Blood and Cerebrospinal Fluid Biomarkers in the Correlations of Clinical Outcomes in Pediatric NMDARE

SPECIFIC AIMS

Anti-NMDA receptor encephalitis (NMDARE) is a recently described autoimmune encephalitis associated with the production of antibodies that target against the NMDA receptor, resulting in a characteristic neuropsychiatric syndrome. Initial symptoms include psychiatric and/or behavioral changes followed by the emergence of seizures, movement disorders, language dysfunction, insomnia, and autonomic instability. Patients often require prolonged hospitalization, including admissions to the intensive care unit (ICU) and/or inpatient rehabilitation unit. Patients typically respond to immunotherapy, but the response can be variable.[1] The pathogenesis of NMDARE has been attributed predominantly to B cells; however, B cell targeted therapies are sometimes not adequate, suggesting that additional immune cells are likely involved. While the NMDAR antibody serves as a diagnostic biomarker,[1-3] no biomarkers have been identified that predict disease course and/or response to treatment. Therefore, we **propose a pilot study to investigate potential biomarkers in pediatric patients with NMDARE to obtain preliminary data and to examine the feasibility for conducting a larger, multisite study**. Our long-term goal is to investigate immunological mechanisms in NMDARE in order to identify biomarkers that predict severity, treatment response, and clinical outcomes. Our specific aims include:

Aim 1: Identify immune cells in pediatric NMDARE compared to controls.
Aim 2: Identify proinflammatory markers (cytokines and/or chemokines) in pediatric NMDARE compared to controls.
Aim 3: Identify immune cells and/or inflammatory markers that associate with outcomes at 3 months after treatment initiation.

BACKGROUND AND SIGNIFICANCE

NMDARE is a type of autoimmune encephalitis associated with the production of antibodies that target the NMDA receptor. NMDARE is suspected if a patient presents with at least 4 of 6 core symptoms: abnormal behavior or cognitive dysfunction, speech changes, seizures, movement disorder, decreased level of consciousness, or autonomic dysfunction. Diagnostic criteria also include either an abnormal EEG or CSF with pleocytosis or oligoclonal bands. A diagnosis is confirmed by the presence of NMDA receptor antibodies in either serum and/or CSF.[4] The NMDA receptor antibody test is specific as it is absent in healthy controls.[5] While the incidence is unknown, NMDARE is the most frequent cause of encephalitis in pediatric patients reported in the California Encephalitis Project. NMDARE is more common than viral encephalitis, including enterovirus, herpes simplex virus (HSV) type 1, varicella-zoster virus, and West Nile Virus.[6] Children comprise about one-third of all patients with NMDARE. [7]

The clinical course of NMDARE is highly variable and often requires prolonged hospitalization. While a majority of patients demonstrate a positive response to immunotherapy within the first several months of treatment initiation, treatment response is also unpredictable. First-line treatments include intravenous (IV) steroids, IV immunoglobulin (IVIG), or plasma exchange (PLEX). Second-line treatments may also be needed, such as rituximab or cyclophosphamide. Early initiation of treatment has been shown to be associated with more favorable outcomes over time.[7,8] Approximately 15% of patients with NMDARE will have a recurrent episode.[7,8]

Few studies have addressed outcomes of these patients, particularly within pediatrics. In the original case series with adults, approximately 75% of patients had a “full recovery” or mild persistent deficits.[1] Better outcomes and fewer relapses are associated with tumors and early treatment. In children, NMDARE is less likely to occur as a paraneoplastic syndrome and can also occur as a post-HSV autoimmune encephalitis. For many, however, the trigger is unknown.[8,9]

We recently examined early functional outcomes of pediatric patients with NMDARE who required inpatient rehabilitation (N=27).[10] The WeeFIM is a functional scale that is often used in

the rehabilitation setting that consists of 18-items assessing the domains of mobility, self-care, and cognition (Raw=18-126).[11] Early functional outcomes were also assessed using the Cognitive & Linguistic Scale (CALS),[12] a 20-item measure designed for serial assessment of cognitive-linguistic recovery following acquired brain injury during inpatient rehabilitation. Significant improvements were noted between admission and discharge WeeFIM scores (Figure 1a) and CALS (Figure 1b) in a subset of patients. However, a small group of patients exhibited a limited or flat change profile in WeeFIM and CALS scores between admission and discharge, deemed "low responders" (CALS score ≤ 35). Younger age, seizures, and more treatments were associated with worse functional outcomes at discharge from inpatient rehabilitation.[10]

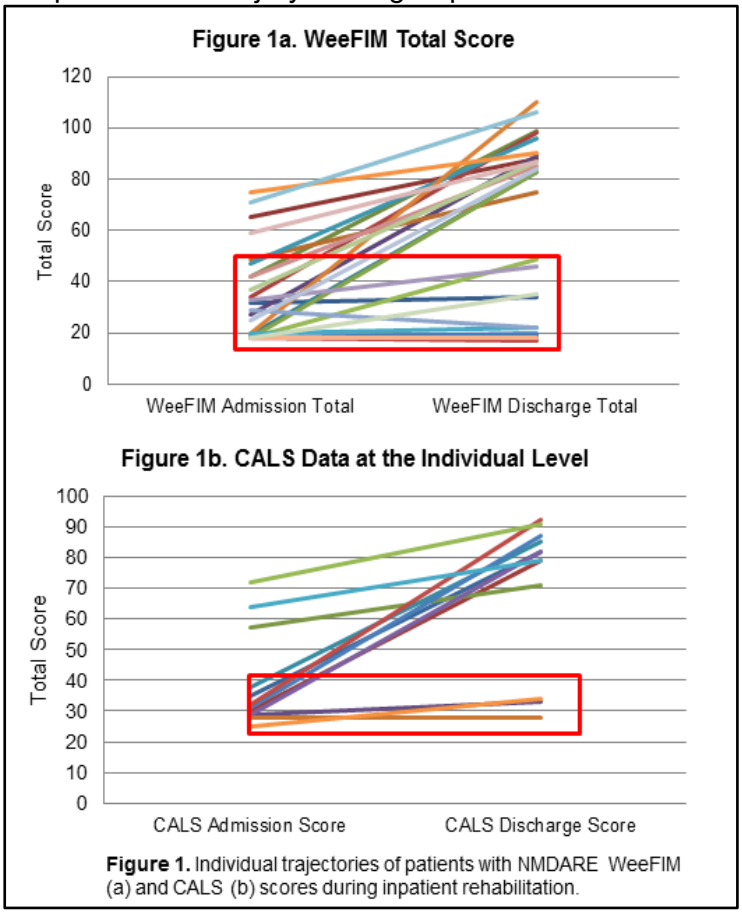

Figure 1. Individual trajectories of patients with NMDARE WeeFIM (a) and CALS (b) scores during inpatient rehabilitation.

Despite these predictors of early outcome in pediatric NMDARE, there is still unexplained heterogeneity in disease course and recovery, and thus a need to explore additional biological explanations for outcomes. The overall aims of the current study are to examine: 1) the underlying immune cell biology and cytokines as well as 2) identify a prognostic biomarker by examining which immune cells and cytokines may be related to early functional outcomes in pediatric patients with NMDARE. Better understanding the underlying mechanisms of NMDARE in pediatrics may help to better determine severity, inform appropriate treatments, predict outcomes, as well as extrapolate to treatment targets in other neuroimmunological diseases.

EXPERIMENTAL DESIGN AND METHODS

Recruitment and Enrollment Plan. Families of patients ages 2 to 18 years presenting with probable NMDARE (≥ 4 core symptoms) to CHOA will be identified by the inpatient neurology team who will notify the research team. Our goal is to recruit a total of 10 patients for this pilot study: 5 patients with NMDARE and 5 controls. Between 2012-2017, CHOA saw 27 patients with 4-7 patients presenting each year with NMDARE.[10] Controls will be age- and gender-matched patients undergoing lumbar puncture (LP) for clinical care in non-inflammatory neurological diseases, such as idiopathic intracranial hypertension, which could either inpatient or outpatient. Exclusion criteria will include patients receiving immunotherapy within 30 days of initial samples.

Measures. *Demographics.* Demographic information (e.g., age, sex, race) will be obtained.

Outcomes. As part of standard of care, serial cognitive assessments (Cognitive & Linguistic Scale - CALS) will be collected. Scores range from 20-100. In our study on early functional outcomes in pediatric NMDARE, we found that "low responders" had a CALS score of ≤ 35. "Responders" will be defined as patients with improved CALS score from admission to 3-

months. Caregivers will complete ratings of executive functioning (Behavior Rating Inventory of Executive Function, Second or Preschool Edition; BRIEF-2/P) and behavioral/emotional functioning (Behavior Assessment System for Children, Third Edition; BASC-3).

Experiments. We will collect 20 ml of blood in two tubes (one for serum and one for peripheral blood mononuclear cells-PBMCs) at admission and 3 months following treatment. We will also collect 1 ml of cerebrospinal fluid (CSF) at admission. Repeat CSF samples will not be obtained.

Immune Cell Markers. We will use high-dimensional flow cytometry (30-parameter, BD FAC Symphony) to examine B cell subtypes, T cell subtypes, monocytes, dendritic cells, and natural killer cells (see Figure 2). Antibodies detecting the following markers will be used: Surface markers: CD45RA, CD3, CD4, CD5, CD8, CD14, CD16, CD25, CD127, CD19, CD20, CD27, CD38, CD138, HLA-DR, CD16, CD56, CD14, CD69, viability dye (Zombie Aqua), IgD, IgM, B220, CD33, CD34, CD21, CD11c, CD1c and intracellular staining markers: FOXP3, IFNg, IL-17, and T-bet. The flow cytometry will be done in collaboration with Dr. Eliver Ghosn and the Pediatric Research Alliance Flow Cytometry Core.

Statistics: For all biomarkers, including cytokine, chemokine, B cell and T cell profiles, we will provide descriptive statistics for all continuous and discrete variables of interest. For continuous measures, median, 25th and 75th percentiles along with mean and standard deviation will be calculated. All discrete characteristics will be estimated with frequency counts and proportions.

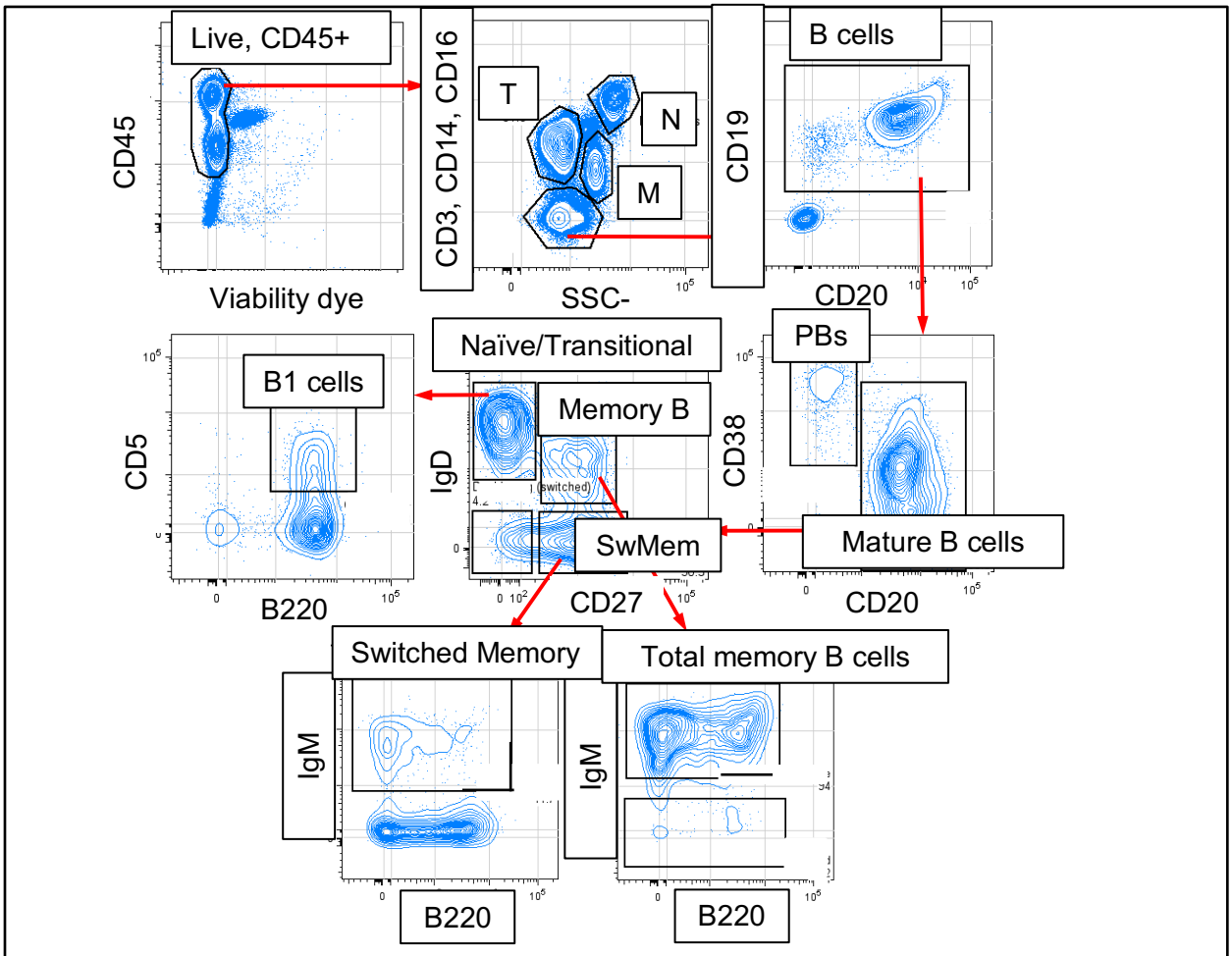

Figure 2. *Flow cytometry analysis of peripheral blood cells. CD45+ cells identify white blood cells. CD3, CD14 and CD16 help to identify T cells (T), neutrophils (N), and monocytes (M). CD19 and CD20 are markers of B cells. CD38 positive identify immature B cells or plasmablasts (PBs) as CD20 identifies mature B cells. IgD and CD27 identifies subsets of B cells including naïve, B1, and switched memory B cells (SwMem). B220 and IgM distinguish different types of antibody producing memory B cells.*

Aim 1: Identify immune cells in pediatric NMDARE compared to controls.

Hypothesis. We hypothesize that patients with NMDARE will have higher levels of immune cell subsets (B- and/or T-cells) in blood and CSF at presentation compared to controls.

Rationale. The pathophysiology of NMDARE is an immune response to a trigger, which may include tumors (most commonly ovarian teratomas), viruses (e.g., HSV), and other unknown stimuli.[7,13,14] B cells are thought to be critical in the pathogenesis of NMDARE. The anti-NMDA receptor antibody has been shown to be pathogenic, as demonstrated by antibody transfer causing symptoms in mice.[15] Upon activation, B cells create antibodies against the NMDA receptor. Once the antibodies bind to the NMDA receptor, the receptor is endocytosed into neurons. The loss of NMDA receptors at the cell surface disrupts their ability to send signals between neurons, which leads to symptoms.[16] Once the antibodies disappear, the receptors are recycled back to the surface and thus, the symptoms are thought to be reversible. Still, it takes time to re-establish the connections between neurons, which contribute to a prolonged recovery.[16,17] However, this reversible process does not explain the patients with persistent poor recovery ("low responders") in NMDARE or patients who do not respond to rituximab, a treatment that targets B cells. Additionally, antibodies can still be present in patients who have recovered from NMDARE and do not correlate with clinical outcomes.[3] Moreover, if NMDARE were purely antibody-mediated, then the addition of second line, T cell targeted immunotherapies (e.g., cyclophosphamide; mycophenolate mofetil), would not be necessary. While antibodies and B cells are thought to be pathogenic, other immune cells are likely involved, namely T cells. T cells were present in an NMDARE brain biopsy[18] but no studies have examined the role of T cells in NMDARE or type of B cells in NMDARE.

Analysis Plan for Aim 1. Both proportions and absolute numbers of cells will be examined comparing 1) patients versus controls and 2) monitoring changes in cells within patients at 3 months compared to baseline using two sample t-test with an overall alpha=0.05. Treatment effect will also be taken into consideration. Historically, most CHOA patients with NMDARE received IV steroids and/or IVIG, 50% plasma exchange, 70% rituximab, and 15% cyclophosphamide.[10]

Potential Pitfalls. We may not observe any differences in the proportion of activated T-cell subsets, monocytes, NK cells, and dendritic cells in NMDARE when compared to controls. However, no one has examined or reported on these cell subtypes in NMDARE; thus, findings could provide novel evidence to support a hypothesis that NMDARE is exclusively B cells, as well as identify the type of B cell involved in NMDARE, allowing for focused, future research.

Aim 2: Identify proinflammatory markers (cytokines and/or chemokines) in pediatric NMDARE compared to controls.

Hypothesis. We hypothesize that patients with NMDARE will have higher proinflammatory cytokine /chemokine levels in the serum and CSF at presentation compared to controls.

Rationale. Cytokines are intracellular signals that modulate immune cells and chemokines recruit immune cells into the central nervous system. Cytokines and chemokines can either be pro-inflammatory or anti-inflammatory. Chemokine (C-X-C motif) ligand 13 (CXCL13) is a chemoattractant for B cells and has been associated with increased synthesis of NMDAR antibody in NMDARE.[19] Other B cell chemokines in a proliferation-inducing ligand (APRIL) and chemokine (C-C motif) ligand 9 (CCL9) have also been shown to be increased in NMDARE.[20] Interestingly, T cell related cytokines and chemokines, including CXCL9, CXCL10,[20] interferon-gamma (IFNg), tumor necrosis factor alpha (TNFa), and interleukin-17A (IL17A)[21] are also elevated in NMDARE, suggesting T cell involvement.

However, it is currently unknown how these cytokines and/or chemokines correlate with subtypes of B or T cells in NMDARE. In addition to the cytokines mentioned above, we would also like to examine levels of IL-1, IL-2, IL-6, IL-7, IL-10, IL-17A, IL-23, TNFa, CCL9, CXCL9, CXCL10, BAFF and APRIL, which are independent of each other. If IL17 is involved with

NMDARE, then IL-17 targeted therapies used in the treatment of other autoimmune diseases could be considered, such as secukinumab.[22] IL-6 is a proinflammatory cytokine that has been examined in only two patients with NMDARE.[23,24] Thus, we would like to examine IL6 in a larger group of NMDARE patients. Tocilizumab, an IL-6 inhibitor, has been used successfully in treatment for one patient with NMDARE and could be a treatment option for other patients.[23]

Analysis for Aim 2. We plan to utilize a multiplex immunoassay of CSF and serum samples to examine chemokines and cytokines at baseline and compare NMDARE patients to controls using two sample t-test with an overall alpha=0.05. The assay would be performed through the Emory Mulitplexed Immunoasay Core.

Potential Pitfalls. We may not observe any differences in proportion of chemokines/cytokines in NMDARE compared to controls. We may also discover there is no correlation between chemokines/cytokines and the immune cell subsets associated with this condition. If so, then it would signify that other chemokines/cytokines or intracellular processes contribute to NMDARE.

Aim 3: Identify immune cells and/or inflammatory markers that associate with outcomes 3 months after treatment initiation.

Hypothesis. We hypothesize that higher levels of immune cell subsets and/or proinflammatory cytokines/chemokines at presentation will be associated with lower CALS scores at 3 months. Moreover, we hypothesize that increased immune cells or increased proinflammatory molecules at 3 months from onset will also be associated with lower CALS scores at 3 months.

Rationale. While the CALS was developed to characterize cognitive-linguistic recovery during inpatient rehabilitation following moderate-to-severe TBI, it has also been useful for serially monitoring and assessing early cognitive recovery in pediatric NMDARE. The patterns and predictors of recovery from NMDARE are poorly understood, especially in children. Given the heterogeneity in NMDARE outcomes, determining specific factors that can explain outcomes and could target specific treatment would be significant. The capability to predict the clinical course and functional outcomes by a blood or CSF test would be useful for NMDARE treatment.

Analysis Plan for Aim 3. The immune function markers (B and T cell repertoire, cytokines, chemokines) will be measured at both baseline and 3 months following treatment initiation for NMDARE patients. A paired t-test of either log-transformed or untransformed data will be used to compare the change in immune function among NMDARE patients. We will correlate these changes to neuropsychological outcomes, including the CALS, BRIEF-2/P, and BASC-3.

Potential Pitfalls. First, the sample size for this study is small; however, this is a pilot/feasibility study. Second, many patients with NMDARE require prolonged hospitalization and recovery time; hence, the proposed time frame may not capture meaningful change. We would then examine if there is any correlation with long-term outcomes.

CONCLUSIONS AND FUTURE STUDIES

We have motivated and described a pilot study for pediatric patients with NMDARE to obtain preliminary data to conduct a larger, multisite study. The investigation of biomarkers, together with neurocognitive tests, will provide important new insights in the field of pediatric NMDARE. If the specific aims yield positive results, we will initiate two follow-up studies. **First**, we will apply for additional funding to support the continuation of this study to longitudinally track trends in immune cells, cytokines/chemokines and cognitive outcomes over time. **Second**, we plan to apply for additional funding to support a multi-site study that will include a larger sample size as well as immunophenotyping and chemokine/cytokine assays at multiple time points to further examine the prognostic value of blood or CSF profiling to predict outcomes in pediatric NMDARE over time. We would further pursue transcriptomics, to assess intracellular changes that lead to the pathogenic cells in NMDARE. Moreover, our findings could be the target of new treatments to treat NMDARE, but also other neuroinflammatory conditions.

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