Application of Immunosignatures to the Assessment of Alzheimer's Disease

Lucas Restrepo, MD, MS,[1,2,3] Phillip Stafford, PhD,[1] D. Mitch Magee, PhD,[1] and Stephen Albert Johnston, PhD,[1,3]

Objective: Accurate assessment of Alzheimer's disease (AD), both presymptomatically and at different disease stages, will become increasingly important with the expanding elderly population. There are a number of indications that the immune system is engaged in AD. Here we explore the ability of an antibody-profiling technology to characterize AD and screen for peptides that may be used for a simple diagnostic test.
Methods: We developed an array-based system to profile the antibody repertoire of transgenic mice with cerebral amyloidosis (TG) and elderly individuals with or without AD. The array consists of 10,000 random sequence peptides (20-mers) capable of detecting antibody binding patterns, allowing the identification of peptides that mimic epitopes targeted by a donor's serum.
Results: TG mice exhibited a distinct immunoprofile compared to nontransgenic littermates. Further, we show that dementia patients, including autopsy-confirmed AD subjects, have distinguishable profiles compared to age-matched nondemented people. Using antibodies to different forms of Aβ peptide and blocking protocols, we demonstrate that most of this signature is not due to the subject's antibodies raised against Aβ.
Interpretation: We propose that "immunosignaturing" technology may have potential as a diagnostic tool in AD.
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Currently, there are no accurate means to establish the diagnosis of Alzheimer's disease (AD).[1–5] Physicians base their diagnosis on the exclusion of other neurological disorders, rather than testing directly for AD, an exercise that misdiagnoses about 1 in 5 patients.[1–5] Hence, substantial interest exists in the development of techniques that may help diagnosing specific dementias. A test for AD is needed in several contexts: (1) during the presymptomatic stage, (2) mild cognitive impairment (MCI), (3) overt symptomatic stage, and (4) disease progression monitoring. Of many biomarkers surveyed to date, none is used routinely in these scenarios. Historically, AD biomarkers have derived from the amyloid cascade, cytokine signaling, and neurotubule biology.[6–8] More recently, the diagnostic merits of autoantibodies have been investigated.[7,9–22] However, the premise of robust, simple, and cost-effective immunodiagnostic techniques to assist in AD assessment remains elusive.

Immunoglobulins are encountered in senile plaques, the distinctive histopathological feature of AD. Many individuals have circulating autoantibodies targeting different molecules, including β-amyloid (Aβ) and tau.[7,9–22] It is possible that the neurodegenerative process of AD offers a growing assortment of epitopes to the immune system, predating the symptomatic stage. Exposure of brain antigens to immune surveillance is facilitated by the progressive derangement of the blood-brain barrier that accompanies AD. Therefore, a test capable of assessing such humoral response may become a useful diagnostic platform. Here we describe a novel strategy for the assessment of AD called "immunosignature," which employs a customized microarray with 10,000 random-sequence peptides. We show that this platform is capable of detecting antibody binding patterns, allowing the identification of peptides that mimic actual epitopes targeted by a donor's plasma.

Subjects and Methods

Microarray Production

Our microarray consists of a solid phase with 10,000 random-sequence 20-mers covalently attached to glass slides, which can be probed with any antibody of interest.[23–26] Peptide sequence

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Address correspondence to Dr Johnston, Center for Innovations in Medicine, The Biodesign Institute, Arizona State University, Tempe, AZ 85287-5901. E-mail: stephen.johnston@asu.edu

From the [1]Center for Innovations in Medicine, Biodesign Institute, [2]Molecular and Cell Biology Program, and [3]School of Life Sciences, Arizona State University, Tempe, AZ.

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and location in the array is known. The 10,000 peptides were designed using custom software that randomly picked 19 natural amino acids (except cysteine) to build stochastic sequences consisting of 17 residues. All peptides have glycine-serine-cysteine linkers at the carboxyl terminus to space main amino acid sequence from the glass slide. Peptides were synthesized by Alta Biosciences (Birmingham, UK) and spotted in duplicate using a NanoPrint LM60 microarray printer (ArrayIt, Sunnyvale, CA). Slides were stored under argon at 4°C until used.

Microarray-Based Immunoassay

Microarray slides were blocked with 3% bovine serum albumin/phosphate-buffered saline (BSA/PBS), then washed with trishydroxymethylaminomethane-buffered saline Tween 20 (TBST) and distilled water. Primary antibodies diluted to 10μM were allowed to react with the arrays in duplicates for 1 hour at 37°C. A biotinylated, species-specific antibody was allowed to incubate with the slides, followed by 5μM Streptavidin conjugated to Alexa 555. Arrays were scanned with a laser to generate digital images that were processed using GenePix Pro v6 (Molecular Devices, Sunnyvale, CA).

Microarray Analysis

Scanned data was loaded into GeneSpring 7.2.1 (Agilent Technologies, Santa Clara, CA) and analyzed. For preprocessing, the slides' signal intensity was $\log_{10}$ transformed and median normalized. Signals were deemed present when intensities were >1 standard deviation from mean local background. Peptide identification was done using *t* tests,[27–29] Model I (fixed effects) 1-way or multiway analysis of variance (ANOVA), and correlation to specific expression patterns. Clustering techniques, including k-means, hierarchical clustering, and self-organizing maps were used for identifying antibody binding patterns. We screened for technically irreproducible values during data preprocessing. Each peptide array replicate provides a 1.5-fold minimum average detectable fold change at $\alpha = 0.05$ and $\beta = 0.20$. False-positive corrections with 5% false discovery rate were carried out using family-wise multiple testing.[30]

Antibodies

We purchased the following monoclonal antibodies: (1) 4G8, which targets the juxtamembrane extracellular domain (residues 17–24) of Aβ (MAB1561SP; Millipore, Billerica, MA); (2) 2B9, raised against amino acids 1–17 of Aβ (sc-70355; Santa Cruz Biotechnology, Santa Cruz, CA); (3) DE2, raised against residues 1–16 of Aβ (MAB 5206SP; Millipore); (4) BAM-10, which recognizes residues 1–12 of Aβ (A3981; Sigma-Aldrich, St. Louis, MO); (5) anti-tau Asp 421 (caspase-cleaved region; MAB5430SP; Millipore), and (6) anti-tau 210-241 (MAB361SP; Millipore). The following polyclonal antibodies were purchased: (1) anti-carboxyl-terminus of Aβ 1–40 (PC149; Calbiochem, San Diego, CA), (2) anti-carboxyl-terminus of Aβ 1–42 (A1976; Sigma-Aldrich), (3) anti-Aβ oligomer, which detects Aβ octamers but not fibrils or monomers (AHB0052; Biosource, Camarillo, CA), and anti-tau phosphothreonine 231 (AB9698SP; Millipore). An anti-human albumin polyclonal antibody raised in goat (A7544; Sigma) was also acquired. Biotinylated antibodies targeting rabbit, mouse, goat, and human immunoglobulin G (IgG) were purchased from Bethyl (Montgomery, TX). Streptavidin-Alexa 555 was purchased from Invitrogen (Carlsbad, CA).

Mice

APPswe/PSEN1-1dE9 transgenic (TG) mice were purchased from Jackson Laboratories (Bar Arbor, ME), as well as nontransgenic controls (B6C3F1/J). Plasma from vaccinated TG mice was provided by Dr Roger N. Rosenberg (Department of Neurology, University of Texas-Southwestern Medical School, Dallas, TX). Five TG mice were vaccinated with a plasmid encoding Aβ 1–42, while 7 were vaccinated with mock DNA. All plasmids were delivered through a gene gun for 10 doses. Two nontransgenic, nonimmunized BALB/c mice were used as additional controls. Plasma samples were obtained at the time the mice were sacrificed (15 months of age).

Human Plasma

Plasma from 12 patients with probable AD and 12 age-matched controls without cognitive derangement were provided by Alex Roher (Cohort A; Banner's Sun Health Research Institute, Phoenix, AZ). All patients were enrolled into a brain-bank program. Postmortem examination was performed by a neuropathologist on 9 patients (5 with and 4 without dementia). Samples were acquired after written consent and approval of the Banner Institutional Review Board (IRB). Plasma from a second cohort of elderly patients (Cohort B) was provided by Roger N. Rosenberg (UT Southwest Medical Center, Dallas, TX). Profiling studies were approved by ASU's IRB (protocol #0912004625).

Blocking Experiments with Aβ-Coated Beads

Synthetic Aβ 1–40 covalently attached to TantaGel S NH2 polystyrene beads (Advanced ChemTech, Louisville, KY) were used, carrying approximately 0.2mmol antigen/gm. To decrease nonspecific binding, various bead concentrations ranging from 1 to 0.01mM were preblocked with 5% BSA-PBS. Beads were stored at 4°C overnight and rinsed with 3% BSA-PBS-0.05% Tween20 prior to mixture with plasma pools dissolved 1:500 in 3% BSA-PBS-0.05% Tween20. This mixture was incubated at 37°C, centrifuged, and the supernatant was assayed on microarray slides as previously described. Blank beads similarly treated were used as controls.

Results

Binding Pattern of Antibodies Against Aβ and Tau

First, we endeavored to determine whether specific antibodies targeting peptides relevant to AD pathophysiology showed distinctive microarray binding patterns. We analyzed the signature of 11 monoclonal or affinity-purified antibodies: 7 against Aβ (4 monoclonal, 3 polyclonal) and 3 against tau (2 monoclonal, 1 polyclonal,

summarized in Supporting Table 1). A polyclonal antibody against human albumin was also included. Each antibody bound different microarray peptides above median signal threshold (3-sigma). Binding intensity and the order in which reactive peptides are ranked yielded specific information regarding each antibody. Peptides bound by each antibody were distinct. The microarray segregated the signature of every individual antibody from the secondary biotinylated antibody by itself (anti-rabbit or anti-mouse) and from other monoclonal and polyclonal antibodies (Fig 1 and Supporting Fig 1). The signature of the secondary antibody can be subtracted from the primary to enhance the specificity of patterns. Results were reproducible, with good agreement between duplicates run by the same individual ($r = 0.846$–0.966) and different operators ($r = 0.95$ for first slide, 0.94 for second slide). Polyclonal antibodies targeting the carboxyl-terminus of Aβ shared binding pattern similarities with an antibody that recognizes Aβ oligomers and an antibody raised against phosphorylated tau. Other antibodies, mainly monoclonal IgG targeting the amino-terminus of Aβ, shared no binding similarities. These experiments show that the microarray platform can detect distinctive patterns of antibody reactivity.

Immunosignature of APPswe/PSEN1-1dE9 Transgenic Mice

These mice are engineered with 2 human mutations found in familial AD, affecting the amyloid precursor protein (APP) and presenilin-1 (PSEN1) genes. The resulting phenotype is well characterized, consisting of progressive amyloidosis involving cerebral cortex, astrocytosis, neurodegeneration, and cognitive impairment, beginning at about 6 months of age.[31–33] The microarray signature of 10-month-old TG mice was different from 4 age-matched B6C3F1/J nontransgenic littermates (Fig 2A, B). Furthermore, the microarray detected a change in the signature of TG mice immunized with a plasmid coding for human Aβ 1–42 (see Fig 2C, D). Aβ immunohistochemistry revealed heavy amyloid deposition in the brain parenchyma of mock-vaccinated TG mice, whereas TG mice treated with Aβ plasmid had reduced amyloid deposits (data not shown). Three microarray peptides avidly bound by plasma from mice vaccinated with Aβ also were among the top binders of the 7 commercial anti-Aβ antibodies. These experiments demonstrate that TG mice have a distinctive immunosignature that can be altered by genetic immunization, although a minimal component of the signature is shared with specific anti-Aβ antibodies.

Immunosignature of AD

Plasma samples from 8 AD patients and 9 age-matched controls without dementia (Cohort A) were assayed on the microarray. Postmortem examination was carried out in 9 of these patients, showing signs of AD in 4 patients (Braak scores IV–V), while insufficient criteria to diagnose AD was noted on 4 cognitively-normal controls (Braak scores II–III). The ninth patient, diagnosed in life with probable AD, received a final diagnosis of progressive supranuclear palsy (PSP) on autopsy (Braak score of III). We detected 3 microarray binding patterns: 1 common to all AD patients and 2 patterns that grouped all control samples, which we term normal and intermediate (Fig 3). The PSP patient had a unique pattern that cosegregated with the normal pattern. We also found that plasma pools from 11 patients with AD and 12 nondemented controls segregate with and are representative of individual plasma samples from either group (see Fig 3C, D). Using ClustalW 2.0, an automatic program for global multiple alignment of amino acid sequences,[34] we found that none of the 50 higher ranking peptides (Supporting Table 2) bound by the autopsy-proven AD plasma pool had sequence similarity with Aβ 1–40 or Aβ 1–42. Eleven microarray peptides highly bound by the AD autopsy plasma pool were also top binders of the 7 commercial anti-Aβ antibodies. The predictive capacity of the immunosignature was assessed by retesting 8 random samples (5 with AD and 3 controls) in blinded fashion. Using GeneSpring GX, we established a learning data set using known binding patterns exhibited by the complete sample set of human IgG. With this training set, blinded samples were assigned to any of the patterns, which correctly recognized 4 AD and 2 control cases but misclassified 2 samples (1 erroneously assigned to AD). We assayed an additional set of plasma samples (12 AD patients and 12 elderly controls) from a different source (Cohort B), using another microarray platform featuring a different assortment of 10,000 random-sequence peptides (10K 2.0). Two plasma pools from the patients from Cohort A who underwent autopsy were used as additional controls. Once again, AD plasma segregated from control samples, while the autopsy pools grouped appropriately with the individual samples according to group. While these are early results, our data supports the concept that different antibody binding patterns are detectable and reproducible, and that the immunosignaturing technique could be developed to assist in the classification of patients with dementia.

Blocking Experiments with Aβ-Coated Beads

To determine whether the immunosignatures observed in humans are partly due to Aβ immunoreactivity, we

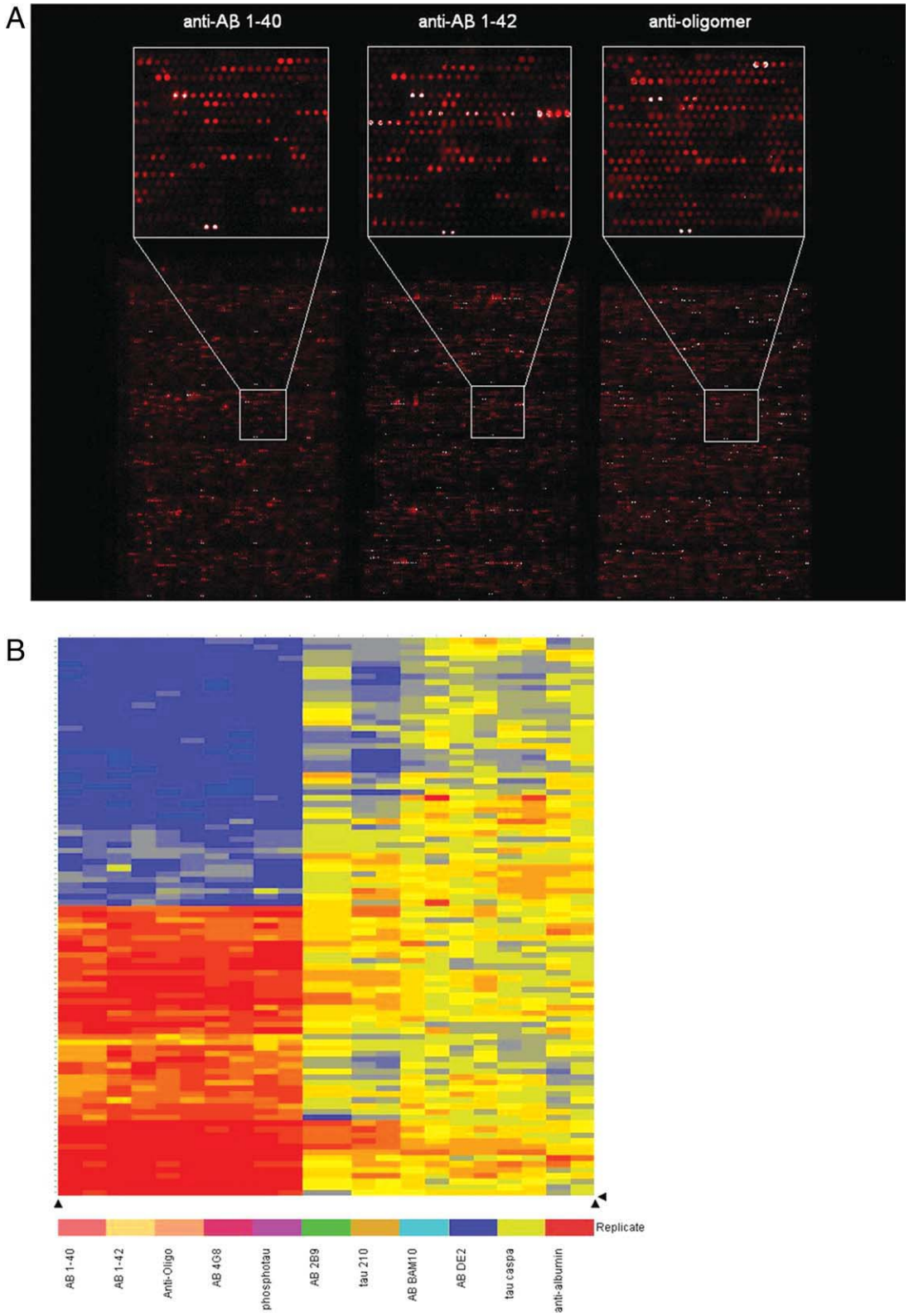

FIGURE 1: Microarray signatures of anti-Aβ antibodies. (A) Scanned image of peptide microarray hybridization of 3 rabbit polyclonal antibodies against Aβ. The white boxes represent equivalent areas within the array, which are expanded above for greater detail. Spots represent individual peptides organized in the array; white, red, and black colors indicate strong, medium, and low antibody binding, respectively. (B) Heat map showing high correlation between antibodies targeting the carboxyl-terminus of Aβ and the anti-oligomer and anti-phospho-tau antibodies. This particular heat map features 93 peptides deemed informative by ANOVA. Each antibody pattern is represented in duplicate.

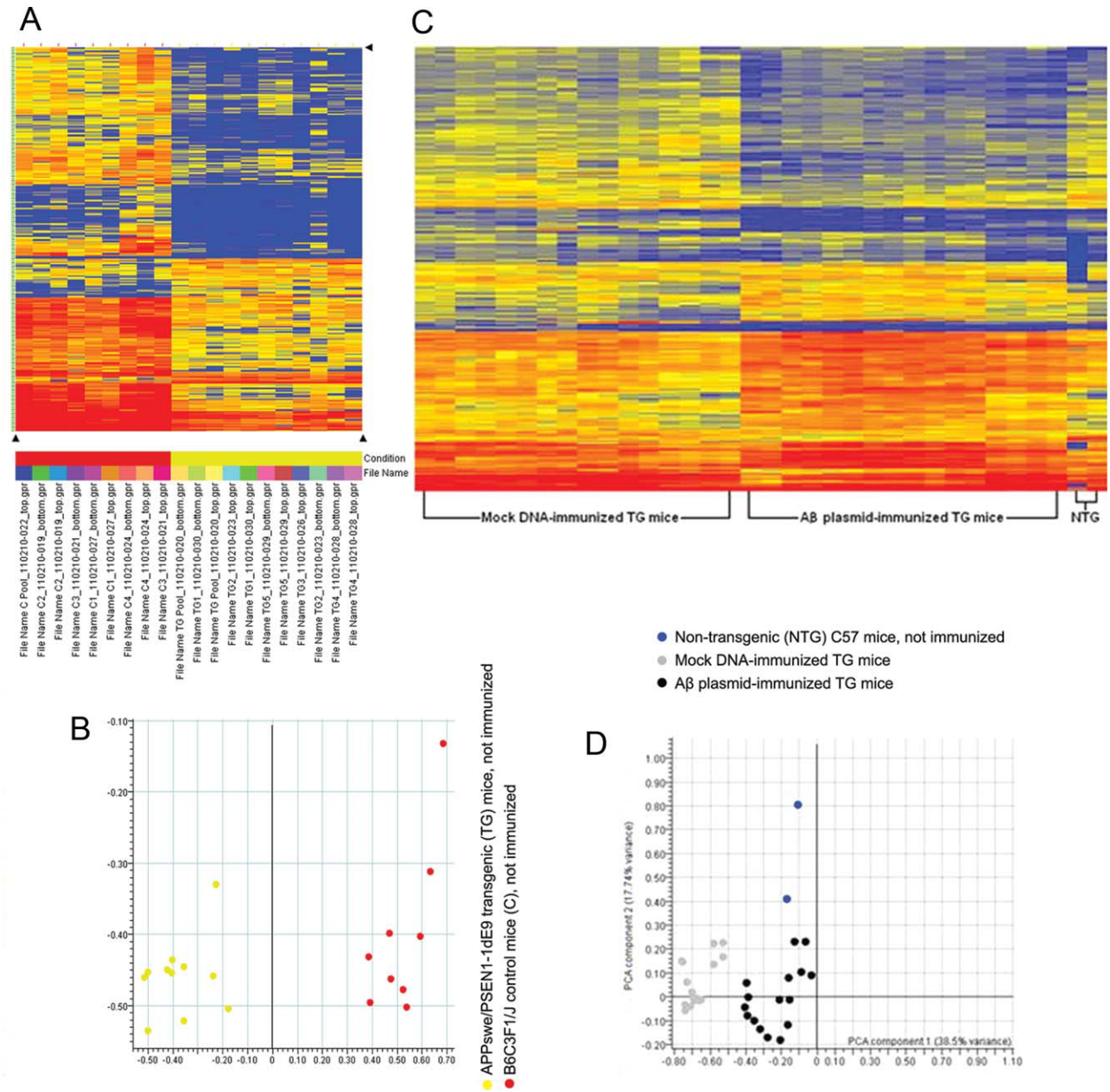

FIGURE 2: Immunosignature of transgenic mice. (A) Heat map of 113 microarray peptides that can discriminate between plasma signatures of *APPswe/PSEN1-1dE9* transgenic (TG) mice (n = 5) and nontransgenic B6C3F1/J littermates (n = 4). Blue tones indicate low binding and red colors indicate avid binding (more antibodies bound per spot), whereas yellow hues designate intermediate binding. Note that plasma pools segregate with individual samples. (B) Principal component scatter (PCA) plot showing same mice plasma samples. (C) Heat map encompassing the entire 10,000-peptide array signature of serum samples from 15-month-old TG mice. The heat map sets apart 3 groups: on the far left, TG vaccinated with mock DNA; center-right, TG mice vaccinated with a plasmid coding for Aβ 1–42; and to the far right, serum samples from nontransgenic nonvaccinated C57 mice (NTG). (D) Principal component scatter plot, demonstrating segregation of plasma signature from mock DNA-treated, Aβ 1–42 plasmid-treated TG and NTG mice.

carried out blocking experiments using synthetic Aβ 1–40 covalently attached to polystyrene beads to pretreat plasma pools before being assayed on microarrays. Untreated plasma pools and pools treated with blank beads were used as controls. The overall signature of plasma pools did not change after blocking with Aβ-coated beads. However, pretreatment with Aβ beads decreased the immunoreactivity of 4 microarray peptides, and completely abolished the signal of 2 peptides (Fig 4). Using ClustalW 2.0, we found no sequence similarity between these peptides and human Aβ 1–40 or Aβ 1–42. Some of these peptides strongly bound polyclonal

anti-Aβ 1–42, anti-Aβ oligomer, and anti-phospho-tau antibodies (Supporting Fig 2). These experiments suggest that only a small portion of the signature is driven by anti-Aβ antibodies, and that blocked microarray peptides may behave as epitope mimetics, given the lack of sequence homology with the blocking antigen. However,

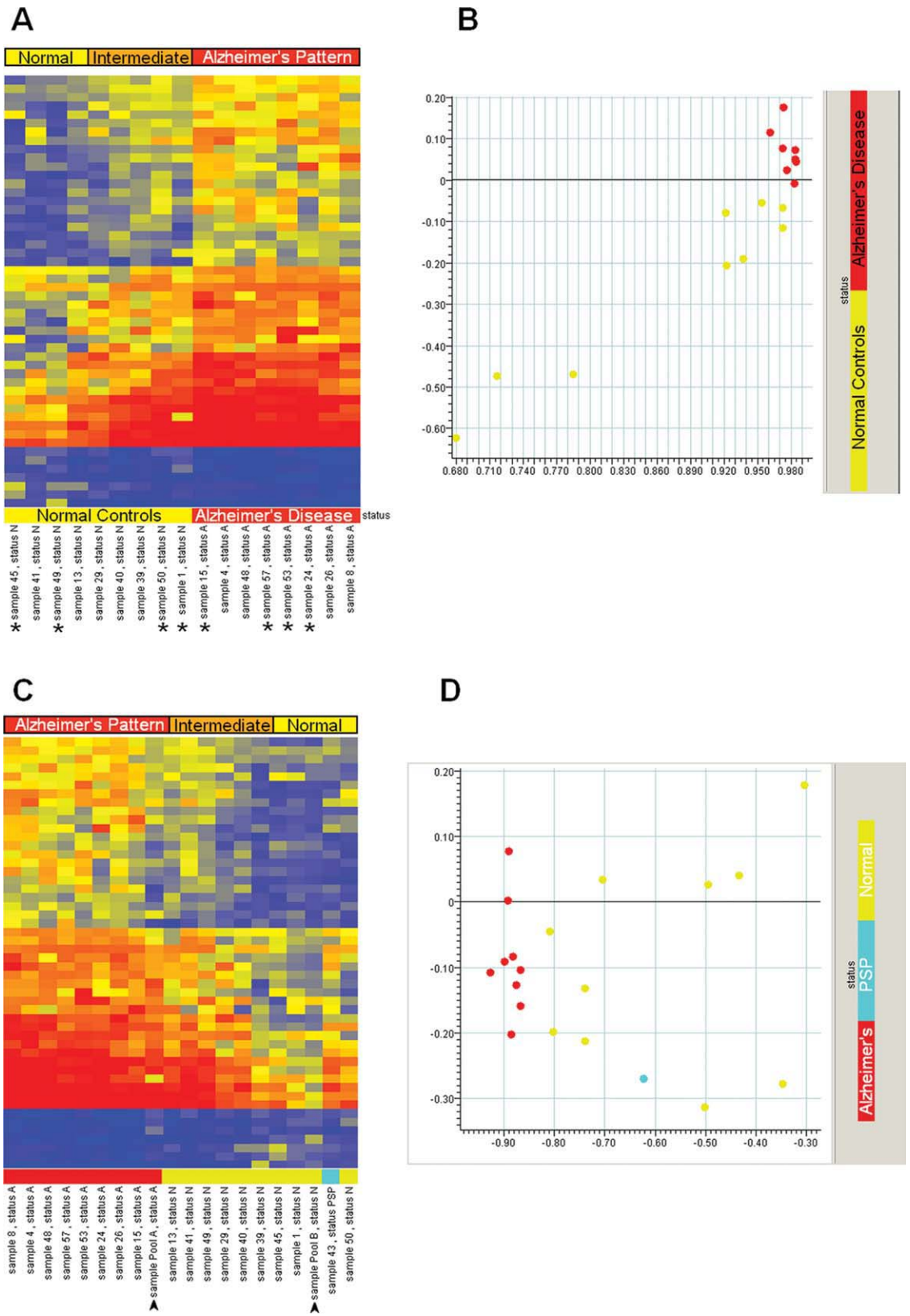

FIGURE 3

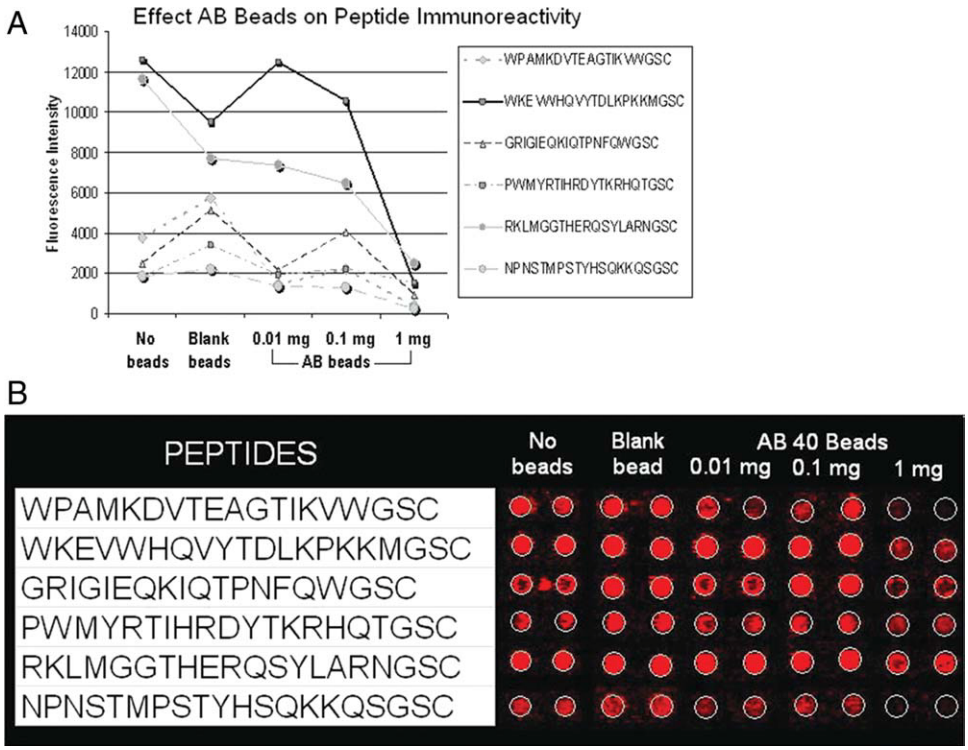

FIGURE 4: Variation in the immunoreactivity of specific microarray peptides elicited by Aβ pretreatment of plasma pools. A plasma pool from AD patients was treated with different concentrations of Tantagel beads. (A) Intensity of fluorescence declined for a few array peptides as the concentration of Aβ 1–40 beads increased. There was minimal variation with blank beads, whereas minimal decline in fluorescence intensity was noted in a plasma pool from normal cognitive controls. (B) Microarray scan showing effects of Aβ 1–40 bead treatment on fluorescence intensity of the specific peptides shown above. The immunoreactivity of 2 of these peptides exhibited marked decline after Aβ 1–40 treatment.

it is possible that an anti-Aβ antibody that conveyed a small portion of the signature or 1 whose removal was masked by binding of another antibody would not be detected.

Cross Reactivity Between AD Plasma, TG Mice, and Anti-Aβ Oligomer Antibodies

Thirty-three peptides were preferentially bound by the anti-oligomer antibody and AD plasma, whereas 19 peptides were specifically bound by plasma of AD patients and TG mice (Supporting Fig 3). Two peptides were avidly bound by the 3 groups: KKNFKTFGFDPLVT WSWGSC and GLPWTLYYLWMRPTYVRGSC. The probability of this occurring by chance is 8.894×10^{-6}. Inquiry with ClustalW 2.0 found no sequence homology between these 2 peptides and human Aβ. Several peptides bound predominantly sera from the PSP patient (29 peptides), the plasma pool from autopsy-confirmed AD cases (22 peptides), and the plasma pool from elderly controls without signs of AD on autopsy (34 peptides; Supporting Fig 3). The probability of this occurring by chance is 1.25×10^{-7}.

Discussion

We have described herein a novel method to assess the immunoreactivity patterns of antibodies targeting

FIGURE 3: Human immunosignature. (A) Heat map depiction of a reduced signature set of 169 peptides that helped distinguishing AD plasma from age-matched nondemented controls. This representation demonstrates patient clustering into 3 separate patterns (*upper box*): AD-type, intermediate, and nondemented control. Asterisks denote individuals who had autopsy, which confirmed AD in 4 patients, while 4 nondemented controls did not exhibit AD pathology. (B) Principal component scatter plot analysis of same plasma samples as in A, demonstrating that individual plasma samples from AD patients (*red dots*) tend to cluster together, whereas samples from nondemented controls (*yellow*) are widely scattered. (C) Heat map demonstrating that plasma pools (*arrowheads*) from AD patients and cognitively normal controls are also correctly discriminated by the platform. The plasma signature of a patient deemed to have AD in life but received diagnosis of progressive supranuclear palsy (PSP) on autopsy, migrated with the pattern of normal controls. (D) Principal component analysis of same patients in C. Notice the close topographical aggregation of the AD and normal cognitive control pools with their respective autopsy-proven counterparts.

different forms of Aβ and tau, as well as plasma samples from *APPswe/PSEN1-1dE9* TG mice and humans with or without AD. The microarray platform used in this study features 10,000 random-sequence peptides that appear to behave as mimetics of the original targets of tested antibodies. We demonstrated that plasma of elderly patients with or without dementia reacts with microarray peptides, and this reaction takes the form of different patterns that allowed us to discriminate, to a certain degree, between patients with or without disease. Furthermore, we demonstrated that the bulk of the immunosignature is independent of Aβ.

We identified a set of random peptides from the array with the highest binding by particular plasma samples, allowing plans for development of arrays with reduced number of peptides, or individual enzyme-linked immunosorbent assays (ELISAs) using random peptides as antigen. This high-throughput screening platform has been used for identifying surface-immobilized peptides that specifically bind bacterial lipopolysaccharides,[23,25] guiding production of synthetic antibodies,[26] and characterizing humoral response to infections and vaccination,[24] but it has not been employed until now to evaluate a chronic disorder such as AD. In another approach to the assessment of dementia, a double-sandwich ELISA microarray featuring plasma cytokines was used to classify blinded samples from patients with clinical diagnosis of AD with almost 90% accuracy.[6] Compared to such platform, our microarray has 3 distinct advantages: (1) it multiplies by 83.3 the number of analytes; (2) it assays antibodies, which are more stable than cytokines; and (3) it is inexpensive, with an average slide cost of $50.

AD diagnosis is an imprecise process of exclusion of other neurological entities, as illustrated by the misdiagnosis of the PSP patient. The gold standard of AD diagnosis is its characteristic neuropathology, which is rarely available to physicians. Autopsy endorses the clinical diagnosis of probable AD in only 65% to 80% of cases.[2] Correct disease classification is imperative for many reasons: first, some dementias do not respond to the treatment recommended for AD or may even become worse with it; second, the prognosis of several dementias is different from that of AD; finally, AD clinical trials cannot be considered definitive considering that 20% to 25% of enrolled subjects may not have the disease. Therefore, a simple test that helps refine the classification of dementia is needed.

A constant finding in AD is inflammation involving brain and plasma. The phagocytic clearance of misfolded proteins and cellular debris can be construed as a beneficial aspect of neuroinflammation, whereas the release of cytokines by activated microglia and complement activation may be detrimental whenever neurotoxicity is promoted.[8] The immune system can be harnessed to clear cerebral Aβ deposits,[8,20] while circulating autoantibodies are proposed as potential biomarkers that may be deployed in dementia clinics.[9–19,21] Plasma and cerebrospinal fluid contain naturally-occurring anti-Aβ antibodies in normal and pathological conditions, but it is debated whether these are protective or deleterious.[9–19,21] Although no explanation for Aβ immunoreactivity is universally accepted, exposure to environmental Aβ mimotopes (ie, the potato virus Y) is a possible mechanism.[13] Both AD patients and healthy elderly individuals possess circulating antibodies that react against tau protein.[21] Autoantibodies (anti-nuclear, anti-parietal cell, anti-thyroid microsomal, and anti-nuclear) are found in about one-third of normal elderly individuals at low titers.[15,22] It is unclear whether titers change overtime or correlate with different clinical stages. We speculate that autoantibodies react to the microarray peptides, accounting in part for the observed signatures. This assertion is based on our finding of microarray peptides that bound commercial anti-Aβ antibodies and AD plasma, while a small portion of the AD immunosignature was blocked with Aβ.

We found that affinity purified antibodies targeting the carboxyl-terminus of Aβ and antibodies against Aβ oligomer and phospho-tau have similar signatures. The carboxyl-terminus of Aβ is crucial for its polymerization, while additional amino acid residues in this region translate into greater aggregation, which provides a potential reason for the similarity between the Aβ antibodies. However, the striking similarity with the phospho-tau antibody pattern is enigmatic. The phospho-tau antibody used in this study reacts with a form of tau that is prone to aggregation within neurons. Although tau and Aβ do not share sequence similarity, it is conceivable that aggregated tau may share a conformational epitope with Aβ oligomers. Interestingly, the anti-Aβ oligomer used herein cross-reacts with several amyloidogenic proteins, including α-synuclein, islet amyloid polypeptide, prion protein, human insulin, lysozyme, and polyglutamine, suggesting a common conformation-dependent structure, regardless of sequence.[35] These issues will be subject of future investigation.

This study has limitations. The animal model used does not fully recapitulate all features of AD; in particular, *APPswe/PSEN1-1dE9* mice do not develop neurofibrillary tangles. Given the limited patient cohort, our results are considered a preliminary proof of principle. We are currently assaying more plasma samples from AD patients and normal elderly controls to answer whether our microarray platform can be used to assist in the clinical classification of dementia. We will also examine whether an immunosignature precedes the onset of

cognitive impairment in TG mice and humans. Given the slow progression of AD pathology (thought to develop many years in advance of symptom onset), an emerging humoral immune response, if any, could be detected and tracked in plasma. In summary, the evaluation of immunosignatures using random-sequence peptide arrays is a promising technique that can be applied to AD research. Future studies with more patients are needed to appraise the merits of immunosignaturing as a potential screening method for AD biomarkers. These studies will be based on informative peptides resulting from preliminary plasma screening on the microarray platform.

Note Added

While this work was under review, Reddy and colleagues[36] and Lindstrom and Robinson[37] reported the feasibility of finding candidate AD biomarkers by screening a peptoid library, a technique related to but different from our microarray-based platform.

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Potential Conflicts of Interest

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