Short communication

Immunosuppression alters disease severity in juvenile Batten disease mice

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**Abstract**

Autoantibodies to brain proteins are present in Juvenile Neuronal Ceroid Lipofuscinosis (Batten disease) patients and in the Cln3−/− mouse model of this disease, suggesting an autoimmune component to pathogenesis. Using genetic or pharmaceutical approaches to attenuate this immune response in Cln3−/− mice, we demonstrate decreased neuroinflammation, decreased deposition of immunoglobulin G in the brain and protection of vulnerable neuron populations. Moreover, immune suppression results in a significant improvement in motor performance providing for the first plausible therapeutic approach for juvenile Batten disease.

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1. Introduction

Mutations in CLN3, which encodes an endosomal/lysosomal transmembrane protein of unknown function, form the genetic basis of Juvenile Neuronal Ceroid Lipofuscinosis (JNCL or Batten disease). JNCL is the most prevalent subtype in a family of lysosomal storage disorders known as the neuronal ceroid lipofuscinoses (NCL), of which there are at least 8 genetic variants with similarities in their clinical features and pathologies including progressive loss of vision, seizures, and severe cognitive and motor decline, eventually leading to premature death (Goebel and Wisniewski, 2004; Mole, 2004; Siintola and severe cognitive and motor decline, eventually leading to premature death (Goebel and Wisniewski, 2004; Mole, 2004; Siintola et al., 2006).

Individuals with JNCL raise autoantibodies against a variety of brain antigens, two of which have been identified as glutamic acid decarboxylase 65 kDa (GAD65) and α-fetoprotein (Castaneda and Pearce, 2008; Chattopadhyay et al., 2002a, b; Ramirez-Montealegre et al., 2005). Immunostaining rat and human brain tissue using JNCL patient derived sera revealed distinct patterns of immunoreactivity that was not restricted to GAD65 positive neuronal populations, suggesting multiple antigenic targets for these circulating autoantibodies (Lim et al., 2006). Autoantibodies are also present in Cln3−/− mice (Mitchison et al., 1999), thus providing a valuable tool for studying the contribution of autoimmunity in JNCL pathogenesis (Chattopadhyay et al., 2002a). Previous studies to have demonstrated an inhibitory effect of GAD65 autoantibodies on glutamate metabolism in vitro (Chattopadhyay et al., 2002a), a compromised blood brain barrier (BBB) and infiltration of immunoglobulins (IgG) into the brain (Lim et al., 2007, 2006). Using both genetic and pharmaceutical approaches, we demonstrate that immune suppression alleviates behavioral and pathological deficits in Cln3−/− mice.

2. Materials and methods

2.1. Animals

C57Bl/6 congenic μMT mice were purchased from Jackson Labs (Bar Harbor, ME) and subsequently backcrossed with 129SvEv wildtype or Cln3−/− mice for 10–12 generations. Mouse strains used in this study were 129SvEv, Cln3−/−, μMT, and Cln3−/−/μMT. Mice were genotyped by PCR as described previously (Kitamura et al., 1991). Rat tissue for immunoblotting was harvested from Sprague–Dawley rats obtained from the Jackson Labs. All procedures were carried out following NIH guidelines and under the guidance of the university committee on animal resources at the University of Rochester.

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2.2. Mycophenolate motefil treatments

Mycophenolate motefil (MMF) was a kind gift from Roche Pharmaceuticals (Nutley, NJ). Daily drug treatments were started at P32 for wildtype and Cln3−/− mice by gavage feeding with 60 mg/kg MMF dissolved in non-fat milk or with non-fat milk alone (placebo) for 30, 70, or 150 consecutive days.

2.3. Motor performance testing

Motor performance was assessed using an accelerating rotarod (AccuScan Instruments, Columbus, OH) (0 to 30 rpm over 240 s) at P62, P102, and P182 following the completion of the daily drug regimen. On the day of testing, mice were subjected to a training period consisting of 3 independent trials (3 runs per trial) with an interval of 10–15 min between trials. The mice were then rested for a period of 3 h, following which they were tested in 3 independent trials (3 runs per trial, 10–15 min interval between trials) and the latency to fall was recorded and averaged over a total of 9 runs.

2.4. Immunohistochemical staining

Immunostaining was performed on free-floating sections as previously described (Bible et al., 2004) using the following antibodies; rabbit anti-Fab2 fragment of the mouse IgG (1:500, AbD Serotec, Oxford, UK); rabbit anti-GFAP antibody (1:4000, Dako, Cambridgeshire, UK); rat anti-mouse F4/80 antibody (1:100, AbD Serotec); rat anti-mouse CD68 antibody (1:100, AbD Serotec).

2.5. Quantification of neuronal number

Unbiased optical fractionator estimates of the total number of neurons from Nissl stained dorsal lateral geniculate nucleus (LGNd) and medial deep cerebellar nuclei (DCN) sections were obtained using

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Fig. 1. B-cell deficient Cln3−/− mice exhibit reduced neuroinflammatory responses and improved motor performance. Male mice were used in this study. Double mutant Cln3−/−/μMT mice do not have circulating autoantibodies in comparison to Cln3−/− mice as shown by immunoblotting (A) and by the lack of IgG deposition in cortical sections (B). (C) Optical fractionator counts of neuronal numbers in the DCN and LGNd at P180. Cln3−/−/μMT mice have significantly more neurons in the DCN in comparison to Cln3−/−, and are statistically indistinguishable from WT mice. (D) Expression of GFAP and F4/80, markers of activated astrocytes and microglia respectively, is markedly reduced in the cortex and the cerebellum of P180 Cln3−/−/μMT in comparison to Cln3−/−. Inset, higher magnification image showing the morphology of GFAP-positive astrocytes and F4/80-positive microglia in the cortex. (E) Motor performance, as measured by the accelerating rotarod, is improved in P60 and P100 Cln3−/−/μMT mice as compared to Cln3−/− mice, and is indistinguishable from WT. At P180, the performance of both Cln3−/−/μMT and μMT mice is markedly reduced in comparison to WT and Cln3−/− mice, likely due to the detrimental long-term effects of immune suppression. * indicates p < 0.05 in comparison to WT.

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Stereoinvestigator™ (MBF Bioscience, Williston, VT) as previously described (Bible et al., 2004). Mean co-efficient of error for all individual optical fractionator estimates was calculated according to Gundersen and Jensen (1987) and was less than 0.09 in all analyses. Data were analyzed by one-way ANOVA with Bonferroni’s post-hoc t-test.

2.6. Immunoblotting

Autoantibodies were detected by western blotting as previously described (Chattopadhyay et al., 2002a). Briefly, brain homogenates from embryonic day 18 (E18), postnatal day 8 (P8) and adult Sprague–Dawley rats were probed with mouse serum (diluted 1:1000) collected at P182. Bound autoantibodies were visualized using anti-mouse IgG-HRP and ECL Plus reagent (GE Healthcare, Piscataway, NJ).

3. Results

Immune deficient Cln3−/− mice were generated by backcrossing Cln3−/− mice with B-cell deficient μMT mice (Kitamura et al., 1991). These Cln3−/−/μMT mice were incapable of generating endogenous IgGs as shown by reduced serum immunoreactivity to rat brain protein extracts in comparison to wildtype (WT) and Cln3−/− mice (Fig. 1A). Immunohistological staining demonstrated a lack of IgG deposition in the brain of Cln3−/−/μMT mice, a phenotype that is readily observed in Cln3−/− mice (Fig. 1B) and JNCL patients (Lim et al., 2007). This was accompanied by a reduction in glial fibrillary acidic protein (GFAP) and F4/80 staining, markers of astroglial and microglial activation respectively, indicative of reduced neuroinflammation (Fig. 1D). Cln3−/− mice display a late onset neurodegeneration, but populations of thalamic relay neurons and deep cerebellar neurons are already lost by 6 months of age (Weimer et al., 2009, 2006). Optical fractionator counts from Cln3−/−/μMT, Cln3−/− mice and WT controls revealed significantly more deep cerebellar nuclei neurons in Cln3−/−/μMT mice (P<0.05, Two-way ANOVA), with similar numbers of these neurons as seen in wild type mice at P180 (Fig. 1C). An increased number of thalamic LGNd neurons was evident in Cln3−/−/μMT mice, but this did not reach statistical significance.

In the accelerating rotarod test (Kovacs and Pearce, 2008), Cln3−/−/μMT mice performed significantly better than Cln3−/− mice at P60 (p<0.001, Two-way ANOVA with Tukey’s post-hoc test) and were statistically indistinguishable from WT and μMT mice (Fig. 1E). At P100, Cln3−/−/μMT mice again out-performed Cln3−/− but did not reach statistical significance, although their performance was lower than that of WT mice.

Fig. 2. Oral administration of the immunosuppressant mycophenolate mofetil (MMF) improves motor performance and reduces neuroinflammation in Cln3−/− mice. Male mice were used in this study. Mycophenolate-treated Cln3−/− mice have reduced serum immunoreactivity to autoantigens in comparison to Cln3−/− placebo-treated mice (A) with no apparent deposition of IgG in the brain (B). (C) Expression of GFAP and CD68, markers of activated astrocytes and microglia respectively, is markedly reduced in the cortex and the cerebellum of MMF-treated P182 Cln3−/− mice in comparison to Cln3−/− placebo-treated mice. Inset, higher magnification image showing the morphology GFAP-positive astrocytes and CD68-positive microglia in the cortex. (D) Motor performance, as measured by the accelerating rotarod, demonstrates a performance vs. group effect with significant improvements in MMF-treated Cln3−/− mice that are indistinguishable from WT placebo in all age groups.

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performance was statistically indistinguishable from WT. At P180, we observed a decreased performance of both Cln3−/−/μMT and μMT mice on the rotarod, likely resulting from the detrimental effects of prolonged immune deficiency on the general health of these mice.

Collectively, these data provide support to the notion that autoantibodies present in JNCL have a pathological role, since genetic blockade of their production in Cln3−/−/μMT mice ameliorates both neurologic and reactive changes associated with CLN3 deficiency and offers some protection to vulnerable neuron populations.

Since Cln3−/− mice raise a wide repertoire of brain-directed autoantibodies (Castaneda and Pearce, 2008; Lim et al., 2006), we opted to test Mycophenolate motefill (MMF), known commercially as CellCept (Roche Pharmaceuticals). MMF inhibits inosine monophosphate dehydrogenase, an enzyme involved in the de novo pathway of purine synthesis in proliferating B and T lymphocytes, thereby attenuating any immune response. MMF (60 mg/kg) dissolved in non-fat dry milk was administered to WT and Cln3−/− mice by daily gavage feeding for a total of 150 days, with placebo receiving only non-fat dry milk. Drug treatments were started at postnatal day 32 (P32), and rotarod testing was performed at P62, P102, and P182. Cln3−/− mice treated with MMF showed significantly improved rotarod performance compared to Cln3−/− mice treated with placebo showing a performance vs. group effect (Fig. 2D, Mauchly χ2 = 0.279, χ2 > 0.75, Hung–Feldt F = 2.023, p < 0.001, repeated measures MANOVA). MMF treatment attenuated the immune response with a marked reduction in serum immunoreactivity for many but not all autoantibodies in MMF-treated mice (Fig. 2A) and in the absence of IgG deposits in the brain (Fig. 2B). MMF-treated Cln3−/− mice also have reduced neuropil inflammation as indicated by a decrease in GFAP and CD68 immunoreactivity and a decreased number of activated CD68+ microglia in widespread brain regions (Fig. 2C). MMF also produced a positive effect upon both DCN and LGNd neurons, but due to the wide variation in MMF-treated Cln3−/− mice this neuroprotective effect was not statistically significant (not shown).

4. Discussion

Our data provide the first direct evidence for a positive effect of any therapeutic treatment upon the neurologic, reactive and neurodegenerative phenotypes of murine JNCL. We demonstrate that immune suppression with MMF results in improvements in motor function in Cln3−/−, and a reduction in circulating autoantibodies directed toward brain antigens. Although it is unclear whether autoimmunity is a cause, or a consequence, of the symptoms observed in JNCL, it is possible that deficits in motor function could be related to the presence of autoantibodies. While much attention has focused on the presence of the GAD65 autoantibody in JNCL, we cannot rule out the possibility that other autoantibodies directed toward brain antigens (Castaneda and Pearce, 2008; Lim et al., 2006) may, either individually or in concert, also play a role in pathogenesis. These results are particularly encouraging, given that MMF has a safe pediatric profile and paves the way for testing whether this or other immunosuppressive approaches will be beneficial in a clinical setting. Although our results indicate a potentially adverse effect of continued immune suppression on WT and Cln3−/− mice, an effect observed with both genetic and pharmacological suppression, it should be noted that the MMF dosage we used was based upon the recommended starting dose in children. Currently, children treated with MMF have their dosage adjusted to the level of immunosuppres-

sion required, while minimizing potentially harmful side effects. With this in mind, it may be possible to administer a beneficial dosage of MMF to JNCL patients for prolonged periods of time while avoiding any potentially deleterious effects associated with immune suppression.

Compared to the progress made towards effective therapies in enzyme based lysosomal storage disorders, these are the first data to provide a realistic hope for those affected by this most prevalent form of profoundly disabling and fatal pediatric disorder.

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References


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