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# Antigen presenting cell abnormalities in the *Cln3*$^{-/-}$ mouse model of juvenile neuronal ceroid lipofuscinosis

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ABSTRACT

Mutations of the *CLN3* gene lead to juvenile neuronal ceroid lipofuscinosis (JNCL), an autosomal recessive lysosomal storage disorder that causes progressive neurodegeneration in children and adolescents. There is evidence of immune system involvement in pathology that has been only minimally investigated. We characterized bone marrow stem cell-derived antigen presenting cells (APCs), peritoneal macrophages, and leukocytes from spleen and blood, harvested from the *Cln3*$^{-/-}$ mouse model of JNCL. We detected dramatically elevated CD11c surface levels and increased total CD11c protein in *Cln3*$^{-/-}$ cell samples compared to wild type. This phenotype was specific to APCs and also to a loss of CLN3, as surface levels did not differ from wild type in other leukocyte subtypes nor in cells from two other NCL mouse models. Subcellularly, CD11c was localized to lipid rafts, indicating that perturbation of surface levels is attributable to derangement of raft dynamics, which has previously been shown in *Cln3* mutant cells. Interrogation of APC function revealed that *Cln3*$^{-/-}$ cells have increased adhesiveness to CD11c ligands as well as an abnormal secretory pattern that closely mimics what has been previously reported for *Cln3* mutant microglia. Our results show that CLN3 deficiency alters APCs, which can be a major contributor to the autoimmune response in JNCL.



## 1. Introduction

Juvenile neuronal ceroid lipofuscinosis (JNCL), or Batten disease, is an autosomal recessive lysosomal storage disorder that causes progressive neurodegeneration in children and adolescents. It is caused by mutation of the *CLN3* gene, which encodes a protein product of the same name (CLN3). Patients present around age 5, typically with visual deterioration, followed by blindness, behavioral, cognitive, and psychiatric disturbances, progressive motor dysfunction, seizures, and eventually death in the third decade of life [1]. The signs and symptoms of disease come from the extensive loss of neurons seen in multiple brain regions, the magnitude of which correlates with widespread proliferation and activation of glial cells [2–4]. The pathogenesis of JNCL is not well-characterized, however, nor is the role of the CLN3 protein. While CLN3 has been extensively implicated in intracellular trafficking, potentially involving the endosomal/lysosomal system and Golgi transport, its function remains incompletely understood [reviewed by Cotman and Staropoli (2012)] [5].

There is evidence of an autoimmune component in JNCL. Patients and *Cln3*$^{-/-}$ mice produce IgG autoantibodies reactive to brain proteins [6–9]. While there is no direct evidence that autoantibodies are harmful in JNCL, IgG deposition can be appreciated in multiple brain regions in human autopsy and genetic mouse model specimens [9]. Moreover, administration of immunosuppressant drugs ameliorates the characteristic motor phenotype of *Cln3*$^{-/-}$ mice and reduces glial activation [10]. These discoveries have led to a clinical trial of mycophenolate mofetil as a treatment for JNCL, which is currently in progress.

Immune cell abnormalities at the cellular level have been only minimally investigated; although several lines of evidence suggest there is potential for cellular dysfunction. Microglia are the resident mononuclear phagocytes of the central nervous system (CNS). *Cln3* mutant microglia, which show abnormal secretion of pro-inflammatory cytokines, appear to contribute directly to neuronal loss [11,12]. Macrophages (Mφ) have a secretory defect that has not been well-defined, and they accumulate particularly large amounts of lysosomal storage material [11–13]. Also suggestive is that Mφ and the other primary antigen presenting cell (APC), the dendritic cell (DC), rely heavily on

*Abbreviations:* APCs, antigen presenting cells; APhC, allophycocyanin; BM, bone marrow; BMCCs, bone marrow cultured cells; BM-DCs, bone marrow dendritic cells; BM-Mφ, bone marrow macrophages; BSA, bovine serum albumin; CHX, cycloheximide; CTB, cholera toxin B; DCs, dendritic cells; DPBS, Dulbecco's phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN-γ, interferon-γ; JNCL, juvenile neuronal ceroid lipofuscinosis; LPS, lipopolysaccharide; LSD, least squares difference; LUTs, lookup tables; MFI, median fluorescence intensity; Mφ, macrophages; NCL, neuronal ceroid lipofuscinosis; PBS, phosphate buffered saline; PDL, poly-D-lysine; PE, phycoerythrin; PerCP, peridinin–chlorophyll protein; PIC, poly I:C; ROI, region of interest; SBDS, Shwachman–Bodian–Diamond syndrome protein; TLR, toll-like receptor; WT, wild type.

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processes in which CLN3 and its interacting partners and associated regulatory proteins are implicated. Shwachman-Bodian-Diamond Syndrome protein (SBDS), myosin IIb, and Cdc42 variably regulate endocytosis of extracellular antigens and subsequent trafficking through the endosomal/lysosomal system, polarization, migration, and chemotaxis, and are needed for normal hematopoiesis [14–27]. Of particular interest is the possibility of leukocyte dysfunction in JNCL via perturbation of surface protein homeostasis; surface protein abnormalities have been described in CLN3-deficient cells, including a reduction of lipid raft membrane microdomain associated proteins, inappropriate $Na^+/K^+$ ATPase subunit composition, and decreased surface expression of rho GTPase activating protein ARHGAP21 [24,28–30].

Immune cells rely on the proper repertoire of surface proteins to mediate interactions with other cells and the environment, with altered levels affecting leukocyte function in a variety of autoimmune diseases [31–38]. The β2-integrins are a leukocyte specific family of adhesion molecules comprised of a common CD18 beta subunit that binds to one of four alpha subunits—CD11a, CD11b, CD11c, and CD11d. While all heteromers function primarily in adhesion-based processes, CD11b and CD11c have additional roles in phagocytosis and inflammatory signaling [reviewed by Mazzone and Ricevuti (1995) and Tan (2012)] [39,40]. CD11c is of particular interest as an overall increase in CD11c is observed in chronic inflammation and in autoimmune diseases associated with autoantibody production (i.e. multiple sclerosis and rheumatoid arthritis) [31,41–47]. It serves as the main dendritic cell marker and is also found on microglia, subsets of Mφ and neutrophils, and on limited numbers of natural killer cells and lymphocytes [46, 48–51].

With this in mind, we hypothesized that CLN3 deficiency might be associated with an aberrant phenotype in APCs, potentially through alteration of cell surface proteins and subsequent perturbation of downstream function. We characterized surface proteins as well as function for APCs from wild type mice and the *Cln3*$^{-/-}$ mouse model of JNCL, which recapitulates several neuropathological and behavioral phenotypes of human JNCL patients [52]. We looked at DCs and Mφ derived from culture of bone marrow stem cells—BM-DCs and BM-Mφ, respectively—as well as elicited peritoneal Mφ and a wider variety of leukocytes harvested from blood and spleen. We also set out to better characterize potential secretory defects in APCs to the extent this has previously been done in microglia. We believe that a better understanding of the mechanisms underlying potential immune dysfunction in JNCL will further the development of more targeted immunotherapies and also help elucidate further the function of CLN3.

## 2. Materials and methods

### 2.1. Mice

All experiments used the *Cln3*$^{-/-}$ (*Cln3*$^{\Delta ex1-6}$) mouse model of juvenile Batten disease inbred on the C57BL/6J background [52]. *Ppt1*$^{-/-}$ and *Tpp1*$^{-/-}$ mice, mouse models of infantile and late infantile Batten disease, respectively, were assessed for CD11c in the comparative NCL mouse model study only [53,54]. Control animals were the C57BL/6J strain. Mice were male and aged 70 to 120 days as younger mice lack a fully developed immune system that includes impaired function of both lymphocytes and APCs [see Adkins, Leclerc et al. (2004) for review] [55]. Injections and euthanasia procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals [56] and approved by the institutional animal care and use committee for Sanford Research, Sioux Falls, South Dakota.

### 2.2. Bone marrow cultured cells

Bone marrow stem cells were cultured as previously described in RPMI 1640 medium with 2.05 mM L-glutamine supplemented with 1 μg/ml amphotericin B, 50 IU/ml penicillin, 50 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum (FBS), and 20 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) (Life Technologies, Carlsbad, CA) [57]. For cells cultured beyond 8 days, GM-CSF was reduced to 10 ng/ml. Where indicated, cells were stimulated on day 8 of culture with addition of toll-like receptor (TLR) ligands lipopolysaccharide (LPS) (100 ng/ml) (Sigma-Aldrich, St. Louis, MO) or poly I:C (PIC) (50 μg/ml) (Sigma-Aldrich). Cells were harvested on days 10 to 12 of culture by fraction — non-adherent and lightly adherent cells comprised the dendritic cell fraction, while firmly adhered cells dislodged with a cell scraper comprised the Mφ fraction. Mixed bone marrow cultured cell (BMCC) populations included both adherent and non-adherent fractions. Previous research has demonstrated the fractions are distinct populations, although phenotypes overlap somewhat [57–59]. Our own experiments consistently showed nearly 100% CD11b positivity and slightly less CD11c positivity in all bone marrow populations (some data not shown). A previous study indicates that few neutrophils should be present [57], consistent with our observation based on morphology.

### 2.3. Elicited peritoneal Mφ

Peritoneal Mφ were elicited with Bio-Gel P100 and harvested as previously described [60]. Cells were seeded into 6-well tissue culture plates, $2.5 \times 10^6$ cells per well, with some activated by priming with 10 ng/ml interferon-γ (IFN-γ) (Novus Biologicals, Littleton, CO) for 6 h followed by addition of 100 ng/ml LPS or 50 μg/ml PIC (LIγ = IFN-γ + LPS; PIγ = IFN-γ + PIC). Cells were harvested 48 to 96 h later by first vacuuming off any non-adherent cells (including any residual, non-apoptosed B1 lymphocytes) and then detaching adherent Mφ by incubating in 6 mM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS) at 37 °C for 10 min.

### 2.4. Splenocytes

Mice were euthanized and spleens excised and macerated with a 5 ml syringe plunger in 10-cm dishes with PBS. Cell suspensions were transferred to 50 ml conical tubes, avoiding larger debris, and washed with PBS. Red blood cells were lysed by resuspending the cell pellet in 9 ml of molecular grade water. After 25 s, 1 ml of 10× PBS was added to the tube followed by 30 ml of 1× PBS. The suspension was filtered through a 40 μm cell strainer and then washed again with PBS.

### 2.5. Peripheral blood cells

Syringes (1 ml) were prepared by flushing with heparinized saline (1000 U/ml), leaving a residual of 10 μl. Mice were euthanized, the chest cavity opened, and blood withdrawn from the right ventricle. Samples were refrigerated at 4 °C for 2 to 4 h followed by pelleting of cells by centrifuging for 10 min at 200 xg. Red blood cells were lysed and the cells filtered and washed as described above.

### 2.6. Gene expression

Day 10 and 11 wild type or *Cln3*$^{-/-}$ mixed BMCCs, with or without activation by LPS for 24 h or 48 h, were harvested as described above, washed three times with PBS, and the cell pellet snap frozen on dry ice. Total RNA was isolated with the Maxwell 16 LEV simplyRNA Cells Kit (Promega, Madison, WI) on a Maxwell 16 Instrument (AS2000) (Promega), according to the manufacturer's instructions. Quantification and assessment of RNA integrity was performed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Relative integrity numbers (RIN) for all samples were >7.0. Reverse transcription to cDNA and real-time quantitative PCR (qPCR) were performed as previously described [61]. TaqMan hydrolysis assays (Life Technologies) included the following: *Cln3* (Mm00487021_m1), *Itgam* (Mm00434455_m1),