HHS Public Access

Author manuscript

Alzheimers Dement. Author manuscript; available in PMC 2022 December 01.

Published in final edited form as:

Alzheimers Dement. 2021 December ; 17(12): 2030–2042. doi:10.1002/alz.12342.

Knowledge gaps in Alzheimer's disease immune biomarker research

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Abstract

Considerable evidence has accumulated implicating a role for immune mechanisms in moderating the pathology in Alzheimer's dementia. However, the appropriate therapeutic target, the appropriate direction of manipulation and the stage of disease to begin treatment remain unanswered questions. Part of the challenge derives from the absence of any selective pressure to develop a coordinated beneficial immune response to severe neural injury in adults. Thus, immune responses to the prevailing stimuli are likely to contain both beneficial and detrimental components. Knowledge gaps include 1) how a biomarker change relates to the underlying biology; 2) the degree to which pathological stage group differences reflect a response to pathology versus trait differences among individuals regulating risk of developing pathology; 3) the degree to which biomarker levels are predictive of subsequent changes in pathology and/or cognition; and 4) experimental manipulations in model systems to determine if differences in immune biomarkers are causally related to pathology.

Keywords

Biomarkers; Immunity; Spinal Fluid; Plasma; Alzheimer's disease; natural selection; Protease inhibitors

1. INTRODUCTION

This manuscript is an opinion piece developed through the ISTAART personal interest area (PIA) Immunity and Neurodegeneration. A series of these review-like articles are being developed collectively with the purpose of highlighting perceived GAPS in knowledge regarding the goal of the ROADMAP established as part of the Alzheimer's Disease Plan to develop disease-modifying treatments for the disease by 2025. This specific manuscript will focus on knowledge gaps regarding the role of immune biomarkers in developing

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Competing Interests Statement. Dr Morgan serves on the Speaker's Bureau for Biogen and is a consultant with Abbvie. Dr Morgan is an MPI on an SBIR with Hesperos Inc. Dr Mielke is a consultant for Biogen and for the Brain Protection Company.

therapeutics. This review is not intended to provide a comprehensive summary of our current knowledge regarding immune related biomarkers in dementia research, and the reader desiring such a summary is referred here [1, 2]. Instead this review will select items illustrating what we don't know that is key to developing effective dementia interventions targeting immunity. The opinions expressed in the manuscript are solely those of the authors and do not represent the opinions of the Alzheimer's Association or ISTAART. A bulleted summary of the key points in each section of the manuscript is presented in Table 1.

A major objective of the ROADMAP is to prevent or effectively treat Alzheimer's dementia. One viable therapeutic strategy would be to manipulate the innate immune system in a manner that benefits clinical outcomes. However, at present we simply do not know how we should try to manipulate the innate immune response to benefit those at risk of, or living with, Alzheimer's disease. It seems unlikely that across-the-board manipulations, which either suppress (or activate) innate immunity, will be very effective. Certainly experience with steroids or NSAIDs has failed to support broad scale immunosuppression as a viable therapy [3–6]. We need targeted manipulations of proteins or pathways that are linked to disease risk (most probably through genetic association), predictive of prospective clinical and/or pathological outcomes in longitudinal cohorts and demonstrated to be beneficial in experimental models. These proteins may then be considered rational targets for therapeutic interventions.

2. GENERAL CONSIDERATIONS REGARDING INNATE IMMUNE BIOMARKERS

One major question is the role of innate immunity in the pathogenesis of Alzheimer's disease. Initial observations suggested that the response of glial cells to amyloid deposits would be detrimental; that any condition resulting in prolonged "inflammation" would promote tissue damage [7–9]. The term "inflammation" is inherently theory-laden as being deleterious. Inflammatory chemical reactions tend to be destructive and inflammatory speech spurs hostile emotions. More recently, there have been suggestions that the "nurturing instincts" of the microglia may be resisting accumulation of Alzheimer's pathology and protecting the brain, thus preventing the emergence of clinical symptoms [10, 11]. The large numbers of polymorphisms found in innate immune genes that modify risk for Alzheimer's disease [12], support the argument that some of these innate immune responses have a causal role in the initiation and/or progression of Alzheimer's pathology. Theoretically, immune gene polymorphisms linked with Alzheimer's disease may alternatively modify baseline immune system traits that influence initiation of the disease (presumably regulating amyloid deposition), or they may moderate the response of the brain to abnormal forms of amyloid/tau and the associated neural damage, influencing resilience to the pathology and disease progression.

A priori, it is virtually impossible that natural selection honed the response programs of the innate immune system to benefit neural injury in adults [13]. Except for perhaps rams establishing dominance [14] or woodpeckers whittling nests [15], animals suffering brain injury most probably did not survive to reproduce and pass on any fortuitous genetic

programs promoting this survival. For the most part, the brain's early innate immune response is largely sculpted by developmental requirements to help regulate neurogenesis, guide neural migration, winnow the surfeit of neurons and synapses initially produced [16, 17], and, after development, to contribute to synapse formation and elimination and promote synaptic plasticity [18]. Throughout life the brain's immune cells also protect from the relatively constant assault by bacteria, viruses and parasites that, until recently, were common chronic human conditions [19–22]. The genetic programs available for the brain's innate immune response are designed and coordinated to succeed in these circumstances, not in response to brain injury caused by amyloid, tau, vascular damage or myelin degeneration. Thus, the innate immune response to neurodegeneration is a hodge-podge of different signaling pathways elicited somewhat haphazardly by combinations of signals never anticipated by evolution. These signals likely change during the course of the disease, implying disease stage may be crucial when considering an intervention.

The research identifying a role for microbes to increase the risk for development of Alzheimer's disease highlights this point. Studies demonstrating infections by HSV1 and other viruses increase risk of Alzheimer's imply normally beneficial immune responses to infection may have deleterious impacts on amyloid/tau biology [23, 24]. Similar observations have been made for *Porphyramonas Gingivalis* and other bacterial infections increasing risk of dementia [25, 26]. Experimental support for a possible pleiotropic effect of the brain's battles with pathogens leading to inadvertent Alzheimer's pathology is the demonstration in mice and 3D-human cultures that infections with fungi, bacteria or viruses stimulate the secretion of Aβ amyloid as an antimicrobial response.[27].

Thus, the concept that immune responses in Alzheimer's disease are an orchestrated, well-intended series of gene expression changes is unlikely to be correct. Levels of some immune proteins may be beneficial and others detrimental to the initiation or progression of the disease. Critically, some differences in immune protein expression may represent inherent traits that vary across individuals. Other differences in immune protein levels, especially at later stage disease, may be a response to signals resulting from the accumulating neurodegeneration. Moreover, the same immune response may be beneficial early in disease when there is minimal brain pathology, but detrimental later when the brain is replete with amyloid plaques and neurofibrillary tangles with significant neurodegeneration. The critical concept is that the innate immune cells in the brain are simply responding to the pattern of signals impinging upon them. They are responding to these signals in a genetically determined manner, without the intention of helping or harming. Manipulating so-called "hub" genes that synchronously regulate multiple proteins to orchestrate coordinated responses during development or infections may not be appropriate because some of the regulated proteins may be beneficial and prevent disease progression whereas other proteins may accelerate it. Our task is to identify which immune proteins are helpful, which are harmful and manipulate them to the patient's advantage.

Each method of studying immune markers in Alzheimer's disease has distinct advantages. A great deal has been learned about the innate immune response in Alzheimer's disease by examining post-mortem brains. The abundant brain tissue permits detailed histological, transcriptomic, and proteomic analysis of the biological condition. By estimating the stage

of disease progression from clinical and pathological markers, some dynamic interpretations may be drawn from the static post-mortem data. However, post-mortem analysis is limited to a single time point in the disease for each individual so the information collected cannot be used prospectively, but rather retrospectively in relation to rate of cognitive decline before death. Moreover, the results need to be conditioned by the desperate (and in the end, futile) medical lifesaving interventions applied near the end of the lifespan. Even if interventions are not provided, there is an underlying death process that affects many pathways and proteins in the brain. The time from death to autopsy also may impact protein and lipid levels. Nonetheless, critical details about the progression of the disease can be learned, especially by examining features that change slowly (e.g. amyloid deposits) rather than quickly (e.g. neurotransmitter release). The wealth of information obtained from these autopsy studies, combined with clinical and genetic work, has provided immense insight with regards to the mechanisms likely to contribute to Alzheimer's disease, and the levels of fluid biomarkers.

Complimentary approaches to autopsy studies for understanding Alzheimer's disease mechanisms are prospective clinical cohort studies or clinical trials. These studies can allow for serial, concurrent assessments of in vivo neuroimaging measures of Alzheimer's pathology and CSF or blood biomarkers. Indeed, CSF and, to a lesser but still significant extent, blood, harbor molecules that reflect some of the biological conditions of the brain tissues. Fluid markers can be collected serially and, compared to most neuroimaging biomarkers, are less expensive and more feasible at the population level.

Fluid biomarker studies compliment postmortem analyses of innate immune activity in several ways. First, fluid biomarkers can be obtained from relatively healthy individuals without the complications of lifesaving interventions (N.B. co-morbid disease and its treatment may still confound fluid biomarker studies and should be considered as a co-variate). Second, measurements can be obtained and compared from individuals at multiple stages across the disease spectrum, including presymptomatically. Third, biofluids can be serially collected to estimate intra-individual change in relation to the pathological and/or clinical disease progression. Last, prognostic studies of a fluid biomarker can be conducted, including among cognitively unimpaired individuals, to help identify which cases are at increased risk of developing Alzheimer's disease or have a faster rate of disease progression.

However, there are also limitations of using fluid biomarkers to understand Alzheimer's disease pathogenesis. First, the linkage between the change in the fluid biomarker level with the underlying biology is often uncertain and, in some cases, opposite (lower CSF Aβ indicates elevated Aβ in brain tissue). For many blood biomarkers it is unclear how changes map onto changes in the brain. Although blood and CSF IL-6 and MCP-1 were found to correlate among AD patients [28], plasma and CSF inflammatory markers have been found to independently relate to AD pathology among non-demented individuals [29]. Second, there are a number of chronic conditions and medications that can influence markers of innate inflammation in the blood and CSF. Thus, it can be difficult to determine whether an innate immune marker, even if previously found in the brain, is changing because of brain changes or due to a peripheral cause. Third, associations between a biomarker and disease states or subsequent outcomes do not demonstrate causality. With the exception of

interventional trials, experiments demonstrating causal relationships in humans are generally not feasible. Thus, to begin implying causal impact of the biomarker change upon disease biology, experimental model systems should be used, with all the caveats associated with translational research. Fourth, fluid sample amounts are limited and relevant biomarker concentrations may be diluted below limits of detection.

3. DIAGNOSTIC BIOMARKERS

Certainly one well regarded role of biomarkers is to aide in the diagnosis of a wide range of diseases. In dementia research, CSF and, increasingly, blood biomarkers are highly predictive of the presence of Alzheimer's disease pathology in the brain. Several excellent reviews have recently been published on this topic and some include results for innate immune marker proteins [30–32]. This ability to differentially diagnosis Alzheimer's disease from other forms of dementia without removing the brain and analyzing it histologically has dramatically improved our ability to identify subjects for clinical research and interventional trials. Using combinations of fluid biomarkers, imaging (PET and MRI) and detailed cognitive evaluations, a series of steps in the progression of Alzheimer's pathology have been recognized. The National Institute on Aging-Alzheimer's Association amyloid-tau-neurodegeneration (ATN) research framework [33] is providing the scaffolding on which to layer and understand other biological markers that may be associated with the development and progression of Alzheimer's disease pathology and the emergence of clinical symptoms. The ATN research framework is a considerable advance over the use of clinical symptoms to establish a differential dementia diagnosis and ascertain disease stage. Previously, both pathological and PET imaging studies reported that the clinical diagnosis of Alzheimer's disease was not accurate in 20–25% of cases, even among top-rated academic institutions [34–36]. As a result, patients with a clinical diagnosis of Alzheimer's disease but without Alzheimer's pathology were historically enrolled in clinical trials of potentially disease-modifying Alzheimer's therapies. It was also difficult to identify biomarkers of disease pathogenesis and progression without knowing what the underlying pathology was. More recent clinical trials require evidence of Alzheimer's pathology, via neuroimaging or fluid biomarkers, using the ATN framework, as part of enrollment eligibility to test the drug among those people most likely to receive benefit.

An increasing number of CSF studies have found multiple immune markers associated with Alzheimer's disease pathology utilizing the ATN research framework. However, one GAP in knowledge regarding these biomarkers is whether they represent trait variables that modify risk of disease, or whether they indicate an immune response to the presence of pathology. A recent cross-sectional CSF study combined three study cohorts to examine 227 samples with 21 immune-related analytes [37]. They found multiple immune markers that increased among those with elevated p-tau (T+) compared to unaffected cases including YKL-40, MIF, sTyro3, sAXL, sTNFR2, ICAM, sTREM2, C1q, C3b, and C4; only complement C3 was different in amyloid positive (A+) cases relative to cognitively unimpaired older adults (A-). In a study of 827 participants, Whelan et al [38] measured 270 proteins in CSF using OLINK panels. They reported cross-sectional elevations of 10 proteins comparing A+ Alzheimer's disease patients to cognitively unimpaired A- controls including CHIT1, MMP-10, and SMOC2 and decreased LDLR levels as the largest effects. Using LASSO

regression, models that included over 30 proteins provided high accuracy (AUC>90%) for diagnosing either A+ Alzheimer's disease or A+ mild cognitive impairment patients compared to A- cognitively unimpaired participants. Notably, over the age of 75, most dementia patients have multiple pathologies but few studies have considered the contribution of vascular pathology, which is also linked to inflammation, when examining immune markers for the diagnosis of Alzheimer's dementia.

Other studies have used unbiased approaches to detecting changes in CSF biomarkers using mass spectrometry techniques. Wesenhagen et al [39] published a meta-analysis of these studies, reporting results on over 600 proteins. More recently, Zhou et al [40] published a study focusing on a targeted subset of CSF proteins, but included data in a supplemental Table S8 on over 2700 proteins. Remarkably, in both studies, many innate immune markers of interest were absent. For example, IL-1β, TNFα, IL-10, IL-13, MCP-1, G-CSF were not in any of the tables of CSF proteins. They were also absent in a table of 5700 proteins from postmortem tissue studies [41] (Table S2A). However, as mentioned above, a limitation was many of the CSF studies included in these meta-analyses lacked information on Alzheimer's pathology and only compared Alzheimer's disease patients to cognitively unimpaired individuals. Another consideration is that although unbiased studies permit the identification of interacting networks of coordinately regulated genes that comprise the brain response to Alzheimer's pathology, many innate immune markers of interest appeared to be below the limits of detection by these unbiased approaches. In fact, many immune markers have been undetectable in most samples using the standard commercially available multiplex ELISA kits [42–44]. Although some of these markers were available in the OLINK platform assays performed by Whelan et al [38], most samples were below limits of detection for these key immune markers on this platform as well. Our own unpublished work, which has detected these same markers using SIMOA assays, suggests there is some opportunity to detect these key innate immune regulatory molecules in the CSF. Table 2 indicates the range of detection for several key immune biomarkers for different platforms according to the platform manufacturer's specification sheets. However, it appears that to fully appreciate the innate immune changes found in the progression of Alzheimer's disease there will need to be combinations of both untargeted and targeted approaches for those analytes present at the lowest levels in the fluid.

In CSF immune biomarker studies focusing on the presymptomatic stages of disease, when individuals are cognitively unimpaired but have amyloid with or without tau pathology and neurodegeneration, some anomalous observations have been described. One surprise is that some immune markers that increase in later, symptomatic Alzheimer's disease, are lower in the cognitively unimpaired A+T-N- cases compared to cases that are A-T-N-. Conversely, some immune markers that are decreased in Alzheimer's dementia patients, have increased levels among cognitively unimpaired A+T-N- individuals compared to A-T-N-. Meyer et al [45] investigated the association of innate immune activity with biomarker indicators of Alzheimer's disease pathology in PREVENT-AD, a well characterized cohort with a confirmed parental history [45]. Among cognitively impaired individuals, six analytes, IL-12 (p40 and p70), IL-15, IL-8, ICAM-1 and VCAM-1 were increased when A+T+ compared to A-T-. Yet, surprisingly, these analytes were distinctly lower for participants with only amyloid pathology (A+T-) compared to those without pathology (A-T-). Similar findings

were corroborated in the ADNI database, where 23 proteins linked to innate immunity followed a similar “V-shaped” (down then up) pattern from A-T- to A+T- to A+T+. Notable in the ADNI dataset were innate immune markers AXL, VEGF, CgA, IL-3, M-CSF, and CD40a. Despite differences in markers assayed, many of the ADNI markers of immune activation again showed a “V-shaped” pattern [45]. Similar reductions in A+T- versus A-T- participants have now been observed for YKL-40 [46], sTYRO3, ICAM-1, C3, C3b, Factor H [37], sTNFRII, TGFβ1, and VCAM-1, [47]. These noted changes in immune markers with Alzheimer’s pathology among cognitively unimpaired individuals further highlight the necessity to not just compare Alzheimer’s dementia patients to controls but to also consider the underlying pathology. This biphasic pattern of change to the progression of Alzheimer’s disease pathology may reflect different innate immune responses to the signals associated with amyloid deposition versus signals associated with tau deposition and/or neurodegeneration. An alternative explanation is that A-N- individuals who have low baseline levels of these immune markers establishes an environment permissive for amyloid deposition. This may reflect either low basal expression or possibly impaired expression due to immune-senescence. In this context, Streit [48] has argued that in aged human brain the microglia become dystrophic or senescent. Bachstetter et al [49] classified microglial in multiple disease conditions and reported large increases in microglia with this dystrophic morphology in cases of Alzheimer’s disease pathology or dementia.

One approach that can resolve these alternative explanations is to perform longitudinal studies of A-T-N- adults and monitor the intra-individual changes in immune markers that occur as amyloid pathology becomes detectable. If the immune markers that are lower in A+T-N- cases compared to A-T-N- cases represent a trait that makes them permissive for amyloid deposition, we would expect A-T-N- older adults with lower biomarker values to have a higher rate of converting to amyloid positive. If instead the lower levels indicate a response to amyloid deposition, the markers should decline as an individual transitions from amyloid negative to amyloid positive. Some longitudinal studies have recently reported collecting multiple CSF samples from late onset AD cases [50] and familial AD cases [51]. Although these studies have primarily focused on Aβ and tau biomarkers, as they accrue more samples over longer time frames and begin to examine the innate immune system biomarkers we should begin to resolve the alternatives posed above. The key observation would be to detect markers that associate with risk to develop amyloid deposits, as opposed to a response to the development of amyloid deposits. Model systems could then be manipulated to identify the underlying biology those markers reflect, potentially supporting development of pharmacological interventions to promote or inhibit that biology.

Several studies have also examined blood-based immune markers for the diagnosis of Alzheimer’s disease. For example, using samples from AddNeuroMed, a model combining Factor B, FS, soluble complement receptor 1, MCP-1, and eotaxin-1 in the test sample was validated in another sample with 73% sensitivity and 77% specificity [52]. However, despite multiple reports of blood markers, some positive and others negative, there is currently no consensus as to what immune blood biomarker can be used for diagnostic purposes [53]. As with CSF, the vast majority of blood-based studies have been limited by the comparison of AD dementia cases and controls and have not considered underlying pathology. Moreover, levels in the blood of some innate immune markers, especially from

those that are brain-derived, may be even lower than in the CSF so sensitive assays are essential. In addition, aging is the biggest risk factor for sporadic Alzheimer's disease dementia, but also associated with multi-morbidity and the use of multiple medications. It has been historically difficult to separate the contributions of peripheral immune markers to Alzheimer's pathogenesis from comorbid peripheral conditions and treatments.

Two recent lines of research, focused on the microbiota-gut-brain axis and exosomes, may provide better specificity between peripheral markers of inflammation and brain Alzheimer's pathogenesis. With regards to the gut-brain axis, animal studies have demonstrated that gut microbiota regulate microglial maturation and function, and may contribute to the development of amyloid plaques and to neurodegeneration [54–56]. Exosomes are secreted membrane vesicles (40–100 nm in diameter) of endosomal origin released from various peripheral and brain cells including neurons, astrocytes, microglia and oligodendrocytes. Because of their small size, the exosomes released from these brain cells can be identified in the blood so may be considered a more direct indicator of brain tissue function [57]. Although most exosomal work to date in the Alzheimer's field has focused on amyloid and tau from neuronally-derived exosomes, the examination of microglial exosomes is ongoing [58]. In addition, multiple studies are now reporting alterations in immune cells from exosomes in AD patients compared to controls. For example, AD patients had higher levels of many complement proteins in astrocyte-derived exosomes compared to controls [59]. However, despite the promising results, the field is currently limited by challenges in validating brain-derived exosomes, contamination of peripheral exosomes, and the need for standardization in surface protein markers and preparations.

For the most part, markers associated with innate immune activity seem to increase in the later stages of Alzheimer's disease and there are fewer differences between MCI A+ and cognitively unimpaired A- individuals. A similar outcome is found using PET imaging for the mitochondrial protein Translocator Protein 18 (TSPO), for which a series of different radioligands have been developed. Although individual studies using relatively small sample sizes were sometimes conflicting, a recent meta-analysis of over 20 studies examining TSPO PET imaging in unimpaired, MCI and AD cases reports that PET signals increase moderately in MCI and more strongly in AD [60]. One disadvantage of this target for microglial activation is the dearth of experimental model data studying this protein. TSPO is not selective for microglia. Immunostaining for TSPO can also be detected in astrocytes, endothelial cells and smooth muscle cells in human brain tissue [61]. Boche et al [62] have recently commented that this target is not coupled to specific microglial functional states such as phagocytosis, antigen presentation, response to immunoglobulins or homeostasis. They suggest a need for new ligands with greater selectivity for specific functional states of the innate immune reaction.

4. PROGNOSTIC BIOMARKERS.

Although identifying biomarkers that aide in diagnosing Alzheimer's disease are useful, this is an association and not suggestive of a moderating effect on the disease. One advantage of fluid and imaging biomarkers is that they can be obtained from living cases. Biomarkers whose levels predict the development or progression of Alzheimer's disease pathology

and/or clinical symptoms might be considered as therapeutic targets. As examples, several studies have reported that higher levels of MCP-1 (CCL2) in CSF predict more rapid rates of cognitive decline [63, 64]. A similar prediction was found using plasma MCP-1 levels [65, 66]. Higher levels of the CSF protein VILIP-1 were associated with faster rates of brain atrophy and cognitive decline [67]. Apolipoprotein J (clusterin) levels are elevated in CSF of A+ individuals and predict rate of entorhinal cortex atrophy [68]. YKL-40, ICAM-1 and Flt-1 were also elevated in A+ individuals. The levels of these proteins also predicted the rate of decline on the MMSE and the risk of converting to Alzheimer's dementia. Meyer et al [69] recently used ADNI data to combine several innate immune biomarker values using LASSO analysis to predict cognitive decline. They found that the model built with CSF innate immune markers was as good as that using core AD biomarkers (Aβ and tau) and when added together could predict 40% of the variance in ADAS-Cog decline. The results for the examination of immune markers in blood for prognosis have been more mixed than for CSF. As an example, elevated IL-6 levels were associated with greater cognitive decline among 1,224 Northern Manhattan participants [70]. In contrast, a study with 1,602 community-dwelling Minnesotans did not find an association between plasma IL-6, IL-10 or TNF-alpha and global or domain specific cognitive decline or risk of MCI among A- or A+ individuals [71].

The increasing number of longitudinal studies being conducted and continuing to collect data will permit even finer discrimination of the predictive value of certain immune biomarkers, especially those studies that incorporate concurrent fluid or imaging assessments of Alzheimer's pathology. From the perspective of drug development, one would expect a protein which is regulating pathology or cognition (either positively or negatively) would correlate with progression within the same pathological stage, rather than just between stages with different levels of pathology and rates of decline. Markers fulfilling this prognostic relationship, combined with data from experimental models and genetics, can help identify rational candidates for potential drug development.

Even the PET imaging agent TSPO has been found to predict subsequent cognitive change. Hamelin et al [72] found correlations among TSPO signals, amyloid PET signals and baseline MMSE, in early stage Alzheimer's cases. They subsequently monitored cognitive function over 2 years and identified fast decliners and slow decliners within the A+ cases. They found that the slow decliners were the cases with higher TSPO signals, implying some protective function of the microglial activation state measured by this ligand. PET Aβ SUVR had no relationship to cognitive decline in this group. Clearly, the development of PET imaging ligands with greater functional specificity will be important to compare with CSF and plasma biomarker signatures in longitudinal studies of both early stage and later stage Alzheimer's disease.

5. HOW DO FLUID BIOMARKER CHANGES RELATE TO CHANGES IN TISSUES?

The drainage of proteins in biological fluids like CSF and blood are typically interpreted as representations of the amount of that protein within the tissue of origin. This is

almost certainly the case for cytokines, chemokines, hormones and other secreted proteins; increases in the fluid reflect increased secretion and presumably increased signaling on the cells bearing receptors. Changes in levels of proteins typically found within cells are sometimes interpreted to represent toxicity in the cells, releasing these cytosolic proteins as the cells degenerate. Such is now believed the case for CSF levels of total tau and neurofilament light-chain [31]. However, through studies of other brain disorders, and using Tau PET and autopsy studies, it now appears that some phosphorylated forms of tau in CSF and blood are selective for Alzheimer's disease, while increased levels of total tau represent neurodegeneration generally (summarized in [33]). Thus, in the ATN framework, phosphorylated T181-tau is a marker for the T+ condition while total tau is a marker for the N condition [73]. However, recent studies in blood and CSF suggest that phosphorylated T217-tau may be even more specific for Alzheimer's disease than phosphorylated T181-tau [74, 75].

One anomaly in relating biomarker levels to the underlying Alzheimer's pathology regards interpreting CSF and, now, blood Aβ levels. *A priori*, 30 years ago, were one to predict a change in CSF Aβ levels in Alzheimer's disease, the most common expectation would have been an increase. It increases in the parenchyma and the parenchyma drains into the CSF and blood. However, both the postmortem histopathology and the amyloid PET ligand data identify Aβ reduction in CSF as the direction of change in Alzheimer's. Thus, when amyloid begins to aggregate there is apparently less available to escape into the CSF, as more is retained in the parenchyma. However, this reduction appears to reflect a state variable rather than a continuous reflection of the amount of amyloid deposition. While PET amyloid signals increase with disease progression, CSF levels remain relatively stable [32]. Thus, unless the protein measured is primarily a secreted protein, the relation of a change in CSF to the underlying biological processes giving rise to it need to be investigated cautiously. See the discussion below regarding the interpretation of sTREM2 levels.

6. TREM2 AS AN EXAMPLE OF A DRUG TARGET FOR ALZHEIMER"S DISEASE

There are certainly hundreds of innate immune proteins that might be manipulated in Alzheimer's disease in an attempt to improve patient outcomes. Clearly to justify making such attempts, strong evidence demonstrating a cause and effect relationship to the disease needs to be collected. One source of such causal information is the genetic variants that modify disease risk. Without necessarily explaining the biology, genetic risk argues that the protein itself or downstream effectors moderate either the initiation or progression of the disease.

TREM2 is probably the most studied innate immune marker that has variants associated with AD risk. The risk variants appear to be loss of function mutations [76]. Although some early studies reported decreased or unchanged TREM2 in Alzheimer's disease CSF [77, 78], most cross-sectional studies find increases in solubleTREM2 (sTREM2) in CSF with advanced disease compared to older cognitively unimpaired A- adults [79–81]. Recent studies using the ATN framework, find significant increases in sTREM2 only

when neurodegeneration is present, including the condition called suspected non-amyloid pathology (SNAP; A-T-N+). In general, sTREM2 correlates with p-tau and with total tau, but not Aβ in CSF [47, 82], arguing it is a neurodegeneration marker, rather than an Alzheimer's pathology marker.

However, it is not clear how this elevation in the CSF sTREM2 relates to the TREM2 on the surface of microglia. The general interpretation is that elevated sTREM2 indicates increased TREM2 expression and signaling in microglia. Increased TREM2 expression provides increased substrate for the extracellular proteases that cleave many surface proteins near the membrane (sheddase enzymes; ADAM 10 and 17) generating sTREM2. However, it is now recognized that there is an alternatively spliced form of TREM2 which lacks the membrane binding domain and could contribute up to 25% of the sTREM2 found in CSF [83]. Increased sTREM2 may alternatively reflect an increased activity of the sheddases, without increased expression and may, in fact, indicate reduced signaling. It is also the case that many pathways are regulated by soluble/secreted receptors, which act as decoys to bind ligands and decrease signaling through the membrane bound receptor (Type II IL-1R, sIL6R, sRAGE, [84]. These decoy receptors can be produced by sheddases, and/or alternative splicing of the receptor. Both mechanisms are responsible for sTREM2 production in brain, implying it may function as a decoy for TREM2 ligands, reducing TREM2 signaling. There is some evidence that sTREM2 itself has activity [85, 86]. Moreover, intact membrane bound TREM2 is challenging to detect histologically in human postmortem brain tissue [87]. Early data from mouse models also found little TREM2 inmmunostaining of cells in brain except in the vicinity of amyloid plaques [88]. Thus, another option is that most TREM2 is rapidly shed from normal brain microglia, and the elevation near plaques would reflect increased TREM2 expression, or interference with sheddase activity possibly by bound ligand.

Additional evidence suggesting TREM2 as a potential therapeutic target for treating Alzheimer's disease has developed from experimental mouse studies. Given the argument that TREM2 variants conferring risk appear to be loss of function mutations, initial studies used TREM2 deficient mice crossed with mice depositing amyloid or tau. Although initially results were inconsistent, TREM2 deficiency appears to increase the deposition of Aβ and produces more compact neuritic plaques [89–91]. A study of 5xFAD mice (APP mice with 5 mutations), found increased TREM2 expression reduced amyloid deposition and other aspects of the 5xFAD phenotype [92]. These data support increasing TREM2 signaling as a potential drug target. Somewhat surprisingly, studies of the TREM2 signaling protein DAP12/TYROBP deficiency in amyloid depositing mice had no major effects on amyloid deposition, but appeared to ameliorate some aspects of the mouse phenotype [93].

Studies examining TREM2 deficiency on tau pathology have not been as supportive of TREM2 as a drug target. Jiang et al [94] found that reducing TREM2 in a tauopathy model (PS19; P301S) with siRNA increased the tau phenotype. Similarly, Bemiller et al [95] found that TREM2 deficiency worsened the tau phenotype in the h-tau mouse model. Conversely, Leyns et al, [96] reported that TREM2 deletion in the PS19 mouse mode protected mice from neurodegeneration. Sayed et al [97] found that haploinsufficiency of TREM 2 increased tau pathology in a mouse model while complete loss of TREM2

protected mice from tauopathy. Most recently, examining a mouse with the R47H mutant forms of TREM2 crossed onto a tauopathy model, the R47H TREM2 reduced the tauopathy phenotype [98]. Some of these discrepancies may be attributed to the model used (h-tau mice have wild type human tau and a generally mild phenotype). The results further suggest the homozygous R47H mutation functions similarly to the complete TREM2 deficiency. However, if TREM2 agonism has opposing effects on amyloid versus tau pathology (similar to some other interventions modulating innate immune activity; Lee et al,[99]), the disease stage in which to evaluate the treatment may need to be selectively chosen.

Recently, two studies reported on the use of two monoclonal antibodies against TREM2 in mouse models of amyloid deposition [100, 101]. Although the premise for the design of the two antibodies was different, they shared several important features. First, the antibodies appeared to inhibit the sheddase cleavage of TREM2 from the membrane, presumably increasing levels on the membrane and increasing ligand driven signaling. Second, the antibodies appear to directly activate TREM2 signaling independent of ligand binding, possibly through dimerization. In mouse models of amyloid deposition, both antibodies reduced amyloid deposition and modified microglial gene expression. Wang et al further reported early results from a phase 1 human study where they observed a dose dependent decrease in CSF sTREM2. This target engagement biomarker is consistent with antibody masking of the sheddase binding site.

A final piece of evidence supporting TREM2 as a therapeutic target relates to the prognostic capacity of CSF sTREM2. Ewers et al [102] reported that in A+T+ cases, those with higher CSF sTREM2 had slower rates of cognitive decline over an average of 4 years. They also were less likely to convert from normal to MCI or from MCI to AD when the ratio of sTREM2/ptau was elevated, although this correlation could also be caused by less p-tau in the early stage cases that decline less rapidly. Nonetheless, assuming elevated sTREM2 indicates greater sTREM2 expression, this is consistent with increased TREM2 signaling being beneficial and supports TREM2 as a drug target.

Thus, there is a broad range of support to examine TREM2 as a therapeutic target in Alzheimer’s. Genetics strongly imply that modification of the protein can have large impacts on risk of developing the disease. Levels of the protein in CSF are associated with diagnosis of Alzheimer’s, although it is uncertain if this implies increased expression and signaling, or elevated shedding and decoy activity with reduced signaling. Manipulation of the protein in experimental models is generally supportive of increased TREM2 signaling reducing amyloid deposition and/or toxicity, although data regarding tau pathology are mixed. Finally, individuals positive for Aβ and p-tau have a better prognosis if their sTREM2 in CSF is elevated. Assuming this reflects elevated expression and signaling, this supports the ongoing studies with activating TREM2 antibodies.

7. METHODOLOGICAL CONSIDERATIONS

One of us (DM) has for most of his career identified as a neurochemist. He has worked with rat tissues, mouse tissue and postmortem human material. Recently he was asked to collaborate with a group from McGill on CSF specimens [69]. Some CSF samples

were sent and analyzed using the SIMOA platform. For most studies of brain tissue, it is standard to add inhibitors of various catabolic processes to preserve the state of the tissue proteins during sample preparation. These include protease inhibitors (at minimum) and often phosphatase inhibitors, deacetylase inhibitors and/or other known inhibitors of ATP-independent catabolic processes modifying proteins. In CSF, it is possible that the levels of the enzymes performing these catabolic reactions are sufficiently low that inhibitors are irrelevant. However, each protein should probably be checked for stability adding inhibitors at the time of specimen collection to half the sample and incubating at room temperature. Some publications referenced here have collected CSF samples from multiple sites and have commented that even though measurements were all performed at one location, data required correction for the site where the sample was collected (like age, sex, and other variables; [37, 79]). A recent study suggests protease inhibitors may not be required for measurements of CSF Aβ as it remains stable for 3 days at room temperature [103]. However, this may not be the case for other proteins. The addition of EDTA to plasma, to chelate magnesium and other divalent cations, may greatly reduce or eliminate most protease activity. However, other catabolic enzymes may still be active. Serum, on the other hand, likely contains multiple proteases responsible for coagulation. Use of consistent preanalytical procedures make it unlikely that degradation produces differences between diagnostic groups, but it may introduce variance and degrade rare proteins below limits of detection.

A second consideration in methodology is the denominator used. For the most part it is the volume of the fluid. However, this assumes that the production and clearance of fluid from the choroid plexus and capillary spaces is constant and in balance with the rate of lymphatic clearance, which may not always be the case [104]. The normal range of CSF protein is listed as 15–45 mg/dl, possibly reflecting different rates of CSF turnover. Some studies carefully measure the ratio of albumin in the CSF and in the blood from the same donor to provide an estimate of the blood brain barrier integrity, but this is not performed routinely, or at least not reported. In the protein immunoblot (western) literature, it is typically required to use a “housekeeping protein”, presumed not to change with treatments (although rarely verified) as the denominator to normalize values for the protein of interest. For studies in Alzheimer’s disease, this may be particularly critical as the blood brain barrier is known to be variably leaky as a result of the disease [105]. Recently, it was suggested to use PDGFβR levels to estimate blood brain barrier breakdown [106]. Because of this variable penetration of blood proteins into brain, there is concern that some of the CSF analytes may be of systemic origin, since blood levels are generally higher than CSF for most analytes. Possibly measuring levels in both fluids, and attempting to correct for the blood contribution to CSF levels by using platelet derived growth factor receptor and/or albumin ratios may lead to more precise estimates of the brain contribution to the analyte levels. This problem of variability in the mixing of proteins from the choroid filtrate with proteins from the brain parenchyma is very challenging to correct in the analysis. Additionally, in Alzheimer’s disease, there is enlargement of the ventricles averaging 9% annually [107], possibly diluting CSF analytes. Still, finding a means to normalize for individual differences in CSF production and clearance rates may reduce the extreme variance found in levels of some CSF analytes. Some analytes require log transformations to display individual data

points when normalized to CSF volume (although analyte levels within a donor appear largely stable over time for most proteins [108, 109]. This may be part of the reason that using protein ratios (Aβ42/Aβ40) provide greater discrimination than the raw values, because they are less sensitive individual differences in the rate of CSF turnover or presence of proteases. Developing and validating similar ratios for immune markers may yield more precise estimates of biomarker contributions form brain.

A third consideration is the effect of the platform used for measurement and other preanalytical variables. Differences in measured values from the same samples across platforms (SIMOA, OLINK, Luminex, Elecsys etc) are often observed, and performance of assays on different platforms will require some level of correction if they are to be combined. For preanalytical variables, The Alzheimer's Association Global Biomarker Standardization Consortium (GBSC) has focused on standardizing fluid markers, specifically CSF, across laboratories. Historically, the focus has been on Aβ42, p-T181-tau, and total tau. Additional future work will be needed for immune-related markers. A subgroup of the GBSC focused on blood-based biomarkers has been developed, Standardization of Alzheimer's Blood Biomarkers (SABB). The SABB presented initial results on some markers and pre-analytic variables at the 2020 Alzheimer's Association International Conference. It was noteworthy that the two immune markers examined, IL-6 and IL-7, were more sensitive than Aβ42 or tau to time and temperature between collection, centrifugation, and freezer. As the development of immune assays move forward, additional consideration of time and temperature, as well as other pre-analytic variables in the interpretation and reporting of the data will be required.

8. SUMMARY AND CONCLUSIONS.

Discovering drugs modifying the immune response to amyloid, tau and associated neurodegeneration is a rational approach to developing agents that slow or prevent Alzheimer's pathology and disease. However, before we can design these medications, we must identify drug targets that can moderate the progression or initiation of pathology. Immune system gene variants linked to risk of Alzheimer's disease are confirmed to play a moderating role in the initiation or progression of the disease. TREM2 mutation variants have a strong association with Alzheimer's disease risk. In addition to postmortem studies, fluid biomarkers are one means by which to identify such immune drug targets. However, there are key gaps in our knowledge regarding biomarkers for these targets that need to be overcome. One gap regards the relationship between differences in the levels of the marker in the fluid, and the differences in the levels or function of the marker within the tissue. For TREM2, an increase in sTREM2 may reflect either increased expression and enhanced signaling, or increased production of a decoy for TREM2 ligands, and decreased signaling. A second gap regards the degree to which differences in levels of a fluid analyte in cross-sectional studies reflect variations in baseline differences that may moderate initiation of disease (a trait variable), versus differences in response to the pathology at different stages of the disease (a response variable). This gap can be at least partially addressed by collecting extensive longitudinal fluid biomarkers samples covering the same person from before onset of amyloid pathology through its progression to tau pathology, neurodegeneration and clinical symptoms. A third gap is identifying those

biomarkers that predict the progression of the disease pathologically and/or cognitively. When the level of an immune marker can estimate prognosis, this becomes a marker that may be causally related to progression of disease. Elevated CSF sTREM2 predicts slower rates of cognitive decline. The fourth gap is to specifically identify a mechanistic causal role for the immune protein drug target in disease initiation or progression. This requires use of experimental systems that model select aspects of the Alzheimer's pathology, and multiple means of manipulating of the proposed target while monitoring the outcome on the disease phenotype in the model system. For TREM2 these results are mixed, but generally favor it as a therapeutic target. However, it may be that TREM2 therapy is effective for one stage of the disease, but ineffective or even detrimental at another stage. Part of the challenge in establishing the causal effects of specific manipulations experimentally is the incomplete nature of the models being examined. For the most part, the mouse models are reasonable approximations of amyloid deposition, or tauopathy, or even inflammation, but none recapitulate the full spectrum of Alzheimer's disease. Further caveats arise from the possibility that human transgenes interact with murine proteins in a different manner than human versions. Judicious considerations of model systems and pathologies as well as improved models of the disease will be required to address this issue.

One major point of this review is to argue that the immune response to neuropathology is largely accidental. Natural selection had no opportunity to guide a beneficial response to brain injury in most adult organisms. While it is conceivable that coordinated regulation of specific gene pathways designed for development or to fight infectious disease may have benefits, it is just as likely different components of these pathways could have counter-acting influences on outcomes. Most drugs are designed to impact a single target, often a protein. Thus, we need to make certain we do not over-interpret the systems biology results, and ignore individual proteins as potential candidates for drug targeting.

One model of Alzheimer's pathology progression suggests that amyloid accumulates over decades and by itself is not particularly toxic. At some point, it reaches a stage where it activates an immune response. Components of this immune response may be the link that promotes the expansion of tau pathology out of the entorhinal cortex and into the rest of the brain causing degeneration and ultimately brain atrophy. We envision a time when we have a meaningful drug armamentarium to prevent and treat Alzheimer's disease. Some medications may be used early in disease to modify amyloid deposition. Others may be used later in the disease to mitigate the effects of tau toxicity. Still others may be used at an intermediate stage, to optimize the immune response to amyloid, and minimize the effects on tau pathology. At the moment this remains speculative. However, overcoming the knowledge gaps described in this review should aide in deciding if this is speculation or a meaningful approach to ending Alzheimer's.

ACKNOWLEDGEMENTS.

We thank Richard Ransohoff for multiple informative discussions and for sharing the concept that natural selection had no influence on the immune reaction to neurodegeneration. We thank Elana Perry for assisting with the search for blood-based biomarkers and reviewing the manuscript for clarity to readers outside the biomarker field. We further thank Brianne Bettcher for reading and commenting upon the manuscript and coordinating the series of reviews from the Immunity and Neurodegeneration PIA from ISTARRT. DGM was supported by grants from the National Institutes of Health (R01 AG 051500, R56 AG058234, R44 AG058330, R01 AG062217 and R01

AG051674) and funds from the Spectrum-MSU Alliance. MMM was supported by grants from the National Institutes of Health (U01 AG06786, R01 AG049704, P30 AG062677).

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Research in Context

Systematic review:

The authors reviewed the published literature on immune biomarkers in Alzheimer's disease. The major consistent observations and a few anomalies are highlighted. Specific attention is paid to the relationship of potential drug targets to the clinical and pathological presentation of the disease.

Interpretation:

We believe there are several gaps in knowledge regarding specific proteins and their potential for development as therapeutic targets and recommend steps needed to close these gaps

Future directions:

We believe there is need for more ion depth longitudinal study of biomarkers during the disease course and increased attention paid to the reproducibility of biomarker measurements

TABLE 1.

SUMMARY OF THE REVIEW

GENERAL CONSIDERATIONS REGARDING BIOMARKERS • Immune responses to neural injury in adults are not guided by natural selection, but simply responses to the prevailing stimuli without regard to benefit or exacerbation. • The discovery that many genes influencing risk of Alzheimer's disease are immune-related validates the innate immune response as potential drug targets • Biomarker studies are one means by which to begin understanding how specific immune proteins interact with the initiation and/or progression of Alzheimer's disease pathology and clinical symptoms
DIAGNOSTIC BIOMARKERS • Most studies of immune biomarkers in Alzheimer's disease report that some biomarkers change at different stages of disease, whether based on clinical symptoms or pathological stages • Some biomarkers that increase in advanced disease, are lower in those who only have amyloid (A+T-N-) compared to amyloid negative cases (A-T-N-). These biomarkers may signal a) individual basal levels (traits) that are permissive for amyloid deposition, or b) different immune responses to amyloid versus tau/degeneration. • Longitudinal studies can resolve whether the biomarker differences in early stages of Alzheimer's pathology reflect traits or response differences. • The success of using blood to measure markers of Alzheimer's pathology (Aβ, p-tau) is encouraging studies of immune markers in blood, even though immune markers have greater challenges to interpretation.
PROGNOSTIC BIOMARKERS • One means by which fluid biomarkers compliment postmortem tissue studies is the ability to predict future changes in pathology or cognitive progression. • Biomarkers reflecting potential drug targets should have a moderating influence on disease and correlate with future progression of pathology and/or cognition
DETERMINING THE RELATIONSHIP OF BIOMARKER CHANGES WITH CHANGES IN TISSUES • An increase or decrease in CSF or blood biomarkers may not reflect a similar change in tissues/organs contributing the biomarker (e.g. CSF Aβ declines when brain levels increase). • Increased sTREM2 in Alzheimer's CSF may reflect a) increased expression and receptor signaling or b) increased shedding and decoy production leading to decreased signaling. • Multiple experiments in model systems are critical for demonstrating cause-effect relationships between potential drug targets and a) the different pathologies found in Alzheimer's disease, and b) the relationship between biomarker changes in fluids and the biological changes in tissues.
METHODOLOGICAL CONSIDERATIONS • Biological fluids should be checked for the stability of biomarkers with and without inhibitors of catabolic processes (e.g. protease inhibitors) to confirm they are not needed for the analyte being measured. • There are considerable individual differences in CSF turnover and total CSF protein levels, yet most studies use volume as the denominator. Efforts should be made to identify some minimally changing CSF markers derived from brain that might be used to normalize individual differences. Even total protein may reduce variance over volume as the denominator. • More work is needed regarding pre-analytical variables that might impact immune biomarkers in biological fluids through the Global Biomarker Standardization Consortium.
CONCLUSIONS • Immune activation by amyloid may be one process linking amyloid deposition to the expansion of tau pathology. • The goal is the development of multiple effective agents to regulate amyloid deposition, immune activation and/or tau pathology to prevent and/or treat Alzheimer's disease, each of which may be optimally prescribed based on the pathological stage of the disease guided by biomarker evidence.

Table 2.

Range of assay sensitivity for six cytokines of interest a fluid biomarkers in Alzheimer's

Analyte	**IL-1β**	**IL-6**	**TNF**	**IL-4**	**IL-10**	**IL-13**
Source	**Single Assays**					
Thermo Standard	4–250	2–20	4–500	8– 500	3– 200	1–100
Thermo ProQuantum	0.06– 10,000	0.06 – 10,000	0.02–5,000	0.02– 5,000	0.06 – 5,000	0.02–5,000
Quanterix SIMOA	0.02–240	0.005–120	0.02–200	0.005–200	0.004–120	0.002–30
MSD V-Plex	NA	0.001–6	0.007–75	NA	0.002–19	NA
	Multiplex Assays					
BioRad XMAP	0.3–4600	0.4–6200	1.1–54,000	0.09–3000	1.4–35,000	0.2–5,000
MSD T-Plex	0.2–10,000	0.1–10,000	1.1–10,000	0.2–10,000	0.3–10,000	0.7–10,000
OLINK	0.5–30,000	0.1–4,000	0.9–30,000	0.2–8,000	0.5–60,0000	7.6–60,000

Data obtained from manufacturer's specification sheets. Data are assay range sensitivity in pg/ml. NA indicates assay kit not available for this analyte.