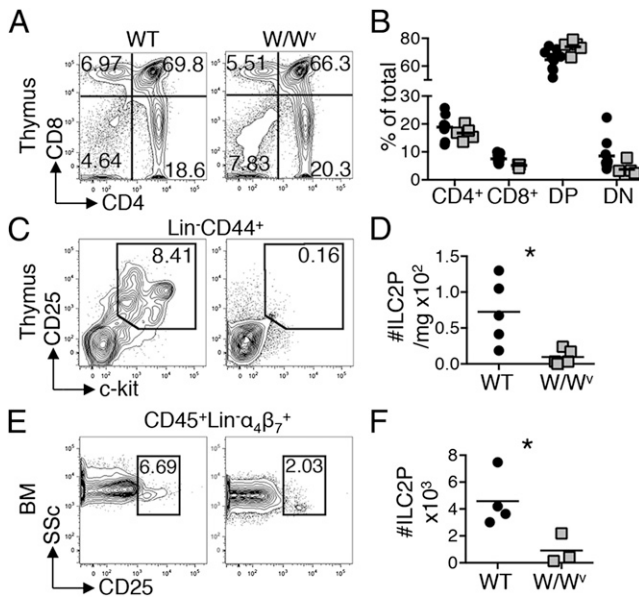


FIGURE 4. SJL-W/W^v males are deficient in ILC2Ps. (A and B) Percentage of CD4⁺ or CD8⁺ single-positive, double-positive (DP), and double-negative (DN) thymic T cells of WT (*n* = 9) and W/W^v (*n* = 5) mice. (C and D) Percentage and number of thymic ILC2Ps (Lineage⁻CD4⁺CD8⁻CD44⁺CD25⁺c-Kit⁺) of total thymic cells (three experiments). (E and F) Percentage and number of bone marrow ILC2Ps (CD45⁺Lineage⁻α₄β₇⁺CD25⁺) (two experiments). **p* < 0.05 by Student *t* test.

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 ILC2 precursors

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

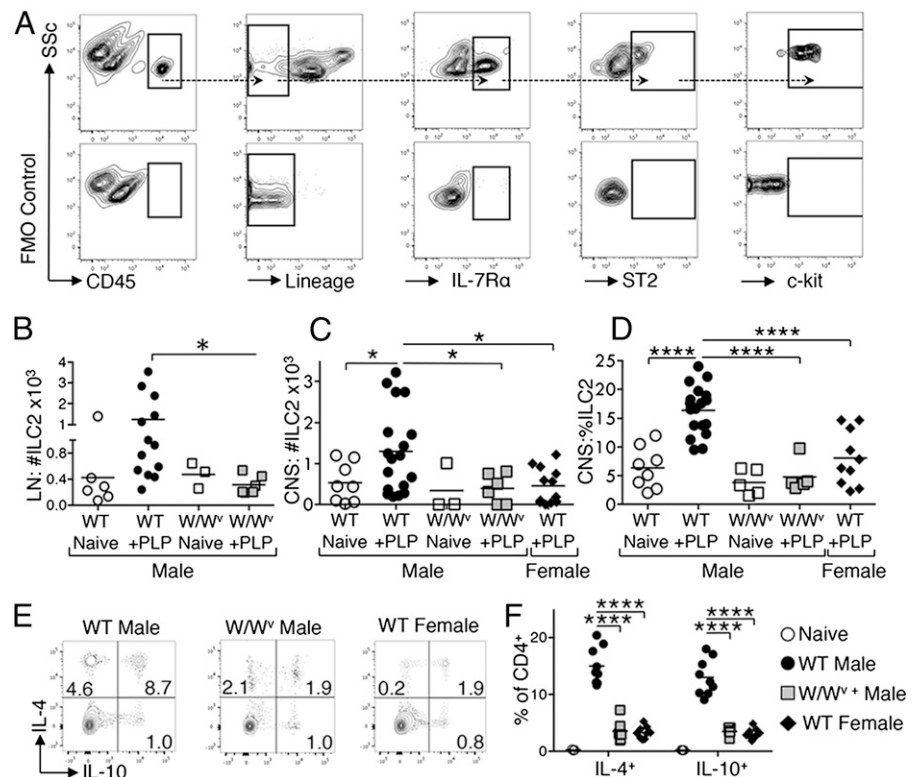
mice. Although we have eliminated mast cells as sole contributors to protection in males (Fig. 1F), subsets of both dendritic cells and ILCs also retain c-Kit expression as mature cells. ILC2s are particularly attractive candidates for providing such an attenuating influence. Defined as CD45⁺Lineage⁻IL-7Rα⁺ST2⁺c-Kit⁺, ILC2s produce IL-5, IL-9, and IL-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 proinflammatory 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 (ILC2Ps) (19). Despite comparable frequencies of CD8⁺ and CD4⁺ single- and double-positive cells (Fig. 4A, 4B), there is a significantly reduced frequency of Lineage⁻CD44⁺CD25⁺c-Kit⁺ cells in W/W^v mice (Fig. 4C, 4D). W/W^v mice also have deficits in the proportion of CD45⁺Lineage⁻α₄β₇⁺CD25⁺ cells in the bone marrow (Fig. 4E, 4F). 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 ILC2Ps (20).

Mature ILC2s are reduced in EAE-susceptible SJL-W/W^v males and WT females compared with 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 Fig. 5A. Ten dpi, ILC2 (CD45⁺Lineage⁻IL-7Rα⁺ST2⁺c-Kit⁺) numbers significantly increased in the draining lymph nodes of WT, but not W/W^v, male mice (Fig. 5B). A similar and more striking increase was observed in the CNS. Again, ILC2s accumulated in EAE-resistant WT but not EAE-susceptible

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 fluorescence minus one (FMO) negative controls (bottom panels). (B and C) Number of mature ILC2s (CD45⁺Lineage⁻IL-7Rα⁺ST2⁺c-Kit⁺) in the draining LNs (B) and CNS (C) 10 dpi. (D) The percentage of ILC2s of the total ILC population (CD45⁺Lineage⁻IL-7Rα⁺) in the CNS 10 dpi (three experiments). (E and F) IL-4 and IL-10 production by infiltrating CD45^{hi}CD3⁺CD4⁺ T cells after a 5-h restimulation period with PLP_{139–151}. (E) Percentage of CD4⁺ cells producing IL-4 and IL-10. For (F), *n* = 3 for naive and *n* > 6 for immunized groups (three experiments). **p* < 0.05, *****p* < 0.001 by Student *t* test.



W/W^v males (Fig. 5C, 5D). Surprisingly, ILC2s also fail to significantly accumulate in the CNS of EAE-susceptible WT females (Fig. 5C, 5D).

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 (Fig. 5E, 5F). 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 are 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 (e.g., hormones, microbiota, chromosomes). 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.

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Disclosures

The authors have no financial conflicts of interest.

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