Feasibility of an early Alzheimer's disease immunosignature diagnostic test

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ABSTRACT

A practical diagnostic test is needed for early Alzheimer's disease (AD) detection. Immunosignaturing, a technology that employs antibody binding to a random-sequence peptide microarray, generates profiles that distinguish transgenic mice engineered with familial AD mutations (APPswe/PSEN1-dE9) from non-transgenic littermates. It can also detect an AD-like signature in humans. Here, we assess the changes in the immunosignature at different time points of the disease in mice and humans. We also evaluate the accuracy of the late-stage signature as a test to discriminate between young mice with familial AD mutations from non-transgenic littermates. Plasma samples from AD patients were assayed 3–12 months apart, while APPswe/PSEN1-dE9 and non-transgenic controls supplied plasma at monthly intervals until they reached 15 months of age. Microarrays with 10,000 random-sequence peptides were used to compare antibody binding patterns. These patterns gradually changed over the life-span of mice. Strong, characteristic signatures were observed in transgenic mice at early, mid and late stages, but these profiles had minimal overlap. The signature of young transgenic mice had an error rate of 18% at classifying plasma samples from late-stage transgenic mice. Conversely, the late-stage transgenic mice signature discriminated between young transgenic mice and littermates with an error rate of 21%. Less distinctive profiles were recognizable throughout the transgenic mice lifespan, being detectable as early as 2 months. The human signature had minimal change on short-term follow-up. Our results call for a reappraisal of the way incipient AD is studied, as biomarkers seen in late-stages of the disease may not be relevant in earlier stages.

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1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia, affecting about 35.6 million people world-wide (Chui and Lee, 2002; Abbott, 2011). Because AD cannot be prevented or cured, the number of affected persons doubles every two decades, causing crippling cognitive disability and economic losses in excess of $604 billion per year. Early detection and treatment will be essential to control this problem (Buckholtz, 2011). In spite of recent advances (Shaw et al., 2007, 2009; Ewers et al., 2011), no specific tests are universally used to diagnose AD. As pathology slowly progresses for decades before initial symptoms emerge (Shaw et al., 2009), and since initial manifestations are generally subtle (Morris et al., 2001; Kawas, 2003; Grundman et al., 2004), a potential diagnostic test for AD must be highly sensitive. Given that future treatments are likely to target people with mild or no symptoms (Shaw et al., 2007, 2009; Buckholtz, 2011), the test must also be highly specific. Considering the challenges involved in obtaining samples from subjects with early AD stages, we explored the utility of a test developed using plasma samples from the terminal phase of the illness as a pre-symptomatic diagnostic tool.

Immunosignaturing is a general diagnostic technology which involves diluting blood and applying it to an array consisting of 10,000 random-sequence peptides (Legutki et al., 2010; Restrepo et al., 2011; Kukreja et al., 2012b; Stafford et al., 2012; Hughes et al., 2012). Antibodies bind to the array revealing a signature affected by the health status of the individual. The initial application of this technology showed that both transgenic mice with cerebral amyloidosis and humans with AD have distinctive immunosignatures relative to healthy age-matched controls (Restrepo et al., 2011), but no investigation of the signature stability over time was undertaken. Since the clinical diagnosis of AD is corroborated by autopsy in 65–80% of cases (Chui and Lee, 2002), a non-invasive blood test could be useful in clinical practice. More importantly, the application of this technology to the pre-symptomatic diagnosis of AD could help prevent or delay the onset of dementia if disease-modifying therapies become available. The simplest approach to developing such a test is to use the signature of autopsy-confirmed AD to create an indicator for early stages of dementia. Here we use a mouse model of AD, APPswe/PSEN1-dE9 mice, to explore this possibility.

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2. Materials and methods

2.1. Microarray

The protocols, performance, sample preparation methods and statistical analyses of the technology are described elsewhere (Brown et al., 2011; Restrepo et al., 2011; Halperin et al., 2012; Hughes et al., 2012; Kroening et al., 2012; Kukreja et al., 2012a,b; Stafford et al., 2012). Briefly, an immunoassay was developed using 10,000 random-sequence 20-mers covalently attached to a glass slide. Peptides were designed with random sequences, except for glycine–serine–cysteine linkers at the carboxyl (peptide library 1) or amino (library 2) terminus. Library 1 peptides were synthesized by Alta Biosciences (Birmingham, UK), spotted in duplicate using a NanoPrint LM60 microarray printer (Arrayit, Sunnyvale, CA). Library 2 peptides were synthesized by Sigma Genosys (St. Louis, MO), printed by Applied Microarrays (Tempe, AZ) using a piezo non-contact printer in a two-up design. Slides were pre-washed with 33% isopropanol/7.5% acetonitrile/0.5% trifluoroacetic acid, and blocked with 0.015% mercaptohexanol/3% BSA/0.05% Tween 20 in PBS prior to adding plasma at 1:500 dilution in 3% BSA/0.05% Tween 20 PBS. Experiments were carried out in a TECAN HS4800-Pro automated incubator (Tecan, Männedorf, Switzerland). Biotinylated anti-human (Bethyl, Montgomery, TX) or anti-mouse (KPL, Gaithersburg, MD) antibodies were incubated with slides, washed, then followed by incubation with streptavidin-Alexa-647 (Invitrogen, Carlsbad, CA). Slides were scanned with an Agilent 'C' scanner (Agilent Technologies, Santa Clara, CA), generating digital images that were subsequently processed with GenePixPro6.0 (Molecular Devices, Palo Alto, CA) and analyzed in GeneSpring 7.3.1 (Agilent, Santa Clara, CA).

2.2. Analysis

Once data was imported into GeneSpring, signal intensity was $\log_{10}$-transformed and median-normalized before analysis. Pearson's correlation coefficient was calculated across replicate slides to ensure reproducibility ≥ 0.85. Technical replicates < 0.85 were reprocessed. Multivariate clustering (two-way hierarchical clustering) was used to generate heatmaps using Euclidean distance with complete linkage as the measure of similarity, while principal component analysis (PCA) was used to display relative differences across samples. Figs. 1–3 utilize plots of the first three principal components. Peptide microarrays provided < 1.3-fold minimum average detectable fold change at $\alpha = 0.05$ and $\beta = 0.20$ per 2 technical replicates. For classification we used linear discriminant analysis (LDA) in R with leave-one-out cross validation to estimate error. T-test with FWER (family-wise error rate) of 5% is used to correct for false positives due to multiple testing. The p-values presented in this paper are not raw but have been adjusted by the Benjamini and Hochberg false discovery rate algorithm (Benjamini and Hochberg, 1995).

2.3. Human plasma

Plasma samples from 6 AD patients and 5 age-matched controls without cognitive derangement enrolled in a brain-bank program were provided by Alex Roher (Banner Sun Health Research Institute, Phoenix, AZ). Postmortem examination was performed by a neuropathologist on 9 patients. Samples were acquired after written consent and approval from the Banner Institutional Review Board (IRB). Profiling studies were approved by ASU's IRB (protocol# 0912004625).

2.4. Mice

Female APPswe/PSEN-1dE9 TGM and B6C3F1/J non-transgenic controls (n = 5/group) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed with standard chow and water provided ad libitum. Plasma samples were processed from blood obtained via sub-mandibular puncture at monthly intervals beginning at age 2 months and stored at −80 °C. Mice were sacrificed at 15 months of age through intra-peritoneal injection of tribromoethanol (5 mg) followed by intra-cardiac exsanguination and cold PBS perfusion prior to decapitation for brain harvesting. Brain axial sections (3–4 mm thick) were treated with 10% formaldehyde overnight, followed by paraffin processing for immunostaining. Murine experiments were conducted under a protocol approved by the Arizona State University Institutional Animal Care and Use Committee.

3. Results

Results from immunosignaturing assays require an understanding of the characteristics of the technology. While expression or SNP microarrays demonstrate a one-to-one binding between RNA or DNA and the target probe, the immunosignaturing peptide arrays enable multiple specificities of antibody to bind a single peptide while a single antibody may bind multiple peptides (Kukreja et al., 2012b). This effect is accommodated by the statistical methods used to select peptides and is noted as the "Immunosignaturing Effect" (Stafford et al., 2012).

3.1. Stability of human immunosignature

We first asked whether the AD immunosignature in humans is stable over time. To answer this question, we assayed two plasma samples collected between 3 and 12 months apart from 5 patients with AD (4 autopsy-confirmed), 6 normal elderly controls (4 autopsy-confirmed), and a demented patient with signs of progressive supra-nuclear palsy (PSP) on autopsy. The time 0 samples were used in a previous paper which examined whether a signature of AD existed at all (Restrepo et al., 2011). Fifty peptides were selected by a two-tailed T-test with FWER = 5% ($p < 4.28 \times 10^{-10}$) patients from age-matched controls using both time 0 and follow-up samples. LDA using these peptides yielded a 0% misclassification rate. We also tested for differences between time 0 and follow-up using a T-test between 'early' and 'late' time points. No peptides passed multiple testing criteria. Fig. 1 (left panel) shows the resulting heatmap where hierarchical clustering was done using Euclidean distance as the measure of similarity. Clustering was performed on the peptides (vertical axis) and patients (horizontal axis) with the colored bars representing the patient class. Patients showed a strong tendency to group with their follow-up sample. A PCA demonstrates this effect in Fig. 1 (right panel). A secondary effect, which is a trait of the immunosignaturing technology, is the higher dispersion of points in healthy cohorts, and the relatively tighter grouping of patients in the disease cohorts (Stafford et al., 2012). This demonstrates that the immunosignature of Alzheimer's disease is stable over at least the time course of this collection study. This has not been shown for any other ongoing or completed immunosignaturing study of chronic disease. The PSP patient exhibited an intermediate pattern, although peptides were selected for resolving AD, not PSP. Eight out of these fifty peptides also bind antibodies raised against Aβ, suggesting that part of the signature could involve anti-Aβ immune-reactivity (data not shown and Restrepo et al., 2011). These observations suggest that AD plasma contains an immunosignature that can distinguish AD people from non-AD controls reproducibly over time.

3.2. Time course of immunosignatures in APPswe/PSEN1-dE9 mice

Because human plasma samples spanning the entire AD's time course are rare and difficult to obtain, we used APPswe/PSEN1-dE9 transgenic mice, a well-characterized animal model of AD engineered with two human mutations leading to accelerated cerebral amyloidosis (Jankowsky et al., 2004; Qu et al., 2004, 2006, 2007; Reiserer et al., 2007; Gotz and Ittner, 2008) to ask two related questions relevant to
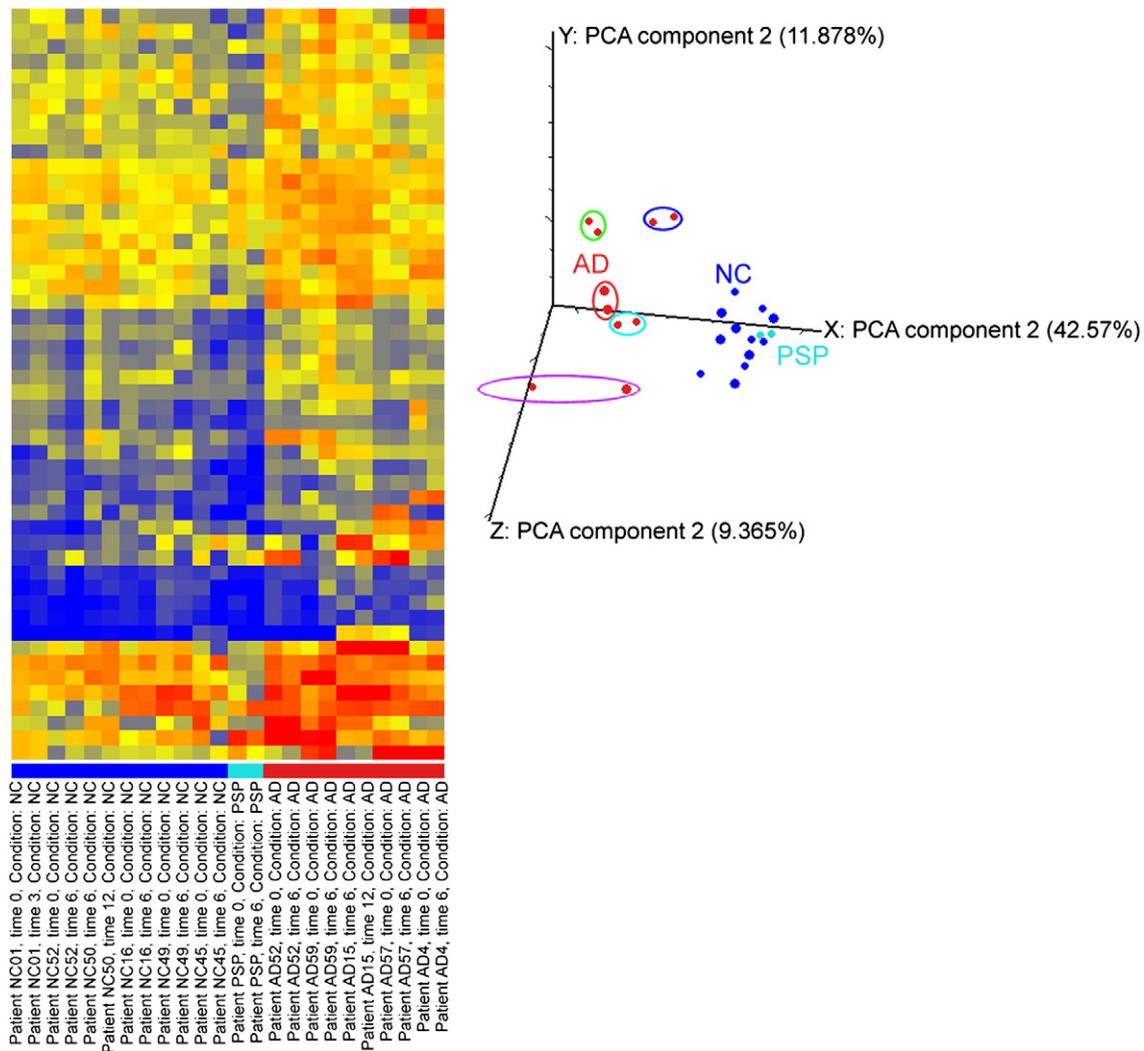
Fig. 1. Stability of human immunosignature. Left: heatmap of peptides selected by FWER-corrected T-test ($p<4.28\times10^{-10}$) and plasma samples from AD (red bar), normal controls (blue bar) and a PSP patient taken at time 0 and follow-up (between 3 and 12 months later). LDA classification error was 0%. Right: PCA of the same plasma samples from AD (red dots) and controls (blue dots). Note that plasma from a patient with progressive supranuclear palsy (PSP, cyan dots) aggregate with controls, and samples from each patient tended to migrate with follow-up samples from that same individual.

developing an early detection diagnostic test: first, how early can a characteristic signature be detected in transgenic mice relative to age-matched littermates? Second, can an immunosignature that was optimized to detect late-stages of the illness be used to diagnose early phases of the disease? To answer these questions, we assayed plasma pools from APPswe/PSEN1-dE9 mice and B6C3F1/J non-transgenic controls drawn monthly, starting at 2 months of age and ending 13 months later (2–15 months). To confirm the expected pathological changes in APPswe/PSEN1-dE9 mice, we performed hematoxylin–eosin and immunostaining on all mice after their sacrifice at 15 months of age. Compared to B6C3F1/J controls, transgenic mice had heavy cerebral amyloid deposition and robust astrocytosis (see Supplementary Fig. 1). We first used a two-tailed T-test ($p = 6.6\times10^{-7}$ to 3.12×10^{-5}) to find peptides that discriminated *all* transgenic mice from the non-transgenic controls, yielding a total of 39 peptides (Supplemental Table 1). This separation is displayed in the left panel of Fig. 2 and the PCA in the right panel of Fig. 2. Most of the 39 peptides showed higher binding in the transgenic mice, with a trend for increased peptide signal with age. The signature was evident but faint, even at two months of life.

In another approach, we determined whether peptides that distinguished APPswe/PSEN1-dE9 from B6C3F1/J at late life stages, when cognitive problems and AD-like neuropathology are florid,

could be used to discriminate transgenic mice at early disease stages. Conceptually, this would be analogous to using late-stage dementia samples to train a system to detect presymptomatic AD. Mice were divided into three groups according to age: early (2–5 months), mid (6–9 months) and late (10–15 months). These time-points are biologically relevant, considering that neurocognitive function in APPswe/PSEN1-dE9 mice begins deteriorating at 8–9 months of age (Jankowsky et al., 2004; Qu et al., 2004, 2006, 2007; Reiserer et al., 2007; Gotz and Ittner, 2008) and their characteristic neuropathology (cortical plaque formation and astrocytosis) is first observed from 6 to 7 months of age (Jankowsky et al., 2004; Qu et al., 2004, 2006, 2007), while no neurocognitive or pathological abnormalities are apparent before four months (Reiserer et al., 2007).

Supplemental Table 2 is a list of the 35 most significant peptides for each of the three time points and the associated FWER-corrected *p*-values. Fig. 3 shows two distinct results: the top panels of Fig. 3 contain the heatmaps and PCA plots for early, mid and late plasma samples plotted against early, mid and late peptides, respectively. The classification error for this analysis was 0%. The Venn diagram shows the overlap in peptides selected by T-test between CP and TGP across early to mid, and mid to late. There were no peptides that overlapped early and late, suggesting differences in ongoing pathological processes exacerbated by
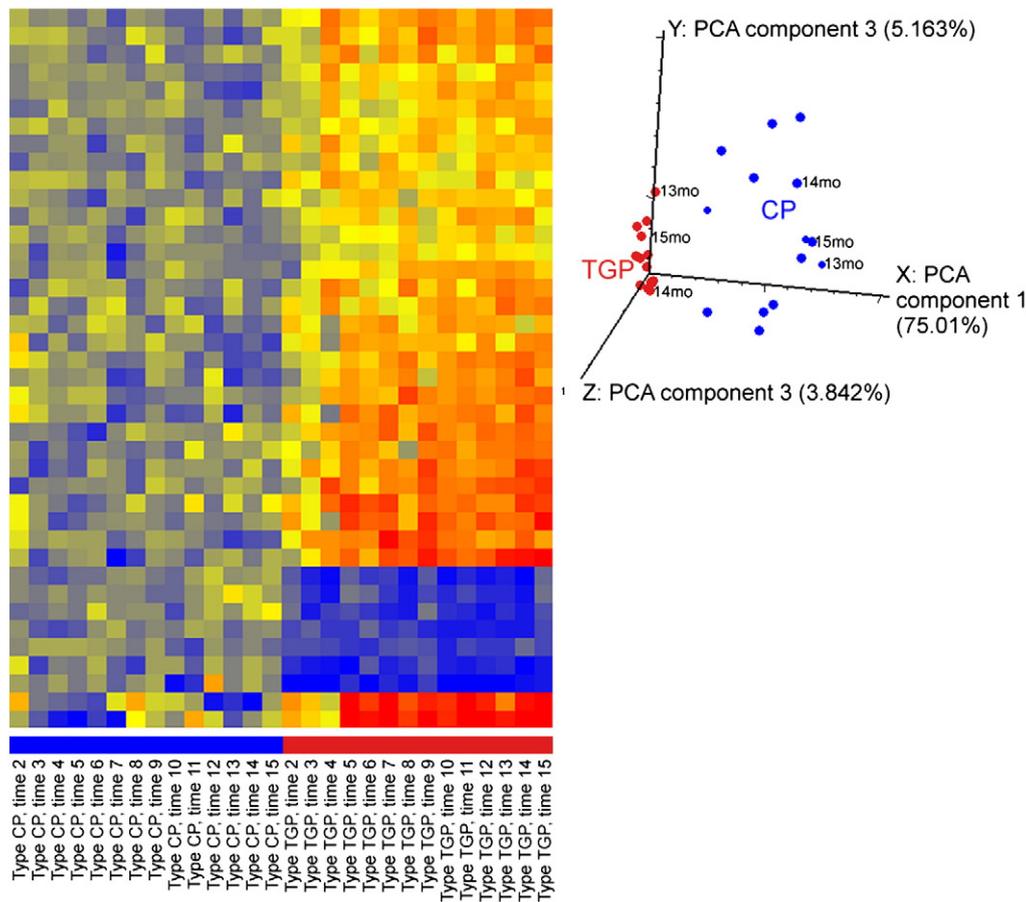
Fig. 2. Antibody signature of transgenic mice. Left: heatmap with 39 peptides ($p < 1.67 \times 10^{-11}$) that showed sustained immune-reactivity overtime in transgenic mice (TG) as compared with B6C3F1/J controls. The y axis lists the different peptides, whereas the x axis depicts plasma pools from TG mice and age-matched controls. (b) Shows a principal component analysis (PCA) graph of plasma pools belonging to TG (yellow) or B6C3F1/J controls (red), while (c) depicts a PCA of plasma pools drawn early in life (red) versus late in life (yellow). Together, these diagrams show that plasma pools segregate according to group, but not according to time point.

time. The analysis was done this way for three reasons: first, in all cases there were at least 35 peptides that survived multiple-testing correction (FWER = 5%) for each time point. Second, it is easier to demonstrate overlap in peptides from one time-point to another when a fixed number of peptides are used. Third, the classifier (LDA) works best when <100 features are used, and 35 features suit this algorithm well. The three 35-peptide sets chosen using a FWER-corrected two tailed T-test (early $p < 1.40 \times 10^{-8}$, mid $p < 1.03 \times 10^{-8}$, late $p < 8.7310^{-7}$) readily separated transgenic from non-transgenic mice at each specific time-point.

We then asked whether the 35 peptides that distinguished transgenic from non-transgenic littermates at late-stages could differentiate the early-stage mice. The bottom panels of Fig. 3 show the second set of results, where non-age-matched peptides are asked to classify disease state. When asked to predict the status of transgenic and non-transgenic mice, late peptides predicted early stage mice with a 21% misclassification rate. When late peptides were asked to predict mid-stage mice, the misclassification rate was 12%. When early peptides were used to classify late samples, the error rate was 18%. None of the 39 peptides from Fig. 2 that generally discriminate the transgenic and non-transgenic mice across *all* time-points appeared in the list of the 35 optimized for each stage, suggesting that there may be antibodies specific to each disease stage. When asked to find antibodies present evenly and reproducibly throughout the entire disease, the array may have identified lower affinity and perhaps lower specificity antibodies than the stage-specific ones.

We next asked whether the resolving power of late-stage peptides on early-stage samples could be improved by including more

peptides that were informative against late-stages. Indeed, the top 130 predictive peptides ($p < 0.000117$) for late-stage discrimination included all 35 early peptides. When these 130 peptides were used to distinguish transgenic mice at early-stages, the error rate was zero. Since late-stage peptides had positive predictive power for early-stages, we asked whether the 35 early peptides predicted mouse groups when mixed with non-informative peptides. When we added 95 randomly-chosen peptides to the 35 early peptides (total = 130), LDA misclassification rate increased to 10%, suggesting that the 35 early peptides perform well even in the presence of random noise. This also indicates that there is predictive power for early disease in the larger list of late-stage peptides.

4. Discussion

Evaluating the potential of immunosignaturing as a diagnostic test for early AD, we first looked at the stability of the signatures in age-matched people with and without AD. We observed a distinctive AD signature, which remained stable in samples taken 3–12 months apart in the same person. We previously identified an AD-specific signature (Restrepo et al., 2011), but now show that the signature remains stable over the short term. A relative proportion of the signature is personal, while another part is related to the disease state. An early criticism of the technology was based on the diverse range of antibodies that different individuals can raise to the same immunogen, leading to the likelihood that disease-specific signatures would be overwhelmed by the personal signatures. We show that the disease
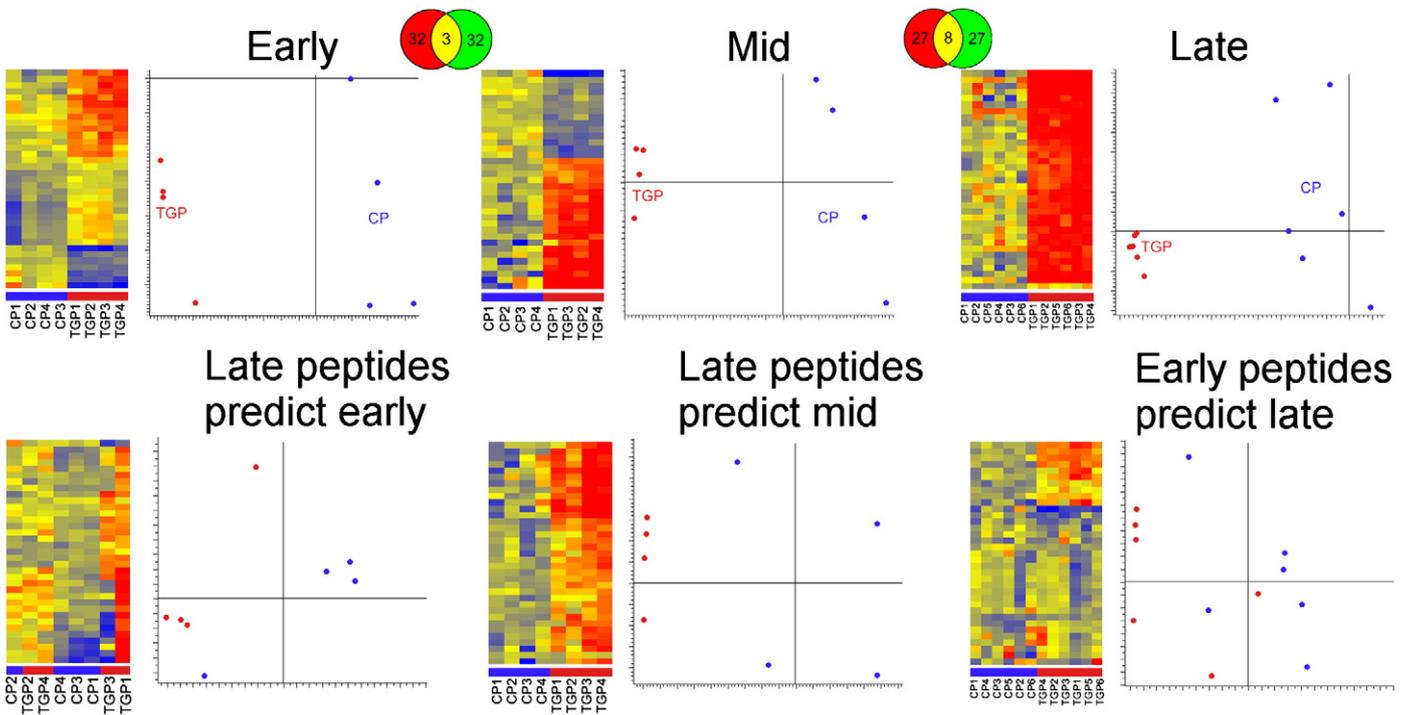
Fig. 3. Temporal profile of antibody binding patterns in mice. We tested the classification performance of immunosignatures obtained from mice at different time-points during disease manifestation. We defined 'early' as 2–5 months, 'mid' as 6 to 9 months and 'late' as 10–15 months of age and selected 35 of the most significant peptides using FWER-corrected T-test. This figure displays the separation between transgenic mice and their age-matched controls using the three different time-point-specific signatures. Top right: heatmap depicting plasma pools obtained from APPswe/PSEN1-dE9 transgenic mice (TGP) and B6C3F1/J non-transgenic controls (CP). The immunoreactivity of the 35 random-sequence early peptides was plotted for the control and transgenic mice at the same time point. Immediately below the heatmap is a principal component plot of the same mice, and the same peptides. The PCA plot shows the relative difference between individuals using the first 2 principal variance components on the x and y axes respectively. The x axis also lists the proportion of total variance in the first principal component. Center top: heatmap and principal component display of mid-stage mice showing separation between TGP and CP. Top right: late individuals plotted using late peptides. The overlap between the peptides selected for each of the time points is shown in the Venn diagrams. There was no overlap between the 35 peptides selected from early and late stages. When the appropriate stage-specific peptides were used to predict their cognate stage, the leave-one-out cross-validation error rate was 0%. Lower left: when the late peptides were used to discriminate the early samples, the classification error rate was 21%. Lower middle: when the late-peptide set was used to classify the mid samples, the LDA error rate was 12%. When the early peptides were used to classify the late samples, the error rate was 18%.

signature is identifiable over the personal one, and that persons with the same disease tend to become more immunologically similar than healthy persons, who seem more immunologically diverse. If a diagnostic for AD is to succeed, it should detect early signs of the emerging pathology. Because well-curated human time course studies for AD are difficult to obtain, we collected blood from APPswe/PSEN1-dE9 mice and age-matched controls from 2 to 15 months of life. When considering only transgenic and non-transgenic groups without incorporating time of collection as a factor, we found that 39 peptides could separate the two groups, with a signature that increased over time (Fig. 2). However, when mice were divided by age into early, mid and late stages, we found 35 highly-significant peptides capable of distinguishing between transgenic and non-transgenic mice at each life-stage. There was minimal overlap between the peptide sets that characterized each stage, and none at all between late and early stages. Late-stage peptides did not separate mice at early stages of the disease, while mid-stage peptides performed slightly better. This implies that there is a constantly refurbished set of antigens that are presented to the immune system as the disease progresses, perhaps as a result of increasing cellular pathology. By lowering the stringency for selecting peptides from the late cohort, we began to include peptides that were specific to early stages of disease, but in an actual clinical setting we would not know which peptides would be best for early diagnostic. There is an indication that mid-stage peptides have some resolving power for early diagnosis, but it is far from perfect. However, if our mice data could be extrapolated to humans, patients with mild cognitive impairment (MCI) destined to develop dementia could be used in the selection of AD-specific peptides. It is possible, considering the unique PSP signature, that

immunosignaturing can distinguish MCI patients who will develop AD from those developing other neurological diseases.

Antibody binding patterns to microarrays may have diagnostic potential, but immunosignature stability is important for two reasons: first, if the variation caused by time is small, then larger sample pools could be used without concerns about noise dampening the signature over time. Second, if there is a large personal component of the signature, it could be useful for monitoring disease progression or response to treatment without regard for commonality to other AD patients. Relative to the first issue, AD signatures were highly distinguishable from age-matched controls regardless of whether they were early or late samples. Relative to the second issue, there was clearly a personal component. Mathematically, samples from the same individual were most similar to each other, indicating that each person may have a distinctive and stable immunosignature, analogous to a fingerprint. Immunologically, even the personal component could not overwhelm the similarity to other AD patients.

The ability to define an AD signature could have value in several ways, including confirmation of diagnosis, enrollment in clinical trials, and monitoring responses to treatment. Lacking practical tests to diagnose AD is not only problematic for patient care, it also represents a barrier for clinical trials, since many enrolled subjects will not have the disease of interest and therefore, would not expect benefit from the studied intervention. Antibody-based diagnostic tests have experienced renewed interest with the development of microarrays featuring plasma cytokines (Ray et al., 2007) and random-sequence peptides (Caiazzo et al., 2007; Roche et al., 2008; Reddy et al., 2011; Restrepo et al., 2011; Vuong, 2011). On the other hand, surveying the antibody

repertoire of individuals with or without a disease has many advantages. There are 10 (Hughes et al., 2012) estimated different antibody specificities, reflecting exposure to various antigens with B-cells amplifying the signal thousands of times over circulating biomarker levels (Sulzer et al., 1993; Cooperman et al., 2004; Cenci and Sitia, 2007). Antibodies are produced early in the course of diseases and are easily retrieved from body fluids. Antibodies are relatively durable and easily stored, being suitable for retrospective analysis. Until recently, immunoassays were limited by the traditional view that the eliciting antigen needs to be known and immobilized to detect an antibody response. However, we and others have developed unbiased platforms to evaluate antibodies using random-sequence peptides, which principally behave as mimetics of unknown antigens. This technology only requires that a significant antibody response has been made — it need not be overwhelming or protective. Ideally, AD should be detected at the pre-symptomatic or early symptomatic stages, when promising disease-modifying therapies are expected to exert the greatest benefit (Buckholtz, 2011). Unfortunately, these stages are also the least understood aspects of the disease, and the most susceptible to diagnostic misclassification with current standards. This explains the impetus to test late-stage signatures for early-stage diagnosis.

We have demonstrated several unique aspects that relate immunosignaturing and AD. First, there is an early immunological response that is detectable by our peptide microarray. It is clearly distinct from late-stage immune response, as a late-stage response can be seen steadily increasing from early to late time points. We also noted that 7/39 peptides also reacted with purified anti-Aβ antibodies, the concentration of which progressively increases with age in the brain and plasma of transgenic mice. These changes in the antibody repertoire of transgenic mice illustrate the complexity of their pathological process, with amyloid overproduction setting off a cascade of events where additional epitopes become targeted by the immune system as animals grow older. Notably, there is also a robust early-stage signature, which fades during the full duration of the experiment (data not shown), suggesting that a population of antigens – that might be potential drug targets – decrease or disappear as symptoms emerge. With proper biochemical purification procedures, peptides from early-stage AD could be used to physically purify early-stage antibodies. These early antibodies can then be used to identify or purify the eliciting antigens, provided an appropriate cohort of patients could be found.

From the practical point of view of developing a human diagnostic, it is challenging in the short-term to acquire samples from all AD stages. Using the mouse model does not circumvent this issue, but provides relevant insights. For instance, by dividing the mice into early, mid and late life stages, we found peptides from each stage that separated transgenic mice from littermates with 100% accuracy. There was no overlap between the 35 peptides between late and early stages. The 35 most informative peptides for early-stages were also included in the top 130 late-stage predictive peptides ($p<0.000117$) but were not at the top of the list. Obviously, this test is artificial because we knew where to draw the threshold to include the 35 early-stage peptides, but this demonstrates that inclusive rather than exclusive strategies for choosing late-stage peptides for a diagnostic are more likely to succeed.

It may also be possible to utilize MCI cases that eventually progress to AD. While no overlap occurred between early and late-stage peptides in mice, there was overlap between early and mid-stage peptides. It may be useful to employ MCI samples to define early-stage peptides for the diagnostic test.

What are the implications for developing a diagnostic test for early-stage AD? To the extent that the mouse model and its caveats can guide this effort, it is encouraging that the disease signature starts early in life. This work also implies that optimizing the diagnostic test using late-stage AD or MCI patients may not provide much overlap with early immunological response but it also suggests that late-stage immunosignatures should be used very broadly when searching for an early AD diagnostic. This has the shortcoming of introducing non-informative peptides and noise in the analysis, but our mouse experiments indicate that this may not be prohibitive in developing an accurate diagnostic test.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2012.09.014.

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