Self-Assembling Peptides Form Immune Suppressive Amyloid Fibrils Effective in Autoimmune Encephalomyelitis

Michael P. Kurnellas, Jonathan B. Rothbard and Lawrence Steinman

Abstract Amyloidogenic proteins have long been linked to neurodegenerative diseases. However, amyloid fibrils composed of six amino acids are protective in an animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE). The reduction of pro-inflammatory cytokines, decrease in the number of inflammatory foci in the parenchyma and meninges of the brain and spinal cord, and amelioration of the neurological signs of EAE when amyloid fibril-forming hexapeptides are administered reveal that some fibrils provide benefit. The therapeutic activity of the amyloid fibrils arise from diverse pathways that include binding of pro-inflammatory mediators in the plasma, reduction of IL-6, TNF-α, and IFN-γ levels, and induction of type 1 interferon (IFN). Type 1 IFN has been used widely as a therapeutic agent for the treatment of MS and has been shown to be therapeutic in EAE with adoptive transfer of Th1 lymphocytes. However, type 1 IFN is known to exacerbate EAE with adoptive transfer of Th17 lymphocytes. Indeed, the amyloid fibril-forming peptide Tau 623–628 was therapeutic in Th1 adoptively transferred EAE, but ineffective in Th17 adoptively transferred EAE. However, the therapeutic effect of Tau 623–628 was restored in IFN-α/β receptor (IFNAR) knockout mice, indicating that other immune pathways independent of type 1 IFN induction play a role in the amelioration of EAE. Moreover, Amylin 28–33, a polar, non-ionizable peptide that does not form fibrils as rapidly as Tau 623–628, induces a small fraction of type 1 IFN compared to Tau 623–628 and is therapeutic in Th17 EAE. The diverse immunological pathways modulated by the self-assembling hexapeptides are under investigation with a goal to develop novel, safe, and potent therapeutics for neuroinflammation.

Keywords Self-assembling peptides · Amyloid fibrils · Immunosuppression · Multiple sclerosis · Neurodegeneration · Molecular chaperones · Anti-inflammatory

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Contents

1 Introduction

The accumulation of amyloid proteins has long been associated with neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's disease; type 2 diabetes; and transmissible spongiform encephalopathy. The proteins associated with these diseases, such as amyloid beta 1–40 and 1–42, Tau, synuclein, huntingtin, islet amyloid polypeptide (IAPP, or amylin), and prion protein, can form insoluble aggregates that play a role in neuronal pathology and degeneration. However, the biological functions of amyloid-forming proteins and the pathophysiological role of amyloid fibrils are not well defined. Amyloidogenic proteins, including alpha B crystallin (HspB5), amyloid precursor protein (APP), Tau, and serum amyloid P (SAP), were all found in MS lesions (Han et al. 2008). However, permanent deletion of HspB5 (Ousman et al. 2007), APP (Grant et al. 2012), major prion protein (PrP) (Gourdain et al. 2012), serum amyloid P (Ji et al. 2012), and Tau (Weinger et al. 2012), resulted in more severe clinical scores and increased neuronal damage in EAE. HspB5 has been shown to be an effective anti-inflammatory agent that has been therapeutic in animal models of multiple sclerosis (Ousman et al. 2007), stroke (Arac et al. 2011) and cardiac and retinal ischemia–reperfusion injury (Pangratz-Fuehrer et al. 2011; Velotta et al. 2011). Moreover, beta-amyloid 1–40 and 1–42 peptides were effective anti-inflammatory agents, ameliorating paralysis, and inflammation in EAE (Grant et al. 2012). The absence of these proteins is detrimental, but administration of amyloid fibril-forming peptides has been shown to be therapeutic in EAE and have an anti-inflammatory capacity. The ability of amyloid-forming peptides and proteins to modulate the immune system may lead to new therapeutic targets for the treatment of MS and other neuroinflammatory diseases.

2 Peptides from Small Heat-Shock Proteins Have Chaperone Activity and Form Amyloid Fibrils

The small heat-shock protein (sHsp), alpha B crystallin (HspB5), is a temperature-sensitive molecular chaperone that binds partially unfolded proteins and prevents deleterious aggregation during heat and cellular stress (Jakob et al. 1993). HspB5 was found to be the most abundant transcript in MS lesions, and the protein levels were found in copious amounts in acute and chronic active lesions (Han et al. 2008). HspB5 levels were elevated in the plasma of MS, neuromyelitis optica (NMO), and stroke patients, as well as in mice with EAE (Arac et al. 2011; Rothbard et al. 2012). The increase in plasma levels of HspB5 may be a protective mechanism to mitigate damage during inflammation, ischemia, and neurodegeneration.

The protective nature of HspB5 is evident in mice lacking this protein when induced with EAE. Mice deficient in HspB5 exhibited more severe clinical symptoms during EAE, with increased glial apoptosis and production of pro-inflammatory cytokines (Ousman et al. 2007). Administration of exogenous alpha B crystallin to mice with EAE reduced the disease severity, suppressed immune cell proliferation, and decreased the production of pro-inflammatory cytokines (Ousman et al. 2007; Rothbard et al. 2012). Cessation of the administration of the protein resulted in a rebound of clinical symptoms during EAE, which indicated that HspB5 was a biological inhibitor and must be maintained at a certain serological level to have its therapeutic effect (Rothbard et al. 2012).

HspB5 did not affect B and T cell proliferation, and it appears that the suppression of inflammation does not occur through direct inhibition on the adaptive immune response (Rothbard et al. 2012). The mechanism of action for the efficacy of HspB5 was based on its ability to bind pro-inflammatory proteins in the plasma (Rothbard et al. 2012). Proteins bound to HspB5 from plasma of MS patients and mice with EAE were identified by mass spectroscopic analyses. Approximately seventy proteins were enriched in the HspB5 precipitate with a temperature-dependent sensitivity. Elevated temperature increases the molecular chaperone activity of the sHsps. Temperature dependence makes HspB5 particularly effective at sites of inflammation, where temperature is known to be elevated compared to non-inflamed tissue. Interestingly, the majority of the proteins identified as binding partners of HspB5 were acute-phase proteins, complement proteins, and coagulation factors (Rothbard et al. 2012). HspB5 not only bound these proteins, but also could decrease the levels of the inflammatory proteins in the plasma and thereby affect the innate immune system. The anti-inflammatory capacity of HspB5 was evident by the reduction in the plasma levels of IL-6, a pleiotropic cytokine with multiple functions in the immune system, and plays a role in the pathogenesis of EAE (Rothbard et al. 2012).

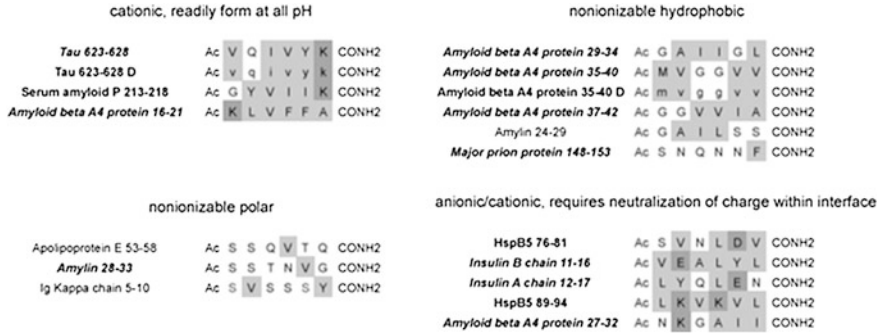
Additional structure activity correlations between chaperone activity and therapeutic function were established when linear peptide regions within HspB5 were examined (Kurnellas et al. 2012). Only the region corresponding to residues 73–92

of HspB5 exhibited chaperone activity. More importantly, the 73–92 peptide was therapeutic in EAE, which had efficacy similar to the full-length protein, and could reduce the production of pro-inflammatory cytokines from stimulated splenocytes and lymph node cells from EAE mice (Kurnellas et al. 2012). Tanaka et al. (2008) have shown that the chaperone activity of alpha A crystallin (HspB4) 73–92 corresponded with the formation of amyloid fibrils and that the loss of amyloid formation also correlates with a loss of chaperone function. In our work, amyloid fibril formation was measured by incubating thioflavin T (ThT) with residues 73–92 from HspB1, 4, and 5 and measuring the fluorescence at 485 nm. Binding of ThT by amyloid fibrils results in ThT fluorescence, which has long been used as a measure of amyloid fibril formation of proteins and peptides (Naiki et al. 1989). Consistent with this measurement, amyloid fibrils were visualized by atomic force microscopy for these peptides. However, removal of a single hydrophobic amino acid at position 77, 79, or 81 of HspB5 73–92 peptide and replacement with a lysine was able to eliminate amyloid formation as represented by ThT binding (Kurnellas et al. 2012). The altered peptides also lost their chaperone activity and effectiveness in EAE treatment (Kurnellas et al. 2012). Amyloid fibril formation was required for the chaperone function and the therapeutic efficacy of the peptides. The necessity to form amyloid fibrils to retain chaperone activity may explain how a relatively short peptide can have an equivalent effect as a full-length protein. To reiterate, chaperone activity is based on the formation of amyloid fibrils, which perhaps unexpectedly provide protection from neuroinflammation. The juxtaposition of amyloid and "protection from neuroinflammation" in the same sentence is certainly unexpected.

3 Hexameric Amyloidogenic Peptides Are Therapeutic in EAE

Amyloid fibrils, which are composed of two self-complementary beta-pleated sheets whose side chains interdigitate to form a zipper-like configuration (Sawaya et al. 2007), are capable of forming pores in biological membranes and are pathogenic to cells (Chiti and Dobson 2009; Jang et al. 2010; Kayed et al. 2003; Quist et al. 2005; Xue et al. 2009). To further reduce the complexity of the structure and eliminate the toxicity of amyloid fibrils formed by larger peptides and proteins, smaller peptides can be utilized. Six amino acids are capable of forming amyloid fibrils (Thompson et al. 2006), but do not form toxic pore-forming structures (Laganowsky et al. 2012). Several groups have written algorithms to predict the amyloid-forming regions based on beta-sheet propensity (Fernandez-Escamilla et al. 2004; Nelson et al. 2005). The Rosetta-Profile algorithm, developed by Eisenberg and colleagues, allows for the quantification of the probability that any six amino acid sequence within a protein is capable of forming a steric zipper spine of an amyloid fibril (Goldschmidt et al. 2010). In the 73–92 peptide, two regions that exhibit the highest propensity to aggregate and form amyloid are residues

Table 1 The amyloidogenic peptides used to treat EAE are segregated by composition and their propensity to form fibrils (Eisenberg and Jucker 2012; Sawaya et al. 2007)



The amyloidogenic hexapeptides in bold font have been confirmed to reduce the symptoms of EAE. The hexapeptides whose crystal structure have been published are in italics. The hydrophobic amino acids are highlighted in light gray and acidic residues and basic amino acids in dark gray. D-amino acids are listed in lower case

76–81 and 89–94 (Goldschmidt et al. 2010). These regions contain alternating hydrophilic and hydrophobic amino acids that correspond to a beta-pleated sheet.

HspB5 76–81 and 89–94 are located within the region with the capacity to act as a molecular chaperone. These and other hexapeptides derived from amyloidogenic proteins, whose crystallographic solution have been determined, including Tau 623–628, beta-amyloid A4 16–21, 27–32, 29–34, 35–40, and 37–42, major prion protein PrP 148–153, Amylin 28-33, insulin B chain 11–16, and insulin A chain 12–17 all form amyloid fibrils (Table 1; Kurnellas et al. 2014) (Eisenberg and Jucker 2012; Sawaya et al. 2007), were shown to act as molecular chaperones as assessed by the inhibition of insulin aggregation (Kurnellas et al. 2013). These peptides are known to have the propensity to form amyloid fibrils, which was confirmed to occur when assessed by ThT staining (Kurnellas et al. 2013). The amyloid fibril-forming hexapeptides tested were therapeutic in EAE, reducing the neurological impairment from the disease (Kurnellas et al. 2013). The shuffled sequences of HspB5 76–81 and Tau 623–628 did not form amyloid and were not able to modulate the disease (Kurnellas et al. 2013), indicating the importance of the sequence of the hexapeptides. Cessation of treatment resulted in a return of paralytic symptoms of EAE, as observed with the small heat-shock proteins and residues 73–92 of HspB5 (Kurnellas et al. 2012). The therapeutic peptides were found to be anti-inflammatory, resulting in the decrease in pro-inflammatory cytokines. Although no direct evidence suggests the peptides enter the central nervous system (CNS), the hexapeptides are able to reduce the number of inflammatory foci in the meninges and parenchyma of brains and spinal cords (Kurnellas et al. 2013), perhaps by their effects on the peripheral immune system.

4 Mechanisms of Action of the Anti-Inflammatory, Therapeutic Amyloid Fibrils

4.1 Chaperone Function

The mechanisms of action of the hexapeptides include a capacity to act as a molecular chaperone, a similarity shared with HspB5 and the other sHsps (Rothbard et al. 2012). The amyloid fibril-forming peptides were incubated with bovine insulin under reducing conditions with dithiothreitol (DTT), and time-dependent light scattering produced by the association of the reduced B chain of insulin was monitored at 360 nm (Bhattacharyya et al. 2006). The hexapeptides were found to inhibit the aggregation of the B chain of insulin, consistent with molecular chaperone function (Kurnellas et al. 2013). The shuffled sequences of Tau 623–628 and HspB5 76–81, which do not form fibrils, lost the ability to inhibit insulin aggregation (Kurnellas et al. 2013). Although Tau 623–628 was able to inhibit the formation of insulin aggregates when added at time 0, it was unable to prevent aggregation following the initiation of aggregation (Kurnellas et al. 2013). Tau 623–628 inhibited fibril formation during the initial step of the process, but not during the nucleation of aggregates.

Tau 623–628 peptide, through its molecular chaperone activity, was able to bind proteins from the plasma of MS patients and mice with EAE. Biotinylated Tau 623–628 was incubated in the plasma, and the proteins bound were identified by mass spectral analysis. Forty-nine proteins were found enriched in the Tau 623–628 precipitate and with 41 of the proteins (84 %) also identified by precipitation with HspB5 (Kurnellas et al. 2013; Rothbard et al. 2012). Among the proteins found precipitated with amyloid fibrils, a high percentage were acute-phase proteins (19 of 49, 39 %), complement factors (11 of 49, 23 %), and members of the coagulation cascade (13 of 49, 27 %), which together comprised 33 of the 49 proteins (67 %). The proteins bound by Tau 623–628 are biologically relevant ligands that are known to bind amyloid fibrils, including apolipoproteins A-I, A-IV, and E (Strittmatter et al. 1993), clusterin (Ghiso et al. 1993), and transthyretin (Velayudhan et al. 2012). Twenty-nine of the forty-nine proteins (59 %) most prominent proteins bound to Tau 623–628 are known to be associated with HDL, including apolipoproteins A-IV, A-I, B-100, and E, clusterin, vitronectin, transthyretin, serum paraoxonase, angiotensin, and prothrombin (Kurnellas et al. 2013). Many of these proteins are known to modulate the disease course during EAE. Mice with genetic deletions of apolipoprotein E (Karussis et al. 2003), Tau (Weinger et al. 2012), HspB5 (Ousman et al. 2007), and APP (Grant et al. 2012) all exhibit exacerbated EAE. Earlier studies have shown that inhibition of angiotensin converting enzyme or angiotensin receptor and inhibition of prothrombin potently inhibit EAE (Han et al. 2008; Platten et al. 2009). The binding of pro-inflammatory mediators may be one factor involved in the immunosuppressive function of the amyloidogenic peptides.

4.2 Modulation of Peripheral Blood Cells

To discern the pathways responsible for the reduction of pro-inflammatory cytokines by the amyloidogenic hexapeptides, gene expression of peripheral blood cells (PBCs) from EAE mice treated with Tau 623–628 was evaluated. Blood was collected after 3 days of treatment, just prior to the reduction of disease, and after 10 days of treatment, in which clinical signs were diminished. The analysis of differential gene expression revealed that IL-6, TNF-α, IL-8, IFN-γ, serum amyloid A (SAA), and members of the S-100 family were decreased (Kurnellas et al. 2014). Gene expression of TNF-α and IFN-γ in PBCs and IL-6 in hepatocytes, as measured by qPCR, was decreased not only when the clinical signs were reduced, but also prior to reduction of the clinical signs after 3 treatments, indicating that their reduced expression might well be a central factor in the abrogation of the disease. Pathway analysis revealed significant reduction in lymphocyte, dendritic cell, and macrophage activation, and neutrophil and phagocyte chemotaxis. The pattern of gene expression at the later time point when the neurological signs were resolved displayed reduced activity of transcriptional factors NFκB, AP-1, NFAT, Spl1, E2F1, and ETS1, which was not seen prior to the reduction of clinical signs (Kurnellas et al. 2014). The pathways indicate that diverse and broad immune suppression occurs following the treatment with Tau 623–628 peptide.

4.3 Type I Interferon Induction

The analysis of gene expression prior to the reduction of symptoms revealed additional pathways modulated by the administration of Tau 623–628. A reduction in the expression of neutrophil-related genes and the induction of a type 1 IFN pathway were observed (Kurnellas et al. 2014). The expression levels of IFN-α4, 5, 9, 12, and 13, and IFN-β were all significantly increased after 3 treatments of Tau 623–628, which is just prior to the modulation of clinical signs. A change in the expression levels of 124 type 1 IFN inducible genes, including Oas1, 2, Isg15, Mx1, Rsad2, Herc5, Aqp3, Ifit1, 3, and IRF1, supports the idea that type 1 IFN plays a role in the therapeutic benefit of the amyloidogenic peptides.

The capacity of amyloid fibrils to decrease neutrophil-related genes has precedent based on earlier work showing that beta-amyloid can be endocytosed by neutrophils leading to the formation of neutrophil extracellular traps (NETs) (Azevedo et al. 2012). NETS are composed of DNA, elastase, and histones, which are released by neutrophils as a response to pathogen or amyloid in order to initiate a response gainst pathogens. Tau 623–628 also induced NETosis following its exposure to human neutrophils (Kurnellas et al. 2014). The formation of NETs also plays an important role in activating plasmacytoid dendritic cells (pDCs), which, in turn, produce type 1 IFN (Azevedo et al. 2012). Indeed, this was corroborated by

the ability of Tau 623–628 to induce the production of type 1 IFN from pDCs (Kurnellas et al. 2014).

The type 1 IFN, IFN-β, has long been used as a treatment for MS, yet varies in its therapeutic efficacy in different models of EAE, with the cytokine being efficacious in Th1-induced disease, but deleterious when the disease is induced by Th17 lymphocytes (Axtell et al. 2010, 2011, 2012). Tau 623–628 was found to follow a similar pattern, in which it was therapeutic in active and in Th1 adoptively transferred EAE, but was ineffective in Th17 adoptively transferred EAE (Kurnellas et al. 2014). However, not all amyloidogenic peptides induce equivalent amounts of type 1 IFN. Amylin 28-33 is a polar peptide that forms fibrils at a slower rate than Tau 623–628 and induces lower levels of type 1 IFN and was able to reduce the clinical signs of EAE (Kurnellas et al. 2014). This indicated that other immune pathways play a role in the amelioration of EAE. This was confirmed by the capacity of Tau 623–628 and Amylin 28–33 to ameliorate the symptoms of Th17 EAE in IFN receptor α/β mutant mice. Although type 1 IFN may play a role in the efficacy of the amyloidogenic peptides in active and Th1 EAE, it was shown to be unnecessary for the therapeutic response.

4.4 Other Pathways Involved in Therapeutic Efficacy

The mechanisms of action of the amyloidogenic peptides are diverse, including the reduction of pro-inflammatory cytokines in PBCs and the induction of type 1 IFN by pDCs activated by NET formation. Although type 1 IFN has been shown to play a role in the modulation of the disease, other pathways appear to have greater importance. The mechanisms responsible for the decrease in pro-inflammatory cytokines are the subject of further experiments and analyses. One such mechanism may be the ability of different immune cells to endocytose the fibril particles, which has been shown to be possible by neutrophils.

Endocytosis of the hexapeptides results from the formation of amyloid fibrils, independent of steric specificity. The D-amino confirmation of Tau 623–628 was able to form fibrils as effectively as the L-Tau 623–628, as measured by ThT staining (Kurnellas et al. 2014). The stereoisomeric fibrils were also able to inhibit insulin aggregation similarly when compared in an insulin chaperone assay (Kurnellas et al. 2014). As expected, D-Tau 623–628 was able to reduce the paralytic symptoms of EAE as well as the L-Tau 623–628. The therapeutic equivalence of the two isomeric peptides indicates that their mode of action will not be dependent on their binding by a stereoselective receptor. This corresponds with gene chip microarray data that the types of blood cells whose populations were most affected by administrations of the fibrils were those known to endocytose particles (Kurnellas et al. 2014), such as insoluble amyloid fibrils (Doherty and McMahon 2009; Sokolowski and Mandell 2011). Future studies will determine whether cell types other than neutrophils, including macrophages and B cells, are capable of endocytosis of the fibril particles, thereby mediating immunosuppression.

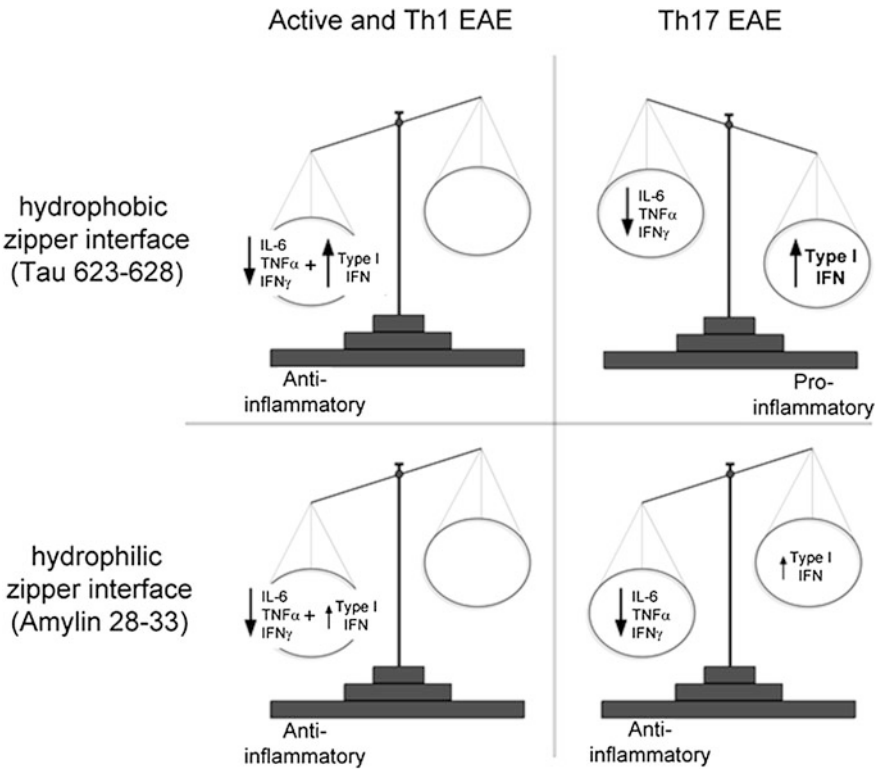


Fig. 1 Amyloid fibrils composed of hexapeptides modulate diverse immunological pathways. Tau 623–628 and Amylin 28–33 are able to reduce the levels of the pro-inflammatory cytokines, IL-6, TNF-α, and IFN-γ, which can lead to the amelioration of paralysis during EAE. Both peptides are able to induce type 1 IFN. However, Amylin 28–33, which forms fibrils more slowly, results in less type 1 IFN, which can be overcome by other anti-inflammatory pathways in the Th17 adoptive transfer model of EAE

5 Conclusions

The cell types affected and pathways modulated by amyloid fibril-forming peptides yielded insights into the mechanisms of action of amyloidogenic peptides and their ability to suppress the immune system. The peptides are able to modulate several pathways, including the reduction of pro-inflammatory cytokines, including IL-6, TNF-α, and IFN-γ, potentially through its chaperone activity. The fibrils can also induce type 1 IFN, which can enhance or limit the effect of the chaperone function depending on whether the disease is dominated by Th1 or Th17 lymphocytes (Fig. 1; Kurnellas et al. 2014). The pleiotropic effects of the hexapeptides may potentially lead to new treatment strategies in inflammatory and neurodegenerative diseases and a better understanding of the role of amyloid proteins and peptides in human disease and health. The discordance between therapeutic amyloid fibrils and

the deleterious effect of amyloidogenic proteins in neurodegenerative disease may be due to several factors, including the formation of toxic structures by larger peptides and proteins.

Although the therapeutic benefit is counter to the general consensus of the research and clinical communities, it is consistent with the experimental data establishing that only those aggregates within mixtures of amyloid fibrils capable of forming pores in biological membranes are pathogenic (Chiti and Dobson 2009; Jang et al. 2010; Kayed et al. 2003; Quist et al. 2005; Xue et al. 2009). Amyloid fibril-forming peptides composed of six amino acids do not form toxic structures (Eisenberg and Jucker 2012; Greenwald and Riek 2010; Sawaya et al. 2007). The simplified structure of these self-assembling peptides is sufficiently different from the naturally occurring amyloidogenic proteins making them not toxic and suitable for safe use as therapeutic molecules. Indeed, amyloid fibrils composed of hexapeptides were significantly less toxic to human monocytes than fibrils composed of beta-amyloid 1–40 and 1–42 (Kurnellas et al. 2014). Additionally, Tau 623–628 did not lead to a decrease in lymphocyte numbers in mice with EAE, but beta-amyloid 1–40 and 1–42, which was therapeutic in EAE, was deleterious to these cells (Grant et al. 2012; Kurnellas et al. 2014). Another factor that may lead to differences between the hexapeptides injected in the peritoneal cavity as compared to endogenous amyloidogenic proteins is the lack of formation of large insoluble aggregates. The self-assembling peptides provide their therapeutic effect in the periphery and likely do not enter the CNS, which may have an inability to clear the large aggregates that form over time.

Despite a growing amount of data supporting the therapeutic efficacy and immunosuppressive properties of the amyloid fibril-forming peptides, some questions do remain. The exact mechanisms of action of the self-assembling peptides are the subject of further experiments. Moreover, the pharmacokinetics and pharmacodynamics of the peptides need to be evaluated. Finally, the determination of whether the peptides cross the blood-brain barrier and enter the CNS must be examined. By answering these questions, the self-assembling peptides may become a useful therapeutic agent in treating MS and other neurodegenerative diseases. It would be a rather surprising reversal in perspective, if short, self-assembling, and amyloid hexapeptide structures were to become therapeutics for some of the diseases where amyloid is considered the basis of pathology.

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