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Full-length Article

Identification of the antigenic epitopes of maternal autoantibodies in autism spectrum disorders

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ABSTRACT

Several groups have described the presence of fetal brain-reactive maternal autoantibodies in the plasma of some mothers whose children have autism spectrum disorder (ASD). We previously identified seven autoantigens targeted by these maternal autoantibodies, each of which is expressed at significant levels in the developing brain and has demonstrated roles in typical neurodevelopment. To further understand the binding repertoire of the maternal autoantibodies, as well as the presence of any meaningful differences with respect to the recognition and binding of these ASD-specific autoantibodies to each of these neuronal autoantigens, we utilized overlapping peptide microarrays incubated with maternal plasma samples obtained from the Childhood Autism Risk from Genetics and Environment (CHARGE) Study. In an effort to identify the most commonly recognized (immunodominant) epitope sequences targeted by maternal autoantibodies for each of the seven ASD-specific autoantigens, arrays were screened with plasma from mothers with children across diagnostic groups (ASD and typically developing (TD)) that were positive for at least one antigen by western blot (N = 67) or negative control mothers unreactive to any of the autoantigens (N = 18). Of the 63 peptides identified with the discovery microarrays, at least one immunodominant peptide was successfully identified for each of the seven antigenic proteins using subsequent selective screening microarrays. Furthermore, while limited by our relatively small sample size, there were peptides that were distinctly recognized by autoantibodies relative to diagnosis For example, reactivity was observed exclusively in mothers of children of ASD towards several peptides, including the LDH-B peptides DCIIIVVSNPVDILT (9.1% ASD vs. 0% TD; odds ratio (95% CI) = 6.644 (0.355–124.384)) and PVAEEEATVPNNKIT (5.5% ASD vs. 0% TD; odds ratio (95% CI) = 4.067 (0.203–81.403)).These results suggest that there are differences in the binding repertoire between the antigen positive ASD and TD maternal samples. Further, the autoantibodies in plasma from mothers of children with ASD bound to a more diverse set of peptides, and there were specific peptide binding combinations observed only in this group. Future studies are underway to determine the critical amino acids necessary for autoantibody binding, which will be essential in developing a potential therapeutic strategy for maternal autoantibody related (MAR) ASD.

1. Introduction

Autism spectrum disorders (ASD) are a set of neurodevelopmental disorders characterized by social interaction and communication deficits that are accompanied by the presence of repetitive and restrictive behaviors (APA, 2013). It is currently estimated that ASD affects 1 in 68 children in the United States, with the average age of diagnosis at approximately 4 years of age (Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators and (CDC), 2014). Despite increases in the prevalence estimates of ASD, the etiology of the disorder remains elusive. Genetic factors are thought to have an important role, with a recent study using estimating heritability to be 83% (Sandin et al., 2017). However, there is ample evidence suggesting that environmental influences, particularly during gestation or the early postnatal period, may also play a contributing factor in the development of ASD (Gronborg et al., 2013; Hallmayer et al., 2011; Kim and Leventhal, 2015).

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Emerging studies suggest immune dysfunction is also a risk factor contributing to the neurodevelopmental deficits observed in ASD. The notion that immune system dysfunction could be a contributing factor in the etiology of ASD stems from the recent recognition of the importance of the maternal immune system in healthy neurodevelopment, and alterations to the gestational immune environment have been demonstrated to produce significant neurodevelopmental consequences in exposed offspring (reviewed in (Meltzer and Van de Water, 2017)). Most notably, several investigators have now identified a strong association between maternal autoantibodies reactive towards fetal brain proteins and risk of ASD (Braunschweig et al., 2007; Braunschweig et al., 2011; Diamond et al., 2013; Singer et al., 2008; Zimmerman et al., 2007). Maternal immunoglobulin G (IgG) antibodies transfer at high concentrations across the placenta beginning around mid-gestation in humans (Garty et al., 1994), thereby providing the newborn with a passive defense mechanism against pathogens. Maternal IgG is also transferred to the newborn during lactation through breast milk, although at much lower levels than IgA, enabling maternal IgG to persist in the newborn through early infancy (Van de Perre, 2003). Under normal conditions, antibodies are unable to cross the blood-brain barrier (BBB) to access the brain. However, the BBB is permissive during early brain development and thus permits maternal antibodies access to the fetal brain (Saunders et al., 2012). Therefore it is not surprising that prenatal exposure to maternal antibodies that bind to fetal brain has been suggested as a mechanism for altering normal brain development (Diamond et al., 2013).

Our laboratory first described a specific pattern of autoantibody reactivity to fetal proteins at approximately 37 and 73 kDa, as well as to fetal proteins at 39 and 73 kDa, that was uniquely found among mothers of children with ASD (Braunschweig et al., 2007; Braunschweig et al., 2011). Furthermore, the same pattern of reactivity at 37 and 73 kDa has been observed in prospectively collected mid-gestation blood samples from mothers who went on to have a child with ASD (Croen et al., 2008), supporting the possibility that these autoantibodies may be pathogenic for at least one form of ASD. Several preclinical animal models have been conducted in mice and non-human primates in support of this hypothesis, finding ASD-relevant behavioral alterations in offspring exposed to the autoantibodies from mothers of children with ASD (Bauman et al., 2013; Braunschweig et al., 2012; Camacho et al., 2014; Martin et al., 2008; Singer et al., 2009). As findings from these studies strongly suggest a role of maternal autoantibodies in the etiology of ASD, the identification of the target antigens for maternal autoantibody related (MAR) ASD was a critical step in advancing this area of ASD research. In the first study of its type, we successfully determined the identity of several proteins targeted by the candidate antigens in fetal brain tissue (Braunschweig et al., 2013). Through tandem mass spectrometry sequencing, the target proteins were identified as: lactate dehydrogenase A and B (LDH-A, LDH-B) (37 kDa band), Y-box binding protein 1 (YBX1) (39 kDa band), stress-induced phosphoprotein 1 (STIP1) (upper 73 kDa band), collapsin response mediator proteins 1 and 2 (CRMP1, CRMP2) (lower 70 kDa band, and guanine deaminase (GDA) (a 44 kDa band not observed in our initial studies) (Braunschweig et al., 2013). When all antigen patterns were combined, a total of nearly 23% of mothers of ASD children had one of the ASD-specific autoantibody patterns containing two or more of the target proteins in comparison to only 1% of control mothers (Braunschweig et al., 2013). Interestingly, each of the identified target proteins is expressed at significant levels in the human fetal brain and has an established role in neurodevelopment (Braunschweig et al., 2013). While the identification of these antigenic proteins further supports the potential role of maternal autoantibodies in the etiology of a sub-type of ASD, the precise mechanism(s) underlying alterations to neurodevelopment and behavior are currently under investigation.

Autoantibodies recognize and bind to a particular region of their antigenic protein target (epitope), and it is this binding specificity that often plays a role in the pathologic effect of the autoantibody. Furthermore, the way in which a self-protein is presented to the immune system can affect what epitopes are exposed and therefore targeted by autoantibodies. Thus, efforts toward characterizing the targeted interaction between the various autoantigens and the maternal autoantibodies are extremely important to gain an understanding of the underlying mechanism(s) of MAR ASD. The identification of the peptide epitope sequences targeted in MAR ASD could additionally be utilized for the establishment of highly translational preclinical animal models, and the development of potential therapeutic strategies specific for one subtype of ASD. Finally, the information gleaned from this study may promote the development of more accurate biological markers for diagnostic purposes, which is becoming exceedingly important as a ASD is highly heterogeneous neurodevelopmental disorder and there are likely multiple, biologically defined subgroups within the ASD spectrum (McDougle et al., 2015; Ousley et al., 2013).

Therefore, the present study aimed to identify the antigenic epitopes recognized by maternal autoantibodies associated with MAR ASD. In order to delineate the specific epitopes of each protein, overlapping peptide microarrays were synthesized and incubated with pooled maternal plasma samples and several candidate peptide epitopes were elucidated. These peptides were then used to create screening microarrays that enabled discovery of the most frequently recognized epitopes for each protein using a larger sample set.

2. Materials and methods

2.1. Study subjects

The study sample included a subset of the 2300 mothers enrolled in the Childhood Autism Risks from Genetics and the Environment (CHARGE) study, an ongoing population-based case-control study designed to evaluate a broad range of risk factors for ASD and other neurodevelopmental disorders (Hertz-Picciotto et al., 2006). The CHARGE study participants in this study included mother-child pairs of children diagnosed with ASD and children selected from the general population (typically developing (TD)) with the average age for all mothers of 31.5 years (Range: 19–47) and an average age of all children at the time of blood draw of 3.5 years (Range: 2–5) (Table 1). Recruitment, eligibility, and psychometric assessment procedures have been previously described (Braunschweig et al., 2011; Hertz-Picciotto et al., 2006). All study participants completed a series of standardized assessments administered by trained clinicians at the UC Davis Medical Investigations of Neurodevelopmental Disorders (MIND) Institute

Table 1
Demographics of study population.

Diagnosis[a]	N	Average Child Age at time of draw (yrs)	Average Maternal Age at birth of child (yrs)
ASD	55	4	29
Severe[b]	41		
Mild	14		
TD	30	3	34

Abbreviations: ASD, Autism Spectrum Disorders; TD, Typically Developing.

[a] Subjects from Childhood Autism Risk from Genetics and the Environment (CHARGE) study.

[b] Based on ADOS comparison scores: range 1–10 with scores ≥7 indicating severe symptoms.

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to confirm diagnoses. ASD diagnosis was verified using gold standard instruments, the Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 2000; Lord et al., 2012) and Autism Diagnostic Interview – Revised (ADI-R) (Le Couteur et al., 2003), using criteria described by Risi et al. (2006) and in accordance with the *Diagnostic and Statistical Manual of Mental Disorders-5* (DSM-5) (American Psychiatric Association, 2013). ADOS comparison scores (Gotham et al., 2009; Lord et al., 2012) (range 1–10) were used to determine ASD intensity, with scores $\geq$7 indicating severe symptoms.

All study procedures were approved by the institutional review boards of the University of California in Davis and Los Angeles and the State of California Committee for the Protection of Human Subjects. Written informed consent was obtained prior to participation.

2.2. Sample collection and preparation

Maternal blood was collected in acid citrate dextrose tubes (BD Diagnostic, Franklin Lakes, NJ). Plasma was separated from cells, coded, and aliquoted to minimize freeze/thaw cycles then stored at −80 °C until use. Prior to their use in the experiments described below, all maternal plasma samples were centrifuged at 10,000×*g* for five minutes to separate lipids and cellular debris from the plasma before dilution.

For the initial epitope discovery screening, we utilized CHARGE plasma samples from mothers of children with ASD and mothers of TD children that were previously determined to be highly reactive to one or more of the candidate protein autoantigens by Western blot analysis (N = 29) (Table 2). To validate the peptides identified within the discovery arrays, we expanded our CHARGE sample set in the screening peptide microarrays to include plasma from mothers of children with ASD (n = 55), and from mothers of TD children (n = 30); the latter numbers are inclusive of the original 29 samples, as these samples were tested in both the discovery and validation phases of this study. Further, in order to ensure peptide binding was antigen-specific, these samples were also characterized by their reactivity to the full-length protein antigens (antigen positive samples n = 67, antigen negative samples n = 18).

2.3. Western blot

Antibody reactivity to LDH-A, LDH-B, GDA, YBX1, STIP1, CRMP1 (Novus Biologicals, Littleton, CO) and CRMP2 (Expression Systems, Davis, CA) full-length proteins in maternal plasma samples was determined via western blot. 1.5–3 μgs of each recombinant protein was separated under reducing conditions in a 12% SDS-PAGE mini-gel (Invitrogen, Carlsbad, CA) and transferred electrophoretically to 0.2 μm pore-size nitrocellulose. MagicMark molecular weight marker (Invitrogen, Carlsbad, CA) was loaded in the single marker lane allowing chemiluminescent visualization of marker bands. The nitrocellulose membrane was then cut into 3 mm wide strips and probed with maternal plasma diluted 1:800. After washing, strips were incubated with horseradish peroxidase conjugated goat anti-human IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MA) diluted 1:20,000. The strips were then washed, incubated with SuperSignal West Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA), and aligned on a glass plate for imaging. Chemiluminescent images were acquired with a FluorChem 8900 imager using AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

2.4. Discovery peptide microarrays

The amino acid sequences of the following candidate autoantigens were obtained from the National Center for Biotechnology Information (NCBI) protein database before their use in the discovery peptide microarrays: LDH-A (GenBank Accession No. AAH67223), LDH-B GenBank Accession No. CAA32033), STIP1 (GenBank Accession No. AAH39299), CRMP1 (GenBank Accession No. NP_001014809), CRMP2 (GenBank Accession No. NP_001184222), GDA (GenBank Accession No. AAH53584), and YBX1 (GenBank Accession No. AAI06046). The full sequence of each antigenic protein was then translated into 15-mer peptides with a

Table 2
Characterization of maternal plasma samples used within the discovery microarrays.

Microarray scheme	Microarray array number	Diagnostic population	Antigen reactivity
Scheme 1: *LDH-A, STIP1, CRMP1*	1	Sample 1: ASD	Sample 1: LDH-A, STIP1, CRMP1
	2	Sample 1: ASD	Sample 1: LDH-A
		Sample 2: ASD	Sample 2: STIP1
		Sample 3: ASD	Sample 3: CRMP1
	3	Sample 1: TD	Sample 1: LDH-A
		Sample 2: TD	Sample 2: STIP1, CRMP1
		Sample 3: TD	Sample 3: STIP1, CRMP1
Scheme 2*: LDH-B, GDA, YBX1, CRMP2*	1	Sample 1: ASD	Sample 1: LDH-B
		Sample 2: ASD	Sample 2: GDA, CRMP1
		Sample 3: ASD	Sample 3: STIP1, YBX1
		Sample 4: ASD	Sample 4: CRMP2
	2	Sample 1: ASD	Sample 1: LDH-B, STIP1, GDA
		Sample 2: ASD	Sample 2: GDA, CRMP2
		Sample 3: ASD	Sample 3: LDH-A, STIP1, YBX1
		Sample 4: ASD	Sample 4: GDA, STIP1, YBX1, CRMP2
	3	Sample 1: ASD	Sample 1: LDH-B, STIP1
		Sample 2: ASD	Sample 2: GDA, STIP1
		Sample 3: ASD	Sample 3: YBX1
		Sample 4: ASD	Sample 4: CRMP2
	4	Sample 1: TD	Sample 1: LDH-B, STIP1, YBX1
		Sample 2: TD	Sample 2: GDA
		Sample 3: TD	Sample 3: STIP1, CRMP1, YBX1
		Sample 4: TD	Sample 4: LDH-A, LDH-B, STIP1, YBX1, CRMP2
	5	Sample 1: ASD	Sample 1: LDH-B, STIP1, YBX1
		Sample 2: ASD	Sample 2: GDA, CRMP1
		Sample 3: ASD	Sample 3: LDH-B, STIP1, YBX1
		Sample 4: ASD	Sample 4: LDH-A, LDH-B, GDA, CRMP1, CRMP2
	6	Sample 1: ASD	Sample 1: LDH-B, GDA, STIP1, YBX1
		Sample 2: ASD	Sample 2: GDA, STIP1, CRMP2

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peptide-peptide overlap of 14 amino acids (aa). Two separate microarray schemes were created for the discovery microarrays: microarray scheme 1 containing the peptide epitope sequences of LDH-A, STIP1, and CRMP1, whereas microarray scheme 2 contained the peptide epitope sequences of LDH-B, GDA, YBX1, and CRMP2. Control peptides, which included the polypeptide protein tag Flag (DYKDDDDKGG) and a peptide derived from human influenza hemagglutinin (HA) (YPYDVPDYAG), framed each array. Furthermore, neutral GS linkers were added to the C- and N-terminus of each protein to avoid truncated peptides. The discovery peptide microarrays were synthesized by PEPperPRINT as previously described, in which the targeted 15-mer peptide sequences are directly printed in duplicate onto a glass slide using solid-phase Fmoc chemistry (PEPperPRINT, Heidelberg, Germany) (Schirwitz et al., 2012). A total of 1537 different peptides for the proteins LDH-A, STIP1, and CRMP1 were printed in duplicate in microarray scheme 1, whereas microarray scheme 2 contained 1810 peptides corresponding to the proteins LDH-B, GDA, YBX1, and CRMP2.

2.5. Screening peptide microarrays

Based on peptide reactivity profiles identified in the discovery microarray analysis, 75 peptides were selected for the screening peptide microarray, including all peptides found to be highly reactive to pooled maternal plasma samples and a selection of negative control peptides. Negative control peptides were identified as those that were not bound by any maternal plasma samples within the discovery microarrays (Supplemental Table 1). Positive control peptides additionally framed each array, including peptides derived from Poliovirus (KEVPALTAVETGAT), the polypeptide protein tag Flag (DYKDDDDKGG), and human influenza HA (YPYDVPDYAG). Identical copies of the resulting screening microarray were synthesized by PEPperPRINT, containing the targeted and negative control peptides in duplicate and distributed randomly on each slide.

2.6. Microarray staining

Following their synthesis, discovery and screening peptide microarrays were stained in accordance with protocols provided by PEPperPRINT (PEPperCHIP® Peptide Microarray; PEPperPRINT). Microarrays first were incubated with standard buffer (PBS containing 0.05% Tween 20, pH 7.4) for 10 min then with blocking buffer (Rockland Blocking Buffer MB-070; Rockland Immunochemicals Inc.) for 45 min at room temperature (RT). To ensure that the secondary antibodies used within these experiments do not interact with antigen-derived peptides on any of the microarrays, one copy of each of the discovery peptide microarrays (microarray scheme #1 and microarray scheme #2) and of the screening peptide microarray were pre-stained with goat anti-human IgG (H + L) DyLight680 secondary antibody (Rockland Immunochemicals Inc., Limerick, Pennsylvania) diluted 1:5000 in staining buffer (standard buffer with 10% blocking buffer) for 45 min at RT (Loeffler et al., 2016). We did not observe any background due to non-specific binding of the secondary antibody.

All microarrays were then incubated overnight on an orbital shaker at 4 °C with either pooled or individual maternal plasma samples diluted 1:250 in staining buffer. The discovery peptide microarrays were incubated with pooled maternal samples, comprised of a mix of 2–4 plasma samples from mothers of children with ASD or TD that had been determined via western blot to be highly reactive to at least one of the candidate autoantigens represented in the microarray. The screening peptide microarrays were instead incubated with a single maternal plasma sample; these samples were representative of both TD and ASD sample populations and were pre-determined via western blot to be either highly reactive to at least one of the candidate protein autoantigens (Autoantibody positive, N = 67) or unreactive to any of the autoantigens (Autoantibody negative, N = 18). After three short washes in standard buffer, microarrays were incubated for 30 min at RT with goat anti-human IG (H + L) secondary antibody conjugated with DyLight680 (Rockland Immunochemicals Inc.) at a dilution of 1:5000 in staining buffer (standard buffer with 10% blocking buffer). Following secondary antibody incubation, discovery and screening peptide microarrays were imaged as described below. Finally, the HA and Flag control peptides framing the microarrays were stained with the provided corresponding control antibodies (mouse anti-HA-Cy5; mouse anti-FLAG M2-Cy3) diluted 1:1000 in staining buffer as an additional internal quality control to confirm the assay quality and the peptide microarray integrity (PEPperCHIP® Staining Kit; PEPperPRINT).

2.7. Peptide microarray spot quantification

Fluorescence signals on all microarrays were detected with a GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, California), and quantification of spot intensities and peptide annotation was performed using PepSlide Analyser software (PEPperPRINT, Heidelberg, Germany). A software algorithm broke down fluorescence intensities (FIs) of each spot into raw, foreground, and background signals, as well as calculated the standard deviation of median foreground intensities. The foreground median FI of each peptide was averaged over duplicates, reflecting the extent of autoantibody binding to the selected peptides. Signal to noise ratios (foreground/local background signal) were additionally calculated for each peptide spot.

2.8. Statistical analysis

To identify the candidate peptide epitope sequences for their subsequent use in the screening microarrays, discovery microarrays were first qualitatively assessed for regional artifacts (dust, lint, etc.) and staining abnormalities. Only regions with peptides exhibiting the highest median FIs and minimal spot aberrations were then quantitatively assessed for peptide immunoreactivity. Peptides with the highest median foreground intensities ($\geq$600 FI) and a signal to noise ratio $\geq$5 for at least one of the pooled maternal samples within the discovery peptide microarrays were considered as highly reactive for this study and were thus included in subsequent screening peptide microarrays (Nagele et al., 2013). These cut-off values were selected based on cutoff ranges reported in similar autoantibody epitope mapping studies that also used PEPperPRINT microarray technology (Hamilton et al., 2015; Korkmaz et al., 2013).

The duplicate coefficient of variation (CV) was calculated for each peptide epitope, and peptides with a CV greater than 50% were set to missing. Peptides that were not bound by any pooled plasma samples in the discovery microarrays were identified and selected to serve as negative controls for the screening peptide microarrays.

Within the screening peptide microarrays, a peptide was determined to be positive (reactive) for a given maternal plasma sample if both of the following criteria were met:

1. The Chebyshev Inequality Precision Value (CI-p-Value), calculated with the red foreground median fluorescent data, was less than 0.05 for both spots for that peptide. The CI-p-Value is defined as

$$\text{CI-p-Value} = \begin{cases} 1 & Y_k \leq \bar{X} + s \\ \left(\frac{s}{(Y_k - \bar{X})}\right)^2 & Y_k > \bar{X} + s \end{cases}$$

where Y_k is the observed FI for a peptide spot, s is the standard deviation of control spots on the array, and $\bar{X}$ is the sample mean of control spots on the array (Love, 2006).
2. The CV between duplicate spots was less than 50%.

After determining the positive/negative status of individual peptides for each sample via CI-p-Values, we first excluded all peptides that were negative against 100% of the 85 maternal samples in an effort to select for robust peptide reactivity profiles. Peptides that were identified as positive for more than 5% of all maternal samples were considered to be immunodominant (Maksimov et al., 2012). To determine whether reactivity to the individual peptide epitopes of interest differed across maternal sample populations, the resulting positive/negative peptide reactivity data was then compared between maternal subjects. Given our relatively small sample size, we deemed it inappropriate to calculate statistical significance across maternal sample groups with either chi-squared test of independence or Fisher's exact test at this time. Instead, odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated for each individual peptide in two distinct sets of preliminary comparative analyses. In the first set of calculated ORs (Set 1), individual peptide reactivity of all 85 maternal samples were compared across mothers of children with ASD and of mothers of TD children. These initial comparisons included maternal samples previously identified by western blot to be non-reactive (negative) to any of the seven protein antigens of MAR ASD. The second set of comparative analyses (Set 2) was calculated only in mothers previously determined via western blot to harbor autoantibodies specific the antigenic protein that corresponds to the peptide epitope of interest (ASD mothers, N = 11–20; TD mothers, N = 3–9). For example, only mothers that were determined to be reactive against LDH-A were included in the third set of OR calculations for the corresponding LDH-A peptide epitopes. A 0.5 continuity correction was applied to all OR calculations for observations with zero cell counts (Subbiah and Srinivasan, 2008). Statistical analyses were performed with PepSlide Analyser software (PEPperPRINT) and SPSS (Version 23, IBM Corporation, Armonk, NY). All graphs were creating using GraphPad Prism software (GraphPad Software, San Diego, CA).

3. Results

3.1. Peptide epitope identification

To identify the candidate peptide epitope sequences recognized by the maternal autoantibodies of interest, we incubated each of the discovery peptide microarrays with a pool of maternal plasma samples that were determined via western blot as highly reactive to one or more of the autoantigenic proteins. Our initial strategy was to be inclusive during the discovery phase to make sure that all possible positive peptides were included in the screening phase. Of the 3347 peptides represented on the discovery overlapping peptide microarrays (schemes #1 and #2), we identified a total of 63 peptide epitopes that exhibited both very high fluorescence affinity with a strong spot FI ($\geq$600), and a signal to noise ratio $\geq$5 (Fig. 1). Of these 63 identified peptide epitopes, 7 peptides were specific to LDH-A, 13 peptides for LDH-B, 4 peptides for GDA, 8 peptides for YBX1, 5 peptides for STIP1, 9 peptides for CRMP1, and 17 peptides for CRMP2 (Supplementary Table 2).

3.2. Identification of immunodominant peptide epitopes

To determine individual reactivity profiles against the antigen peptides identified in the discovery arrays, a total of 85 maternal plasma samples were tested on the screening peptide microarray containing a total of 75 peptides, including the 63 highly reactive 15-mer peptide sequences, 7 elongated peptides that contained 7 of the 62 peptides, but were 1–2 aa longer in their sequence (e.g. 16–17 aa long), as well as a selection of negative control peptides identified within the discovery microarrays. The peptides with the longer sequences were included as they were sequential on the discovery array and we elected to have them made as a combined peptide to determine if the reactivity was independent or inclusive. Further, as mothers of children with ASD often have reactivity to combinations of two or more of the MAR-specific autoantigens and thus could be positive for peptides contained within array schemes 1 and 2 of the discovery microarrays, the validation arrays also enabled us to test reactivity to the seven autoantigens simultaneously. The screening peptide microarray confirmed that the maternal autoantibodies recognized unique, discrete peptide sequences within the antigenic proteins of interest and did not react to the control peptides (Fig. 2). Additionally, mothers whose plasma demonstrated reactivity to a given protein often had reactivity to more than one peptide contained within that protein (Supplementary Fig. 1). Following the calculation of CI-p-values during the more stringent analytical phase, a total of 29 peptides were identified as non-reactive against all 85 maternal samples and were thus excluded from subsequent comparative analyses (Supplementary Table 3). Maternal reactivity towards the remaining 46 peptides was then compared across diagnostic groups, calculating two separate sets of odds ratios to best quantify the association between maternal peptide reactivity and having a child with ASD (Table 3; Supplementary Tables 4–10). Of the 46 peptide epitopes analyzed, 4 peptides were specific to LDH-A, 10

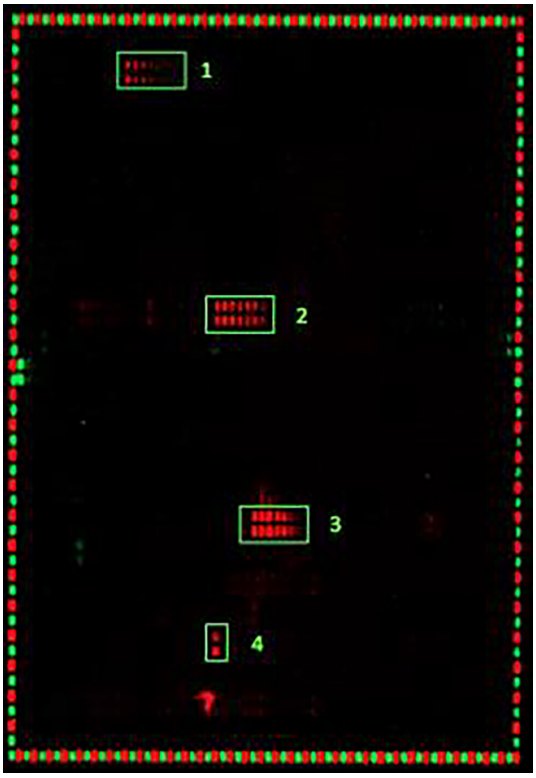

Fig. 1. Representative discovery array with LDH-A, STIP1 and CRMP1 15-mer amino acid sequences probed with a maternal sample positive for all three proteins. Each box shows the region of antibody reactivity (Box 1 = LDH-A; Box 2 = STIP1; Boxes 3 and 4 = CRMP1). The microarray frames (controls) in each were stained with anti-Flag (shown in green) and anti-HA antibodies (shown in red), while the red spots within the array represent areas of antibody recognition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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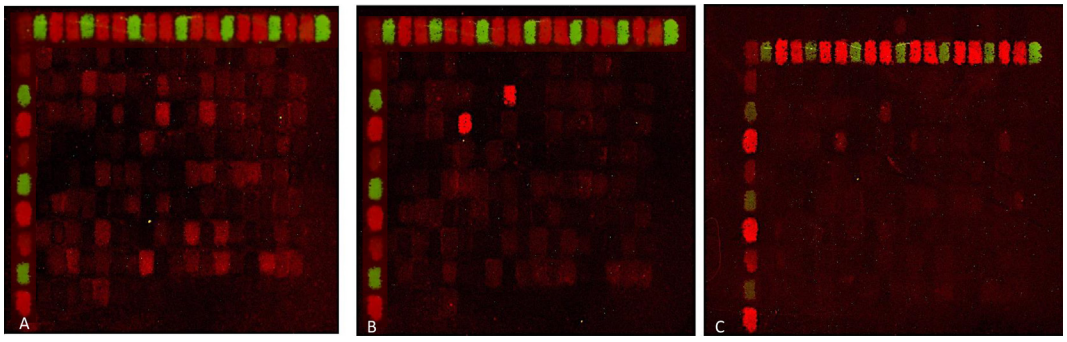

Fig. 2. Representative figure of screening arrays with all peptides represented. A) Sample from a mother whose child has severe ASD, and has reactivity to 5 of the 7 antigens; B) Sample from a mother whose child has mild ASD and has reactivity to one antigen; and C) Sample from a mother whose child is typically developing and has no reactivity to any of the seven protein antigens. The microarray frames (controls) in each were stained with anti-Flag (shown in green if present) and anti-HA antibodies (shown in red), while the red spots within the array represent areas of antibody recognition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Screening microarray – proportion of maternal reactivity to individual peptides.

Protein	Peptide	TD (N = 30)		ASD (N = 55)		ASD vs TD
		N	%	N	%	OR (95% CI)[a]
LDH-A	ADELALVDVIEDKLK	1	3.3%	1	1.8%	0.537 (0.032–8.905)
	CHGWVLGEHGDSSVP	2	6.7%	1	1.8%	*0.259 (0.023–2.985)*
	DLADELALVDVIEDK[b]	4	13.3%	3	5.5%	0.375 (0.078–1.801)
	VDVIEDKLKGEMMDL[b]	3	10.0%	8	14.5%	1.532 (0.374–6.267)
LDH-B	CIIIVVSNPVDILTY	0	0.0%	1	1.8%	1.679 (0.066–42.490)[a]
	CIIIVVSNPVDILTYVT	1	3.3%	0	0.0%	*0.177 (0.007–4.486)*[a]
	DCIIIVVSNPVDILT[b]	0	0.0%	5	9.1%	**6.644 (0.355–124.384)**[a]
	EKLIAPVAEEEATVP[b]	3	10.0%	4	7.3%	0.706 (0.147–3.386)
	ESMLKNLSRIHPVSTMV	1	3.3%	1	1.8%	0.537 (0.032–8.905)
	IAPVAEEEATVPNNKIT	0	0.0%	2	3.6%	**2.851 (0.133–61.327)**[a]
	LIAPVAEEEATVPNN	1	3.3%	1	1.8%	0.537 (0.032–8.905)
	LQTPKIVADKDYSVTAN	1	3.3%	1	1.8%	0.537 (0.032–8.905)
	PVAEEEATVPNNKIT	0	0.0%	3	5.5%	**4.067 (0.203–81.403)**[a]
	TPKIVADKDYSVTAN	1	3.3%	1	1.8%	0.537 (0.032–8.905)
STIP1	PPPPPPKKETKPEPM[b]	5	16.7%	2	3.6%	*0.189 (0.034–1.040)*
	PPPPPPKKETKPEPME[b]	5	16.7%	7	12.7%	0.729 (0.210–2.533)
	VDLGSMDEEEEIATP[b]	1	3.3%	7	12.7%	**4.229 (0.495–36.140)**
	VLLGVDLGSMDEEEE[b]	8	26.7%	15	27.3%	1.031 (0.378–2.813)
GDA	MDLNDTFPEYKETTE[b]	1	3.3%	8	14.5%	**4.936 (0.587–41.527)**
YBX1	ETVEFDVVEGEKGAE[b]	3	10.0%	8	14.5%	1.532 (0.374–6.267)
	PAAPPAAPALSAADT[b]	8	26.7%	19	34.5%	1.451 (0.544–3.874)
	PPRQRQPREDGNEED[b]	19	63.3%	28	50.9%	0.600 (0.241–1.494)
	RRPYRRRRFPPYYMR	1	3.3%	0	0.0%	*0.177 (0.007–4.486)*[a]
	TVKWFNVRNGYGFIN	1	3.3%	0	0.0%	*0.177 (0.007–4.486)*[a]
CRMP1	QSNFSLSGAQIDDNN	1	3.3%	1	1.8%	0.537 (0.032–8.905)
	VDITSWYDGVREELE[b]	5	16.7%	13	23.6%	1.548 (0.493–4.859)
	VGSDADVVIWDPDKL[b]	5	16.7%	5	9.1%	0.500 (0.132–1.889)
	VTSPPLSPDPTTPDY[b]	8	26.7%	5	9.1%	0.275 (0.081–0.936)
	VVPEPGSSLLTSFEK	0	0.0%	2	3.6%	**2.851 (0.133–61.327)**[a]
CRMP2	ELRGVPRGLYDGPVC[b]	4	13.3%	6	10.9%	0.796 (0.206–3.075)
	ELRGVPRGLYDGPVCE[b]	5	16.7%	11	20.0%	1.250 (0.390–4.010)
	GENLIVPGGVKTIEA	1	3.3%	0	0.0%	*0.177 (0.007–4.486)*[a]
	GVPRGLYDGPVCEVS[b]	4	13.3%	3	5.5%	0.375 (0.078–1.801)
	HNSSLEYNIFEGMEC[b]	6	20.0%	17	30.9%	1.789 (0.619–5.174)
	KTISAKTHNSSLEYN	0	0.0%	2	3.6%	**2.851 (0.133–61.327)**[a]
	LRGVPRGLYDGPVCE[b]	7	23.3%	12	21.8%	0.917 (0.317–2.649)
	MAERKQSGKAAEDEE[b]	4	13.3%	12	21.8%	1.814 (0.529–6.218)
	QKAVGKDNFTLIPEG	1	3.3%	0	0.0%	*0.177 (0.007–4.486)*[a]
	RGVPRGLYDGPVCEV[b]	3	10.0%	5	9.1%	0.900 (0.200–4.058)
	RGVPRGLYDGPVCEVSV[b]	2	6.7%	3	5.5%	0.808 (0.127–5.123)
	TSPPLSPDPTTPDFL	1	3.3%	0	0.0%	*0.177 (0.007–4.486)*[a]
	VGSDADLVIWDPDSV[b]	11	36.7%	14	25.5%	0.590 (0.226–1.539)
	VKTISAKTHNSSLEYN[b]	2	6.7%	3	5.5%	0.808 (0.127–5.123)
	VNDDQSFYADIYMED[b]	14	46.7%	20	36.4%	0.653 (0.265–1.612)
	VPEPGTSLLAAFDQW[b]	8	26.7%	11	20.0%	0.688 (0.242–1.954)
	VPRGLYDGPVCEVSV[b]	1	3.3%	8	14.5%	**4.936 (0.587–41.527)**

Abbreviations: TD, typically developing; ASD, autism spectrum disorder; OR (95% CI), odds ratio (95% confidence interval).
Observed ORs with values above 2.0 are highlighted in bold; ORs with values less than 0.3 are italicized.
[a] A 0.5 continuity correction was applied to all OR calculations for observations with zero cell counts.
[b] Indicates immunodominant epitope, as defined as binding to at least 5% of all 85 maternal samples (N ≥ 5).

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peptides for LDH-B, 1 peptide for GDA, 5 peptides for YBX1, 4 peptides for STIP1, 5 peptides for CRMP1, and 17 peptides for CRMP2. Furthermore, at least one peptide was identified for each of the seven antigenic proteins with more than 5% of maternal samples demonstrating positive reactivity (immunodominant). As indicated in Table 3, 28 immunodominant epitopes were identified in total (LDH-A = 2; LDH-B = 2; STIP1 = 4; GDA = 1; YBX1 = 3; CRMP1 = 3; CRMP2 = 13).

In addition to identifying the dominant epitopes recognized by the maternal autoantibodies, we performed a preliminary set of comparison analyses to evaluate whether the proportion of maternal reactivity for each peptide varied across the diagnostic populations (Table 3; Supplementary Tables 4–10). While limited by our relatively small sample size, there were several peptides differentially recognized by autoantibodies for both diagnostic populations. For example, maternal reactivity was observed exclusively in mothers of children of ASD towards several peptides, including the LDH-B peptides DCIIIVVSNPVDILT (9.1% ASD vs. 0% TD; OR (95% CI)$_{Set\ 1}$ = 6.644 (0.355–124.384)) and PVAEEEATVPNNKIT (5.5% ASD vs. 0% TD; OR (95% CI)$_{Set\ 1}$ = 4.067 (0.203–81.403)). For other proteins, there was only one peptide that had significantly higher reactivity in the ASD population such as STIP1 (VDLGSMDEEEEIATP; 15% ASD vs. 0% TD; OR (95% CI) = 3.800 (0.177–81.585)), and CRMP1 (VDITSWYDGVREELE; 33% ASD vs. 0% TD; OR (95% CI) = 5.720 (0.272–120.327)). While significant exclusive peptide reactivity was not observed for any of the 30 TD mothers, several peptides were identified as positive for a larger proportion of mothers of TD children relative to mothers of children with ASD (Table 3). Maternal reactivity against the STIP1 peptide PPPPPPKKETKPEPM, for example, was identified as positive in 16.7% of TD mothers yet only 3.6% of ASD mothers (OR (95% CI)$_{Set\ 1}$ = 0.189 (0.034–1.040)).

In addition to looking at the ASD group as a whole, we also separated those mothers whose children had a diagnosis of ASD into ASD-severe and ASD-mild based on the ADOS severity scale. While we did not have enough samples for both groups to accurately analyze the relationship between ASD severity and peptide reactivity pattern, examination of the data in this manner provided preliminary data suggesting that there could be epitope reactivity differences associated with child outcome for some antigens (Supplementary Tables 4–10). For example, there were several LDH-B peptides for which only the mothers of children with mild ASD were reactive (and reactive to multiple peptides within LDH-B). However, for the LDH-B peptide DCIIIVVSNPVDILT, for which reactivity is seen only for ASD samples, all reactivity was driven by the ASD severe cases. This was also noted for the STIP1 peptide, VDLGSMDEEEEIATP (ASD vs. TD OR (95% CI)$_{Set\ 2}$ = 3.800 (0.177–81.585)), the YBX1 peptide ETVEFDVVEGEKGAE (ASD vs. TD OR (95%CI)$_{Set\ 2}$ = 3.148 (0.134–73.856)), and the CRMP1 peptide VDITSWYDGVREELE (ASD vs. TD OR (95%CI)$_{Set\ 2}$ = 5.720 (0.272–120.327)).

4. Discussion

Previous studies have demonstrated that plasma from some mothers of children with ASD contained autoantibodies reactive to 7 neurodevelopmental proteins (Braunschweig et al., 2013). It is currently hypothesized that autoantibodies present in some mothers of children with ASD may have deleterious neurodevelopmental consequences, such as directly interfering with the function and/or decreasing the availability of proteins critical for neurodevelopment. While the ontogeny of maternal autoantibody generation in the mothers is unclear, studies that aim to gain an understanding of the detailed pathogenic mechanisms are currently underway. Thus, the primary goal of the current study was to determine and verify the epitopes for each autoantigen using maternal samples with known reactivity, as well as autoantibody negative maternal samples as controls. To better understand MAR ASD and the manner in which maternal autoantibodies interact with their target proteins, we successfully determined the immunodominant epitopes for each autoantigen. In addition, differential reactivity to some peptide epitopes was noted between mothers of children with ASD and mothers of TD children. For example, there were individual peptides that were only recognized by the ASD maternal samples and not bound by the autoantibodies in the TD maternal plasma samples, even though they recognized the same full-length autoantigen. As these findings would have previously been recognized as falsely positive, our data herein suggests that target specificity is important for risk identification and might be important for disease pathogenesis. How this repertoire difference arises is currently unknown. One possibility is that self-antigens can be processed and presented differently depending on the route and circumstances of exposure. In addition to the differential peptide recognition between the ASD and TD maternal samples as a whole, some interesting differences between the ASD severe and ASD mild samples arose.

Interestingly, it appears that slight shifts in the amino acid sequence lead to differential binding between the two groups, as was observed for STIP1. This suggests that there are binding repertoire differences even to a similar linear peptide sequence between the ASD and TD sample sets indicative of determinant spreading in the MAR ASD autoantibody-positive mothers. The phenomenon of epitope spreading is a well-known but not totally understood feature of autoimmune disorders (Xu et al., 2016). It was first described in a murine model of the T-cell-mediated demyelinating disease multiple sclerosis in which immunization of susceptible mouse strains with myelin basic protein (MBP), or the immunodominant MBP peptide, induces experimental autoimmune encephalitis with associated clinical paralysis. Studies show that during the inductive phase of disease, the initial T-cell response is directed towards a single MBP peptide, but this response expands to include several other cryptic peptides of MBP as disease progresses (Lehmann et al., 1992). Cryptic epitopes by definition are not naturally presented by antigen-presenting cells, thereby implying that events associated with inflammation and immune activation that were triggered by the initial insult make the cryptic epitopes visible to the autoreactive T cells.

Studies in autoantibody-mediated diseases such as rheumatoid arthritis have shown that the number of peptides recognized by autoantibodies increased prior to disease onset. During active disease, patients with undifferentiated arthritis who later developed rheumatoid arthritis recognized significantly more peptides than that those who did not progress (van der Woude et al., 2010), suggesting that the expanded repertoire contributes to disease. Thus, the examination of determinant spreading might be useful for predicting onset and disease severity of an autoimmune disorder. Our preliminary examination of severity of the child's ASD relative to maternal autoantibody reactivity suggests that there could be differences in peptide epitope reactivity associated with outcome in the child for some antigens. Further expanded studies will be necessary to confirm this initial finding.

Future studies will be conducted to determine the critical amino acids necessary for autoantibody binding, as this may further segregate the ASD population from the TD population and is essential in developing a potential therapeutic strategy for MAR ASD. Additionally, we aim to develop a peptide ELISA with which to conduct blocking studies, similar to those in Braunschweig et al. (2013). We will utilize the full-length proteins as well as peptide sequences to determine if peptide reactivity is lost, which would further demonstrate that maternal autoantibodies are specifically recognizing and binding to peptide epitopes.

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Defining the mechanisms through which these ASD-specific maternal autoantibodies lead to alterations in neurodevelopment is an area of active investigation. Numerous animal model studies using gestational transfer of purified IgG from mothers of children with ASD have demonstrated that brain-reactive maternal autoantibodies induce long-term behavioral changes in exposed offspring exposed during gestation (Bauman et al., 2013; Martin et al., 2008; Martinez-Cerdeno et al., 2016; Singer et al., 2009). Beginning with the first passive transfer study in rhesus monkeys and continuing on with murine passive transfer models, these models suggest that there is pathologic significance associated with the ASD-specific maternal autoantibodies. However, a more stringent animal model in which tolerance is broken to the defined autoantigens is needed to truly recapitulate the clinical phenotype in an endogenous model of MAR ASD. For this model to be both successful and relevant, the immunodominant peptides recognized by ASD-specific human maternal autoantibodies must be known in order to create the autoantibodies in the animal. Based upon the findings of the current study, the next generation animal model of MAR ASD is currently underway and will allow us to develop a clearer understanding of the mechanism responsible for MAR ASD as well as assist in finding new ways of treating and preventing this disorder.

In addition to a providing a more useful tool for the study of MAR ASD autoantibody pathology, looking at the target peptides in terms of cross-reactivity with critical ligands or receptors is also of interest. For example, there is evidence that autoantibodies from patients with systemic lupus erythematosus (SLE) directed against double stranded DNA cross-reacts with the NMDA receptor and has been implicated in the neuropsychiatric symptoms observed in some patients with SLE (Diamond et al., 2009; Huerta et al., 2006). The neuropathologic significance of these cross-reactive autoantibodies was further established in a murine model, thereby demonstrating the importance of understanding the peptide specificity of clinical autoantibodies.

Finally, we should note that there were several limitations for the current study. While we determined the relevant epitopes for MAR ASD, use of these peptides in determination of differential reactivity are preliminary in terms of clinical significance and need to be confirmed using a more quantitative and reliable assay, as well as a larger sample population. Moreover, there is currently no standard for processing the results of peptide microarrays, which are still plagued by issues with batch and inter-assay variability making interpretation of the data more challenging (Zhu et al., 2015). In the future, we will determine the critical amino acids needed for autoantibody binding to provide the information necessary to create individualized therapeutic strategies. Additionally, succeeding experiments with larger sample sizes will enable the detection of associations between maternal autoantibody reactivity to individual or combinations of peptides and increased aberrant behaviors and/or increased cognitive and social deficits in children with ASD. Though studies utilizing a larger sample size must be conducted to verify these results, the identification of the peptides described herein are the first step towards the development of an endogenous and clinically relevant animal model for MAR ASD. Further, these peptides have the potential to be used as a more robust set of biomarkers for ASD risk assessment and subphenotype stratification. Finally, efforts are underway to determine if the location and/or amino acid sequences of the ASD-specific epitopes have functional significance.

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Declarations

The authors (JV and EE) have a patent application involving the peptides described herein. The other authors have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbi.2017.12.014.

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