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Meningeal Mast Cells Affect Early T Cell Central Nervous System Infiltration and Blood-Brain Barrier Integrity through TNF: A Role for Neutrophil Recruitment?

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Mast cells contribute to the pathogenesis of experimental autoimmune encephalomyelitis, a rodent model of the human demyelinating disease multiple sclerosis. Yet their site and mode of action is unknown. In both diseases, myelin-specific T cells are initially activated in peripheral lymphoid organs. However, for disease to occur, these cells must enter the immunologically privileged CNS through a breach in the relatively impermeable blood-brain barrier. In this study, we demonstrate that a dense population of resident mast cells in the meninges, structures surrounding the brain and spinal cord, regulate basal CNS barrier function, facilitating initial T cell CNS entry. Through the expression of TNF, mast cells recruit an early wave of neutrophils to the CNS. We propose that neutrophils in turn promote the blood-brain barrier breach and together with T cells lead to further inflammatory cell influx and myelin damage. These findings provide specific targets for intervention in multiple sclerosis as well as other immune-mediated CNS diseases. ***The Journal of Immunology*****, 2010, 184: 6891–6900.**

Experimental autoimmune encephalomyelitis (EAE) is a demyelinating disease of the CNS and the prototypic animal model of multiple sclerosis (MS) (1). Like MS, EAE is characterized by immune destruction of the myelin axonal sheath, leading to loss of saltatory nerve conduction and multiple neurologic deficits. Although the initiating events in MS remain unknown, EAE can be induced by immunization with myelin peptides in CFA or by adoptive transfer of myelin-reactive T cells. In both diseases, autoreactive Th1 and Th17 cells, which are activated in the peripheral lymphoid organs, must enter the immunologically privileged CNS to initiate local inflammation (2). However, myelin-specific T cells have been detected in similar frequency in peripheral tissues of patients with MS and healthy individuals (3, 4) indicating that the generation of these cells in the periphery is not sufficient for disease. Rather, it is the passage of these pathologic cells through the blood-brain barrier (BBB), specialized vasculature that is relatively impermeable to cells and macromolecules, into the CNS that is one of the major determinants of disease progression (2).

Once activated, encephalitogenic T cells traffic within hours into the CNS via two specific routes: the cerebrospinal fluid-producing choroid plexus (5) and directly through the vasculature of the meninges (6). Recent studies have also implicated the meninges, structures that surround and invest the CNS and house the cerebral spinal fluid, as a site of early T cell reactivation. The meninges consist of three distinct layers: the outermost dura mater as well as the arachnoid mater and the pia mater, collectively termed the leptomeninges (Fig. 1*A*). T cells entering the CNS are first arrested in the leptomeninges, where they scan the luminal surface of the blood vessels at this site prior to diapedesis (6). These cells can be detected interacting with APCs and proliferating in the subarachnoid or leptomeningeal space around the brain and spinal cord (6, 7).

The myriad of factors that regulate CNS entry of inflammatory cells and BBB permeability are still ill defined. The localization of early T cell immune events in the meninges is of interest given that the outermost layer of the meninges, the dura mater, as well as some regions of the pia mater, are densely populated with mast cells, a cell population implicated in both EAE and MS (8). They are often found near meningeal vessels (Fig. 1*A*, 1*B*). Mast cells are multifunctional innate immune cells perhaps best known for their role as the dominant effector cells in allergic disease following IgE/FcεRI-mediated activation and for promoting vascular permeability via production of histamine (for review, see Ref. 9). Yet mast cells also express many receptors that facilitate IgE-independent modes of activation and are the source of numerous other immunomodulatory molecules. A growing body of evidence shows that mast cell-T cell interactions influence the activation phenotypes of both these cell types (10–13). We previously demonstrated that mast cells contribute to severe disease in EAE. Mast cell-deficient WBB6ckit-W/W^v mice develop attenuated and delayed EAE. This phenotype is reversed in nonirradiated W/W^v mice by i.v. mast cell reconstitution with wild-type (WT) bone marrow-derived mast cells (BMMCs) (14). However, the relevant mast cell populations in this disease, as well as their mode and site of action, has remained elusive.

The location of mast cells at the site of initial T cell entry into the CNS led us to ask whether meningeal mast cells affect events that regulate disease severity, and, if so, how they contribute. Through selective reconstitution of mast cells to the dura mater and pia mater of W/W^v mice as well as the cervical lymph nodes (LNs), we

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Abbreviations used in this paper: BBB, blood-brain barrier; BMMC, bone marrow-derived mast cell; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; HDC, histidine decarboxylase; i.c., intracranially; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PE, pinacyanol erythrosniate; PMN, polymorphonuclear cell; WT, wild-type.

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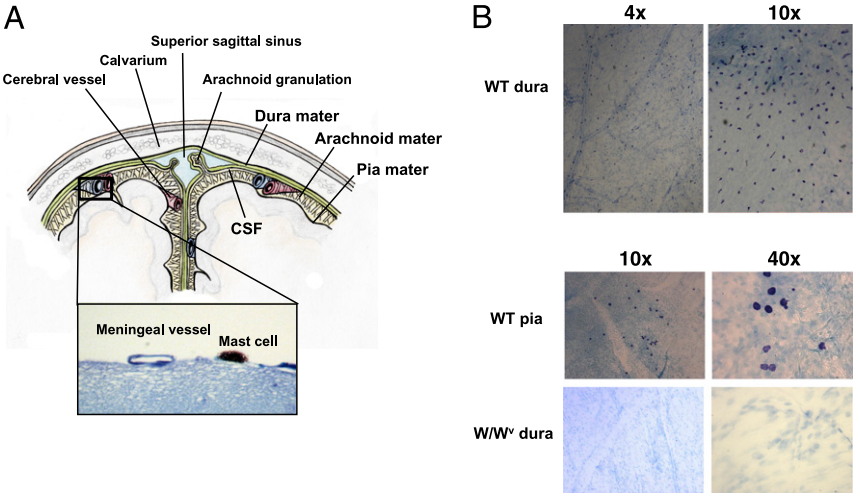

FIGURE 1. Mast cells are normal residents of the dura and pia mater within the meninges of WT mice and are absent in WBB6ckit$^{W/W^v}$ mice. *A*, Illustration of a coronal section of the brain displaying the position of the three meningeal membranes with respect to the CNS parenchyma. Note the mast cell [stained with PE] observed in proximity to a cerebral vessel in a sagittal section of a naive WT mouse brain (original magnification ×100). *B*, Toluidine blue staining of mast cells in the dura and pia mater of WT mice (original magnification ×4, ×10, and ×40). Mast cell-deficient W/W^v-derived samples are shown for comparison. Representative of samples taken from at least 20 mice of each genotype.

demonstrate that these cells are necessary and sufficient for the restoration of WT levels of disease, disease-induced BBB permeability, and both early and late T cell infiltration and T cell reactivation. The influx of other cells, particularly an early population of neutrophils, is also affected by meningeal mast cells, and this recruitment is dependent on the production of mast cell-derived TNF. Surprisingly, meningeal mast cell-produced histamine has no role in these events. Given that TNF is a potent neutrophil (polymorphonuclear cell [PMN]) chemoattractant (15, 16), that neutrophils make up a substantial proportion of the early inflammatory cell infiltrate in EAE (17, 18), and that neutrophils have an established role in regulating endothelial cell integrity and vascular permeability (19), we propose that mast cells act indirectly through PMNs to promote the breach of the BBB and inflammatory cell influx to the CNS in EAE.

Materials and Methods

Mice

Female W/W^v mice (WBB6F1-Kit^W/Kit$^{W\text{-}v}$), littermate controls, and TNF$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 3–5 wk of age and housed in the barrier facility at Northwestern University Feinberg School of Medicine (Chicago, IL). Histidine decarboxylase-deficient (HDC$^{-/-}$) mice (20) were originally generated by Dr. Hiroshi Ohtsu (Tohoku University, Sendai, Japan). 2D2 TCR transgenic mice (21) were originally generated by Dr. Vijay Kuchroo (Harvard University, Cambridge, MA) and crossed in our laboratory with Thy1.1 (B6.PL-Thy1^a/CyJ) congenic mice purchased from The Jackson Laboratory. All experiments were approved by the Northwestern University Animal Care Committee, and all mice were

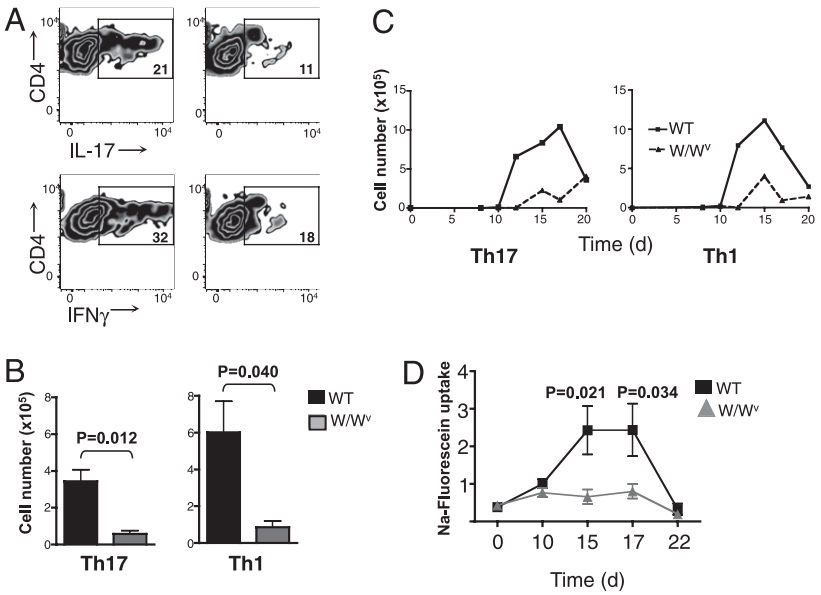

FIGURE 2. WBB6ckit$^{W/W^v}$ mice exhibit reduced Th1 and Th17 cell infiltration in the CNS after active EAE disease induction. EAE was induced in age-matched WT and W/W^v recipients. At the indicated days, cells were isolated from the CNS and T cells were analyzed by flow cytometry. Percentages (*A*) and numbers (*B*) of IL-17– and IFN-γ–producing CD4$^+$ T cells isolated from the CNS of WT and W/W^v animals and restimulated ex vivo with MOG$_{35–55}$. Cells were analyzed by flow cytometry at day 17. Representative FACS experiment is shown. Data in *B* are expressed as mean ± SD and are representative of results from three individual experiments. n = 3–4 mice/group, CNS pooled. *C*, Total numbers of endogenous IL-17– and IFN-γ–producing CD4$^+$ T cells isolated from the CNS of WT and W/W^v animals, restimulated ex vivo with MOG$_{35–55}$, and analyzed by flow cytometry at days 0, 8, 10, 12, 15, 17, and 20 post EAE induction (n = 4 individual mice/time point). *D*, Detection of sodium fluorescein penetrance into the CNS as a measure of BBB permeability. Analysis of BBB permeability from day 0 (naive) through day 22; data are expressed as mean ± SD of samples taken from two independent experiments. n = 13 individual animals assessed/ time point (days 15 and 17). n = 5 individual animals assessed/time point (days 0, 10, and 22). Significance determined by unpaired Student two-tailed t test.

housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility.

EAE induction and clinical scoring

Mice were immunized s.c. at two injection sites on the posterior flank with 100 μg myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide (Emory University Microchemical and Proteomics Facility, Atlanta, GA) emulsified in 5 mg/ml CFA (IFA with desiccated *Mycobacterium tuberculosis* H37 RA, VWR) in conjunction with 250 ng pertussis toxin (List Biological Laboratories, Campbell, CA) administered i.p. On days 0 and 2, Animals were scored daily for signs of clinical disease according to the following criteria: 0, no disease; 1, tail flaccidity; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis with muscle wasting and inability to right from supine; and 5, moribund. Disease incidence is defined as a score of 1 or greater for 2 consecutive days or a score of 2 or more for at least 1 d.

BMMC derivation and reconstitution

Bone marrow was harvested from the femurs of 4–6-wk-old mice and cultured with 5 ng/ml recombinant murine IL-3 (Invitrogen, Carlsbad, CA) and 12.5 ng/ml recombinant murine stem cell factor (Invitrogen) in complete RPMI 1640 (15% heat-inactivated FBS, 2 mM glutamine, 1% penicillin-streptomycin, 1 mM sodium pyruvate, and 50 μM 2-ME) for 6–8 wk, at which time >95% of these cells are c-kit$^+$ FcεRI$^+$. Four-week-old W/W^v mice were reconstituted i.v. in the tail vein with 4×10^6 or intracranially (i.c.) with 10^6 BMMCs in 50 μl PBS. Reconstitution of the mast cell compartment in W/W^v mice does not require prior irradiation. Intercranial injections were performed on anesthetized mice to a depth of 2 mm in the center of the right cerebral hemisphere with a 25-gauge needle. In i.c. reconstitution experiments, nonreconstituted WBB6 littermates were sham i.c. injected with 50 μl PBS. Reconstitution was confirmed in a subset of mice by histology at >8 wk posttransfer.

Isolation of CNS leukocytes

Mice were anesthetized at indicated day and perfused by injection of 30 ml PBS into the left ventricle. The CNS (cerebrum, cerebellum, and spinal cord) was removed, pooled, and incubated in HBSS (without Ca^{2+}/Mg^+) with 300 U/ml type IV collagenase (Worthington Biochemical, Lakewood, NJ) at 37˚C for 1 h. CNS was then triturated and homogenized, and leukocytes were enriched on a 40% Percoll gradient.

Transfer of naive transgenic T cells

CD4$^+$ T cells were isolated with CD4$^+$ MACS beads (Miltenyi Biotec, Auburn, CA) from homogenized LNs of naive F1 2D2$^+$ Thy1.1$^+$ females. A total of 1×10^6 CD4$^+$ T cells was transferred via i.v. tail vein injection. After 48 h, disease was actively induced as described above.

Adoptive transfer of encephalitogenic T cells

Three- to 5-wk-old Thy1.1 congenic mice were immunized to induce disease as described above and sacrificed at day 10, and total cells from the draining LNs were isolated. Cells were cultured at 10^7 cells/ml for 4 d in complete DMEM (10% heat-inactivated FBS, 2 mM glutamine, 1% penicillin-streptomycin, 1 mM sodium pyruvate, and 50 μM 2-ME) with $MOG_{35–55}$ peptide (50 μg/ml), recombinant human IL-12 (12 ng/ml; PeproTech, Rocky Hill, NJ) and IL-23 (10 ng/ml; R&D Systems, Minneapolis, MN) for 4 d. Four days later, blasts were counted, and 4×10^6 blasts was transferred to each Thy1.2-recipient animal (day 0). Pertussis toxin 250 ng (List Biologicals) was administered i.p. on days 0 and 2 relative to cell transfer.

Flow cytometry

For ex vivo cell-surface staining, cells were prepared from homogenized spleens, LNs, and CNS (CNS cell preparations performed as described above). Cells were aliquoted into 96-well plates, blocked with anti-CD16/32 Fc Block (eBioscience, San Diego, CA), and stained with the appropriate extracellular Ab (eBioscience and R&D Systems). For intracellular cytokine staining, cells were restimulated with $MOG_{35–55}$ (50 mg/ml) peptide instead of PMA and ionomycin. Cells were assayed for cytokine production using the Fixation & Permeabilization Kit (eBioscience) or for Foxp3 expression using a Mouse Regulatory T Cell Staining Kit (eBioscience).

BBB permeability assay

Naive or immunized mice were injected ip with 10% sodium fluorescein (NaFlu, Sigma-Aldrich, St. Louis, MO). After 10 min, blood was drawn from the right cardiac ventricle followed by PBS perfusion through the left cardiac ventricle. Spinal cords were removed, weighed, homogenized, and processed with the cardiac blood for fluorimetry. Fluorescence was measured by a SpectraMax Gemini XS fluorimeter (Molecular Devices, Sunnyvale, CA). The presence of NaFlu in the samples was quantified using a standard curve. The uptake of NaFlu into the CNS was calculated by the following equation: (micrograms NaFlu in spinal cord/weight of spinal cord)/(micrograms NaFlu in cardiac blood/amount of cardiac blood).

Isolation and staining of the dura and pia mater

Following perfusion, both the brain and the calvarium of the skull were fixed in formalin for 24 h and then transferred to PBS. The calvarium was stained with acidic toludine blue for 10 s, and the dura was then dissected from the bone while submerged in water and floated onto slides. Brains were stained with acidic toludine blue for 30 s and submerged in water, and a scalpel was used to cut a shallow rectangle on the dorsal side of the cortex and a second rectangle around the vermis of the cerebellum. The pia mater was then carefully separated from the tissue of the brain using the edge of the scalpel and then floated onto slides.

Histology of other tissues

CNS, spleen, LNs, and gut tissue was fixed in 10% buffered formalin for 24 h and transferred to tissue cassettes. Tissue was paraffin embedded, sectioned, and stained with pinacyanol erythrosniate (PE). All sectioning and staining was performed by Histo-Scientific Research Laboratories (Mount Jackson, VA).

Real-time PCR

For CNS mediator expression assays, total CNS leukocytes were isolated as described above, and RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI). mRNA was quantified using iCycler iQ5 Real Time PCR Detection System (Bio-Rad, Hercules, CA), PerfecTA SYBR Green SuperMix (Quanta BioSciences, Gaithersburg, MD), and the following primers: IL-1 (forward primer: 5′-GACGGCACACCCACC-CT-3′ and reverse primer: 5′-AAACCGTTTTTCCATCTTCTTCTTT-3′), TNF (forward primer: 5′-GCCACCACGCTCTTCTGTCT-3′ and reverse

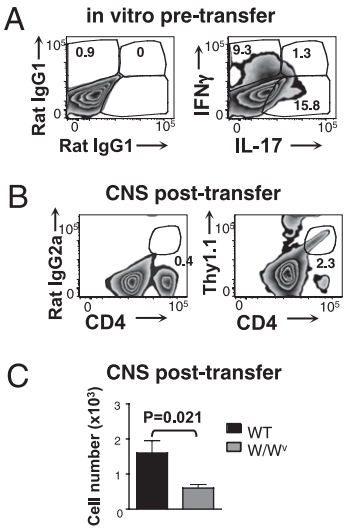

FIGURE 3. The early CNS entry of adoptively transferred encephalitogenic cells is compromised in WBB6ckit$^{W/W^v}$ mice. Encephalitogenic Thy 1.1$^+$ $MOG_{35–55}$-specific T cells were derived in WT mice and transferred to age-matched WT and W/W^v recipients. *A*, Representative flow cytometric analysis of IL-17 and IFN-γ production prior to adoptive transfer (*right panel*). Isotype control staining is shown in the *left panel*. Cells were restimulated in vitro with PMA and ionomycin prior to analysis. *B*, Representative analysis of CNS accumulation of adoptively transferred Thy1.1$^+$ CD4$^+$ cells (*right panel*) in CNS of recipient WT mice at 60 h posttransfer by flow cytometry. Isotype control staining is shown in the *left panel*. *C*, Quantification of Thy1.1$^+$ CD4$^+$ T cells 60 h posttransfer in the CNS of recipient WT and W/W^v mice. Data are expressed as mean ± SD. These experiments are representative of two independent experiments; Experiment 1 (shown): n = 6/group (WT or W/W^v recipients). Experiment 2: n = 5 mice/group. Significance determined by unpaired Student two-tailed t test.

primer: 5′-GGTCTGGGCCATAGAACTGATG-3′), CCL3 (forward primer: 5′-CCAAGTCTTCTCAGCGCCAT-3′ and reverse primer: 5′-GAATCTTCCGGCTGTAGGAGAAG-3′), and CCL4 (forward primer: 5′-TCTGCGTGTCTGCCCTCTC-3′ and reverse primer: 5′-TGCTGAGAACCCTGGAGCA-3′).

For detection of TNF mRNA in the dura mater of naive and immunized animals, EAE was induced in WT and W/W^v mice as described previously. Six days following immunization, naive and immunized mice were perfused, and the dura mater was removed from the calvarium using forceps. RNA was isolated and quantified using RT-PCR as described above using the TNF primers listed above.

Statistics

All statistics were performed using Prism 4 software (GraphPad, San Diego, CA). For comparison of two separate groups, a two-tailed *t* test was used. For comparison of three or more groups, one-way ANOVA was used.

Results

Despite similar peripheral activation profiles, myelin-specific Th1 and Th17 cells generated in W/W^v mice fail to efficiently enter the CNS

Previous studies demonstrated that WBB6 ckit$^{W/Wv}$ mast cell-deficient mice do not develop severe EAE after active disease induction with $MOG_{35–55}$-derived peptide plus CFA or when encephalitogenic cells are adoptively transferred (14, 22). A comprehensive analysis of peripheral T cell responses revealed that the generation of Ag-specific Th1 and Th17 cell responses is largely normal with respect to cytokine production and activation marker status (Supplemental Fig. 1). The exception is a small but reproducible defect in early IFN-γ production by CD4$^+$ Th1 cells in W/W^v mice (Supplemental Fig. 1) (22).

We previously demonstrated that the total numbers of CD4$^+$ and CD8$^+$ T cells are reduced in the CNS at day 11 post disease induction (22). However, these studies did not enumerate Ag-specific T cells nor did they determine whether the reduced cell numbers observed at day 11 represent merely a delay or a real defect in CNS entry in mast cell-deficient mice. To address this question, we examined Th1 and Th17 cell entry into the CNS after active immunization. At day 17, the peak of disease, ~20% of WT CD4$^+$ T cells isolated from the brain and spinal cord express IL-17, and 30% express IFN-γ after Ag-specific restimulation (Fig. 2*A*) compared with roughly 10% and 18%, respectively, in W/W^v mice. The total numbers of CNS-infiltrating Th17 and Th1 cells are drastically reduced at peak

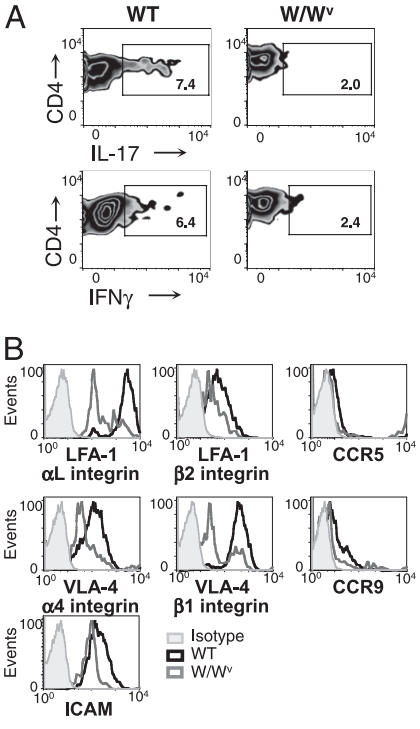

FIGURE 4. T cells that can enter the CNS of W/W^v mice fail to be reactivated. Mice were immunized to induce EAE, and at day 17 post-immunization, CNS cells were isolated, and T cells were analyzed directly ex vivo without in vitro peptide restimulation. *A*, IL-17 and IFN-γ production by CNS-infiltrating CD4$^+$ cells in WT and W/W^v mice. These data are representative of four independent experiments in which CNS samples from four mice/group were pooled. *B*, Flow cytometric analysis of activation molecules expressed on CNS-infiltrating CD4$^+$ T cells in WT and W/W^v mice at day 10 post EAE induction. These data are representative of two independent experiments in which CNS samples from four mice/group were pooled.

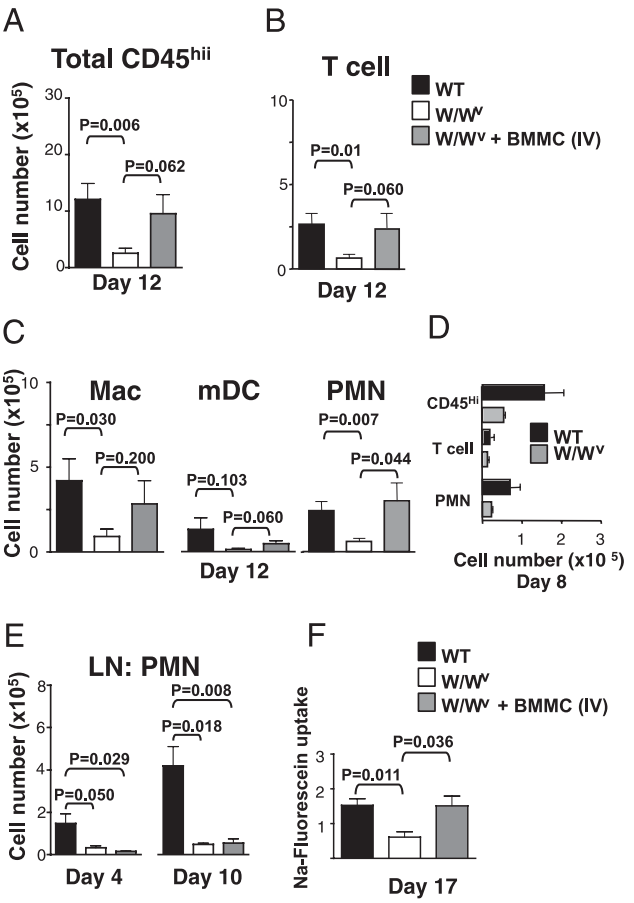

FIGURE 5. BMMC reconstitution of WBB6ckit$^{W/Wv}$ mice restores WT levels of inflammatory cell infiltration to the CNS and BBB permeability. Age-matched mice were immunized to induce disease and sacrificed at indicated day for analysis. Quantification of total CNS infiltrating CD45^{hi} cells (*A*), T cells (CD45^{hi}, CD3$^+$, CD4$^+$) (*B*), macrophages (CD45^{hi}, CD11b$^+$, CD11c$^-$), myeloid DCs (CD45^{hi}, CD11b$^+$, Cd11c$^+$), and neutrophils (CD45^{hi}, Ly6G$^+$, CD11c$^-$) (PMN) (*C*) by flow cytometry at day 12 in WT, W/W^v, and i.v. BMMC-reconstituted W/W^v mice. *D*, Comparison of infiltrating CNS cell types at day 4. Data in *A–D* represent mean numbers ± SD from eight mice/WT or W/W^v group analyzed individually. *E*, Quantification of neutrophils in LNs of WT, W/W^v, and BMMC-reconstituted W/W^v mice at days 4 and 10 post EAE induction. Data are expressed as mean ± SD and are representative of two independent experiments with $n = 4–5$ mice/group. *F*, BBB permeability of age-matched WT and W/W^v and W/W^v-reconstituted mice with WT BMMCs mice as measured by sodium fluorescein penetrance into the CNS at day 17 post disease induction. Data represent mean ± SD from the analysis of five mice in each experimental group analyzed individually. Significance determined by unpaired Student two-tailed *t* test.

disease as well (Fig. 2*B*). Also striking are the differences in the kinetics of CNS-infiltrating $CD4^+$ T cells. Small differences are detected as early as day 8 (not shown), but the disparity in numbers of Th17 and Th1 cells in W/W^v mice is most notable at day 12 (Fig. 2*C*) and later, suggesting there is an absolute defect in T cell entry. Similar disparities in $CD8^+$ IFN-γ^+ cell entry are also observed, but myelin-specific $CD8^+$ IL-17^+ cells are not detected in any site (data not shown).

Perturbations of the BBB are intimately associated with MS and EAE disease progression and severity (23). Although it is well established that mast cells contribute to general vascular permeability through the production of histamine, serotonin, and other vasoactive molecules (24), their role at the BBB has never been documented. We compared the integrity of the BBB in WT and W/W^v animals after EAE induction by utilizing an in vivo sodium fluorescein assay. Notably, a small increase in permeability is detected in WT mice by day 10 and peaks between days 15 and 17, just prior to and at the time of maximal disease and cellular infiltration (Fig. 2*D*). Of interest, this permeability is transient and undetectable by day 22, at the same time a decline in CNS T cells in observed. W/W^v animals never display appreciable BBB permeability as measured by this assay.

The entry of adoptively transferred encephalitogenic cells into the CNS is also compromised in W/W^v mice

The results of previous adoptive transfer experiments show that encephalitogenic T cells can be detected in the subarachnoid regions of the meninges and the CNS within 24 h of transfer, prior to any signs of overt inflammation (6, 25). We determined whether early T cell entry was affected in mast cell-deficient mice by comparing the ability of passively transferred encephalitogenic Thy 1.1^+ $CD4^+$ WT T cells to enter the CNS of

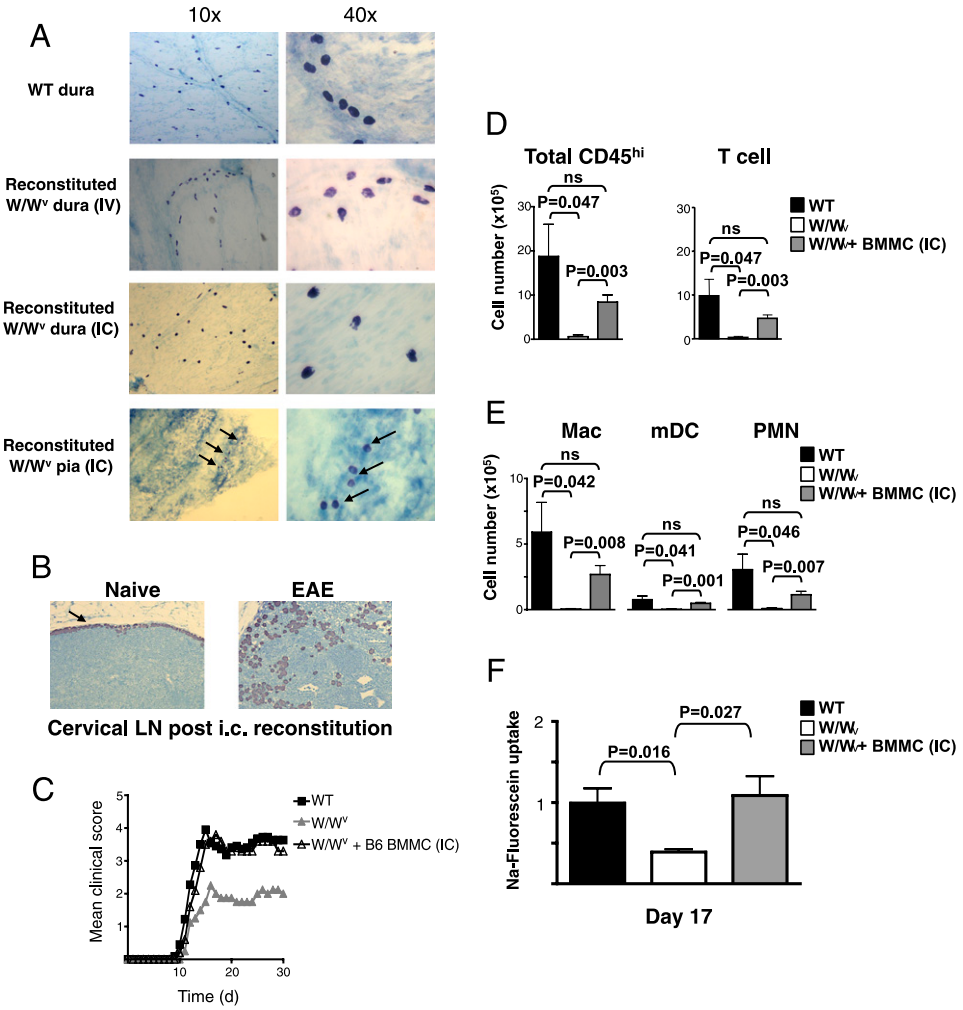

FIGURE 6. Selective reconstitution of mast cells to the dura and pia mater via i.c. injection restores CNS cellular influx and severe disease. Age-matched mice were reconstituted i.v. or i.c. with WT BMMC. In i.c. reconstitution experiments, control mice received equal volumes of PBS i.c. *A*, Mast cells present in the dura and pia mater of i.v. and i.c. BMMC-reconstituted W/W^v mice. Tissues were stained with toluidine blue (original magnification ×10, ×40). Data are representative of >12 each of i.v.- and i.c.-reconstituted mice. *B*, Histological sections of cervical LNs from naive and immunized i.c. BMMC-reconstituted W/W^v mice at day 30 post-EAE induction stained with PE (original magnification ×10) (n = 6 [immunized], n = 3 [naive]; representative images shown). *C*, EAE disease course in WT, W/W^v, and i.c. WT BMMC-reconstituted W/W^v mice (results representative of two independent experiments: experiment 1: WT, n = 11; W/W^v, n = 7; i.v.-reconstituted W/W^v, n = 6; i.c.-reconstituted W/W^v, n = 4; experiment 2: WT, n = 15; W/W^v, n = 8; and i.c.-reconstituted W/W^v, n = 8). Statistics shown in Table I. Quantification of total CNS-infiltrating $CD45^{hi}$ cells and T cells ($CD45^{hi}$, $CD3^+$, $CD4^+$) (*D*) and macrophages ($CD45^{hi}$, $CD11b^+$, $CD11c^-$), myeloid DCs ($CD45^{hi}$, $CD11b^+$, $CD11c^+$) and neutrophils ($CD45^{hi}$, $Ly6G^+$, $CD11c^-$) (PMN) (*E*) by flow cytometry at day 12 post EAE induction in WT, W/W^v, and i.c. WT BMMC-reconstituted W/W^v mice. n = 8/group analyzed individually. *F*, Detection of sodium fluorescein penetrance into the CNS of WT, W/W^v, and i.c. WT BMMC-reconstituted W/W^v mice at day 17. n = 5/group. Significance determined by unpaired Student two-tailed t test.

naive Thy1.2$^+$ WT and W/W^v recipients. Prior to transfer, both IFN-γ– and IL-17–producing cells are evident, as well as a small double-positive population (Fig. 3*A*). At 60 h posttransfer, Thy1.1$^+$ CD4$^+$ cells are clearly distinguishable in the CNS of recipient mice (Fig. 3*B*). However, significantly fewer total numbers of encephalitogenic cells gain CNS access in W/W^v mice (Fig. 3*C*), a finding that corresponds with increased peripheral cell numbers, particularly in the spleen (data not shown). Numbers of Thy1.1$^+$ CD4$^-$ cells, presumably CD8$^+$ T cells, entering the CNS were also reduced (data not shown). Therefore, under conditions of equivalent T cell activation, encephalitogenic T cells are less likely to gain early entrance to the CNS of W/W^v mice, suggesting that mast cells are playing a major role in regulating this infiltration.

CNS-infiltrating T cells fail to be reactivated in W/W^v mice

In addition to Ag encounter in the leptomeninges (6, 7), encephalitogenic T cells in the CNS parenchyma encounter both resident and infiltrating myelin-loaded APCs, allowing reactivation and initiating the local inflammatory response (26, 27). CNS-derived T cells were analyzed on day 17 postimmunization. We observed that the few T cells that are able to enter the CNS of W/W^v mice fail to be strongly reactivated. CNS-derived W/W^v T cells assayed directly ex vivo without peptide restimulation show reduced expression of cytokines (Fig. 4*A*). The relative expression of hallmark cell-surface activation markers that direct cell trafficking is also lower compared with the equivalent population of WT CNS-derived T cells (Fig. 4*B*).

Efficient CNS cellular infiltration in EAE is dependent on mast cells

Definitive evidence that mast cells are regulating CNS inflammatory cell entry is dependent on the ability to re-establish normal cellular infiltration with specific mast cell reconstitution (28). We previously demonstrated that i.v. BMMC reconstitution of W/W^v mice restores WT disease susceptibility (14). In this study, we observed that by day 12, the disease-associated CNS CD45^{hi} cellular influx has dramatically increased in mast cell-reconstituted animals (Fig. 5*A*). Total numbers of all types of infiltrating cells, including CD3$^+$ T cells (Fig. 5*B*), macrophages, myeloid dendritic cells (DCs), and neutrophils (Fig. 5*C*), approach those observed in WT CNS isolates at day 12. Of note, relatively large numbers of neutrophils are detected in CNS preparations from WT mice at day 8, earlier than the major DC and macrophage influx (Fig. 5*D* and data not shown), suggesting that, after T cells, they are the earliest inflammatory cell to enter.

This early disease-induced influx of neutrophils to the CNS is of particular interest because neutrophils are a prominent component of the CNS infiltrate in EAE, and depletion of these cells can delay and even prevent disease development (17, 18). In addition, mast cells recruit neutrophils to sites of inflammation in a variety of disease models (29–32). W/W^v mice characteristically exhibit a mild peripheral neutropenia (33) (Fig. 5*E*). Yet, despite the ability of mast cell reconstitution to restore the neutrophil trafficking to the CNS, reconstituted W/W^v mice continue to exhibit peripheral neutropenia (Fig. 5*E*). I.v. BMMC reconstitution also restores WT-like permeability in these mice (Fig. 5*F*). Taken together, these data support the idea that the breach of the BBB, allowing significant immune cell influx, is strictly dependent on the presence of mast cells.

Meningeal mast cell populations are restored by i.v. mast cell reconstitution

Our data indicate that the major mechanism of mast cell action in EAE is to regulate cellular influx into the CNS by acting locally at a site proximal to the BBB. Yet, there is no evidence of mast cells in the parenchyma of either the brain or spinal cord following i.v. BMMC reconstitution of W/W^v mice despite re-establishment of disease susceptibility (14, 34 and data not shown). These previous studies did not specifically examine the meninges, and the normal presence of numerous mast cells at these sites (Fig. 1*A*, 1*B*) suggests meningeal mast cells are a significant population in EAE. Thus, we asked whether i.v. BMMC reconstitution can restore these cells to this site. As shown in Fig. 6*A*, systemically transferred mast cells populate the dura mater. Consistent with previous results, no mast cells were found in the parenchyma of either the brain or spinal cord.

Intracranial mast cell reconstitution results in selective population of meningeal mast cells in W/W^v mice that restores WT-like disease parameters

Mast cell reconstitution of W/W^v mice to local sites has been used in various disease models to definitively identify their site of action (28). To determine whether we could achieve selective restoration of meningeal mast cells, BMMCs were injected i.c. Eight weeks posttransfer, mast cells are detected in both the dura and pia mater of the meninges (Fig. 6*A*) but not the parenchyma of the CNS, spleen, or stomach or inguinal, axillary, and brachial LNs

Table I. *Intracerebral mast cell reconstitution restores disease susceptibility in W/W^v mice*

	n	Mean High Score (Mean ± SEM)	*p* Value	High Score ≥4
Group 1				
WT	11	4.227 ± 0.1561	WT versus W/W^v: $p < 0.01$	10/11
W/W^v	7	2.857 ± 0.4461		1/7
W/W^v + WT BMMC (i.v.)	6	4.167 ± 0.2789	W/W^v + WT BMMC i.v. versus W/W^v: $p < 0.01$	5/6
W/W^v + WT BMMC (i.c.)	4	4.500 ± 0.2041	W/W^v + WT BMMC i.c. versus W/W^v: $p < 0.01$	4/4
Group 2				
WT	15	3.800 ± 0.1069	WT versus W/W^v: $p < 0.001$	11/15
W/W^v	9	2.056 ± 0.2561		0/9
W/W^v + WT BMMC (i.c.)	8	4.375 ± 0.2266	W/W^v + WT BMMC i.c. versus W/W^v: $p < 0.001$	7/8
W/W^v + HDC$^{-/-}$ BMMC (i.c.)	6	3.917 ± 0.3270	W/W^v + HDC$^{-/-}$ BMMC i.c. versus W/W^v: $p < 0.001$	4/6
W/W^v + TNF$^{-/-}$ BMMC (i.c.)	4	2.375 ± 0.3146	W/W^v + TNF$^{-/-}$ BMMC i.c. versus W/W^v: NS W/W^v + TNF$^{-/-}$ BMMC i.c. versus WT: $p < 0.001$ W/W^v + TNF$^{-/-}$ BMMC i.c. versus W/W^v + B6 BMMC i.c.: $p < 0.001$	0/4

Mean high disease scores of MOG$_{35–55}$ immunized WT, W/W^v, and W/W^v mice reconstituted i.c. or i.v. with WT BMMCs or BMMCs deficient in TNF or HDC (*p* value analysis by one-way ANOVA).

(data not shown). The numbers and distribution of mast cells are not identical to WT with fewer mast cells (~25–50 of WT numbers) and a patchier pattern of engraftment observed (compare Fig. 6*A*, *top left* and *right panels*). Following disease induction, i.c.-reconstituted W/W^v mice develop disease equivalent to WT and i.v.-reconstituted W/W^v animals (Fig. 6, Table I), a finding that correlates with restored cellular infiltration (Fig. 6*D*, 6*E*) and restored BBB permeability (Fig. 6*F*).

Of note, mast cells are also present in the cervical LNs of i.c.-reconstituted mice. In naive mice, these cells are observed primarily in the subcapsular space and occasionally the cortical sinuses (Fig. 6*B*). In animals with disease, mast cells are distributed throughout the cortex and medulla, often in close contact with lymphoid nodules.

Local restoration of HDC$^{-/-}$ mast cells to the meninges does not restore severe disease susceptibility

Histamine is perhaps the most prominent of mast cell-produced molecules that have been implicated in vascular permeability and cellular trafficking (35). To determine whether meningeal mast cell-derived histamine has an effect on disease progression, W/W^v mice were locally reconstituted via i.c. injection with either WT or HDC$^{-/-}$ (20) mast cells. After 8 wk, disease was induced. Some mice in the cohort were examined for the presence of mast cells. The numbers of mast cells found in the dura mater of those that received histamine-deficient cells were comparable to mice receiving WT cells, indicating that the inability to express this factor does not impair growth or long-term establishment at this site (data not shown). As shown in Fig. 7*A* and Table I, local reconstitution with WT and HDC-deficient mast cells restores susceptibility to WT levels of disease. Early disease scores were delayed compared with WT animals (days 11, 13, and 14: $p < 0.5$; day 15: $p < 0.01$), but no statistically significant differences in cumulative or mean high disease scores were observed (Table I).

Mast cell-derived TNF regulates disease through neutrophil recruitment

The lack of a role for mast cell-derived histamine was surprising and led us to consider other molecules. We examined the contribution of mast cell-derived TNF for a number of reasons. TNF mRNA levels were among those increased in diseased animals in both the

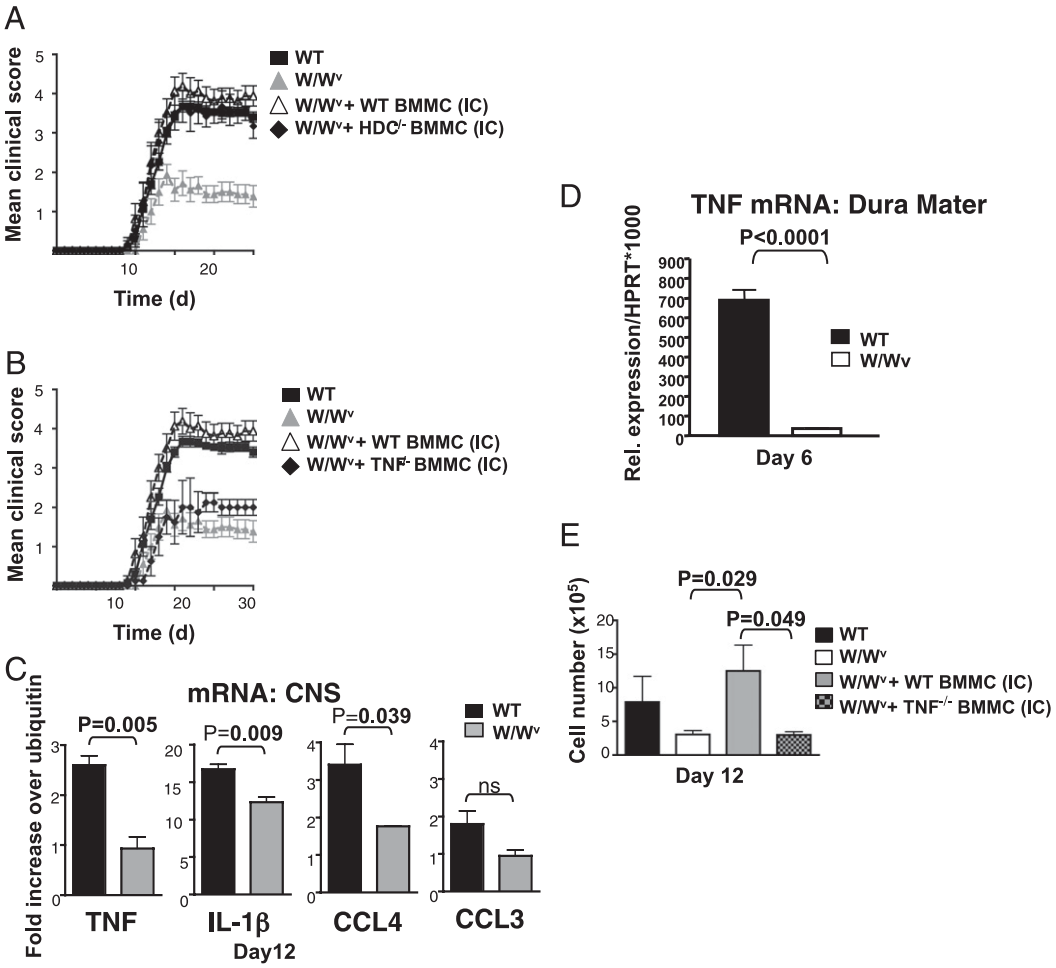

FIGURE 7. Meningeal mast cell-produced TNF is a critical determinant of fulminant disease and neutrophil recruitment to the CNS. EAE disease course in WT, W/W^v, and HDC$^{-/-}$ i.c. BMMC-reconstituted W/W^v mice (*A*) and TNF$^{-/-}$ i.c. BMMC-reconstituted W/W^v mice (*B*) (statistics shown in Table I). Mice were reconstituted with indicated mast cells i.c. TNF$^{-/-}$ reconstitution performed via i.c. (one time) or i.v. was repeated two independent times with at least four animals each. HDC$^{-/-}$ reconstitutions represent one experiment with six individual animals. As controls, age-matched WT and W/W^v mice received i.c. injections of an equal volume of PBS. After 8 wk, disease was induced in mice and scored as described in *Materials and Methods*. *C*, Cytokine and chemokine mRNA expression in total cells isolated from the CNS of WT and W/W^v mice at day 11 post EAE induction as assessed by real time-PCR (data represent results from two independent CNS RNA isolations from pooled cells of five mice/group; each sample was run in duplicate and repeated three times in independent runs). *D*, Expression of TNF mRNA assessed by real time-PCR in the total calvarial dura of naive animals and at day 6 post EAE induction. Representative of two independent experiments, run in duplicate, and repeated three times in independent runs. $n = 5$/group. *E*, Quantification of total CNS-infiltrating neutrophils (PMN) in WT, W/W^v, and TNF$^{-/-}$ i.c. BMMC-reconstituted W/W^v mice by flow cytometry at day 12 post EAE induction. Data from a single experiment with five mice/group. Significance determined by unpaired Student two-tailed *t* test.

CNS at day 11 and the dura mater at day 6 (Fig. 7*C*, 7*D*). As discussed earlier, neutrophils make up a major proportion of the early inflammatory cell infiltrate in the CNS in EAE (17, 18, 36) (Fig. 5*D*). Furthermore, we show that this neutrophil trafficking is dependent on the presence of meningeal mast cells and is selective for the CNS. It is well established that neutrophil interactions with vascular endothelium lead to barrier dysfunction and increased permeability in many inflammatory diseases including EAE (19, 35). Importantly, mast cell TNF production is also required for efficient neutrophil recruitment in many disease models (29–32). To test its possible contribution to disease, i.c. injection of TNF-deficient mast cells was performed on a cohort of W/W^v mice. As shown in Fig. 7*C*, despite the efficient establishment of these cells in the dura mater, there is an inability to induce WT disease levels in these animals. There is also a selective inability to recruit neutrophils to the CNS in TNF$^{-/-}$ mast cell-reconstituted mice (Fig. 7*E*). Taken together, these data implicate TNF produced by meningeal mast cells as a major mediator of disease progression. A model for mast cell action through TNF is shown in Fig. 8.

Discussion

Since the first in vivo demonstration of a role for mast cells in EAE, there have been many examples of the contribution of these cells to both human and murine forms of disease (8). However, mast cells are ubiquitously distributed and multifunctional, making it a major challenge to define the relevant populations and determine their mechanisms of action. The inability of BMMC reconstitution to populate the CNS parenchyma of W/W^v mice despite restoration of WT disease susceptibility originally suggested to us that mast cells exert a major effect in the periphery during the induction phase of disease. Several studies have since established that mast cells have direct influences on T cell activation and function, consistent with this hypothesis (11, 12, 37, 38). We previously observed a small but reproducible defect in early IFN-γ production by CD4$^+$ Th1 cells in W/W^v mice following induction of EAE (22). However, subsequent and comprehensive studies reported in this paper reveal no appreciable differences in the ability to prime Th17 responses or in the general activation state of encephalitogenic T cells. Thus, it is unlikely that this relatively limited difference in peripheral response can fully account for the pronounced disease disparity observed between W/W^v and WT mice.

Our data do demonstrate that the major disease-related alteration in mast cell-deficient mice is the inefficient entry of very early T cells into the CNS as well as the later trafficking of T cells and other inflammatory cells to this site. The consequence of this dearth of cell entry is a reduction in inflammatory myelin destruction and less severe disease. Those encephalitogenic T cells that do ultimately access the CNS of W/W^v mice are unable to be fully reactivated, a consequence likely due to lower numbers of infiltrating APCs, particularly DCs.

These data point to a local gatekeeping function for mast cells at the interface of the periphery and CNS. The conclusion that meningeal mast cells are the relevant population in this process is based on several observations. First, mast cells are very abundant in the meninges, perhaps more densely distributed within the dura mater than within any other tissue except the skin. In addition, the meninges are immunologically relevant structures in EAE and MS. As described earlier, the meninges are sites of initial T cell entry, where myelin-specific cells undergo reactivation and proliferation prior to accessing the CNS parenchyma through meningeal blood vessels (6, 39). In fact, the presence of multifocal perivascular infiltrates and demyelination is most pronounced in areas bordering the meninges (39). Most compelling is our finding that localized transfer of mast cells via i.c. injection, which establishes mast cell populations in the meninges but not within any CNS parenchymal sites, also restores susceptibility to severe disease and results in WT levels of cellular CNS infiltration and BBB permeability in W/W^v mice.

The BBB establishes a physical and metabolic barricade that insulates the CNS from the systemic circulation and protects it in noninflammatory conditions (23). However, this is not a completely impermeable barricade. Under basal conditions, both naive and activated T cells filter in and out of the CNS in a pattern of Ag-seeking behavior, albeit in more limited numbers than in peripheral tissues (40). Our data suggest mast cells play a role in this immune surveillance mechanism and allow this two-way passage of cells prior to the establishment of major inflammation. The inefficient entry of adoptively transferred T cells into the CNS of mast cell-deficient mice at 60 h posttransfer, a time point that encompasses the earliest events in the initiation of inflammation in EAE, indicates that T cell activation alone is not sufficient for CNS entry. The presence of mast cells at the meningeal junction appears to create a more permeable basal environment for early T cell entry into the CNS.

The frank breach of the BBB, permitting more extensive cellular infiltration, is a hallmark feature of both MS and EAE (23). We show in this study that meningeal mast cells govern this breach, although it appears their action is, at least in part, indirect. Mast cell production of histamine at the CNS-BBB interface does not appear to be necessary for disease development, a somewhat surprising observation given its potent effect on generalized vascular permeability. The mast cell-derived mediator of most interest in this regard is TNF. TNF is highly expressed by mast cells and exists as a preformed entity in granules (41). It can be released within seconds of cellular activation or can be induced via new synthesis. We show that TNF mRNA is strongly expressed at day 6 in the dura mater of WT, but not W/W^v mice, and most importantly, reconstitution with TNF-deficient meningeal mast cells cannot restore disease to WT levels.

The variety of previously defined functions associated with TNF is consistent with it playing a critical role in EAE. For example, abrogated EAE is observed in TNFR p55/p75$^{-/-}$ and TNFR p55$^{-/-}$ mice (42), and blocking TNF can inhibit disease (43, 44). In addition, TNF has potent effects on enhancing T cell activation (12) and

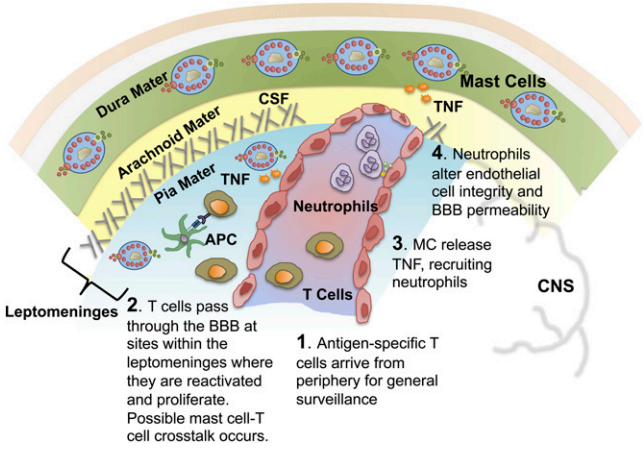

FIGURE 8. Model of meningeal mast cell action in EAE. Recent studies have documented the ability of T cells to traffic into the leptomeninges early in disease (6, 7) [(1) and (2)]. There, they encounter APCs, become reactivated, and proliferate (2). We propose that these proliferating T cells activate mast cells, located within the leptomeninges and the dura mater, to produce and release TNF early in disease. This TNF recruits neutrophils to the region (3). Neutrophils act to alter endothelial cell integrity and BBB permeability, leading to further inflammatory cell influx (4).

altering the activation state of the vascular endothelium (45, 46), although we saw no difference in the expression of VCAM-1 on BBB vasculature of W/W^v mice after disease induction (B. Sayed and M. Walker, unpublished observations). However, TNF also plays a major role in the recruitment of neutrophils as well as DCs (15, 47–50). Although we cannot exclude the possibility that TNF directly enhances the reactivation of T cells in the meninges or alters the activation state of the vascular endothelium, a major function of TNF in this setting is likely to be early neutrophil recruitment to the CNS and the meninges.

Neutrophils have only recently been appreciated as contributors to EAE. Although these cells are prevalent in early CNS infiltrates, they do not comprise a major proportion of inflammatory cells in later disease (17, 36). We also observe numerous neutrophils in the early infiltrate in WT mice after EAE induction. However, the numbers are significantly decreased in W/W^v mice. Furthermore, the clear restoration of disease-induced neutrophil influx to the CNS in the face of continuing peripheral neutropenia in reconstituted W/W^v mice strongly argues that meningeal mast cells are selectively recruiting these cells to the vicinity of the BBB. We propose mast cells may indirectly control early breaches of the BBB through TNF by regulating neutrophil chemotaxis and activation. A report by Carlson et al. (36) supports this idea of direct neutrophil-mediated BBB breakdown. Using the $PLP_{139–151}$-induced relapsing remitting model of EAE in SJL mice, they demonstrate that blockade of CXCR2 in vivo abrogates disease, BBB integrity, and early CNS PMN influx. PMN depletion also prevents BBB disruption and mitigates clinical and histological features of disease despite the presence of activated myelin-specific T cells in the periphery (36). They hypothesize that neutrophils mediate BBB breakdown at a time point between the reactivation of myelin-specific T cells and the massive influx of other inflammatory cells.

It is still unclear how mast cells become activated in this setting. There is evidence that myelin can directly induce mast cell degranulation and superoxide production (51). In EAE, the mycobacterial components of CFA could act as TLR agonists to activate mast cells (reviewed in Ref. 8). However, mast cell effects are apparent in adoptively transferred disease in which TLR signals are not relevant. We speculate that initial T cell localization at meningeal sites allows for mast cell-T cell crosstalk and subsequent mast cell activation, leading to the release of mast cell factors, such as TNF, that affect neutrophil recruitment and ultimately the BBB breach. A number of studies demonstrating such crosstalk are support this idea (10–13, 52–58). In addition, such a scenario is consistent with the time frame proposed by Carlson et al. (36). A model for these T cell-mast cell interactions and effects on neutrophil recruitment, as well as T cell reactivation in the leptomeninges, is shown in Fig. 8.

Our study raises other interesting issues. Both myelin Ags and inflammatory cells drain to the cervical LNs during EAE, where secondary autoreactive T cell activation presumably occurs (59). Although we find little evidence of a robust mast cell influence on primary T cell responses, their presence and disease-induced migration from the capsular region to the T and B cell zones within the cervical LNs suggests a role in subsequent T cell activation. TNF production by mast cells may also be extremely relevant in recruiting DCs and enhancing T cell activation at this site. The results of recent studies strongly support this hypothesis. Tight spatial interactions exist between mast cells and Tregs and Th17 cells in the LNs following disease induction (37). Neutralization of IL-9, a cytokine that mediates mast cell-T regulatory cell crosstalk, and an IL-9R deficiency attenuate EAE (60). This correlates with decreased numbers of Th17 cells and IL-6–producing macrophages in the CNS, as well as decreased mast cell numbers in the cervical LNs. These data indicate that mast cells in the regional LNs are important for amplifying the inflammatory response, perhaps through augmentation of Th17 cell function.

The transient nature of BBB permeability and robust cellular infiltration in EAE is also worth noting. During the initial phases of disease, frank permeability coincides with massive infiltration. Following peak disease, however, there is an apparent return to steady-state conditions despite the ongoing CNS inflammatory response, suggesting specific compensatory mechanisms are in place allowing a return of the vascular barrier to a basal state. As mast cells are clearly involved in the opening of the BBB, it will be important to determine whether a modulation of mast cell function is a necessary part of this compensatory process.

In conclusion, we provide strong evidence that the mast cell populations in the meninges are critical cells for fulminant EAE. To date, the study of meningeal mast cells has been limited to investigations of migraine pain and wound healing after CNS trauma. These data may contribute to the derivation of strategies to alter inflammatory cell trafficking to the CNS. Targeted therapies directed at blocking mast cell action at the CNS interface, in conjunction with the standard disease therapies, may provide more efficacious treatment options for MS as well as other CNS inflammatory diseases.

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Disclosures

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