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Amyloid beta acts synergistically as a pro-inflammatory cytokine

Thomas J. LaRocca[a,b,*], **Alyssa N. Cavalier**[a,b], **Christine M. Roberts**[a], **Maddie R. Lemieux**[a], **Pooja Ramesh**[a], **Micklaus A. Garcia**[a], **Christopher D. Link**[a,**]

[a]Department of Integrative Physiology, University of Colorado Boulder, Boulder, CO, United States of America

[b]Department of Health and Exercise Science, Center for Healthy Aging, Colorado State University (Current), Fort Collins, CO, United States of America

Abstract

The amyloid beta (Aβ) peptide is believed to play a central role in Alzheimer's disease (AD), the most common age-related neurodegenerative disorder. However, the natural, evolutionarily selected functions of Aβ are incompletely understood. Here, we report that nanomolar concentrations of Aβ act synergistically with known cytokines to promote pro-inflammatory activation in primary human astrocytes (a cell type increasingly implicated in brain aging and AD). Using transcriptomics (RNA-seq), we show that Aβ can directly substitute for the complement component C1q in a cytokine cocktail previously shown to induce astrocyte immune activation. Furthermore, we show that astrocytes synergistically activated by Aβ have a transcriptional signature similar to neurotoxic "A1" astrocytes known to accumulate with age and in AD. Interestingly, we find that this biological action of Aβ at low concentrations is distinct from the transcriptome changes induced by the high/supra-physiological doses of Aβ often used in in vitro studies. Collectively, our results suggest an important, cytokine-like function for Aβ and a novel mechanism by which it may directly contribute to the neuroinflammation associated with brain aging and AD.

*Correspondence to: T. LaRocca, Department of Health and Exercise Science, Center for Healthy Aging, Colorado State University, 1582 Campus Delivery, Fort Collins, CO 80523-1582, United States of America., tom.larocca@colostate.edu (T.J. LaRocca).
**Correspondence to: C. Link, Department of Integrative Physiology, Institute for Behavioral Genetics, University of Colorado Boulder, 354 UCB, Boulder, CO 80309, United States of America., linkc@colorado.edu (C.D. Link).

Authors' contributions

TJL and CDL designed the study and wrote the manuscript; TJL, ANC, CMR, MRL, PR, MAG and CDL generated and analyzed data; TJL, ANC, CMR, MRL and CDL provided conceptual insight and edited the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All RNA-seq data has been deposited in the Gene Expression Omnibus (accession GSE157461).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2021.105493.

Keywords

Amyloid beta; Alzheimer's disease; Astrocytes; Inflammation; Transcriptomics

1. Introduction

The amyloid beta (Aβ) peptide is centrally involved in Alzheimer's disease (AD), the most common age-related neurodegenerative disorder (Xia et al., 2018). However, the exact biological function and role of Aβ in the development of AD remain poorly understood. In fact, although aggregated Aβ is a well-known feature of AD pathology (i.e., in senile plaques), the peptide itself has been reported to act as a regulator of synaptic function, an antimicrobial peptide, a tumor suppressor, and a modulator of blood-brain barrier permeability (van der Kant and Goldstein, 2015; Brothers et al., 2018; Reinhard et al., 2005). Among these and other possible biological roles for Aβ, one compelling (and potentially unifying) idea is that Aβ may be an immune modulator. Indeed, evidence suggests that Aβ may activate pro-inflammatory signaling pathways in multiple CNS cell types (Kinney et al., 2018), and this observation is consistent with the fact that neuroinflammation is a central feature of brain aging and AD (Xia et al., 2018; Heneka et al., 2015a).

Neuroinflammation is characterized by increased production of pro-inflammatory cytokines in the CNS, especially by microglia and astroglia (Heneka et al., 2014; Ransohoff, 2016). It occurs in response to many stimuli (e.g., pathogens, injury, etc.) and plays an important role in protecting neurons and the brain. Growing evidence demonstrates that *chronic* neuroinflammation develops with aging (Barrientos et al., 2015; Di Benedetto et al., 2017), and the neurotoxic cytokine milieu associated with this persistent neuroinflammation contributes to the development of AD (Heneka et al., 2015a; Calsolaro and Edison, 2016). Innate immune activation in glial cells appears to be particularly important in this context (Heneka, 2017; Heneka et al., 2015b), and recent data demonstrate a specific accumulation of pro-inflammatory "disease-associated astrocytes" in both brain aging and AD (Habib et al., 2020). Still, the possible mechanisms underlying astroglial activation in brain aging/AD are unclear.

One potentially simple explanation for glial cell innate immune activation in brain aging/AD could be a direct, cytokine-like effect of Aβ, which is thought to be produced largely by neurons (Vassar et al., 1999; LeBlanc et al., 1997). This idea has been suggested before (Bales et al., 2000), and there have been some reports of immune activation in response to Aβ (Barrientos et al., 2015; Speciale et al., 2003; Gitter et al., 1995; Lotz et al., 2005). The general concept is also consistent with: 1) the fact that Aβ shares structural similarities (e.g., small amphipathic structure, expression in multiple tissues) with anti-microbial peptides like cathelicidin, which has been reported to stimulate pro-inflammatory cytokine secretion in glial cells (Lee et al., 2015); 2) numerous studies showing that Aβ can induce pores in cell membranes (Stewart and Radford, 2017), which is a common characteristic of immune-activating anti-microbial peptides; 3) reports that LPS (a classic innate immune stimulus) increases Aβ in the brain (Zhu et al., 2014); and 4) growing evidence that, similar

to pro-inflammatory cytokines, Aβ levels increase with aging in the human brain (Denver and McClean, 2018; Rodrigue et al., 2009). Collectively, these observations suggest that the Aβ peptide itself could have important, pro-inflammatory signaling effects in the CNS that are highly relevant to brain aging/AD. However, while aggregated Aβ can clearly induce microglial immune activation (Hemonnot et al., 2019), the effects of soluble Aβ on human glial cells have not been extensively investigated. Here we used transcriptome analyses to investigate the global effects of soluble Aβ on primary human astrocytes. We observed that exposing these cells to 1 μM $A\beta_{1-42}$ (the most neurotoxic form of the peptide) caused transcriptional changes indicative of immune activation. However, this response was absent with more physiological levels of Aβ (10 nM), leaving open the question of whether Aβ can truly act as a pro-inflammatory cytokine in the early stages of brain aging/AD (i.e., before higher concentrations/aggregates of the peptide develop).

Interestingly, others have shown that a cocktail of microglial-produced cytokines (tumor necrosis factor α [TNF-α], Interleukin 1α [IL-1α], and complement protein 1q [C1q]) can convert mouse astrocytes into an immune activated, neurotoxic "A1" state (Liddelow et al., 2017). The same group also demonstrated that these "A1" immune-activated astrocytes (marked by expression of complement protein C3) increase with aging in mice (Clarke et al., 2018) and are abundant in AD patient brains (Liddelow et al., 2017), suggesting that reactive astrocytes could directly contribute to age-related AD. These observations highlight the point that pro-inflammatory cytokines act in concert in vivo (Gouwy et al., 2005), which could suggest that the pro-inflammatory activity of Aβ might require other, synergistic cytokines. Only one previous study has investigated this idea, but the authors used immortalized astrocytes and high/non-physiological concentrations of Aβ (Rossi and Bianchini, 1996). Here, we show that primary human astrocytes can be converted to a reactive, A1-like state using either the originally reported TNF-α + IL-1α + C1q cocktail, or substituted cocktails in which 10 nM Aβ replaces C1q or IL-1α. Our findings suggest that immune activation may be a natural biological function of Aβ that contributes to coordinated pro-inflammatory responses in the CNS, and this could have important implications for our understanding of early mechanisms in age/AD-related neuroinflammation.

2. Results

2.1. Immunomodulatory effects of Aβ on human astrocytes

In initial studies, we treated primary human astrocytes with 1 μM $A\beta_{1-42}$ (a high but commonly used dose) and performed RNA-seq to profile changes in gene expression. We found that this high dose of Aβ differentially affected transcript levels of numerous genes. In fact, Aβ significantly increased the expression of >2600 genes and reduced >2700 genes/transcripts vs. controls (Fig. 1A, next page). Consistent with previous reports that Aβ may be pro-inflammatory, gene ontology analyses indicated that these Aβ-induced gene expression changes included increased levels of inflammatory genes/transcripts, marked by enrichment for gene ontology terms related to immune/defense responses, cytokine signaling and interferon responses (Fig. 1B). A coherent biological response was not as obvious in the downregulated transcriptional modules, although several gene ontology terms were associated with ion transport and neurotransmitter/synapse modulation. These findings are

in line with studies that have documented the production of pro-inflammatory cytokines and reactive oxygen species (immune defense mediators) by astrocytes in response to high dose Aβ (White et al., 2005; Johnstone et al., 1999; Smits et al., 2002), and with more recent reports of immune/inflammatory gene expression in astrocytes isolated from AD patient brains (Sekar et al., 2015). However, when we replicated these studies using a more physiological level of $A\beta_{1-42}$ (10 nM), we observed no transcriptome/inflammatory gene expression changes (Fig. 1C).

2.2. Evidence for cytokine-like effects of Aβ

We considered the possibility that 10 nM $A\beta_{1-42}$ failed to elicit immune activation in astrocytes because, in vivo, Aβ might act in concert with other pro-inflammatory cytokines. Building on work by Liddelow et al. (Liddelow et al., 2017), who demonstrated that a cocktail of three microglial-produced cytokines (TNF-α, IL-1α and C1q) was required for strong immune activation in murine astrocytes, we tested the idea that more physiological levels of Aβ might act synergistically with pro-inflammatory cytokines linked with aging/AD and astrocyte activation (Clarke et al., 2018). First, we tested permutations of the TNF-α + IL-1α + C1q cocktail (Liddelow et al., 2017) in which we substituted Aβ for individual cytokines (all in serum-free media). Using immunofluorescence staining for CD44, a cellular adhesion molecule that tracks with astrocyte activation (Liddelow et al., 2017; LaRocca et al., 2019; Girgrah et al., 1991), we found that: 1) the cytokine-only TNF-α + IL-1α + C1q cocktail activates human astrocytes; and 2) we could substitute Aβ for C1q or IL-1α and achieve similar astrocyte activation (Fig. 2A, next page). We then repeated these experiments and immunoblotted for additional proteins, and we found that the synergistic effect of Aβ in this cytokine cocktail was greatest when substituting it for C1q (i.e., TNF-α + IL-1α + Aβ), as indicated by greater expression of glial fibrillary acidic protein (GFAP, a common marker of reactive astrocytes) and IL-1β (a pro-inflammatory cytokine released by immune-activated glial cells) (Heneka et al., 2015b) (Fig. 2B). In these exploratory trials, we used both Aβ and oligomerized Aβ, which may be more toxic (Cline et al., 2018), but that we had not tested at first. We found greater activation with non-oligomerized peptide and therefore used this in subsequent, quantitative studies, as follows: Using IL-1β and intercellular adhesion molecule 1 (ICAM-1) as indicators of AD-relevant immune activation (Akiyama et al., 1993; Lee and Benveniste, 1999; Frohman et al., 1991), we found in a dose/response experiment that low concentration $A\beta_{1-42}$ (but not as low as 1 nM) could fully substitute for C1q in activating human astrocytes (Fig. 2C). In fact, we found that 10 nM Aβ combined with similar concentrations of TNF-α and IL-1α markedly increased levels IL-1β and ICAM-1, neither of which increased in response to high-dose 1 μM Aβ (i. e., in Fig. 1). In follow-up experiments, we also found that cytokine cocktails with 10 nM $A\beta_{1-40}$ (the less neurotoxic and aggregate-prone isoform) caused similar immune activation (Supplementary Fig. 1), suggesting that while Aβ can have effects at high concentrations, the peptide itself may also have a cytokine-like function at lower, physiologically relevant doses, independent of its tendency to aggregate.

2.3. Aβ/cytokine-induced transcriptome changes

To extend our analysis of human astrocyte activation beyond the limited set of markers employed in Fig. 2, we repeated our experiments using RNA-seq to capture the global

effects of Aβ/cytokine exposure. In these studies, we also used astrocytes from three different donors to identify the most conserved effects of the treatment. As we previously observed in replicate cultures from a single donor (Fig. 1C), we found that 10 nM $A\beta_{1-42}$ alone had minimal effects on transcript accumulation (Fig. 3A, next page). Similarly, and in agreement with previous reports (Liddelow et al., 2017), TNF-α combined only with IL-1α also caused minimal transcriptome changes (Fig. 3B). However, the addition of either C1q or Aβ to this cocktail resulted in a major biological response, reflected by significantly increased/decreased expression of ~2500 genes (Fig. 3C,D). Gene expression patterns induced by the cytokine-only cocktail (TNF-α + IL-1α + C1q) and the Aβ/cytokine cocktail (TNF-α + IL-1α + Aβ) were strikingly similar, as reflected by no major gene-by-gene differences (Fig. 3E) and strong inter-treatment correlation (Fig. 3F), again suggesting that Aβ could have cytokine-like effects. Moreover, as expected from the highly similar transcriptome patterns induced by these two treatments, gene ontology analysis revealed very similar gene set enrichments (Fig. 3G). In particular, both treatments upregulated transcriptional modules related to immune system activation, cytokine/interferon signaling and inflammatory responses, whereas common downregulated transcriptional modules included gene sets modulating neurogenesis, synaptic structure, and other cellular health/development pathways. Interestingly, gene expression changes induced by the Aβ/cytokine cocktail only correlated weakly with those induced by high dose (1 μM) Aβ alone (Fig. 3H). Many of the genes most affected by the Aβ/cytokine cocktail but not by Aβ alone were mediators of innate immune responses (e.g., C3 and IL-1β) and/or established markers of reactive astrocytes (e.g., lipocalin-2, LCN2) (Liddelow et al., 2017; Bi et al., 2013), further suggesting that physiological Aβ may have a distinct role in immune activation.

2.4. Aβ/cytokine-activated astrocytes are similar to disease-associated astrocytes

To determine specifically which biological pathways may be activated when Aβ synergizes with cytokines, we examined the genes that were differentially expressed in response to the Aβ/cytokine cocktail vs. TNF-α and IL-1α alone (Fig. 4A). While this approach yielded fewer significant hits, gene ontology analysis of these transcripts pointed clearly to immune activation and cytokine signaling (Fig. 4B).

We next asked if these Aβ/cytokine-activated human astrocytes could be equivalent to the reactive, neurotoxic “A1” astrocytes induced by cytokine treatment of murine astrocytes. To test this possibility, we performed RNA-seq on Aβ/cytokine-treated astrocytes from one biological donor in triplicate (as in Fig. 1), in order to reduce variability and increase resolution of key biological pathways. We found that these cells responded to Aβ/cytokine treatment with transcriptomic changes similar to those observed in our three-donor experiments (Supplementary Fig. 2), and that gene expression differences in single vs. three-donor experiments were highly correlated ($R^2 = 0.84$, $p < 0.000001$), supporting this approach. We then confirmed changes in key genes that increased/decreased in both these and our three-donor data by both immunoblotting and RT-PCR, with a particular focus on mediators of innate immune activation and established markers of reactive astrocytes. We confirmed increased protein levels for two key reactive astrocyte markers (C3 and ICAM-1) in response to Aβ/cytokine treatment and the cytokine-only cocktail (Fig. 4C). We also confirmed that Aβ/cytokine treatment caused significant increases in mRNA for:

IL1B (IL-1β) and *C3* (innate immune response mediators); nuclear factor κ B (NFκB, a major pro-inflammatory transcription factor); and *ICAM-1*, serpin G1 (*SERPING1*) and *LCN2*, all markers of reactive astrocytes (Liddelow et al., 2017; Bi et al., 2013) (Fig. 4D). Reduced transcripts confirmed by RT-PCR included excitatory amino acid transporters 1 and 2 (*SLC1A2*/*GLUT1* and *SLC1A3*/*GLUT2*), in line with our gene ontology analyses showing altered expression of synaptic function modulators. Interestingly, we noted that 1 μM Aβ did not affect the expression of IL-1β and C3 (Fig. 4C), which is consistent with our initial RNA-seq data on this treatment and further supports the idea of distinct, cytokine-like effects for the Aβ peptide itself (i.e., at lower doses less likely to involve toxic aggregates).

Finally, having confirmed that human astrocytes treated with the Aβ/cytokine cocktail expressed classic markers of astrocyte activation, we analyzed our RNA-seq data to determine if the treated astrocytes resembled "A1" reactive astrocytes that are neurotoxic, or "A2" activated astrocytes that are reportedly protective (Liddelow et al., 2017). Both types of astrocyte are marked by activation of specific genes/proteins, as well as a suite of common response factors ("pan-reactive" astrocyte markers). As observed in previous mouse studies, we found that astrocytes treated with TNF-α + IL-1α + C1q increased expression of the full suite of A1 astrocyte markers. Moreover, in support of the idea that Aβ may contribute to astrocyte toxicity, we also found that Aβ/cytokine-treated astrocytes increased expression of this same set of markers (Fig. 4E).

3. Discussion

Our studies extend on previous data (Liddelow et al., 2017; Clarke et al., 2018) by showing that a synergistic TNF-α + IL-1α + C1q cytokine cocktail induces immune activation in human astrocytes, and by demonstrating that Aβ may have an important, previously unrecognized role in neuroinflammatory signaling. This finding is highlighted by our observation that Aβ, at plausible physiological levels, can substitute for C1q in the activation of human astrocytes.

The idea that a potential "natural" function of Aβ contributes to AD pathology is new but gaining traction. Indeed, many earlier studies have focused on the pro-inflammatory, cellular effects of Aβ at high micro-molar (aggregation-prone) concentrations. Here, we extend on those data with a whole-transcriptome (RNA-seq) analysis of biological pathways that may be involved in the response to high-dose Aβ. For example, we note that major transcriptional modules upregulated by Aβ in our data include numerous genes related to interferon response factors (IRFs), TNF proteins and receptors, and NF-κB signaling—many of which have been linked with Aβ before, but not in the same dataset. These and others' data on the effects of high concentration Aβ may be relevant in the context of late-stage AD (i.e., when Aβ aggregates). However, many groups are beginning to reconsider the role of Aβ in AD through the lens of its potential/reported physiological functions (Brothers et al., 2018), and the number of studies investigating the effects of Aβ at physiologically relevant concentrations is growing. These more recent investigations have shown that nano- and even picomolar concentrations of Aβ can modulate neuronal signaling and microglial activity (Lazarevic et al., 2017; Maezawa et al., 2011). Our data are consistent with these recent reports, but they also provide new evidence for distinct biological effects of low

concentration Aβ on astrocytes, which are increasingly implicated in brain aging and neurodegenerative diseases like AD (Habib et al., 2020; Clarke et al., 2018; Garwood et al., 2017). In fact, taken together with recent evidence that innate immune activation may also drive Aβ production (Hur et al., 2020), our data could suggest a feed-forward model in which Aβ exacerbates immune activation in astrocytes and immune-activated astrocytes produce more Aβ. Our studies also raise a number of important questions:

First, to what extent do these cell culture experiments reflect in vivo cell physiology? Estimates of soluble Aβ peptide concentrations in the brain/cerebrospinal fluid are typically in the picomolar range (Seubert et al., 1992; Roher et al., 2009). However, the existence of Aβ aggregates in senile plaques, coupled with biochemical studies demonstrating that concentrations of ~100 nM are required for Aβ aggregation (Novo et al., 2018), suggest that local Aβ concentrations significantly exceed the average brain Aβ concentration (Raskatov, 2019). We note that we have used ~10 nM levels of both cytokines and Aβ in our studies, and thus the synergistic pro-inflammatory effects of these peptides in cultured astrocytes are occurring at equivalent concentrations. We administered these treatments for 24 h, as this timepoint caused the greatest activation in preliminary studies, similar to what others have observed, but future studies could explore even lower concentrations and longer duration treatments that might further mimic in vivo physiology. The primary human astrocytes used in our study were from fetal sources, raising the possible concern that they may not fully reflect adult brain astrocytes. However, these primary cells are arguably: 1) more reflective of in vivo physiology than any related cell lines; and 2) a better model than aged and/or IPSC-derived astrocytes for investigating the *basic* biological effects of Aβ/cytokines, as their fundamental biological signaling pathways may be more intact than cells subjected to aging/reprogramming/other changes. Indeed, fetal CNS cells are typically used in cell culture experiments (e.g., rat embryonic hippocampal cultures) for these and other reasons. We cannot exclude the possibility that adult astrocytes would respond differently, but we are unaware of a specific mechanism that would produce this result. We also note that a recent report demonstrates that hIPSC-derived astrocytes from adult donors can be converted to neurotoxic, A1 astrocytes with the same TNF-α + IL-1α + C1q cocktail used in our studies (Barbar et al., 2020), and all of the gene/transcript changes we confirmed in our data (Fig. 4) follow the same pattern in RNA-seq data from that study. Interestingly, in both our data and this recent publication, some A1 astrocyte genes/transcripts reported in mice (e.g., *GFAP*) are not increased with cytokine treatment; this may be an important area for future study (i.e., determining specific differences between murine and human reactive astrocytes). Indeed, recent evidence suggests that AD-associated glial gene expression patterns may be markedly different in humans vs. mice (Zhou et al., 2020) (although we note that all A1 astrocyte genes identified in mice that also have human orthologs were increased in cytokine-treated cells in our data).

Next, what species of Aβ are responsible for its pro-inflammatory effects? Our studies have employed nominally monomeric $A\beta_{1-42}$, freshly prepared from dried HFIP-solubilized synthetic peptide. As we cannot control multimerization of the peptide during its 24 h incubation with cultured astrocytes, we cannot directly infer that the Aβ monomer is the pro-inflammatory species. We do note that treatment with intentionally oligomerized Aβ using the media pre-incubation protocol originally described by Klein and colleagues

(Lambert et al., 2001) caused similar levels of immune marker induction (Fig. 2B), as did treatment with cytokines plus $A\beta_{1-40}$ (the less neurotoxic and aggregation-prone isoform of the peptide). Taken together, these observations suggest a cytokine-like function for the peptide that may be independent of its oligomerization activity at higher concentrations. However, a more comprehensive characterization of the specific Aβ species that lead to immune activation will likely require additional studies that employ: 1) other versions of the Aβ peptide believed to have reduced (e.g., Aβ G37L) or increased (e.g., Aβ E22 delta) oligomerization capacity (Hung et al., 2008; Chen et al., 2017; Melchor et al., 2000); and 2) additional markers of astrocyte activation (e.g., beyond ICAM-1, IL-1β and others used in our study), which could provide insight on more nuanced, Aβ isoform-specific effects. Given the very low (nM) concentrations we have used here, new/advanced methods for detecting Aβ monomers/oligomers may be needed in such studies, as standard approaches (e.g., immunoblotting) would be unfeasible.

How could Aβ (or specific Aβ species) act in concert with cytokines to synergistically promote astrocyte activation? This is the salient question arising from our studies. Conceptually, Aβ could be acting through two different (but not mutually exclusive) types of mechanism: 1) direct interaction via an Aβ-specific receptor, activating an intracellular signaling cascade that converges with pathways stimulated by TNF-α, IL-1-α and C1q; or 2) by enhancing signaling downstream of these cytokines, perhaps by sensitizing the relevant receptors. There is a complex and sometimes contradictory literature on possible Aβ receptors (Jarosz-Griffiths et al., 2016; Mroczko et al., 2018), and few of the associated studies have used astrocytes. Receptors and intracellular signaling pathways for the microglial cytokines we have used are much better understood, although their complexity confounds the ready identification of an obvious target for intervention. We did note slight differences in astrocyte activation markers in cells treated with Aβ/cytokines vs. C1q/cytokines (e.g., somewhat greater GFAP expression in Fig. 2B). However, these effects were small and not significant, and the transcriptomes of cells treated with C1q/cytokines vs. Aβ/cytokines were highly similar (Fig. 3F), suggesting that the observed differences could be related to temporal differences (e.g., in protein processing, although this may warrant further investigation). In fact, we only detected significant differential gene expression with Aβ/cytokines vs. C1q/cytokines when studying cells from one donor (Supplementary Fig. 2 and Data file). These differences were minimal and mapped only to GO terms related to cytokine signaling, and > 95% of the genes/transcripts involved were also increased/decreased with C1q/cytokine treatment vs. TNF-α + IL-1α or controls. Such findings could be more consistent with a model in which Aβ modulates downstream signaling of cytokines, but it may be necessary to conduct a large-scale, unbiased genetic screen (e.g., a full-genome lentivirus-based CRISPR study) to more specifically determine the mechanisms by which Aβ may act as a pro-inflammatory cytokine.

Finally, are the pro-inflammatory effects of Aβ/cytokines on astrocytes relevant to Alzheimer's disease? Recent findings associating late onset AD (LOAD) risk alleles (e.g., in TREM2, CD33, ApoE, etc.) with microglial function (Gratuze et al., 2018; Karch and Goate, 2015) have highlighted the likely role of neuroinflammation in AD. Astrogliosis is a common component of AD pathology, and the detection of putative neurotoxic "A1" astrocytes in aging and AD brains (Liddelow et al., 2017; Clarke et al., 2018) suggests

a mechanism by which neuroinflammation could have a primary role in neuropathology (i.e., immune-activated glial cells causing neuronal death, rather than the other way around). Moreover, a specific population of pro-inflammatory "disease-associated astrocytes" has recently been detected in the context of both brain aging and AD (Habib et al., 2020), and we note that the most significantly upregulated genes in these disease-associated astrocytes (e.g., *GFAP*, *APOE*, *CST3*, etc.) are also significantly upregulated by the Aβ/cytokine cocktail used in our experiments. Thus, our studies suggest a model in which the accumulation of Aβ in AD could have a dual role in inducing neuroinflammation: accumulation of insoluble Aβ in senile plaques can lead to microglial immune activation, while synergy between soluble Aβ and microglial-secreted cytokines promotes the induction of neurotoxic "A1" astrocytes. Interestingly, deletion of the TNF-α receptor (*TNFR1*) has been reported to prevent the memory and neuronal dysfunction induced by injection of Aβ oligomers into mice (Lourenco et al., 2013), which is consistent with the hypothesis that the neurotoxicity of Aβ could in part stem from its synergistic pro-inflammatory interaction with TNF-α.

Collectively, our findings have potentially broader implications in that they could relate to the etiology of age-related (sporadic) AD. While there is a strong consensus that Aβ accumulation plays a central role in the initiation of AD pathology, the causes of this accumulation have not been established. If the Aβ peptide is indeed a natural, evolutionarily selected immune modulator, insults that provoke an innate immune response might be expected to induce increased production of Aβ. Numerous studies have associated bacteria or viruses with AD (Allnutt et al., 2020; Itzhaki, 2019; Sochocka et al., 2017). These studies have largely relied on analysis of postmortem brain samples, and thus cannot demonstrate causality. However, a recent study has shown that low level infection of a human cortical brain model with herpes simplex virus type 1 (HSV-1) results in gliosis accompanied by increased expression of both $A\beta_{1-42}$ and TNF-α (Cairns et al., 2020). It is therefore tempting to speculate that microbial exposure in the brain could play a role in AD pathology via innate immune activation that upregulates Aβ, and that the age-related accumulation of Aβ (which eventually leads to aggregation/plaques) might be a consequence of these events.

4. Materials and methods

4.1. Astrocyte cell culture and treatments

Primary human astrocytes were used for all experiments. Cells from three different donors were purchased from different vendors (Lonza, lot number 0000647218; ScienCell, lot 22156; and Fisher/Gibco, lot 1948466). Vendors confirmed similar procedures for astrocyte isolation based on previously established protocols (Sharif and Prevot, 2012), in which the cerebral cortex was dissected, washed, minced, enzymatically digested to remove connective tissue and dissociated into astrocyte-specific medium in culture flasks and grown for several weeks, followed by isolation of adherent astrocytes by removing floating neuro-spheres (including via agitation), replating and expansion. All cells had typical astrocyte morphology and tested ~90% positive for GFAP (Supplementary Fig. 3). We confirmed GFAP expression and also immuno-stained cells for fibroblast-specific protein 1 (FSP-1), but we observed minimal/no staining for this non-astrocyte marker in any cultures.

Astrocytes were maintained in complete astrocyte growth medium (ScienCell) at 37 °C and 5% CO_2 in a humidified incubator. Cells were subcultured when confluent and grown to ~90% confluency for all experiments. All experiments were performed in serum-free media on passage 2–3 cells; treatments were applied by adding fresh medium containing pre-mixed reagents, and cells were harvested after 24 h incubation as previously described (LaRocca et al., 2019). Lyophilized cytokines (TNF-α, IL-1α, C1q) were obtained from Sigma-Aldrich, resuspended in molecular biology grade water to make stock solutions, aliquoted and frozen for use in experiments (final experimental concentrations: 0.03 ng/μL [TNF-α], 0.30 ng/μL [IL-1α] and 4 ng/μL [C1q]) (Liddelow et al., 2017). Aβ peptides (Anaspec and Abcam) were prepared following standard protocols (Stine et al., 2011) as dried HFIP films, then dissolved in fresh, anhydrous DMSO (Sigma) to make a ~ 5 mM solution, diluted in cold F12 media at target concentrations either for immediate use (monomers) or incubation overnight to produce oligomers when indicated, as previously described (Fonte et al., 2011). All experiments were repeated 3–5 times.

4.2. RNA-seq and gene expression analyses

RNA-seq and gene expression analyses were performed using standard methods as previously described (LaRocca et al., 2019). After the indicated treatments, astrocyte cultures were rinsed with DPBS and lysed in Trizol (Thermo), and RNA was recovered with an RNA-specific spin column kit (Direct-Zol, Zymo Research) including a DNase I treatment to remove genomic DNA. Poly(A)-selected libraries were generated using sera-mag magnetic oligo(dT) beads (Therma Scientific) and Illumina TruSeq kits. All libraries were sequenced on an Illumina NovaSeq 6000 platform to produce >40 M 150-bp paired-end fastq reads per sample. These reads were trimmed and filtered with the fastp program (Chen et al., 2018), then aligned to the hg38 *H. sapiens* genome using the STAR aligner (Dobin et al., 2013). Gene/read counts were generated using HTseq-count (Anders et al., 2015), and differential gene expression was analyzed with Deseq2 (Love et al., 2014). Differentially expressed genes were then analyzed for gene ontology enrichment using both the GOrilla algorithm (Eden et al., 2009) and DAVID (Dennis Jr. et al., 2003), and visualized using ReviGO (Supek et al., 2011). To confirm RNA-seq results by RT-PCR, cDNA was generated from ~1 μg RNA per condition with random hexamers using Super Script IV First-Strand cDNA Synthesis Reaction (Invitrogen) according to manufacturer's instructions, and a linearity curve was generated for all replicates. 5 ng cDNA was then used for PCR reactions (PerfeCTa SYBR Green FastMix Low ROX; Quanta Biosciences), which were quantified on an ABI 7900 instrument and normalized to GAPDH for analysis/presentation. Primer sequences were: IL-1β forward ATGCACCTGTACGATCACTG, reverse ACAAAGGACATGGAGAACACC; C3 forward AGGC CAAAGATCAACTCACC, reverse ATAGTGTTCTTGGCATCCTGAG; ICAM-1 forward CAATGTGCTATTCAAACTGCCC, reverse CAGCGTAG GGTAAGGTTCTTG; LCN2 forward TGAGCACCAACTACAACCAG, reverse AGAGATTTGGAGAAGCGGATG; NFKBIA-1 forward GTCTACACTTAGCCTCTATCCATG, reverse AGGTCAGGATTTTGCAGGTC; SERPING1 forward GTCCTCCTCAATGCTATCTACCTG, reverse GTTTGGTCAATGAAATGGGCCAC; SLC1A2 forward TCTTCCCT

GAAAACCTTGTCC, reverse AGTCACAGTCTCGTTCAACAG; SLC1A3 forward CCATGTGCTTCGGTTTTGTG, reverse AATCAGGAAGAGAAT ACCCACG.

4.3. Immunofluorescence staining

Astrocytes were grown and treated on glass chamber slides (Nunc Lab-Tek) for immunofluorescence staining and imaging as previously described (LaRocca et al., 2019). After 24 h treatments, medium was removed and cells were washed in DPBS. Cells were then fixed in 4% paraformaldehyde, washed and permeabilized in 0.25% Triton-X, washed again and blocked in 3% fetal bovine serum/3% normal goat serum (Jackson ImmunoResearch). Primary antibody (CD44, Abcam; 1:500 dilution) was diluted in 5% normal goat serum and added to slide wells for overnight incubation at 4 °C, after which slides were washed in DPBS with 0.1% tween and incubated with secondary antibody (Alexafluor 555, Invitrogen; 1:1000 dilution in 5% normal goat serum) for 1 h, followed by DAPI (Sigma-Aldrich; 1 μg/mL in DPBS) for 10 min at room temperature. Cells were washed again before mounting with Prolong mounting medium (Invitrogen) and then imaged on a Zeiss Axioskop fluorescence microscope at 40× magnification. Per cell fluorescence was measured in five images per slide well for each treatment and normalized to the mean of all conditions.

4.4. Immunoblotting

Western blotting was performed on whole-cell lysates as previously described (LaRocca et al., 2019). Semi-confluent cultures were lysed in ice cold radio-immunoprecipitation assay lysis buffer with protease and phosphatase inhibitors (Roche), and 10 μg protein was separated by electrophoresis on 4–12% polyacrylamide gels then transferred to nitrocellulose membranes (all reagents from Bio-Rad). Membranes were blocked (5% milk in TBS-Tween buffer) and incubated for 18 h with primary antibodies: CD44 (Abcam, 1:1000 dilution); GFAP (Thermo-Fisher, 1:3000); ICAM-1 (Novus Biologicals, 1:2000); C3 (Novus, 1:2000); IL-1β (Novus, 1:1000). Proteins were then detected with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) and ECL chemiluminescent substrate (Pierce) on a ChemiDoc imager (Bio-Rad), and signal (protein expression) was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling, 1:1000).

4.5. Statistical analyses

All figures were prepared with GraphPad Prism software, which was also used to perform statistical tests indicated in figure legends. Differentially expressed genes shown in MA plots were detected using Deseq2 software as described above (Love et al., 2014), and gene ontology overlap significance (hypergeometric probability) was calculated using the GeneOverlap program (Shen, 2014).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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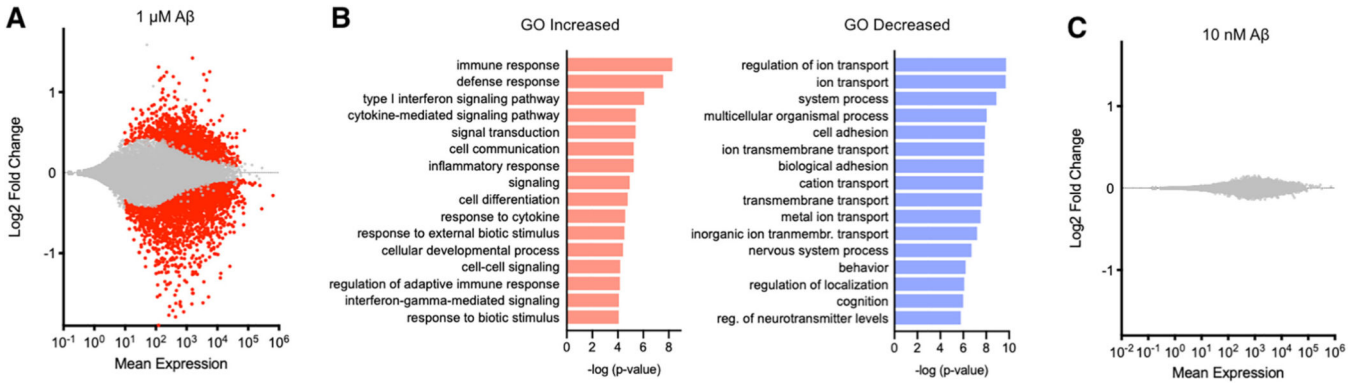

Fig. 1. Transcriptome analysis of human astrocytes treated with high and low concentration Aβ.

(A) MA plot showing Log2 fold change and mean gene expression levels in primary human astrocytes treated with 1 μM Aβ for 24 h. Note >5000 significantly increased/decreased genes shown in red (FDR < 0.1). (B) Top transcriptional modules increased/decreased in gene ontology (GO) analysis of 1 μM Aβ-treated astrocytes. (C) MA plot showing Log2 fold change and mean gene expression (no significant changes) in astrocytes treated with 10 nM Aβ for 24 h. All experiments performed in triplicate with cells from one donor.

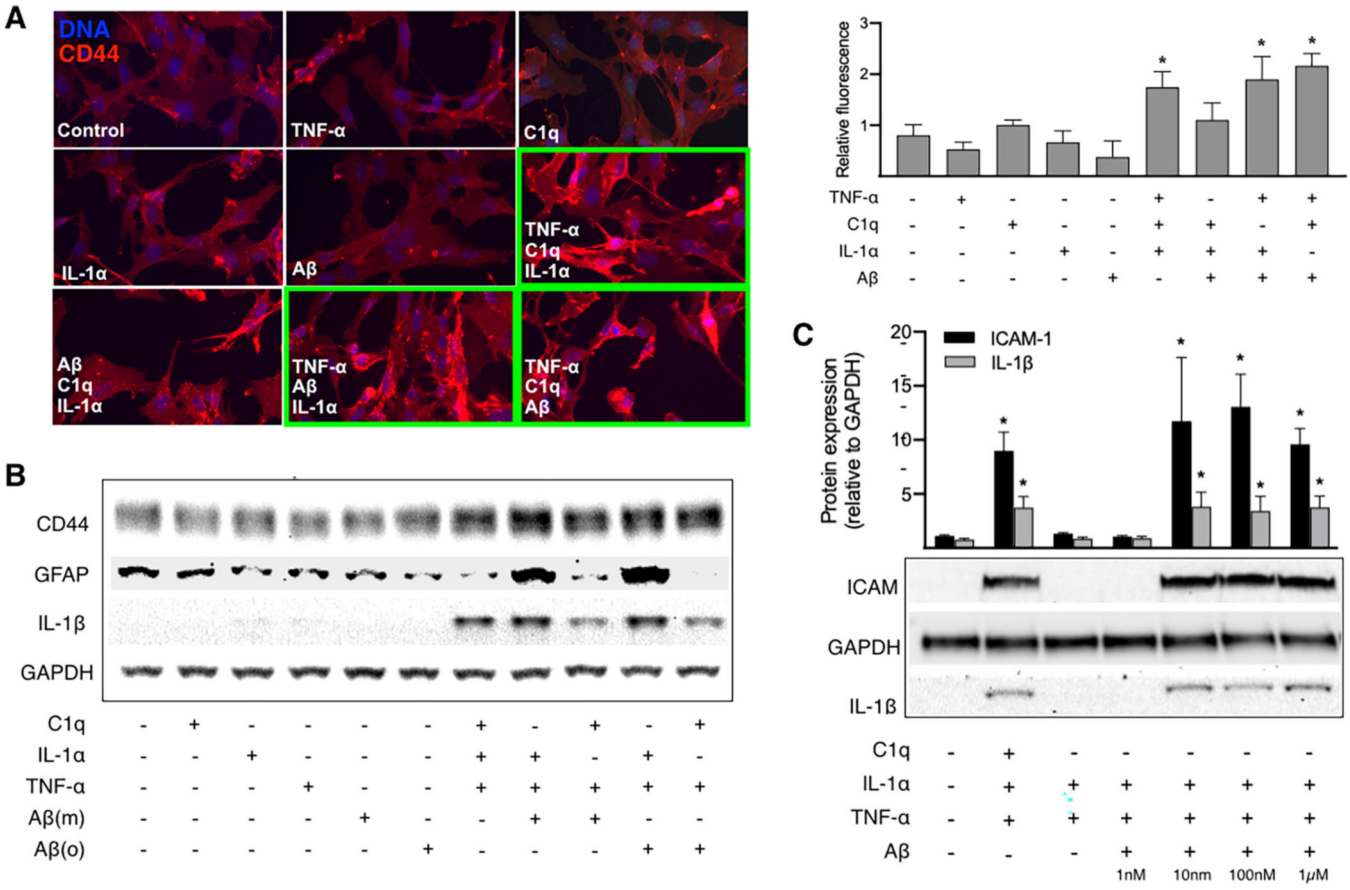

Fig. 2. Astrocyte immune activation induced by cytokines and Aβ/cytokine cocktails.

(A) Representative immunofluorescence images of the astrocyte activation marker CD44 in cells treated with TNFα, IL-1α and C1q, as well as Aβ (all alone or in combination). Note greater staining with all-cytokine cocktail and when Aβ is substituted for IL-1α or C1q (relative signal quantified at right). (B) Western blots showing that Aβ monomers (m) and oligomers (o) can substitute for IL-1α or C1q to induce immune activation, marked by increased expression of the pro-inflammatory cytokine IL-1β and the astrocyte activation marker GFAP. Note somewhat greater activation when Aβ is substituted for C1q. (C) Dose-response experiments showing that nanomolar concentrations of Aβ cause astrocyte activation (increased expression of IL-1β and the reactive astrocyte marker ICAM-1) when combined with TNF-α and IL-1α. All experiments performed 3–5 times in primary human astrocytes from one donor. $*P < 0.05$ vs. control, unpaired two-tailed *t*-test.

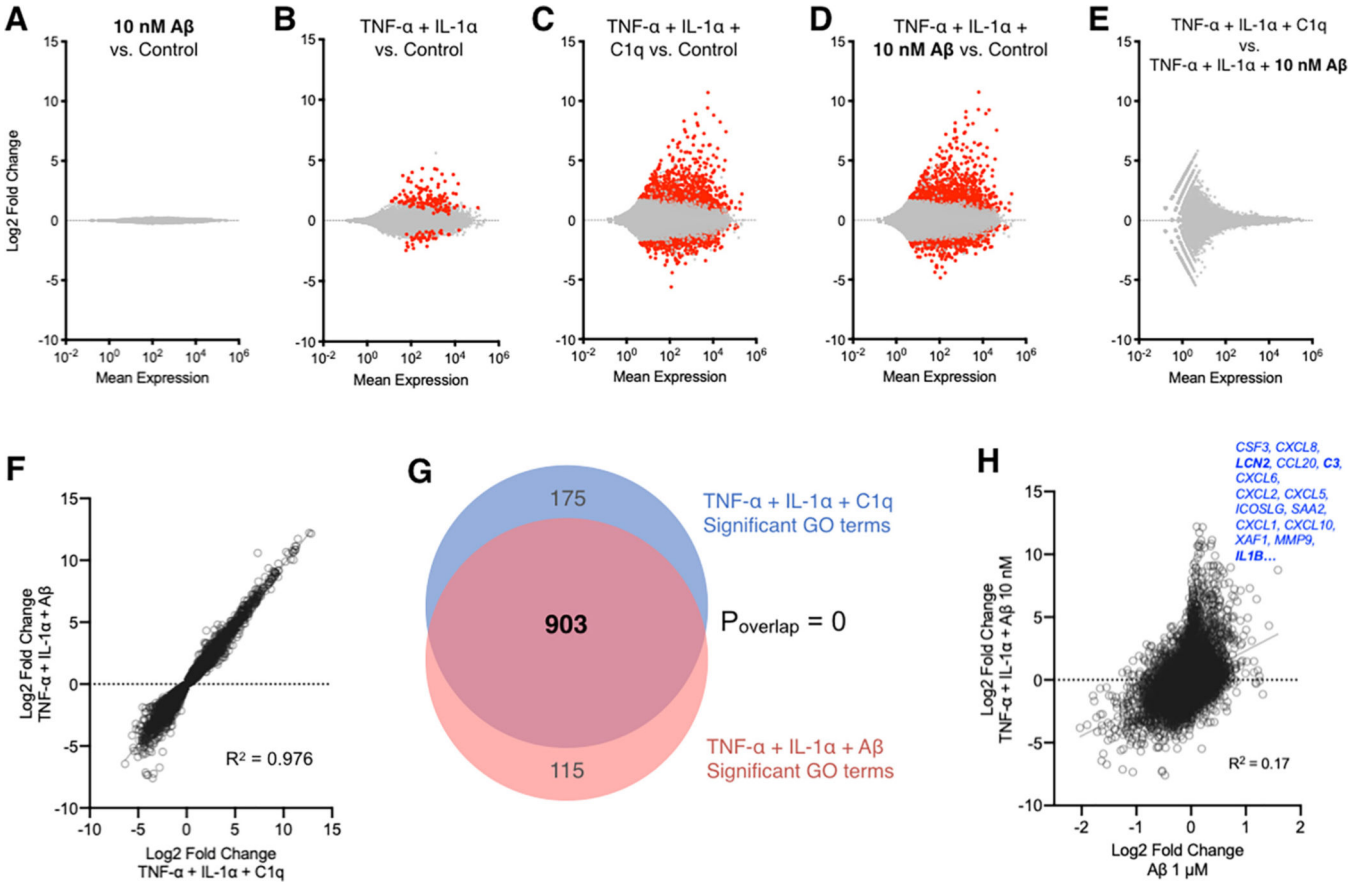

Fig. 3. Similar transcriptional changes with cytokines and Aβ/cytokine cocktails.

(A-E) MA plots showing Log2 fold change and mean gene expression in astrocytes treated with low-dose (10 nM) Aβ and/or combinations of TNF-α, IL-1α or C1q. Note no transcriptome changes with 10 nM Aβ alone and modest changes with TNF-α + IL-1α, but major and similar differences with both TNF-α + IL-1α + C1q and TNF-α + IL-1α + Aβ. Significantly increased/decreased genes in red (FDR < 0.1). (F) Correlation between gene expression changes induced by cytokine cocktail vs. Aβ/cytokine cocktail. (G) Comparison of gene ontology (GO) terms significantly increased/decreased by cytokine-only and Aβ/cytokine cocktail. (H) Weak correlation between gene expression changes induced by Aβ/cytokine cocktail vs. high concentration (1 μM) Aβ alone; transcripts most different with Aβ/cytokine cocktail vs. Aβ alone in blue. All experiments performed in primary human astrocytes from three different donors.

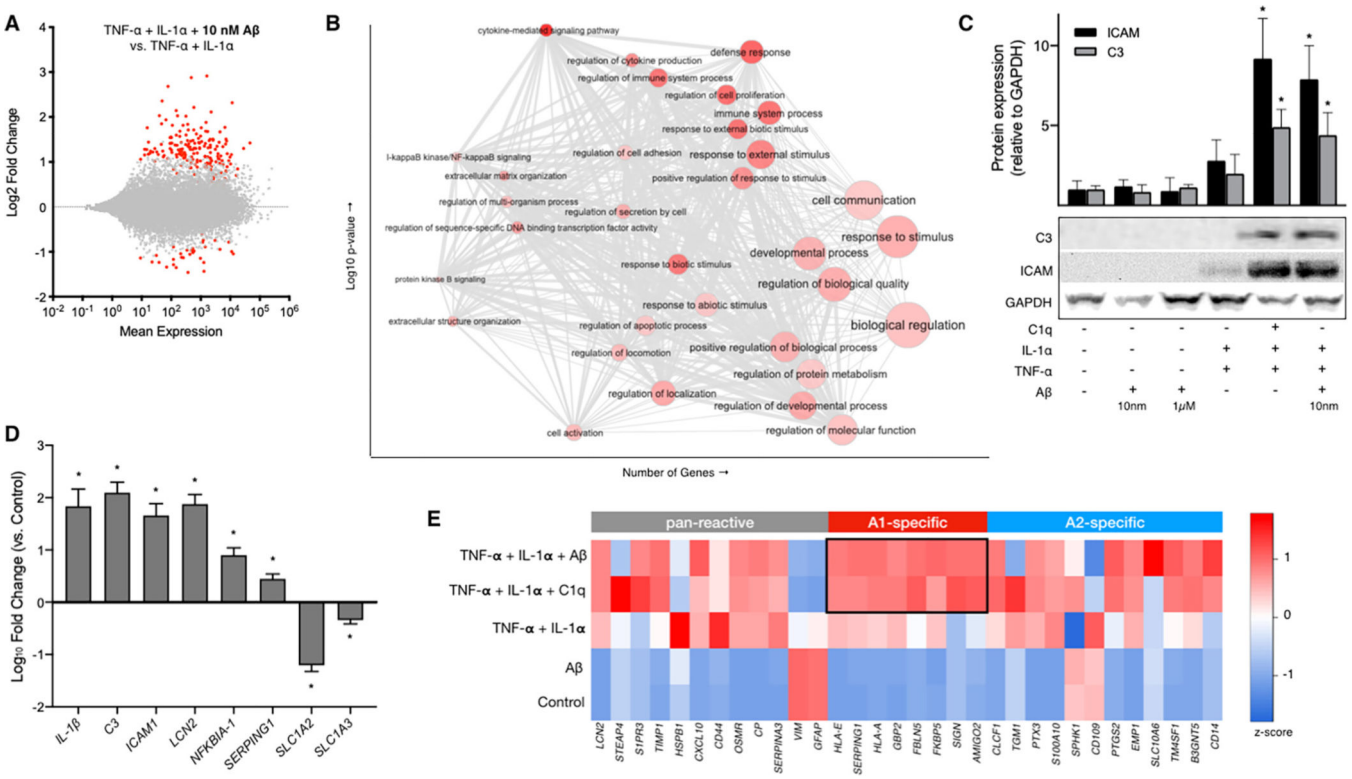

Fig. 4. Upregulation of immune response and reactive astrocyte genes by Aβ/cytokine cocktail.

(A) MA plot showing Log2 fold change and mean gene expression in astrocytes treated with TNF-α + IL-1α + Aβ vs. TNF-α + IL-1α alone (significantly increased/decreased genes in red, FDR < 0.1; data on primary human astrocytes from three different donors, as in Fig. 3). (B) Enhanced immune activation/cytokine signaling indicated by gene ontology analysis of genes differentially expressed when Aβ is added to TNF-α + IL-1α (based on significantly modulated transcripts in A). (C–D) Western blot and quantitative RT-PCR conifrmation of reactive astrocyte markers and innate immune mediators identified as increased/decreased with Aβ/cytokine cocktail treatment in RNA-seq data. Data collected using RNA and protein lysate from the same cells (one donor in triplicate) analyzed in Fig. 1 and Supplementary Fig. 1. *$P < 0.05$ vs. control, unpaired two-tailed *t*-test. (E) Heat map showing relative expression of reactive astrocyte markers in response to Aβ and/or cytokines based on RNA-seq data from primary human astrocytes (one donor in triplicate as in Fig. 1 and Supplementary Fig. 2).