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MYELIN ANTIGEN LOAD INFLUENCES ANTIGEN PRESENTATION AND SEVERITY OF CENTRAL NERVOUS SYSTEM AUTOIMMUNITY

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Abstract

This study was designed to understand the impact of self-antigen load on manifestation of organ specific autoimmunity. Using a transgenic mouse model characterized by CNS hypermyelination, we show that larger myelin content results in greater severity of experimental autoimmune encephalomyelitis attributable to an increased number of microglia within the hypermyelinated brain. We conclude that a larger self-antigen load affects an increase in number of tissue resident antigen presenting cells (APCs) most likely due to compensatory antigen clearance mechanisms thereby enhancing the probability of productive T cell-APC interactions in an antigen abundant environment and results in enhanced severity of autoimmune disease.

Keywords

EAE; microglia; antigen load; hypermyelination; myelin content; Akt

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Introduction

Total antigenic content within primary lymph nodes constituted by density of cognate peptide-MHC (pMHC) complexes per dendritic cell (DC) as well as total number of antigen presenting DCs determines the efficiency of T cell activation and speed of transition to the effector mode (Henrickson et al., 2008) against foreign antigens. However, there still remains a lack of understanding regarding the significance of self antigen load characterized by unlimited availability within a targeted tissue and its impact on activation of the self-reactive T cell repertoire and progression of autoimmunity. We hypothesized that ‘although the content of a self-antigen within the targeted tissue can be considered “infinite” or “unlimited”, a larger self-antigen load will lead to more efficient antigen presentation and transition of autoreactive T cells to the activated state thereby predisposing to more severe organ specific autoimmunity under conditions favoring breakdown of tolerance’.

To test our hypothesis and analyze the impact of self antigen content within the tissue on severity of organ specific autoimmunity, we studied the onset and progression of experimental autoimmune encephalomyelitis (EAE) in transgenic mice characterized by CNS hypermyelination (Plp-Akt-DD mice). The Plp-Akt-DD transgenic mouse model was previously designed by us to express the anti-apoptotic serine threonine protein kinase encoding *Akt* gene under transcriptional control of the myelin proteolipid protein (PLP) promoter that restricts its expression to oligodendrocytes and is characterized by increase in total myelin content in the brain beginning at early stages of development (Flores et al., 2008). Increase in protein production due to constitutively active Akt expression in various cell types has been previously demonstrated especially in the cardiac hypertrophy models of Akt expression (Shioi et al., 2000; Condorelli et al., 2002; Matsui et al., 2002) and is most likely a consequence of its role in the mammalian target of rapamycin (mTOR) signaling pathway for protein synthesis (Narayanan et al., 2009; Bodine et al., 2001; Rommel et al., 2001).

Results from our study demonstrate significantly more severe EAE associated pathology in hypermyelinated Plp-Akt-DD transgenic mice compared to their wild type (WT) littermates with “normal” myelin content. Further characterization of disease etiology reveals that increased severity of EAE in hypermyelinated transgenics was due to the presence of a larger number of tissue resident APCs, the microglia within the brain. We conclude that an increase in self antigen load within a tissue even if it is to an already abundant pool of self antigen can affect the onset and progression of autoimmunity. The immunological impact of the increase in antigenic load is a side effect of compensatory antigen clearance mechanisms to maintain tissue homeostasis via increasing the number of phagocytic resident microglia. In addition, we show that a mere increase in the number of resident APCs in the brain or other tissue which may occur due to factors related or unrelated to antigen load has the potential to result in detrimental autoimmune consequences thereby reiterating their critical role in generation and progression of CNS targeted autoimmunity and as therapeutic targets for immunomodulation and treatment of autoimmunity.

Material and Methods

Generation of Plp-Akt transgenic mice

Transgenic mice expressing constitutively active Akt driven by the *Plp* promoter (Wight et al., 1993) were generated as described earlier (Flores et al., 2008). Briefly, Akt cDNA was inserted into the *AscI/PacI* sites of a modified *Plp* promoter cassette as described (Fuss et al., 2000). The *Plp* promoter/Akt gene was injected in SWR/J F1 mice to generate transgenics and positive founders identified by PCR amplification of DNA from tail snips using intron SV40 upper (5'-GCAGTGGACCACGGTCAT-3') and Akt lower (5'-

CTGGCAACTAGAAGGCACAG-3') primers. Plp-Akt-DD heterozygous SWR/J males were mated with wild type (WT) SWR/J female littermates to generate only heterozygous Plp-Akt-DD transgenic offspring in order to maintain consistency in Akt expression levels. Plp-Akt-DD transgenic mice show enhanced myelination compared to WT littermates beginning as early as P10 and increasing with age as described earlier both by immunostaining of cerebrum samples and real time PCR analysis for myelin proteins and RNA at different time points of development (Flores et al., 2008). The Plp-Akt-DD mouse model is characterized by an increase in total myelin in the brain without any imbalance in proportions of myelin component proteins as in other currently available models of myelin overexpression in the CNS (Ip et al., 2008; Karim et al., 2007; Leder et al. 2007).

Induction of experimental autoimmune encephalomyelitis

Since the Plp-Akt-DD transgene has been established on the SWR murine background (H2-IA^q) we used the PLP-104-117, IA^q/SWR restricted model which has been extensively used and established as a valid model for EAE studies. IA^q restricted PLP-104-117 peptide was synthesized at the molecular biotechnology core of the Lerner Research Institute. 6-8 week old Plp-Akt-DD transgenic female mice or age and sex matched WT littermates were immunized by *s.c.* injection of 100 µg of PLP 104-117 peptide in CFA in the abdominal flank. Each mouse also received 200 ng pertussis toxin by *i.p.* injections at days 0 and 3 of immunization. All mice were weighed and evaluated daily for signs of neurological deficit. Mice were scored daily for clinical disease severity according to the following criteria: 0, no disease; 1, decreased tail tone or slightly clumsy gait; 2, tail atony and/or moderately clumsy gait and/or poor righting ability; 3, limb weakness; 4, limb paralysis; 5, moribund state.

Immunohistochemical analysis of brain tissue

During acute or chronic stages of EAE, Plp-Akt-DD and WT female littermates were anaesthetized with isoflurane inhalation and brain tissue fixed by intracardiac perfusion with chilled PBS followed by 4% paraformaldehyde. Brain tissue was extracted from the cranial cavity, fixed overnight in 4% paraformaldehyde and cryoprotected in 30% sucrose before mounting in optimum cutting temperature medium (Triangel Biomedical services, Durham, NC). As required, 10 µm or 30 µm serial sections of the entire brain were taken. Every twentieth serial section was stained with Harris hematoxylin (Sigma Aldrich, St Louis, MO) and eosin Y (EMD Chemicals, Gibbstown, NJ). Serial sections adjoining those identified to be positive for mononuclear cell infiltrates by H&E staining were immunostained with mouse CD3 antibody (MCA 1477, Serotec, Raleigh, NC) to confirm presence of T cell infiltrates. Brain sections around the hippocampus region were stained for the rabbit ionized calcium binding adaptor molecule 1 (Iba1) microglial cell surface markers using HRP conjugated Iba1 antibody (Wako, Richmond, VA). All images were acquired using 10× or 20× objectives on a Leica DMR microscope with an Optronics Magnafire digital camera.

In vivo magnetic resonance imaging (MRI) of EAE mice

All MRI studies were performed at the Case center for imaging research (CCIR) at Case Western Reserve University. Plp-Akt-DD and WT female littermates with chronic EAE (n=2) were anesthetized by isoflurane/oxygen inhalation 8 weeks post EAE onset and scanned using conventional T2 weighted spin echo acquisitions (TR/TE=2000/30ms, resolution: 260 × 260 × 1000 µm; field of view: 3.35*3.35cm; matrix: 128*128) on a Bruker Biospec 7T MRI scanner. Vital signs such as body temperature, heart and respiratory rate were controlled and monitored throughout the duration of the imaging session. The T2-weighted images generated excellent contrast between ventricle and surrounding brain tissue. Scanner host software (Paravision, Bruker, Billerica, MA) was then used to calculate the ventricular volume and total brain volume for each animal using a simple region of interest (ROI) analysis. For each imaging slice, the ventricle and brain areas were assessed

by manually drawing an ROI over each region. The ROI areas in each slice were summed and then multiplied by the slice thickness to calculate the respective volumes.

Preparation of brain and lymph node derived cell populations

As required, naïve or PLP 104-117 immunized Plp-Akt-DD and WT female mice were perfused through the intracardiac route with PBS to eliminate hematogenously derived cells. Brains were extracted, homogenized through a wire mesh and further treated with 0.2 mg/ml of Collagenase Type II and 50 KU/ml of DNase I for 1 hour in HBSS.

For preparation of brain derived total mononuclear cells—Single cell suspensions were prepared from brains derived from naïve mice as described above. Cells were resuspended in 6 ml of 70% percoll per brain. Density gradient centrifugation was carried out on a 70%-30% percoll gradient, at 2400 rpm for 45 minutes at 20°C. The mononuclear cell layer obtained at the 30% and 70% percoll interface was collected and washed twice to further remove any debris and myelin protein contaminants.

For purified microglia preparations—Microglia were further purified from the mononuclear cell layer obtained above from naïve brains using positive selection with CD11b antibody labeled magnetic beads (Miltenyi Biotech, Auburn, CA). Although the brain tissue was thoroughly perfused to remove any blood derived infiltrating cells, CD11b expression is not exclusive to microglia. Hence the microglia preparations isolated as described above may have a minor component of contaminating brain infiltrating monocytes. However, since all microglia preparations were derived from naïve mice with no EAE we expect this contamination to be minimal and not significant for the experimental results presented. For activation of MHC class II expression, microglia were stimulated with 10 ng/ml murine IFN γ in overnight cultures at 37°C.

For preparation of brain infiltrating T cells—WT and Plp-Akt-DD mice were immunized with PLP 104-117 to induce EAE. At peak of EAE onset, brains were homogenized and a single cell suspension obtained as described above. Pellets were resuspended in 4 ml of 30% percoll and overlaid on 3.5 ml each of 37% and 70% percoll. Discontinuous density gradient centrifugation was carried out as described above. The lymphocyte cell layer at the 37%-70% interface was collected, washed, resuspended in DMEM with 10% FBS and incubated in a nylon wool column (Polysciences, Warrington, PA) at 37°C for 0.5 hours. The column was washed with twice its volume of warm media to collect all T cells. The cell population obtained after nylon wool elution was 99% pure for T cell (data not shown).

For preparation of PLP104-117 primed peripheral T cells—6-8 week old SWR/J WT female littermates were immunized with PLP 104-117 peptide in CFA as described above. Ten days after immunization peripheral lymph nodes were removed and homogenized to form a single cell suspension and T cells positively selected using Thy1.2 antibody labeled magnetic beads (Miltenyi Biotech, Auburn, CA).

ELISPOT Assays

Primed peripheral lymph node T cells from WT females were purified using Thy1.2 magnetic bead and cocultured with Plp-Akt-DD transgenic or WT brain derived mononuclear cells or CD11b positively selected purified microglia as APCs in ratios of 1:5, 1:10, 1:20 and 1:40 T cells:APCs. Cells were co-cultured for 72 hours in IFN γ or IL-17 antibody (Ebioscience, San Diego, CA) coated ELISPOT plates (Millipore, Billerica, MA), washed and colored spots developed using streptavidin-alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride substrate color development

module (R&D Systems, Minneapolis, MN). Number of spots per well were analyzed on an immunospot image analyzer using the Immunospot v4.0 software (Cellular Technologies, Cleveland, OH).

Quantitative RT-PCR for gene expression

Mice were anaesthetized and quickly perfused through the intracardiac route with 10 ml of ice-cold PBS. Brain tissue extracted was snap frozen in liquid nitrogen and mRNA extracted using the Trizol method (Invitrogen, Carlsbad, CA). cDNA was generated using the Retrotranscriptase II reaction (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was carried out using specific primers for the microglia specific Iba1 marker (lower primer: 5' CTGAGAAAGTCAGAGTAGCTGA 3'; upper primer: 5' GGATCAACAAGCAATTCCTCGA 3'). Relative gene expression was calculated as fold increase of gene specific mRNA expression relative to GAPDH expression.

Flow cytometry

Mononuclear cells derived from the brain of Plp-Akt-DD and WT animals by 30%-70% percoll density gradient centrifugation were cultured with or without 10 ng/ml of IFN γ overnight. Cells were stained for microglia cell surface markers with anti-CD11b-FITC, anti-CD45-FITC and anti IA^q-PE labeled antibodies (BD Pharmingen, San Jose, CA). Cells were acquired on BD FACS Scan flowcytometer and data analyzed using the Cell Quest Pro software.

Statistical analysis

The Mann Whitney U test was conducted to evaluate the significance of differences in daily mean clinical EAE scores. All other probability values were calculated using the independent Students t test. mRNA data were analyzed using the GraphPad Software (GraphPad Prism, La Jolla, CA).

Results

Hypermyelinated transgenic mice develop more severe EAE compared to their WT littermates

In our earlier studies, we demonstrated hypermyelination in the CNS of Plp-Akt-DD transgenic mice compared to their WT littermates as a result of constitutive Akt expression in oligodendrocytes (Flores et al., 2008). To test if increase in myelin content can influence onset and progression of autoimmune disease, 6-8 week old PlpAkt-DD transgenic females and their age and sex matched WT littermates (n=8) were induced with EAE by immunization with the encephalitogenic PLP 104-117 peptide. Since the plp-Akt-DD transgene has been established on the SWR background we used the IA^q restricted, PLP-104-117/SWR murine model of EAE for our studies. Mice were weighed and evaluated daily for neurologic signs over a period of 30 days after immunization. Plp-Akt-DD mice with EAE showed significantly higher disease severity as determined by increased mean clinical scores over time ($p < 0.026$; z score of 2.218; Figure 1a) and significantly greater body weight loss during the course of EAE ($p < 1.6 \times 10^{-4}$; Figure 1b), compared to their WT littermates with EAE. No significant differences were observed in time to onset of EAE (Time to onset 14.5 ± 0.5 days for Akt transgenics and 15.5 ± 3.5 days for WT mice; $p < 0.49$). The range of disease scores within the WT and transgenic groups from onset (day 12 after disease induction) till about day 18 after disease induction was similar with non-significant differences in mean clinical scores ($p = 0.06$). By day 19 after disease induction (approximately 1 week after onset), 5 out of 9 transgenic mice had progressed to a clinical score of 4 or more at one point in their disease progression history compared to only 1 of 8

WT mice. The greatest and most significant differences between disease scores were seen beginning at day 23 after disease induction (10-11 days after disease onset) as the clinical scores in the WT mice start improving and the transgenic mice continue progressing ($p < 4.1 \times 10^{-5}$).

The Plp-Akt-DD transgenic mouse EAE model shows unique brain involvement and associated pathology similar to that seen in human MS

Unlike conventional EAE models in the murine SWR/SJL strains, where CNS pathology is mainly restricted to the spinal cord, Plp-Akt-DD mice with EAE displayed unique disease pathology with brain involvement similar to that seen in human MS. During acute EAE hypermyelinated transgenics showed significant CD3+ T cell infiltration (Figure 2a, left panel) in the brain unlike their WT littermates with similar disease severity where no significant T cell presence in the brain was observed (Figure 2a, right panel). During the acute phase of EAE, T cell infiltrates in the Plp-Akt-DD transgenic brain were restricted to perivascular spaces and the hippocampal fissure between the hippocampus and thalamus, adjacent to the third ventricle as shown in figure 2a. Upon progression to chronic phase of disease, 8 weeks post immunization, the mononuclear cell infiltrates persisted around the hippocampal fissure with further extension into the brain parenchyma as shown (left panel Figure 2b). Mononuclear T cell infiltrates were also observed around the lateral ventricles, fimbria and the adjacent brain parenchyma (left panel Figure 2c). On the other hand, in age and sex matched WT littermates, no significant T cell infiltration into the brain parenchyma was observed both during acute and chronic EAE (right panels in Figures 2). The clinical scores of mice subjected to immunohistochemical evaluation of brain infiltration ranged from 3-3.5 for Plp-Akt-DD transgenics and from 0-3 for WT littermates.

Although we see hypermyelination in the spinal cords of the Plp-Akt-DD transgenics, and extensive infiltration of the spinal cords both in the WT and transgenic mice during acute EAE, no differences in severity of cellular infiltration were observed between the transgenic and WT spinal cords at the single time point (during the acute phase of EAE) examined in the current study (data not shown). Examination of spinal cord infiltrates later during disease progression is required. Since more obvious and significant differences were observed in inflammation in the brain between WT and hypermyelinated transgenics we focused on the contributory factors in the brain for the rest of the study.

EAE associated pathology in Plp-Akt-DD transgenic brain can be documented by MRI

During the chronic phase of EAE, histological evaluation of the PLP-Akt-DD transgenic brain revealed presence of bilateral, periventricular and hippocampal region T cell infiltrates (Figure 2b, 2c) similar to that seen in human MS. To document and correlate histologically defined brain pathology with MRI, Plp-Akt-DD and WT mice were imaged during the chronic phase of EAE, 8 weeks post disease onset by DTI weighted MRI. DTI images of Plp-Akt-DD transgenic mice show visibly larger ventricle diffusion areas (Figure 3, upper panel) when compared to images from WT animals with comparable severity of EAE. (Figure 3, lower panel). The relative brain to ventricular volume ratio in the transgenic animals was quantified by drawing regions of interest for the outer ventricle diffusion areas of the brain. The mean ventricular to brain volume ratio in Plp-Akt-DD transgenics was calculated to be 0.0202 compared to 0.0071 in WT littermates reflecting an increase of 185% in the ventricular volume of transgenic animals, indicating substantial loss of brain parenchyma. Although the observed increase in ventricle size by MRI in transgenic mice could be attributed to numerous structural reasons such as elevated CSF pressure, our immunohistochemistry data showing significant cellular infiltration corresponding to the same area (Figure 2) is compelling evidence in favor of an immunological cause leading to loss of brain parenchyma.

Increased severity of EAE in Plp-Akt-DD transgenics is not due to enhanced pro-inflammatory immune responsiveness of peripheral lymph node cells

To establish an etiology for the enhanced severity of EAE in the hypermyelinated Plp-Akt-DD mice, we compared peripheral lymph node immune responses in transgenics and their WT littermates. Briefly, ten day PLP 104-117 primed lymph nodes from female Plp-Akt-DD and WT littermates (n=5) were tested for IL-17 and IFN γ production in recall response to different doses of PLP 104-117 or Ovalbumin by ELISPOT assays. No significant differences in IL-17 (p=0.98; Figure 4a) or IFN γ (p=0.947; Figure 4b) production in response to PLP 104-117 was observed in peripheral lymph nodes of transgenic or WT mice suggesting that enhanced disease severity in the hypermyelinated mice is primarily caused by factors characteristic of the Plp-Akt-DD CNS with minimal or no contribution by cells derived from the periphery.

Plp-Akt-DD transgenics displayed more efficient antigen presentation of intrinsically derived CNS antigens compared to WT

To test for differences in antigen presentation within the CNS, brain derived mononuclear cells from naïve Plp-Akt-DD and WT littermates, were tested for their efficiency of presenting intrinsically derived self-antigen to primed T cells from WT mice. Briefly, any hematogenously derived cells in the naïve brain were cleared by thorough intracardiac perfusion with PBS. Brain tissue was homogenized and subject to discontinuous density gradient centrifugation. Brain derived total mononuclear cells at the 30%-70% percoll interface were collected and activated with 10ng/ml of murine IFN γ overnight to enhance MHC class II expression. Total mononuclear cells derived from naïve brain were co-cultured for 72 hours in pre-coated ELISPOT plates with Thy1.2 positively selected, PLP 104-117 primed T cells derived from WT mice at ratios of 1:5, 1:10, 1:20 and 1:40 brain mononuclear cells:T cells, keeping the number of primed T cells constant at 2×10^5 T cells per well. No antigen was exogenously added to the cultures in order to measure the intrinsic potential of brain resident APCs to present endogenously derived MHC bound antigens without disturbing their native, tissue characteristic cell surface peptide-MHC composition. Analysis of IL-17 (Figure 5a) and IFN γ (Figure 5b) spot forming units revealed that the brain derived total mononuclear cell population from the Plp-Akt-DD mice, when presenting endogenously derived antigens *in vitro*, elicited significantly higher recall responses from primed T cells compared to equal numbers of brain derived total mononuclear cells from WT mice. In other words, a smaller number of mononuclear cells was required to present antigen and activate T cells when the mononuclear populations was derived from the brain of hypermyelinated transgenic mice.

Antigen presenting microglial cells purified from naïve Plp-Akt-DD transgenic and WT mice do not differ in their antigen presenting capacity and elicit similar T cells recall responses

To determine if the enhanced efficiency of antigen presentation in Plp-Akt-DD transgenics is a property of individual brain resident APCs or merely due to differences in number of APCs within the brain of the hypermyelinated transgenics, we positively selected microglia (the resident APCs in the brain) from the total brain mononuclear cells population described above using CD11b magnetic beads. Since expression of CD11b is not exclusive to microglia there might be a contaminating component of blood derived monocytes and macrophages in the microglia preparations. However, since the microglia were derived from naïve mice minimal brain infiltration and insignificant contamination of the microglia preparation is expected. Purified microglia were cultured overnight with or without 10 ng/ml IFN γ , washed and co-cultured in defined ratios with PLP 104-117 primed peripheral T cells from WT animals in antibody coated ELISPOT plates. Both the naïve Plp-Akt-DD and WT brain resident microglia elicited comparable T cell recall responses to endogenous self

antigens. No significant differences in T cell recall responses were observed when antigen was presented in context of brain resident microglia whether activated with IFN γ or not-activated *in vitro* (Figure 6a and b; $p=0.66$ and 0.8 respectively). The apparent lack of differences in antigen presentation between microglia with or without *in vitro* IFN γ activation is either due to the pre-activated state of microglia derived from the CNS of transgenic mice or procedure induced activation of microglia during isolation. These data show that upon normalization of absolute numbers of antigen presenting microglia, no differences in antigen presentation efficiency were evident between transgenic and WT animals, indicating lack of any major contributions related to differences in activation status or cell surface pMHC complex density per microglial cell. These findings strongly suggest that observed enhanced efficiency of antigen presentation in the transgenics is due to presence of a higher proportion of competent APCs (resident microglia) in the brain derived mononuclear cell population of hypermyelinated transgenics.

Higher numbers of activated infiltrating T cells are observed in the brain of Plp-Akt-DD transgenic mice during EAE compared to wild type animals with EAE

An increase in number of APCs in the Plp-Akt-DD brain would provide increased availability of cognate pMHC interactions and create an optimal environment for quicker transition and greater activation of T cells infiltrating the brain during EAE. In order to identify differences in number of activated T cell, brain infiltrating T cells were isolated from Plp-Akt-DD mice and WT mice at peak of EAE using percoll density gradient centrifugation and further enriched by nylon wool column elution. Equal numbers of T cells isolated from WT and hypermyelinated transgenics were cultured in IFN γ antibody coated ELISPOT plates for 72 hours, without restimulation in order to assess their native activation state. Although very few brain infiltrating T cells were obtained from WT animals at peak of disease, comparison with those derived from hypermyelinated mice with EAE revealed approximately 3 fold higher frequencies of IFN γ producers in the Plp-Akt-DD brain infiltrating T cells (Figure 7) indicating enhanced numbers of activated CNS infiltrating T cells in the transgenics correlated with enhanced disease severity in the transgenics. Typically, we get greater than 90% purity of T cells with the nylon wool column purification method. However, there is the possibility of co-purification of a minor population of antigen presenting cells with the isolated T cell population by this method. In that event, these data would still reflect a higher frequency of activated, endogenous myelin reactive T cells infiltrating the brain of PLP-Akt-DD transgenics compared to the WT mice.

PLP-Akt transgenic mice have increased numbers of CD45, CD11b, Iba1 positive microglia in the brain compared to their wild type littermates

To finally establish that the observed increase in EAE severity and enhanced activation of infiltrating cells in the hypermyelinated brain is due to the presence of a larger number of resident microglia, we analyzed the number of CD45, CD11b, Iba1 positive microglia in the brain parenchyma of naïve disease free Plp-Akt-DD and naïve disease free WT mice by flow cytometry, immunohistochemistry and quantitative RT-PCR. Mice were perfused and mononuclear cells isolated from the brain on a discontinuous 30%-70% percoll gradient as described above. Brain derived total mononuclear cell preparation were stained for microglial surface markers with CD11b and CD45, FITC labeled antibodies and analyzed by flow cytometry. The flow histogram plots reveal an approximately two fold increase in number of CD11b (Figure 8a, left panel) and CD45 (Figure 8a, right panel) positive cells in the Plp-Akt-DD transgenic brain compared to WT animals. The finding was further confirmed by demonstration of a significant increase in number of Iba1 positive cells in coronal brain sections of Plp-Akt-DD mice compared to age and sex matched WT littermates by immunohistochemistry (Figure 8b). Further, expression of Iba1 relative to GAPDH was increased in brain tissue from two month old naïve Plp-Akt-DD mice

compared to their age and sex matched naïve WT littermates ($n=3$; $p<0.029$; Figure 8c). Increased number of microglia were also observed in the spinal cords of the Plp-Akt-DD transgenic mice compared to WT mice (data not shown).

Discussion

The current study was designed to determine the impact of self-antigen load on onset and progression of organ specific autoimmunity. Results from our study demonstrate that a larger self-antigen load within a tissue can predispose to enhanced autoimmunity by facilitating increased antigen presentation and self-reactive T cell activation at the site of inflammation. Using a transgenic mouse model characterized by CNS hypermyelination, we show that increase in severity of EAE in hypermyelinated transgenics is mostly attributable to a concurrent increase in the number of CNS resident microglia, likely due to activation of antigen clearance mechanisms triggered by the increased antigen load. Our study is the first to establish and provide experimental evidence for the concept that any increase in antigen load even if it is to an already abundant pool of antigen can influence autoimmunity; an effect that can be mediated merely by an increase in the number of scavenger APCs within the target tissue.

The relationship between total antigen content, its presentation by APCs and downstream T cell responses has been a key focus of cellular immunology. It has been suggested that induction of T cell activation and effector differentiation requires a threshold antigen dose whereby serial encounters of recirculating T cells with APC, as a means to ‘measure’ the overall amount of antigen in lymph nodes, incrementally contribute to the probability of reaching the activation threshold. Therefore, the kinetics of T cell transition to the activation phase is directly correlated to the number of cognate pMHC complexes per DC as well as with the density of antigen-presenting DCs per lymph node (Henrickson et al., 2008). Along the same lines we hypothesized that during progression of autoimmune diseases, similar T cell-APC interaction dynamics are likely to exist at the site of inflammation within the target tissue and “increase in load of self antigen within the targeted tissue will lead to more severe autoimmune disease”.

It is compelling to consider the myelin content in the CNS to be a large, unlimited source of antigen where any further increase in the antigenic supply may apparently not be of much immunological consequence. However, in spite of being the most abundant protein in the CNS, myelin gene expression is regulated in a spatiotemporal manner with maximal levels of expression occurring in oligodendrocytes during the active myelination period of CNS development (Wight and Dobretsova, 2004). It has been documented through numerous studies in mouse models that overexpression or deficiency of one or more myelin component in the CNS can result in induction of compensatory protein clearance mechanisms such as increase in numbers of CD11b positive microglia, phagocytosis and subsequent autoimmune inflammation (Ip et al., 2008; Karim et al., 2007; Leder et al., 2007; Muller et al., 2007). Earlier studies by our group revealed an increase in microglia numbers concurrent with increase in myelin content with progression of age in the Plp-Akt-DD transgenic mice (Flores et al., 2008). In the current study we show that a larger self antigen load can influence an increase in the number of resident APCs predisposing to enhanced CNS autoimmunity. The reasons for increase in number of microglia in a hypermyelinated CNS instead of increase in density of pMHC complexes per microglial cell are not entirely clear to us. We hypothesize that a higher than normal protein content in the transgenic brain starting at early stages of development could trigger homeostatic mechanisms within the organ for enhanced protein clearance through increased phagocytosis by scavenging microglia. Whether, the increased number of microglia in the brain stems from proliferation

of resident microglia or recruitment from the periphery is not defined in the current study and will be studied as part of subsequent investigations.

The importance of resident microglia in initiating and propagating adaptive immune response in the CNS is well recognized (Mack et al., 2003; Ponomarev et al., 2005; Cassiani-Ingoni et al., 2007; Olson and Miller, 2004). It is well recognized that microglia can function as APCs for memory T cells or T cells activated in the periphery to further propagate the immune response within the CNS. Activation of autoreactive T cells by APCs is a complex phenomenon that might be limited by various factors such as repertoire availability of self-reactive T cell, density of pMHC complexes per APC, activation status of APCs and variations in activation thresholds for self vs infectious antigens. We demonstrate here that a mere increase in numbers of tissue resident APCs allows increased access to the unlimited self-antigen pool predisposing to more severe autoimmunity and extensive cellular infiltration of the brain. Although, the current study did not show any differences in the severity of cellular infiltration in the spinal cord of the hypermyelinated transgenics compared to the WT mice during acute EAE, further studies examining the cords at later time points during EAE progression are required to confirm this observation. Recent studies have demonstrated differences between brain and spinal cord microglia in expression of surface molecules, inflammatory mediators, infiltration of Th1 vs Th17 cells and contribution to pathology of disease study (Olson JK, 2010; Batchelor et al., 2008; De Haas et al., 2008; Murphy et al., 2010), all or some of which could explain the lack of enhanced infiltration in the hypermyelinated transgenic spinal cords at the time point examined in the current study.

Myelin phagocytosis by infiltrating macrophages and activated microglia is a key determinant of disease progression. Phagocytic activity of microglia involving clearance of apoptotic cells and debris from the CNS is a well controlled process of maintaining the balance between repair and regeneration within the CNS (Napoli and Neumann 2010; Kigerl et al., 2009; Gitik et al., 2011). In addition, microglia have been shown to function as divergent subsets (M1/M2) that play a critical role in maintaining the balance between tissue homeostasis/regeneration and inflammatory immune responses within the CNS (Merson et al., 2010; Kiegl et al., 2009). In view of the existence of this homeostatic balance between antigen load, remodeling, and scavenging of the protein from within the tissue it is reasonable to assume that any disruption in the cellular machinery maintaining this balance such as increase in antigen load, increase in APC numbers or both would have detrimental immunological consequences. Under this caveat it might be important to proceed with caution in application of regenerative therapeutic strategies for autoimmune demyelinating diseases involving myelin overexpression or constitutive activation of factors enhancing oligodendrocyte or progenitor cell survival. Such strategies might be beneficial under conditions of prior extensive demyelination but would require careful control of the balance between damage reduction and myelin accumulation, so as to maintain a total myelin load not more than that in an uninjured brain. In the current study, we demonstrate that the critical balance between self-antigen load and APCs numbers is crucial for onset and progression of autoimmunity in the CNS, a concept that may be extended to autoimmune diseases affecting tissue other than the brain. Current need for treatments aimed at manipulation of numbers (Heppner et al., 2005), activation status and phagocytic capacity of macrophages/microglia has been suggested (Koning et al., 2009). Results from our study provide experimental evidence for the key role played by microglia in CNS targeted autoimmunity and highlight the immense potential of such pharmacological or immunomodulatory strategies targeted to specific characteristics of tissue resident APCs for halting progression of MS.

Finally, the Plp-Akt-DD transgenic EAE mouse model by virtue of its unique pathology with brain involvement similar to that seen in human MS and its amenability to MRI imaging provides a novel EAE model system, more translational for MS research than conventional EAE mouse models. As previously described by us, the Plp-Akt-DD mice show CNS hypermyelination beginning at early stages of development with no effect on the myelin content of the peripheral nervous system and no increase in number of oligodendrocytes at any stage of development (Flores et al., 2008). Upon induction of EAE, the Plp-Akt-DD transgenic mice present with brain pathology characterized by substantial mononuclear infiltration in and around the hippocampus as well as the classical “lymphocyte cuffs” around small blood vessels in the brain parenchyma, with chronic progression to bilateral periventricular infiltrates and substantial loss of white matter quantifiable as significantly increased ventricle to brain volume ratios by high resolution MRI. The transgenic Plp-Akt-DD mouse model is unique in its characteristic increase of total CNS myelin content beginning from embryonic stages rather than an imbalance in selected myelin protein components and therefore represents a more balanced physiological state of the CNS to test our hypothesis on the impact of total self-antigen content on onset and progression of autoimmunity. Also, the increase in CNS myelin content in the transgenics due to constitutively active Akt expression is restricted by the PLP promoter only to cells of the oligodendrocyte lineage and therefore excludes the impact of any other anti-apoptotic or immunomodulatory effects of Akt other than the consequence of its expression in oligodendrocytes i.e. hypermyelination in the brain.

In conclusion, our study is the first to experimentally define the impact of self antigen content within a targeted tissue on onset and progression of autoimmunity. Any increase in self antigen load even to an already “unlimited” pool of antigen is associated with an increase in phagocytic tissue resident APCs in order to maintain tissue structure and homeostasis. However, under additional conditions associated with breakdown of tolerance these otherwise benign changes can have detrimental immunological consequences such as heightened reactivity to self and more severe autoimmunity. In addition, our study highlights the importance of the role played by tissue resident APC in onset and progression of autoimmune responses and brings to light the significance of therapeutic strategies targeted towards manipulation of tissue resident APC function as treatment for autoimmune diseases such as EAE/MS.

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Figure 1. Plp-Akt-DD transgenic mice develop more severe EAE compared to WT littermates
 WT and Plp-Akt-DD transgenic female mice (n=8) were induced with EAE. Mice were weighed and evaluated daily for neurologic signs over a period of 30 days after immunization. Compared to WT mice transgenic mice showed significantly more severe disease as determined by (a) significantly greater increase in mean clinical scores over time ($p < 0.026$; z score 2.218) (b) reduction in body weight (gms) during the course of disease progression ($p < 1.6 \times 10^{-4}$). Error bars depict $\pm$ SE

Figure 2. Significantly increase CD3 positive T cell infiltrates are seen in the brain of Plp-Akt-DD transgenic mice compared to WT during acute and chronic phases of EAE

At peak of EAE onset (a) and during chronic EAE, 8 weeks post onset of EAE (b and c), brain tissue from Plp-Akt-DD transgenic and WT mice was perfused and sliced into 10 μ m frozen sections. Immunohistochemical staining with CD3 antibody shows significantly increased CD3+ mononuclear cell infiltrates around the perivascular spaces in the brain of Plp-Akt-DD transgenic mice (*left panel*) compared to WT (*right panel*) during acute phase of EAE (a) and progressing during the chronic phase of the disease to the hippocampus region extending into the brain parenchyma (b) as well as the fimbria and adjoining parenchyma (c). Representative sections presented in figure 2b and 2c to depict brain infiltration during chronic EAE were derived from mice with clinical scores ranging from 3-3.5 for Plp-Akt-DD transgenics and 0-3.5 for WT mice. Size bar =100 μ m.

Figure 3. Alterations in brain morphology can be documented by MRI in Plp-Akt-DD transgenic mice with EAE

Eight weeks post onset of EAE (chronic phase EAE) Plp-Akt-DD transgenic mice with and without EAE, were imaged using a 7T magnet. T2 weighted MRI images (n=2) show visibly larger ventricle diffusion areas in Plp-Akt-DD transgenic mice (*upper panel*) compared to WT littermates with similar disease severity (*lower panel*). The relative brain to ventricular volume ratio in Plp-Akt-DD transgenic animals, quantified by drawing ROIs for the outer ventricle diffusion areas and the brain was found to be 0.0202 compared to 0.0071 in WT littermates reflecting an increase of 185% in transgenic animals consistent with substantial loss of brain parenchyma.

Figure 4. Plp-Akt-DD transgenic and wild type littermates have similar peripheral lymph node recall responses to the priming PLP 104-117 antigen

Ten days after priming with PLP 104-117 peptide, lymph node cells from Plp-Akt-DD transgenic and WT female mice (n=5) were restimulated with PLP 104-117 or OVA peptide in precoated ELISPOT plates for 72 hours. No significant differences were found in peripheral recall immune responses to the priming PLP 104-117 epitope between Plp-Akt-DD transgenic and WT animals as evidenced by frequency of (a) IL-17 (p=0.98) and (b) IFN γ (p=0.947) producing lymph node cells. Error bars depict $\pm$ standard error.

Figure 5. Mononuclear cell population derived from the naïve Plp-Akt-DD transgenic brain can activate T cells with higher efficiency compared to that derived from the WT brain

Naïve Plp-Akt-DD and WT female mice were perfused through the intracardiac route with PBS (n=8). Mononuclear cells were extracted from brain homogenates by discontinuous density gradient centrifugation on a 30%-70% percoll gradient of brain homogenates and further activated with 10 ng/ml of IFN γ overnight. Pooled mononuclear cell preparation from n=8 naïve mice was used as antigen presenting cells (APC) to primed T cells derived from wild type littermates. T cells were purified using Thy1.2 positive magnetic beads from PLP 104-117 primed, ten day peripheral lymph nodes from WT SWR female mice. Brain derived mononuclear cells from naïve Plp-Akt-DD transgenic and wild type littermates and 3×10^5 primed T cells from wild type mice were co-cultured at ratios 1:5, 1:10, 1:20 and 1:40 (APC:T cells) in antibody coated ELISPOT plates. Equal numbers of naïve Plp-Akt-DD transgenic brain derived mononuclear cells used as APCs can elicit significantly stronger recall responses from primed T cells when compared to mononuclear cells derived from naïve wild type animals as evident by higher frequencies of (a) IL-17 (p=0.036) and (b) IFN γ (p=0.038) spot forming units per 2×10^5 T cells after 72 hours of culture.

Figure 6. Microglia selected from naïve Plp-Akt-DD transgenic or WT brain do not differ in their antigen presenting capacity and elicit similar T cell recall responses

Microglia were purified using CD11b coated magnetic beads from the mononuclear cell population derived from brains of naïve Plp-Akt-DD transgenic and WT animals (n=8). Purified and pooled populations of positively selected microglia were cultured overnight with (a) or without (b) 10 ng/ml IFN γ . Defined ratios of naïve microglia to PLP-104-117 primed peripheral T cells from WT mice were co-cultured in antibody pre-coated ELISPOT plates and evaluated for presence of IL-17 specific spot forming units. No significant differences were observed in primed T cell recall responses when antigen was presented by equal numbers of (a) activated or (b) non activated-Plp-Akt-DD transgenic or WT derived microglia (p=0.66 and 0.8 respectively).

Figure 7. Higher numbers of activated infiltrating T cells are observed in the brain of Plp-Akt-DD transgenic mice during EAE compared to wild type animals with EAE

At peak of EAE onset, brain infiltrating T cells were isolated from WT and Plp-Akt-DD mice (n=6) using percoll density gradient centrifugation and further enrichment by nylon wool columns. Infiltrating T cells from different mice were pooled together and cultured in IFN γ antibody coated ELISPOT plates for 72 hours without any antigenic stimulation and frequencies of IFN γ spot forming units were determined. Higher frequencies of IFN γ producing T cells were observed in the brain infiltrates derived from Plp-Akt-DD transgenics compared to those derived from WT littermates.

Figure 8. Plp-Akt-DD transgenic mice have increased numbers of CD45, CD11b and MHC class II positive cells compared to SWR WT mice

Mononuclear cells were isolated from the brain of naïve Plp-Akt-DD transgenic and naïve WT female mice and stained for CD11b-FITC and CD45-FITC microglial surface markers. (a) Flow cytometry analysis shows an approximate two fold increase in number of CD11b (*left panel*) and CD45 (*right panel*) positive cells in the naïve PLPAkt-DD transgenic brain (black line) compared to naïve WT animals (grey line). An increase in number of microglia was demonstrated in the naïve PLP-Akt-DD transgenic brain compared to naïve wild type animals by (b) immunohistochemical staining for Iba1 expression in the hippocampal region (c) Real time quantitative PCR for Iba1 gene expression normalized to GAPDH expression

(n=4; $p<0.029$). The expression of Iba-1 mRNA in the WT brain has been assigned an arbitrary value of 1.