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Apolipoprotein E isoforms and regulation of the innate immune response in brain of patients with Alzheimer's disease

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Abstract

The largest genetic risk for late-onset Alzheimer's disease (AD) resides at the apolipoprotein E gene (*APOE*) locus, which has three common alleles (ε2, ε3, ε4) that encode three isoforms (apoE2, apoE3, apoE4). The very strong association of the *APOE* ε4 allele with AD risk and its role in the accumulation of amyloid β and animal models solidify the biological relevance of apoE isoforms but do not provide mechanistic insight. The innate immune response is consistently observed in AD and is a likely contributor to neuronal injury and response to injury. Here we review emerging data showing that apoE isoform regulation of multiple facets of the innate immune response in the brain may alter AD not only through amyloid β-dependent mechanisms, but also through other, amyloid β-independent mechanisms.

Introduction

Alzheimer's disease (AD) has rare autosomal dominant forms. However, the form of AD causing a major public health problem is late-onset AD, which is not caused by dominantly inherited mutations but rather by genes conferring genetic risk in combination with other processes that are currently ill-defined. Although the etiology and pathogenesis of late-onset AD remains obscure, a key component of all forms of AD appears to be accumulation of Aβ peptides: endoproteolytic fragments of the product of the amyloid precursor protein gene, *APP*. The risk of the common late-onset form of AD, likely arising from modulated age of onset, has been repeatedly associated with the apolipoprotein E gene (*APOE*) following pioneering work that now has been replicated in genome-wide association studies (GWAS) for AD [1–3]. The strength of the association of the *APOE* locus with AD is orders of magnitude greater than that of other loci. Humans have three common alleles of *APOE*, unlike most other mammals who possess only one allele. AD risk is greatest with inheritance of ε4 allele, less with ε3 allele, and least with ε2 allele, and there is a gene dosage effect. The corresponding human apoE isoforms are 299-amino acid proteins that differ in amino acids at positions 112 and 158 (Table) [4] and possess very well-characterized isoform-specific actions in a variety of biological contexts. ApoE is an integral constituent of many lipid transport lipoproteins, playing key roles in both particle assembly and structure, as well as receptor-mediated lipoprotein uptake via the family of cell surface LDL receptors. ApoE

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is synthesized principally in the liver, with a discrete pool synthesized, secreted and maintained in the CNS.

Genetic associations establish biological relevance but not mechanism of action. Although the contribution to AD risk of neighboring chromosome 19 genes for translocase of outer mitochondrial membrane (TOMM) 40 and apoC-I has not been formally excluded [5, 6], the vast majority of work has focused on APOE. The model that has emerged from studies to elucidate mechanism of action is that apoE isoform-dependent effects, with apoE4 > apoE3 > apoE2, lead to increased aggregation and deposition, decreased clearance, or both, of Aβ peptides in the cerebrum [7].

In spite of the wealth of mechanistic data for apoE isoform-specific effects in experimental models of AD and in human neuroimaging and cerebrospinal fluid (CSF) biomarker studies, our knowledge is incomplete [8–16].

How apoE isoforms lead to altered Aβ aggregation and deposition or clearance is not entirely clear. Several groups have suggested that differential binding of apoE isoforms to Aβ influences these processes through receptor-mediated mechanisms, thereby modulating biological activity of Aβ peptides [17–19]. However, the fundamentals of apoE isoform-dependent binding to Aβ peptides *in vivo* are just beginning to be explored and appear to have quite different characteristics than *in vitro* [20].

Here we review evidence in support of an additional, or perhaps alternate, mechanism of action: that apoE isoforms differentially regulate the innate immune response to Aβ peptides and other relevant activators in AD, which in turn influences Aβ peptide aggregation, deposition, and clearance.

Innate immunity and AD

Innate immunity is the portion of the immune system that defends the host through non-pathogen-specific pathways. In contrast, T cells, B cells, and antibody-secreting plasma cells of the adaptive arm mobilize in response to specific antigenic determinants. This characteristic restriction of specific pathogen recognition by adapative immune system effectors arises through complex genetic reorganization during development (Figure 1). The net effect of the innate immune response may be beneficial, deleterious, or variable depending on the repertoire of components activated and the intensity, duration, and repetition of the response. It is not surprising then that some of the work in this area has revealed components of the innate immune response that suppress processes of AD or neuron damage, while other components have the opposite effects in experimental models (reviewed in [21]). Two major components of the innate immune response are the complement cascade and a class of pattern recognition receptors called Toll-like receptors (TLRs).

The complement system comprises over 30 proteins and protein fragments that are activated and greatly amplified through a tightly regulated proteolytic cascade [22]. The complement system is designed to assist with opsonization and phagocytosis, chemotaxis, and modification and lysis of the pathogen by the membrane attack complex (MAC). The complement system can be activated by three upstream pathways—classical, alternative, and mannose-binding lectin binding—which in turn are activated by different endogenous and exogenous molecules. Activation via any of the three pathways leads to C3 convertase-catalyzed hydrolysis of C3 to C3a and C3b, promoting further amplification of the complement cascade. More distal components of the complement cascade include C5a, a potent chemotaxis factor, and C5b, which initiates assembly of MAC. Aβ has been shown to directly bind C1q, activating the complement cascade via the classical pathway. In addition,

critical players in the alternative complement pathway, namely C3 and C5, as well as their activated forms and cognate receptors, are implicated in altered plaque deposition in AD mouse models, likely through changes to AB clearance by microglia [23]. Some GWAS also have associated the complement receptor 1 gene (*CR1*) with increased AD risk [1, 2]. While not as strong as *APOE*, the association between *CR1* and AD reached genome-wide significance [2] and has been replicated and proposed to be related to a particular CR1 isoform [24]. CR1 regulates the complement cascade by modulating phagocytosis and activating distal complement components.

In the CNS, microglia appear to be the major source of complement proteins, whose transcription is increased in regions affected by AD [25]. Numerous groups have reported the association of Aβ plaques and neurofibrillary tangles in AD brain specimens with complement proteins in the classical pathway, particularly the MAC [26–29]. In addition to these observational data, functional data on the role of complement in AD pathogenesis comes from biochemical and *in vivo* studies. Interestingly, the classical complement pathway, traditionally activated by antigen-antibody complexes, can also be activated by other molecules, including Aβ [30]. *In vivo* studies with mouse models reveal that the perturbation of specific complement proteins can either promote or suppress the pathogenesis of Aβ deposition. For example, when C3 activation was suppressed, either by genetic ablation (C3−/−) [31] or by expression of soluble complement receptor-related protein y (sCrry; a complement inhibitor) [32], neuronal damage and Aβ accumulation increased. Conversely, when interferon-γ enhanced the activation of the complement system in another transgenic model of AD, Aβ deposition decreased [33]. In contrast to these studies indicating that the activation of some complement proteins may be useful in suppressing some AD processes, a transgenic mouse model of AD lacking C1q showed neuroprotection and fewer activated microglia without modification of Aβ [34]. In a different transgenic model of AD a C5a receptor (CD88) antagonist reduced Aβ accumulation and microglial activation [23], likely by binding to microglial CD88 [35] Future studies will be needed to elucidate whether the net effect of complement is to promote or suppress AD pathogenesis in vivo.

TLRs comprise a family of plasma and nuclear membrane glycoprotein receptors that recognize exogenous structures present in microorganisms and endogenous structures produced from tissue injury or disease [36]. Once activated, these pattern recognition receptors coordinate gene transcription through several pathways, including those involving NF-κB, p38 MAP kinase (K), to initiate innate immune responses. Key effectors of the innate immune response include the inducible form of cyclooxygenase (COX2), tumor necrosis factor α (TNFα), interleukin 6 (IL-6), and inducible nitric oxide synthase (iNOS), among many other protein molecules. Not all of these molecules are activated directly by TLRs but rather follow in waves of derivative activation. For example, secretion of TNFα and IL-6 following TLR3 or TLR4 activation is dependent upon microglial activation of PGE_2 receptor subtype 1 (EP1) [37]. Moreover, while ample data support a role for TNFα as a key effector of microglial-mediated neuron damage, recent work also indicates that increased expression of TNFα in mouse hippocampus suppresses Aβ accumulation in a model of AD [38]; however, it is not clear whether this constitutes neuroprotection in this model.

Microglia express TLR2, TLR3, TLR4, and TLR9 and respond with varying degrees of effectiveness to selective activation of each receptor [37]. Myeloid differentiation factor 88 (MyD88) is a required signal transducing adaptor protein for TLR2, TLR4, and TLR9, but not TLR3. The activation of microglial TLR2 damages the brain in experimental animal models of multiple sclerosis [39, 40], head trauma [41, 42], cerebral ischemia [43], and AD [44, 45]. The activation of microglial TLR4 injures brain tissue in models of AD [45–47]

and head trauma [41] In transgenic models of AD the activation of microglial TLR9, TLR2, or MyD88 suppresses Aβ oligomerization and reduces Aβ accumulation while also improving cognitive performance [34, 48–50]. Direct activation of microglial TLR3 or TLR4 produces paracrine damage to neurons in cell culture and *in vivo* [51–53].

Aggregated Aβ peptides activate microglia in large part through CD14/TLR4-dependent mechanisms [54]. Conversely, microglial CD14 activation modulates Aβ deposition [55]. One group has associated a polymorphism in TLR4 with risk for AD [56]. Moreover, since mRNA is an endogenous ligand for TLR3 [57], nucleic-acid mediated activation of TLR3 may potentially occur in neurodegenerative diseases such as AD, Parkinson's disease, and amyotrophic lateral sclerosis [58], in which mRNA accumulates in pathologic lesions.

Taken together, observations from human autopsy material and GWAS point to a potential role for the innate immune response in AD pathogenesis. Experimental studies from animal models highlight multifaceted mechanisms by which specific components of innate immunity not only act as indirect effectors of neuron damage, but also influence the amount and form of Aβ accumulation. The overall effect of innate immune activation or suppression in AD likely will vary between individuals and brain regions, representing a delicate balance between activation sufficient to suppress Aβ accumulation and activation that unleashes neurotoxicity (Figure 2).

ApoE isoforms in the regulation of innate immunity

While associations between *APOE* and amyotrophic lateral sclerosis, multiple sclerosis, Parkinson's disease, stroke, vascular dementia, and other disorders have been proposed, the strongest evidence to date is between *APOE* and disease risk for AD [7]. ApoE isoforms have been proposed to possess Aβ-dependent and Aβ-independent mechanisms by which they influence the initiation and progression of AD [59]. Immune activation is a prominent feature of AD in autopsy material, in which microglia invade and surround senile plaques. Intense APOE immunoreactivity colocalizes with plaque-associated amyloid and microglia [60], suggesting a role for apoE in regulating the innate immune response in AD. The following sections review data supporting a role for apoE isoforms as key regulators of the innate immune response in three areas: (1) microglial activation, (2) immune-mediated toxicity to neurons, and (3) potential modulators of Aβ peptide deposition and clearance.

1. Microglial Activation

Most investigations of apoE isoform-specific regulation of the innate immune response in brain have focused on microglial activation, since these are the major resident immune effector cells in the CNS. Primary adult rat microglia treated with HDL-like particles were used by Chen and colleagues to investigate apoE-specific differences in inflammatory mediators secreted by microglia [61]. ApoE3- and apoE4-containing HDL-like particles were partially purified by size exclusion chromatography from conditioned medium of HEK cells stably transfected with cDNA encoding either apoE isoform. HEK-apoE4 and human HDL spiked with recombinant apoE4 dramatically induces microglial PGE_2 and IL-1β secretion compared to HEK-apoE3.

Microglia from targeted replacement (TR) APOE mice show similar isoform effects. TR APOE mice derived by homologous recombination were developed by Sullivan and colleagues and contain chimeric genes consisting of mouse 5' regulatory sequence continuous with mouse (noncoding) exon 1 followed by human exons (and introns) 2–4 to produce TR APOE2/2, TR APOE3/3, or TR APOE4/4 mice [62, 63]. Using these mice, Brown, Colton, and colleagues have shown that that activated TR APOE4/4 microglia secrete more NO than TR APOE3/3 microglia [64, 65] and have expanded their studies to

include peritoneal macrophages and PIC, a selective activator of TLR3 [66]. TR APOE4/4 primary microglia display enhanced activation, including increased secretion of TNFα and IL-6, with subsequent induction of NOS2 expression and activity; similar effects were seen in peritoneal macrophages from TR APOE3/3 and TR APOE4/4 mice. They also generated mice that express only one copy of APOE3 (TR APOE3/0) whose macrophages display an intermediate pro-inflammatory status between TR APOE3/3 and TR APOE4/4. In combination, these data confirm the pro-inflammatory nature of TR APOE4/4 microglia/macrophages compared to TR APOE3/3, suggesting inherent differences in the activity of apoE3 and apoE4 following activation of these cells by TLR3 or TLR4 ligands. They further suggest that gene dosage may be critical in the regulation of these components of the innate immune response.

This previous study [66] also included *in vivo* experiments using peripheral LSP injection to activate systemic innate immunity and then examined responses in mouse cerebral cortex. Using TR APOE3/3 and TR APOE4/4 mice, they observed significantly greater TNFα and IL-12 transcription of genes for TNF in APOE4/4 mice than APOE3/3 by quantitative PCR. While this important experiment demonstrates the relevance of *APOE* alleles in systemic immune activation, interpretation of the results is limited by the complexities of transduction of peripheral inflammation to central inflammation [69]. The reduced immune response observed in cerebral cortex from TR APOE3/3 mice might be due to reduced peripheral immune activation, reduced efficiency of signal transduction from periphery to CNS, or the inherent differences in microglial activation demonstrated in cell culture experiments.

In another *in vivo* study, Ophir and colleagues used C57Bl/6 transgenic (tg) mice that lacked *apoE* but expressed a single copy of *APOE3* or *APOE4* [67] to investigate whether regulation of brain inflammation is affected by *APOE* [68]. Immune activation was achieved by intracerebroventricular (ICV) injection of LPS, and gliosis was assessed by immunohistochemistry followed by morphometry [68]. Maximal hippocampal gliosis is observed 3 days after LPS injection in 3-month-old mice, with similar results in 6-month-old mice but much less activation in 12-month-old mice. LPS activation leads to increased hippocampal apoE production that is not isoform-specific. Interestingly, the authors observed that astrogliosis in younger mice is apoE-dependent and greater in apoE3 tg mice than apoE4 tg mice. In contrast, microglial activation is apoE-independent.

2. Innate Immune-Mediated Toxicity To Neurons

While some components of the innate immune response are neurotrophic or protective, intense or protracted activation of the innate immune response leads to elaboration of neurotoxic species. The exact composition of the neurotoxic effectors is not clear; thus, understanding apoE effects on the innate immune response includes determining whether differences in microglial activation manifest as differences in neurotoxicity. Our group tested the hypothesis that apoE isoform modulation of glial innate immune response may differentially alter bystander or paracrine damage to neurons [70]. Using mixed primary co-cultures of cerebral microglia from the TR mice mentioned above [62, 63] and cerebral cortical neurons from either wild type (wt) or apoE−/− mice, we observed that paracrine damage to neurons from microglia is greatest with TR APOE4/4, intermediate with TR APOE3/3, and least with TR APOE2/2 microglia [70]. Importantly, there is no difference among TR APOE cultures in expression of TLR4 or binding of LPS by apoE isoforms. We confirmed the results mentioned above [64, 65] that activated TR APOE4/4 microglia secrete more NO than TR APOE3/3 microglia and extended them to show that activated TR APOE2/2 are no different from wt [70]. However, in our study we also determined that detectable increase in medium NO occurs *after* paracrine neurotoxicity, thereby questioning the relevance of NO as neurotoxic effector in this model. Similarly, when microglial secretion of 10 cytokines and chemokines is screened, only TNFα and IL-6 are significantly

elevated coincident with neurotoxicity. Both show TR APOE4/4 > TR APOE3/3 > TR APOE2/2. A P38-MAPK inhibitor ablates TR APOE-dependent microglial TNFα and IL-6 secretion and protects neurons. Using primary cultures of TNFα astrocytes for comparison, we also observed TR APOE-dependent differences in cytokine secretion; however, these are NF-κB- and not p38-MAPK-dependent [71].

While primary cultures of enriched cell types, including cocultures, have the advantage of being a relatively pure population of cells, they also have several disadvantages with respect to understanding CNS physiology, since interactions and direct connections among cells is lost. This can be addressed not only *in vivo* but also in organotypic cultures that retain physiologically relevant proportions of cells and their local contacts. The studies by us described in the previous paragraph were repeated using organotypic cultures of hippocampus from TR APOE mice activated with the same preparation of LPS [70]. The same rank order for hippocampal pyramidal neuron damage and TNFα and IL-6 secretion is observed, with TR APOE4/4 > TR APOE3/3 > TR APOE2/2. Importantly, pharmacologic inhibition of p38MAPK suppresses these TR APOE-dependent differences and protects neurons from the bystander damage caused by microglial activation in organotypic cultures.

These studies indicate that some important elements in this neurotoxic response are apoE isoform-dependent: p38MAPK, PGE_2, NO, TNFα, and IL-6. Activated microglia-mediated neurotoxicity in multiple models is greatest with TR APOE4, followed by TR APOE3, and least with TR APOE2, and apparently is related to expression of apoE isoforms, since in at least some models the difference among TR APOE cells can be blocked with low density lipoprotein receptor-related protein associated protein 1 (RAP).

Interestingly, while LPS is commonly used to activate specifically the innate immune response, the source and purity of the LPS preparation used can be critical to its specificity of activation [72]. In culture, several groups have shown that high quality LPS preparations have no demonstrable effect on neurons because these cells lack functional CD14 and TLR4. In an *in vivo* study using direct activation of CD14/TLR4 co-receptors in cerebrum by ICV injection of LPS into TR APOE mice, we demonstrated specificity of TLR4 activation by observing no effect of ICV LPS in mice genetically lacking CD14 or TLR4 but full effect in mice lacking TLR2 [73]. Damage to hippocampal pyramidal neurons was assessed following activation of microglial CD14/TLR4 by measuring changes in dendrite length using Golgi staining. We and others have found that TR APOE4/4 mice have slightly but significantly shorter dendrites at baseline (6 weeks of age), a result confirmed by others [74]. Following exposure to ICV LPS, there is comparable loss of dendrite length at 24 hr among the three TR APOE mice.

Recovery of dendrite length over the next 48 hr is greater in TR APOE2/2 than TR APOE3/3 mice, while TR APOE4/4 mice show failure of dendrite regeneration. Accompanying cell culture experiments suggested that the enhanced neurotrophic effect observed in TR APOE2/2 is RAP-dependent [73].

Overall, the *in vivo* studies largely validate the cell culture studies and indicate that apoE isoform-specific modulation of microglial innate immunity and the resulting damage to neurons is modulated by expression of different apoE isoforms.

3. Aβ Peptide Modulation

Sequential proteolysis of the amyloid precursor protein to generate Aβ peptides is promiscuous at the C terminus and generates a family of peptides that vary in the number of amino acids; the most abundant contain 40 or 42 amino acids. One group investigated the modulatory role of apoE isoforms in $A\beta_{40}$-mediated complement activation [75]. $A\beta_{40}$ of

unclear aggregation state adhered to the plastic of a microwell followed by addition of human serum displays robust activation of C3. ApoE3, apoE4, or BSA substituted for $A\beta_{40}$ results in no C3 activation. When $A\beta_{40}$ is followed by apoE isoforms and a polyclonal apoE antibody to detect binding, apoE2 and apoE4 appear to bind more rapidly and perhaps more avidly than apoE3. While apoE4 bound to $A\beta_{40}$ increases Aβ-mediated complement activation by about 25%, there was no significant effect of apoE2 or apoE3 in this *in vitro* assay.

Microglia possess many actions, one of which is activation to a neurotoxic phenotype, as reviewed above. Microglia also are the major phagocytic cell in brain. We explored *in vitro* possible mechanisms to explain apparent decreased clearance of Aβ peptides in animal models that express apoE4 [7]. Using a phagocytosis assay in which we demonstrated that the PGE_2 receptor subtype 2 (EP2) suppressed mouse microglial phagocytosis of Aβ peptides from AD brain sections [76], we attempted to determine if microglia from TR APOE mice might also show differential phagocytosis. We found there was no difference in microglial phagocytosis when comparing TR APOE4, TR APOE3, and TR APOE2 mice (unpublished data). Microglia are migratory cells and part of their overall effectiveness resides in their ability to move towards lesions or areas of damage. Indeed, microglia migrate toward Aβ deposits in brain in mice and apparently do so in humans with AD. Recently we demonstrated that microglial migration *in vitro* is highly TR APOE-dependent. Using standard activators of microglial migration, C5a and ATP, we showed that rank order for microglial migration *in vitro* was TR APOE3/3 > TR APOE4/4 ≈ TR APOE2/2 for both activators [77]. Interestingly, the mechanisms for TR APOE-dependent differences in microglia migration differed for complement-mediated activation vs. ATP with the former related to RAP-dependent processes and the latter to alteration in second messenger signaling. While these results do not follow the order of genetic risk for AD they do mirror the results for complement activation. Moreover, these results clearly demonstrate apoE isoform-specific effects on microglial migration, which in turn might influence the processes that underlie Aβ clearance.

Conclusion

Accumulation of Aβ peptides in cerebrum appears to be a central event in the initiation or progression of AD. Aβ peptides are pleiotropic neurotoxins that can directly damage neurons; they also can activate microglia to adopt a neurotoxic phenotype, and thus indirectly damage neurons.

ApoE also is a pleiotropic molecule with multiple actions, at least some of which vary among its three common isoforms. Several laboratories have now reproducibly shown that microglial activation to a neurotoxic phenotype is apoE isoform-dependent and greatest with apoE4 expressing cells, less with apoE3, and least with apoE2. Indeed, this apoE isoform-dependent microglial activation translates into apoE isoform-dependent (microglial-mediated) damage to neurons in primary culture, organotypic culture, and *in vivo*. Thus, under conditions when the innate immune response has become neurotoxic, greatest neuron damage is observed in those models expressing apoE4, less in those expressing apoE3, and least in those expressing apoE2.

As reviewed earlier, expression of different *APOE* alleles influences Aβ peptide accumulation in brain. One mechanism underlying this effect may be direct apoE isoform-specific binding to and subsequent apoE receptor-mediated events. We have reviewed data to support another mechanism: apoE isoform-specific modulation of components of the innate immune response that modulate the amount and quality of Aβ deposition. We return to the fact that genetic associations define biological relevance but not mechanism of action.

Perhaps the outstandingly strong association of *APOE* alleles with risk of AD derives from multiple apoE isoform-specific mechanisms that include both direct binding of Aβ and modulation of the innate immune response.

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Highlights

- Largest genetic risk for late-onset Alzheimer's disease resides at the apoE gene
- Innate immune response is consistently observed in AD and a likely contributor
- apoE isoforms regulate multiple facets of innate immune response in brain
- apoE may alter AD through both Aβ-dependent and independent mechanisms

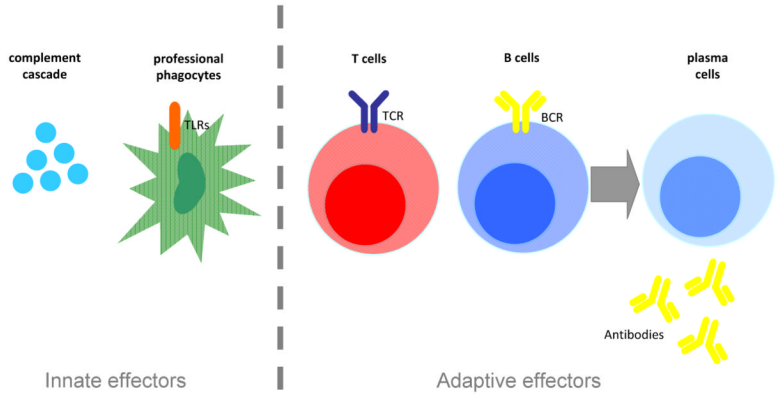

Figure 1. Innate and Adaptive Immunity

Innate immune system effectors include components of the complement cascade (C1q, C3(a), C5(a)) as well as professional phagocytes (macrophages, tissue-specific dendritic cells). The adaptive arm of the immune system includes T cells, B cells, and the antibody-secreting plasma cells. Functions of innate immune system effectors are not burdened by genetic restriction as seen in adaptive responses.

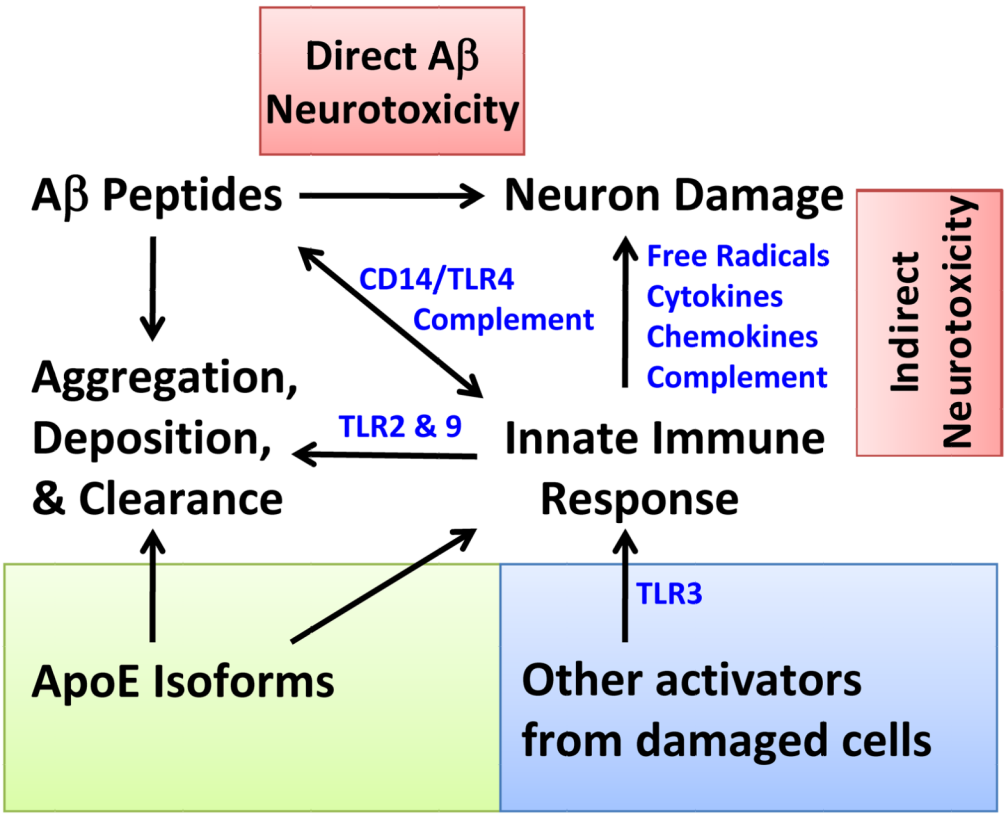

Figure 2. Interaction between Aβ, Innate Immune Response, and *APOE*

Schematic of complex interaction between Aβ and the innate immune response and subsequent effect on Aβ aggregation, deposition and clearance. Other activators contribute as well, resulting both direct and indirect effects on neurons. There is evidence that apoE isoforms differentially regulate these processes, and thus the extent of neuron damage and Aβ burden.

Table 1
Common isoforms of human apolipoprotein E

Common allelic variants of the apolipoprotein E gene (*APOE*), called ε2, ε3, and ε4, encode for three different 299 amino acid proteins called apoE2, apoE3, and apoE4 that differ only at amino acid positions 112 and 158. For comparison, corresponding amino acids for the sequence of most other mammals and for apes are included. Most other mammals, including apes, have threonine (T) at position 61 and arginine (R) at positions 112 and 158; the exceptions are rabbit (Rb) and bovine (Bo) apoE that have cysteine (C) at position 112. Humans differ from other mammals in having R at position 61. Human apoE4 has R at positions 112 and 158, making it the "simian" form of human apoE. ApoE3 differs from apoE4 by a single amino acid substitution: C for R at position 112 that derives from the transition of CGC to TGC in the codon for this amino acid. Human apoE2 differs from apoE3 by the same transition in the codon for amino acid 158. Thus, human apoE apparently is evolving from the "simian" apoE4 to apoE2 with progressive substitutions of C for R at positions 112 and 158.

apoE	aa61	aa112	aa158
Other Mammals	T	R (C in Rb & Bo)	R
Apes	T	R	R
Hu ε4	R	CGC R	R
Hu ε3	R	TGC C	CGC R
Hu ε2	R	C	TGC C