Published in final edited form as:
J Neuroimmunol. 2008 October 15; 203(1): 23–32. doi:10.1016/j.jneuroim.2008.06.034.

MHC class II exacerbates demyelination *in vivo* independently of T cells

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Abstract

We have shown previously the importance of MHC class II for central nervous system remyelination; however, the function of MHC class II during cuprizone-induced demyelination has not been examined. Here, we show that *I-A*$_{\beta}^{-/-}$ mice exhibit significantly reduced inflammation and demyelination. *RAG-1*$^{-/-}$ mice are indistinguishable from controls, indicating T cells may not play a role. The role of MHC class II depends on an intact cytoplasmic tail that leads to the production of IL-1β, TNF-α, and nitric oxide, and oligodendrocyte apoptosis. Thus, the function of MHC class II cytoplasmic tail appears to increase microglial proliferation and activation that exacerbates demyelination.

1. Introduction

The Major Histocompatibility Complex class II (MHC II) molecules present antigenic peptides to CD4^{+} T cells through interactions with both the T cell receptor (TCR) and the CD4 molecule (Doyle and Strominger, 1987; Germain, 1994; Jones et al., 2006; Konig et al., 1992; Konig et al., 1995). Enhancement of MHC class II has been found to be important for the education of CD4^{+} T cells and T cell-mediated immune responses, while the elimination of MHC II is frequently associated with the ablation of such responses (Grusby and Glimcher, 1995). Previous *in vivo* studies have primarily focused on the effects of MHC II antigens as ligands for the TCR and not as a receptor for signaling cell activation (Thomson, 1995; Zamvil et al., 1985).

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MHC II molecules have been observed in mouse brains (Hauser et al., 1983; Hickey and Kimura, 1988; Ting et al., 1981; Wong et al., 1984) and in a number of neurological disorders (Hickey, 1991; Mattiace et al., 1990; McGeer et al., 1988a; McGeer et al., 1988b). In diseases involving viral infections or experimental autoimmune encephalitis (EAE), where T cell involvement is prominent, the function of MHC II is primarily that of antigen presentation by microglia/macrophages (Fritz and McFarlin, 1989; Zamvil and Steinman, 1990). Unlike normal mouse brain, there is little to no expression of MHC II in healthy human brain (Kreutzberg, 1996); yet in neurological disorders, such as Alzheimer's disease, Huntington's disease, and Parkinson's disease, MHC II^+ microglial/macrophage cells are present. Interestingly, T cells do no appear to be involved in these CNS disorders (Hickey et al., 1991; Mattiace et al., 1990; McGeer et al., 1988a; McGeer et al., 1988b; Zamvil et al., 1985). In addition, even in MS lesions, microglia/macrophages have been found in the absence of significant T cell infiltration (Barnett et al., 2006; Li et al., 1993; Prineas, 2002; Raine, 1994). Thus, the function of MHC II and the significance of T cells in these diseases are important issues that need to be clarified.

We previously used *I-*$A_\beta^{-/-}$ *twitcher* mice, a murine model for human globoid cell leukodystrophy or Krabbe's disease, to show that the absence of MHC II on microglia/macrophages reduces CNS demyelination. In the wild type (*I-*$A_\beta^{+/+}$) *twitcher* mice, demyelination is accompanied by increases in microglia/macrophages with few detectable T cell infiltrates (Matsushima et al., 1994). Due to the near absence of T cells in the demyelinating brain of these mice, it is plausible that MHC II may not be functioning in antigen presentation. An alternative role for MHC II is in signal transduction. There are ample reports that show the triggering of MHC II on T cells, B cells, and monocytes leads to results in intracellular signaling, activation, differentiation, and/or the production of proinflammatory cytokines (Andre et al., 1994; Bishop and Haughton, 1986; Cambier et al., 1987; Espel et al., 1996; Fast et al., 1989; Harton and Bishop, 1993; Mehindate et al., 1996; Nabavi et al., 1992; Nabavi et al., 1989; Odum et al., 1991; Trede et al., 1991; Wade et al., 1989). However, all of these studies were performed *in vitro* with cultured cells, and the *in vivo* physiological significance is less clear.

To further investigate the role of MHC II on microglia/macrophages during CNS disease, we used cuprizone intoxication, a copper-chelating neurotoxin, to induce demyelination (Blakemore, 1972; Blakemore, 1973; Ludwin, 1978; Suzuki and Kikkawa, 1969). We have previously shown that C57BL/6 mice that were exposed to a cuprizone-supplemented diet resulted in a predictable course of demyelination and pathology (Hiremath et al., 1998; Morell et al., 1998). During cuprizone intoxication, the perturbation of oligodendrocytes is evident by the dramatic inhibition of transcripts for myelin-basic protein (MBP), myelin-associated glycoproteins (MAG), and ceramide galactosyl transferase (Morell et al., 1998). Subsequently, the GST-pi mature oligodendrocytes are depleted from the demyelinating lesion by apoptosis (Mason et al., 2000). Cuprizone-induced demyelination is similar to the *twitcher* model in that microglia/macrophage accumulate at the site of demyelination (Hiremath et al., 1998; Morell et al., 1998). However, in contrast to the *twitcher* mouse model, cuprizone-induced demyelination is characterized by the accumulation of resident microglia with very few (< 4%) peripheral macrophages (McMahon et al., 2002; Yagi et al., 2004). Moreover, this is without obvious T cell accumulation presumably due to the intactness of the blood-brain-barrier (Bakker and Ludwin, 1987; Kondo et al., 1987). The cuprizone intoxication model can be conveniently studied for mutant mice on the C57BL/6 background (Hiremath et al., 1998).

The current report shows cuprizone-induced demyelination and pathology in *I-*$A_\beta^{-/-}$ mice (Grusby et al., 1991) demonstrated a significant decrease in acute demyelination and pathology compared to wild type mice. Interestingly, mice with a truncated *I-*A_β (*I-*A_β^{tr})

which have intact extracellular and transmembrane sequences but lacks the cytoplasmic domain (Smiley et al., 1995; Smiley et al., 1996) exhibited similar responses as *I-A*$_{\beta}^{-/-}$ mice. Furthermore, *I-A*$_{\beta}^{-/-}$ and *I-A*$_{\beta}^{tr}$ mice had fewer proliferating microglia/macrophages and reduced amounts of TNF-α, IL-1β, and nitric oxide in the brain compared to wild type mice, suggesting a role for MHC II in the activation of microglia/macrophages. Additionally, demyelination and pathology studies from cuprizone-treated recombinase activating gene-1 knockout (*RAG-1*$^{-/-}$) mice show that the role of MHC II is *likely* independent of T cells. The data provide *in vivo* evidence for a role for MHC II molecules other than antigen presentation to T cells.

2. Materials and Methods

Mice and tissue preparation

C57BL/6J, *I-A*$_{\beta}^{-/-}$, *I-A*$_{\beta}^{tr}$, and *RAG-1*$^{-/-}$ male mice at five to six weeks old were either purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) or bred and maintained in our pathogen-free animal facility at UNC. Only eight to ten weeks old age-matched animals were used in an experiment. Cuprizone was mechanically mixed into ground Breeder chow 2000 (Purina, Richmond, IN) to make a 0.2% mixture by a weight-to-weight ratio and mice were treated as previously described (Hiremath et al., 1998). Mouse brains were harvested at appropriate time points and processed for paraffin sections as previously described (Hiremath et al., 1998). Briefly, paraffin-embedded tissues were sectioned at 5 μm in thickness at the level of section 251 according to Sidman's mouse atlas (Sidman et al., 1971). For frozen sectioning, brains were harvested and frozen in an isopentane freezing bath. They were then sectioned by a cryostat at 5 μm in thickness at the level of section 241-251 according to Sidman's mouse atlas (Sidman et al., 1971).

Evaluation of demyelination

For routine histology, the sections were stained with Luxol fast blue-periodic acid Schiff (LFB-PAS) stain and demyelination was evaluated as previously described (Hiremath et al., 1998). Briefly, three independent readers in a double-blind fashion scored LFB-PAS-stained sections from zero to three. Zero was equivalent to the myelin status of a mouse not treated with cuprizone, whereas a score of three was total demyelination usually observed at six weeks of treatment with cuprizone. In addition, cellularity was scored on a scale of zero to three in these samples as an estimate of the number of nuclei-containing cells infiltrating demyelinating regions. Zero represented few nuclei as in the untreated animal and three represented the heavy accumulation of nuclei in the myelin tract following complete demyelination.

Immunohistochemical staining

MHC II staining was performed according to manufacturer's protocol (Serotec, Raleigh, NC). Briefly, 5 μm thick fresh frozen sections from brains of mice exposed to cuprizone for 3 weeks were post-fixed with acetone. Sections were treated with 0.3% peroxide for 5 min at 4° C to eliminate endogenous peroxidase activity. Sections were then treated with primary or isotype control antibody overnight at 4°C, followed by FITC-conjugated secondary antibody for 2 hrs at room temperature. To verify the degree of microglial/macrophage response, paraffin sections of the cerebrum were processed for immunocytochemical detection of microglia/macrophages with a lectin RCA-1 (Sigma, St. Louis, MO), using the Autoprobe III kit with streptavidin-conjugated peroxidase base (Biomed Co., Foster City, CA) as previously described (Hiremath et al., 1998). All scores and quantitation of cells in each coronal section of the corpus callosum were typically conducted at two sites, immediately lateral of midline (see labeled box in Figure 1).

GST-pi staining for mature oligodendrocytes was performed on paraffin sections. Sections were deparaffinized, permeabilized with 0.1% Triton-X-100, and unmasked with 1 mg/ml trypsin in 0.05M HCl solution at 37° for 15 minutes. Sections were then blocked and treated with a 1:1000 dilution of rabbit anti-pi GST (Biotrin International Inc., Newton, MA) overnight at 4° C. Sections were then washed with PBS and treated with FITC conjugated anti-rabbit antibody (Vector Laboratories) for 30 minutes at room temperature. Sections were washed with PBS and mounted with Vectashield (Vector Laboratories) prior to analysis.

To determine the number of proliferating microglia/macrophages, bromodeoxyuridine (BrdU) incorporation in microglia/macrophages was monitored by immunohistochemical colocalization. BrdU was injected intraperitoneally three times daily 2 days and 1 day prior to harvest, and once on the day of harvest as previously described (Taniike and Suzuki, 1995). Detection of BrdU was performed according to the manufacturer's instructions (Zymed, San Francisco, CA). Percentage indicated is the number of double-labeled BrdU and RCA-1^+ cells was divided by the number of RCA-1^+ microglia/macrophages.

Anti-nitrotyrosine staining was performed on paraffin sections. Sections were deparaffinized and treated with the sodium citrate unmasking solution from Vector Laboratories (Burlingame, CA). Sections were then blocked and incubated overnight at 4°C with 1:100 anti-nitrotyrosine antibody (Upstate Biotechnologies, Lake Placid, NY). Sections were then washed with PBS and treated with 1:100 anti-rabbit biotinylated antibody (Vector Laboratories) for 2 hours at room temperature. Sections were washed, treated with avidin-FITC (Vector Laboratories) for 30 min at room temperature, and washed prior to analysis.

Morphometric and immunohistochemical quantification of tissue

The immunohistochemical-stained sections were used for the quantitative analysis of cells accumulating the corpus callosum during demyelination as previously described (Hiremath et al., 1998). Briefly, individual cells were counted based on nuclei in the corpus callosum using a morphometry system based on a Nikon FXA microscope (Garden City, NY) equipped with an Optronics TEC-470 CCD Video Camera System (Goleta, CA). Image processing, analyses, and measurements were carried out using the public domain NIH image program (http://rsb.info.nih.gov/nih-image/). The counted cells or stained tissue area for nitrotyrosine staining from *I-A*$_\beta^{-/-}$ and *I-A*$_\beta^{tr}$ cuprizone-treated animals were compared to wild type cuprizone-treated animals.

Brain protein extracts and quantitation of cytokines

Brains from cuprizone treated mice were harvested and sectioned to isolate the region containing the corpus callosum using a brain block. This brain section was frozen in liquid nitrogen and homogenized with a tissue homogenizer in a PBS/protease inhibitor cocktail. The homogenate was spun at maximum speed in a microfuge at 4°C and the supernatant was analyzed for presence of IL-1β and TNF-α by a standard sandwich EIA.

Statistical Analyses

All statistical analyses were conducted by one-way analysis of variance (ANOVA) test followed by the appropriate multiple comparisons test when appropriate.

3. Results

MHC II expression increases with demyelination

Brains from cuprizone-treated C57BL/6 mice were routinely analyzed for demyelination in the corpus callosum at levels 241-251 according to Sidman's atlas (Figure 1A) (Sidman et

al., 1971). MHC II^+ cells were detected early during cuprizone-induced demyelination and they remained significantly higher in numbers between weeks 3 and 6 (Figures 1B and 1C). An immunohistochemical colocalization analysis showed that MHC II is expressed exclusively by microglia/macrophages (data not shown).

MHC II cytoplasmic tail exacerbates demyelination

To determine if MHC II antigens exacerbates CNS histopathology, *I-*$A_\beta^{-/-}$ C57BL/6 mice were exposed to the cuprizone diet, and the brains from these mice were analyzed for demyelination that is typically detectable by week 3. Luxol fast blue-periodic acid Schiffs (LFB-PAS) stains myelin blue, and counterstains the remaining tissue pink (Figure 2A). Cuprizone-fed *I-*$A_\beta^{-/-}$ mice had significantly less demyelination at 3 ($p<0.0003$) and 3.5 ($p<0.01$) weeks than wild type mice (Figures 2A and 2B). Concomitant with the reduced demyelination, there was also a significant reduction in cellularity at the 3 and 3.5 weeks time points (Figure 2B). Thus, the reduction in demyelination and cellularity in cuprizone intoxication corroborates our previous report that MHC II exacerbates demyelination in the *twitcher* mouse (Matsushima et al., 1994) and demonstrates a similar role for MHC II in two distinct models of demyelination.

Structure-function studies of $H\text{-}2^b$ MHC II have shown that the amino acids of the β-chain transmembrane and cytoplasmic domains are critical to signaling function of this molecule. To assess whether the cytoplasmic tail of MHC II is important in demyelinating disease, *I-*A_β^{tr} C57BL/6 mice that lack 13 amino acids of the carboxy-terminus of the β chain were exposed to cuprizone (Smiley et al., 1995; Smiley et al., 1996). Although antigen presenting cells (APCs) from these mice show some alterations in *in vitro* antigen presentation, they do not exhibit any anomalous responses to *in vivo* antigenic challenges and express similar levels of MHC II antigen as wild type mice (Smiley et al., 1995; Smiley et al., 1996). Cuprizone-treated *I-*A_β^{tr} mice exhibited a dramatic reduction in demyelination at 3 ($p<0.0003$) and 3.5 ($p<0.001$) weeks compared to wild type mice (Figures 2A and 2B). This reduction is similar to that observed with *I-*$A_\beta^{-/-}$ mice. However, at week 4, differences among the mice are not significant and by week 5, *I-*A_β^{tr} or *I-*$A_\beta^{-/-}$ mice were fully demyelinated similar to wild type mice (data not shown). In parallel with reduced demyelination, there was also a significant reduction in cellularity at the 3 and 3.5 weeks treatment time points (Figure 2B). Differences between the wild type mice and *I-*A_β^{tr} or *I-*$A_\beta^{-/-}$ mice were greatest at 3.0 weeks, hence most of the subsequent studies were performed at this time point.

During cuprizone intoxication, mature oligodendrocytes undergo apoptosis and their depletion is believed to be the primary cause of demyelination (Mason et al., 2000). To determine whether the presence of MHC II on microglia/macrophages affects the myelinating cells, we quantified mature oligodendrocytes by immunohistochemistry using a GST-Pi stain (Cammer and Zhang, 1993; Mason et al., 2000). The number of GST-pi mature oligodendrocytes was significantly decreased in cuprizone-treated *I-*$A_\beta^{+/+}$ mice (approximately $530/mm^2$) at week 3 compared to untreated mice (approximately ($1610/mm^2$). In contrast, the number of mature oligodendrocytes in 3.0 week cuprizone-treated *I-*$A_\beta^{-/-}$ and *I-*A_β^{tr} mice was approximately two-fold greater than wild type mice (approximately $1160/mm^2$ and $1366/mm^2$ vs. $530/mm^2$, respectively) at 3.0 weeks. The number of oligodendrocytes in the $I\text{-}A_\beta^{-/-}$ and $I\text{-}A_\beta^{tr}$ mice was only slightly reduced when compared to untreated mice. These results implicate that the expression of MHC II molecules by microglia/macrophages may adversely affect the survival or maturation of mature oligodendrocytes.

MHC II cytoplasmic tail increases microglial proliferation, inflammatory cytokines, and oxygen radicals

The pathology differences in the survival of mature oligodendrocyte in MHC II mutant mice were accompanied by differences in microglial proliferation, inflammatory cytokine synthesis, and the presence of oxygen radicals as shown below. Immunohistochemical staining for the RCA-1 lectin (Figure 3A), a marker for microglia/macrophages (Hiremath et al., 1998), revealed that wild type mice treated with cuprizone for 3 weeks had significantly increased numbers of microglia/macrophages compared to *I-A*$_{\beta}^{-/-}$ and *I-A*$_{\beta}^{tr}$ mice (Figure 3B). MHC II signaling has been shown to trigger proliferation of cells (Baluyut et al., 1993; Cambier and Lehmann, 1989; Lane et al., 1990; Mooney et al., 1990; Spertini et al., 1992). We find the diminished numbers of microglia/macrophage in the *I-A*$_{\beta}^{-/-}$ and *I-A*$_{\beta}^{tr}$ mice can be partly explained by the reduced number of proliferating microglia/macrophages in these mice as measured by BrdU incorporation (Figure 4). The engagement of MHC II on monocytes is known to result in cytokine production, such as TNF-α and IL-1β (Scholl et al., 1992; Trede et al., 1991) which are likely detrimental to oligodendrocyte survival (Loughlin et al., 1994; Merrill, 1991; Selmaj and Raine, 1988). Significantly greater amounts of TNF-α and IL-1β were produced in the brains of cuprizone-treated wild type mice when compared to *I-A*$_{\beta}^{-/-}$ or *I-A*$_{\beta}^{tr}$ mice (Figure 5). Finally, activation through MHC II can result in increased nitric oxide synthesis *in vitro* (Hauschildt et al., 1993; Zembowicz and Vane, 1992). Nitrotyrosine reflects the presence of nitric oxide that reacts with free radicals to form peroxynitrites that bind proteins and convert tyrosine residues to nitrotyrosine (Beckman and Koppenol, 1996). An anti-nitrotyrosine staining (Figure 6) demonstrates increased nitric oxide activity in the cuprizone-treated wild type mice compared to cuprizone-treated *I-A*$_{\beta}^{-/-}$ and *I-A*$_{\beta}^{tr}$ mice. Thus, the cytoplasmic tail of MHC II expressed on microglia/macrophages appears to function in proliferation and in the production of noxious substances that correlates to mature oligodendrocyte depletion during demyelination.

T cells are not required for activating MHC II

T cells are rarely detected in the demyelinating lesions of cuprizone-treated mice, but they could engage MHC II molecules on microglia/macrophages via their T cell receptors. To definitively assess the role of lymphocytes, *RAG-1*$^{-/-}$ mice that are deficient in T and B cells were exposed to cuprizone. The extent of demyelination and cellularity in *RAG-1*$^{-/-}$ mice was similar to wild type mice (Figure 7A). Furthermore, perivascular cuffing characteristic of lymphocyte infiltration was not observed in wild type mice or I-A mutant mice. $CD3^+$ T cells were not detected in corpus callosum samples of 8 different wild type mice and only one T cell was detected in 2 of 7 *I-Aβ*$^{-/-}$ mice. These data would suggest that T cells do not play a significant role in cuprizone-induced demyelination. In addition, the number of microglia/macrophages was also similar (Figure 7B). These results suggest exclusion of lymphocytes in this disease model and suggest MHC II molecules on microglia/macrophages do not function in antigen presentation.

4. Discussion

We have demonstrated that MHC II exacerbates cuprizone-induced demyelination. A likely role for MHC II molecules is to act as signaling receptors, presumably activating microglia/macrophages during acute inflammation of the CNS. This is supported by the observation that the absence of the cytoplasmic tail of MHC II reduces histopathology similarly to mice devoid of MHC II molecules. However, this reduction is a delay in demyelination as I-A mutant mice are fully demyelinated by week 5 similar to wild type mice. In *I-A*$_{\beta}^{tr}$ and *I-Aβ*$^{-/-}$ mice undergoing demyelination, there are fewer proliferating $BrdU^+$ microglia and lower amounts of IL-1β, TNF-α and nitric oxide, each of which may accelerate the depletion

of mature oligodendrocytes. Lastly, the exacerbation of demyelinating disease appears to occur independently of T cells.

In diseases like MS, MHC II functions as an antigen presenting molecule to activate autoreactive $CD4^+$ T cells. In fact, a link between MHC II and a propensity for diseases like MS has been established with a familial rate of occurrence approaching 20% (Kalman and Lublin, 1999). Various MHC II molecules, such as DR15, DQ6, and Dw2, have been associated with either an increased or decreased propensity for MS in various populations (Kalman and Lublin, 1999). In the murine model for MS, EAE, susceptibility to disease is dependent on the MHC II haplotype, such as $I\text{-}A^u$ and $I\text{-}A^s$, of a given animal (Fritz et al., 1985), and other studies using T cell receptor transgenic mice and various encephalitogenic proteins have demonstrated a key role for antigen presentation in EAE (Wucherpfennig, 1994). The propensity for disease susceptibility in various haplotypes suggests a role for antigen presentation in MS and EAE.

A major function of microglia/macrophages in acute CNS inflammation is believed to be phagocytosis of myelin (Flaris et al., 1993; Nabavi et al., 1989; Perry, 1998; Perry and Gordon, 1988; Streit, 2002; Streit et al., 1988; Streit and Kreutzberg, 1988). Phagocytosis activates microglia/macrophages that results in the upregulation of various activation markers, such as MHC II, and secretion of proinflammatory cytokines (Gehrmann et al., 1995). It has been shown that phagocytised myelin peptides increase autoreactive T cells (Zamvil et al., 1985; Zamvil and Steinman, 1990). However, microglia/macrophages expressing MHC II are also found in MS lesions where there are few T cells present (Li et al., 1993; Raine, 1994). Additionally, a similar lack of T cells is found in neurological disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Hickey et al., 1991; McGeer et al., 1989; McGeer et al., 1988a; McGeer et al., 1988b; McGeer et al., 1994). In such cases, the role of MHC II on microglia/macrophages is not clear.

The cuprizone-induced model for demyelination is ideal because the time course of cellular, biochemical, and morphological events is predictable and easily reproduced. In contrast to EAE, the lack of T cell infiltrates in cuprizone-induced demyelination minimizes the possibility of the effect of antigen presentation on disease and eliminates confounding interpretation of results that might be attributed to T cells. In cuprizone-induced demyelination, MHC II is detected only on microglia/macrophages *in vivo* (Hiremath et al., 1998) (Arnett et al., 2003). Astrocytes can express MHC II *in vitro*, but these cells do not express MHC II *in vivo* during cuprizone intoxication (Arnett et al., 2003). We have shown in this study that T cells do not play a significant role in the disease process. Additionally, the C57BL/6 background ($H\text{-}2^b$) of the mice treated with cuprizone (Hiremath et al., 1998) is ideal for determining a possible *in vivo* signaling function for MHC II because only the I-A molecule is present. The I-E MHC II molecule is not expressed due to a mutation in the promoter of the α chain of the I-E molecule (Mathis et al., 1983). The $I\text{-}A_\beta$ cytoplasmic tail deletion mutation has previously been shown to eliminate a majority of the signal transduction function *in vitro* in B cell lines (Harton and Bishop, 1993), although *in vitro* evidence suggests that antigen-presenting cells function remains intact in $I\text{-}A_\beta^{tr}$ mice (Smiley et al., 1995).

We speculate that MHC II may serve in signal transduction that activates microglia/macrophages. Recently, several *in vitro* analyses have demonstrated that T cell-independent activation of MHC II on human monocytes and macrophages results in the secretion of various proinflammatory cytokines such as TNF-α and IL-1β (Espel et al., 1996; Fast et al., 1989; Matsuyama et al., 1993; Palkama and Hurme, 1993; Trede et al., 1991; Zembala et al., 1994). Furthermore, ample evidence from *in vitro* analyses suggest MHC II can signal to

activate B cells or T cells and cause either differentiation, proliferation, or secretion of cytokines (Ahsmann et al., 1997; Baluyut et al., 1993; Bishop, 1992; Bishop and Frelinger, 1989; Bishop and Haughton, 1986; Cambier and Lehmann, 1989; Cambier et al., 1987; Chia et al., 1994; Damle et al., 1993; Harton and Bishop, 1993; Kanner et al., 1995; Lane et al., 1990; Mollick et al., 1991; Mooney et al., 1990; Naitoh et al., 1994; Newell et al., 1993; Spertini et al., 1992; Spertini et al., 1991; Wade et al., 1995). Thus, it is plausible that MHC II expressed on microglia/macrophages may signal similar activation with consequences that exacerbates disease. Our data showing reduced numbers of proliferating microglia/macrophages and lower NO, TNF-α, and IL-1β in the absence of the cytoplasmic tail in the *I-A*$_{\beta}^{tr}$ mice supports this hypothesis and suggest this may be a novel mechanism by which microglia/macrophages become activated and exacerbate CNS pathology.

The results presented in this report demonstrate for the first time that the cytoplasmic domain of MHC II has a crucial role *in vivo*, and in our particular assays, it exacerbates CNS demyelination. Specifically, the cytoplasmic domain of MHC II appears to be important in activation of microglia/macrophages. These results are consistent with our previous findings in the murine model of Krabbe's disease, the *twitcher* mouse. By crossing the *I-A*$_{\beta}^{tr}$ transgenic mice to *twitcher* to obtain *I-A*$_{\beta}^{tr}$ *twitcher* mice, we found a similar reduction in the severity of demyelination compared to *I-A*$_{\beta}^{+/+}$ *twitcher* mice (data not shown). Both cuprizone-induced and *twitcher* models for demyelination corroborate the importance of the MHC II cytoplasmic tail in demyelinating disease.

These results are consistent with our hypothesis that during demyelination, MHC II molecules expressed on microglia/macrophages may serve as signaling molecules that lead to the activation and proliferation of microglia/macrophages accompanied by synthesis of cytokines associated with inflammation. The production of detrimental proinflammatory cytokines, such as TNF-α or IL-1β, and substances such as nitric oxide may lead to oligodendrocyte perturbations that further exacerbate pathogenesis. These substances have repeatedly been shown to be produced by activated microglia/macrophages and to cause oligodendrocyte death *in vitro* (Merrill, 1991; Merrill et al., 1993; Selmaj and Raine, 1988). Our results in Figures 5 and 6 suggest that the increased presence of proinflammatory products in the cuprizone-treated *I-A*$_{\beta}^{+/+}$ mice compared to *I-A*$_{\beta}^{-/-}$ and *I-A*$_{\beta}^{tr}$ mice might be contributing to the concomitant increased depletion of mature oligodendrocytes in the cuprizone-treated *I-A*$_{\beta}^{+/+}$ mice. This depletion of mature oligodendrocyte numbers is attributed to perturbed cells that show severe inhibition of MBP and MAG transcription (Morell et al., 1998), and subsequently undergo apoptosis (Mason et al., 2000). Our findings are in support of other investigators that have also postulated that microglia/macrophages may be detrimental to demyelinating disease (Stoll and Jander, 1999) (Gonzalez-Scarano and Baltuch, 1999).

We additionally demonstrate that T cells likely do not participate in cuprizone-induced demyelinating disease; hence the conventional role of MHC II as a ligand for T cells may not be occurring in this model. In this model, there are very few T cells as mentioned above in Results (McMahon et al., 2002) and negligible amounts of T cell-associated cytokines (IFN-γ) are present in the brain (unpublished results). Furthermore, we do not observe perivascular cuffing associated with conventional T cell-mediated demyelination in EAE or viral models and the blood-brain barrier remains intact in C57BL/6 mice during cuprizone exposure (McMahon et al., 2002). Finally, we do not observe CD4 expression at any time point during cuprizone-induced demyelination or remyelination in our microarray analyses (data not shown). These findings suggest T cells are not likely contributing to this disease process. To definitively determine whether T cells have a role in this disease, we have treated *RAG-1*$^{-/-}$ mice with cuprizone and found no differences in demyelination, in cellularity, or in the number of RCA-1^{+} microglia/macrophages (Figure 7B and data not

shown). This demonstrates that microglia/macrophages *per se* in the absence of T lymphocytes may be equally prominent effector cells in exacerbating demyelinating disease. We have also demonstrated that MHC II is important for remyelination at week 7 and week 8; however, there, too, T cells do not play a role in remyelination (Arnett et al., 2001). Currently, it is not known how MHC II is being activated in our model in the absence of T cell function. However, we presume that activation through MHC II may cause the elevated release of proinflammatory substances that may be detrimental to mature oligodendrocytes during demyelination. Interestingly however, TNF-α appears to be detrimental to mature oligodendrocytes during demyelination, but essential for oligodendrocyte precursors during remyelination based on the respective cell type expression of TNFRI and TNFRII (Arnett et al., 2001). This depletion of mature oligodendrocytes is followed by an accumulation of $NG2^+$ oligodendrocytes precursor cells (Mason et al., 2000); however, by week 5, the number of oligodendrocytes precursor cells in $I\text{-}A_\beta^{-/-}$ mice was similar to $I\text{-}A_\beta^{+/+}$ mice (data not shown). It is plausible that the residual levels of TNF-α remaining in I-A mutant mice is sufficient to sustain oligodendrocytes precursor cells (Figure 5) or that factors other than TNF-α may be supporting oligodendrocytes precursor cell recruitment in $I\text{-}A_\beta^{-/-}$ mice. Thus, we suggest that MHC II serves to activate microglia and secrete TNF-α and other factors, which at first may enhance demyelination but later contributes to facilitate remyelination. Further investigations may provide insights for the role of MHC II on microglia and potential oligodendrocyte interactions.

Acknowledgments

We acknowledge the generosity of Dr. L. Glimcher, Dr. M. Grusby, and Dr. S. Smiley for providing us with the $I\text{-}A_\beta^{-/-}$ and $I\text{-}A_\beta^{tr}$ mice. We further thank Dr. R. Bagnell for his help with microscopy, and Dr. C. Lawler for her help and expertise in statistical analysis. MMH was supported by the NIAID grant AI07273, the UNC Graduate Dissertation Completion Fellowship, and the UNC Medical Alumni Association Endowment Fund. VSC was supported by NIAID T32 AI07273. KS was supported by NINDS grants NS24453 and HD03110. JPYT was supported by NINDS grant NS35372 and NMSS grant RG7295. GKM was supported by NAID grant AI51770 and NMSS grant RG3898A.

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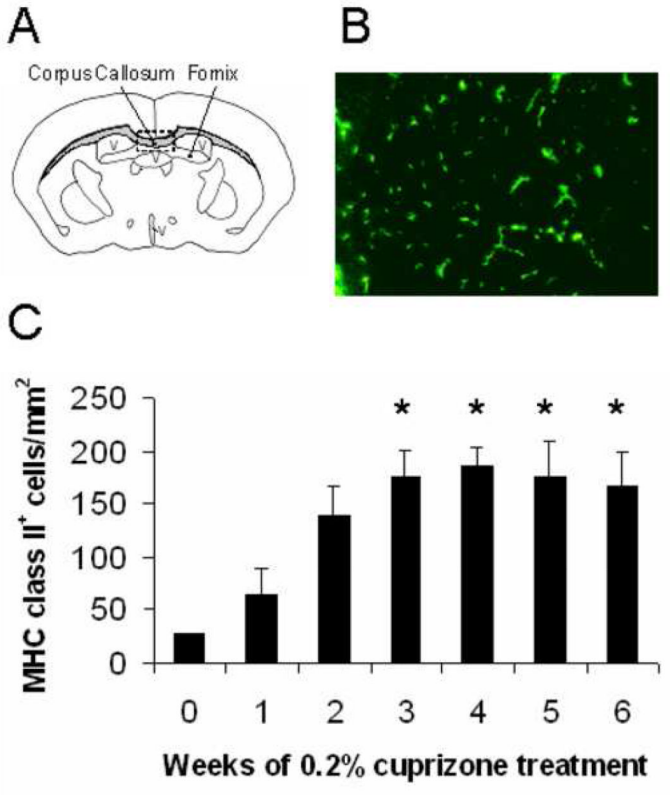

Figure 1.

MHC class II^+ cells were detected in the corpus callosum of cuprizone-treated mice.

(A) Illustration of the corpus callosum from which all coronally-sectioned mouse brains were analyzed near midline within the rectangular box. V = ventricle

(B) Brain sections were stained with anti-I-A^b antibody to identify MHC class II^+ cells in the demyelinated corpus callosum at week 3.0 (200X magnification).

(C) MHC class II^+ cells were quantified at weekly intervals during the 6 week exposure to cuprizone and were compared to untreated mouse brains (week 0). *Denotes $p<0.05$ by one-way analysis of variance (ANOVA) test followed by Dunnett multiple comparisons test comparing all time points to 0 week timepoint; n=3 mice each week

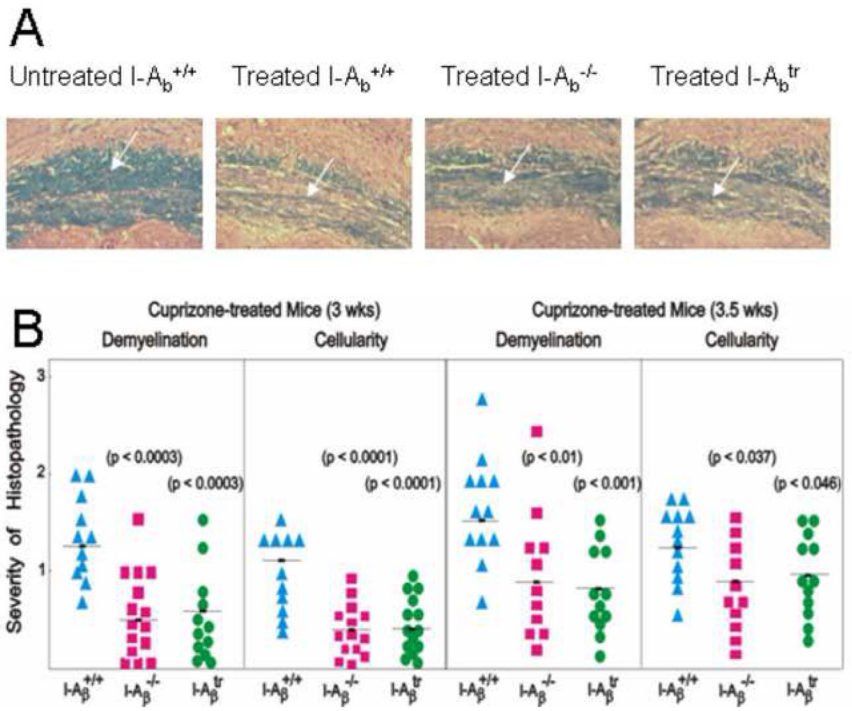

Figure 2.

Mice exhibited reduced demyelination in the corpus callosum when MHC class II was absent or when the cytoplasmic tail was truncated.

(A) LFB-PAS-stained coronal brain sections exposing the corpus callosum (indicated by the arrow) showed blue-stained myelin in untreated *I-A*$_{\beta}^{+/+}$ mice. Treatment of wild type, *I-A*$_{\beta}^{-/-}$, and *I-A*$_{\beta}^{tr}$ mice with cuprizone for three weeks induced demyelination that is indicated by the lack of blue and the presence of pink counterstained fibers. Wildtype mice had significantly greater demyelination (indicated by the decreased blue staining) compared to *I-A*$_{\beta}^{-/-}$ and *I-A*$_{\beta}^{tr}$ mice. All pictures are shown at 100X magnification.

(B) The histopathology of the corpus callosum in the *I-A*$_{\beta}^{-/-}$ and the *I-A*$_{\beta}^{tr}$ mouse was significantly reduced when compared to the wild type mouse. Brain sections were scored by three independent readers in a double-blind manner for the severity of demyelination and cellularity on a scale of zero to three and averaged as previously described (Hiremath et al., 1998). A score of 3 indicated complete demyelination, and a score of 1.5 indicated demyelination of 50% of the fibers. A score of zero indicated the lack of demyelination. Similarly, peak cellularity indicated by numbers of nuclei at week 5 of cuprizone treatment was taken as a score of 3 while untreated control was given a score of zero. Statistical analyses were conducted by ANOVA followed by the Dunnett multiple comparisons test which compared the wild type to *I-A*$_{\beta}^{-/-}$ or *I-A*$_{\beta}^{tr}$ mice. Results are from three independent experiments using 4 mice per group per time point.

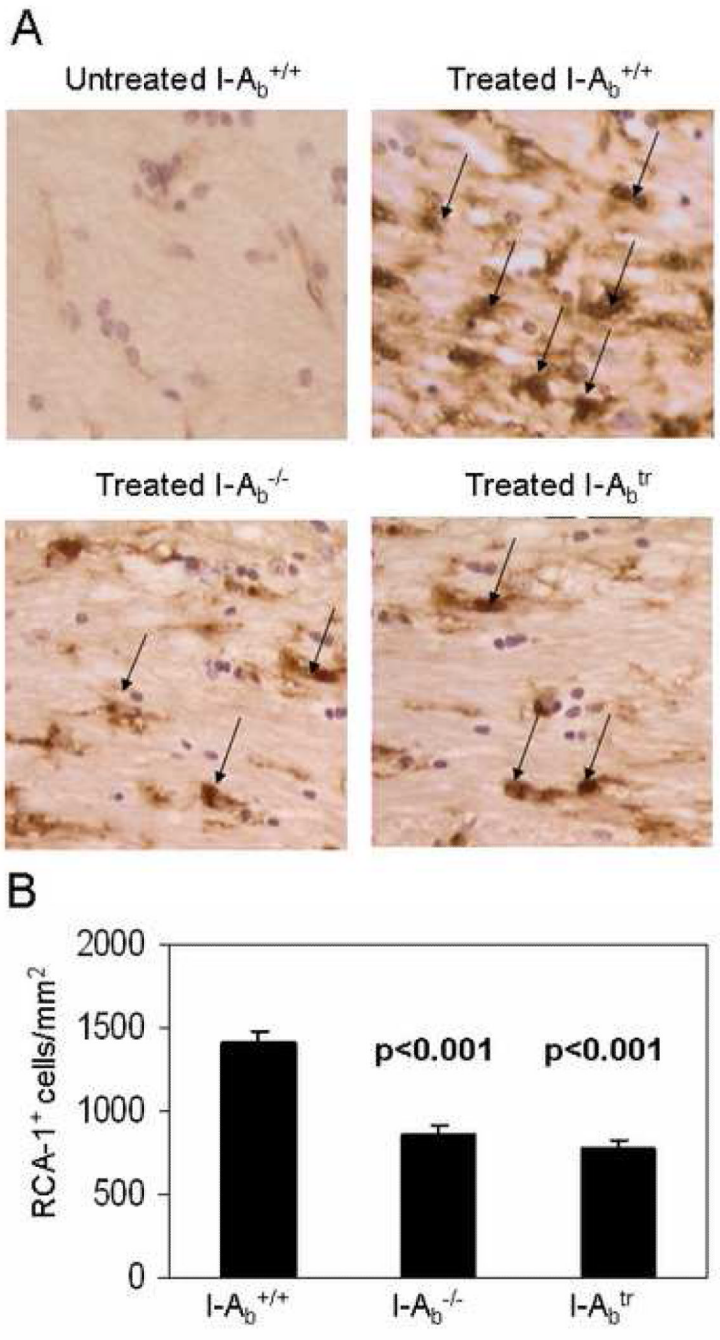

Figure 3.

The absence of MHC class II or the cytoplasmic tail of MHC class II showed a reduced microglia/macrophage accumulation in the corpus callosum.

(A) Representative RCA-1-stained microglia/macrophages (brown-stained cells with nuclei and indicated by arrows) in the corpus callosum of untreated controls wild type, *I-A*$_{\beta}^{-/-}$, and *I-A*$_{\beta}^{tr}$ mice treated for 3 weeks with 0.2% cuprizone at 400X magnification are shown.

(B) Morphometric quantitation of RCA-1^{+} microglia/macrophages were facilitated by using the NIH Imaging software (Hiremath et al., 1998). The corpus callosum of wild type mice treated with cuprizone (n = 12 per genotype) for three weeks had greater numbers of

microglia/macrophages than *I-*$A_\beta^{-/-}$ or *I-*A_β^{tr} mice. Statistical analyses were conducted by ANOVA using the Dunnett multiple comparison test comparing to wild type mice.

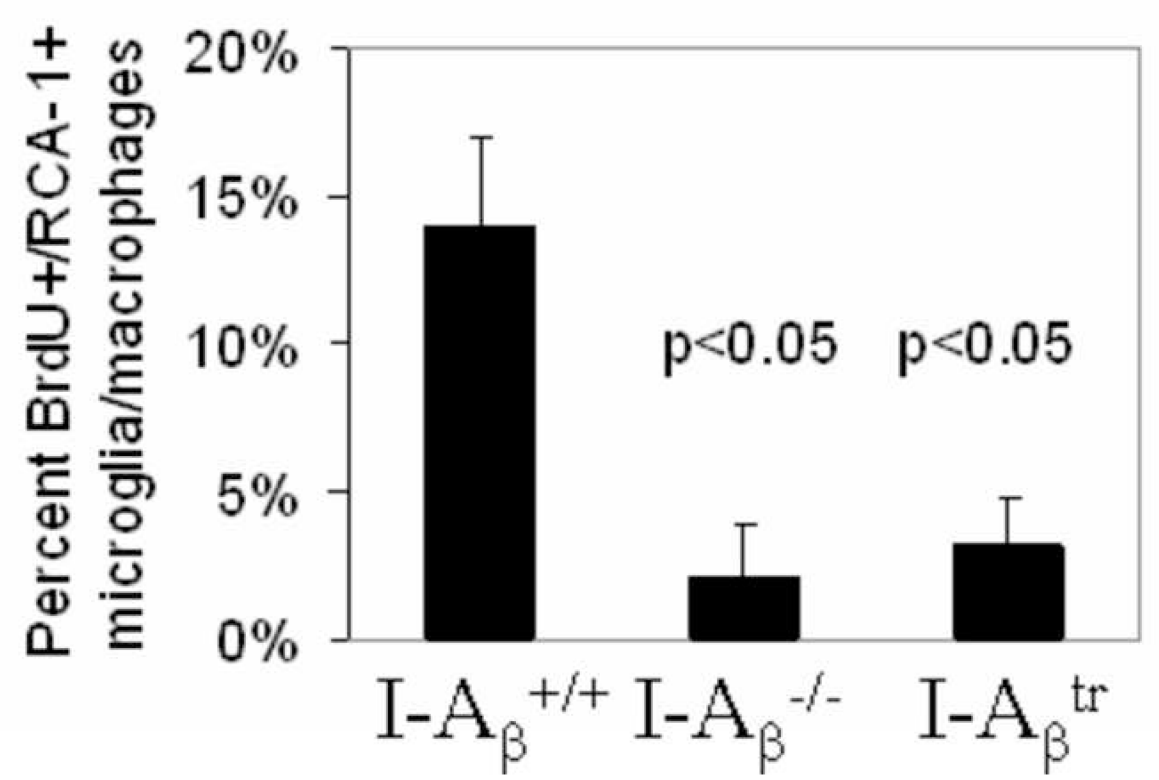

Figure 4.

BrdU incorporation is increased in wild type microglia/macrophages following cuprizone treatment.

The percent of RCA-1^{+} microglia/macrophages which incorporated BrdU at the 3 week treatment point (n = 4 per genotype) was greater in wild type mice than in *I-A*$_{\beta}^{-/-}$ or *I-A*$_{\beta}^{tr}$ mice. Statistical analysis was conducted using the Kruskal-Wallis nonparametric ANOVA test because the values did not fall within a normal U-shaped distribution.

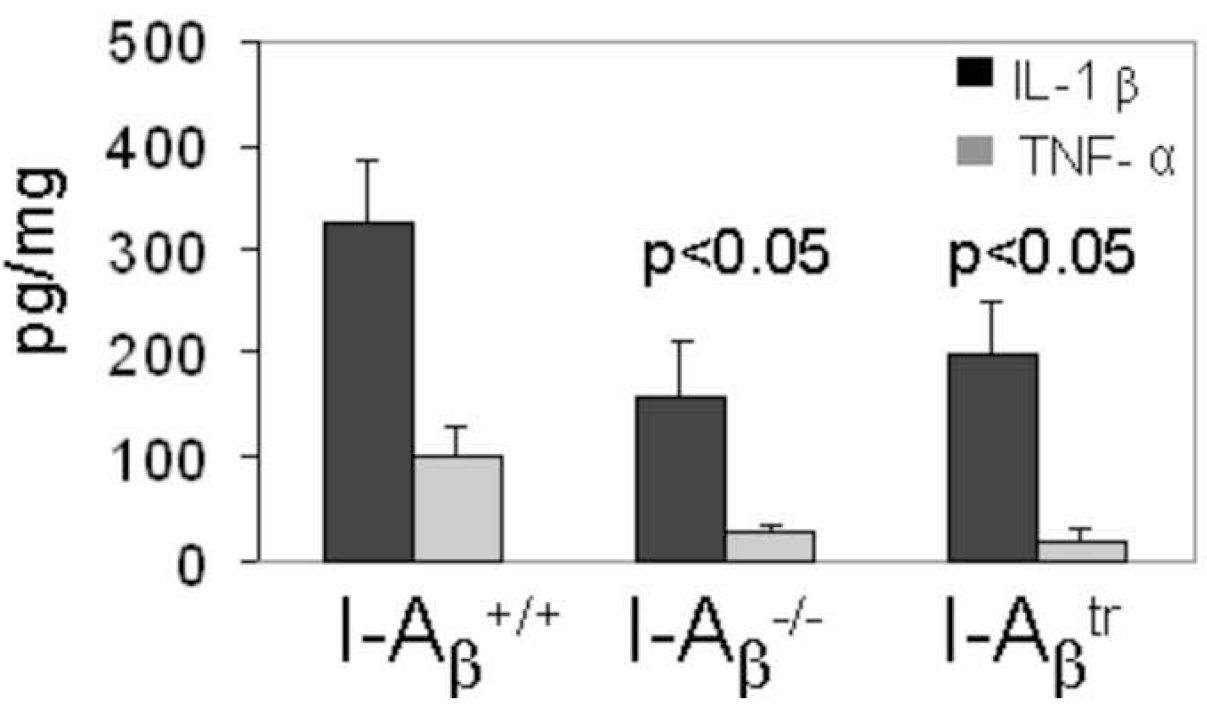

Figure 5.

TNF-α and IL-1β production in the brains of wild type mice was greater than *I-*$A_{\beta}^{-/-}$ and *I-*A_{β}^{tr} mice (n = 9-12 mice per genotype).

Brains of cuprizone-treated mice were homogenized in the presence of protease inhibitors and extracts were assayed for TNF-α and IL-1β using EIA. Statistical analyses were conducted by ANOVA using the Dunnett multiple comparison test comparing to wild type mice.

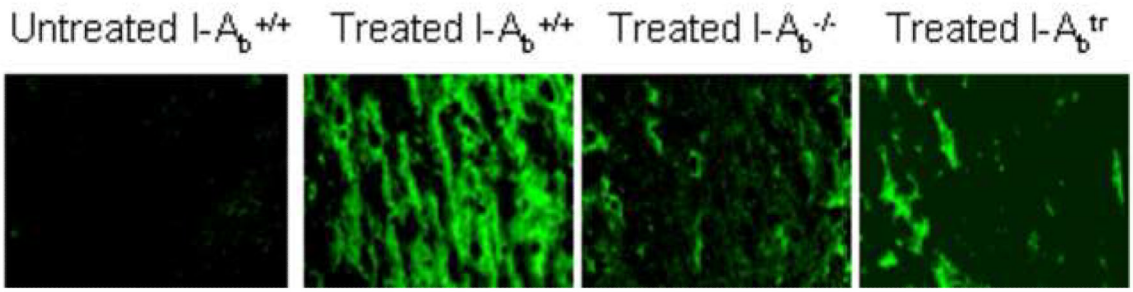

Figure 6.

Nitrotyrosine staining was increased in cuprizone-treated wild type mice. The lack of nitrotyrosine staining indicated the absence of nitric oxide in the corpus callosum of untreated control mice, but its presence in 3 week, cuprizone-treated wild type mice. The level of nitrotyrosine staining was reduced in cuprizone-treated *I-A*$_\beta^{-/-}$ and *I-A*$_\beta^{tr}$ mice (n = 4-5 mice per genotype).

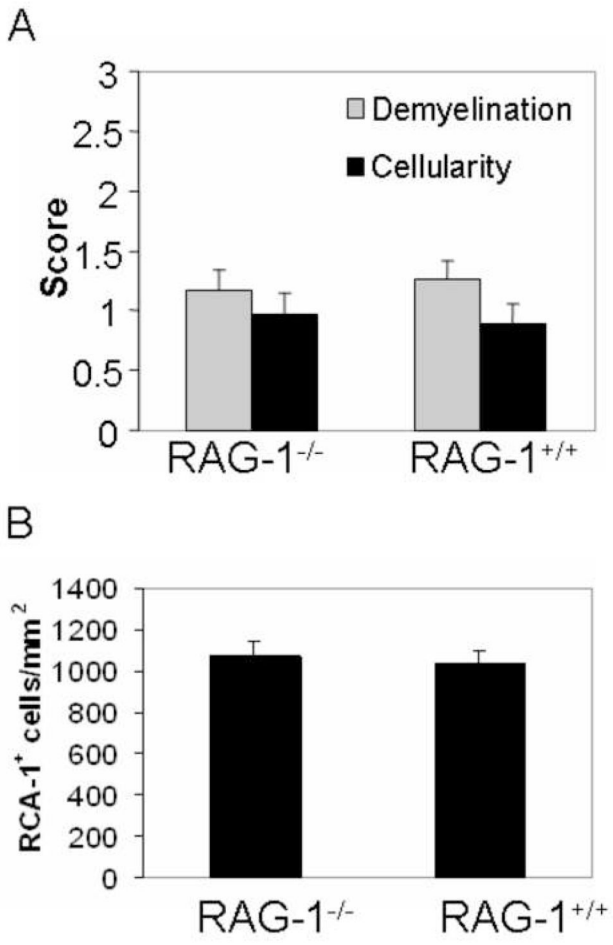

Figure 7.

Cuprizone-induced neuropathology and microglia/macrophage numbers were not affected by the *RAG-1*$^{-/-}$ mutation.

(A) *RAG-1*$^{-/-}$ and *RAG-1*$^{+/+}$ mice exhibit a similar degree of demyelination and cellularity at 3 weeks (n = 11-12 per genotype). Three independent experiments were conducted using 3 or 4 mice per genotype. LFB-PAS- and RCA-1-stained coronal brain sections were scored as described in Figure 2 and Figure 3, respectively.

(B) Quantitation of the RCA-1^{+} microglia/macrophages revealed no statistical difference in the brains of *RAG-1*$^{-/-}$ and *RAG-1*$^{+/+}$ mice (n = 11-12 per genotype) as described in Methods section. Statistical analysis was conducted using ANOVA comparing wild type to *RAG-1*$^{-/-}$ mice.