Published in final edited form as:

J Autoimmun. 2016 September ; 73: 100–110. doi:10.1016/j.jaut.2016.06.015.

Meningeal mast cell-T cell crosstalk regulates T cell encephalitogenicity

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Abstract

GM-CSF is a cytokine produced by T helper (Th) cells that plays an essential role in orchestrating neuroinflammation in experimental autoimmune encephalomyelitis, a rodent model of multiple sclerosis. Yet where and how Th cells acquire GM-CSF expression is unknown. In this study we identify mast cells in the meninges, tripartite tissues surrounding the brain and spinal cord, as important contributors to antigen-specific Th cell accumulation and GM-CSF expression. In the absence of mast cells, Th cells do not accumulate in the meninges nor produce GM-CSF. Mast cell-T cell co-culture experiments and selective mast cell reconstitution of the meninges of mast cell-deficient mice reveal that resident meningeal mast cells are an early source of caspase-1-dependent IL-1β that licenses Th cells to produce GM-CSF and become encephalitogenic. We also provide evidence of mast cell-T cell co-localization in the meninges and CNS of recently diagnosed acute MS patients indicating similar interactions may occur in human demyelinating disease.

Keywords

Experimental autoimmune; encephalomyelitis (EAE); Multiple sclerosis (MS); Meninges; Mast cells; GM-CSF; IL-1beta; Inflammasome; Caspase-1; T helper cells; T cell licensing; Myeloid cells

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Author contributions

AER, MEWC, CL and MAB designed the experiments. AER and MEWC performed and analyzed the mouse experiments. YG performed the human immunohistochemistry analysis. AER, MEWC and MAB wrote the manuscript. CL edited the manuscript.

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Conflict of interest

All authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2016.06.015.

1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) [1]. Immune-mediated damage to the myelin sheath and myelin-producing oligodendrocytes leads to focal demyelinating lesions, neuronal and axonal damage, and variable neurological deficits. Although the etiology is still unknown, it is generally accepted that MS is initiated by the migration of peripherally primed myelin-specific Th1 and Th17 cells into the immune specialized CNS [2]. However, studies in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), reveal that the transit of Th cells from priming sites in the peripheral lymphoid organs to the CNS parenchyma is not direct. Myelin-primed Th cells have been shown to localize in the lungs for 2–3 days after adoptive transfer where they undergo reprogramming and up-regulate genes that facilitate CNS homing and entry [3]. Th cell transit through the gut-associated lymphoid tissues and exposure to commensal bacteria has also been reported to promote the differentiation and expansion of Th17 cells in a spontaneous model of EAE [4]. These data have lead to the idea that newly primed autoreactive T cells are not inherently pathogenic, but must first be “licensed” in peripheral tissues through interactions with other resident immune cells as in the lung or with commensal bacteria in the gut [5].

Yet several features of the meninges, highly vascularized mast cell rich tissues that surround both the brain and spinal cord and enclose the cerebrospinal fluid (CSF), suggest they are more relevant sites for promoting T cell encephalitogenicity. In preclinical EAE, myelin-specific T cells interact with myelin-loaded resident antigen presenting cells (APCs) in the meninges days before autoreactive T cells are detected in the CNS [6,7]. In contrast, Th cell accumulation in the lungs is non-specific and lung-residing myelin-reactive Th cells first localize almost exclusively in the meninges and blood 48 h after a secondary transfer to naïve mice [3]. Resident mast cells in the meninges play an essential role in regulating early inflammation in EAE as well. Activated within a day of disease induction, mast cells express multiple pro-inflammatory mediators including IL-1β, a cytokine that promotes T cell production of GM-CSF and is essential for EAE development [8,9]. Mast cells also express TNF, which stimulates an early influx of neutrophils to the meninges and loss of blood brain barrier (BBB) integrity, resulting in large-scale immune cell infiltration into the CNS [8,10].

The reports of coincident early activation of mast cells and T cells in the meninges as well as the expression of IL-1β by meningeal mast cells [8] lead us to hypothesize that interactions between these cells has consequences for promoting T cell encephalitogenicity. There is substantial *in vitro* and *in vivo* data demonstrating that mast cell-T cell interactions occur and result in cross activation. Perhaps most relevant to EAE is the demonstration that mast cells co-localize with Th17 and regulatory T cells (Tregs) in the draining lymph nodes and CNS of mice with EAE [11]. Through the production of IL-6 and engagement of OX40L, mast cells counteract effector Th cell suppression by Tregs, thus amplifying the autoreactive response.

In this report, we demonstrate that in the absence of mast cells, Th cells do not accumulate in the meninges nor do they generate a robust GM-CSF response. Mast cell-T cell co-culture experiments and selective meningeal reconstitution of mast cell-deficient mice reveal that

resident meningeal mast cells are an early source of caspase-1-dependent IL-1β, which in turn licenses Th cells to produce GM-CSF and become encephalitogenic. We also provide evidence of mast cell-T cell co-localization in the meninges in a subset of acute MS patients with prominent meningeal inflammation, suggesting that interactions between these cell types occur *in vivo* in human disease. These data have implications for developing meningeal mast cell targeted therapies that inhibit IL-1β production in early MS.

2. Methods

2.1. Mice

C57BL/6; WBB6F$_1$-*Kit*$^{W/Wv}$ and control WBB6; B6.129S2-Casp1tm1Flv/J and control C57BL/6NCr; and B6.PL-Thy1a/CyJ were purchased from Jackson Laboratory. The *Il1b*$^{-/-}$ mice were a kind gift from Dr. Thirumala-Devi Kanneganti. 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.

2.2. Active EAE induction

Four to eight week old mice were immunized with 100 μg MOG$_{35–55}$ emulsified in Complete Freund's Adjuvant and subject to blinded scoring as follows; 0: healthy, 1: flaccid tail, 2: ataxia, 3: full hind limb paralysis, 4: moribund, 5: death.

2.3. Generation of effector T cells

Ten to twelve days post immunization, the inguinal, axillary, and brachial lymph nodes were isolated from donor mice and homogenized. Isolated cells were cultured at 6 × 10^6 cells/mL for four days in 15% complete RPMI with 50 μg/mL of MOG$_{35–55}$ or OVA$_{323–339}$ supplemented with 10 ng/mL rmIL-23 (R & D Systems) and 12 ng/ml rmIL-12 (R & D Systems).

2.4. Adoptive transfer

Four × 10^6 T cell blasts were transferred intraperitoneally into recipient mice. No pertussis toxin was administered. Mice were scored as described above.

2.5. Generation of bonemarrow-derived mast cells (BMMCs) and meningeal reconstitution

BMMCs were generated as previously described [12]. Purity was determined by flow cytometry (>94% live cells co-expressing c-kit and FcεRIα). For reconstitution, 3–5 week old *Kit*$^{W/Wv}$ mice were reconstituted intracranially at the cranial vertex with 1 × 10^6 BMMCs.

2.6. CNS and meningeal leukocyte isolation

Mice were perfused by injection of 30 mL PBS into the left ventricle. Brains and spinal cords were dissected out and incubated in serum free RPMI with 300 U/mL Type IV collagenase (Worthington Biochemical Corp.) at 37 °C for 90 min. The samples were then homogenized over metal screens and leukocytes were enriched on a 40% Percoll (VWR)

gradient for flow cytometry analysis. The meninges were dissected from the calvarium under a light microscope. The meninges from 3 to 5 mice were pooled, digested with 1 mM EDTA (VWR) and 300 U/mL Type IV collagenase (Worthington Biochemical Corp.) at 37 °C for 30 min. The pooled samples were then homogenized over 70 μM cell strainers (MidSci) with plastic syringe plungers and processed for flow cytometry.

2.7. Flow cytometry

Cells were treated with FcBlock (anti-CD16/32-Biolegend) and stained with the indicated antibodies (eBioscience and Biolegend). For intracellular cytokine staining, cells were restimulated with 50 mg/mL $MOG_{35–55}$ for 5 h with 3 μg/mL Brefeldin A (eBioscience) for the last 3 h. Cells were assayed for cytokine production using the Fixation & Permeabilization Kit (eBioscience).

2.8. Quantitative real-time PCR analysis

RNA was isolated from co-cultured cells using the SV Total RNA Isolation System (Promega) and from the pooled meningeal samples using a hand held tissue homogenizer and the RNAeasy Fibrous Tissue Mini Kit (Qiagen). cDNA was generated using SuperScript III First-Strand Synthesis System (Invitrogen). Quantification was performed with iCycler iQ5 Real Time PCR Detection System (Bio-Rad) using PerfecTA SYBR Green SuperMix (BioRad). For each sample, the cycle number (Ct) at which the analysis threshold was reached, set in the linear range of the amplification curve (fluorescence = f[cycle]), was calculated. The relative expression of the cDNA template was calculated as the ratio of 2ΔCt for each transcript tested relative to a control housekeeping gene (*Hprt*). PCR primers are listed below.

Gene	Forward	Reverse
Tpsab1	CTGTTGGTGTTAGGAACCAGG	GTGCTGAATGCTTGTTTGCCA
Hdc	CGTGAATACTACCGAGCTAGAGG	ACTCGTTCAATGTCCCCAAAG
Tnf	GCCACCACGCTCTTCTGTCT	GGTCTGGGCCATAGAACTGATG
Il1b	GACGGCACACCCACCCT	AAACCGTTTTTCCATCTTCTTCTTT
Il17	AGCTCATCCGAGTGGTCCAC	GCTTCCTGAGGCTGGATTCC
Csf2	ATGCCTGTCACGTTGAATGAAG	GCGGGTCTGCACACATGTTA
Ifng	ACGTCATCCGAGTGGTCCAC	GCTTCCTGAGGCTGGATTCC

2.9. Preparation and toluidine blue staining of meninges

Following perfusion, the calvarium (containing the meninges) was dissected and fixed in formalin for 24 h before transfer and storage in PBS. The calvarium was then stained with acidic toluidine blue for 15 s and rinsed three times in distilled water. The meninges were separated from the calvarium while submerged in distilled water and floated onto glass slides. After the tissue was dried and adhered to the slide, it was covered with a coat of clear nail polish. Mast cells are visualized as granulated purple chromatic cells under 10 × magnification with a Primo Plan ACHROMAT 10 ×/0.25 lens at room temperature with a Zeiss Primo Star light microscope using IC Capture 2.0 (The Imaging Source). The average

number of mast cells in 18 1 mm × 1 mm squares in 6 different regions was calculated and expressed as number of mast cells per mm^2.

2.10. Mast cell: T cell co-culture

Effector T cells were generated as described above. $CD4^+$ T cells were isolated either with Dynabeads® FlowComp™ Mouse CD4 positive isolation beads (Invitrogen) or CD4 (L3T4) MicroBeads (Miltenyi). BMMCs were generated as described above and cultured with isolated $CD4^+$ Th cells at a 1:1 ratio for 2–6 h at 37 °C with or without plate-bound anti-CD3 (2 μg/mL) and anti-CD28 (5 μg/mL). Following co-culture, T cells were separated from the BMMCs by bead separation. Recombinant mouse IL-1β (R &D) was used at 2, 10, and 50 ng/ml. Recombinant mouse IL-18 (R &D) was used at 2 and 50 ng/ml.

2.11. Histology

Formalin fixed, paraffin embedded, 5 mm thick sections from archival MS CNS tissue were analyzed for the presence of meningeal and parenchymal mast cells. Inclusion criteria (Supplemental Fig. 1) were: i) pathological diagnosis of confluent demyelinating disease consistent with MS; ii) presence of meninges; iii) autopsy performed within 3 years of symptom onset; and iv) sufficient tissue for additional histopathological analysis. Among 20 MS cases, 11 blocks from 11 cases (5 females, 6 males, median age: 36, range: 18–71 years) met the inclusion criteria. Sections were stained with hematoxylin-eosin, Luxol fast blue, and by immunohistochemistry with antibodies against proteolipid protein (Serotec, MC839G) and CNPase (Convance, SMI91) for myelin, CD3 (Serotec, MCA1477) for T cells, and tryptase (Abcam, ab2378) for mast cells. Demyelinating activity was classified according to previously published criteria [13].

2.12. Statistics

All statistics were performed with GraphPad Prism 6.0. Specific statistical tests are provided in each figure legend.

3. Results

3.1. Myelin specific Th cells accumulate in the meninges in early EAE

To study T cell-mast cell interactions in the meninges we utilized an adoptive transfer system that allows tracking of MOG-primed T cells. $Thy1.1^+$ donor mice were immunized with $MOG_{35–55}$ peptide. Ten days post immunization, lymph node cells were isolated and cultured for 4 days with $MOG_{35–55}$ peptide under conditions that induce Th1 and Th17 cells. Four × 10^6 T cell blasts were then transferred to $Thy1.2^+$ recipients. As a control, cells from $OVA_{323–339}$-immunized $Thy1.1^+$ mice were also expanded *in vitro* and transferred to $Thy1.2^+$ recipients. $Thy1.1^+$ cells were examined in the meninges (Fig. 1a,c) and the CNS (Fig. 1b,c) at days 3 and 6 post transfer. At both time points significantly more MOG-specific Th cells than OVA-specific Th cells were detected in the meninges and the CNS (Fig. 1c). This selective accumulation of MOG-specific Th cells likely reflects their interaction with myelin-bearing APCs in the meninges that serve to activate and retain MOG-specific T cells as previously reported [6,7,14].

Consistent with the idea that T cells traffic through the meninges prior to their entry into the CNS [14–16], it is notable that 30 times more MOG-specific Th cells are detected in the meninges compared to the CNS ($4.81 \times 10^4 \pm 1.14 \times 10^4$ vs. $0.16 \times 10^4 \pm 0.05 \times 10^4$ respectively) at day 3 (Fig. 1c). However, this ratio changes by day 6, when similar numbers of Th cells are observed in the meninges and CNS ($4.71 \times 10^4 \pm 1.56 \times 10^4$ vs. $3.63 \times 10^4 \pm 1.00 \times 10^4$ respectively).

3.2. Mast cells are activated by T cell transfer and regulate the accumulation of myelin-specific Th cells in the meninges

Many of the insights into mast cell contributions to EAE were gained in studies utilizing $Kit^{W/Wv}$ mice, which exhibit an 80–90% reduction in c-kit signaling and lack mast cells [17]. We have previously shown that female $Kit^{W/Wv}$ mice are protected from severe EAE and this protection is lost upon the systemic or local meningeal reconstitution of mast cells [10,12,18]. To determine whether mast cells influence T cell transit to the meninges, $MOG_{35–55}$-primed T cells from Thy1.1$^+$ wild type donors were transferred to either wild type or $Kit^{W/Wv}$ Thy1.2$^+$ recipients and their presence in the meninges was assessed 1 day post transfer. As shown in Fig. 2a,b, a discrete population of Thy1.1$^+$ CD4$^+$ T cells is observed in wild type recipients, whereas a much smaller population was detected in $Kit^{W/Wv}$ recipients. Intracranial injection of mast cells, which selectively reconstitutes mast cells in the meninges and cervical lymph nodes of $Kit^{W/Wv}$ mice [10], restores T cell influx to wild type levels (Fig. 2a,b). This deficit in early T cell accumulation in the meninges of $Kit^{W/Wv}$ mice corresponds with the failure of these T cells to cause disease upon adoptive transfer (Fig. 2c).

Early T cell accumulation in the meninges of wild type mice corresponds to a dramatic induction of *Ccl1*, *Ccl2* and *Ccl17* (Fig. 2d–f), genes that encode T cell-attracting chemokines, as early as 12 h post T cell transfer. The expression of these chemokines is reduced in $Kit^{W/Wv}$ mice consistent with previous reports that they are expressed by mast cells [19,20]. We also detect c-kit-dependent expression of *Icam1*, encoding a molecule expressed by mast cells and other cells that mediates T cell activation through interaction with LFA-1 (Fig. 2g) [21].

The c-kit-dependent expression of chemokines and adhesion molecules indicates that T cell transfer alone can elicit mast cell gene expression in the absence of adjuvants commonly used in active disease induction. To further test this possibility, graded numbers of $MOG_{35–55}$-primed T cells were transferred to wild type recipients. Mast cell numbers and the expression of mast cell-associated gene transcripts within the meninges were assessed 1 day post transfer. As shown in Fig. 2h and i, mast cell numbers do not increase upon T cell transfer at this time point. However, several inflammatory transcripts are significantly induced in a dose dependent manner with the highest expression in mice that received the most T cells. These include transcripts not expressed by T cells such as the mast cell-specific gene, *Tpsab1*, which encodes tryptase (Fig. 2j) and *Hdc*, which encodes histidine decarboxylase (Fig. 2k). *Il1b* and *Tnf*, which are expressed by multiple cell types including mast cells, T cells and macrophages, are also induced (Fig. 2l,m). Similar to chemokine

genes, these transcripts are induced as early as 12 h post transfer and this expression is largely c-kit dependent (Fig. 2n–q).

3.3. Robust GM-CSF production by Th cells is acquired post priming in wild type but not $Kit^{W/Wv}$ mice

Myelin-specific Th cells accumulate in the meninges of disease-susceptible wild type but not disease-resistant mast cell-deficient recipients as early as day 1 (Fig. 2a–c). This observation suggests that T cell reactivation in the meninges enhances the expression of pathogenic cytokines such as GM-CSF, conferring encephalitogenicity. To test this possibility, T cells were primed in $Thy1.1^+$ wild type mice and reactivated *in vitro* under conditions that do not favor robust GM-CSF production. T cell cytokine expression was compared prior to transfer and 12 days post transfer in both the meninges and CNS of $Thy1.2^+$ wild type and $Kit^{W/Wv}$ adoptive transfer recipients. Prior to transfer, T cell blasts express both IL-17 and IFNγ, but negligible GM-CSF (Fig. 3a). Upon transfer fewer Th cells are present in the meninges and CNS of $Kit^{W/Wv}$ mice relative to wild type mice as expected (Fig. 3b,d) and only a small proportion of these are $Thy1.1^+$ (Fig. 3c,e). Notably, the frequency of $GM\text{-}CSF^+$ and $IL\text{-}17^+$ T cells in both the meninges and CNS is dramatically reduced in $Kit^{W/Wv}$ recipients, while the frequency of IFNγ-producing T cells does not differ (Fig. 3f, g). Although these experiments cannot distinguish whether the enhanced GM-CSF and IL-17 expression by T cells is acquired in the meninges or during transit to the meninges, they confirm that post-priming T cell licensing in the meninges is c-kit dependent.

3.4. Mast cell-derived IL-1β directly augments GM-CSF production by Th cells

T cell transfer elicits c-kit dependent Il1b expression in the meninges (Fig. 2m,q) and Th cells in the meninges produce a strong GM-CSF response (Fig. 3f). Thus we hypothesized that IL-1β expressed by meningeal mast cells drives Th cell GM-CSF expression. IL-1β-driven GM-CSF production by Th cells was previously shown to be dependent on caspase-1, an enzyme that cleaves pro-IL-1β, as well as pro-IL-18, to their active mature forms [22]. Although the cellular source of IL-1β was not identified in these experiments, T cells isolated from the draining lymph nodes of immunized $Casp1^{-/-}$ mice express significantly reduced GM-CSF compared to those from wild type mice.

We first tested whether direct interactions between these cell populations result in cross-activation by establishing an *in vitro* mast cell: T cell co-culture system (Fig. 4a). T cells from $MOG_{35–55}$-immunized mice were cultured under polarizing conditions to expand Th1 and Th17 cells. Purified $CD4^+$ T cells, with or without anti-CD3/CD28 re-stimulation, were subsequently co-cultured with bone marrow-derived mast cells for 4 h. Upon re-isolation, the purity of the mast cell and Th cell fractions was greater than 94% (Fig. 4b). Cytokine gene expression by each individual population was evaluated by RT-PCR (Fig. 4c–g). As a control for inducible *Il1b* expression, wild type mast cells activated via cross-linking of the high affinity FcεR1 showed an average 6-fold increase in expression over unstimulated mast cells (Fig. 4c, lanes 1,2). The co-culture of anti-CD3/CD28-activated Th cells and wild type mast cells results in an approximately 12-fold increase in *Il1b* expression compared to co-cultures with resting Th cells (Fig. 4c, lanes 3, 4). As expected, neither $Il1b^{-/-}$ mast cells nor

activated wild type Th cells express appreciable *Il1b* mRNA (Fig. 4c, lanes 5,6 and Supplemental Fig. 2a).

We next examined whether mast cells can directly elicit *Csf2* expression by Th cells. As shown in Fig. 4d, lanes 1,2, activated Th cells show an average 6-fold increase in *Csf2* expression over resting Th cells and expression is significantly increased by the addition of wild type mast cells (Fig. 4d, lane 3). Co-culture with $Il1b^{-/-}$ mast cells or with $Casp1^{-/-}$mast cells, which cannot produce bioactive IL-1β or IL-18, does not induce increased *Csf2* expression over anti-CD3/CD28 activation alone (Fig. 4d, compare lanes 2 and 4,5). GM-CSF production by mast cells was not detected in these experiments (Supplemental Fig. 2b).

Because caspase-1 has a role in regulating the expression of bioactive IL-1β as well as IL-18 [23,24], we asked if mast cell-derived IL-1β alone is sufficient to increase *Csf2* expression in Th cells. $Casp1^{-/-}$ mast cells and activated Th cells were co-cultured with increasing concentrations of either recombinant IL-1β or IL-18 (Fig. 4d,e). Addition of IL-1β (Fig. 4d, lanes 6–8) but not IL-18 (Fig. 4e, lanes 5,6) to the $Casp1^{-/-}$ mast cell: T cell co-culture restores the Th cell *Csf2* response. We conclude that activated Th cells exert a direct effect on mast cells by stimulating the caspase-1 pathway to induce bioactive IL-1β expression. This in turn promotes increased T cell GM-CSF production.

Mast cell derived IL-1β also impacts *Il17* expression. As shown in Fig. 4f, lanes 3,4, co-culture with wild type, but not $Casp1^{-/-}$ mast cells, induces increased *Il17* expression. The addition of exogenous IL-1β, but not IL-18 (data not shown), restores this response to T cell-$Casp1^{-/-}$ mast cell co-cultures (Fig. 4f, lanes 5e7). Neither wild type nor $Casp1^{-/-}$ mast cells significantly affect the expression of *Ifng* by re-activated Th cells (Fig. 4g, lanes 3,4).

3.5. Caspase-1 expression by meningeal mast cells is sufficient for encephalitogenic Th cell generation

To determine if a meningeal mast cell-specific deficit in IL-1β production alters Th cell GM-CSF production and encephalitogenicity *in vivo*, $Kit^{W/Wv}$ mice were selectively reconstituted with wild type or $Casp1^{-/-}$ mast cells by intracranial transfer as previously described [10] (Fig. 5a). Eight weeks post-reconstitution, EAE was induced by $MOG_{35–55}$ peptide immunization and disease indices were compared (Fig. 5b and Table 1). $Kit^{W/Wv}$ mice reconstituted with wild type mast cells show disease onset and severity similar to that of wild type controls. Both groups show significantly more severe disease than $Kit^{W/Wv}$ mast cell deficient mice as previously reported [12]. However, reconstitution with $Casp1^{-/-}$ mast cells does not restore disease severity to wild type levels. In fact, the early disease course of the $Kit^{W/Wv}$ + $Casp1^{-/-}$ mast cells is indistinguishable from that of the $Kit^{W/Wv}$ non-reconstituted controls indicating that caspase-1 activity in meningeal mast cells is essential for severe disease.

The cytokine response of CNS-infiltrating Th cells was next assessed in these cohorts (Fig. 5cek). The frequency and number of GM-CSF-producing infiltrating Th cells is significantly reduced in the $Kit^{W/Wv}$ + $Casp1^{-/-}$ mast cells cohort relative to both wild type mice and $Kit^{W/Wv}$ mice reconstituted with wild type mast cells (Fig. 5c–e). IL-17 production is also

diminished in *Casp1*$^{-/-}$ mast cell reconstituted *Kit*$^{W/Wv}$ mice (Fig. 5f–h), supporting the co-culture data that indicates a role for mast-derived caspase-1 in Th cell IL-17 production. Although the frequency of IFNγ-producing Th cells was similar among all immunized mice (Fig. 5i–j), the number of IFNγ-producing Th cells is reduced in the *Kit*$^{W/Wv}$ + *Casp1*$^{-/-}$ mast cells cohort compared to the wild type reconstituted *Kit*$^{W/Wv}$ mice, perhaps suggesting an *in vivo* effect of IL-18, an IFNγ -inducing cytokine, that was not detected in the *in vitro* co-culture experiments (Fig. 5k). Taken together, the above data strongly support a critical role for caspase-1-dependent mast cell-derived IL-1β in encephalitogenic Th cell generation in EAE.

3.6. Evidence of meningeal mast cell-T cell co-localization in a subset of acute MS patients

Although mast cells are also normal residents in the meninges and CNS of humans, they are somewhat rare and difficult to image under homeostatic conditions [25–28]. In MS, most studies have focused on the identification of meningeal mast cells from tissues of patients with late/progressive disease [29–32]. Yet there is a paucity of data that addresses the presence of mast cells and T cells in the meninges during early disease. We examined meningeal tissues from a small cohort of acute MS patients that were within 2–3 years of diagnosis. Of 11 cases that showed frank meningeal inflammation as previously defined [13] (Fig. 6a and Supplemental Fig. 1), four of these (36.4%) were positive for mast cell tryptase staining (Fig. 6b). All were positive for CD3$^+$ T cells. We observed meningeal mast cell-T cell co-localization topographically associated with subpial cortical demyelination (Fig. 6c–e). Mast cells were also present in white matter parenchymal lesions of differing demyelinating stages (early active, inactive, and remyelinated), often in close proximity to infiltrating T cells [data not shown]. As shown in (Fig. 6f–h), perivascular mast cells and T cells co-localized within an early active MS lesion, characterized by myelin-laden macrophages. These observations suggest that, as in EAE, there is the potential for similar mast cell-T cell crosstalk in the meninges of MS patients.

4. Discussion

The precise mechanisms that lead to oligodendrocyte and neuronal damage in EAE and MS are still unclear. However, it is now generally accepted that CNS-infiltrating Th cells are not the direct effectors of CNS pathology. Rather Th cells orchestrate a local and damaging inflammatory response mediated largely by myeloid cells [33]. T cell-derived GM-CSF, which in this setting acts predominantly on CCR2$^+$ monocytes, is critical for this myeloid cell recruitment and activation in EAE [9,34,35]. Based on this data, together with the finding that GM-CSF-deficient (*Csf2*$^{-/-}$) Th cells fail to transfer EAE, current models equate Th cell encephalitogenicity with GM-CSF expression [9,33–36]. In MS, the frequency of GM-CSF-producing Th cells corresponds with more severe disease suggesting a similar mechanism exists in humans [37].

Our studies implicate the relatively large resident mast cell population in the meninges as promoters of T cell encephalitogenicity and thus establish the meninges as critical sites of T cell licensing. Although once considered solely as physical protection for the CNS, the

meninges are now appreciated as locations of immune activity in both health and disease. Similar to immune border sites such as the skin, gut and lungs, mast cells, DCs, macrophages and innate lymphoid cells are normal residents [38,39]. Thus, it has been proposed that the meninges provide a first line of defense from infections that threaten the CNS (Fig. 7a). However, we and others have shown that the meninges are sites of pathological immune responses in EAE and MS (reviewed in Ref. [39]). This concept is embodied in the "two wave model," which proposes that peripherally-primed myelin specific Th cells gain access to the immune specialized CNS by first accessing the meninges [15,40]. In a first wave, primed myelin-specific Th cells enter the meninges where they encounter resident myelin-loaded APCs and are retained and reactivated. The resulting sustained inflammation in the meninges is thought to promote a second wave of pathologic immune cell infiltration into the CNS parenchyma [6,13,39]. In an extension of this model, we propose that mast cell activation is a critical component of the first wave. Mast cells release T cell-attracting chemokines, amplifying T cell influx to the meninges, and IL-1β, which drives GM-CSF production by Th cells (Fig. 7b). GM-CSF- producing Th cells enter the CNS in the second wave where they elicit the recruitment and survival of inflammatory monocytes.

While there are likely other cellular sources of IL-1β in the meninges that contribute to an ongoing Th cell GM-CSF response, including $CCR2^{+}$ monocytes [35], we contend that mast cells are an early and essential source in the first wave of EAE. In the absence of IL-1β-producing mast cells in mast cell deficient $Kit^{W/Wv}$ mice or in $Kit^{W/Wv}$ mice reconstituted with $Casp1^{-/-}$ mast cells, GM-CSF production by CNS-infiltrating T cells and disease severity is profoundly attenuated (Fig. 5b–e and 7c). The attenuated disease course of $Kit^{W/Wv}$ mice or $Kit^{W/Wv}$ mice reconstituted with $Casp1^{-/-}$ mast cells aligns with the results of previous studies. Th cells that are unable to respond to IL-1β ($Il1r^{-/-}$) have similar deficits in GM-CSF production and pathogenicity [22]. Furthermore, $Il1b^{-/-}$, $Il1r1^{-/-}$, $Casp1^{-/-}$, $Asc^{-/-}$ and $Nlrp3^{-/-}$ mice, which have deficiencies in IL-1β signaling or in components of the inflammasome that regulates IL-1β processing, are protected from EAE [22,41–44].

Caspase-1-independent production of IL-1β by Th17 cells has recently been shown to be relevant in the second wave of T cell CNS infiltration [45]. While T cell-intrinsic IL-1β affects the survival and proliferation of these cells, it exerts only a modest effect on GM-CSF production. These data raise the intriguing possibility that distinct IL-1β sources provide unique functional influence in disease.

We have demonstrated that there is bidirectional activation of mast cells and T cells in early EAE. Mast cells exert their influence on T cells through *Il1b* expression, but what is less clear is how T cells affect mast cell inflammasome activity. Our adoptive transfer experiments show that T cell transfer alone is sufficient for mast cell activation. However whether this requires direct cell-cell interactions or is mediated by soluble factors released by T cells such as ATP has not been determined. Class II MHC expression can be induced in mast cells, which confers the ability to present antigen to T cells [46,47]. This suggests that T cells may cross-activate mast cells that are serving as APCs. Studies showing that direct interactions between LFA-1/ICAM-1 and OX40/O40L trigger both mast cell and T cell

activation [11,21,48] as well as our observation of mast cell-T cell co-localization in the inflamed meninges of MS patients provide evidence for a contact-dependent mechanism. Ongoing studies are investigating these questions.

Aberrant inflammasome activation is associated with several other CNS inflammatory conditions including stroke, Alzheimer's disease, and traumatic brain injury [49]. In MS, the elevated expression of IL-1β and caspase-1 by peripheral blood monocytes correlates with increased severity and progression of disease [50–54]. Elevated levels of IL-1β are also reported in the CSF of MS patients before clinical relapses and *CASP1* expression is detected in MS plaques [55–58]. Finally, there is evidence that meningeal inflammation is often pronounced in disease and precedes the formation of CNS demyelinating lesions in some patients [13,59]. Thus, our data suggest new therapeutic possibilities that target meningeal mast cells and mast cell-derived IL-1β in MS and other myeloid cell-driven CNS inflammatory diseases. Importantly, because the meninges have less restrictive vasculature than the CNS, the need for modulating therapies to cross the BBB is eliminated [reviewed in Ref. [39]]. There is clinical evidence to support this type of therapeutic approach. Although not its primary mechanism of action, the FDA-approved MS drug teriflunomide (Aubagio) induces mast cell apoptosis through its effects on c-kit [60]. Another non-specific c-kit inhibitor, imatinib mesylate (Gleevec), reduces EAE severity [61]. Based on their efficacy in other autoimmune diseases, therapies that interfere with IL-1β signaling may also be of use in MS patients [62]. For example, a large-scale retrospective analysis of rheumatoid arthritis patients who received anakinra, an IL-1R antagonist, revealed an odds ratio of 0.80 (95% CI 0.29 to 2.24) for developing a demyelinating disorder in this cohort [63]. Finally, a recent report demonstrated the effectiveness of an NLRP3 inhibitor in reducing circulating IL-1β and ameliorating EAE [64].

5. Conclusions

Our study extends the growing body of evidence implicating IL-1β in EAE/MS pathophysiology by identifying a mechanism for IL-1β pathogenicity and defining mast cells as a critical source of this cytokine in early disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Blayne Sayed for designing the mast cell-T cell co-culture system, Kelly Foy for assistance with mast cell quantification, Mark Ebel for assistance with RT-PCR analyses and Thirumala-Devi Kanneganti for the kind gift of the *Il1b*$^{-/-}$ mice. Grant support: NMSS RG 4684A5/1 (M.A.B), NIH F31 NS084691 (A.E.R.), NIH F31 NS068031 (M.W.-C.), Department of Immunology T32 (5 T32 AI 7047–33) and a grant from the Mayo Clinic Center for MS and Autoimmune Neurology (CMSAN) (M.W.-C.).

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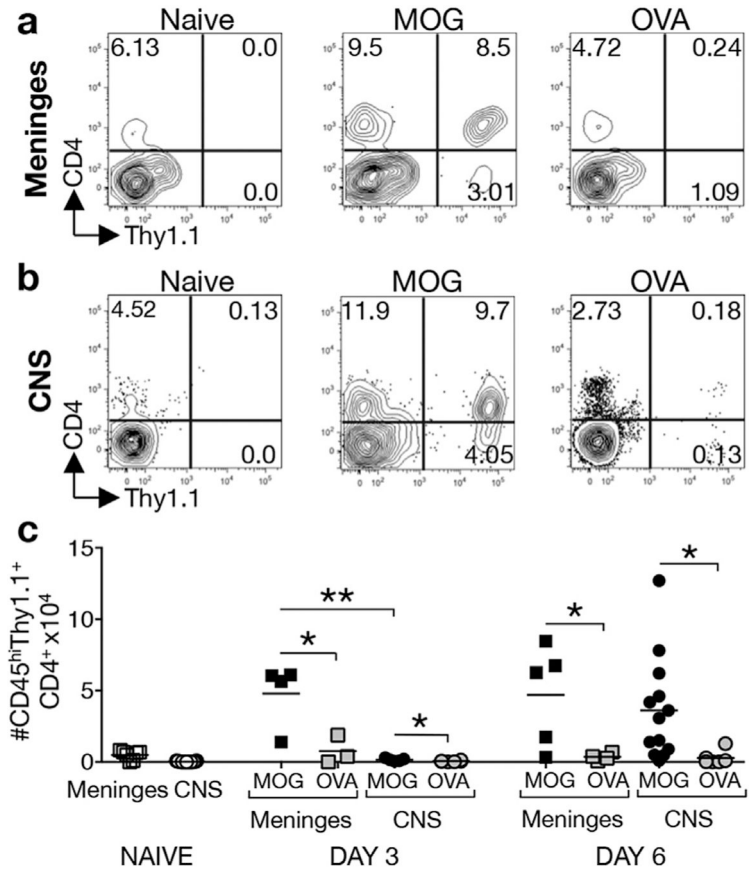

Fig. 1. $MOG_{35–55}$-primed, but not $OVA_{323–339}$-primed, Th cells accumulate in the meninges and CNS early in EAE.

Four x10^6 $MOG_{35–55}$- or $OVA_{323–339}$-primed T cell blasts from Thy1.1$^+$ donor mice were restimulated with peptide *in vitro* and transferred to congenic Thy1.2$^+$ recipients. The frequency and numbers of Thy1.1$^+$CD45$^+$CD4$^+$ cells in the meninges (**a,c**) and CNS (**b,c**) was determined 3 or 6 days post transfer. (**a**) Representative analysis of $MOG_{35–55}$ or $OVA_{323–339}$-primed CD4$^+$Thy1.1$^+$ T cells detected in the pooled meninges of Thy1.2$^+$ recipients at day 6 post transfer. (**b**) Representative analysis of $MOG_{35–55}$ or $OVA_{323–339}$-primed CD4$^+$Thy1.1$^+$ T cells detected in the CNS (pooled brain and spinal cord) of Thy1.2$^+$ recipients 6 days post transfer. Percentages of the CD45$^{+/hi}$ parent gate are shown. (**c**) Numbers of CD45$^+$CD4$^+$Thy1.1$^+$ $MOG_{35–55}$ or $OVA_{323–339}$-primed T cells in the meninges and CNS at indicated days post T cell transfer. For meninges samples, each point represents the analysis of a pool of tissues from 3 to 5 mice and is expressed as numbers/mouse. CNS data points represent the analysis of individual mice. *$p < 0.05$ and **$p < 0.01$ by Student's *t*-Test. 4 independent experiments.

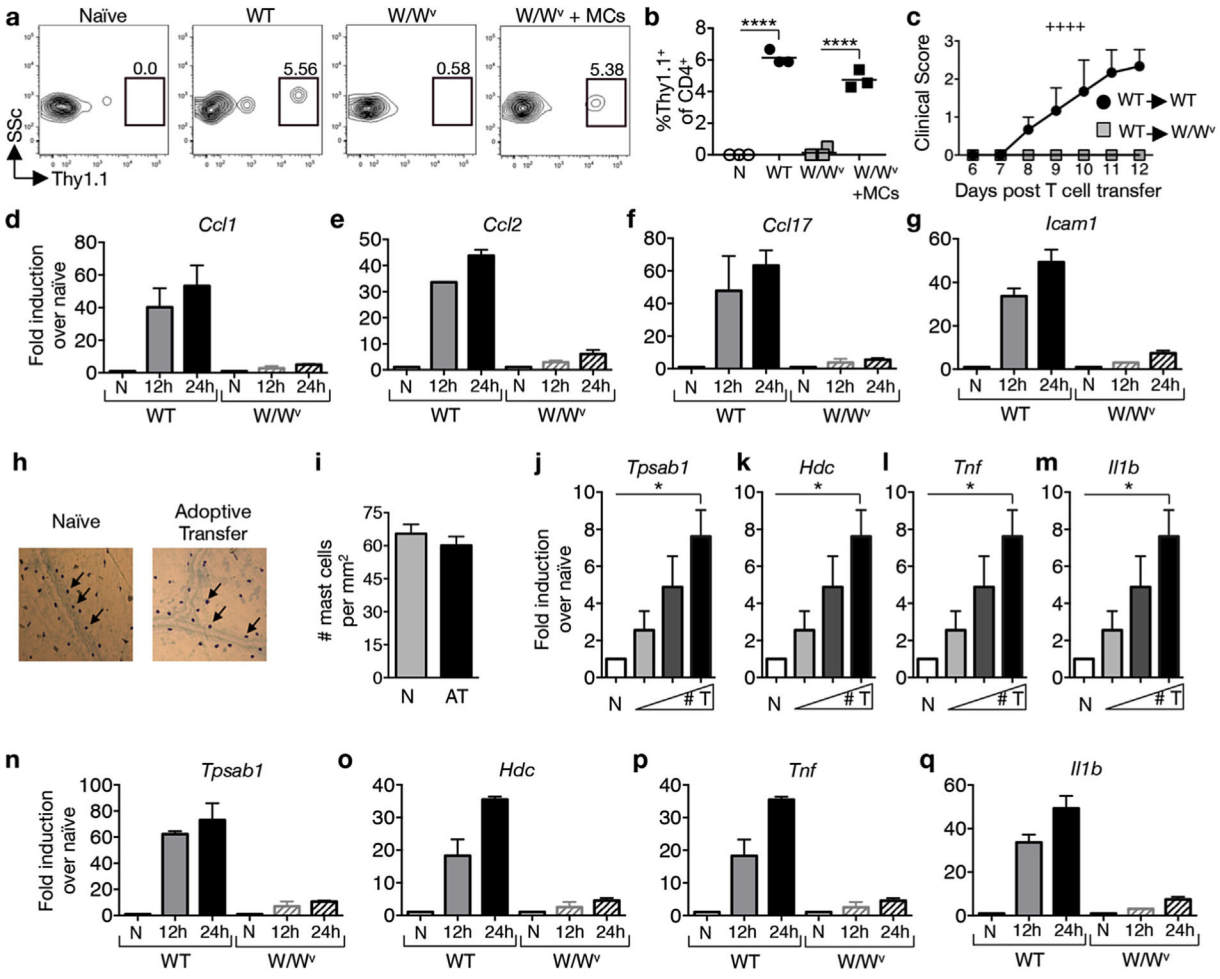

Fig. 2.

Mast cells are activated by T cell transfer and regulate the accumulation of myelin-specific Th cells in the meninges. $MOG_{35–55}$-primed T cells from Thy1.1$^+$ mice were reactivated with $MOG_{35–55}$ peptide *in vitro* for 4 days before transfer of 4 × 10^6 blasts to Thy1.2$^+$ wild type (WT), mast cell-deficient *Kit*$^{W/Wv}$ (W/W^v), or meningeal mast cell reconstituted *Kit*$^{W/Wv}$ (W/W^v + MCs) recipients. (**a**) Representative flow cytometric analysis of Thy1.1$^+$ CD4$^+$ T cell infiltration in the meninges 24 h post transfer. Numbers represent percentage of CD45$^+$CD4$^+$ cells that are Thy1.1$^+$.(**b**) Frequencies of Thy1.1$^+$ T cells detected in the meninges of indicated recipients. Each data point represents the analysis of pooled meningeal tissue from 4 mice. ****p < 0.0001 by Student's *t*-Test. (**c**) Clinical scores of WT and *Kit*$^{W/Wv}$ recipients after adoptive transfer of 4 × 10^6 WT T cell blasts. $^{++++}$p < 0.0001 by two way ANOVA. 2 independent experiments using 4 mice each. (**d–g**) RT-PCR analyses of pooled meninges tissues from WT and W/W^v recipient mice at indicated time points. The expression of indicated genes was determined relative to *Hprt* and expressed as fold induction over naïve. n = 2 pooled samples of 5 mice each for each time point. 2 independent experiments. (**h**) Meningeal mast cells identified by toluidine blue staining and (**i**) mast cell numbers in naïve (N) and T cell recipient mice (AT) at 24 h post transfer, n = 9 mice. (**j–m**) RT-PCR analysis of pooled meninges samples as described in (d–g) 24 h after

transfer of T cell blasts [0 (N), 2, 4, and 8×10^6] to wild type recipients. *$p < 0.05$ by Student's *t*-Test. n = 4 per group, 2 independent experiments. (**n–q**) RT-PCR analyses of pooled meninges as described in (d–g). n = 2 pooled samples of 5 mice for each time point. 2 independent experiments. All values are expressed as mean ± SEM.

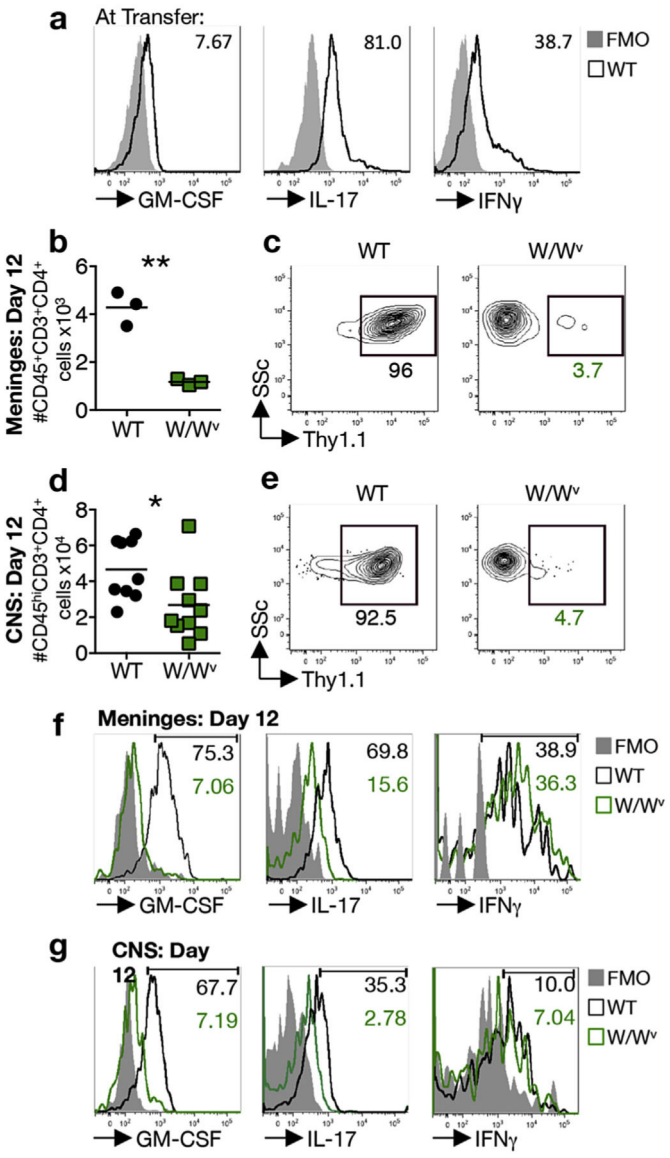

Fig. 3.

Robust GM-CSF production by Th cells is acquired post priming in wild type, but not *Kit*$^{W/Wv}$ mice. (**a**) Representative analysis of cytokine production by $MOG_{35–55}$ eprimed wild type donor T cell blasts prior to adoptive transfer. Numbers denote frequency of cytokine-expressing $CD3^+CD4^+$ blast cells and are representative of 3 independent experiments. (**b**) Accumulation of $CD45^+CD3^+CD4^+$ T cells in the meninges at day 12 post transfer of 4×10^6 $Thy1.1^+$ T blasts to wild type (WT) and *Kit*$^{W/Wv}$ (W/W^v) recipients. Each data point represents analysis of meningeal tissue pooled from 3 mice and is expressed as average cell number per mouse. 3 independent experiments. **$p < 0.01$ by Student's *t*-Test. (**c**) Representative analysis showing the frequency of $Thy1.1^+$ T cells of the $CD45^+CD3^+CD4^+$ population in the meninges of indicated recipients at day 12 post T cell transfer. (**d**) Accumulation of $CD45^{hi}CD3^+CD4^+$ T cells in the CNS of indicated recipients at day 12 post T cell transfer. Each data point represents analysis of pooled brain and spinal

cord tissue from one mouse, 3 independent experiments. *$p < 0.05$ by Student's *t*-Test. (**e**) Representative analysis showing the frequency of Thy1.1^{+} T cells of the CD45^{hi}CD3^{+}CD4^{+} population in the CNS of indicated recipients. (**f**) Representative analysis of cytokine production by CD45^{+}CD3^{+}CD4^{+} Th cells in the meninges of pooled samples from 4 mice at day 12 post transfer of T cell blasts. Numbers denote frequency of cytokine expressing CD45^{+}CD3^{+}CD4^{+} cells in WT (black) and W/W^{v} (green) meninges. (**g**) Representative analysis of cytokine production by CD45^{hi}CD3^{+}CD4^{+} T cells in the CNS of indicated recipients at day 12 post transfer of T blasts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

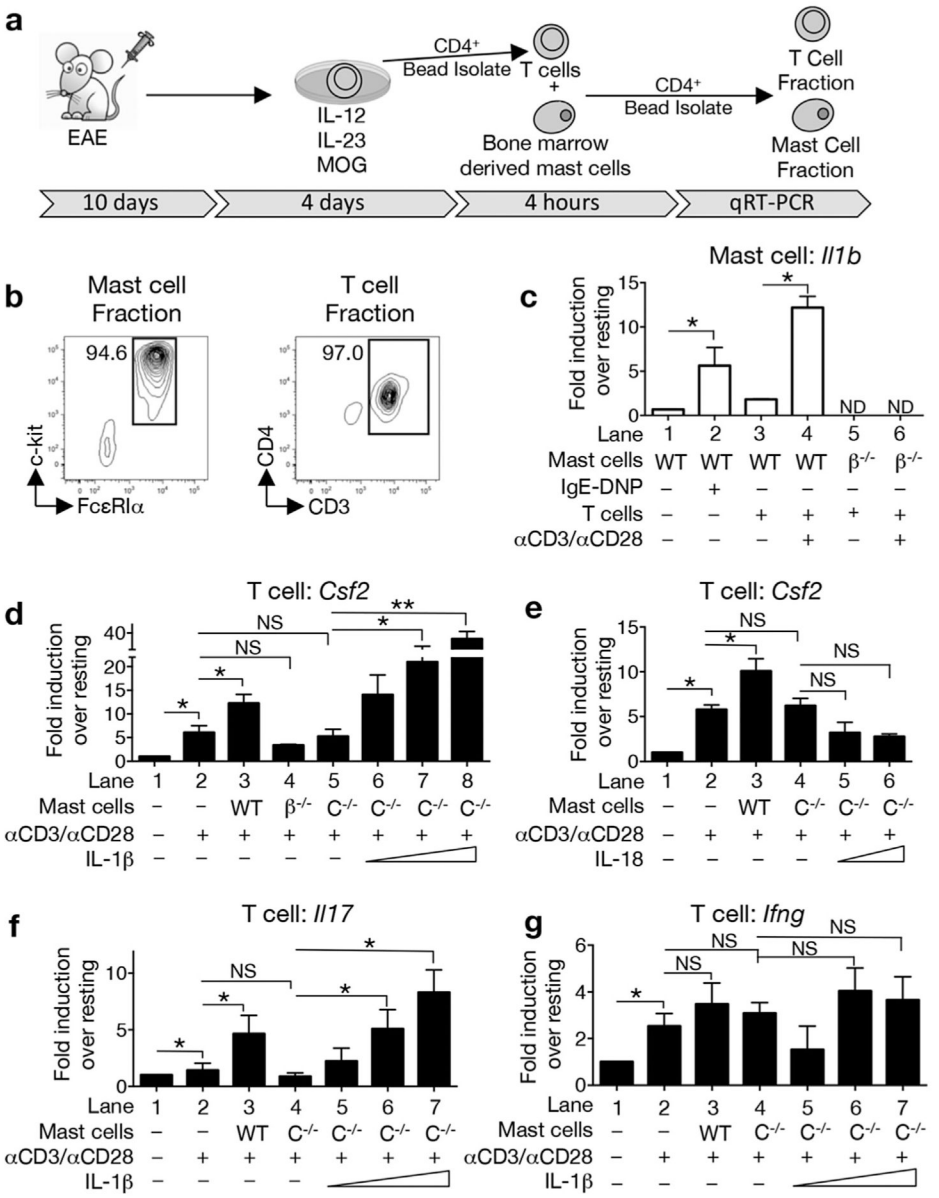

Fig. 4.

Mast cell-derived, caspase-1-dependent IL-1β directly augments GM-CSF production by Th cells. (**a**) Lymph node cells from $MOG_{35–55}$- immunized donor mice were collected at day 10 post immunization and cultured with $MOG_{35–55}$ peptide under Th1/Th17 polarizing conditions for 4 days. T cells were isolated using $CD4^+$ magnetic beads and then co-cultured under various conditions with bone marrow-derived mast cells. After 4 h, $CD4^+$ magnetic beads were used to separate the T and mast cell fractions, which were then assessed for gene expression by quantitative real time PCR. (**b**) Representative FACs analysis showing the purity of each fraction. The percentage of c-kit^+Fcε$RI\alpha^+$ or $CD3^+CD4^+$ cells is shown. Data is representative of 4 independent experiments. (**c**) *Il1b* expression by WT and *Il1b* $^{-/-}$($\beta^{-/-}$) mast cells was analyzed in the presence or absence of Th cells. ND-not detected. (**d, e**) *Csf2* induction by resting or reactivated (αCD3 and αCD28) Th cells in the absence of mast cells

or in the presence of WT, $\beta^{-/-}$, or *Casp1*$^{-/-}$($C^{-/-}$) mast cells. (**d**) Exogenous IL-1β (2, 10, and 50 ng/ml) or (**e**) IL-18 (2 and 50 ng/ml) was added to the *Casp1*$^{-/-}$ mast cell: T cell co-cultures. (**f,g**) Induction of *Il17* and *Ifng* by Th cells co-cultured with WT or $C^{-/-}$ mast cells. Gene expression is relative to *Hprt* and expressed as fold induction over resting cells. *$p < 0.05$ and **$p < 0.01$ by Student's *t*-Test. n = 2 independent cultures for panel (**e**) and n = 4 for remaining panels (**c,d,f,g**). All values are expressed as mean ± SEM.

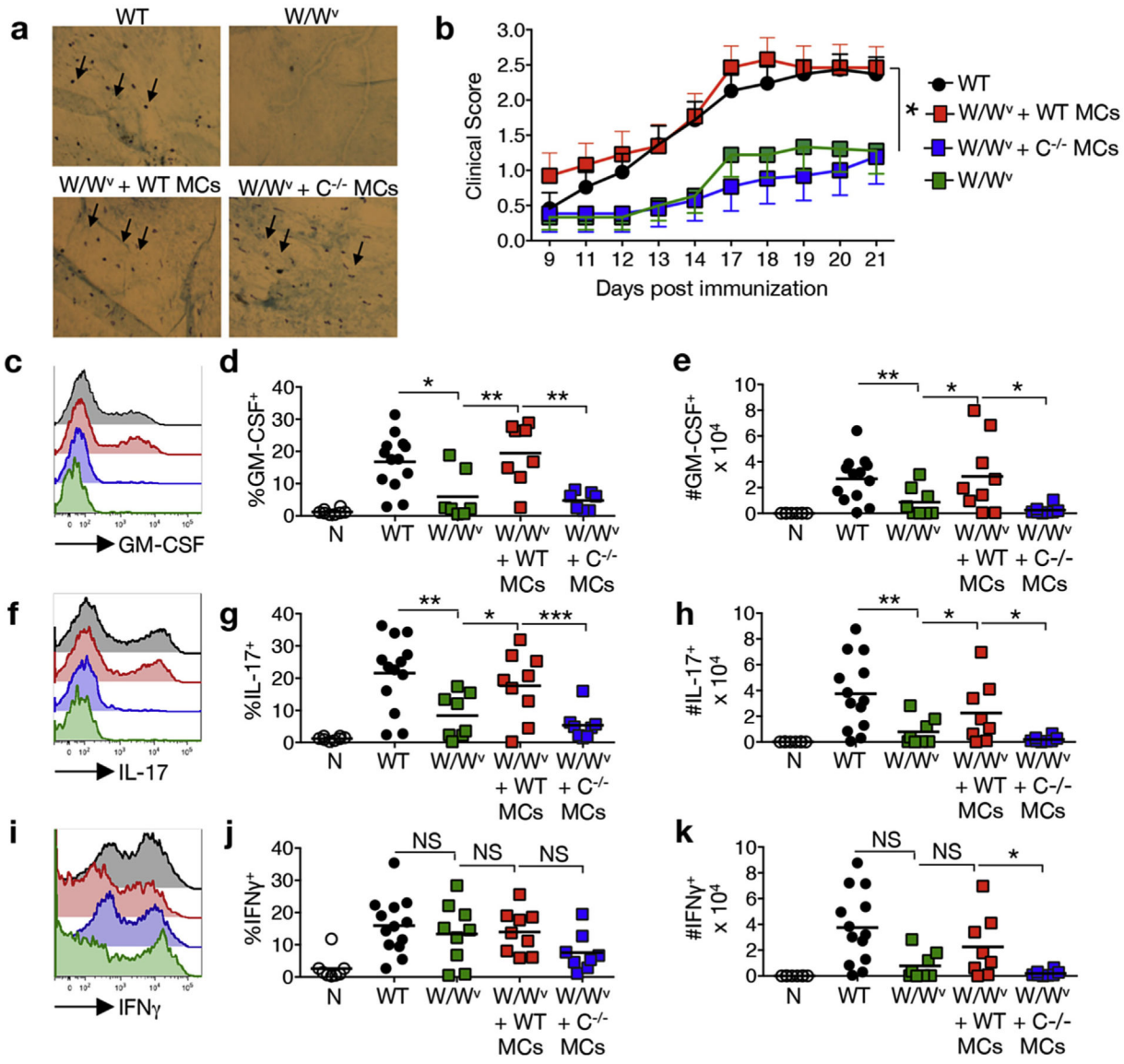

Fig. 5.

Meningeal mast cell-derived caspase-1 is necessary for EAE development and Th cell encephalitogenicity in the CNS. The meninges of naïve *Kit*$^{W/Wv}$ mice were selectively reconstituted with wild type (W/W^{v} + WT MCs) or *Casp1*$^{-/-}$(W/W^{v} + C$^{-/-}$ MCs) bone marrow-derived mast cells. (**a**) Reconstitution was confirmed in a subset of mice by toluidine blue staining of the calvarial dura mater. Arrows denote toluidine blue-positive mast cells. (**b**) Eight weeks post reconstitution, EAE was induced by $MOG_{35–55}$ peptide immunization and the disease course was compared to non-reconstituted controls. *$p < 0.05$ by 2-way ANOVA. $n \geq 12$ for each group. 4 independent experiments. Data points are expressed as mean ± SEM. (**c–k**) Twelve days post immunization, CNS infiltrating leukocytes were isolated from a subset of the immunized mice and analyzed by flow cytometry. Representative analyses of GM-CSF (**c**), IL-17 (**f**), and IFN**γ** (**i**) production by CD45^{hi}CD3^{+}CD4^{+} cells are shown. FMO negative controls were used to set gates.

Histogram colors correspond to experimental groups designated in (b). (**d,g,j**) Frequency and (**e,h,k**) numbers of $CD45^{hi}$ $CD3^{+}CD4^{+}$ cells that express cytokines *p < 0.05, **p < 0.01, and ***p < 0.001 by Student's *t*-test. n ≥ 8 for each group, 3 independent experiments.

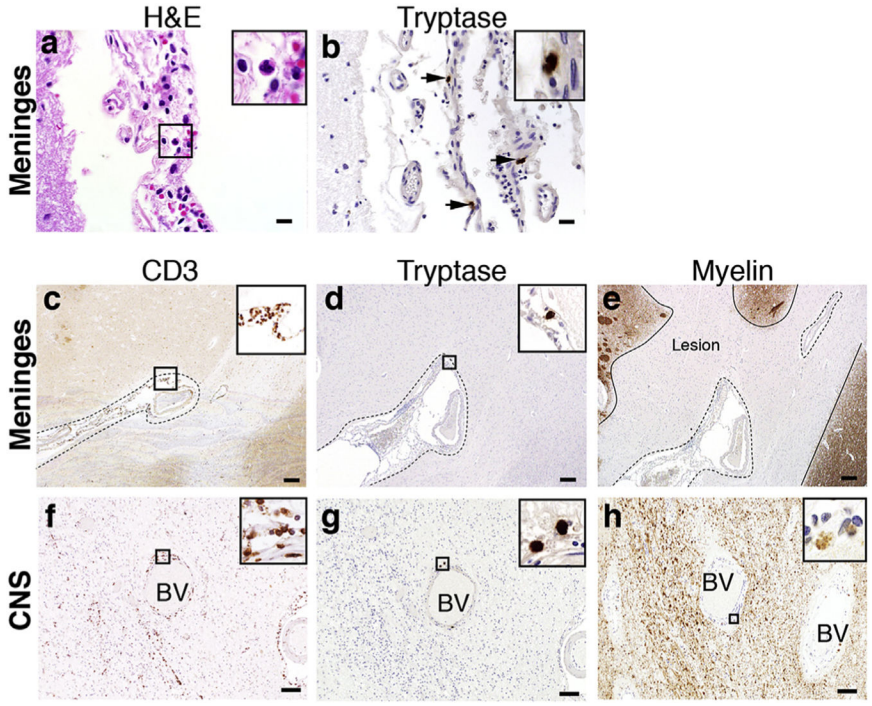

Fig. 6.

Mast cell-T cell co-localization in the meninges and CNS of acute MS patients. (**a**) H&E staining shows brain-associated meningeal granulocyte infiltration in an acute MS autopsy case. (**b**) Immunohistochemistry analysis of tryptase positive mast cells (arrows) present within the meninges of the same case. (**c**) Immunohistochemistry analysis of $CD3^+$ T cells identified in the meninges (designated by dashed outline) of a brain sulcus of an acute MS autopsy case. (**d**) Immunohistochemistry analysis of tryptase positive mast cells on a consecutive section of (c) reveals mast cells and T cells co-localized in the meninges. (**e**) Myelin PLP staining on a consecutive section of (c,d), shows subpial cortical demyelination associated with meningeal inflammation. Lesion borders are designated by a solid line. (**f**) Immunohistochemistry analysis of infiltrating perivascular $CD3^+$ T cells identified in the CNS parenchyma of an acute MS autopsy case. BV = blood vessel. (**g**) Immunohistochemistry analysis of perivascular tryptase positive mast cells identified in a consecutive section of (f) shows mast cell-T cell co-localization within an active demyelinating lesion. (**h**) Myelin CNPase staining of consecutive sections of (f,g) within the CNS parenchyma shows myelin debris within macrophages suggesting early demyelinating activity. Enlarged views shown in panel insets. Scale bars in A–B: 20 μm, C–E: 200 μm, F–H: 100 μm.

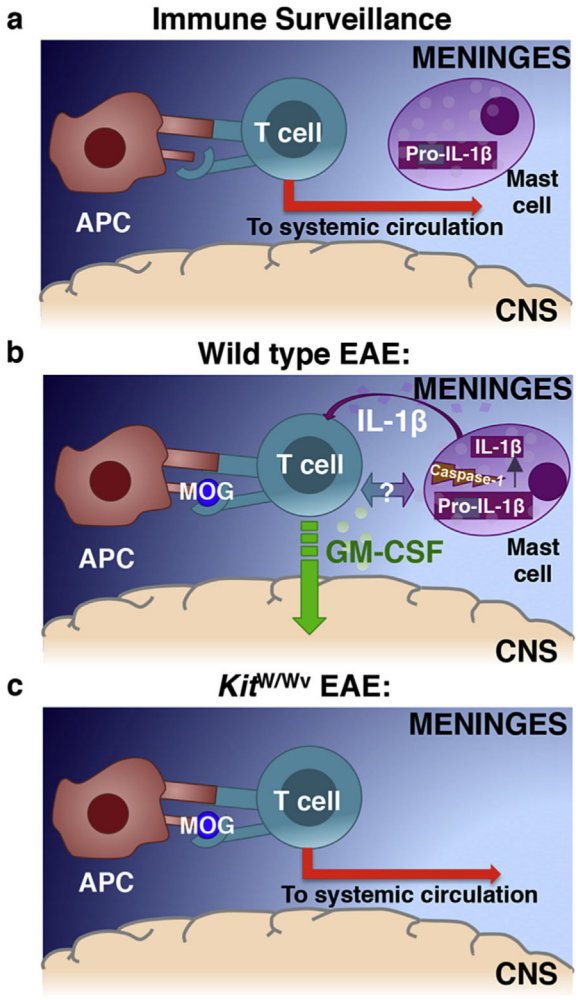

Fig. 7.

A Model: Meningeal mast cell derived, inflammasome-dependent, IL-1β is necessary for T cell encephalitogenicity and infiltration into the CNS. (**a**) During physiological immune surveillance, T cells transit through the meninges. Under steady-state conditions, the surveying T cells do not interact with resident antigen presenting cells in the meninges and instead re-enter systemic circulation. (**b**) In EAE/MS, autoreactive transiting T cells interact with cognate antigen presenting cells. Activated T cells also interact with and activate mast cells resulting in caspase-1-dependent cleavage of pro-IL-1β to its mature form. Upon release, mast cell-derived IL-1β licenses T cells to produce GM-CSF and become encephalitogenic. (**c**) In the absence of meningeal mast cells (*Kit*$^{W/Wv}$ mice), mast cell: T cell interactions are nonexistent, resulting in decreased GM-CSF production by transiting T cells, less efficient CNS-entry of T cells and other cells and reduced clinical disease severity.

Table 1

Selective meningeal reconstitution of *Kit*$^{W/Wv}$ mice with *Casp1*$^{-/-}$ mast cells fails to restore severe EAE.

The meninges of *Kit*$^{W/Wv}$ (W/Wv) mice were selectively reconstituted with wild type (W/W^{v} + WT MCs) or *Casp1* $^{-/-}$ (W/W^{v} + C$^{-/-}$MCs) mast cells and disease indices were compared. Day of onset was determined in mice exhibiting clinical signs for ≥2 days. Peak score represents the average highest clinical score observed. Cumulative disease represents the sum of all clinical scores per mouse in each group over the observation period. Values are expressed as mean ± SEM.

Group	Day of onset	Peak score	Cumulative disease, days 0–35	Cumulative disease, days 0–21	Cumulative disease, days 22–35
WT	12.91 ± 2.00	3.25 ± 0.13	37.79 ± 2.47	15.29 ± 1.63	22.50 ± 1.39
W/W^{v}	15.73 ± 1.09 $^{**++}$	1.82 ± 0.39 $^{***+}$	21.90 ±5.06 $^{**+}$	9.60 ± 2.378 $^{+}$	13.15 ± 2.76 **
W/W^{v} + WT MCs	12.17 ± 0.39	3.42 ± 0.32	41.25 ±6.125	18.67 ± 2.12	22.58 ± 4.10
W/W^{v} + C$^{-/-}$ MCs	16.75 ± l.84 $^{++}$	2.06 ± 0.50	24.31 ±6.76	8.19 ± 3.12 $^{+}$	16.13 ± 4.35

** p < 0.01

*** p < 0.001 compared to WT by Student's *t*-Test.

$^{+}$ p < 0.05

$^{++}$ p < 0.01 compared to W/W^{V} + WT MCs by Student's *t*-Test. n ≥ 8 for all groups. 3 independent experiments.