European Journal of Immunology

Mast cells are required for optimal autoreactive T cell responses in a murine model of multiple sclerosis

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Received 15/7/05
Revised 25/8/05
Accepted 12/10/05

[DOI 10.1002/eji.200535271]

Once considered to be of sole importance in allergy and parasitic infections, the role of mast cells in other pathologic and protective immune responses is becoming increasingly evident. We previously demonstrated that mast cells contribute to the severity of EAE, the rodent model of multiple sclerosis. Here we show that one mode of mast cell action is through effects on the autoreactive T cell response. Early indices of both peripheral CD4 and CD8 T cell activation, including IFN-γ production and increases in CD44 and CD11a expression, are attenuated in mast cell-deficient (W/W^v) mice after myelin oligodendrocyte glycoprotein$_{35–55}$ priming when compared to WT animals. Reduced infiltrates of activated T cells in the central nervous system are also observed. Importantly, selective repletion of the mast cell compartment restores most T cell responses in the lymph nodes and the central nervous system, correlating with reconstitution of severe disease. The adoptive transfer of WT-derived encephalitogenic T cells results in significantly less severe disease in W/W^v recipients, indicating that mast cells also exert potent effects after the initial T cell response is generated. Our data provide the first *in vivo* evidence that mast cells can significantly influence T cell responses and suggest that mast cells exacerbate disease during both the inductive and effector phases.

Key words: Autoimmunity · EAE/MS · Mast cells · T cells

Introduction

Experimental autoimmune/allergic encephalomyelitis (EAE) is the rodent model of multiple sclerosis (MS), a debilitating demyelinating disease of the central nervous system (CNS) [1, 2]. We previously demonstrated that mast cells (MC) influence myelin oligodendrocyte glycoprotein (MOG)$_{35–55}$-induced EAE disease course [3]. WBB6/F$_1$-Kit^W/Kit^{Wv} MC-deficient (W/W^v) mice exhibit delayed onset and reduced severity of EAE compared to their wild-type (WT) littermates. Selective repletion of the MC populations by transfer of *in vitro* differentiated bone marrow-derived MC restores early and severe disease. Though a role for MC was definitively established, their mode of action has remained elusive. It was originally assumed that MC were exerting their primary effects on EAE within the CNS through the local expression of cytokines, chemokines and proteases that promote immune cell influx and myelin damage. However, re-establishment of clinical disease can occur without the appreciable restoration of CNS MC, indicating that MC also act in the periphery to affect disease course [4].

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Abbreviations: **CNS:** central nervous system · **MC:** mast cell · **MOG:** myelin oligodendrocyte glycoprotein · **MS:** multiple sclerosis · **W/W^v:** WBB6/F$_1$-Kit^W/Kit^{Wv}

One potential site of action is through either direct or indirect influence on the generation of the primary T cell response. MC reside in close proximity to dendritic cells (DC) in many tissues and are present at sites of initial T cell activation including the secondary lymphoid organs [4, 5]. They express several molecules known to influence both DC and T cell maturation including the cytokines IL-4, IL-12, IL-15 and TNF-α and the costimulatory molecules CD154 and OX40L [5–10]. In addition, several recent *in vitro* studies have confirmed that MC can regulate T cell function [7]. In this study, we investigated the possibility that MC regulate EAE disease severity through their influence on the autoreactive T cell response. The results provide the first *in vivo* evidence that MC can amplify the magnitude of the early T cell response and promote disease progression after initial priming.

Results

W/W^v-derived T cells are not inherently defective and mediate equivalent disease in a mast cell-competent environment

MC development initiates in the bone marrow and requires the interaction of the c-kit receptor, expressed by hematopoietic precursors, with its ligand, stem cell factor (SCF), produced by stromal and other cells [11]. W/W^v mice contain two distinct mutations in *c-kit*, c-kit^W and c-kit^{Wv}, that compromise signaling and result in MC deficiency and are commonly used to demonstrate the *in vivo* contribution of these cells to physiologic responses. Yet, SCF-c-kit interactions also influence the development of other hematopoietic cells, including T cells [12]. Because EAE is dependent on a functional autoreactive T cell response, we considered the possibility that intrinsic defects in W/W^v T cell populations contribute to attenuated disease. To test this possibility, thymic T cell populations of W/W^v mice and WT littermates were analyzed for indices of normal T cell development. As shown in Fig. 1A, the CD4/CD8 thymocyte profile of W/W^v mice is normal, consistent with a previous report [13]. In addition, equivalent numbers of CD4 and CD8 T cells are present in the peripheral lymph nodes (LN) (Fig. 1B) and spleen (data not shown) of naive W/W^v and WT mice.

We next determined whether W/W^v T cells are intrinsically defective in their ability to become activated and mediate disease. One day prior to EAE disease induction, naive W/W^v or WT splenocytes were transferred i.v. into TCRβ-deficient mice. These animals contain a genetically targeted deletion of the TCR β chain that results in the absence of CD4 and CD8 T cells. Mice were immunized with MOG$_{35–55}$ and clinical disease was scored daily (Fig. 2). As expected, TCRβ-deficient animals did not develop any clinical signs of disease. Mice that received either WT or W/W^v T cells exhibited an identical mean day of disease onset (day 21.8±0.4) and similar disease severity (mean high score WT: 3.5±0.6; W/W^v: 3.6±0.6; p=0.85). In an independent set of experiments, proliferation of naive WT and W/W^v T cells in response to Con A was found to be equivalent (data not shown). These results confirm normal T cell development in W/W^v mice and demonstrate that W/W^v-derived T cells are functionally equivalent to WT T cells in their ability to mediate disease if primed in an MC-competent environment.

Normal lymph node hypertrophy in response to MOG$_{35–55}$ peptide immunization is compromised in W/W^v mice

To examine the potential effects of MC deficiency on T cell function *in vivo*, several indices of T cell activation were assessed. LN hypertrophy is a common sequela to immunological challenge and reflects, in part, the expansion of antigen-specific T cells after antigen encounter. In bacterial infections and dinitrofluoroben-

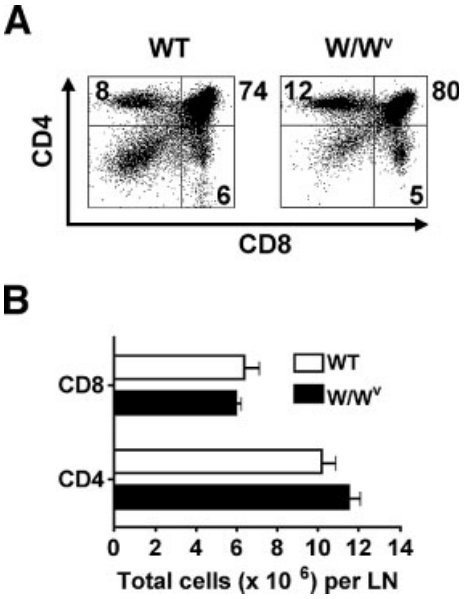

Figure 1. Normal numbers of thymic and peripheral T cells are present in naive W/W^v mice. (A) Thymocytes from naive age-matched WT and W/W^v littermates were analyzed by flow cytometry to evaluate CD4 and CD8 T cell development. Numbers indicate the percentage of cells in each quadrant. The data are representative of three independent analyses. (B) Total CD4 and CD8 T cell numbers in the inguinal LN of 4–6-wk-old naive mice. LN cells were counted and stained with anti-CD4 and anti-CD8 antibodies and analyzed by flow cytometry. Total CD4$^+$ and CD8$^+$ cell numbers were calculated by multiplying the percentage of CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ T cells with the total numbers of LN cells. Data represent the average of LN cells from four individual mice.

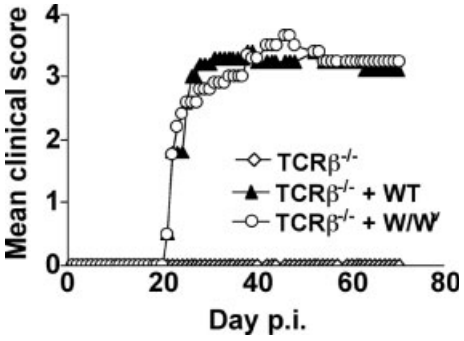

Figure 2. W/W^v- and WT-derived T cells elicit a similar EAE disease course in an MC-competent environment. Naive WT or W/W^v splenocytes (5×10^7) were transferred i.v. into TCR$\beta^{-/-}$ mice 1 day prior to immunization with MOG_{35-55} plus CFA to induce EAE. Mice were scored daily for clinical signs of disease. All mice exhibited comparable mean high scores (3.5±0.6 and 3.6±0.6, respectively) and the same mean day of disease onset (day 22±0.4). Data are representative of two independent experiments with *n*=5 mice/group per experiment. Mean high score represents the average of the highest daily disease score recorded for each group of mice. Mean day of onset represents the average day at which first symptoms of clinical disease were observed in each experimental group.

zene sensitization, LN hypertrophy is MC-dependent [14, 15]. We asked whether there was evidence for decreased LN cellularity in W/W^v mice after peptide immunization as well. Draining inguinal LN and the spleen were harvested from mice at various times post-immunization. By day 20, the LN of W/W^v mice but not the spleen exhibited significantly decreased cellularity when compared to WT mice (Fig. 3A, B). Consistent with this observation, total numbers of W/W^v-derived CD4 and CD8 T cells were significantly reduced (Fig. 3C, D).

Several indices of cellular activation are altered in splenic and lymph node T cells of immunized W/W^v mice

We hypothesize that MC provide a setting that enables optimal T cell activation and next examined whether initial T cell activation is compromised in an MC-deficient environment. The expression of hallmark T cell activation markers and antigen-induced cytokines was assessed at early times post-immunization in spleen- and LN-derived cells. CD44 and CD11a are adhesion molecules required for the initial entry of encephalitogenic T cells into the CNS [16]. Although both these molecules are expressed at low levels in naive T cells, activated T cells express demonstrably higher levels. CD44^{hi} and CD11a^{hi} expression was determined by comparison to naive controls. At day 8 post-immunization, the peak of peripheral T cell responses in WT mice, significantly fewer CD44^{hi} and CD11a^{hi} T cells were

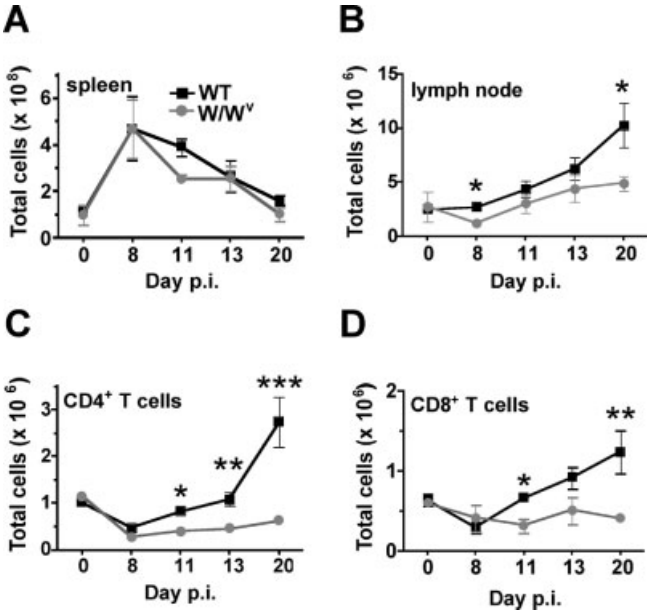

Figure 3. Reduced LN cellularity is observed in primed MC-deficient mice. Total spleen (A) and LN (B) cellularity was analyzed in WT and W/W^v mice at 8, 11, 13 and 20 days post-immunization (p.i.) by Trypan blue exclusion (*n*=4–6 mice/group; $^*p$=0.0025, $^{**}p$=0.02). (C) Total numbers of CD3$^+$ CD4 T cells in the inguinal LN (*n*=6–8 mice/group) as determined by flow cytometric analysis; $^*p$=0.019, $^{**}p$=0.01, $^{***}p$=0.004. (D) Total numbers of CD3$^+$ CD8 T cells in the inguinal LN as in (C); $^*p$=0.076, $^{**}p$=0.01, one-tailed Student's t-test. Total numbers of CD4$^+$ and CD8$^+$ T cells were calculated as described in Fig. 1B.

observed in W/W^v mice as compared to WT controls (Fig. 4A, B). In addition, the normal decrease in CD62L expression associated with the initial exit from the LN [17], was not observed (data not shown). Notably, these differences are consistently most pronounced in the CD8 T cell population.

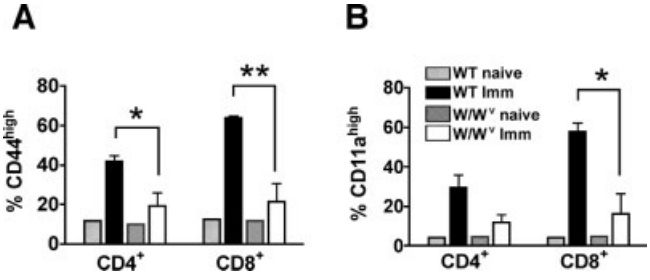

Figure 4. W/W^v-derived T cells exhibit reduced activation-dependent expression of the hallmark activation markers CD44 and CD11a. Several indices of T cell activation were analyzed directly *ex vivo* by flow cytometry after priming with MOG_{35-55} peptide. (A) Percentage of CD44^{hi} CD4$^+$ and CD8$^+$ T cells in the LN at day 8 post-immunization; $^*p$=0.047, $^{**}p$=0.0228. Day 8 represents the peak of the WT response. (B) Percentage of CD11a^{hi} CD4$^+$ (p=0.0753) and CD8$^+$ T cells ($^*p$=0.033) as described in (A).

To evaluate MOG-specific IFN-γ responses, cells were restimulated directly *ex vivo* with $MOG_{35–55}$ prior to analysis by intracellular staining and flow cytometry. This peptide contains both the CD4 and CD8 T cell epitopes [18]. Delayed kinetics and an overall lower frequency of IFN-γ^+ T cells were observed in $MOG_{35–55}$-primed W/W^v mice (Fig. 5A, B). Again, this diminished response was most striking in the CD8 T cell population. Cytokine levels were measured in supernatants from W/W^v spleens restimulated with MOG peptide. The amount of IFN-γ and IL-4 was significantly lower than WT levels, indicating an overall attenuation in MOG-specific cytokine responses and not a shift from Th1 to Th2 (Fig. 5C). Expression of GM-CSF and TNF-α, cytokines associated with severe disease [19, 20], was comparable in W/W^v and WT supernatants (data not shown). Of note, selective MC reconstitution restored antigen-specific W/W^v T cell responses thus verifying the MC dependency of these responses (Fig. 5D).

IL-12 can overcome deficits in $MOG_{35–55}$-specific W/W^v T cell responses and encephalitogenicity

Suboptimal Th1 cell activation in W/W^v mice may be due to the lack of costimulatory molecules such as CD40 and/or cytokines including IL-4 expressed by MC. These factors could directly alter the phenotype of proximal DC and subsequently change the context of antigen presentation to T cells. For example, DC-derived IL-12 acts to promote Th1 cell differentiation during initial antigen encounter and is required for the generation of encephalitogenic T cells *in vivo* and *in vitro* [21]. Early exposure of DC to IL-4 during their initial activation/maturation can inhibit IL-10 expression and promote IL-12 production [22]. One possible explanation for reduced disease is that a lack of MC-derived IL-4 in W/W^v mice leads to a deficit in IL-12 production and less efficient Th1 cell priming. Thus, we investigated whether deficits in T cell activation could be overcome by providing IL-12 *in vitro*.

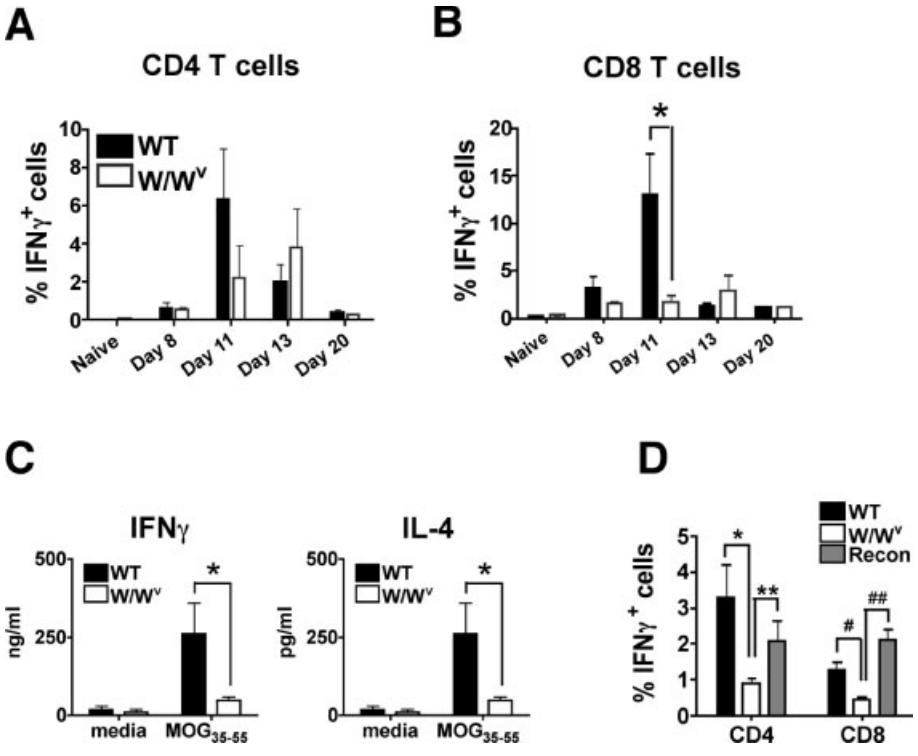

Figure 5. The antigen-specific expression of IFN-γ, a hallmark Th1 cytokine, is reduced in W/W^v-derived T cells after $MOG_{35–55}$ immunization. Inguinal LN cells were harvested from naive and immunized mice at days 8, 11, 13 and 20 after priming with $MOG_{35–55}$ and the percentage of IFN-γ^+ cells was analyzed by flow cytometry in $CD3^+$ $CD4^+$ (A) and $CD8^+$ (B) T cell populations. Data are representative of four independent experiments using two to six mice per group. Percentages were calculated as [(samples restimulated with 30 μg/mL $MOG_{35–55}$ *in vitro* for 3 h) – (samples measured directly *ex vivo*)]; $^*p$=0.0237, one-tailed Student's *t*-test, unpaired. IFN-γ expression by CD4 T cells in $MOG_{35–55}$-primed W/W^v mice was not statistically different from WT mice at any time point (day 11, p=0.1173). (C) Splenocytes from day 11-primed mice were stimulated *in vitro* with $MOG_{35–55}$ peptide and supernatants were analyzed for IFN-γ ($^*p$=0.0004) and IL-4 ($^*p$=0.0486) after 72 h using a multiplex cytokine assay. (D) MC reconstitution restores the ability of W/W^v T cells to generate a strong antigen-specific IFN-γ response. WT, W/W^v and MC-reconstituted W/W^v mice were immunized as described in Materials and methods and LN cells were harvested at day 11 and analyzed as in (A, B); n=4–5/group; $^*p$=0.0182, $^{**}p$=0.0490, $^{\#}p$=0.096, $^{\#\#}p$=0.0012, one-tailed Student's *t*-test, unpaired.

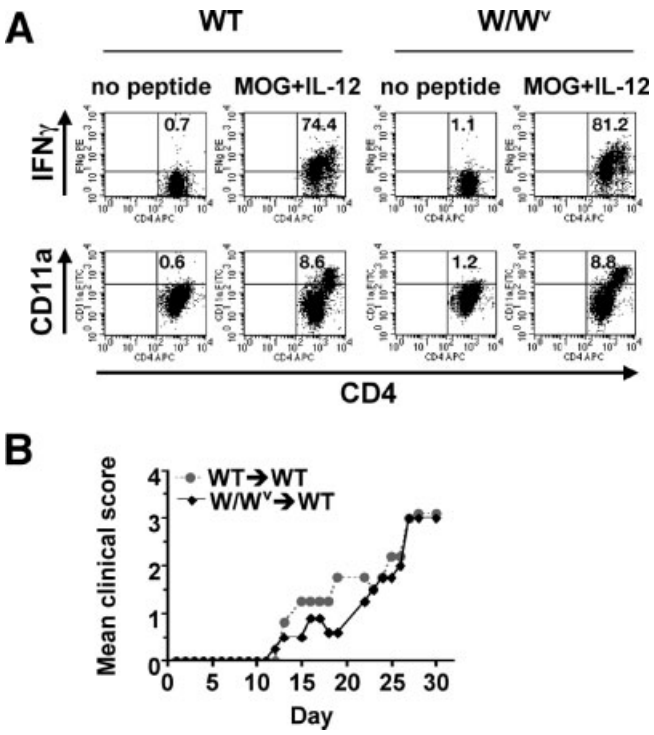

Figure 6. *In vitro* culture of W/W^v T cells with IL-12 restores indices of activation to WT levels and results in comparable encephalitogenicity. (A) WT and W/W^v mice were immunized in the rear flanks with 300 μg $MOG_{35–55}$ emulsified with CFA. Ten days later, inguinal LN were harvested individually from each mouse and the pooled cells were assessed for IFN-γ production after 72 h of culture in the presence or absence of 30 μg/mL $MOG_{35–55}$ and 20 ng/mL rhIL-12. All samples were gated on $CD3^+$ $CD4^+$ cells. Numbers represent the percentage of cells in the quadrant indicated. Data shown are representative of two independent experiments with four mice/group. (B) Equivalent numbers of T cell blasts (2×10^6) generated in (A) were adoptively transferred i.v. to WT and W/W^v mice (n=4).

LN cells were isolated from immunized WT and W/W^v mice and cultured *in vitro* with $MOG_{35–55}$ in the presence of IL-12. The phenotype of the cells was assessed after 72 h. Although W/W^v mice have significantly attenuated T cell responses directly *ex vivo* (see Fig. 4, 5), the frequency of IFN-γ^+ WT and W/W^v CD4 T cells is essentially equivalent after *in vitro* culture in the presence of IL-12 (81.2 vs. 74.4% in W/W^v and WT, respectively; Fig. 6A). Similar percentages of $CD11a^{hi}$ and $CD44^{hi}$ T cells were also observed in both groups after *in vitro* culture (8.8 vs. 8.6% $CD11a^{hi}$ and 74.1 vs. 68.5% $CD44^{hi}$ CD4 T cells in W/W^v and WT cultures, respectively). Transfer of these *in vitro* cultured W/W^v T cells into WT mice elicits similar disease as compared to WT cells (Fig. 6B and Table 1, compare row A to B). The finding that IL-12 can compensate for the MC deficiency is suggestive of an MC-dependent effect on IL-12 production by DC, possibly through MC release of IL-4 during initial DC activation.

Mast cells contribute to disease severity after T cell priming has occurred

Our data thus far confirm that optimal T cell priming is strictly dependent upon the presence of peripheral MC and provide one mechanism through which MC influence disease severity. However, MC are normal residents within the CNS and are found associated with plaques in active MS, suggesting that they also directly influence acute disease [23, 24]. To assess the potential contribution of MC in events that occur subsequent to T cell priming, adoptive transfers of encephalitogenic T cells into WT and W/W^v recipients were performed. WT T cells elicit a disease course with the same day of onset in W/W^v and WT recipients, but mediate significantly less severe disease in W/W^v mice (Table 1,

Table 1. Mast cells influence T cell encephalitogenicity

	Donor	Recipient	Mean day of onset ± SEM[a)]	p value[b)]	Mean cumulative score[c)] ± SEM	p value[b)]
A	W/W^v →	WT	16±2.5		42.3±2.7	
				n.s.[d,e)]		n.s.[d)]
B	WT →	WT	13.5±0.5		45.3±3.9	
				n.s.[d)]		$p<0.01$
C	WT →	W/W^v	13.8±0.8		35.3±4.7	
				$p<0.001$		$p<0.001$
D	W/W^v →	W/W^v	21±2.4		20.8±2.9	

[a)] n=4, representative of two experiments.
[b)] Statistical significance was determined using one-tailed Student's t-test.
[c)] Mean cumulative score is the average sum of all daily scores for each group of mice.
[d)] n.s. = not significant.
[e)] p=0.1823.

compare row B to C). It is notable that despite the apparent recovery of functional encephalitogenic W/W^v T cells after culture in the presence of IL-12, transfer of W/W^v-derived encephalitogenic T cells into MC-deficient hosts results in the least severe disease (Table 1, compare row C to D). This likely indicates a requirement for the continuous involvement of MC *in vivo* for fulminant T cell encephalitogenicity.

Mast cells influence CNS T cell infiltrates

A checkpoint in EAE and MS disease progression is the entry of autoreactive T cells into the CNS through the normally impermeable blood-brain barrier where they orchestrate the inflammation-mediated damage to the myelin sheath [25]. MC could exert effects on these processes through alteration of vascular permeability via histamine release or recruitment of inflammatory cells through release of chemotactic factors [26, 27].

To determine whether resident CNS MC affect T cell infiltration in the brain and spinal cord, WT, W/W^v and reconstituted W/W^v mice were primed and T lymphocyte populations in the CNS were analyzed by flow cytometry. A reduction in the percentage and absolute numbers of both CD4 and CD8 T cells was observed in the CNS of W/W^v mice as compared to WT controls, likely reflecting, in part, the attenuated phenotype observed in the periphery (Fig. 4, 5). In reconstituted mice, a similar profile of CNS-infiltrating CD4, but not CD8 T cells, was observed in the brain and spinal cord as compared to WT animals (Fig. 7A, B). Because MC do not repopulate the brain during reconstitution [4], this selective deficit in CD8 T cell infiltrates indicates that there are distinct requirements for the efficient establishment of these T cell subsets in the CNS compared to CD4 T cells. The restoration of peripheral MC through reconstitution is sufficient for the activation and infiltration of CD4 T cells. In contrast, CD8 T cells also appear to require signals provided by CNS MC.

In line with these findings, we observed that virtually all of the CD8 T cells in the brain and spinal cord of primed WT mice are CD44^{hi}, whereas in W/W^v mice, a significant percentage had a naive CD44$^{lo/-}$ phenotype (Fig. 7C). CD8 T cells in the CNS of reconstituted mice are fully activated, an observation consistent with the repletion of CD4 T cell infiltrates to the CNS, and suggesting that CD4-mediated inflammation within the CNS is required for the efficient activation of CD8 T cells.

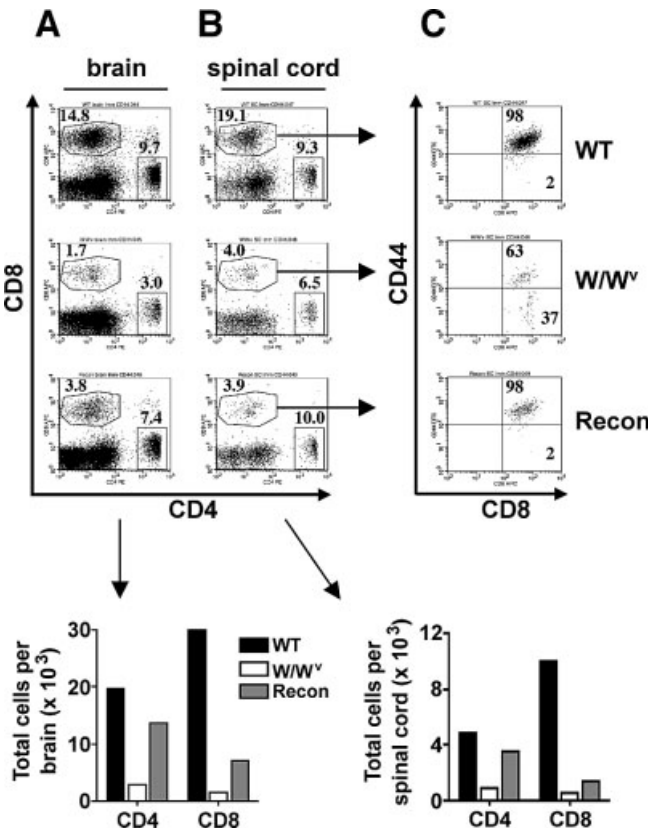

Figure 7. Repletion of the peripheral MC compartment results in normal infiltrates of activated CD4$^+$, but not CD8$^+$ T cells, in the CNS of W/W^v mice. Naive WT, W/W^v and W/W^v MC-reconstituted mice were primed with MOG$_{35–55}$ and infiltrating leukocytes were isolated from the spinal cord and brain (collectively the cerebellum and brainstem) at day 11 and analyzed by flow cytometry. Cells were gated on forward and side scatter to include lymphocytes present in the CNS and the percentages of CD4$^+$ and CD8$^+$ T cells in the brain (A) and spinal cord (B) were determined. Total infiltrating CD4 and CD8 T lymphocytes per brain and spinal cord per mouse are shown in lower panels. (C) Percentage of CD44^{hi} CD3$^+$ CD8$^+$ T cells in the spinal cord. Each experiment represents CNS samples pooled from three to five mice and data are representative of two to four independent experiments per group. The MOG$_{35–55}$ specificity of CNS-infiltrating CD8 T cells was confirmed in independent experiments using MOG$_{37–50}$ H2-D^b tetramers as previously described [18].

Discussion

The role of MC in non-allergic inflammatory responses has been a subject of many recent investigations. Although *in vitro* evidence of T cell-MC interactions exists, as does proof that MC are normally present in secondary lymphoid organs, little is known about the *in vivo* contribution of these cells to adaptive immune responses [28–31]. The availability of the W/W^v mouse model has been pivotal in definitively establishing a role for MC in innate immune responses that confer, for example, resistance to bacterial infection [32]. However,

because the c-kit mutations present in this mouse can affect the development of other hematopoietic cell lineages, the use of this model requires stringent proof that MC are responsible for any phenotypic differences observed between W/W^v mice and their WT counterparts. This is particularly true in instances such as the present study of EAE where MC effects on T cell function are being investigated. Despite the widespread use of W/W^v mice, a careful analysis of potential inherent defects in lymphoid populations has not been previously reported.

In this study we verify that the T cell compartment of naive W/W^v mice is intact and that there are no intrinsic differences in T cell development in the thymus or in peripheral T cell populations. It is only after immunization that the deficit in T cell responses is evident. Taken together with the ability of MC reconstitution to restore disease and T cell activation phenotypes, these data confirm an *in vivo* role for MC in the generation of antigen-specific T cell responses and are consistent with our hypothesis that MC provide a microenvironment that promotes optimal T cell activation.

How might MC influence Th cell differentiation fates during initial antigen exposure? As previously discussed, at many sites of antigen entry, MC are in close proximity to DC and likely modulate T cell phenotypes indirectly through effects on DC [5]. In addition to IL-4, histamine may be important in this context. Histamine regulates *in vitro* IL-12 production by antigen-presenting cells including DC [33–35], which in turn dictates T cell differentiation and function. MC are also positioned to have a direct effect on the generation of Th cells. Normally present at a low frequency within secondary lymphoid organs, MC numbers increase in LN in response to immune challenge [4, 14, 36]. Histamine can act through histamine H1 (H1R) and H2 receptors (H2R) on naive T cells to promote Th1 differentiation and enhance IFN-γ expression by activated T cells [33]. Both H1R$^{-/-}$ and H2R$^{-/-}$ mice have significantly diminished MOG-induced EAE disease severity [34, 37]. Also relevant is that in addition to IL-4 and histamine, MC express IL-12 and costimulatory molecules such as B7-1 and B7-2, CD40 and CD40L which contribute to full T cell activation (for review, see [38]).

It is also of interest that MOG-specific CD8$^+$ T cell responses appear to be most affected by MC deficiency. This observaion may reflect a general requirement for MC in the optimal functioning of CD8$^+$ T cells in a variety of immune settings, a finding that has a bearing on understanding the regulation of anti-viral and anti-tumor immunity. However, it likely also has relevance to the course of EAE. Several recent publications have reported an important role for CD8 T cell responses in EAE [18, 39–41]. It will be of interest to determine whether and how MC affect the generation of individual effector and memory CD8 T cell subsets. In summary, although it was originally hypothesized by our laboratory that MC exerted their major effects within the CNS in EAE and MS, these findings clearly illustrate a new and more complex role for MC in EAE that is likely operational in MS and many other physiologic responses that rely on the adaptive arm of the immune response.

Materials and Methods

Mice

Three- to five-week-old W/W^v (H-2^{bxj}) female mice, their congenic littermates (WBB6/F$_1$-Kit$^+$/Kit$^+$, WT) and C57BL/6-Tcrb^{tmlMom} (TCR$\beta^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animal care was provided according to protocols approved by the Institutional Animal Care and Use Committee of Emory University and Animal Care and Use Committee of Northwestern University. The MC compartment of W/W^v mice was selectively reconstituted by i.v. transfer of 4×10^6 bone marrow-derived MC as previously described [3].

EAE induction and disease evaluation

Disease was induced by priming with 300 μg of MOG$_{35–55}$ as previously described [3]. Alternatively, adoptive transfer of EAE was performed based on a protocol described by Tompkins *et al.* [42]. Briefly, draining inguinal LN were harvested at day 11 post-immunization and cells were cultured with 30 μg/mL MOG$_{35–55}$ and 20 ng/mL rhIL-12 (R&D Systems, Minneapolis, MN) in complete RPMI. After 72 h in culture, 2×10^6 T cell blasts were injected i.v. (day 0) into the tail vein of either WT or W/W^v mice. Mice were scored daily for clinical signs of paralysis: 0, no physical sign of disease; 1, tail flaccidity; 2, hind limb weakness; 3, hind limb paralysis; 4, fore limb paralysis and/or the inability to right from supine; 5, moribund.

Kinetic analysis of cell surface markers and cytokine production

At various times post-immunization cell surface analyses of draining LN and spleen were performed. Antibodies specific for CD3, CD4, CD8, CD44, CD11a and CD62L were obtained from BD Biosciences (San Diego, CA). To evaluate IFN-γ production, either intracellular cytokine staining (Cytofix/Cytoperm Kit, BD Biosciences) or cytokine secretion assays (Miltenyi Biotech, Auburn, CA) were used as per manufacturer's instructions. Samples were analyzed on a FACS Caliber using CellQuest software (BD Biosciences).

Liquichip analysis of cytokine production

Spleens were harvested from MOG$_{35–55}$-primed mice 11 days post-immunization. Cells were plated at 4×10^6 cells/well in 2 mL of complete Dulbecco's modified Eagle's medium and cultured with medium alone, OVA$_{323–339}$ or MOG$_{35–55}$ peptide

(30 μg/mL), or Con A (1 μg/mL) at 37°C, 5% CO_2. Culture supernatants were collected at 72 h and cytokine levels were assayed using the Beadlyte® Mouse Multi-Cytokine Detection System 2 (Upstate Biotechnology Inc., Lake Placid, NY), according to manufacturer's instructions.

Isolation of CNS leukocytes

Mice were anesthetized at day 11 post-immunization and perfused with phosphate-buffered saline. Brain (combined cerebellum and brainstem) and spinal cord were removed, minced in Hank's balanced salt solution (without Ca/Mg) and treated with 300 U/mL of Collagenase Type 4 (Worthington Biochemical, Lakewood, NJ) for 45 min at 37°C. Lymphocytes were enriched using a 30% Percoll gradient and analyzed via flow cytometry.

Acknowledgements: We would like to thank Dr. Brian Evavold at Emory University (Atlanta, GA) for supplying us with MOG37–50 H2-Db tetramers. This work was supported by a grant from the National Multiple Sclerosis Society (to M.A.B).

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