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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 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.

Keywords

CLN3; JNCL; Batten Disease; immunosuppression

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

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progressive loss of vision, seizures, 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; Chattopadhyay et al., 2002b; 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 autoimmunity in JNCL pathogenesis (Chattopadhyay et al., 2002a). Previous studies have demonstrated an inhibitory effect of the GAD₆₅ autoantibody 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; Lim et al., 2006). Using both genetic and pharmaceutical approaches, we demonstrate that immune suppression alleviates behavioral and pathological deficits in *Cln3*^{-/-} mice.

Materials and Methods

Animals

C57Bl/6 congenic μ MT 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*^{-/-}, μ MT, and *Cln3*^{-/-}/ μ MT. 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.

Mycophenolate mofetil treatments

Mycophenolate mofetil (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 60mg/kg MMF dissolved in non-fat milk or with non-fat milk alone (placebo) for 30, 70, or 150 consecutive days.

Motor performance testing

Motor performance was assessed using an accelerating rotarod (AccuScan Instruments, Columbus, OH) (0 to 30 rpm over 240 seconds) 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 hrs, 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.

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).

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 *Stereoinvestigator*TM (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 (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.

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).

Results

Immune deficient *Cln3*^{-/-} mice were generated by backcrossing *Cln3*^{-/-} mice with B-cell deficient μ MT mice (Kitamura et al., 1991). These *Cln3*^{-/-}/ μ MT 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*^{-/-}/ μ MT 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; Weimer et al., 2006). Optical fractionator counts from *Cln3*^{-/-}/ μ MT, *Cln3*^{-/-} mice and WT controls revealed significantly more deep cerebellar nuclei neurons in *Cln3*^{-/-}/ μ MT mice ($P < 0.05$, Two-way ANOVA), with similar numbers of these neurons as seen in wild type mice at P180 (Figure 1C). An increased number of thalamic LGNd neurons was evident in *Cln3*^{-/-}/ μ MT mice, but this did not reach statistical significance.

In the accelerating rotarod test (Kovacs and Pearce, 2008), *Cln3*^{-/-}/ μ MT 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 μ MT mice (Fig. 1E). At P100, *Cln3*^{-/-}/ μ MT mice again out-performed *Cln3*^{-/-} but did not reach statistical significance although their performance was statistically indistinguishable from WT. At P180, we observed decreased performance of both *Cln3*^{-/-}/ μ MT and μ MT 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 the notion that autoantibodies present in JNCL have a pathological role, since genetic blockade of their production in *Cln3*^{-/-}/ μ MT 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 mofetil (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 placebos 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 $p=0.279$, $\epsilon>0.75$, Hyun-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 absence of IgG deposits in the brain (Fig. 2B). MMF treated *Cln3*^{-/-} also have reduced neuroinflammation as indicated by a decrease in GFAP and CD68 immunoreactivity and a decreased number of activated CD68-positive 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).

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, may 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 recommended starting dose in children. Currently, children treated with MMF have their dosage adjusted to the level of immunosuppression 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 this profoundly disabling and fatal pediatric disorder.

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Figure 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 fractionators 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, are markedly reduced in the cortex and 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 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, performance of both *Cln3*^{-/-}/μMT and μMT mice are 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.

Figure 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, are markedly reduced in the cortex and 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.