School of Medicine and Faculty of Science

PH.D. PROGRAM IN TRANSLATIONAL AND MOLECULAR MEDICINE

DIMET

MAST CELLS IN THE PATHOGENESIS OF EXPERIMENTAL MULTIPLE SCLEROSIS

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CHAPTER 1

INTRODUCTION

1. MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic inflammatory neurodegenerative disorder of the central nervous system (CNS), characterized by the presence of multifocal plaques of demyelination, immune cell infiltration and axonal damage, primarily located in the white matter [1]. MS is the most common cause of neurologic disability in the white young adult population, affecting approximately 2.5 million people worldwide [2]. Since its first description by Carswell, Charcot and Cruvellier in 19th century, several advances have been done in the comprehension of the pathogenesis of MS. Nonetheless the aetiology of this disease is still elusive and therapeutic interventions have limited efficacy [3]. MS is widely thought to occur in genetically predisposed individuals following exposure to an environmental trigger that activates myelin-specific T cells in the peripheral lymphoid organs. Following re-stimulation in the CNS, autoreactive T cells orchestrate an immune-mediated attack against components of myelin, leading to demyelination and axonal injury [4]. Demyelination and damage of axons lead to inefficient propagation of action potentials through the internodes of nerves (loss of saltatory conduction) and result in neurological symptoms [2].

1. 1. Clinical Features and Epidemiology

1.1.1. Clinical patterns of MS

Four clinical patterns of MS are recognized by international consensus (Fig. 1) [5]. The relapsing-remitting form (RR-MS) affects approximately 85% of patients [5]. RR-MS typically starts between 20 and 40 years of age and has a female prevalence of approximately 2:1 [1]. RR-MS is characterized by recurrent acute episodes of neurologic dysfunction (relapses) lasting for several days; they generally occur one or two times per year and are followed by complete or partial recovery (remissions) over several months [1, 5]. In approximately 70% of cases, RR-SM converts to a secondary progressive form (SP-MS) in later stages of disease [1]. Early symptoms in RR-MS include sensory disturbances, unilateral optic neuritis, double vision (diplopia), limb weakness, gait ataxia, trunk and limb paraesthesia on neck flexion (Lhermitte's sign) [1, 2]. Axonal discharge and ephaptic transmission between demyelinated axons are thought to induce paroxysmal symptoms (e.g. trigeminal neuralgia, brief tonic spasms, vertigo) [2]. As the disease worsens bladder dysfunction, fatigue and increase in body temperature (Uhthoff's symptom) are common [1, 2]. In more advanced stages of disease cognitive deficits (e.g. memory loss, impaired attention), dysphagia, progressive quadriparesis and sexual dysfunction can occur. Cortical signs (early dementia, aphasia, seizures) are present in MS patients only rarely [1, 2].

In 10% of patients the disease is progressive from the onset without relapses, therefore called primary-progressive (PP-MS), and displays a similar incidence between females and males [5]. It affects more often

the spinal cord and, less frequently, the optic nerve, cerebrum or cerebellum. PP-MS often presents with an upper-motor neuron syndrome of the legs and gradually worsens leading to cognitive impairment, quadriparesis, visual loss, bladder and sexual dysfunction [1, 5]. Approximately 5% of patients experience a progressive-relapsing form of disease (PR-SM), characterized by a progressive onset, later associated to one or more relapses [5].

The natural history of MS is largely unpredictable. However female sex, sensory or visual symptoms and complete recovery from relapses are more favourable prognostic factors. On the contrary male gender, motor involvement, frequent relapses and development of large number and volume of lesions in early stages of disease are predictive of a more severe progression of MS [6-8].

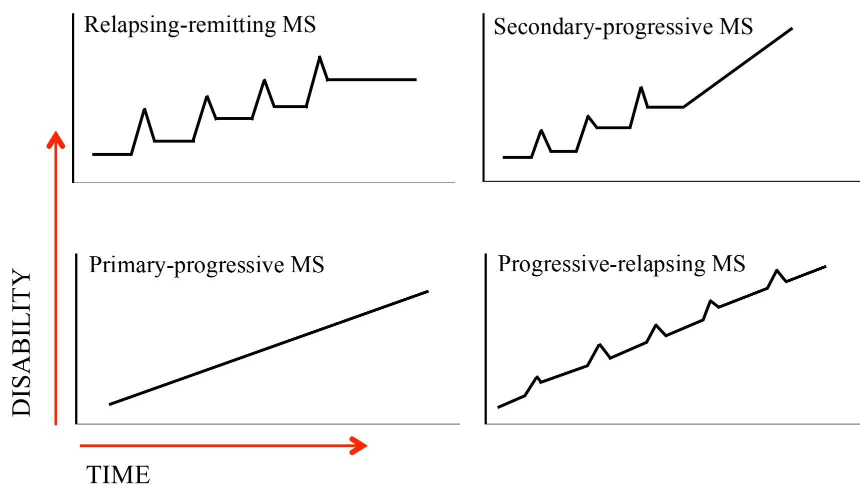

Figure 1. *Clinical Patterns of Multiple Sclerosis.*

1.1.2. Diagnosis

Diagnosis of MS takes advantage of both imaging technologies (magnetic resonance imaging, MRI) and laboratory investigations, that allow establishing “dissemination in space and time” of neurologic dysfunction. After the first clinical attack, dissemination in space and time can be demonstrated by the evaluation of subsequent MRI abnormalities, according to specific criteria [9]. T_2-weighted MRI scans of MS brain highlight multiple bright signal abnormalities in the white matter of the CNS, affecting often the periventricular areas and corpus callosum (Fig. 2A). T_1-weighted MRI after the intravenous administration of a contrast agent (i.e. gadolinium, a heavy metal) identifies areas of breakdown of the blood-brain barrier (BBB), which appear as hyper-intensity regions, due to the extrusion of this metal across the BBB (Fig. 2B).

Approximately 50-90% of MS patients exhibit immunoglobulins (Ig) of restricted specificity (oligoclonal bands, OCB) in the cerebrospinal fluid (CSF) (Fig. 2C). The presence of intrathecal OCBs suggests an inflammatory process occurring in the CNS and can be of help in the diagnostic process [2]. Subclinical lesions can also be identified by the evaluation of latencies of visual, auditory and somatosensory evoked potentials, which may be delayed in MS patients. This test can be useful to provide support for the “dissemination in space” of MS lesions (Fig. 2C) [10].

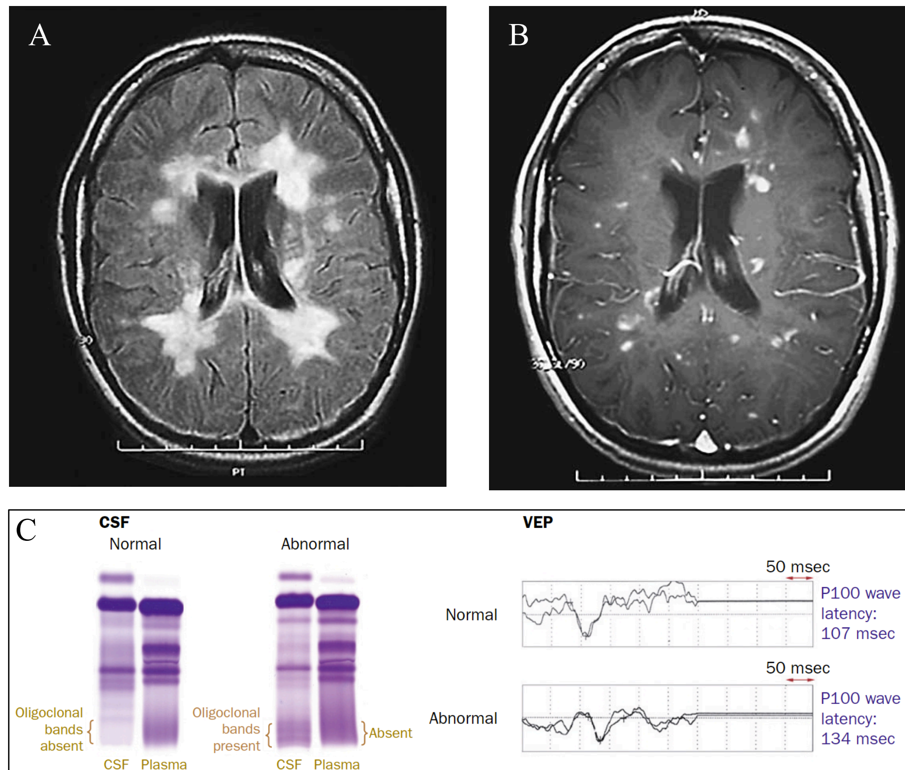

Figure 2. *Clinical investigations for diagnosis of multiple sclerosis*. (A) An axial FLAIR (Fluid Attenuated Inversion Recovery) T_2-weighted MRI scan of MS brain shows multiple ovoid hyper-intense lesions in the periventricular white matter. (B) After the administration of gadolinium, many of the lesions demonstrate ring or peripheral enhancement, indicating the breakdown of the blood–brain barrier. (C) CSF protein electrophoresis highlights oligoclonal Ig bands in 50-90% of MS cases. Latency of evoked visual (VEP), auditory and somatosensory potentials is typically delayed in MS patients. (Adapted from Noseworthy et al., *NEJM* 2000 and Compston and Coles, *Lancet* 2002 [1, 2]).

1.1.3. Epidemiology

Kurtzke has identified areas of MS prevalence. High prevalence regions comprise northern Europe, the northern USA and Canada, New Zealand and southern Australia. Medium prevalence areas include southern Europe, southern USA and northern Australia. Low prevalence zones include Asia and South America (Fig. 3) [11].

Migration studies have shown that people migrating from a low risk to a higher risk area, retain the low risk of their area of origin [12]. However children of immigrants that moved from a low risk area to UK (a high risk area), acquired risk of MS similar to other UK-born children, that is a higher risk than their parents [12]. Some studies also reported that age of migration could influence the acquisition of MS risk. In brief, people migrating from a low to a high risk area of USA had lower risk of MS, if they were migrating before being 15 years old [13]. Also European immigrants to Israel (low risk area) retained a higher risk of MS, if migrating at older ages (>15 years) [12]. The variability of the prevalence and incidence of MS in different regions of the world could be due to combination and cooperation of both genetic and environmental factors.

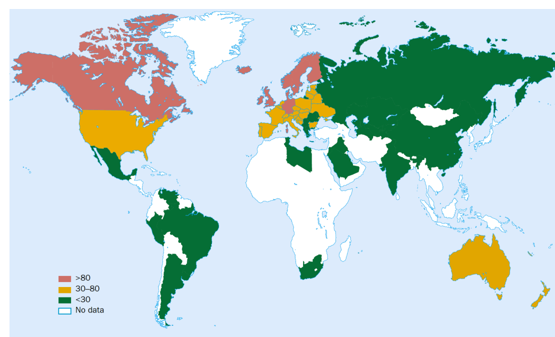

Figure 3. *Worldwide prevalence of multiple sclerosis per 100 000 population* (Marrie, *Lancet Neurol* 2004 [12]).

1.2. PATHOGENESIS

1.2.1. Genetic Factors

Familial aggregations of MS cases and higher (or lower) incidence of disease in specific ethnics have suggested a genetic basis for the development of MS [14]. First-degree relatives of affected individuals have a 20-50 fold (2%-5%) higher risk to develop MS, and monozygotic twins show concordance rate between 20 and 35% [15]. A parent of origin effect in MS has also been described. Maternal half-siblings of MS patients have almost double the risk of developing MS if compared to paternal half siblings (2,35% vs 1,31%), whereas MS risk in maternal half-sibling does not differ significantly if compared to full-siblings (2,35 % vs 3,11%) [14].

Some ethnic groups are at higher risk of MS than others. Higher frequency rate is found in North America, Scandinavia, Iceland, British Isles [5]. Lower prevalence rate is common in Japanese, Chinese, African and Native Americans, Mexicans, Samis, New Zealand Maori and Turkmen [5, 16].

The most important genetic factor affecting MS susceptibility has been identified on chromosome 6p21 and corresponds to major histocompatibility complex (MHC) locus. This locus is believed to account for 10%-60% of the genetic risk for MS [17, 18]. In particular the histocompatibility leukocyte antigen (HLA)-DR15 haplotype in Caucasian (DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602) is considered the strongest “risk allele” for MS [17]. Other genes within the DR15 haplotype include the tumour necrosis factor (TNF) cluster, transforming growth factor (TGF)- β family members, cytotoxic T

lymphocyte-associated antigen (CTLA)-4, interleukin (IL)-1 receptor antagonist, IL-1 and estrogen receptor [15]. In other areas (e.g. Sardinia) other HLA class II alleles have been associated to increased risk of MS and they include HLA-DRB1*0301, DRB1*0405 and DRB1*1303 [19]. Association of HLA class I alleles to MS seems much lower than HLA class II. HLA-A3 (A*0301) and -B7 are reported to increase susceptibility, whereas HLA-A2 (A*0201) confers protection against the disease [15].

Genome-wide association studies have discovered other genes with a modest effect on MS, and they comprise IL-7 receptor α (IL7RA), IL-2 receptor α (IL2RA), tumour necrosis factor receptor superfamily member 1A (TNFRSF1A), C-type lectin-domain family 16 member A (CLEC16A), interferon regulatory factor 8 (IRF8), CD58 and CD6 [20].

The fact that MS incidence is approximately 2-fold higher in women than men has suggested that female physiology, and hormones in particular, could account for this difference [14]. This hypothesis has also been supported by the observation of decreased relapse rate in late pregnancy [21]. However the mechanisms by which hormones could influence the expression of MS are still under investigation.

1.2.2. Environmental Factors

The low concordance of MS in identical twins and recurrence familial rate suggest a contribution of the environment to the aetiology of MS [15]. A huge number of environmental agents have been investigated as potential causative factors in MS pathogenesis.

The north to south decreasing gradient of MS prevalence in the

northern hemisphere has implied latitude as a potential environmental risk factor [14, 15]. One possible explanation of the association between latitude and MS incidence could be sunlight exposure [14]. Several studies reported a strong inverse correlation between levels of ultraviolet radiation or past sunlight exposure and MS susceptibility [12, 14, 22]. The effect of sunlight may potentially be mediated by vitamin D [15]. A prospective, nested case-control epidemiological study in US army has shown that the risk of MS among whites significantly decreased with increasing serum levels of 25-hydroxyvitamin D [23].

Bacteria and viruses have been extensively studied as potential environmental triggers of MS. They include *Chlamydia pneumoniae*, measles, rubella, mumps and several herpes viruses as herpes simplex virus (HSV) 1 and 2, varicella zoster virus, HHV6 and Epstein Barr virus (EBV) [14, 15]. The search for a viral agent responsible for MS has also been encouraged by the viral aetiology of some other human demyelinating diseases, as progressive multifocal leukoencephalopathy induced by papovavirus JC; post-infectious encephalitis and subacute sclerosing panencephalitis, both caused by measles virus; HIV encephalopathy [24, 25]. The most compelling association of a virus with MS has been found for EBV. This virus leads to a latent lifelong infection of B cells. EBV affects almost 100% of MS patients in comparison to 94% of age-matched controls [26]. Serum titres of antibodies (Ab) against the EBV nuclear antigen 1 (EBNA1) seem to be increased several years before the onset of MS [27]. Individuals with a history of infectious mononucleosis have been shown to have an increased risk of developing MS, even though

some studies did not confirm these data [12]. In addition a recent report has shown an abnormal accumulation of EBV-infected B cells in MS brains [28]. Nonetheless the actual causative or pathogenic role of this viral agent in MS is still under debate.

1.2.3. Pathogenic steps in the development of CNS autoimmune response

MS is generally perceived as an inflammatory demyelinating disorder of the CNS, triggered by a $CD4^+$ T cell-driven immune response against components of myelin [29]. MS and its pathogenesis have been widely studied by taking advantage of an animal model, experimental autoimmune (or allergic) encephalomyelitis (EAE) [30]. EAE is induced in susceptible strains either by an active immunization with immunodominant epitopes of myelin antigens supplemented with adjuvants, or by passive transfer of $CD4^+$ T cells activated against myelin antigens [31]. The ability to induce EAE by adoptive transfer of *in vitro*-activated myelin-specific $CD4^+$ T cells has reinforced a prominent role for this T cell subset in the pathogenesis of MS [32, 33]. However many evidences suggest that several other immune players could have a significant role to the elicitation of MS, such as $CD8^+$ T cells, B cells and antibodies or cells of the innate immune system as mast cells [15, 34].

1.2.3.1. Peripheral activation of autoreactive T cells

Autoreactive T cells are present also in healthy individuals and they are supposed to have escaped mechanisms of central tolerance [4, 5]. Central tolerance is the process by which T cells recognizing with

high-avidity self-peptides presented on MHC complexes in the thymus are deleted. However thymocytes expressing a TCR with low affinity for self-antigens-MHC complexes or thymocytes specific for a self-peptide that does not bind well to MHC molecules can escape central tolerance [4]. In physiological conditions these circulating autoreactive T cells are kept under a state of "ignorance" by additional mechanisms of peripheral tolerance and regulatory networks. However the avidity of autoreactive T cells for self-antigens can be augmented under particular conditions, thus leading to their aberrant, pathological activation [4, 5].

In the context of CNS immune responses, it has been shown that nonself-antigens introduced in the CNS can be detected in cervical or paraspinal lymph nodes [35]. Analogously in transgenic mice expressing a TCR specific for myelin proteolipid protein (PLP), which develop spontaneous EAE, myelin-specific T cells seem to be activated first in the CNS-draining cervical lymph nodes [36]. It is still unclear if these antigens are passively drained or actively carried in lymphoid organs by antigen presenting cells (APCs) [35]. The reasons why APCs should present myelin antigens in an immunogenic context and activate self-reactive T cells have not been identified, but several mechanisms have been proposed related to microbial infections.

Viruses and bacteria have been considered by far as environmental triggers with the ability of breaking peripheral tolerance and activate CNS autoimmune responses. However specific pathogens have still not been conclusively identified in MS [14]. It has been shown that transgenic mice expressing myelin- specific TCR develop

spontaneous EAE if housed under nonpathogen-free conditions, whereas if they are kept in a pathogen-free facility they remain disease-free [37]. Several mechanisms have been hypothesised to explain how infectious agents could trigger MS:

a) Molecular mimicry. Structural similarities shared by epitopes of microbial and self-proteins can induce cross-reactivity of T and B cells in the host [15]. It has been shown that MBP-specific T cell clones from MS patients could be activated by viral and bacterial peptides sharing with MBP the MHC-TCR contact amino acid motif [38]. Subsequently Lang et al. have demonstrated that an MBP-specific T cell line from a MS patient was able to recognize both a DRB1*1501-restricted myelin basic protein (MBP) and DRB5*0101-restricted Epstein-Barr virus (EBV) peptide. These findings highlighted that the same TCR can cross-react with different peptides bound on different class II molecules, as long as charge distribution and overall shape are preserved [39].

b) Bystander activation. Another possibility could be that recurrent infections by pathogens may provide adjuvant signals, such as cytokines, superantigens or toll-like receptor ligands, which induce a nonspecific polyclonal activation of autoreactive T cells [15]. In the EAE model it has been shown that a superantigen (staphylococcal enterotoxin B) can induce relapses through interaction with a MBP-specific T cell clone expressing V β8 TCR [40].

A second, alternative proposed mechanism of bystander activation depends on specific TCR recognition.

During a microbial infection, killing of infected cells can result in destruction of tissue and release of autoantigens. The presentation of self-antigens to autoreactive T cells in a pro-inflammatory context, could lead to their aberrant activation and later epitope spreading [15]. In mice persistent infection of the CNS by Theiler's virus induces a virus-specific T cell response that later expands to myelin antigen and induce a demyelinating inflammatory pathology of the CNS [41]. In addition during viral infections the altered and increased processing of self-peptides by APCs, could uncover cryptic epitopes, which are normally not processed or presented to T cells. This process is believed to be more relevant for the epitope spreading [15, 42, 43].

1.2.3.2. Infiltration of autoreactive T cells in the CNS

The CNS has long been considered an "immune-privileged" site of the body. Indeed CNS parenchyma is protected from external stimuli/insult by the blood-brain barrier (BBB), a specialized barrier made of tight junctions between endothelial cells of blood vessels, the basement membrane of vessels, astrocyte feet and microglial cells [4]. The blood-CSF barrier, which is composed of the tight junctions between epithelial cells, surrounds the choroid plexus and meningeal venules [4] (Fig. 4). Recently it has been highlighted that in physiological conditions activated and memory T cells express adhesion molecules and chemokine receptors necessary for crossing these barriers and perform CNS immune surveillance [44].

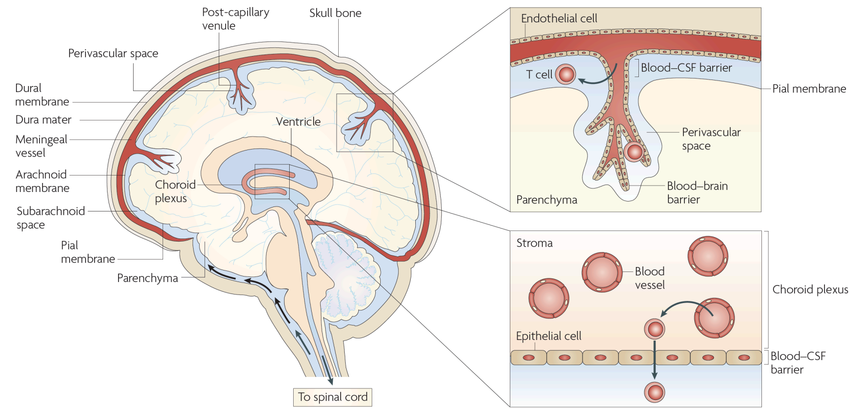

Figure 4. *Possible routes of entry of activated T cells in the brain*. Activated T cells can reach the subarachnoid space by crossing the blood-CSF barrier of meningeal venules or choroid plexus or get into the brain tissue by migrating through the BBB surrounding post-capillary venules which penetrate the brain parenchyma (Goverman, *Nat Rev Immunol* 2009 [4]).

However in absence of an inflammatory response, endothelial cells of the BBB have been shown not to express complementary adhesion molecules that are essential for T cells to adhere to the vessel wall [44]. In contrast, adhesion molecules and selectins are constitutively expressed on the blood-CSF barrier, which therefore has been proposed as the main route of entry of T cells carrying out immune surveillance of the CNS [44, 45]. T cells crossing the blood-CSF barrier get to the region between the pial and arachnoid membranes, namely subarachnoid space (SAS) [4]. In line with this hypothesis, several studies have shown that SAS is the first site of entrance of $CD4^+$ T cells in the CNS during EAE [46, 47]. In this area T cells, re-stimulated by local APCs, proliferate and induce endothelial cell activation, leading to recruitment of other immune cells. The

inflammatory damage occurring in SAS may activate distal microglial cells that stimulate the up-regulation of adhesion molecules on parenchymal vessels and favour the recruitment of other immune cells through the BBB [4]. Activated T cells crossing endothelial cells of BBB, have to digest subendothelial basal lamina containing type IV collagen, before reaching brain parenchyma. Matrix metalloproteases (MMPs) 2 and 9 (also called gelatinase A and B), which specifically degrade collagen type IV, have been found in perivascular infiltrates of MS lesions and in spinal fluid of MS patients [5, 34].

A key molecule regulating the entry of $CD4^+$ T cells in the CNS is the integrin very late antigen (VLA)-4. VLA-4 has been found on perivascular T cells of acute MS lesions [34]. VLA-4 binds vascular cell adhesion molecule (VCAM)-1, that is expressed by activated endothelial cells and mediates adhesion of $CD4^+$ T cell derived from RR-MS patients to inflamed brain endothelium [48, 49]. On the base of promising results obtained in EAE [50], targeting of VLA-4 by a monoclonal antibody (natalizumab, also known as Tysabri) produced a rapid decrease of contrast-enhancing MRI lesions and a reduction of the incidence of relapses in MS subjects [49]. However this treatment unexpectedly resulted also in decrease of immune surveillance and the emergence of multifocal leukoencephalopathy in a small number of individuals [5].

Once the blood-CSF and blood-brain barriers are breached, immune cells (e.g. T cells, B cells, macrophages, APCs, mast cells) can diffuse into the white matter of the CNS, target components of myelin sheath and induce demyelination and axonal damage observed in MS plaques (Fig. 5) [31].

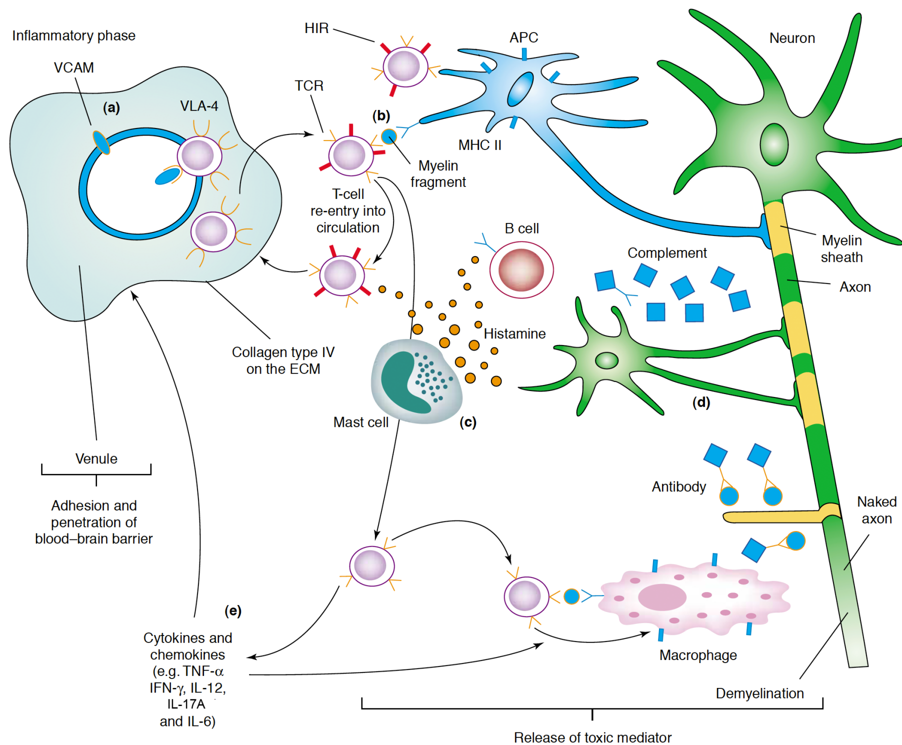

Figure 5. *Pathophysiology of EAE and presumably MS*. (a) Following peripheral activation, T and B cells penetrate the CNS blood vessel endothelium. The key molecule in adhesion is VLA-4 integrin on T and B cells. This integrin binds to VCAM and the lymphocytes diapedese, crawling through and penetrating the extracellular matrix. Matrix metalloproteases (MMPs) are crucial for this process. (b) Once inside the brain, T cells recognize myelin fragments in association with class II molecules of the MHC. (c) Mast cells and histamine may also have a role in modulating the inflammatory response in MS and EAE. (d) Antibodies to protein and lipid components of the myelin sheath can activate complement, culminating in the production of membrane attack complexes, which damage the oligodendrocyte and lead to the stripping of myelin by activated macrophages.

Figure 5 *(continued)*. (e) Inflammatory cytokines, such as IL-6, IL-17A, TNF and IFN-γ, amplify the inflammatory response in the brain. Some of these cytokines have Janus-like activities, both inducing pathology but also having key roles in recovery (modified from Steinman and Zamvil, *Trends Immunol* 2005 [31]).

1.2.4. The MS plaque: neuropathological features.

The pathological hallmark of MS consists of multiple plaques of demyelination, located more often in the optic nerves, periventricular white matter, corpus callosum, brain stem, cerebellum and spinal cord [1]. Several types of lesions have been distinguished and classified, according to structural and immunopathological features [51, 52].

Acute active plaques (Fig. 6) are generally characterized by hypercellularity, infiltration of myelin-laden macrophages, T cells, B lymphocytes and plasma cells in both perivascular areas and parenchyma, loss of myelin, reactive hypertrophic astrocytes, axonal damage [3, 51]. A deeper analysis of acute plaques has identified four pathological subtypes, which seem to vary among different individuals, but not intraindividually [51, 52]. Pattern I is characterized by prevailing infiltration of macrophages and T cells and often remyelination [52]. Pattern II is similar to pattern I with the additional precipitation of activated complement and antibodies on degenerating myelin sheaths [52]. This feature resembles tissue pathology observed in Guillain Barrè syndrome, an acute inflammatory demyelinating disorder of the peripheral nervous system [15, 52].

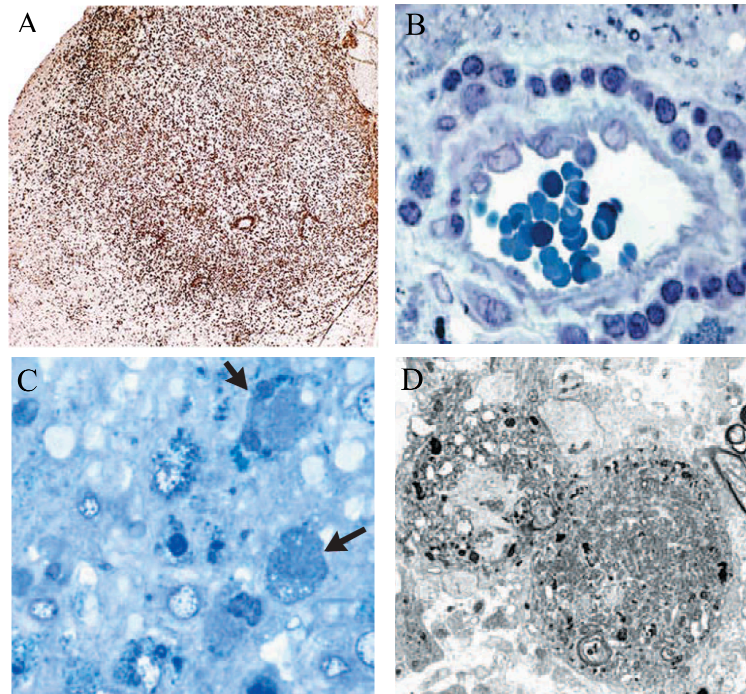

Figure 6. *The acute active plaque.* (A) Extensive macrophage infiltration throughout the extent of the lesion (staining for KiM1P macrophage marker). (B) A blood vessel with red cells is ringed by small lymphocytes and is surrounded by a demyelinated parenchyma (toluidine blue staining). (C) A demyelinated area with numerous damaged transected axons (arrows) (toluidine blue staining). (D) Electron micrograph showing axonal sferoids filled with accumulations of mitochondria, dense bodies and neurofilaments (adapted from Frohman, *NEJM* 2006 and Hu and Lucchinetti, *Semin Immunopathol* 2009 [3, 51]).

Pattern III displays indistinct margins of macrophages, apoptotic oligodendrocytes with preferential loss of myelin-associated glycoprotein (MAG) and significant expression of hypoxia inducible factor (HIF)-1α, a specific marker of hypoxic injury, which is indeed

upregulated in cerebral focal ischemic lesions [15, 51]. Pattern IV shows prominent degeneration of non-apoptotic oligodendrocytes with limited repair in the peri-plaque white matter and occurs mainly in PP-MS [51, 52]. An extensive analysis of early acute MS lesions has highlighted that pattern II is the most frequent (about 58%), followed by pattern III (about 26%), pattern I (about 15%) and IV (about 1%) [51].

Chronic active plaques usually display perivascular infiltrating cells and macrophage immunoreactive for myelin degradation products, which accumulate along the sharply defined edge of the lesion. Demyelination, hypertrophic astrocytes and some damaged axons are also present [3, 51].

Smoldering active plaques show low demyelination, a hypocellular and inactive lesion centre, surrounded by macrophages and microglial cells, few of which are myelin-laden [51].

Chronic silent plaques are characterized by hypocellularity, astroglial scar, no macrophages containing myelin degradation products, a reduced number of demyelinated axons, vessels with thickened walls [3, 51].

1.2.5. $CD4^+$ T cells in CNS autoimmune response

$CD4^+$ T helper cells exhibit a key role in controlling and tuning both acquired and innate immune responses [15]. The demonstration that it was possible to induce an autoimmune response against the CNS in animals by transferring myelin-specific MHC class II restricted T cells, prompted several efforts for clarifying the role of $CD4^+$ T cells in the pathogenesis of MS [5, 32, 33].

1.2.5.1. Evidences for $CD4^+$ T involvement in MS

$CD4^+$ T cells are present both in brain lesions, mainly in perivascular spaces, and in the CSF of MS patients [15]. In most of the studies, the search for antigen-specific $CD4^+$ T cells has strikingly revealed that T cells reactive to myelin antigens, such as myelin basic protein (MBP), are present at the same frequency between MS subjects and healthy controls [53, 54]. It has been proposed that these autoreactive T cells in healthy individuals may provide inflammatory signals and neurotrophic factors important for neuroprotection after injuries [55] or promote neurogenesis and spatial learning abilities in adulthood [56]. A detailed analysis has uncovered that myelin-specific T cells from MS patients are less dependent or independent of co-stimulation, exhibiting a memory or activated phenotype [57, 58]. Further studies have suggested that myelin-specific T cell clones from MS subjects have a more pro-inflammatory phenotype than healthy controls [3]. These $CD4^+$ T cell lines derived from patients preferentially recognize with high-avidity myelin epitopes, which display lower predicted affinity for HLA-DR2 molecules and therefore may be not expressed in the thymus [59]. It has also been reported a higher frequency of T cells expressing degenerate T cell receptor (TCR) (namely, TCR which can bind multiple distinct peptide-MHC complexes) in MS patients if compared to controls [60]. This observation has important implications for the hypothesis of molecular mimicry [4].

The most compelling evidence that myelin-reactive T cells can induce inflammatory demyelination derive from a clinical trial in which it was used an altered peptide ligand (APL) modelled after an immuno-dominant epitope of MBP (MBP_{83-99}). This peptide was

developed to stimulate autoreactive T cells and render them inactive, however several patients unexpectedly experienced either clinical exacerbations or an increase in disease activity, as measured by MRI. Disease flares were associated to an increase of over 1000-fold in T helper (Th) 0 and/or Th1 $CD4^+$ T cells specific for the MBP epitope [61]. This study did not establish that MBP-specific $CD4^+$ T cells were responsible for MS before this trial, however it clearly showed that circumstances that lead to their selective expansion can result in disease flares [62].

An indirect proof of $CD4^+$ T cell role also derives from the observation that the strongest genetic factor conferring risk of MS is the haplotype of HLA-DR and -DQ molecules [34]. In addition the association between particular MHC II alleles and disease susceptibility has been observed also in EAE [4]. Furthermore, humanized transgenic mice expressing patients-derived TCR specific for MBP epitopes (amino acids 84-102, 83-99, 111-129) and their respective restriction elements (DRB1*1501, DRB5*0101 and DRB1*0401) develop spontaneous EAE [62, 63].

Nonetheless it should be reminded that targeting of $CD4^+$ T cell by an anti-CD4 monoclonal antibody did not result in clinical improvement of MS subjects [64, 65]. Instead a wider depletion of lymphomononuclear cells by a monoclonal antibody directed against CD52 (alemtuzumab, also known as CAMPATH-1H), resulted in profound reduction in disability and inflammatory activity in RR-MS, but not SP-MS patients [5].

1.2.5.2. CD4$^+$ T cells effector functions in MS and EAE

Originally it was believed that MS and EAE were CD4$^+$ Th1-mediated diseases [66]. Th1 subset is driven by interleukin (IL)-12, secretes interferon (IFN)-γ, IL-2 and tumor necrosis factor (TNF), and is involved in host defence against intracellular pathogens (Fig. 7) [66, 67].

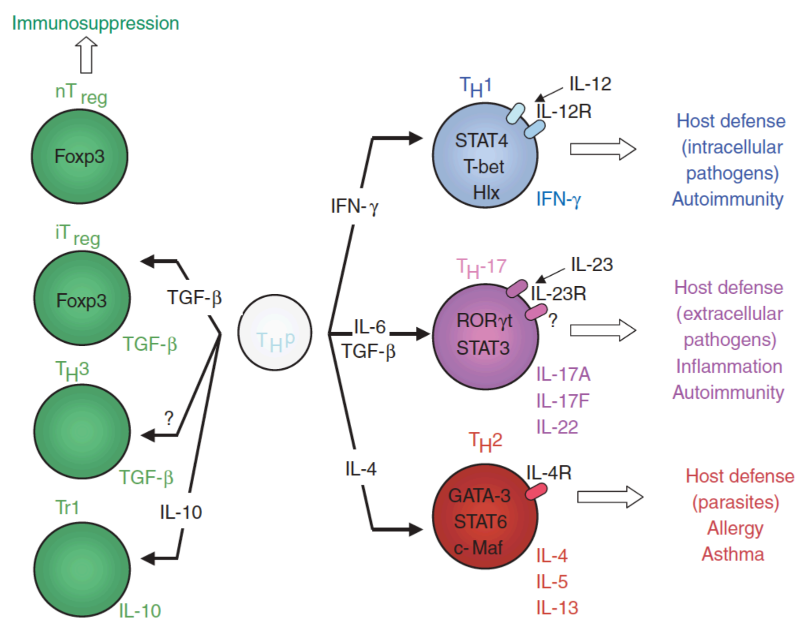

Figure 7. *Differentiation of CD4$^+$ T cell lineages*. Peripheral naïve CD4+ T helper precursor cells (Thp) can differentiate into three subsets of effector T cells (Th1, Th2 and Th17) and several subsets of regulatory T cells (Treg), including induced Treg cells (iTreg), Tr1 cells and Th3 cells. Naturally occurring Treg cells (nTreg) are generated from CD4$^+$ thymic T cell precursors (Bettelli et al. *Nat Immunol* 2007 [67]).

IFN-γ has been found in significant amounts in the CSF of MS patients and the expression of IFN-γ and IL-12 in the CNS and CSF has been correlated with increased disease activity [4, 66].

Moreover administration of IFN-γ exacerbates MS [68]. Considerable quantities of IFN-γ have been found in the spinal cords of EAE mice [66]. Adoptive transfer of myelin-specific Th1 cells induces severe EAE and mice deficient in the "master" transcription factor of Th1 lineage (T-bet) are resistant to EAE [66, 69]. IFN-γ induces MHC class II expression in the CNS and favour recruitment of macrophages and monocytes, which are a substantial component of immune cells infiltrating the inflamed CNS [4]. However paradoxical data have shown that mice with genetic deletions for IFN-γ, TNF and IL-12p35 develop severe EAE [70, 71].

On the contrary genetic deletion of IL-6 or IL-23 protects mice from development of EAE [72, 73]. IL-6, along with TGF-β and IL-23, drives the induction of a recently discovered $CD4^+$ T helper lineage, the Th17 subset. Th17 cells secrete IL-17A, IL-17F and IL-22 and are deputed to host defence against extracellular pathogens [67].

The ability of IL-23 polarized Th17 cells to induce severe EAE after passive transfer into naïve mice has reinforced the idea that Th17 is a highly inflammatory population in CNS autoimmunity [74]. Increased amount of IL-17 mRNA has been found in the blood and CSF mononuclear cells from MS patients [75]. Also IL-17 and IL-6 transcripts are upregulated in MS plaques [76]. IL-17A-producing T cells have been found at high frequencies in the blood and CSF of untreated RR-MS patients [77]. Nonetheless knockout mice for IL-17A and IL-17F develop full EAE and ectopic expression of IL-17A does not exacerbates EAE [78]. Recent studies suggest that T cells that co-express IL-17 and IFN-γ may be critical for development of EAE [4].

Finally, a recent work has shown that Th cells lacking GM-CSF loose their encephalitogenic potential, despite the expression of IL-17 and IFN-γ [79]. GM-CSF synthesis is induced in T cells by IL-23 and to date is the only T cell-derived cytokine with a non-redundant role in EAE [77]. Nonetheless ustekinumab, a monoclonal antibody targeting p40 chain common both to IL-12 and IL-23, has been ineffective for MS patients [80].

Th2 lineage is driven by IL-4, secretes IL-4, IL-5 and IL-13 and is believed to drive host resistance to parasites infection and allergic disorders [67]. Several evidences support a role for Th2 responses in suppressing EAE and MS [81]. Nevertheless, adoptive transfer of myelin-reactive Th2 cells to immunodeficient mice induces EAE with an eosinophilic infiltrate in the brain [82]. Mast cells represent major effector cells of Th2-associated immune responses, and several lines of evidence indicate that mast cells may also contribute to the development of EAE and participate in the pathology of MS [81]. Details of the current literature on Th2 allergic responses in MS and EAE will be discussed in section 4 of the introduction.

1.2.6. $CD8^+$ T cell responses in MS and EAE

$CD8^+$ T cells have been identified in MS lesions, both in perivascular areas and in brain parenchyma, with cytotoxic granules polarized toward oligodendrocytes and axons [4]. $CD8^+$ T cells are generally more numerous than $CD4^+$ T cells in lesions and their clonal expansion is detected more frequently if compared to $CD4^+$ T cells [4]. Also MBP-specific $CD8^+$ T cells isolated from MS patients secrete pro-inflammatory cytokines such as IFN-γ and TNF [83].

Contrarily to what observed for $CD4^+$ T cells, the frequency of $CD8^+$ T cells specific for myelin peptides is higher in MS patients than healthy controls [84]. It has been shown a particular enrichment of memory $CD8^+$ T cells in the CSF and blood of MS subjects [4].

In EAE it has been demonstrated that $CD8^{-/-}$ mice develop an encephalomyelitis with less mortality, but more relapses [85]. Also, adoptive transfer of myelin-reactive $CD8^+$ T induces severe EAE [86].

1.2.7. B cell responses in MS and EAE

MS lesions from several patients are characterized by the deposition of antibodies and complement activation on myelin sheath, thus suggesting a role for B cells and antibody production in the aetiology of the disease [87]. The presence of OCBs in the CSF of 50-90 % of all diagnosed patients further support this hypothesis [87]. Myelin specific antibodies have been found in serum and CSF of MS patients [87], however they have been also found in different neurologic diseases and even healthy controls [88, 89]. Analysis of Ig gene rearrangements has outlined B cell clonal expansion in the CSF of MS subjects, indicating the occurrence of a focused immune response [90]. Therapy of MS patients with a monoclonal antibody depleting B cells (rituximab) has reduced newly emerging lesions [4]. In EAE the injection of monoclonal antibodies directed against myelin oligodendrocyte glycoprotein (MOG), result in clinical exacerbation of the ongoing disease [91]. However B cells are dispensable to the induction of active EAE with myelin peptides, although they are required when EAE is induced by myelin protein, as emerged in studies on B cell-deficient mice [87]. In a recent work, transgenic

mice expressing a MOG-specific TCR (which develop spontaneous optic neuritis in small percentage) were crossed with another transgenic strain expressing the heavy chain of a MOG-specific antibody. These double transgenic mice developed an aggressive autoimmune inflammatory response affecting the spinal cord and optic nerves [92].

2. EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

The origin of EAE dates back to 1925, when Koritschoner and Scheiwenburg reported that repeated inoculations of rabbits with human spinal cord resulted in paralysis [93]. In the 1930s, Thomas M. River was investigating the basis for neurological complications (i.e. encephalomyelitis and ascending paralysis) observed in some individuals treated with Pasteur's rabies vaccines or infected with viruses such as vaccinia and measles [94, 95]. He discovered that repeated injections of normal rabbit brain emulsions in rhesus monkeys induced ataxia and muscle weakness associated to demyelination and perivascular infiltrates in brain and spinal cord [96]. Over the years EAE has been induced in a wide range of species, including mouse, rat, guinea pig, rabbit, hamster, dog, sheep and marmoset [97]. Protocols for EAE induction have improved their efficiency owing to the use of adjuvants such as complete Freund's adjuvant (CFA), consisting of killed *Mycobacterium Tuberculosis* in paraffin oil, and *Bordetella Pertussis* Toxin (PTX) [93, 98]. Also fractionation of spinal cord homogenate led to the identification of encephalitogenic myelin antigens, such as MBP, PLP and MOG [93].

The most common EAE models currently employ rats and mice and different clinical courses have been reported, according to the strain of mice and peptide used (Fig. 8) [93].

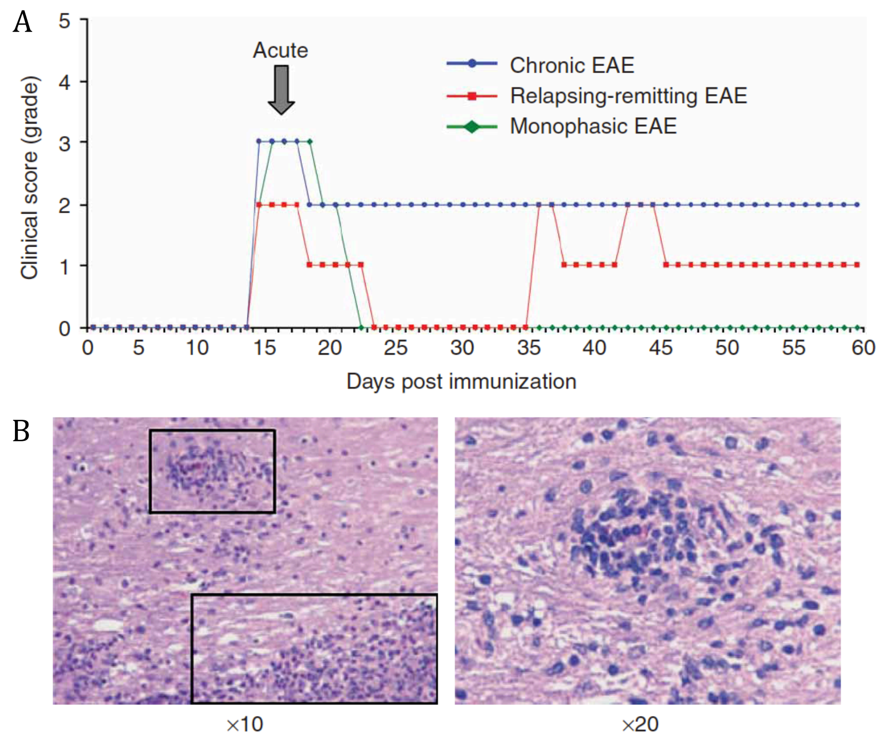

Figure 8. *Examples of different clinical courses of EAE.* (A) Induction of EAE in different mouse strains and with different myelin epitopes result in chronic, relapsing-remitting or acute monophasic disease. Clinical signs are graded according to a 0-5 point scale (B) Representative tissue section of spinal cord from mice at the onset of EAE, stained with haematoxylin and eosin. Inflammatory cells infiltrates in the white matter are boxed, whereas a perivascular lesion is presented at higher magnification (Stromnes and Goverman *Nat Protoc*, 2006 [93]).

Also active and passive methods of EAE induction have been described. In the majority of the models, EAE clinically manifests as ascending-flaccid paralysis starting from the tail and progressing to hind and forelimbs [93]. In C57BL/6 mice (bearing H-2^b haplotype of

MHC), EAE can be induced actively by subcutaneous administration of $MOG_{35\text{-}55}$ peptide in CFA and by intravenous or intraperitoneal injection of PTX. These mice develop EAE with a chronic clinical course of paralysis [99]. Active immunization with $PLP_{139\text{-}151}$ induces a relapsing-remitting EAE in SJL-J mice ($H\text{-}2^s$), while in other strains such as PL/J ($H\text{-}2^u$) an acute monophasic disease is observed [99]. Actively induced EAE consists of an induction phase, which involves the priming and activation of myelin-specific $CD4^+$ Th1/Th17 cells, and an effector phase. During the effector phase, encephalitogenic $CD4^+$ T cells migrate to the CNS, where they are re-activated by APCs, and orchestrate an immune-mediated attack of myelin. Immune cells recruited in EAE lesions include macrophages, $CD8^+$ T cells, B cells and plasma cells, thus resembling the neuroinflammatory milieu observed in MS plaques [93]. Details of the pathogenetic steps occurring in MS and EAE have already been described in the previous section.

The effector phase of disease can be also studied by passive EAE [93]. Initially Paterson showed that injection of lymph node cells from rats immunized with spinal cord homogenate into naïve animals induced EAE [100]. Subsequent studies have demonstrated that transfer of activated myelin-specific $CD4^+$ T cells was able to induce the disease [33].

EAE has been useful for better characterizing the effector mechanisms of CNS autoimmune responses, and exploring new therapeutic strategies for MS. However some limitations have emerged as a model of MS. In fact EAE is induced by administration of non-physiological stimuli and consequently is not useful to

understand the initiating events occurring in MS [33]. Also, the induction protocol of active EAE favours the establishment of $CD4^+$ MHC class II-restricted T cells, underestimating the contribution of $CD8^+$ T cells and B cells, whose role in MS pathogenesis has been increasingly recognized [101]. Therapies proven to be efficacious in EAE have shown in same cases negligible or detrimental effect in MS. Nonetheless it should be kept in mind that four current available therapies for MS have been translated into humans on the basis of the promising results obtained in the EAE model. They include natalizumab, glatiramer acetate (a mixture of polypeptides of 4 amino acids with chemical properties mimicking MBP), mitoxantrone (an anthracenedione cytotoxic and immunosuppressant agent), and fingolimod (a sphingosine-1-phosphate receptor modulator) [97, 102]. A comparison of the outcome of immunotherapies between EAE and MS is reported in Fig. 9 [35].

Substance	Initial therapeutic strategy	Efficacy in MS	Efficacy in EAE	Comments
Interferon-γ	Anti-viral	Exacerbates	Ameliorates or no effect	Opposite effects in MS and EAE
Interferon-β	Anti-viral	Reduces relapse rates and MRI activity	Ameliorates, but efficacy is variable	Efficacy was first demonstrated in MS
Glatiramer acetate	Immunomodulatory	Reduces relapse rates and MRI activity	Ameliorates	Efficacy was first demonstrated in EAE
Lenercept	TNF-α receptor blockade	Exacerbates	Ameliorates	Opposite effects in MS and EAE
Anti-CD4 antibody	Depletion of $CD4^+$ T cells	No effect	Ameliorates	Different outcome in MS and EAE
Oral myelin	Immune tolerance	No effect	Ameliorates	Different outcome in MS and EAE
Mitoxantrone	Immunosuppression	Reduces relapse rates and disease progression	Ameliorates	Efficacy was first demonstrated in EAE
Natalizumab	Anti-migratory	Reduces relapse rates and MRI activity	Ameliorates	Efficacy was first demonstrated with an analog antibody in EAE

Figure 9. *Results of different immunotherapies in MS and EAE* (Hemmer et al. *Nat Clin Pract Neurol*, 2006 [35]).

3. MAST CELLS

3.1. Biology of mast cells

3.1.1. Development and phenotype

In late 1800s Paul Ehrlich provided the first histologic description of a particular subset of cells, that he named “Mastzellen” (or “well fed cells”), that were infiltrating human tissue affected by chronic inflammation [103].

Mast cells (MCs) derive from hematopoietic stem cells (HSCs) and are extensively distributed throughout all vascularised tissues [104]. MCs preferentially reside nearby surfaces exposed to environmental triggers, such as skin, airways and gastrointestinal tract. This location poses MCs in a particularly relevant position, together with dendritic cells, for the initiation and propagation of immune responses [105]. It has been recently proposed that MCs derive from an independent branch of the adult hematopoietic pathway, which is distinct from the branches giving rise to common myeloid progenitors and common lymphoid progenitors [106]. Generally MCs circulate in the blood as precursor cells and mature locally after migration in the tissue where they will ultimately reside [107]. Unlike basophils, which are often associated to MCs, they are long-lived (weeks to month) and can re-enter the cell cycle and proliferate after proper stimulation [107].

Stem cell factor (SCF), also known as the ligand for the receptor c-Kit (CD117) is the main survival and developmental factor for MCs, but also IL-3, IL-4 and IL-9 have been shown to influence the number

and mediator content of MCs [108].

Based on the anatomical distribution and/or mediator content, MCs have been classified into subpopulations summarized in Fig. 10 [108, 109].

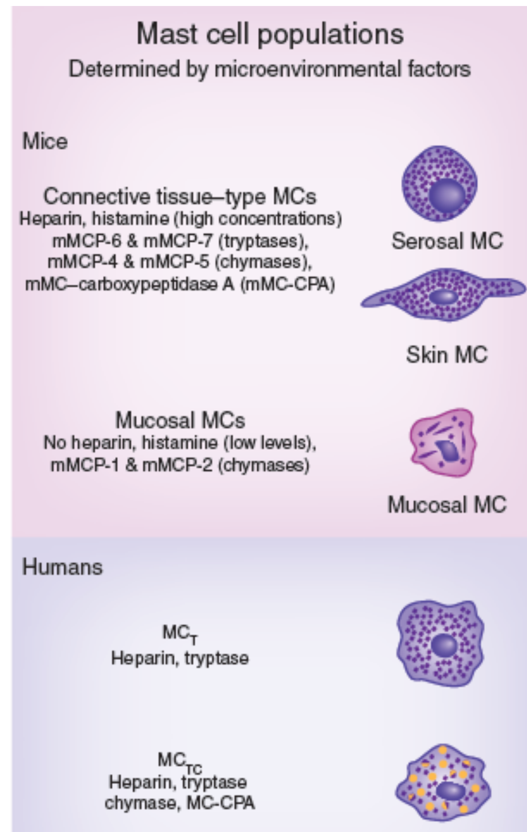

Figure 10. *Mast cell populations in mice and humans*. Mast cells in mice or humans can be subcategorized into populations defined by anatomical location and/or mediator content (such as proteoglycans (heparin versus chondroitin sulfates) or proteases (tryptases, chymases or MC-CPA)) (Galli et al. *Nat Immunol,* 2011[108]).

3.1.2. Activation

MCs are best known as key effector cells in IgE-associated immediate hypersensitivity reactions and allergic disorders, as well as in certain protective immune responses to parasites [110]. During IgE-mediated biological responses, the cross-linking of FcεRI-bound IgE with a multivalent antigen induces aggregation of two or more FcεRI molecules and activates downstream intracellular-signaling events leading to degranulation [110]. MC-granules contain biogenic amines (histamine and, only in mice and rats, serotonin); serglycin proteoglycans (such as heparin and chondroitin sulphate); serine proteases (tryptases, chymases, carboxypeptidases); cytokines and growth factors (such as TNF-α, IL-4 and VEGF) [111]. FcεRI-mediated activation of MCs induces also the *ex novo* synthesis of lipid mediators such as prostaglandins (PGD_2, PGE_2) and leukotrienes (LTB_4, LTC_4) [111]. Also, it has been shown that IgE alone can increase MC survival and promote the production of cytokines, such as IL-4, IL-6 and TNF-α [112]. As well, MCs can be activated to degranulate and release mediators by antigen-IgG_1 through FcγRIII [113]. Furthermore, there is increasing evidence that receptors for other ligands - such as adenosine, complement component 3a (C3a), cytokines, chemokines, pathogen-associated molecular patterns (PAMPs) - can either potentiate FcεRI-mediated mast-cell activation or, by themselves, stimulate the release of selective mast-cell mediators [114]. For example lipopolysaccharide (LPS) binding to toll-like receptor (TLR)-4 induces release of IL-6 rather than preformed granule-associated mediators (Fig. 11) [107].

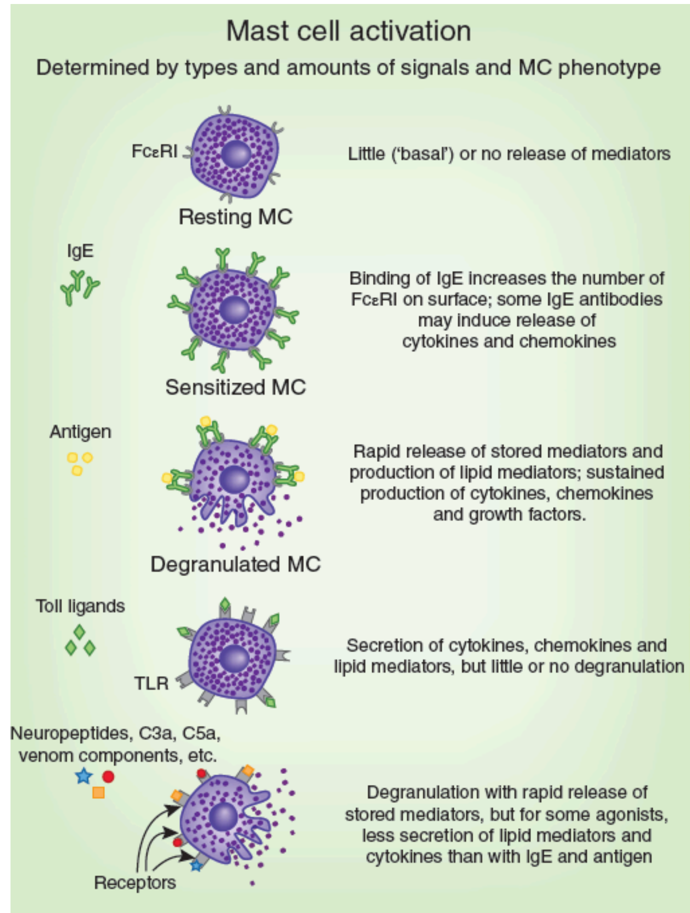

Figure 11. *Patterns of functional activation of mast cells.* In IgE-associated immune responses to allergens or parasites the activation of MCs via crosslinking of IgE bound to high-affinity receptors for IgE (FcεRI) on the cell surface by multivalent antigens results in rapid exocytosis of the cytoplasmic granules (degranulation), the production of lipid mediators (such as leukotrienes and prostaglandins) and secretion of many cytokines, chemokines and growth factors. Also IgE alone can stimulates MCs secretion of cytokines and chemokines [108]. MCs can respond to IgE-independent stimuli, such as the pathogen-associated molecular patterns (through TLRs), C3a and C5a anaphylatoxins of the complement system, venoms (Galli et al., *Nat Immunol* 2011[108]).

According to the type of stimulus and the specific inflammatory milieu, MCs can be "tunable" toward a pro-inflammatory or anti-inflammatory phenotype [107]. However, markers of specific MC subsets have not been identified yet [114]. Due to their plasticity and heterogeneity, it's extremely difficult to evaluate *in vitro* the contribution of MCs in physiological and pathological conditions. Therefore many efforts have been made to identify models suitable for studying *in vivo* MC function [105].

3.2. Mouse models of mast cell function

Mice that specifically lack MCs have still not been reported [104]. However several mouse strains harbouring spontaneous inactivating mutations in the *White spotting (W)* locus, which corresponds to c-Kit gene on chromosome 5, display MC-deficiency and have been used to analyse *in vivo* the properties of MCs [115].

c-Kit is the tyrosine kinase receptor for SCF. In the adult c-Kit is expressed by HSCs and is important for maintenance of normal haematopoiesis and bone marrow mesenchymal stem cells [115]. c-Kit is downregulated during terminal differentiation, but, among hematopoietic cells, MCs retain high levels of expression of c-Kit also at final stage of differentiation and are strongly dependent on SCF for their development, survival and function [116-118].

For several years the most commonly used model for studying MCs has been the $Kit^{W/W\text{-}v}$ strain on $WBB6F_1$ background [109]. This strain bears two mutated alleles at the *W* locus: the *W* mutation is a G to A point mutation at a splice donor site leading to exon skipping and

production of a truncated c-Kit, that lack the transmembrane domain and is not expressed on the cell surface [119]. The *W-v* mutation is a C to T point mutation (resulting in the change Thr660Met) in the c-Kit tyrosine kinase domain that considerable reduces, but not eliminates, receptor signalling [120, 121]. *Kit*$^{W/W\text{-}v}$ mice display profound MC-deficiency but also some other c-Kit dependent abnormalities, such as defective melanogenesis, sterility, anemia, neutropenia and deficiency of interstitial cells of Cajal (ICCs) [122].

In the last years another strain, the *Kit*$^{W\text{-}sh/W\text{-}sh}$, on C57BL/6 background, gained popularity for *in vivo* studying MCs. The W-sash (W-sh) mutation, first described in 1982, consists of an inversion mutation in the *Kit* promoter, spanning 3.1 Mb and encompassing 27 known genes. The 3' end of this inversion is located upstream the coding region of *Kit* and breaks a positive element that controls c-Kit expression specifically in MCs, whereas the 5' breakpoint maps within and disrupts the *Corin* gene [123, 124]. *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice display severe MC-deficiency, lack melanocytes and ICCs, but they are not anaemic, nor sterile [122].

Differences in biological responses between c-Kit mutant and wild-type control mice could be related to any of the abnormalities of these mice. To ascertain the specific contribution of MCs, it's possible to selectively repair the lack of these cells by the adoptive transfer of genetically compatible, *in vitro*-derived wild-type or mutant MCs. MCs can be obtained *in vitro* from embryonic stem cells or adult bone marrow and then injected intravenously, intraperitoneally or intradermally to create the so-called MC "knock-in mice" [107]. The adoptively transferred MCs gradually acquire phenotypic

characteristics of the native mast cell population found in wild-type mice [125]. However number and distribution of injected MCs in some anatomical sites could be different if compared to wild-type mice [107]. Nonetheless, even if MC knock-in mice do not exactly recapitulate the anatomical and numeric distribution of MCs observed in the wild-type counterparts, they can exhibit a biologically response indistinguishable from wild-type mice. The interpretation of possible findings obtained in experiments using MC knock-in mice is summarized in Fig. 12.

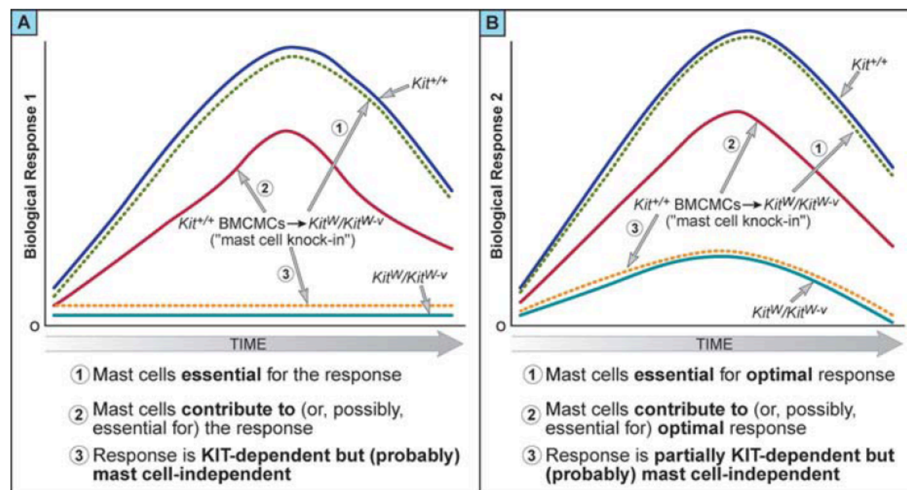

Figure 12. *Interpretation of possible findings in experiments using mast cell knock in-mice*. Biological responses evaluated in WBB6F1–*Kit*$^{+/+}$ versus *Kit*$^{W/W\text{-}v}$ and bone marrow-cultured mast cells (BMMCs)-transplanted *Kit*$^{W/W\text{-}v}$ mice. The same principles apply to responses in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Galli et al., *Annu Rev Immunol* 2005[107].

3.3. Mast cells in innate and adaptive immune responses

3.3.1. Mast cells in innate immunity

In mice there is evidence that MCs can enhance resistance to the venom of poisonous reptiles, arthropods and honeybee or to endogenous toxins as endotoxin-1, whose concentrations increase during bacterial peritonitis and sepsis, by releasing proteases stored in their granules (e.g. carboxypeptidase A3) [126, 127]. MCs have also a key role in recruiting neutrophils to sites of bacterial infection through secretion of TNF-α [128, 129].

3.3.2. Mast cells in adaptive immunity

In the mouse IgE-dependent immune responses are almost completely MC-dependent [107]. MCs can drive the pathology of IgE-mediated type I immediate hypersensitivity reactions. Responses can be localized, as atopic dermatitis (eczema), allergic rhinitis (hay fever), and allergic asthma or systemic as anaphylaxis [104, 105, 130, 131]. MCs can enhance host resistance to certain parasites [132]. Mast-cell-protease (MCP)-6 for example is important for expulsion of intestinal parasite *Trichinella Spiralis* [133].

MCs can exert also negative immunomodulatory effects, contributing to dampen adaptive immune responses. Indeed they can promote skin allograft acceptance by recruiting $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells [134] or limit the magnitude of contact hypersensitivity (CHS) reaction to the haptens DNFB (2,4-dinitro-1-fluorobenzene) or urushiol (the hapten-containing sap of poison ivy or

poison oak) by secreting IL-10 [135]. MCs and mast cell-derived IL-10 suppress innate cutaneous response to chronic low-dose ultraviolet B (UVB) irradiation [135]. Also, systemic immunosuppression of CHS responses induced by UVB irradiation of the skin is dependent on dermal MCs and histamine [136].

3.3.3. Mast cells in autoimmune diseases

MCs may either enhance or suppress the inflammation associated to different types of autoimmune diseases. MC-deficient $Kit^{W/W\text{-}v}$ and $Kitl^{Sl/Sl\text{-}d}$ (a MC-deficient c-Kit ligand mutant strain) mice are protected from autoantibody-mediated models of rheumatoid arthritis and bullous pemphigoid, inflammatory disorders affecting respectively the joint and the skin. Reconstitution of $Kit^{W/W\text{-}v}$ mice with BMMCs restores complete disease susceptibility in both conditions, thus indicating a pro-inflammatory role of these cells in such diseases [137, 138]. However, recent work has shown that, unlike $Kit^{W/W\text{-}v}$, $Kit^{W\text{-}sh/W\text{-}sh}$ mice develop a full clinical and histological autoimmune arthritis, thus questioning the actual involvement of MCs in this disorder [139].

Conversely, MCs seem to exert a protective function in experimental autoimmune glomerulonephritis, by limiting clinical and histological glomerular pathology and mortality [140, 141].

MCs have been implicated also in the pathogenesis of MS and EAE. Evidences for a potential effector and/or immunomodulatory role of MCs in these diseases are detailed in the next section.

4. ALLERGIC RESPONSES IN MS AND EAE

4.1. Th2 responses in MS and EAE

MS and EAE are generally perceived as Th1/Th17-mediated diseases [70]. Conversely, Th2 responses, which are associated to allergic disorders, such as asthma, food allergy or rhinitis, are considered to play a role in suppressing MS and EAE, and a shift of the immune response from Th1 towards Th2 has represented a promising therapeutic strategy for these and other autoimmune diseases [81]. However, recent observations suggest that several components of 'classical' Th2-allergic responses might play a role in the development and progression of EAE [81]. Adoptive transfer of myelin-reactive Th2 cells to immune-deficient mice can induce EAE [82]. Also, in EAE mice the demonstration that anaphylactic shock, the most severe manifestation of allergic reactions, can develop to 'self peptides' that are also targets of autoimmune attack, represents perhaps the most dramatic evidence of the clinically significant occurrence of both Th2-allergic and Th1/Th17-autoimmune effector mechanisms in the same subjects [142].

4.2. Mast cells in MS and EAE

Several reports have documented the presence of MCs within the plaques of MS patients, where they are generally clustered around venules and capillaries [143, 144]. This anatomical location places

MCs at important interface between the general circulation and the brain parenchyma, both in the normal CNS (“the immune gate to the brain”, [145]) and in the vicinity of MS lesions. In brain of MS patients, MCs are activated and express the FcεRI, and their numbers are lower in acute lesions than in chronic active plaques [143, 144]. Gene microarray and quantitative RT-PCR analyses have revealed that transcripts for mast cell-related genes (i.e. tryptase and FcεRI) are up-regulated in MS plaques [76, 146] and mast cell tryptase is also elevated in the spinal fluid during MS relapses [147]. In EAE, numerous studies have reported a correlation between number, distribution and/or activation state of brain MCs and the development and severity of the disease [81]. A significant support for a pro-inflammatory role of MCs in EAE derives from M. Brown’s group, showing that *Kit*$^{W/W-v}$ mice develop EAE with delayed onset and significantly milder severity as compared to congenic *Kit*$^{+/+}$ mice (Fig. 13) [148]. Reconstitution of these mice with BMMCs derived from wild-type mice but not from mice genetically lacking Fc receptors restores typical disease susceptibility [149]. Further studies have demonstrated that MCs may exert their immune functions in this model by influencing autoimmune T cell responses in periphery, rather than in the parenchyma of CNS, which is not repopulated in *Kit*$^{W/W-v}$ mice after BMMC transplantation [150]. In line with these findings, the analysis of draining lymph nodes during acute EAE revealed activated MCs, regulatory T cells (Treg) and Th17 cells establishing tight spatial interactions, suggesting the occurrence of a MC-mediated modulation of Treg and Th17 cells immune responses [151].

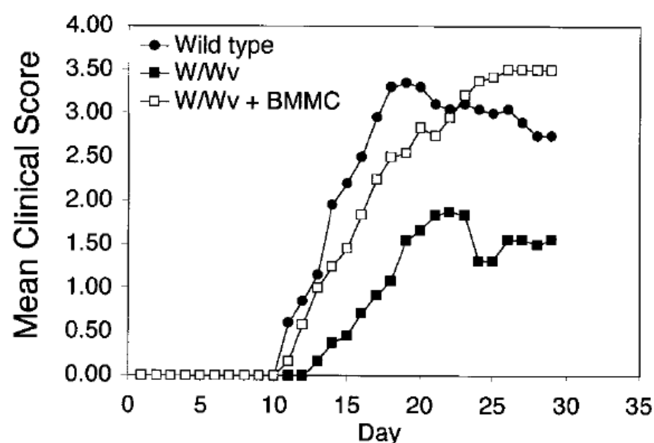

Figure 13. *Clinical expression of EAE in MC-deficient Kit$^{W/W\text{-}v}$, Kit$^{+/+}$ and MC-reconstituted Kit$^{W/W\text{-}v}$ mice* (Secor et al., *JEM* 2000 [148]).

The studies performed on MCs and EAE so far rely on a single model of MC-deficiency, the *Kit*$^{W/W\text{-}v}$, which bears several c-Kit-dependent phenotypic abnormalities other than MC-deficiency, such as neutropenia and anaemia [104]. Also, the work conducted so far on this topic does not allow discriminating how MCs shape the CNS autoimmune response occurring in EAE and by which mechanisms/mediators MCs do activate during brain inflammation. The study of how MCs intervene in the pathology of EAE and MS might importantly contribute to our comprehension of the pathophysiology of these diseases and it might help for designing better therapies in future.

4.3. Histamine in MS and EAE

Histamine is an important mediator in several physiological and pathological processes, including neurotransmission and brain function, hormonal secretion, and gastrointestinal and circulatory function [152, 153]. Synthesized from histidine by a unique enzymatic reaction mediated by histidine decarboxylase (HDC), histamine binds four types of membrane receptors: H1R, H2R, H3R, and H4R. All HRs are heptahelical G-protein coupled receptors with seven putative transmembrane domains and transduce extracellular signals through Gq (for H1R), Gs (for H2R), and Gi/o (for H3R and H4R) [152, 153].

Histamine is one of the main mediators stored in MCs granules and is generally associated to allergic responses [81]. Histamine promotes a Th2 environment in humans and in mice through interaction with monocytes and DCs, but can also influence T and B cell functions directly [154-158]. Indeed, in polarized human T cells, it promotes Th1 responses through H1R and down-regulates Th1 and Th2 responses through H2R, with congruent data in mice lacking H1R and H2R [159, 160].

Several evidences have suggested a role for histamine in MS and in EAE [81]. H1R and H2R are expressed on mononuclear cells within the inflammatory foci in the brain of mice with EAE, whereas encephalitogenic Th1 cell lines activated against $PLP_{139–151}$ express more H1R and less H2R compared with Th2 cell lines [161]. Mice deficient for H1R develop milder $MOG_{35–55}$-induced chronic EAE than wild-type mice and treatment with H1R antagonists reduces the severity of $PLP_{139–151}$-induced RR-EAE [160, 161] and rat EAE [162].

Re-expression in T cells of the H1R allele from mice susceptible to EAE restores full disease expression in resistant H1R-deficient mice [163]. Also Bphs, a gene that is associated with susceptibility to EAE and other autoimmune diseases in animal models, has been identified as H1R gene [160].

However, histamine might also have an important role in dampening autoimmune brain inflammation. Treatment with a specific agonist of H2R prevents chronic EAE [164]. Further $HDC^{-/-}$ mice, which lack histamine, exhibit exacerbation of chronic EAE with an increased T cell production of pro-inflammatory cytokines if compared with wild-type mice, and more diffuse inflammatory CNS infiltrates containing a large polymorphonuclear component and eosinophils [165]. These data emphasize a major regulatory role for histamine and its receptors in EAE. However, the direct effects of histamine on auto-reactive myelin-specific T cells from non-genetically manipulated mice are largely unknown.

5. SCOPE OF THE THESIS

Mast cells (MCs) have been suggested to play a detrimental role in the development of EAE. However studies performed so far on this topic relied on a single model of MC-deficiency, the *Kit*$^{W/W-v}$, which bears several c-Kit dependent phenotypic abnormalities other than MC-deficiency, such as neutropenia and anaemia [104]. Also, it is not clear how MCs shape the CNS autoimmune response occurring in EAE. In the first work, we focused on evaluating the contribution of MCs to EAE in both *Kit*$^{W/W-v}$ mice and in the newly characterized *Kit*$^{W-sh/W-sh}$ strain, which seems to bear fewer c-Kit dependent hematopoietic alterations. We also aimed at characterizing the T cell response to myelin antigen in a context of MC-deficiency. The comprehension of how mast cells intervene in the pathology of EAE and MS might help designing better therapies for these diseases.

Histamine is one of the main preformed mediators stored in MC granules. In the second part of the thesis we evaluated the ability of histamine to modulate the response of myelin-activated T cells. We explored the effect of histamine and specific agonists of histamine receptors 1 and 2 on activation and migratory capacity of myelin-autoreactive T cells through the inflamed BBB.

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CHAPTER 2

Exacerbated experimental autoimmune encephalomyelitis in mast-cell-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice

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Abstract

Mast cell (MC)-deficient c-Kit mutant *Kit*$^{W/W-v}$ mice are protected against experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, suggesting a detrimental role for MCs in this disease. To further investigate the role of MCs in EAE, we took advantage of a recently characterized model of MC deficiency, *Kit*$^{W-sh/W-sh}$. Surprisingly, we observed that myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$-induced chronic EAE was exacerbated in *Kit*$^{W-sh/W-sh}$ compared with *Kit*$^{+/+}$ mice. *Kit*$^{W-sh/W-sh}$ mice showed more inflammatory foci in the central nervous system (CNS) and increased T-cell response against myelin. To understand whether the discrepant results obtained in *Kit*$^{W-sh/W-sh}$ and in *Kit*$^{W/W-v}$ mice were because of the different immunization protocols, we induced EAE in these two strains with varying doses of $MOG_{35–55}$ and adjuvants. Although *Kit*$^{W-sh/W-sh}$ mice exhibited exacerbated EAE under all immunization protocols, *Kit*$^{W/W-v}$ mice were protected from EAE only when immunized with high, but not low, doses of antigen and adjuvants. *Kit*$^{W-sh/W-sh}$ mice reconstituted systemically, but not in the CNS, with bone marrow-derived MCs still developed exacerbated EAE, indicating that protection from disease could be exerted by MCs mainly in the CNS, and/or by other cells possibly dysregulated in *Kit*$^{W-sh/W-sh}$ mice. In summary, these data suggest to reconsider MC contribution to EAE, taking into account the variables of using different experimental models and immunization protocols.

Introduction

MCs are key factors in IgE-associated immediate hypersensitivity reactions, during which they release a wide spectrum of inflammatory mediators in response to IgE and antigen (Ag) stimulation [1]. However, recent findings have pointed out that MCs may exert also important effector and/or immuno-modulatory functions in other physiopathological conditions as venom detoxification, pathogen clearance, tumour growth, contact hypersensitivity, allograft acceptance and autoimmunity [2]. Most of these studies have assessed the *in vivo* role of MCs by the use of mouse models carrying spontaneous mutations in c-Kit receptor (WBB6F1-$Kit^{W/W\text{-}v}$ or C57BL/6- $Kit^{W\text{-}sh/W\text{-}sh}$ mice) or c-Kit ligand (WCB6F1-$Kitl^{Sl}/Kitl^{Sl\text{-}d}$), which show severe deficiency of MC populations [3,4].

MCs may either enhance or suppress the inflammation associated to different types of autoimmune diseases. MC-deficient $Kit^{W/W\text{-}v}$ and $Kitl^{Sl/Sl\text{-}d}$ mice are protected from autoantibody-mediated models of rheumatoid arthritis and bullous pemphigoid, inflammatory disorders affecting, respectively, the joint and the skin. Reconstitution of $Kit^{W/W\text{-}v}$ mice with bone marrow derived, *in vitro*-cultured MCs (BMMCs) restores complete disease susceptibility in both conditions, thus indicating a pro-inflammatory role of these cells in such diseases [5,6]. Conversely, MCs seem to exert a protective function in experimental autoimmune glomerulonephritis, by limiting clinical and histological glomerular pathology and mortality [7,8]. However, a recent work has shown that, unlike $Kit^{W/W\text{-}v}$, $Kit^{W\text{-}sh/W\text{-}sh}$ mice develop a full clinical and histological autoimmune arthritis, thus ques-

tioning the actual involvement of MCs in this disorder [9].

A complex and conflicting scenario emerges from studies on the role of MCs in promoting central nervous system (CNS) autoimmune response occurring in multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE) [10]. Several reports have documented the presence of MCs within the plaques of MS patients, where they are generally clustered around venules and capillaries, in lower numbers in acute lesions than in chronic active plaques [11,12]. Gene microarray and quantitative RT-PCR analyses have revealed that transcripts for MC-related genes (i.e., tryptase and IgE Fcε receptor I) are upregulated in MS plaques [13,14] and MC tryptase is also elevated in the spinal fluid during MS relapses [15]. In EAE, numerous studies have reported a correlation between number, distribution and/or activation state of brain MCs and the development and severity of the disease [10]. A significant support for a pro-inflammatory role of MCs in EAE derives from M Brown's group, showing that *Kit*$^{W/W-v}$ mice develop EAE with delayed onset and significantly milder severity as compared with congenic *Kit*$^{+/+}$ mice [16]. Reconstitution of these mice with BMMCs derived from wild-type (WT) mice but not from mice genetically lacking Fc receptors restores typical disease susceptibility [17]. Further studies have demonstrated that MCs may exert their immune functions in this model by influencing autoimmune T-cell responses in periphery, rather than in the parenchyma of CNS, which is not repopulated in *Kit*$^{W/W-v}$ mice after BMMC transplantation [18]. In line with these findings, the analysis of draining lymph nodes during acute EAE revealed activated MCs, regulatory T cells and Th17 cells

establishing tight spatial interactions, suggesting the occurrence of a MC-mediated inhibition of regulatory T-cell function in support of Th17 immune response [19]. In *Kit*$^{W/W-v}$ mice, MCs have also extra-lymphoid roles in EAE, favouring the access of neutrophils to inflamed CNS by secreting TNF in meninges [20].

In contrast to these previous works, Bennett *et al* [21] have recently shown that MCs are dispensable for EAE development in both *Kit*$^{W/W-v}$ and *Kit*$^{W-sh/W-sh}$ models. Conversely, Stelekati *et al* [22] have recently published that EAE severity is decreased in *Kit*$^{W-sh/W-sh}$ mice but is restored following MC knockin [22], thus confirming the data obtained by Brown and colleagues in *Kit*$^{W/W-v}$ model.

It is possible that discrepant results obtained on this topic by different groups could in part be related to different protocols of immunization used to elicit EAE, as well as to different animal models of MC deficiency. Indeed, at least in the *Kit*$^{W/W-v}$ model, it has been shown that the doses of adjuvant or peptide in immunization protocols can drastically affect MC involvement in the disease under investigation, as emerged in experimental models of asthma [23] and contact hypersensitivity [24].

The aim of our study was to unravel the contribution of MCs to EAE in both *Kit*$^{W-sh/W-sh}$ and *Kit*$^{W/W-v}$ strains by using different protocols of immunization, in order to evaluate if disease outcome was affected by different experimental settings. In this study, we report that, under three different immunization conditions, *Kit*$^{W-sh/W-sh}$ mice develop a more severe EAE and an increased immune response against myelin than *Kit*$^{+/+}$ animals. However, systemic reconstitution of *Kit*$^{W-sh/W-sh}$ mice with BMMCs fails to restore WT

susceptibility, therefore suggesting that in this model peripheral MCs may be redundant for EAE development. Surprisingly, *Kit*$^{W/W-v}$ mice are protected from EAE only when immunized with a 'strong' protocol of immunization (i.e., with higher doses of Ag and adjuvants, similar to the one used by Secor *et al*), but not when immunized with a milder one. Thus, MC contribution to EAE pathogenesis needs to be reconsidered, taking into account the variations because of the different immunization conditions and genetic defects.

Materials and Methods

Animals

c-Kit mutant C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and their WT controls C57BL/6- *Kit*$^{+/+}$, c-Kit mutant WBB6F1-*Kit*$^{W/W\text{-}v}$ mice and their congenic WT littermates WBB6F1-*Kit*$^{+/+}$, and myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$-TCR transgenic 2D2 mice were purchased from Jackson Laboratories. Age-matched female, 8- to 12-weeks-old mice were used in all EAE experiments. Mice were maintained under pathogen-free conditions at the animal facility of Fondazione IRCCS Istituto Neurologico 'Carlo Besta' Milano or of Stanford University. All procedures involving animals were approved by the Institute Ethical Committee and performed in accordance to institutional guidelines and national law (DL116/92) and the Division of Comparative Medicine at Stanford University, and carried out according to the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC) and the National Institutes of Health guidelines.

Peptide Synthesis, Protocols of Active and Passive EAE and Granulocyte Depletion

$MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) and control peptide (rat P0; DGDFAIVKFTKVLLDYTGHI) were synthesized by using standard 9-fluorenylmethoxycarbonyl chemistry on a 433A automated peptide synthesizer (Applied Biosystems) and purified by HPLC. The purity of each peptide was >95% as assessed by analytical reverse-phase HPLC. EAE was induced with different protocols of

immunization. For EAE induction in Figure 1a, $MOG_{35–55}$ peptide was dissolved in PBS to a concentration of 4 mg/ml and emulsified with an equal volume of IFA supplemented with 8 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra (Difco). Mice were injected s.c. in their flanks with 0.1 ml of the peptide emulsion (for a total of 200 μg of $MOG_{35–55}$/mouse and 400 μg of *M. tuberculosis*) and, on the same day and at 48 h later, were injected i.v. with 0.2 ml containing 200 ng of *Bordetella pertussis* Toxin (List Laboratories) dissolved in PBS. Doses of $MOG_{35–55}$ and adjuvants used in other protocols are specified in figure legends. Passive EAE was induced as described in Yang *et al* [25]. Briefly, splenocytes from 2D2 mice were activated with $MOG_{35–55}$ (20 μg/ml), IL-2 (Proleukin) and IL-7 (Peprotech) for 2 days, expanded with IL-2 and IL-7 for 5 days, restimulated overnight with coated anti-CD3 (eBioscience), IL-12 (kindly provided by Dr Maurice Gately, Hoffmann LaRoche, Nutley, NJ) and IL-18 (R&D Systems) and i.p. injected (10^6/mouse). Mice were assessed daily for neurological signs of EAE according to the following five-point scale: (0) healthy; (1) tail weakness or paralysis; (2) paraparesis (incomplete paralysis of one or two hind limbs/plegia of one hind limb); (3) paraplegia extending to the thoracic level; (4) forelimb weakness or paralysis with hind limbs paraparesis or paraplegia; and (5) moribund or dead animal. For granulocyte depletion, mice were treated i.p. with 10 μg anti-Ly6G mAb (clone RB6-8C5) dissolved in saline (200 μl) daily from d0 to d9.

Bone-Marrow-Derived MC Differentiation and Adoptive Transfer into *Kit*$^{W\text{-}sh/W\text{-}sh}$ Mice

BMMCs were obtained by *in vitro* differentiation of bone marrow cells taken from mouse femurs as described [26]. After 5 weeks, BMMCs were monitored for FcεRI expression by flow cytometry using FITC-conjugated anti-mouse FcεRI-α (clone MAR-1, eBioscience) and PE-Cy7-conjugated anti-mouse c-Kit (clone 2B8, eBioscience). Purity was usually > 90%. BMMCs were obtained from pooled three to four mice in each experiment. For MC-engraftment studies, BMMCs derived from *Kit*$^{+/+}$ mice, were injected i.v. into the tail vein (10^7 cells in 400 ml PBS) into 4-weeks-old female *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. EAE experiments were initiated at 6–8 weeks after adoptive transfer of BMMCs.

T-Cell Proliferation Assay and Cytokine Measurement

Draining lymph node cells (LNCs) were isolated from *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice at 10 days after the induction of EAE, and they were cultured *in vitro* with $MOG_{35–55}$, Con A (1 μg/ml; positive control), rat P0 (negative control) or medium alone. Cells were cultured in 96-well microtiter plates at a density of 4-5 x 10^5 cells/well in 200 μl of RPMI 1640 (EuroClone) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-ME (5 x 10^5 M), HEPES buffer (0.01 M) and 10% FCS (enriched RPMI 1640). After 72 h of incubation at 37°C with 5% CO_2, cultures were pulsed for 18 h with 0.5 μCi of [^{3}H]thymidine per well and proliferation was measured

from triplicate cultures on a β-counter (PerkinElmer). Data are expressed as mean c.p.m. ± s.e.m. For cytokine analysis, spleen cells were harvested from *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice at 10 days after the induction of EAE and cultured in 24-well plates (3.5 x 10^6 cells/well) with $MOG_{35–55}$, ConA, rat P0 or medium alone in the same conditions as described above. Supernatants from *in vitro*-cultured spleen cells were analyzed by ELISA for IFN-γ, IL-6, IL-10 (anti-mouse OptEIA ELISA Set; BD Pharmingen), and IL-17 (R&D Systems) according to the manufacturer's protocols. Supernatants were collected from cultured cells at different time points: 24 h for IL-6, 48 h for IFN-γ and 96 h for IL-10 and IL-17. Results are shown as mean of duplicates; s.e.m. were always within 10% of the mean.

Flow Cytometry

PE-Cy7-conjugated anti-CD4 (clone L3T4), PE-conjugated anti-CD11b (clone M1/70), FITC-conjugated anti-Gr1 (clone RB6-8C5) were from eBioscience. FITC-conjugated anti-Ly6G (clone 1A8) and APC-conjugated Ly6C (clone 1G7.G10) were from Miltenyi Biotec. Surface staining reactions were performed in PBS supplemented with 2% FCS on ice for 30 min. Intracellular staining with AlexaFluor647-conjugated anti-IL17A (clone eBio17B7) and PE-conjugated anti-IFN-γ (clone XMG1.2) was carried out after 4h stimulation with Monensin solution (eBioscience), 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich). Staining with PE-conjugated anti-Foxp3 mAb (clone FJK-16S) was performed according to the manufacturer's instructions (eBioscience). Flow cytometry data were acquired on a

FACSCalibur (Becton Dickinson) and analyzed with FlowJo software (version 8.8.6; Treestar).

Histopathology and Immunohistochemistry

For histological evaluation of EAE in *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice, five animals per group were killed at 7 weeks after the induction of EAE, and the brain and spinal cord were removed, fixed in 10% formalin and embedded in paraffin. Sections (4 μm) were cut on a microtome and stained with hematoxylin and eosin to study basic histopathological changes. Sections were analyzed in a blinded manner (PLP). The inflammatory changes were expressed as number of perivascular inflammatory infiltrates per square millimetre in the spinal cord [27] or as number of inflammatory foci per section, respectively, for the spinal cord and the brain.

Histopathological analysis of MC distribution in steady state and inflamed tissues was carried out on toluidine blue-stained sections from formalin-fixed, paraffin-embedded samples. The number of MCs was estimated by counting MCs out of five high-power microscopic fields (X 400) showing the highest MC densities and then summing the counts relative to each HPF. For immunohistochemical detection of IL-10^{+} cells, sections from formalin-fixed, paraffin-embedded lymph nodes underwent immunostaining with the streptavidin biotin peroxidase complex method using a specific primary anti-mouse IL-10 antibody (clone JES5-2A5, eBioscience) and aminoethylcarbazole as chromogen. All the immunostained sections were counterstained with hematoxylin. Slides were analysed under a Leica DM2000 optical microscope, and microphotographs were

collected using a Leica DFC320 digital camera.

Measurement of Serum Antibody Response

Blood was collected from the tail of *Kit*$^{W-sh/W-sh}$ and *Kit*$^{+/+}$ mice before and at 7 weeks after the induction of EAE. $MOG_{35–55}$-specific IgG, IgG1, IgG2a, IgG2b and IgG3 Abs were measured by ELISA as described elsewhere [28]. Briefly, 96-well microtiter plates (Immunol; Thermo Labsystems) were coated overnight at 4°C with 0.1 ml of $MOG_{35–55}$ diluted in 0.1M NaHCO3 buffer (pH 9.5) at a concentration of 0.010 mg/ml. The plates were blocked with PBS/10% FCS (blocking buffer) for 2h. Samples were diluted in blocking buffer at 1/100, and Ab binding was tested by the addition of peroxidase-conjugated monoclonal goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnology Associates), each at a 1/5000 dilution in blocking buffer. Enzyme substrate was added and plates were read at 450 nm on a microplate reader.

Statistical Analysis

Student's t-test, two tailed, was used to compare the results between the two groups. In all tests, $P<0.05$ was considered statistically significant.

Results

MC-Deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ Mice Develop Exacerbated EAE

We first evaluated the involvement of MCs in EAE by using the more recently characterized model of MC deficiency, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, which seems to bear milder c-Kit-related phenotypic abnormalities than *Kit*$^{W/W\text{-}v}$ strain [29]. Indeed, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice are not anemic, neutropenic or sterile as *Kit*$^{W/W\text{-}v}$ animals, although they are affected by some hematopoietic alterations such as splenomegaly with expanded myeloid populations, and increased number of circulating neutrophils and platelets [30]. Chronic progressive EAE was induced in MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice by immunization with $MOG_{35–55}$. This protocol induces in this strain a clinical course classically characterized by the acute onset of neurological symptoms reaching a peak about at 2 weeks after immunization, followed by a short and generally incomplete recovery phase after which symptoms of intermediate gravity are chronically maintained [28,31]. Surprisingly, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice developed exacerbated EAE compared with *Kit*$^{+/+}$ mice (Figure 1a, Table 1), characterized by an anticipated onset and a more severe progression. The clinical severity of EAE was significantly worsened in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice during both recovery and chronic phases of disease (mean disease score was 1.8 ± 0.2 in *Kit*$^{+/+}$ vs 2.8 ± 0.3 in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice ($P<0.05$) at day 20 (recovery phase), and 1.8 ± 0.2 in *Kit*$^{+/+}$ vs 2.6 ± 0.3 in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice ($P<0.05$) at day 43 (chronic phase)). The clinical data were corroborated by a histopathological analysis of CNS inflammation in the chronic phase

that revealed more inflammatory foci in the CNS of *Kit*$^{W\text{-}sh/W\text{-}sh}$ compared with *Kit*$^{+/+}$ mice (2.17 ± 1.15 vs 0.75 ± 0.79/mm2, respectively, P<0.05, in the spinal cord; 2.08 ± 1.49 vs 0.83 ± 0.76/section, respectively, P>0.05, in the brain; Figure 1b).

MCs can limit the magnitude of some adaptive immune responses by establishing functional interactions with regulatory T cells [32] or by secreting IL-10 [33]. Given the increased EAE severity shown by *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, we investigated whether the peripheral immune response against myelin was affected by MC deficiency. For this purpose, we isolated draining lymph nodes and spleens from mice with EAE at 10 days after immunization. LNCs from *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice showed a significantly increased proliferation in response to MOG_{35-55} (Figure 1c) and Ag-stimulated splenocytes from these mice produced significantly more IL-17 and IL-6, but less IL-10, than *Kit*$^{+/+}$ animals (Figure 1d). There were no significant differences in the production of IFN-γ (Figure 1d). Percentages of IL-17^{+} CD4 T cells (0.9 ± 0.4 in *Kit*$^{+/+}$ vs 2.2 ± 0.2 in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, P<0.05) and IFN-γ^{+} CD4 T cells (1.2 ± 0.2 in *Kit*$^{+/+}$ vs 2.5±0.6 in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, P40.05) were also increased in lymph nodes from *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice compared with *Kit*$^{+/+}$ animals (Figure 1e). Immunohistochemical analysis of lymph node sections revealed few scattered IL-10^{+} cells in *Kit*$^{W\text{-}sh/W\text{-}sh}$ and control *Kit*$^{+/+}$ samples from the acute phase. In these samples, a slight difference was observed in the amount of IL-10^{+} mononucleated cells between *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice. Such a difference was more evident in samples from the chronic phase, in which the overall amount of IL-10^{+} cells was higher (Figure 1f). In line with these results, intracellular staining of IL-10 showed a

slightly decreased IL-10 content in CD4 T cells in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice in both the acute and chronic phases (Supplementary Figure S1).

MCs have been described to enhance IgE and IgG1 production by B cells [34, 35] and to secrete cytokines affecting B-cell responses such as IL-4, IL-5 and IL-13 [1]. Analysis of Ab responses in sera from immunized *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice (Figure 1g) revealed a slight but not significant decrease in titers of IgG antibodies specific for $MOG_{35–55}$ in MC-deficient mice (mean OD, 0.190 ± 0.074 in *Kit*$^{+/+}$ vs 0.079 ± 0.047 in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice; P = 0.194). Analysis of the IgG isotypes showed decreased titres of Ag-specific IgG2b Abs in *Kit*$^{W\text{-}sh/W\text{-}sh}$ compared with *Kit*$^{+/+}$ mice (mean OD, 0.890 ± 0.477 in *Kit*$^{+/+}$ vs 0.177 ± 0.124 in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, P=0.178) but not of the other IgG isotypes.

We [19,26] and others [32,36,37] have reported that MCs can arrange several kinds of interaction with Treg. We tested whether *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice harboured some alteration in the Treg compartment. We found a significantly lower Treg frequency in spleens, but not in draining lymph nodes, of *Kit*$^{W\text{-}sh/W\text{-}sh}$ compared with *Kit*$^{+/+}$ mice, at both d10 and d42 after immunization (Supplementary Figure S2). However, the fraction of Treg among CNS-infiltrating CD4 T cells at d10 post immunization did not differ between *Kit*$^{W\text{-}sh/W\text{-}sh}$ and controls (Supplementary Figure S2C). A decrease in Treg frequency was also observed in lymph nodes of naive *Kit*$^{W\text{-}sh/W\text{-}sh}$ compared with *Kit*$^{+/+}$ mice (Supplementary Figure S3).

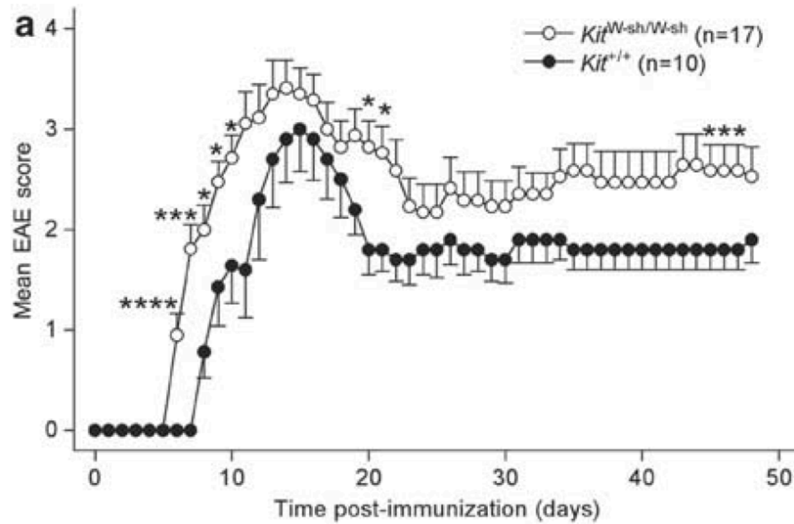


Figure 1 MC-deficient Kit$^{W\text{-}sh/W\text{-}sh}$ mice develop exacerbated EAE associated with increased CNS inflammation and autoimmune response. (**a**) EAE was induced in *Kit*$^{+/+}$ (n=10) and *Kit*$^{W\text{-}sh/W\text{-}sh}$ (n=17) mice with $MOG_{35\text{-}55}$ peptide as detailed in *Materials and Methods* and mice were scored daily as described [28]. Data represent mean clinical score ± SEM. (* $P < 0.05$, ***$P < 0.005$, **** $P < 0.001$; Student's t test, 2 tailed). These data are from a single experiment representative of three independent replicates, each including 9 to17 mice per group.

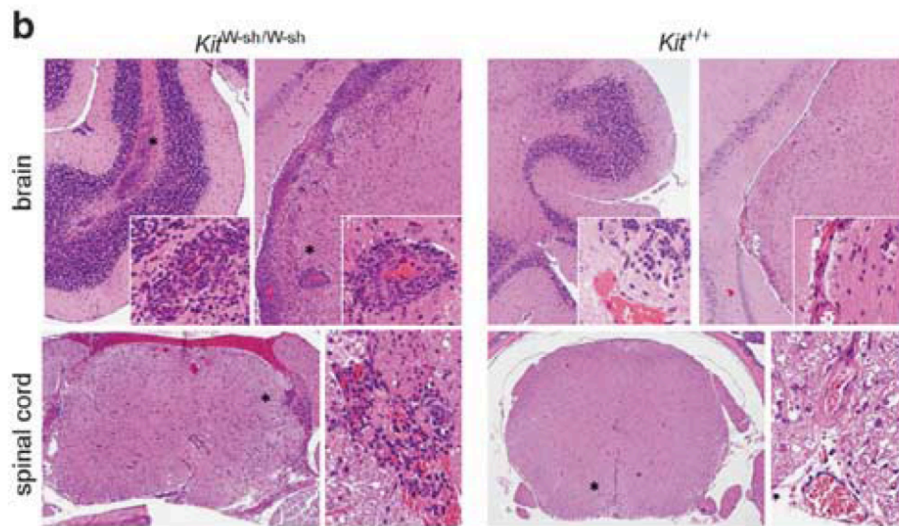


Figure 1 (continued) (**b**) Distribution and phenotype of the lesions in the CNS of *Kit*$^{+/+}$ and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice obtained 7 weeks after EAE induction. The upper left images show representative brain sections from cerebellum (left panel) and hippocampal/thalamic region (right panel) from *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice highlighting severe perivascular infiltrates (asterisk), as shown in detail in the corresponding insets. Conversely, *Kit*$^{+/+}$ mice (upper right images) show no signs of active inflammation with few dilated vessels and rare inflammatory cells (insets) both in the cerebellum (left panel) and in the hippocampal/thalamic region (right panel). Similar findings were observed in the spinal cords, with the *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice showing severe perivascular inflammatory infiltrates (asterisk) (left panels) and the *Kit*$^{+/+}$ mice being substantially free of inflammatory cells with foci of residual myelin damage (asterisk) with microcystic appearance (inset).

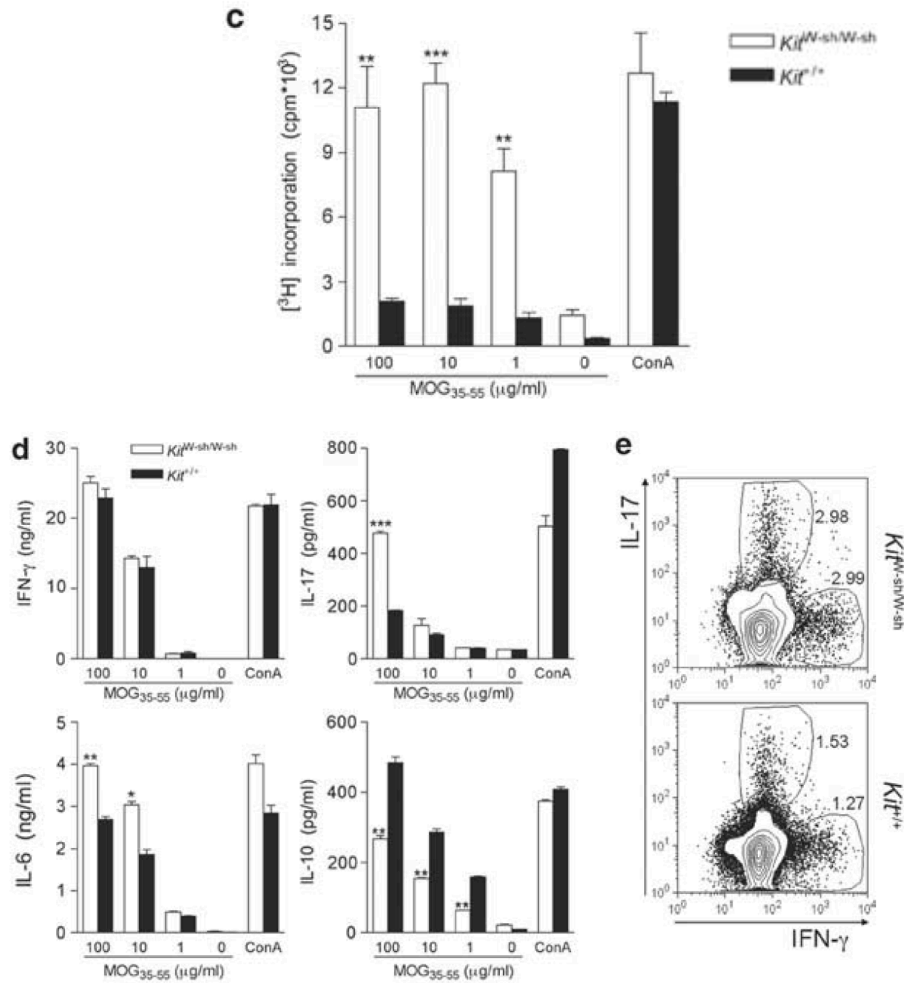
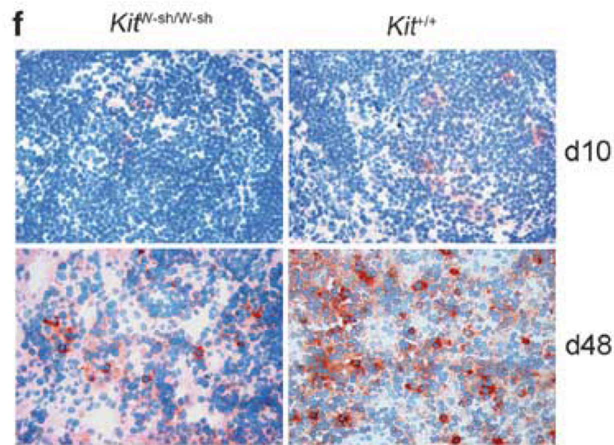


Figure 1 (continued) (**c**) Proliferation rate of LNCs (mean cpm ± SEM, from triplicate wells) and (**d**) cytokine production of splenocytes (means ± SEM, from duplicate wells) isolated from *Kit*$^{+/+}$ and *Kit*$^{W-sh/W-sh}$ mice (n=4 mice per group) 10 days after EAE induction and stimulated with MOG_{35-55}, concanavalin A (conA) or medium alone. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ (Student's *t* test, 2 tailed). (**e**) Representative graphs showing percentages of $CD4^+$ IL-17^+ and $CD4^+$ IFN-γ^+ T cells in draining lymph nodes (DLN) obtained from *Kit*$^{+/+}$ and *Kit*$^{W-sh/W-sh}$ mice 10 days post-EAE induction.



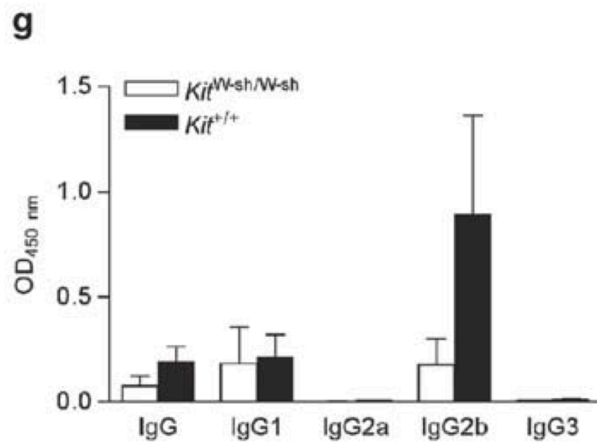


Figure 1 (continued) (**f**) DLN isolated from *Kit*$^{+/+}$ and *Kit*$^{\text{W-sh/W-sh}}$ during acute (d0) and chronic (d48) EAE display a different density of IL-10-espressing mononuclear cells (anti-IL-10, STREPT-ABC complex method. (Original magnification x 200). (**g**) Anti-MOG$_{35-55}$-specific IgG antibodies and IgG antibody isotypes in sera from *Kit*$^{+/+}$ (n=10) and *Kit*$^{\text{W-sh/W-sh}}$ (n=15) mice obtained 7 wks after the induction of EAE. Each mouse was tested individually in duplicate. Bars represent mean titers ± SEM.

Table 1 EAE in *Kit*$^{W-sh/W-sh}$ and *Kit*$^{+/+}$ wild-type mice

Strain	Incidence (%)[a]	EAE onset, day[a]	Peak disease severity[a]	Cumulative disease score[a]
Kit$^{W-sh/W-sh}$	100 (17/17)	7.0 ± 0.5[‡]	3.8 ± 0.2	109.9 ± 9.5[†]
Kit$^{+/+}$	90 (9/10)	9.8 ± 0.8	3.3 ± 0.4	78.9 ± 9.5

Abbreviation: EAE, autoimmune encephalomyelitis.
[a]Data are shown as mean ± s.e.m.
[†]$P < 0.05$.
[‡]$P < 0.01$ (all *P*-values are in comparison with *Kit*$^{+/+}$ wild-type mice by Student's *t*-test, two tailed).

We evaluated the amount and distribution of MCs by histopathological evaluation of toluidine-blue-stained sections. In both naive and immunized *Kit*$^{+/+}$ mice only few MCs were detected in the CNS, mostly associated with the meninges. MCs infiltrating the brain or spinal cord parenchyma were only exceptionally observed in immunized *Kit*$^{+/+}$ mice. The amount and distribution of MCs in draining lymph nodes were affected by immunization, as lymph nodes from naive mice showed few MCs mainly lodged inside the sinuses or within the medullary area (not shown), whereas those from immunized mice showed higher numbers of MCs either scattered or clustered within the T-cell-rich perifollicular areas (Figure 2). In naïve and immunized *Kit*$^{W-sh/W-sh}$ mice, no MCs were detected in the CNS parenchyma. Draining lymph nodes from the same mice were characterized by a hypertrophic medulla with dilated sinuses and by the absence of MCs (Figure 2).

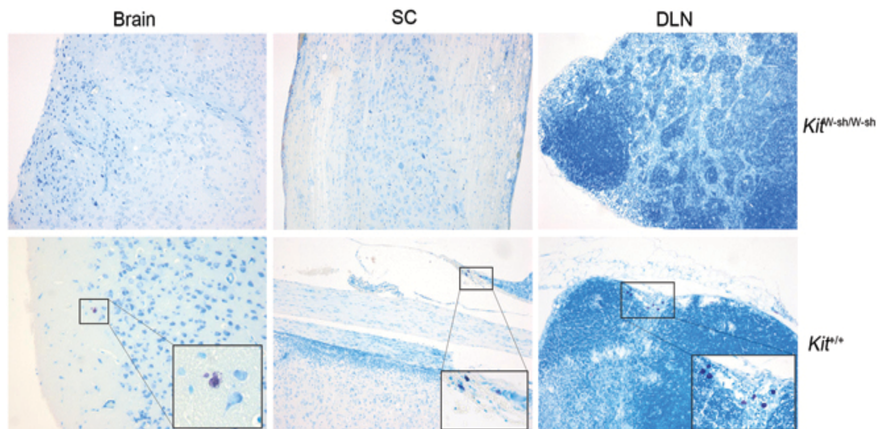


Figure 2 Mast cell distribution in brain, spinal cord and DLN of Kit$^{+/+}$ and Kit$^{W\text{-}sh/W\text{-}sh}$ EAE mice. In immunized *Kit*$^{+/+}$ mice few MCs populate the CNS, mostly localized within the meninges. In DLNs from the same mice, small clusters of MCs can be observed in the peri-follicular areas of the cortex. In *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, no MCs can be detected in either the CNS or DLNs. (Toluidine blue. Original magnification X 100, inset X 400).

Kit$^{W\text{-}sh/W\text{-}sh}$ Mice Develop Severe EAE Regardless the Immunization Protocol

Clinical and immunological findings obtained by us in the *Kit*$^{W\text{-}sh/W\text{-}sh}$ model were in contrast with the work by Secor *et al* [16] showing that MC-deficient *Kit*$^{W/W\text{-}v}$ mice develop a milder EAE associated with a reduced pro-inflammatory autoimmune response [38]. However, in these studies, a ‘strong’ protocol of immunization (i.e., high doses of $MOG_{35–55}$, heat-killed *Mycobacterium tuberculosis* in CFA and *Bordetella pertussis* toxin (PTX)) was used to induce EAE. To evaluate whether the discrepant outcome obtained by us in the *Kit*$^{W\text{-}sh/W\text{-}sh}$ strain could depend on the specific conditions of immunization, we induced EAE in *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice by two additional protocols of immunization stronger than the one used in Figure 1, containing increased doses of PTX or, similarly to Secor et al, higher doses of $MOG_{35–55}$, *M. tuberculosis* and PTX. We observed that *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice developed a more severe EAE than controls (Figure 3a and b) also under these conditions of immunization. Interestingly, immunization with the strongest protocol (Figure 3b) resulted in a more significant clinical difference between *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice. Analysis of the peripheral immune response against $MOG_{35–55}$ during the acute phase of disease induced with both protocols of immunization confirmed an increased pro-inflammatory profile of immune cells from *Kit*$^{W\text{-}sh/W\text{-}sh}$ compared with those from *Kit*$^{+/+}$ mice (data not shown).

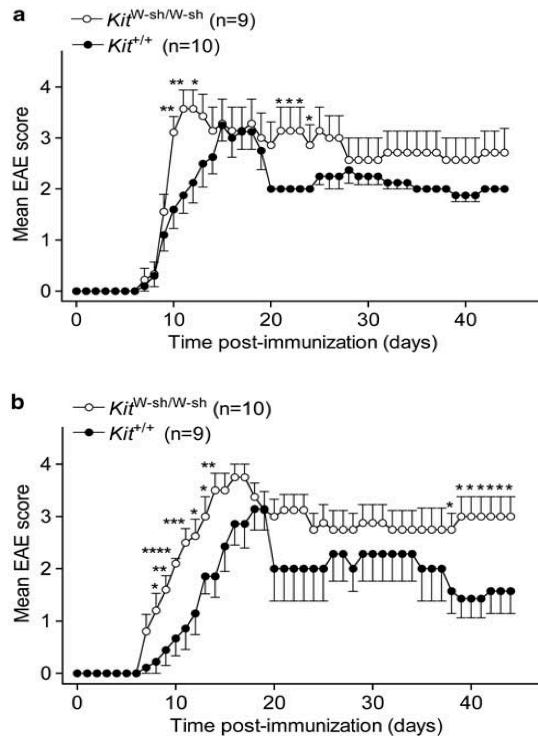


Figure 3 Kit$^{W\text{-}sh/W\text{-}sh}$ mice develop exacerbated EAE under different conditions of immunization. EAE was induced by two additional protocols of immunization bearing increased doses of sole PTX or of both adjuvants and myelin peptide. (**a**) *Kit*$^{+/+}$ (n = 10) and *Kit*$^{W\text{-}sh/W\text{-}sh}$ (n = 9) were immunized by s.c. injection of 200 µg of $MOG_{35\text{-}55}$ emulsified in complete Freund's adjuvant (CFA) containing 400 µg of *M. tuberculosis* (on day 0) and i.v. injection of 400 ng of PTX (on days 0 and +2). (**b**) *Kit*$^{+/+}$ (n = 9) and *Kit*$^{W\text{-}sh/W\text{-}sh}$ (n = 10) were s.c. injected with 300 µg of $MOG_{35\text{-}55}$ emulsified in CFA containing 500 µg of *M. tuberculosis* (on days 0 and +7) and i.v. injected with 400 ng of PTX (on days 0 and +2). Mice were scored daily. Data represent mean clinical score ± SEM from a representative experiment out of two independent replicates, each including 4-10 mice per group, performed for each protocol of immunization (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, **** $P < 0.001$; Student's *t* test, two tailed).

MCs Promote or Inhibit EAE in $Kit^{W/W\text{-}v}$ Mice Depending on the Conditions of Immunization

Given the surprising observation of exacerbated EAE in absence of MCs in the $Kit^{W\text{-}sh/W\text{-}sh}$ mouse model, we decided to go back to the $Kit^{W/W\text{-}v}$ mouse model and to study the disease in these mice by using different protocols of immunization as we did in the $Kit^{W\text{-}sh/W\text{-}sh}$ mice. We induced EAE with our standard protocol of immunization or with a 'stronger' one similar to that used by Secor *et al* [16]. Surprisingly, with lower doses of Ag and adjuvants, $Kit^{W/W\text{-}v}$ mice exhibited a more severe disease course than controls, resembling the phenotype observed in the $Kit^{W\text{-}sh/W\text{-}sh}$ strain (Figure 4a). Conversely, when we applied a 'stronger' protocol of immunization, $Kit^{W/W\text{-}v}$ mice developed a significantly milder EAE compared with $Kit^{+/+}$ mice, confirming a detrimental role for MCs in EAE under these specific experimental conditions (Figure 4b).

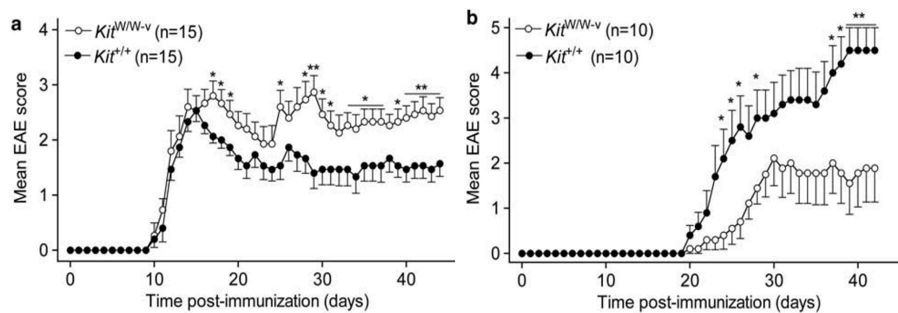


Figure 4 Kit$^{W/W\text{-}v}$ susceptibility to EAE depends on the protocols of immunization. (**a**) $Kit^{+/+}$ (n = 15) and $Kit^{W/W\text{-}v}$ (n = 15) were immunized with 100 µg of $MOG_{35\text{-}55}$ emulsified in complete Freund's adjuvant (CFA) containing 200 µg of *M. tuberculosis* (s.c. on day 0) and 200 ng of PTX (i.v. on days 0 and +2). (**b**) $Kit^{+/+}$ (n = 10) and $Kit^{W/W\text{-}v}$ (n = 10) were immunized as described in Figure 3b.

Figure 4 (continued) Mice were scored daily. Data represent mean clinical score ± SEM from a single experiment performed for each protocol of immunization (**P* < 0.05; **P* < 0.01; Student's *t* test, two tailed).

Systemic Reconstitution of $Kit^{W\text{-}sh/W\text{-}sh}$ Mice with BMMCs Fails to Revert EAE Phenotype

To test whether the different EAE severity of $Kit^{+/+}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice was truly due to the absence of MCs, rather than to other c-Kit-related alterations [30], $Kit^{W\text{-}sh/W\text{-}sh}$ mice were engrafted with BMMCs differentiated from Kit donors. Intravenous route of transplantation allowed repopulation of peripheral lymphoid organs, although, as previously observed [29,39], MC reconstitution did not restore the physiological MC distribution observed in WT mice (Figure 5a). Also, BMMC i.v. transplantation failed to repopulate the CNS (Figure 5a), as already reported in BMMCs-reconstituted $Kit^{W/W\text{-}v}$ mice [18]. Of note, we observed that Treg deficiency was corrected in lymph nodes of BMMCs-reconstituted naïve $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Supplementary Figure S3). As shown in Figure 5c, BMMCs-transplanted $Kit^{W\text{-}sh/W\text{-}sh}$ mice developed EAE similarly to MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice. These data suggest that in this model the restoration of a peripheral pool of MCs is not sufficient to recapitulate WT disease course, at least during the priming phase.

Passive EAE is Exacerbated in $Kit^{W\text{-}sh/W\text{-}sh}$ Mice

The above data indicated that c-Kit mutation affects the development of autoimmune manifestations mainly outside the peripheral lymphoid organs, suggesting that an altered encephalitogenic T-cell priming

was unlikely responsible for the exacerbated disease course observed in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. To confirm this hypothesis, passive EAE was induced in *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice by transferring *in vitro*-primed $MOG_{35–55}$-specific TCR-transgenic 2D2 T cells [25]. This approach allowed uncoupling priming and effector phase of the disease and isolating the role played by c-Kit mutation in inflammation in the CNS. We found that, also in this setting, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice developed clinical signs of disease with anticipated onset and exacerbated severity compared with *Kit*$^{+/+}$ mice (Figure 5d). This result suggested that the major contribution of the *Kit*$^{W\text{-}sh/W\text{-}sh}$ mutation to EAE pathology occurred downstream T-cell priming and concerned probably the effector phase of the disease mounting in the CNS.

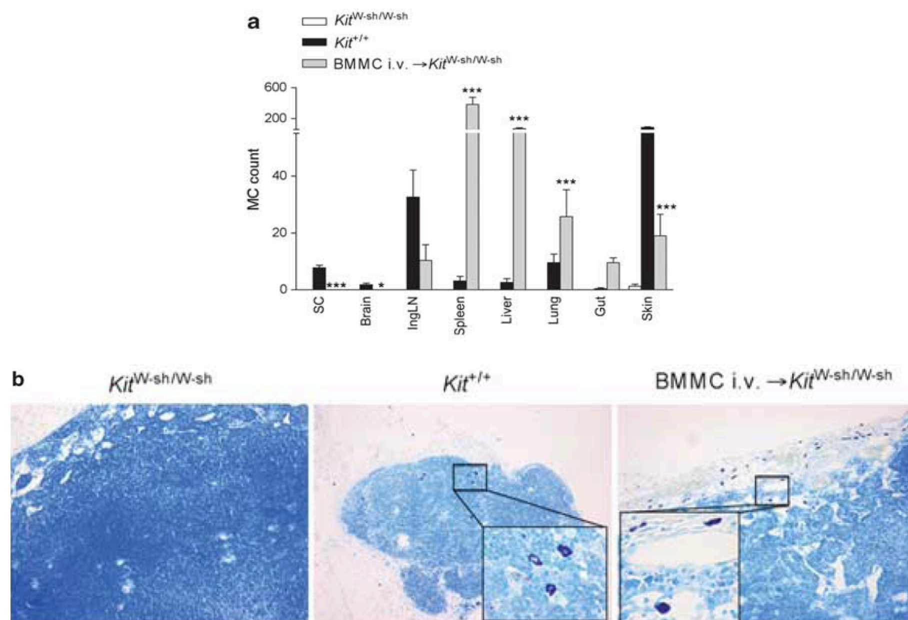


Figure 5 Mast cell reconstitution fails to attenuate EAE clinical severity in Kit$^{W\text{-}sh/W\text{-}sh}$mice. (**a-b**) BMMCs were engrafted i.v. (10^7 cells/mouse) in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and MC distribution in multiple organs was analyzed 6-8 weeks later by toluidine blue staining.

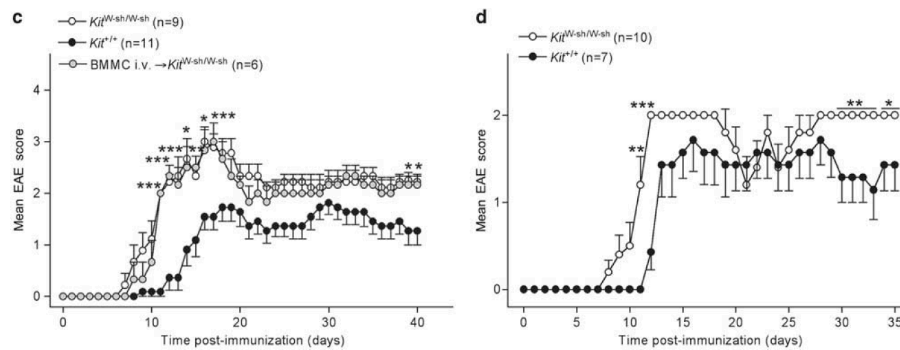


Figure 5 (continued) (**a**) Intravenously-injected MCs repopulated tissues of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and preferentially accumulated in the spleen, liver, and lung. The number of MCs was estimated by counting MCs in five high-power microscopic fields (400x) showing the highest MC densities and then summing the counts relative to each high power field. Data represent mean cell counts in each organ ± SEM. (*P < 0.05, ***P < 0.005; Student's *t* test, two tailed, calculated between *Kit*$^{+/+}$ and BMMCs-engrafted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice). (**b**) Inguinal LN (IngLN) from reconstituted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice showed a comparable amount of MCs to LN from WT mice, though the distribution of MCs in repopulated LNs was mainly intrasinusoidal (Toluidine blue. Original magnification X 100, inset X 400). (**c**) EAE was induced in *Kit*$^{+/+}$, *Kit*$^{W\text{-}sh/W\text{-}sh}$ and BMMCs-transplanted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice with $MOG_{35\text{-}55}$ peptide as described in *Materials and Methods*. BMMCs were injected i.v. 6-8 weeks before immunization. These data are from a single experiment representative of two independent replicates, each including 4 to 10 mice per group. (**d**) Passive EAE was induced in *Kit*$^{+/+}$ (n=7) and *Kit*$^{W\text{-}sh/W\text{-}sh}$ (n=10) mice by i.p. injection of *in vitro* primed $MOG_{35\text{-}55}$-specific TCR-transgenic 2D2 T cells. Mice were scored daily. Data represent mean clinical score ± SEM from a representative experiment out of two independent replicates, each including 7-10 mice per group (*P < 0.05, **P < 0.01, ***P < 0.005; Student's *t* test, two tailed).

Alterations in the Granulocyte Compartment do not Influence EAE in *Kit*$^{W-sh/W-sh}$ Mice

Kit$^{W-sh/W-sh}$ mutation may affect the homeostasis of granulocyte populations expressing c-Kit [29,30]. As granulocytes participate as effector cells in autoimmune inflammation in the CNS during EAE [40, 41], we speculated that enhanced granulocytosis might contribute to disease exacerbation in *Kit*$^{W-sh/W-sh}$ mice. We first characterized the percentage of Gr1^{hi} CD11b^{hi} and Gr1^{int} CD11b^{int} myeloid populations in the lymphoid organs of naïve *Kit*$^{+/+}$ and in *Kit*$^{W-sh/W-sh}$ animals. We observed a higher percentage of both subsets in the spleens of *Kit*$^{W-sh/W-sh}$ mice compared with controls, according to previous findings [30], whereas only the Gr1^{hi} CD11b^{hi} population was expanded in their lymph nodes (Supplementary Figure S4A). The anti-Gr1mAb used for staining (RB6-8C5 clone) recognized both Ly6G^{+} and Ly6C^{+} subsets [42], representing granulocytic and monocytic cells, respectively. The analysis of these two populations showed that both Ly6G^{+} and Ly6C^{+} cells were expanded in the spleens of *Kit*$^{W-sh/W-sh}$ mice, but only the Ly6G^{+} CD11b^{+} subset was amplified in lymph nodes (Supplementary Figure S4B). The Gr1^{hi} CD11b^{hi} population expressed c-Kit at higher level than the Gr1^{int} CD11b^{int} counterpart in *Kit*$^{+/+}$ mice; moreover, c-Kit expression in the Gr1^{hi} CD11b^{hi} compartment was significantly decreased in *Kit*$^{W-sh/W-sh}$ mice, whereas remaining unaltered within the Gr1^{int} CD11b^{int} counterpart (Supplementary Figure S4C). These pieces of evidence suggested that Gr1^{hi} CD11b^{hi} cells (likely encompassing the Ly6G^{+} granulocytes) were more strictly dependent on c-Kit signal for their homeostasis than the Gr1^{int} CD11b^{int} cells (including the Ly6C^{+}

monocytes). To explore the effects of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mutation in the expansion of myeloid cells upon EAE induction, we looked at the frequency of CD11b^{+}, Ly6G^{+} and Ly6C^{+} cells in lymphoid organs and CNS on immunization. The percentage of total CD11b^{+} cells was higher in draining lymph nodes of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice compared with controls at d10 after immunization. A faster accumulation of the Ly6G^{+}, rather than the Ly6C^{+}, fraction mainly accounted for this difference between the two strains (Figure 6a and b). At d10, the leukocytes infiltrating the CNS of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice contained a higher fraction of Ly6G^{+} CD11b^{+} cells, suggesting that an enhanced granulocyte-mediated inflammation might correspond to a peripheral granulocyte expansion (Figure 6c and d). To identify the contribution of granulocyte expansion to the increased disease severity expressed by *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, active EAE was induced in MC-deficient and WT mice under a granulocyte-depleting regimen. To this aim, mice were treated i.p., daily from d0 to d9, with the anti-Gr1 RB6-8C5 clone, which specifically depleted Ly6G^{+} while sparing Ly6C^{+} cells (Figure 6f), according to published results [43]. In line with previous findings [41], granulocyte elimination importantly delayed the onset and attenuated the severity of EAE symptoms in *Kit*$^{+/+}$ mice (Figure 6e). However, although Ly6G^{+}-depleted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice developed a milder disease than *Kit*$^{+/+}$ untreated controls, these mice still developed a more severe disease than Ly6G^{+}-depleted *Kit*$^{+/+}$ controls, suggesting a granulocyte-independent mechanism behind enhanced autoimmune inflammation in c-Kit mutated mice (Figure 6e). In support of this conclusion, we observed that in BMMC-i.v. reconstituted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, in which the normal disease course

was not rescued (Figure 5c), alterations of granulocyte homeostasis were completely recovered in both the $Gr1^{hi}$ and $Gr1^{int}$ compartments (Supplementary Figure S5). We noticed decreased T-cell proliferation and inflammatory cytokine production in both $Ly6G^{+}$-depleted $Kit^{W\text{-}sh/W\text{-}sh}$ and $Kit^{+/+}$ compared with untreated $Kit^{+/+}$ mice. This suggests that the increased disease severity observed in granulocyte-depleted $Kit^{W\text{-}sh/W\text{-}sh}$ compared with granulocyte-depleted $Kit^{+/+}$ mice was not due to differences in T-cell peripheral activation (Figure 6g). Taken together, these data suggest that $Kit^{W\text{-}sh/W\text{-}sh}$ mice developed exacerbated EAE independently from T-cell priming, peripheral MC presence or granulocytic effector cell alterations.

Beside MCs, other cells of the immune system may express c-Kit at variable levels and under different circumstances [44], such as dendritic cells (DCs) [45]. c-Kit pharmacological inhibition fosters a productive cross-talk between DCs and natural killer (NK) cells [46] and promotes the differentiation of a DC subset endowed with cytotoxic ability and IFN-γ competence [47]. However, as previously reported [29], we did not observe any difference between naïve $Kit^{W\text{-}sh/W\text{-}sh}$ and $Kit^{+/+}$ mice in NK or DC frequency in the spleen (Suppl. Figure S6).

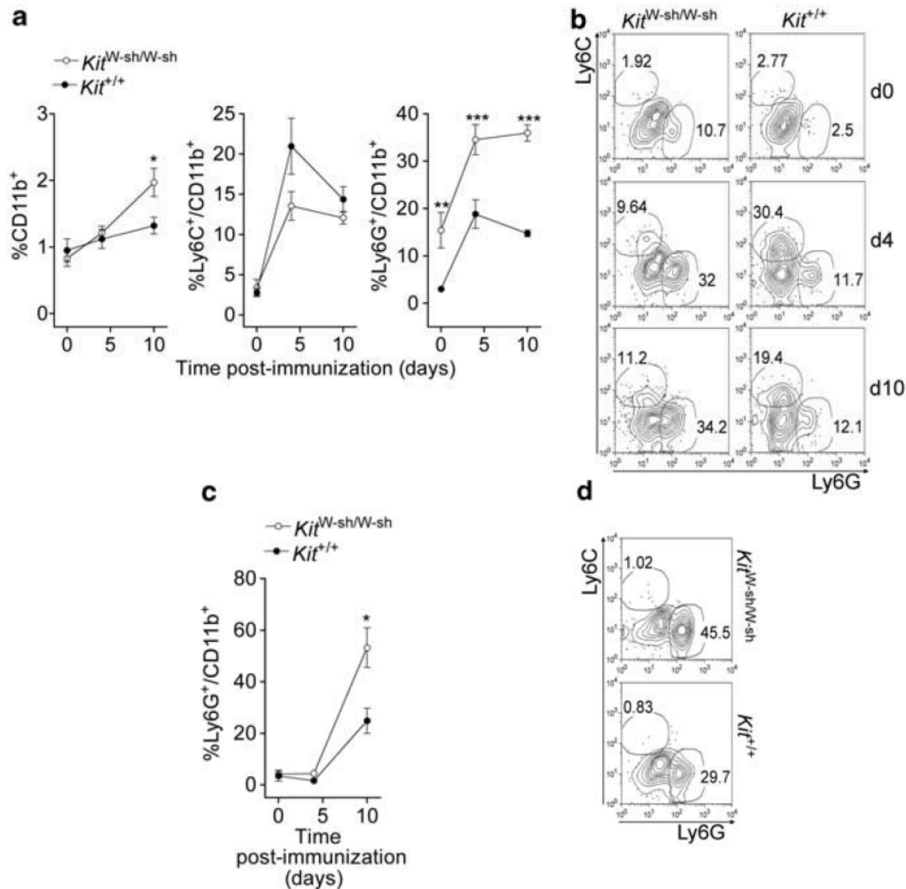


Figure 6 The severe abnormalities in granulocyte compartment of Kit$^{W\text{-}sh/W\text{-}sh}$ mice are not responsible for increased EAE severity. (**a-d**) The percentage of total CD11b$^+$ and the fraction of Ly6G$^+$/Ly6C$^+$ cells among the CD11b+ compartment was evaluated by flow cytometry in inguinal lymph nodes (**a-b**) or CNS (**c-d**) from *Kit*$^{+/+}$ and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice at days 0, 4 or 10 upon immunization. Data in **a** and **c** represent mean percentage ± SEM (*P < 0.05, **P < 0.01, ***P < 0.005; Student's t test, two tailed). Representative contour plots of Ly6C versus Ly6G in gated CD11b$^+$ cells are depicted in **b** and **d**.

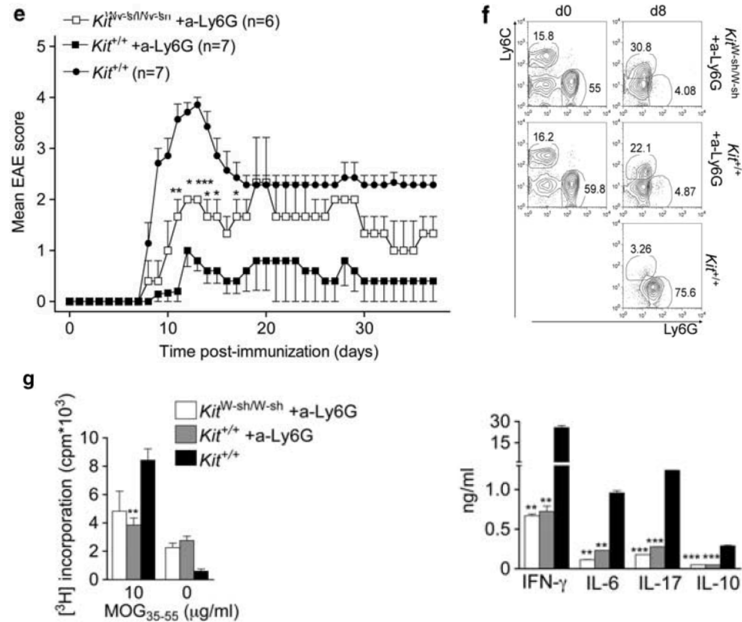


Figure 6 (continued) (**e**) $Kit^{+/+}$ (n=14) and $Kit^{W\text{-}sh/W\text{-}sh}$ (n=6) mice were immunized with $MOG_{35\text{-}55}$ peptide. $Kit^{+/+}$ (n=7) and $Kit^{W\text{-}sh/W\text{-}sh}$ (n=6) were treated with anti-Ly6G mAb as described in *Materials and Methods*. The second cohort of $Kit^{+/+}$ mice (n=7) was not treated and served as an internal control of EAE. Mice were scored daily. Data are from a single experiment representative of two independent replicates, each including 6 to 7 mice per group. Data represent mean clinical score ± SEM. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$; Student's *t* test, 2 tailed, calculated between a-Ly6G-treated $Kit^{+/+}$ and a-Ly6G-treated $Kit^{W\text{-}sh/W\text{-}sh}$ mice). (**f**) Representative contour plots showing depletion of $Ly6G^+$ but not $Ly6C^+$ cells in peripheral blood 8 days after a-Ly6G treatment. (**g**) Proliferation rate of LNCs (mean c.p.m. ± SEM, from triplicate wells) and cytokine production of splenocytes (means ± SEM, from duplicate wells) isolated from untreated $Kit^{+/+}$ mice and from anti-Gr1-treated $Kit^{+/+}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice (n=3-7 mice per group) 10 days after EAE induction and stimulated with $MOG_{35\text{-}55}$ 10 μg/ml or medium alone. **$P < 0.01$, ***$P < 0.005$ (Student's *t* test, 2 tailed, calculated in comparison to untreated $Kit^{+/+}$ mice).

Discussion

Broad evidence suggests that MCs may have multiple and even divergent roles in autoimmune and inflammatory diseases. The vast majority of these observations come from rodent MC-deficient models harbouring different mutations in the c-Kit gene. In this study, we showed that the *Kit*$^{W/W-v}$ animals were differentially susceptible to EAE induction depending on the strength of the immunization protocol. Conversely, *Kit*$^{W-sh/W-sh}$ mice developed anticipated and exacerbated EAE, either passively or actively induced, under all the tested conditions of immunization. Such behaviour could not be attributed to an immunoregulatory role of MCs in T-cell priming occurring in lymphoid organs, as indicated by the failure of recovering the normal disease course by reconstituting the peripheral MC pool or by passively inducing EAE bypassing T-cell priming. We confirmed some defects in the granulocyte compartment in *Kit*$^{W-sh/W-sh}$ mice that might participate to inflammation in the CNS, although granulocyte elimination could not abrogate the difference in disease outcome between c-Kit mutant and WT mice.

Most of the information about the role of MCs in EAE came from the Brown's group that showed decreased EAE onset and severity in *Kit*$^{W/W-v}$ mutant mice [16,38]. In their system, MC systemic reconstitution restored disease severity by recovering an efficient encephalitogenic T-cell response in peripheral lymphoid organs [18] and by restoring neutrophil penetration at the blood-brain barrier [20]. Our results reconciled, in part, the apparently contrasting findings in the two MC-deficient strains. Indeed, we could reproduce

Brown's data in the *Kit*$^{W/W\text{-}v}$ model when a strong immunization protocol was applied, but under milder conditions, which are our standard procedure to induce active EAE [48], *Kit*$^{W/W\text{-}v}$ mice developed an exacerbated rather than an attenuated disease, replicating our results in the *Kit*$^{W\text{-}sh/W\text{-}sh}$ model. However, our results are in contrast with a recent report from the same group, showing decreased EAE symptoms in *Kit*$^{W/W\text{-}v}$ mice compared with *Kit*$^{+/+}$ mice also on the application of a relatively milder immunization protocol (100 μg of $MOG_{35–55}$ and 250 ng of PTX) [20]. Thus, the discrepancies between our results and those reported by the Brown's group remain to be fully understood. However, it is possible that the opposite results observed in *Kit*$^{W/W\text{-}v}$ under different immunization protocols are related to a differential impact of the same mutation on distinct pathological mechanisms depending on the quality/quantity of the immune stimulation and on experimental conditions. Furthermore, different mutations in the same gene/locus can lead to contrasting outcomes under the same conditions of stimulation. Indeed, high doses of $MOG_{35–55}$, CFA and PTX produced a milder outcome in *Kit*$^{W/W\text{-}v}$ mice, but a more severe disease in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice than in *Kit*$^{+/+}$ controls. The reasons for this discrepancy may rely on differences between strains regarding non-MC-related abnormalities. For instance, it has been shown that *Kit*$^{W/W\text{-}v}$, but not *Kit*$^{W\text{-}sh/W\text{-}sh}$, mice are resistant to antibody-induced arthritis because the former is neutropenic, whereas the latter shows splenic myeloid hyperplasia, both at basal level and on immunization [9,30]. Depletion of $Gr1^{+}$ cells showed a prominence of granulocytes, rather than MCs, in the pathogenetic mechanisms driving arthritis in this model [9]. The

divergent susceptibility of the two strains to EAE development may even reside in non-immune-related processes. The observation that the *Kit*$^{W\text{-}sh/W\text{-}sh}$ mutation determines the disruption of the Corin gene [30] suggests that the *Kit*$^{W\text{-}sh/W\text{-}sh}$ model may recapitulate the defects observed in Corin$^{-/-}$ mice. Indeed, *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice show cardiomegaly [30] and may also display spontaneous hypertension, a condition associated to Corin ablation [49] that may favour EAE development, as indicated by the recent observation that anti-hypertensive drugs suppress autoimmune response in EAE [50]. The *Kit*$^{W\text{-}sh/W\text{-}sh}$ mutation may also produce dysregulations in a series of genes included in the inverted region, whose effects in immunity are largely unknown [30]. Finally, the two c-Kit-mutated strains differ in their genetic background, and the WBB6F1-*Kit*$^{+/+}$ is characterized by higher number of peritoneal MCs and neutrophils than the C57BL/6-*Kit*$^{+/+}$ [51]. In summary, our findings underscore the need for clear and doubtless identification of the role of MCs in a certain process by taking advantage of different c-Kit mutants. These variables (the strain of MC-deficient mice, the kind of mutation in c-Kit gene and the experimental model) are well exemplified by the work of Piliponsky *et al* [52] in mouse models of sepsis. This study demonstrates that MCs can promote survival in cecal ligation and puncture (CLP) of moderate severity in WBB6F1-*Kit*$^{W/W\text{-}v}$, C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ and WBB6F1-*Kit*$^{W\text{-}sh/W\text{-}sh}$ strains, but they exert no effect in WBB6F1-*Kit*$^{W/W\text{-}v}$ and WBB6F1-*Kit*$^{W\text{-}sh/W\text{-}sh}$ affected by severe CLP and increase mortality in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ with severe CLP or infected with *Salmonella typhimurium* [52].

Also, indirect support to our results may come from other

mutant mice carrying quantitative or qualitative defects in the MC population. Some of us have recently reported a more severe EAE in histidine decarboxylase (HDC)-deficient mice [28], which are not only genetically unable to produce histamine, but also show paucity in MC number and abnormalities in MC cytoplasmic granules [53]. Therefore, it is possible that these mice share with c-Kit mutants the phenotypical defects that depend on MC ablation. It must be noted that some of the results here reported may be associated also to an alteration of histamine signalling in these MC-deficient mice, as histamine has been lately reported to reduce T cell proliferation and IFN-γ production in autoreactive T cells [54].

Our data also show some discrepancy with the results obtained by other groups in the *Kit*$^{W\text{-}sh/W\text{-}sh}$ strain, showing unchanged [21] or reduced [22] EAE clinical course in these mice in comparison with WT counterpart. The former study highlights that MCs accumulate in the CNS trafficking from the bone marrow during EAE but are dispensable for the development of the disease [21]. Interestingly, although in this study no difference in EAE expression between *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice was observed, an increased Ag-specific T-cell proliferation in immunized *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice was reported, in line with our findings. The latter study shows that milder clinical signs of EAE in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice correlate with decreased $CD8^{+}$ T-cell activation [22]. Although the reasons for these discrepancies still need to be determined, we cannot exclude that different mouse housing conditions may affect the outcome of the *Kit*$^{W\text{-}sh/W\text{-}sh}$ mutation in EAE development, a process dependent on the composition of the gut microflora [55], which in turn may be modulated in a context of

MC deficiency [56]. Moreover, environmental stress may modulate autoimmunity development in experimental models [57] and MCs possess well-known abilities to translate neurological into immunological signals [58].

MC-knockin mouse models are useful tools to attribute a specific role to MCs or to a MC-expressed molecule in a particular biological process or pathological condition [29]. Indeed, reconstitution of c-Kit mutant mice with *in vitro*-differentiated MCs should recover only MC-dependent defects independently from the kind of c-Kit mutation. Different routes can be used to transfer BMMCs attaining different outcomes. Systemic intravenous injection produced MC accumulation but altered distribution in blood-draining organs with only a modest repopulation at priming sites, i.e., the inguinal and axillary lymph node, and in the neighbouring skin, according to previous data [29, 39]. In particular, the spleen has been recognized to trap most of i.v.-injected MCs, thus inhibiting their spreading to other tissues [39]. Thus, intravenous injection of BMMCs may inefficiently reproduce the topography and the quantity of MCs observed in lymphoid organs of $Kit^{+/+}$ mice in physiological conditions. However, we could detect the rescue of some MC-related biological functions, such as the recovery of normal Treg and granulocyte frequency, in lymph nodes and spleens, respectively, of reconstituted mice. In these conditions, MC transfer failed to recover the normal disease course. Although we cannot exclude that the altered MC distribution at priming sites, especially at draining lymph nodes, might have influenced the clinical outcome of EAE in these reconstituted mice, this result suggests that an immunoregulatory role of MCs in the priming phase of EAE is

unlikely. According to previous results in the *Kit*$^{W/W-v}$ strain [18], injected BMMCs failed to repopulate the CNS parenchyma, therefore this experimental setting was not suitable to ascertain whether MCs exerted a regulatory role directly in the inflamed milieu of the CNS. Therefore, tissue-specific MC knockin should be obtained, when possible, by localized MCs injections in order to allow optimal and physiological repopulation. Intravenous or intracranial injection of BMMCs has been recently found to restore the pool of meningeal MCs in *Kit*$^{W/W-v}$ mice, leading to the recovery of a WT-like disease course and to the rescue of neutrophil infiltration in the CNS [20]. In our system, although detecting scattered MCs in *Kit*$^{+/+}$ mice in the context of the reticulum formed by the arachnoid, the subdural neurothelium, and the dura mater, in line with previous observations [29], we could not find MCs populating the meninges in BMMC-reconstituted *Kit*$^{W-sh/W-sh}$ mice (not shown).

To explore the contribution of immune cells other than MCs in the observed phenotype, we checked the defective frequency of other c-Kit-related cells, such as NK and DCs [45–47], in *Kit*$^{W-sh/W-sh}$ mice, without finding any difference, according to previous data [29]. However, we cannot exclude that DC-expressed c-Kit has some role on immunization, as demonstrated in a model of allergic asthma, in which Ag/adjuvant exposure induced on DCs the expression of both c-Kit and its ligand, promoting Th2 and Th17 responses [45]. We then focused our attention on granulocytes because of their reported altered homeostasis in *Kit*$^{W-sh/W-sh}$ mice [30] and their involvement in EAE pathogenesis [41]. Confirming previous results [30], we found an expanded myeloid compartment in the spleens of *Kit*$^{W-sh/W-sh}$ mice,

involving both the $Gr1^{hi}$ $CD11b^{hi}$ and the $Gr1^{int}$ $CD11b^{int}$ subsets. We further characterized these myeloid cells for the Ly6G and Ly6C markers, and found that both subpopulations were expanded in the spleens of $Kit^{W\text{-}sh/W\text{-}sh}$ animals. We looked at the frequency of these cells also in lymph nodes hosting encephalitogenic T-cell priming and found $Gr1^{hi}$ $CD11b^{hi}$ and $Ly6G^{+}$ $CD11b^{+}$ cells, but not $Gr1^{int}$ $CD11b^{int}$ or $Ly6C^{+}$ $CD11b^{+}$ cells, selectively expanded by the c-Kit mutation. As the anti-Gr1 mAb used to stain granulocytes reacts with both Ly6G and Ly6C molecules [42], it was not possible to ascertain whether the $Ly6C^{+}$ entirely corresponded to the $Gr1^{int}$ and the $Ly6G^{+}$ to the $Gr1^{hi}$ subset. We found that the whole $CD11b^{+}$ compartment expressed c-Kit, although at a lower level than MCs (not shown), but the c-Kit expression level varied between different subsets and different genotypes, being higher in WT $Gr1^{hi}$ $CD11b^{hi}$ cells compared with mutated $Gr1^{hi}$ $CD11b^{hi}$, WT $Gr1^{int}$ $CD11b^{int}$ and mutated $Gr1^{int}$ $CD11b^{int}$ cells. These data confirm that, contrary to the $Kit^{W/W\text{-}v}$, the $Kit^{W\text{-}sh/W\text{-}sh}$ mutation does not produce an indiscriminate functional knockdown of the c-Kit receptor. Rather, the inversion in a regulatory element upstream the c-Kit gene may finely regulate the effects of c-Kit expression not only in MCs, but also in other cells expressing c-Kit under different transcriptional stimuli. In line with this hypothesis, we found that the $Gr1^{hi}$ $CD11b^{hi}$ compartment seemed more strictly related to the $Kit^{W\text{-}sh/W\text{-}sh}$ mutation than the $Gr1^{int}$ $CD11b^{int}$ counterpart, in terms of peripheral lymphoid accumulation/homing and c-Kit expression level. However, our data also suggest that granulocyte abnormalities detected in $Kit^{W\text{-}sh/W\text{-}sh}$ mice may be ascribed to granulocyte-extrinsic defects, as we could rescue a

normal myeloid compartment in the spleens following systemic MC reconstitution. On the contrary, in the *Kit*$^{W/W-v}$ model, the failure to correct peripheral neutropenia with BMMC reconstitution suggests a neutrophil-intrinsic role of the *Kit*$^{W/W-v}$ mutation in shaping the granulocyte compartment [20]. It could be proposed that, in *Kit*$^{W-sh/W-sh}$ mice, MCs may actively inhibit granulopoiesis by unknown mechanisms, or passively limit excessive myeloid expansion simply by sequestering the majority of locally available, paracrine or juxtacrine, SCF through the highly expressed c-Kit receptor. This observation suggests to carefully interpret the data obtained in MC-knockin experimental systems, as also non-MC-related defects may be corrected by MC transfer.

We confirmed myeloid cell expansion in the spleen (not shown) and in draining lymph nodes of $MOG_{35–55}$-immunized mice, involving both $Ly6G^+$ and $Ly6C^+$ compartments. The latter has been recently identified as a monocytic immunosuppressive subset that expanded at EAE onset and inhibited *ex vivo* T-cell proliferation, thus being attributed to the family of myeloid-derived suppressor cells [59]. However, the *in vivo* suppressive function of $Ly6C^+$ cells in EAE has been questioned by the observation of an attenuated disease development upon $Ly6C^+$ cell pharmacological elimination [60]. Conversely, the $Ly6G^+$ fraction was not suppressive *ex vivo* [59] and accordingly Ly6G elimination strongly inhibited the development of both active and passive EAE, suggesting an involvement of granulocytes in the effector phase of EAE [41]. $Ly6G^+$ cells, expanded in preimmune *Kit*$^{W-sh/W-sh}$ mice, further increased in lymph nodes on immunization and infiltrated earlier the CNS. We confirmed *in vivo*

the pathogenetic, rather than immunosuppressive, nature of these cells in both c-Kit mutated and WT mice, as both strains showed attenuated and delayed symptoms when treated with anti-Gr1 mAb. However, the alterations carried by *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice in the Ly6G^{+} subset were not causally related to the enhanced EAE clinical course, as granulocyte depletion could not abate the difference between *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice in terms of clinical disease expression, despite it dramatically reduced encephalitogenic T-cell responses at the same extent in both groups compared with untreated controls.

Overall our data tend to exclude a role for peripheral MCs in regulating T-cell priming, even though we could detect, in draining lymph nodes of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice at d10 on immunization, enhanced T-cell proliferation, increased secretion of pro-inflammatory cytokines and decreased IL-10 amount, partially in line with previous data [21]. The frequency of Th17 and Th1 cells was higher in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice at d10 post immunization. Even though the discovery that Th17 cells drove autoimmunity subverted the previous belief that Th1 cells were the most relevant pathogenic cells in EAE, it is now well accepted that both T-helper subsets participates, through distinct mechanisms, to EAE and MS pathogenesis [61]. Indeed, the IL-12-Th1 and the IL23-Th17 axes lead to prominent macrophage or granulocyte infiltrate, respectively, in spite of clinically indistinguishable disease courses [40]. Th17 encephalitogenic cells displayed the unique feature to enter the not-inflamed CNS [62] and to promote the assembly of ectopic lymphoid follicle-like structures in the CNS [63]. The observed increase of Th17 and Th1 cells in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice may be related to the fact that MCs can exert some influence on T-cell

differentiation by releasing a variety of cytokines in response to adjuvants on immunization [64]. In the *Kit*$^{W/W-v}$ model, a central role has been attributed to MCs in promoting T-cell priming, but MCs also enhanced inflammation in the effector phase of the disease [18,38]. Indeed, on one side, MC i.v. reconstitution recovered normal EAE, although without repopulating the CNS [18] on the other side, *Kit*$^{W/W-v}$ mice still developed milder symptoms on passive transfer of encephalitogenic T cells [38]. Others have found that MCs are attracted from the spleens to the draining lymph nodes upon immunization [18]. The cytokine IL-9, produced by Th17 cells in the lymph nodes of $MOG_{35–55}$-primed mice, has been recently identified to recruit MCs in lymphoid organs and to promote EAE development and progression [65]. We have shown *in vitro* that activated MCs inhibit Treg suppression and promote the differentiation of Th17 effector cells [19]. *In vivo*, MCs colocalized with Th17 cells and Treg in draining lymph nodes of $MOG_{35–55}$-immunized mice, thus potentially engaging multiple interactions with cells participating to adaptive immune response [19]. In this study, we found that immunization in WT mice induced an increase in MC infiltration in draining lymph nodes, which was associated with a preferential distribution of MCs in T-cell perifollicular areas of the lymph node cortex, and with a certain degree of MC clustering. By contrast, no significant change in MC infiltration and distribution was observed in the CNS of WT mice following immunization, supporting that MC functions related to Ag stimulation are mainly played in the DLNs. Analysis of naïve and immunized *Kit*$^{W-sh/W-sh}$ mice highlighted the absence of MCs in steady-state and inflamed tissues, respectively.

Grippingly, the absence of MCs from DLNs of immunized *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice was associated with a lower number of IL-10-expressing cells populating DLNs as compared with that observed in WT mice. This was in favour of a role for MCs in regulating IL-10 production during immune response instruction.

A high fraction of degranulated MCs has been detected in the CNS of MBP-immunized mice [66]. In a model of allograft tolerance, MC degranulation has been shown to break Treg-mediated immunological tolerance [37]. However, by toluidine blue staining, we could not detect significant MC degranulation in draining lymph nodes or in the CNS of $MOG_{35–55}$-immunized mice. Accordingly, ultrastructural analysis of MCs infiltrating the CNS in a marmoset model of EAE showed selective secretion, rather than degranulation [67].

Many data suggest that MCs can engage multiple interactions with Treg [19,26,32]. In this study, we found a defect in Treg frequency in lymphoid organs of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice before and during EAE development, at both acute and chronic phases. The reasons for impaired Treg homeostasis in naive *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice remain presently obscure. Contrary to another c-Kit mutant, showing severe defects in thymocytes development [68], we could not detect any major alteration in thymocyte subsets in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (data not shown). Notably, BMMC reconstitution restored a physiological Treg frequency in LN of preimmune *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. This observation points to a Treg-extrinsic role of this c-Kit mutation in shaping Treg compartment and suggests that MCs may not only mold Treg plasticity but also support Treg homeostasis. The OX40–OX40L axis may be involved in a defective Treg expansion in MC-deficient

mice. Indeed, MCs constitutively express OX40L [26] and OX40 is a key signal in supporting Treg fitness especially under inflammatory conditions [69]. Treg are important suppressor cells in both the initiation and the recovery phase of EAE [70], therefore the enhanced EAE disease course in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice could be associated to the decreased Treg accumulation in LN and spleen. However, *Kit*$^{+/+}$ and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice showed the same proportion of CNS-infiltrating Treg and BMMCs-reconstituted mice still developed exacerbated symptoms despite the recovery of a physiological Treg frequency.

MCs residing and/or recruited to the CNS has several roles in non-immunological conditions. *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice show a greater anxiety-like phenotype than WT controls [71]. Located in close proximity with the blood-brain barrier, MCs represent early responders to cerebral ischemia and potent inflammatory effector cells [72]. MCs may even modulate tumor metastasization into the brain [73]. In *Kit*$^{W/W\text{-}v}$, meningeal MCs promote a breach in the blood–brain barrier to allow the entry of inflammatory cells [20]. Overall these functions imply a role for MCs in increasing, rather than regulating, the permeability of blood-brain barrier. On the contrary, we found here an enhanced immune infiltrate in MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, especially granulocytes. Of note, we found more granulocytes infiltrating also the CNS of HDC$^{-/-}$ mice, carrying histamine deficiency and MC paucity [28]. Future experiments will unravel the true role of MCs in promoting or regulating the access of immune cells to inflamed CNS.

Supplementary Information

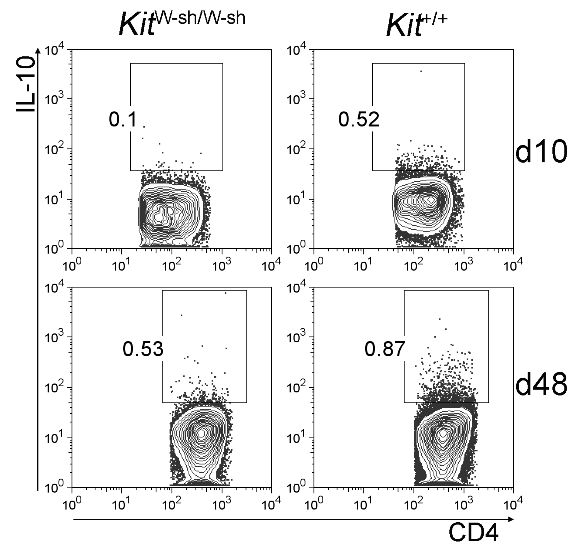


Figure S1 Decreased IL-10 secretion by CD4 T cells in DLN of Kit$^{W-sh/W-sh}$ mice. The percentage of IL-10^{+} cells among CD4^{+} T cells was checked in draining lymph nodes (DLN) of *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice. This analysis was performed at d10 and d48 upon immunization by intracellular staining after a 4-hrs restimulation with PMA, ionomycin and monensin.

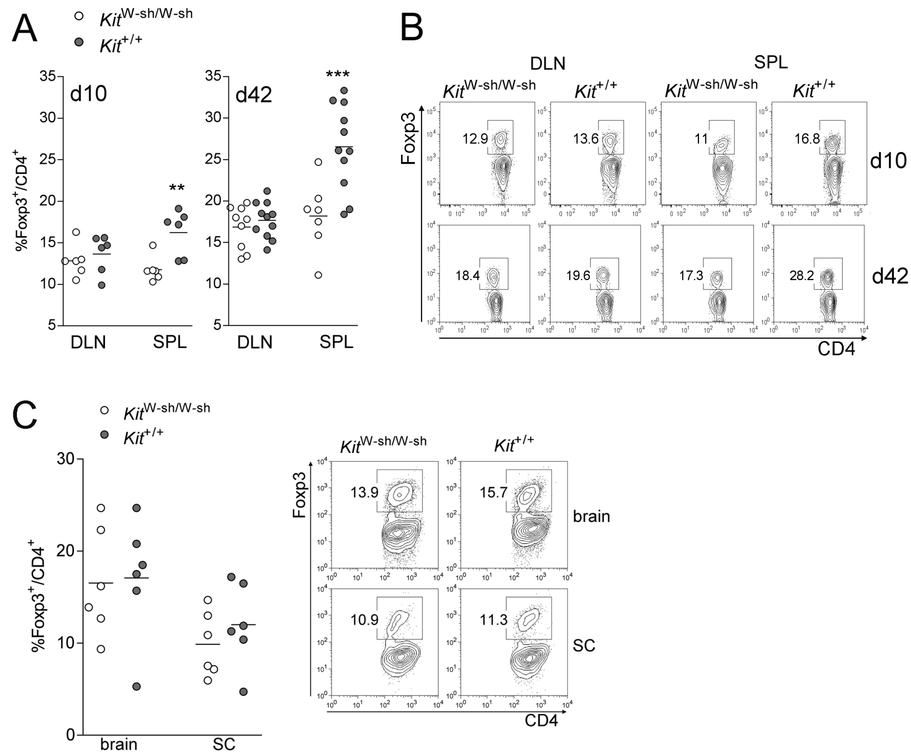


Figure S2 Decreased Treg frequency in immunized Kit$^{W\text{-}sh/W\text{-}sh}$ mice. (A-B) The percentage of Treg was checked in draining lymph nodes (DLN) and spleens (SPL) of *Kit*$^{+/+}$ and *Kit*$^{\text{W-sh/W-sh}}$ mice at d10 and at d42 upon immunization. (A) Percentage of Foxp3$^+$ Treg among CD4$^+$ T cells. $^{**}P < 0.01$, $^{***}P < 0.005$; Student's *t* test, 2 tailed, calculated between *Kit*$^{\text{W-sh/W-sh}}$ and *Kit*$^{+/+}$ controls. (B) Representative contour plots showing Foxp3 staining in gated CD4$^+$ T cells. (C) Treg frequency in brains and spinal cords (SC) analyzed at d10 upon immunization.

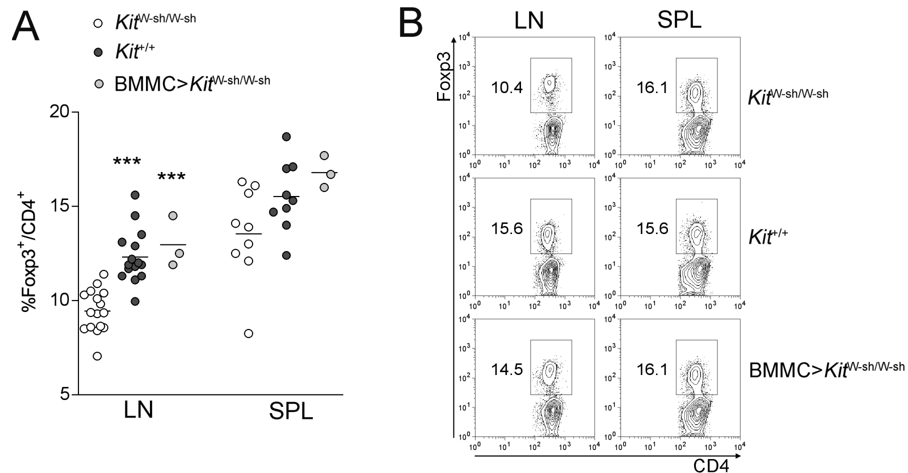


Figure S3 Treg defects in preimmune Kit$^{W\text{-}sh/W\text{-}sh}$ mice are corrected by BMMC reconstitution. (A) The percentage of Foxp3^{+} Treg among CD4^{+} T cells was evaluated by flow cytometry in inguinal lymph nodes (LN) and spleens (SPL) of preimmune *Kit*$^{\text{W-sh/W-sh}}$ *Kit*$^{+/+}$ mice. The same analysis was performed on *Kit*$^{\text{W-sh/W-sh}}$ mice 6 weeks after systemic reconstitution with BMMCs. ***$P < 0.005$; Student's *t* test, 2 tailed, calculated in comparison to untreated *Kit*$^{\text{W-sh/W-sh}}$ mice. (B) Representative contour plots are shown.

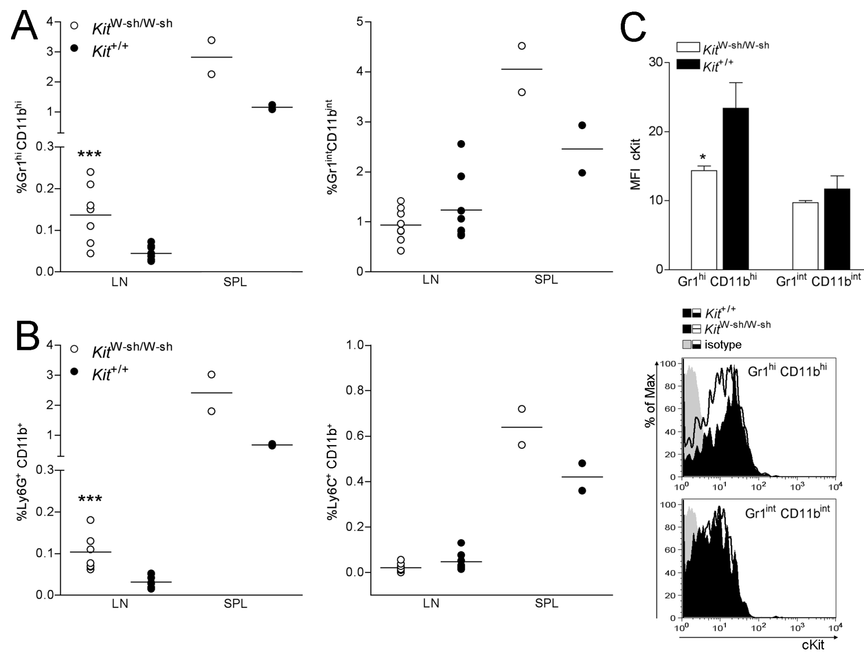


Figure S4 Kit$^{W\text{-}sh/W\text{-}sh}$ mice show basal defects in the granulocyte compartment. The percentage of Gr1^{hi} CD11b^{hi}/Gr1^{int} CD11b^{int} cells (A) and Ly6G^{+} CD11b^{+}/Ly6C^{+} CD11b^{+} cells (B) was evaluated by flow cytometry in lymph nodes (LN) and spleen (SPL) of preimmune 8 wks-old *Kit*$^{+/+}$ and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. (C) Expression level of *c-Kit* was evaluated by flow cytometry on gated Gr1^{hi} CD11b^{hi} or Gr1^{int} CD11b^{int} cells. Mean fluorescence intensity (MFI) and representative histogram plots are shown. Data represent mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.005; Student's t test, 2 tailed).

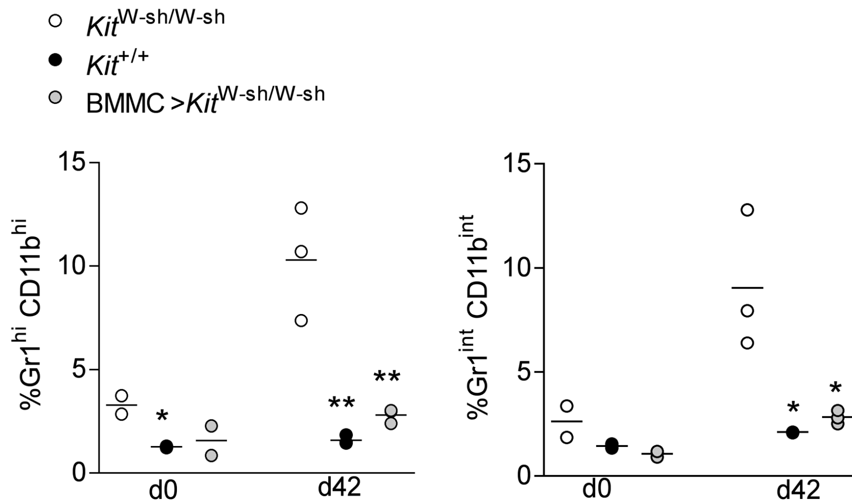


Figure S5 BMMCs i.v. engraftment rescues splenic granulocyte defects in Kit$^{W\text{-}sh/W\text{-}sh}$ mice. The percentage of Gr1^{hi} CD11b^{hi} and Gr1^{int} CD11b^{int} cells has been checked by flow cytometry in the spleen of *Kit*$^{+/+}$, *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{W\text{-}sh/W\text{-}sh}$ i.v. engrafted with BMMCs. Preimmune (d0) or immunized mice in the chronic phase of the disease (d42) were analyzed. Data represent mean ± SEM (*P < 0.05, **P < 0.01; Student's *t* test, 2 tailed, calculated in comparison to untreated *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice).

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CHAPTER 3

Histamine regulates autoreactive T cell activation and adhesiveness in inflamed brain microcirculation

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Abstract

Histamine may contribute to the pathology of MS and its animal model EAE. We explored the effects of histamine and specific HR agonists on activation and migratory capacity of myelin-autoreactive T cells. We show that histamine in vitro inhibits proliferation and IFN-γ production of mouse T cells activated against $PLP_{139-151}$. These effects were mimicked by the H1R agonist HTMT and the H2R agonist dimaprit and were associated with reduced activation of ERK1/2 kinase and with increased levels of cell cycle inhibitor p27Kip-1, both involved in T cell proliferation and anergy. H1R and H2R agonists reduced spontaneous and chemokine-induced adhesion of autoreactive T cells to ICAM-1 in vitro and blocked firm adhesion of these cells in inflamed brain microcirculation in vivo. Thus histamine, through H1R and H2R, inhibits activation of myelin-autoreactive T cells and their ability to traffic through the inflamed BBB. Strategies aimed at interfering with the histamine axis might have relevance in the therapy of autoimmune disease of the CNS.

Introduction

Histamine is an important mediator in several physiological and pathological processes, including neurotransmission and brain function, hormonal secretion, and gastrointestinal and circulatory function [1, 2]. Histamine is also critical in inflammation and a potent regulator of the innate and adaptive immune responses [3]. Synthesized from histidine by a unique enzymatic reaction mediated by HDC, histamine exerts its effects through four types of membrane HRs: H1R, H2R, H3R, and H4R. All HRs are heptahelical GPCRs with seven putative transmembrane domains and transduce extracellular signals through Gq (for H1R), Gs (for H2R), and Gi/o (for H3R and H4R) [1, 2]. In the immune system, histamine influences T cell polarization by having effects on monocytes and DCs, which promote a Th2 environment in humans and in mice [4–10]. Histamine can also influence T and B cell functions directly [11]. Indeed, in polarized human T cells, depending on the receptor engaged, histamine promotes Th1 responses through H1R and down-regulates Th1 and Th2 responses through H2R [12] with congruent data in mice lacking H1R and H2R [12, 13].

A role for histamine in MS and in EAE, an animal model for this disease associated with $CD4^+$ T cells reactive to myelin- secreting IFN-γ (Th1) and/or IL-17 (Th17) [14, 15], has been suggested (reviewed in refs. [3, 16]). We have shown previously that H1R as well as H2R are expressed on mononuclear cells within the inflammatory foci in the brain of mice with EAE, whereas encephalitogenic Th1 cell lines activated against $PLP_{139–151}$

expressed more H1R and less H2R compared with Th2 TCLs [17]. Inhibition of myelin oligodendrocyte glycoprotein$_{35–55}$-induced, chronic EAE has been reported in mice deficient for H1R [13, 18, 19], and treatment with H1R antagonists reduced the severity of PLP$_{139–151}$-induced, relapsing-remitting EAE [17, 20] and rat EAE [21]. Supporting the hypothesis of an important role for H1R in the development of EAE, it has been shown that Bphs, a gene that is associated with susceptibility to EAE and other autoimmune diseases in animal models, is H1R [13]. Re-expression in T cells of the H1R allele from mice susceptible to EAE restores susceptibility to this disease in resistant H1R-deficient mice [18, 19]. However, histamine might also have an important role in limiting autoimmune brain inflammation, as HDC$^{-/-}$ mice, which lack histamine, exhibit exacerbation of chronic EAE with an increased T cell production of proinflammatory cytokines compared with WT mice, and a more diffuse inflammatory CNS infiltrates containing a large polymorphonuclear component and eosinophils [22]. Further, treatment with a specific agonist of H2R prevents chronic EAE [23]. These in vivo data emphasize a major regulatory role for histamine and its receptors in EAE. However, the direct effects of histamine on autoreactive myelin-specific T cells from nongenetically manipulated mice are largely unknown.

In this study, by using histamine and H1R and H2R agonists, we investigated the effects of these molecules on mouse T cells activated against the myelin peptide PLP$_{139–151}$. We show that these molecules modulate autoreactive T cells to reduce antigen-induced proliferation and IFN-γ production. HR agonists inhibit the adhesion of myelin-

autoreactive T cells to mouse ICAM-1 in vitro and block firm adhesion of these cells on the activated brain vessels in an in vivo model of early EAE inflammation. The H1R agonist inhibits integrin activation of myelin-autoreactive T cells. These data show an unexpected role for histamine as an inhibitor of autoreactive T cell activation and reveal for the first time an important role for histamine in the regulation of the ability of myelin-activated T cells to traffic through the inflamed brain microcirculation.

Materials and Methods

Mice

SJL mice (Charles River, Calco, Italy), female, 8–12 weeks old, were used in this study. All procedures involving animals were approved by the Ethical Committee of the Neurological Institute Foundation Carlo Besta (Milan, Italy) and performed according to the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC).

Peptide synthesis and immunization protocol

PLP139–151 (HSLGKWLGHPDKF) was synthesized using standard 9-fluore- nylmethyloxycarbonyl chemistry and purified by HPLC. The peptide purity was > 95%, as assessed by analytical, reverse-phase HPLC. EAE was induced in mice by s.c. immunization in their flanks with $PLP_{139–151}$ (100 μg/ mouse), emulsified in CFA containing 2 mg/ml heat-killed mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI, USA), and assessed daily for neurological signs of EAE, according to a five-point scale [24].

T cell proliferation assays

LNCs or spleen cells were cultured in vitro in 96-well microtiter plates at a density of 500 X 10^3 cells/well (for LNCs) or 350 X 10^3 cells/well (for splenocytes) in 200 μl RPMI 1640, supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-ME (5 X 10 5 M), HEPES buffer (0.01 M), and 10% FCS

(enriched RPMI), and stimulated with $PLP_{139-151}$ (20 μg/ml), Con A (2 μg/ml), anti-CD3 mAb (1 μg/ml; clone 41452C11, BD PharMingen, San Diego, CA, USA), or medium alone. The amount of histamine contained in culture medium was 1.1 X 10^{-9} M, as measured by an enzyme immunoassay kit (ImmunoTech, Beckman Coulter, Brea, CA, USA), as described previously [22]. $CD3^{+}$ T cells were purified from suspensions of splenocytes depleted of B220, CD11b, CD49b, and Ter-119-positive cells by magnetic separation (Miltenyi Biotec, Germany). Cell purity was confirmed by flow cytometric analysis and was determined to be 94% or higher. $CD3^{+}$ T cells (50 X 10^{3}/well) were stimulated in a 96-well plate with $PLP_{139-151}$ (20 μg/ml), anti-CD3 mab (1 μg/ml), or medium alone in enriched RPMI in the presence of 500 X 10^{3} γ-irradiated (3000rad) splenocytes from naïve mice as an APC source [25]. After 72h of incubation (37°C, 5% CO2), cultures were pulsed for 18 h with 0.5 μCi/well [H^{3}]-thymidine, and proliferation was measured from triplicate cultures on a β-counter (Perkin Elmer, Waltham, MA, USA). Data are shown as mean cpm ± SEM. To test the effects of histamine on T cell proliferation, LNCs or purified $CD3^{+}$ T cells were treated with histamine dihydrochloride (Sigma-Aldrich, Seelze, Germany), HTMT dimaleate (Tocris Bioscience, Bristol, UK), dimaprit dihydrochloride (Tocris Bioscience), or medium alone for 2 h at 37°C in RPMI 1640, washed, and in vitro-stimulated as described above. The concentrations of histamine, HTMT, or dimaprit used in the different experiments are reported in figure legends.

Cytokine measurements

Supernatants from LNCs or purified $CD3^+$ T cells cultured in parallel with those cells used in proliferation assays were used for cytokine analysis. IFN-γ, IL-10, IL-6 (anti-mouse OptEIA ELISA set, BD PharMingen), and IL-17 (anti-mouse DuoSet ELISA, R&D Systems, Minneapolis, MN, USA) were analyzed by ELISA, according to the manufacturer's protocols. Supernatants were collected from cultured cells at 48 h for IFN-γ and IL-6 and at 96 h for IL-10 and IL-17. Results are shown as mean of triplicates ± SEM, and SEMs were within 10% of the mean.

Real-time PCR

Total RNA was isolated from LNCs or from magnetically purified $CD3^+$ T cells from LNCs of naïve or immunized mice ex vivo at different time-points during EAE or upon in vitro stimulation using Trizol reagent (Invitrogen, San Diego, CA, USA), following the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA using Superscript II RT (Invitrogen) and random primers, as described by the manufacturers. The expression of H1R and H2R was quantified by using the following commercial primer-probe sets (Applied Biosystems, Foster City, CA, USA): H1R, Mm00434002_s1; H2R, Mm00434009_s1; GAPDH, Mm99999915_g1. A C_T was used to determine H1R and H2R mRNA expression relative to house-keeping GAPDH. The C_T value was normalized for each sample using the formula: $\Delta CT = C_{T\,(target)} - C_{T\,(GAPDH)}$, and the relative expression of the receptors was calculated using the equation $2^{-}\Delta^{CT}$.

Western blot and biochemical analyses

Total cell lysates were obtained from LNCs ex vivo (baseline) and at different time-points during in vitro stimulation in 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 2 μg/ml each aprotinin, leupeptin, and pepstatin, as described previously [26]. Total proteins from each lysate were subjected to SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred onto a nitrocellulose filter membrane (Protan, Schleicher and Schuell BioScience, Dassel, Germany) using a Trans-Blot cell (Bio-Rad Laboratories, Milan, Italy) and transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were placed in 5% nonfat milk in PBS plus 0.5% Tween 20 at 4°C for 2 h to block non-specific binding sites. Filters were incubated with specific antibodies before being washed and then incubated with a peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ, USA). Peroxidase activity was detected by using the ECL system (Amersham Biosciences). The antibodies used were the following: anti-p27Kip-1 (Cell Signaling Technology Inc., Beverly, MA, USA), anti-ERK1/2, and antiphosphorylated ERK1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The filters were also probed with an antitubulin antibody (Sigma-Aldrich) to normalize the amount of loaded protein. All filters were quantified by densitometric analysis of the bands using the program ScionImage (Version 1.63 for Mac, Scion Corp. Inc., Frederick, MD, USA).

Flow cytometry

A TCL activated against $PLP_{139-151}$, prepared as described previously [27], was antigen-stimulated for 4 days and then treated for 2 h with histamine, HTMT, dimaprit, or medium alone before labeling with fluorescent antibody for α4 integrin (VLA-4; PS/2 clone, kindly provided by Dr. Eugene Butcher, Stanford University, Palo Alto, CA, USA), LFA-1 (anti-αL chain; clone TIB-213 from ATCC, Manassas, VA, USA), PSGL-1 (clone 4RA10, kindly provided by Dr. Dietmar Vestweber, Max Plank Institute, Germany), L-selectin (Mel-14 clone, ATCC), and CD44 (IM/7 clone, ATCC). Isotype-matched antibodies were used as controls. Cell suspensions were incubated with 15% adult bovine serum before incubation with the specific mAb at 4°C for 30 min. At least 10,000 events were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA) using the CellQuest software.

In vitro T cell adhesion to mouse ICAM-1

A TCL activated to $PLP_{139-151}$ was treated with histamine, HTMT, or dimaprit or left untreated for 2 h at 37°C and then added in triplicates on slides coated overnight at 4°C with purified mouse ICAM-1 as described [28]. Binding assay medium was represented by DMEM without sodium bicarbonate containing 10 mM HEPES and 5% BCS (HyClone, Logan, UT, USA), pH 7.2. To determine spontaneous adhesion, 100 X 10^3 T cells in 25 μl/well were incubated on ICAM-1-coated slides for 10 min at 37°C, and then slides were washed in PBS and fixed. For chemokine induced adhesion, 50 X 10^3 T cells in 25 μl/well were incubated on ICAM- 1-coated slides for 3 min at

37°C, CCL20 (MIP-3β; 1 μM) was added, and after an additional 3 min, incubation slides were washed in PBS and fixed. Comput- er-assisted enumeration was performed.

Intravital microscopy and image analysis

Lymphocytes of a $PLP_{139-151}$-activated TCL were labeled with green CMFDA (Molecular Probes, Eugene, OR, USA) or orange CMTMR (Molecular Probes). Cells were kept for 1–5 days in antigen-free medium before intravital microscopy experiments to reduce background adhesiveness and to allow inside-out signaling generated by local proadhesive agonists [29]. For the last 2 h, cells were treated with HTMT, dimaprit, or PBS. Mice were surgically treated and injected i.p. with 12 μg LPS (Escherichia coli 026:B6; Sigma-Aldrich) 5–6 h before starting the intravital experiment as described previously [29]. A total of 2 X 10^6 fluorescent-labeled cells/condition was injected slowly into the carotid artery by a digital pump. The images were visualized by using a silicon-intensified target video camera (VE-1000 SIT, Dage-MTI, Michigan City, IN, USA) and a Sony SSM-125CE monitor and recorded using a digital VCR (Panasonic NV-DV10000). Video analysis was performed by playback of digital videotapes as described [29]. No significant differences were observed in hemodynamic parameters during the injection of cells treated with histamine agonists or left untreated. Lymphocytes that remained stationary on a venular wall for ≥ 30 s were considered adherent. At least 140 consecutive cells/venule were examined. Rolling and firm-arrest fractions were determined as the percentage of cells that roll or firmly arrest within a given venule on the total number of cells that

enter that venule during the same period of time.

ImageStream data acquisition and analysis

Lymphocytes of a $PLP_{139-151}$-activated TCL were treated with HTMT for 2 h or left untreated before chemokine stimulation with CXCL12. Cells (10^6) were then incubated with 10 μg/ml TIB-213 (anti-LFA-1; ATCC) for 30 min on ice. After washing, cells were stained with a secondary antibody (goat anti-rat IgG PE-conjugated; Caltag Laboratories, Burlingame, CA, USA). Stained cells were resuspended in PBS for the ImageStream analysis. Images were acquired on the ImageStream imaging cytometer System 100 (Amnis Corp., Seattle, WA, USA). Images of fixed cells were collected and analyzed using ImageStream data exploration and analysis software [30]. LFA-1 clustering was evaluated, analyzing the distribution on the cell surface of the fluorescence, associated with the PE staining specific for the integrin. Uniform (uniform distribution of fluorescence), clustered (small spots of fluorescence), and caps (big clusters of fluorescence) cells were gated using the Area feature versus the Delta Centroid XY feature [31]. The Area feature was calculated for Channel 4 (PE-specific emission; area of fluorescence), applying to the images of a threshold mask; this feature allows us to discriminate between cells with a larger fluorescence area (high Area values) and smaller fluorescence area. The Delta Centroid XY feature calculates the distance between the center of the PE fluorescence image and the center of the brightfield image for each image pair. This feature distinguished images with globally distributed staining (lower Delta Centroid values) from those with capped staining (higher Delta

Centroid values). When plotted versus the Area feature, Delta Centroid XY permits distinguishing between punctate and uniform staining [31]. In our analysis, cells with Area values higher than 30 and Delta Centroid XY values lower than 180 were considered uniform cells for their fluorescence distribution. Cells with Area values lower than 30 and Delta Centroid XY values lower than 2 were considered clustered cells (small spots of fluorescence). Cells with Delta Centroid XY values higher than 2 were considered caps cells (highly polarized fluorescence).

Statistical analysis

Student's t test, two-tailed, was used to compare results between two groups. For intravital microscopy studies, multiple comparisons were performed using the Kruskall-Wallis test with the Bonferroni correction of *P*. Differences were regarded significant with a value of $P \leq 0.05$. Analysis was performed with SPSS software (Chicago, IL, USA). In all tests, $P \leq 0.05$ was considered statistically significant.

Results

Histamine reduces in vitro proliferation and IFN-γ production of myelin-autoreactive T cells

Before exploring the effects of histamine on T cells activated against myelin, we examined the expression of H1R and H2R ex vivo on LNCs and on purified $CD3^+$ T cells derived from SJL mice ($H\text{-}2^s$ haplotype) in which relapsing-remitting EAE was induced with $PLP_{139-151}$. H1R and H2R were expressed at the mRNA level on LNCs (Fig. 1A) and on purified $CD3^+$ T cells (Fig. 1B) of mice with EAE, and transcripts for H2R were always over ten fold more elevated compared with those for H1R. The expression of both receptors varied during the different phases of EAE on LNCs and T cells, a fact that might be related to changes in the cytokine milieu [1] and/or in the activation state of T cells and of other immune cells during the disease. Indeed, it has been reported that H1R mRNA is down-regulated rapidly in unprimed $CD4^+$ T cells upon TCR activation with anti-CD3/CD28 antibody [18]. The reduced transcripts of H1R on LNCs and on purified T cells at the onset of EAE might reflect the activation of T cells during disease priming. Also, as autoreactive T cells after priming migrate rapidly from the LNs toward the CNS, differences in mRNA expression of HRs might also be influenced by the percentage of myelin-specific T cells that are present in LNs at a given time during EAE.

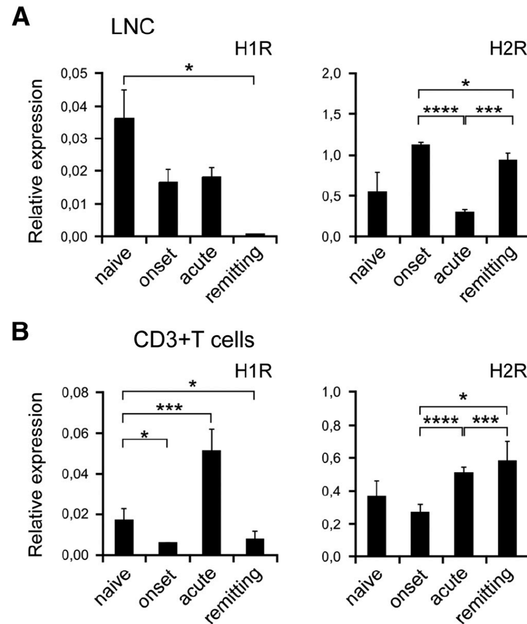


Figure 1. mRNA expression of H1R and H2R during the course of EAE. Freshly isolated LNCs (A) or LNC-purified $CD3^+$ T cells (B) from SJL mice at different phases of $PLP_{139–151}$-induced EAE were used to examine ex vivo mRNA expression of H1R and H2R. Mice were considered as remitting when the EAE score was decreased at least one point for at least two consecutive days. Data represent mean mRNA expression relative to GAPDH mRNA expression of three to four mice at each time-point ± SD and are representative of two independent experiments. *$P < 0.05$; ***$P < 0.005$; ****$P < 0.001$.

We then explored in vitro the effects of histamine on proliferation and cytokine production of LNCs derived from mice with EAE. As shown in Fig. 2A, treatment of LNCs with histamine for 2 h before in vitro stimulation with $PLP_{139–151}$ reduced LNC proliferation and production of the Th1 cytokine IFN-γ, and no significant changes were observed in the production of the suppressor

cytokine IL-10. As reported previously [11], we did not observe in these experiments a typical dose-response curve, a fact that could be related to the different affinity of histamine for H1R, H2R, and probably, for H4R (not evaluated in this study). Reduced proliferation and production of IFN-γ were even more pronounced when histamine was used to treat LNCs derived from mice at later stages of EAE, and IL-10 was also reduced by histamine treatment at those stages of the disease (Supplemental Fig. 1). To evaluate the contribution of H1R and H2R activation to the effects produced by histamine on these cells, we treated LNCs with HTMT, a selective H1R agonist, or dimaprit, a selective H2R agonist, for 2 h before antigen stimulation. As shown in Fig. 2B, HTMT inhibited proliferation and IFN-γ production significantly from LNCs, and IL-17 was also reduced by this treatment. All agonists inhibited the production of IL-6 in these cells, although HTMT was more effective in doing so, and IL-10 was slightly but significantly reduced by dimaprit.

A variety of immune cells expresses the receptors for histamine [1–3], and the effects of histamine that we observed on activated LNCs were likely to be the result of the triggering of this mediator on receptors expressed on different immune cells. We therefore wanted to ascertain the effects of histamine specifically on T cells by treating with histamine and its agonist $CD3^+$ T cells, which were purified magnetically from splenocytes of mice with EAE. As shown in Fig. 2C, treatment of $CD3^+$ T cells with histamine, HTMT, or dimaprit for 2 h before in vitro antigen stimulation inhibited T cell proliferation and IFN-γ production significantly and reduced the secretion of IL-17, IL-6, and IL-10.

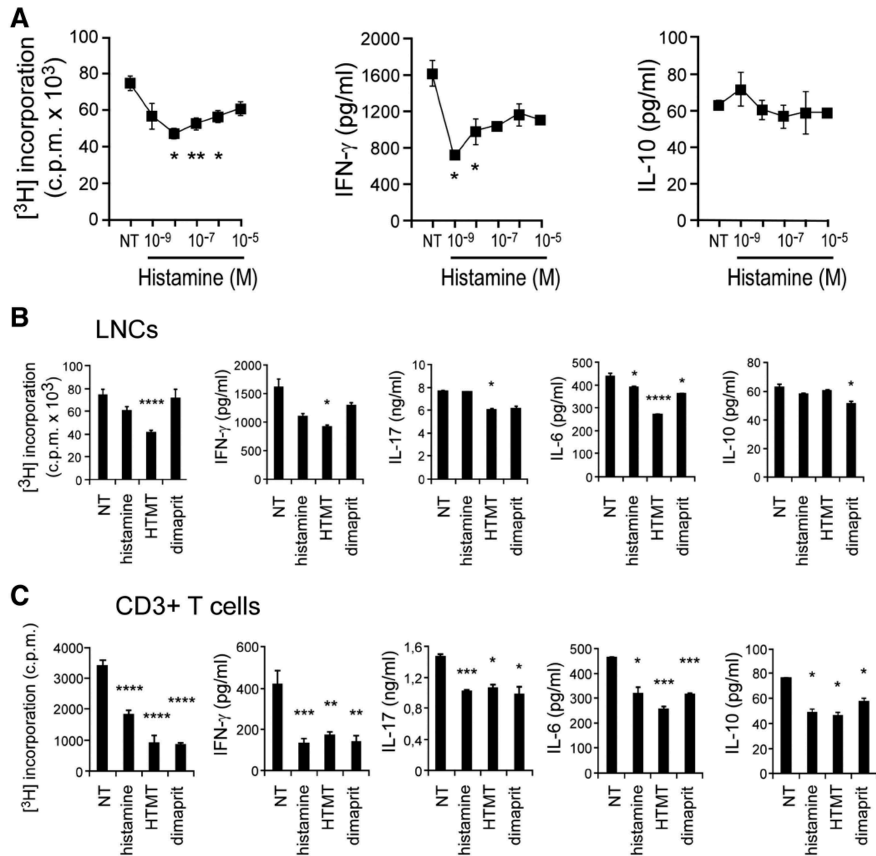


Figure 2. Histamine inhibits proliferation and IFN-γ cytokine production of LNCs and $CD3^+$ T cells activated against $PLP_{139–151}$. (A and B) LNCs obtained from SJL mice 10 days after the induction of EAE (*n*=3–5 mice) were pooled and left untreated (NT) for 2 h before in vitro stimulation with $PLP_{139–151}$ (20 μg/ml) or treated with increasing concentrations of histamine (A) or with histamine, H1R agonist HTMT or H2R agonist dimaprit, all agonists at a concentration of 10^{-5} M (B). (C) $CD3^+$ T cells magnetically purified from splenocytes of SJL mice 10 days after the induction of EAE were pooled (*n*=3–5 mice) and treated with histamine, H1R agonist HTMT or H2R agonist dimaprit (all at a concentration of 10^{-5} M) or left untreated for 2 h before in vitro stimulation with $PLP_{139–151}$ and γ-irradiated splenocytes.

Figure 2 (continued) Proliferation and cytokine production were measured as described in Materials and Methods. Data are shown as mean ± SEM. **P*<0.05; ***P* < 0.01; ****P* < 0.005; *****P* < 0.0001 versus untreated cells. Proliferation of unstimulated (= no antigen) cells was always below 7500 cpm in LNCs (A and B) and below 700 cpm in $CD3^+$T cells (C) and was not modified by any of the pharmacological treatments used (data not shown). This is representative of similar results obtained in four to five independent experiments performed.

Effect of histamine on biochemical pathways involved in T cell activation and anergy

To understand the molecular mechanisms by which histamine modulated proliferation and cytokine production of myelin-activated T cells, we analyzed the ERK1/2 pathway, a known regulator of T cell proliferation and IFN-γ production. As shown in Fig. 3A, treatment with histamine, HTMT, or dimaprit before in vitro antigen stimulation of LNCs from mice with EAE reduced ERK1/2 phosphorylation, and HTMT induced the strongest inhibition. These data were in line with the reduction of IFN-γ production observed by LNCs upon treatment with histamine and its agonists. Also, we studied the modulation of cyclin-dependent kinase inhibitor p27Kip-1, a molecule involved in the control of the cell cycle and in the induction and maintenance of T cell anergy [32, 33]. The expression of p27Kip-1 was increased in antigen-stimulated LNCs that were pretreated with histamine, HTMT, or dimaprit, and dimaprit produced the strongest effect (Fig. 3B), suggesting the induction of a state of hyporesponsiveness in these treated cells.

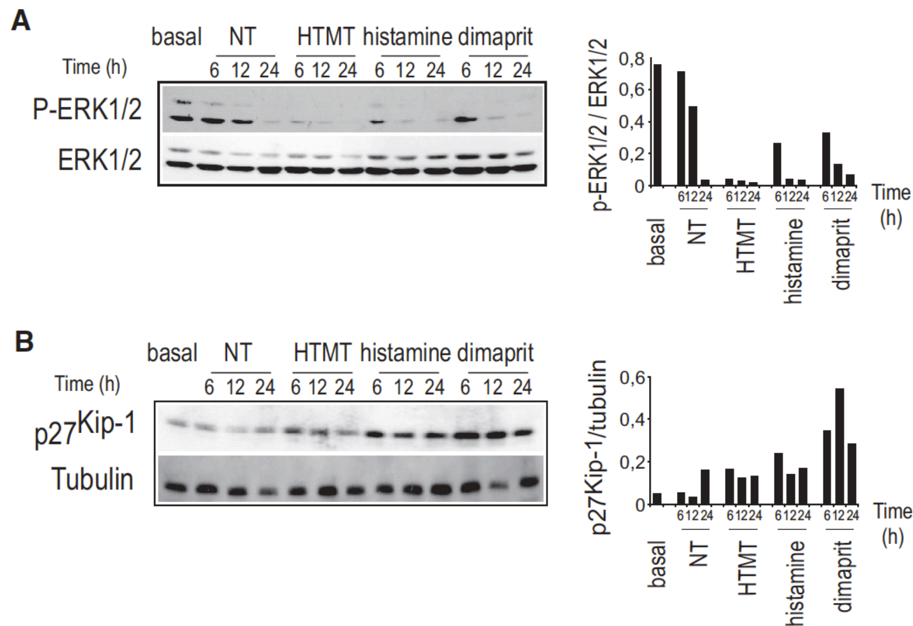


Figure 3. Effect of histamine on biochemical pathways involved in T cell activation and anergy. LNCs obtained from SJL mice 10 days after the induction of EAE (*n*=5 mice) were pooled and treated with histamine, H1R agonist HTMT, or H2R agonist dimaprit (all at a concentration of 10^{-5}) or left untreated for 2 h before in vitro stimulation with $PLP_{139-151}$ (20 µg/ml). Whole cell lysates were prepared from ex vivo untreated cells (basal) and at 6, 12, and 24 h after antigen stimulation and analyzed by Western blot analysis for phospho (P)-ERK1/2 and total ERK1/2 (A) and for p27Kip-1 and tubulin (B). The graphs on the right show quantitation of each specific protein.

Histamine reduces the adhesion capability of myelin-autoreactive T cells in vitro and in an in vivo model of early EAE inflammation

We then wanted to ascertain whether histamine affects the ability of myelin-autoreactive T cells to adhere to brain endothelium and thus, to influence their capacity to migrate through the BBB. We first explored the ability of histamine to modulate the adhesion of myelin-activated T cells to mouse ICAM-1 in vitro. As shown in Fig. 4A and B, treatment of a TCL activated against $PLP_{139–151}$ with histamine for 2 h significantly reduced spontaneous and chemokine-induced adhesion to ICAM-1. HTMT and dimaprit produced effects that were concordant with those produced by histamine, although the more profound inhibition of T cell adhesion to ICAM-1 was observed with the H1R agonist HTMT.

We next examined whether histamine triggering through H1R or H2R affects the ability of myelin-activated T cells to adhere to the endothelium in inflamed brain microcirculation in vivo by using intravital microscopy in an experimental model that mimics early EAE inflammation [29, 34]. As shown in Fig. 4C and D, pretreatment with HTMT and dimaprit of T cells of the TCL activated against $PLP_{139–151}$ had no significant effect on rolling but led to a significant inhibition of firm adhesion to inflamed brain microvessels of myelin-autoreactive T cells.

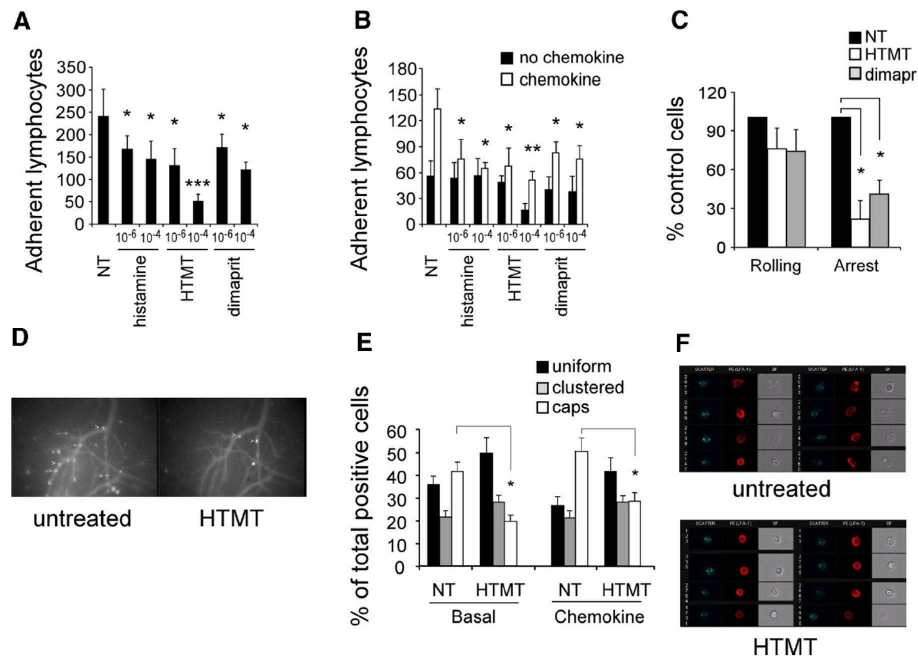


Figure 4. Histamine reduces the adhesion capability of myelin-autoreactive T cells. T lymphocytes of a TCL activated against PLP139–151 were used to perform these experiments. (A and B) T cells were treated with histamine, H1R agonist HTMT, or H2R agonist dimaprit (used at concentrations of 10^{-6} or 10^{-4} M) or left untreated (NT) for 2 h at 37°C and then added in triplicates on slides precoated with purified mouse ICAM-1. (A) For evaluation of spontaneous adhesion, 100 X 10^{3} T cells were incubated in medium culture for 10 min at 37°C. (B) For chemokine-induced adhesion, 50 X 10^{3} T cells were incubated in medium for 3 min at 37°C (no chemokine), followed by an additional 3 min in the presence of CCL20 (1 μM; chemokine). Data are shown as mean counts of adherent T cells/0.2 mm^2 ± SD. **P* <0.05; ***P* <0.005; ****P* <0.001 versus untreated cells (A) or versus chemokine-stimulated, untreated cells (B). Data are representative of at least three independent experiments. (C and D) Fluorescently labeled T cells were treated for 2 h with HTMT or dimaprit (at a concentration of 10^{-5}) or left untreated before the intravital microscopy experiments.

Figure 4 (continued) (C) Rolling and arrest fractions were calculated as described in Materials and Methods in five to seven venules from three to four animals for each experimental condition. Data are shown as mean ± SD. *P <0.05 versus untreated cells. (D) Adherent $PLP_{139–151}$-activated T cells (arrows) that were left untreated (CMFDA-labelled) or treated with HTMT (CMTMR-labelled) in the same brain venules are shown. (E and F) Analysis of LFA-1 clustering was performed on acquired cells with the ImageStream system software as described in Materials and Methods. (E) Mean percentages ± SD of cells presenting LFA-1 integrin distribution in uniform, clustered, and caps patterns in unstimulated (Basal) or chemokine (CXCL12)-stimulated cells in the presence or absence of HTMT from three experiments are shown. *P < 0.05 for distribution in caps in HTMT-treated cells versus untreated cells. (F) Representative cells from the most frequent pattern of LFA-1 distribution in unstimulated cells in the presence or absence of HTMT.

<u>H1R agonist HTMT reduces LFA-1 distribution in clusters and caps</u>

To explore the mechanisms by which histamine modulates the adhesion ability of myelin-autoreactive T cells, we first analyzed the expression of adhesion molecules on T cells of a TCL activated against $PLP_{139–151}$ upon treatment with histamine or its agonists. None of these compounds modified the expression of PSGL-1, VLA-4, L-selectin, LFA-1, or CD44 on $PLP_{139–151}$-activated T cells, as determined by flow cytometry analysis (Fig. 5 and Supplemental Fig. 2).

We then wanted to ascertain if histamine interferes with signal transduction pathways leading to integrin activation and subsequent leukocyte arrest to inflamed brain venules. We analyzed LFA-1 integrin distribution on myelin- activated T cell membranes by

using the ImageStream imaging cytometer, a powerful system that allows analysis of protein distribution and precise localization in different cellular compartments [30]. It has been shown previously that integrin distribution in small clusters or in larger areas ("big polar patches" or "caps") represents a modality of integrin activation leading to lymphocyte adhesion, whereas a more uniform distribution is indicative of a low-adhesion capacity [35, 36]. We observed that HTMT, which presented the highest inhibitory effect on the adhesion of activated T cells to mouse ICAM-1 in vitro and to inflamed brain vessels in vivo, significantly reduced LFA-1 distribution in caps on T cells of the TCL activated against $PLP_{139–151}$ under basal condition and after stimulation with chemokine CXCL12, suggesting a regulatory effect for this molecule on LFA-1 integrin activation (Fig. 4E and F).

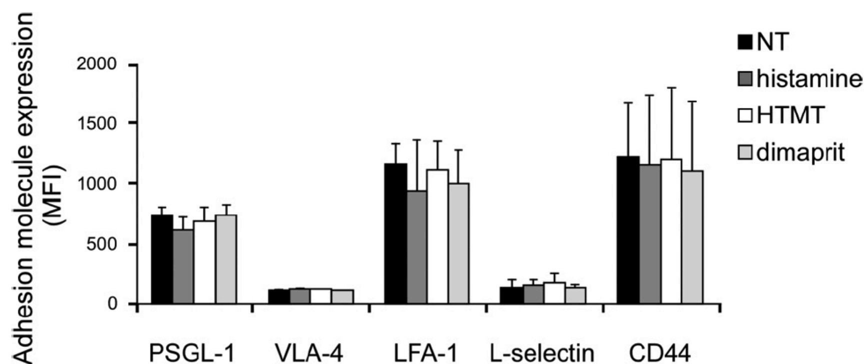


Figure 5. Flow cytometry analysis of the expression of adhesion molecules on myelin-autoreactive T cells upon treatment with histamine, H1R agonist HTMT, and H2R agonist dimaprit. T lymphocytes of a TCL activated against $PLP_{139–151}$ were treated with histamine, HTMT, or dimaprit (all used at concentrations of 10^{-5}) or left untreated for 2 h before labelling with adhesion molecule-specific fluorescent antibodies. Data represent mean fluorescence intensity (MFI) ± SEM from three experiments.

Discussion

The signaling of histamine occurs through each of its four known receptors, and each of these receptors might exert a variety of different effects within the immune response leading to brain inflammation and demyelination of EAE. In this study, we explored the ability of histamine, H1R agonist HTMT, and H2R agonist dimaprit to modulate two important steps of the autoimmune attack leading to EAE: T cell activation and adhesiveness to the inflamed microcirculation, an event that precedes T cell penetration in the brain parenchyma. Our results show that histamine in vitro inhibits antigen-induced proliferation and IFN-γ cytokine secretion of T cells activated against myelin. These effects were induced by HTMT and dimaprit, suggesting that the inhibitory effects induced by histamine on T cell proliferation and cytokine production are associated with histamine triggering of H1R or H2R. Our results were also corroborated by molecular analyses of intracellular pathways involved in differentiation, proliferation, and cell cycle control: ERK1/2 phosphorylation was reduced by histamine treatment, and p27Kip-1 expression increased, suggesting that histamine functionally modulates the activation state and responsiveness of myelin-activated T cells. Our data also indicate that histamine, through H1R and H2R, blocks spontaneous and chemokine-induced adhesion of myelin-autoreactive T cells to mouse ICAM-1 in vitro and reduces firm arrest of these cells to inflamed brain circulation in an in vivo model of early EAE inflammation. Although neither histamine nor its agonists modulated the expression of adhesion molecules on myelin-

autoreactive T cells, HTMT inhibited LFA-1 distribution in clusters and caps, an important mechanism by which histamine might modulate the ability of T cells to adhere to inflamed brain vessels.

Our observation of a reduction of IFN-γ production by LNCs and $CD3^+$ T cells induced by histamine is consistent with the results of the work of Osna and colleagues [10], obtained on mouse splenocytes and Th1 cells with histamine and histamine antagonists, and with those of Sonobe and colleagues [37], obtained on splenocytes from H1R- and H2R- deficient mice. However, in apparent contrast with our data, recent work obtained in H1R-deficient mice has shown that H1R is important for optimal IFN-γ production by $CD4^+$ T cells [18]. It must be noted that in our experimental setting, we explored the effects of histamine and its H1R and H2R agonists on $CD3^+$ T cells purified from mice with EAE and thus activated in vivo with $PLP_{139–151}$, and the work by Noubade and colleagues [18] has been conducted on $CD4^+$ T cells purified from nonimmunized, H1R-deficient mice and activated polyclonally in vitro with the anti-CD3/28 antibody. It is possible that histamine has different effects on in vitro versus in vivo activated T cells. Also, it has to be considered that histamine can affect IFN-γ production by $CD8^+$ T cells [37], so that in our model the effects of histamine on $CD8^+$ T cells might have contributed to the reduced production of IFN-γ that we observed on $CD3^+$ T cells. Last, as discussed by the authors themselves in the work by Noubade et al. [18], it cannot be ruled out that in H1R-deficient mice, differences in H2R (and H4R) expression caused by loss of H1R-dependent cross-regulation may also influence IFN-γ production.

A role for histamine in the adhesion of leukocytes during

inflammation and recruitment of immune cells into inflamed tissues has been suggested previously. Indeed, histamine can up-regulate the expression of vascular endothelial cell-associated molecules that promote trafficking of leukocytes through postcapillary venules [38, 39], an effect probably associated with the ability of this mediator to activate NF-kB through H1R (reviewed in ref. [40]). At the BBB level, histamine can increase vascular permeability by having direct effects on tight junctions (reviewed in ref. [41]). However, in an air pouch-type allergic inflammation model, histamine has been shown to down-regulate leukocyte infiltration through H2R [42], suggesting that depending on the specific receptor engaged, histamine might provide fine-tuning of leukocyte traffic through endothelial cells. The results shown here reveal an inhibitory effect of histamine, HTMT, and dimaprit on the adherence ability of myelin-autoreactive T cells on endothelial cells in vitro and in an in vivo model of EAE early brain inflammation. These effects did not appear to be associated with an effect of histamine on the expression of adhesion molecules on T cells. Instead, we observed that the H1R agonist HTMT reduced LFA-1 distribution significantly in clusters and caps (big polar patches) on activated T cells (Fig. 4), an event that facilitates cell–cell contacts and adhesive interactions, which are required for efficient migration through the endothelium [36]. Integrin distribution in clusters indicates increased lateral mobility and higher binding avidity [35, 36], and it has been shown recently that integrin clusters are associated with integrin-increased affinity, further emphasizing the importance of adhesion molecule distribution on cell surface [43]. Also, the distribution of adhesion molecules in larger areas

(caps/clusters vs. round distribution) allows a higher outside-in signaling and a higher stabilization of cell adhesion required for adhesion strengthening during the migration through the endothelium [44]. Thus, the effects of H1R engagement on integrin activation and distribution on T cells might represent an important mechanism by which histamine modulates myelin-activated T cell adhesiveness in inflamed brain vessels. Further studies are required to explore the effects of HR-generated signaling on integrin affinity, another modality of integrin activation. Supporting our results, it has been shown previously that H2R signals through a PKA-dependent signaling pathway [45], whereas H1R signals through PKC as well as PKA in certain experimental conditions [46, 47]. Indeed, cAMP and PKA activation are able to block integrin activation and leukocyte adhesion through blockade of RhoA small G proteins [48], thus supporting the hypothesis that H1R activation can interfere with the signaling machinery controlling integrin activation and T cell migration into the brain.

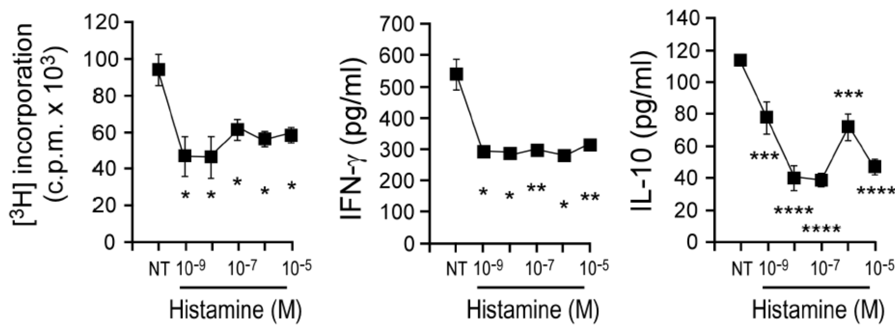
The results presented here have been obtained from in vitro studies and from an in vivo model of early brain inflammation and might help explain the exacerbation of EAE observed in vivo in histamine-deficient $HDC^{-/-}$ mice, including some of the features of the disease expressed by these mice, such as increased T cell production of proinflammatory cytokines and more diffuse inflammatory CNS infiltrates [22]. In this regard, it must be considered that within the four known receptors for histamine, we focused our study on the effects of H1R and H2R on myelin-autoreactive T cells because of the in vivo evidences supporting a role for these two receptors in EAE

and because these receptors are expressed on T cells. Other receptors for histamine are known or are likely to play roles in EAE. Indeed, disruption of H3R, which is expressed on neural cells but not normally on hematopoietic cells [49, 50], leads to exacerbation of EAE associated with dysregulation of the BBB permeability [50], and the role in this disease of H4R, which is expressed on a variety of immune cell types and has been shown recently to be involved in Th2 polarization [51, 52], still needs to be investigated.

In conclusion, although the role of histamine in a disease as complex as EAE, let alone human MS, requires further investigation, our study confirms an important role for this mediator in the activation of myelin-autoreactive T cells and reveals for the first time an important role for histamine in the regulation of the ability of these autoreactive cells to adhere to the inflamed brain endothelium.

Supplementary Information

A



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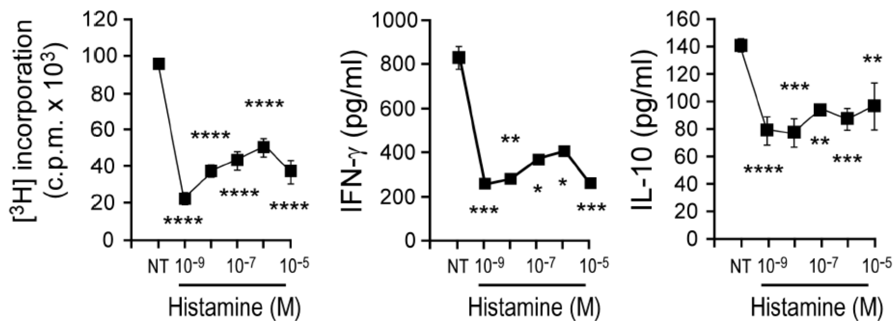
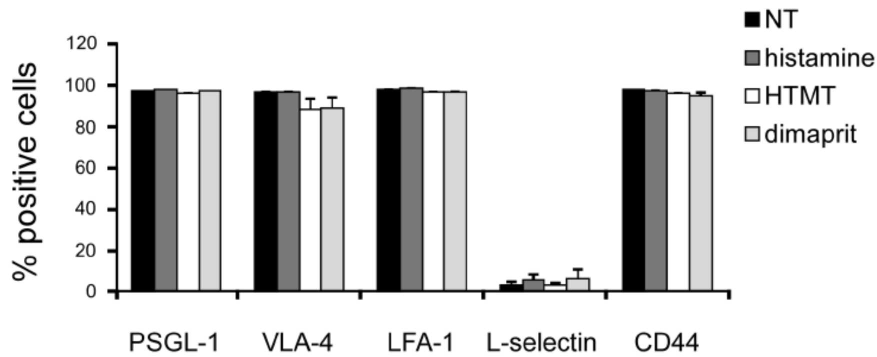


Supplementary Figure 1. Effects of histamine on proliferation and cytokine production of LNCs obtained from mice with $PLP_{139\text{-}151}$-induced EAE at later stages of disease. LNCs obtained from mice 18 days (A) and 24 days (B) after the induction of EAE (n=3-5 mice) were pooled and treated with increasing concentrations of histamine for 2 h before *in vitro* stimulation with $PLP_{139\text{-}151}$ (20 µg/ml). Proliferation and cytokine production were measured as described in Methods. Data are shown as mean ± SEM. **P* <0.05, ***P* < 0.01, ****P*<0.005 and *****P*<0.0001 *vs* untreated (NT) cells. Proliferation of unstimulated (= no antigen) cells was always below 2,500 cpm and was not modified by any of the pharmacological treatments used (data not shown).



Supplementary Figure 2. Flow cytometry analysis of the expression of adhesion molecules on myelin autoreactive T cells upon treatment with histamine, H1R agonist HTMT and H2R agonist dimaprit. T lymphocytes of a TCL activated against $PLP_{139.151}$ were treated with histamine, HTMT, dimaprit (all used at concentrations of 10^{-5}), or left untreated (NT) for 2 h before labelling with adhesion molecule-specific fluorescent antibodies. Data represent percentage positive cells ± SEM from 3 experiments.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

Most of the information about the role of MCs in EAE so far came from the work of Brown's group, which showed decreased EAE severity in *Kit*$^{W/W-v}$ mutant mice [1, 2]. In their experimental setting, MC systemic reconstitution restored disease severity by recovering an efficient encephalitogenic T-cell response in peripheral lymphoid organs and by restoring neutrophil penetration at the blood–brain barrier [3, 4]. We here report that *Kit*$^{W/W-v}$ mice are differentially susceptible to EAE depending on the strength of the immunization protocol. Conversely, *Kit*$^{W-sh/W-sh}$ mice develop anticipated and exacerbated EAE, either passively or actively induced, under all the tested conditions of immunization. Our results reconcile, in part, the apparently contrasting findings in the two MC-deficient strains. Indeed, we could reproduce Brown's data in the *Kit*$^{W/W-v}$ model, by applying a strong immunization protocol, but under milder conditions, which are our standard procedure to induce active EAE, *Kit*$^{W/W-v}$ mice developed an exacerbated rather than an attenuated disease, replicating the results that we obtained in the *Kit*$^{W-sh/W-sh}$ model. It is possible that the opposite outcomes observed in *Kit*$^{W/W-v}$ strain under different immunization protocols may be related to a diverse impact of the same mutation on distinct pathological mechanisms depending on the quality/quantity of the immune stimulation and on the experimental conditions. Nonetheless a recent report from the same group showed decreased EAE symptoms in *Kit*$^{W/W-v}$ mice

compared with $Kit^{+/+}$ mice also on the application of a relatively milder immunization protocol (100 μg of $MOG_{35–55}$ and 250 ng of PTX) [3]. Thus, the discrepancies between our results and those reported by the Brown's group remain to be fully understood.

Furthermore, different mutations in the same gene/locus can lead to contrasting outcomes under the same conditions of stimulation. Indeed, high doses of $MOG_{35–55}$, CFA and PTX produced a milder outcome in $Kit^{W/W-v}$ mice, but a more severe disease in $Kit^{W-sh/W-sh}$ mice if compared to $Kit^{+/+}$ controls. The reasons for this discrepancy may rely on differences between strains regarding the genetic background or also c-Kit-related abnormalities independent of MCs. The two MC-deficient strains differ in their genetic background, and the WBB6F1-$Kit^{+/+}$ is characterized by higher number of peritoneal MCs and neutrophils than the C57BL/6-$Kit^{+/+}$ [5]. Also, it has been shown that $Kit^{W/W-v}$, but not $Kit^{W-sh/W-sh}$ mice are resistant to antibody-induced arthritis because the former is neutropenic, whereas the latter shows splenic myeloid hyperplasia, both at basal level and after immunization [6, 7]. Depletion of $Gr1^{+}$ cells showed a more relevant role for granulocytes, rather than MCs, in the pathogenic mechanisms driving arthritis in this model [7].

As altered homeostasis of granulocytes has been reported in $Kit^{W-sh/W-sh}$ mice [8] and these cells are involved in EAE pathogenesis [9], we characterized the contribution of granulocytes to the clinical and immunological outcome obtained in $Kit^{W-sh/W-sh}$ mice affected by EAE. Confirming previous results, we found an expanded myeloid compartment in the spleens of $Kit^{W-sh/W-sh}$ mice, involving both the $Gr1^{hi}CD11b^{hi}$ and the $Gr1^{int}$ $CD11b^{int}$ subsets [8]. However,

our data also suggest that granulocyte abnormalities detected in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice may be ascribed to granulocyte-extrinsic MC-deficiency-dependent defects, as we could rescue a normal myeloid compartment in the spleens following systemic MC-reconstitution of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. On the contrary, in the *Kit*$^{W/W\text{-}v}$ model, the failure to correct peripheral neutropenia by MC reconstitution suggests a neutrophil-intrinsic role of the *Kit*$^{W/W\text{-}v}$ mutation in shaping the granulocyte compartment [3]. It could be proposed that in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice MCs may actively inhibit granulopoiesis by unknown mechanisms, or passively limit excessive myeloid expansion simply by sequestering the majority of locally available, paracrine or juxtacrine, SCF through the highly expressed c-Kit receptor. Notably, the granulocyte alterations carried by *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were not causally related to the enhanced EAE clinical course, as granulocyte depletion could not abate the difference between *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice in terms of clinical disease expression, despite it dramatically reduced encephalitogenic T-cell responses at the same extent in both groups compared with untreated controls.

We observed an increase of MCs infiltrating draining lymph nodes (DLNs) of wild-type C57BL/6-*Kit*$^{+/+}$ mice following immunization with $MOG_{35\text{-}55}$. MCs displayed a preferential distribution in T-cell perifollicular areas of the lymph node cortex, with a certain degree of clustering. Also, some of us have previously shown that MCs co-localize with Th17 and Treg cells in DLNs of $MOG_{35\text{–}55}$-immunized mice, thus potentially engaging multiple interactions with these cells and participating to adaptive immune response [10]. We could detect enhanced T-cell proliferation, increased secretion of pro-

inflammatory cytokines and decreased IL-10 amount in draining lymph nodes of MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice at d10 after immunization, in line with other reports [11, 12]. The frequency of Th17 and Th1 cells was also higher in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and it may be related to the fact that MCs may exert some influence on T cell differentiation by releasing a variety of cytokines in response to adjuvants on immunization [13].

Many data suggest that MCs can engage multiple interactions with Treg [10, 14, 15]. In this study, we found a defect in Treg frequency in lymphoid organs of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice before and during EAE development, at both acute and chronic phases. The reasons for impaired Treg homeostasis in naïve *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice remain presently obscure. Notably, MC-reconstitution restored a physiological Treg frequency in LN of naïve Kit mutant mice. This observation points to a Treg-extrinsic role of this c-Kit mutation in shaping Treg compartment and suggests that MCs may not only mould Treg plasticity but also support Treg homeostasis. The OX40–OX40L axis may be involved in a defective Treg expansion in MC-deficient mice. Indeed, MCs constitutively express OX40L and OX40 is a key signal in supporting Treg fitness especially under inflammatory conditions [14, 16]. Treg are important suppressor cells in both the initiation and the recovery phase of EAE [17], therefore the enhanced EAE disease course in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice could be associated to the decreased Treg accumulation in peripheral lymphoid organs or CNS.

To confirm that the increased CNS autoimmune response observed in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice was due to MCs we evaluated EAE in MC-reconstituted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice but we were not able to restore

disease susceptibility to wild-type levels. Several reasons could account for this result. We performed MC-reconstitution by intravenous injection of 10^7 MCs into 4 weeks old-mice mice and started EAE experiments after 6-8 weeks after transplantation. The time between MC-reconstitution and the initiation of EAE is a standard period required for *in vitro*-derived MCs (BMMCs) to acquire a phenotype (in terms of morphology and granule content) similar to MCs found in the wild-type tissue [18, Wolters, 2005 #267]. Systemic intravenous injection produced MC accumulation but altered distribution in blood-draining organs with only a modest repopulation at priming sites, i.e., the inguinal and axillary lymph nodes, and in the neighbouring skin, according to previous data [19, 20]. In particular, the spleen has been recognized to trap most of i.v.-injected MCs, thus inhibiting their spreading to other tissues [20]. Thus, intravenous injection of BMMCs may inefficiently reproduce the topography and the quantity of MCs observed in lymphoid organs of $Kit^{+/+}$ mice in physiological conditions. Nonetheless, we could detect the rescue of some MC-related biological functions, such as the recovery of normal frequencies of Treg and granulocytes in lymph nodes and spleens, respectively, of reconstituted mice. It is possible that MCs are not responsible for the exacerbated CNS autoimmune response observed in $Kit^{W\text{-}sh/W\text{-}sh}$ mice. However we cannot rule out that the altered MC number and distribution at priming sites, especially at draining lymph nodes, in reconstituted mice might have failed in promoting a functional recover of Treg or other MC-related immune suppressive properties, ultimately leading to a clinical course of EAE similar to the one displayed by not- reconstituted $Kit^{W\text{-}sh/W\text{-}sh}$ mice.

Also, according to previous results in the *Kit*$^{W/W-v}$ strain, intravenous transplantation of BMMCs failed to repopulate the CNS tissue, therefore this experimental setting was not suitable to ascertain whether MCs exerted a regulatory role directly in the inflamed milieu of the CNS [4, 19]. In *Kit*$^{+/+}$ mice we detected scattered MCs in the context of the reticulum formed by the arachnoid, the subdural neurothelium, and the Dura mater. Located in close proximity with the BBB, MCs represent early responders to cerebral ischemia and potent inflammatory effector cells [21]. MCs may even modulate tumor metastatisation into the brain [22]. In *Kit*$^{W/W-v}$, meningeal MCs promote a breach in the BBB to allow the entry of inflammatory cells [3]. Overall these functions imply a role for MCs in increasing, rather than regulating, the permeability of blood-brain barrier (BBB). On the contrary, we found here an enhanced immune cell-infiltration in MC-deficient *Kit*$^{W-sh/W-sh}$ mice, especially granulocytes. We also showed that passive transfer of myelin-activated T cells resulted in earlier EAE onset in *Kit*$^{W-sh/W-sh}$ mice if compared to controls, similarly to what observed in the active EAE setting. These data suggest that MCs could exert some immune-modulatory functions also in the CNS and may reduce the access of immune cells to the CNS. Remarkably, we found earlier EAE onset and more granulocytes infiltrating the CNS also in histidine decarboxylase (HDC)$^{-/-}$ mice, carrying histamine deficiency and MC paucity [28]. Interestingly histamine, one of the main MC-mediators, has been shown to reduce BBB permeability by stimulating histamine receptor 3 (H3R) on brain presynaptic neurons and histamine receptor 1 (H1R) on brain endothelial cells [23, 24].

Of note, few months after publication of our paper on

MCs and EAE, another report came out showing that *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice developed exacerbated EAE, displaying reduced Treg infiltration in the CNS and enhanced pro-inflammatory response, in line with our data [12]. In this work the authors were able to revert the increased EAE of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice to wild-type levels by MC-reconstitution. However they performed an alternative MC-reconstitution experiment, by injecting MCs into *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice 9 days after immunization of mice, and not 6-8 weeks before the initiation of the MC-knockin experiment, as we did. BMMCs-transplanted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice displayed EAE severity, frequency of Treg in the CNS and peripheral myelin-specific immune response comparable to wild-type mice. Remarkably in this MC-knock-in setting, MCs were found also in the CNS of BMMCs transplanted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice [12].

Indirect support to our results may come from other mutant mice carrying quantitative or qualitative defects in the MC population. Some of us have recently reported a more severe EAE and increased T cell production of pro-inflammatory cytokines in histidine decarboxylase (HDC)$^{-/-}$ mice, which are not only genetically unable to produce histamine, but also show paucity in MC number and abnormalities in MC cytoplasmic granules [25, 26]. Histamine is one of the main preformed mediators stored in MC granules and it is possible that *Kit*$^{W\text{-}sh/W\text{-}sh}$ and HDC$^{-/-}$ mice (both on a C57BL/6N background) share phenotypic defects depending on MC ablation and/or an alteration of histamine signalling.

The signalling of histamine occurs through each of its four known receptors. In the second paper here reported, we outlined that histamine, H1R agonist HTMT, and H2R agonist dimaprit

modulate two important steps of the autoimmune attack leading to EAE: T cell activation and adhesiveness to the inflamed brain microcirculation. Indeed our results show that histamine *in vitro* inhibits antigen-induced proliferation and IFN-γ cytokine secretion of T cells activated against myelin. These results were also corroborated by molecular analyses of intracellular pathways involved in differentiation, proliferation, and cell cycle control: ERK1/2 phosphorylation was reduced by histamine treatment, and p27Kip-1 expression increased, suggesting that histamine functionally modulates the activation state and responsiveness of myelin-activated T cells. Our data also indicate that histamine, through H1R and H2R, blocks spontaneous and chemokine-induced adhesion of myelin-autoreactive T cells to mouse ICAM-1 *in vitro* and reduces firm arrest of these cells to inflamed brain circulation in an *in vivo* model of early EAE inflammation.

Our observation of a reduction of IFN-γ production by LNCs and $CD3^+$ T cells induced by histamine is consistent with the results of the work of Osna and colleagues, obtained on mouse splenocytes and Th1 cells with histamine and histamine antagonists, and with those of Sonobe and colleagues, obtained on splenocytes from H1R- and H2R-deficient mice [27, 28]. However, in apparent contrast with our data, recent work obtained in H1R-deficient mice has shown that H1R is important for optimal IFN-γ production by $CD4^+$ T cells [29]. It must be noted that in our experimental setting, we explored the effects of histamine and its H1R and H2R agonists on $CD3^+$ T cells purified from mice with EAE and thus activated *in vivo* with the myelin peptide $PLP_{139-151}$, and the work by Noubade and colleagues has

been performed on $CD4^+$ T cells purified from non-immunized, H1R-deficient mice and activated polyclonally *in vitro* with the anti-CD3/28 antibody [29]. It is possible that histamine has different effects on *in vitro* versus *in vivo* activated T cells. Also, histamine can affect IFN-γ production by $CD8^+$ T cells, so that in our model the effects of histamine on $CD8^+$ T cells might have contributed to the reduced production of IFN-γ that we observed on $CD3^+$ T cells [28]. Last, as discussed by the authors themselves in the work by Noubade et al., it cannot be ruled out that in H1R-deficient mice, differences in H2R and H4R expression caused by loss of H1R-dependent cross-regulation may also influence IFN-γ production [29].

A role for histamine in the adhesion of leukocytes during inflammation and recruitment of immune cells into inflamed tissues has been suggested previously. Indeed, histamine can upregulate the expression of vascular endothelial cell-associated molecules that promote trafficking of leukocytes through postcapillary venules [30, 31]. At the BBB level, histamine can increase vascular permeability by having direct effects on tight junctions (reviewed in ref. [32]). However, in an air pouch-type allergic inflammation model, histamine has been shown to downregulate leukocyte infiltration [33]. The results shown in our paper reveal an inhibitory effect of histamine, HTMT, and dimaprit on the adherence ability of myelin-autoreactive T cells on endothelial cells *in vitro* and in an *in vivo* model of EAE early brain inflammation. Although neither histamine nor its agonists modulated the expression of adhesion molecules on myelin-autoreactive T cells, HTMT inhibited LFA-1 distribution in clusters and caps. Integrin distribution in clusters indicates increased

lateral mobility and higher binding avidity, and it has been shown recently that integrin clusters are associated with integrin-increased affinity, further emphasizing the importance of adhesion molecule distribution on cell surface [34-36]. Also, the distribution of adhesion molecules in larger areas (caps/clusters vs. round distribution) allows a higher outside-in signalling and a higher stabilization of cell adhesion required for adhesion strengthening during the migration through the endothelium [37]. Thus, the effects of H1R engagement on integrin activation and distribution on T cells might represent an important mechanism by which histamine modulates myelin-activated T cell adhesiveness in inflamed brain vessels. Of note, mice overexpressing H1R only on endothelial cells but deficient for H1R in all other cell types (H1RKO-vWF^{H1R} Tg mice), display reduced BBB permeability and are protected from EAE [23].

FUTURE PERSPECTIVES

Our findings underscore the need for clear and doubtless identification of the role of MCs in several pathological processes by taking advantage of different c-Kit mutants and, if possible, of different experimental models.

Both naïve and MOG_{35-55}-immunized *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice have reduced Treg frequency in lymph nodes, which is restored following MC-reconstitution. This evidence points to a potential cross-talk between MCs and Treg. This relationship has been in part investigated *in vivo* in a model of skin allograft acceptance. In this setting it has been shown that Treg-secreted IL-9 recruits MCs to the transplanted tissue, promoting allograft acceptance [15]. However if and how MCs signal to Treg is still not well understood. MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice mice and mice unable to synthesize histamine ($HDC^{-/-}$), which also display MCs paucity, develop a more severe EAE than wild-type mice and an increased pro-inflammatory response against myelin antigen [25, 26]. Interestingly a recent report has highlighted that histamine contributes to Treg homeostasis by binding H4R [38]. Indeed mice deficient for H4R show reduced Treg frequency in peripheral lymphoid organs and exacerbated EAE [38]. These findings suggest that MCs and histamine, one of the main preformed mediators stored in MC granules, may synergistically contribute to shape the homeostasis and function of Treg and dampen detrimental CNS autoimmune responses. Thus, future work should be undertaken to understand if and how MCs and histamine might influence Treg

functions in the context of the autoimmune processes leading to EAE.

Furthermore, in our study we observed that *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice develop earlier and more severe active EAE in comparison to wild-type mice, associated to an increased immune cell-infiltration in the CNS. Also, when we bypassed the induction phase of EAE by passively transferring myelin-activated T cells, we obtained again an anticipated EAE onset in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. This observation suggests a potential role for MCs in reducing immune cell infiltration and/or re-activation in the CNS. Interestingly histamine has been shown to reduce BBB permeability of the CNS during EAE, by interacting with H1R on brain endothelial cells and with H3R on presynaptic neurons [23, 24]. Thus, further experiments would be helpful to unravel the true role of MCs and MC-secreted histamine in promoting or regulating the access of immune cells to the inflamed CNS.

The comprehension of how MCs and histamine regulate Treg function and BBB permeability will contribute to better understand how it is possible to intervene on these cells and mediator to ameliorate mouse models of MS.

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Histamine regulates autoreactive T cell activation and adhesiveness in inflamed brain microcirculation. Lapilla M, Gallo B, Martinello M, Procaccini C, **Costanza M**, Musio S, Rossi B, Angiari S, Farina C, Steinman L, Matarese G, Constantin G, Pedotti R. *Journal of Leukocyte Biology* 2011 Feb;89(2):259-67

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