Heme and hemoglobin suppress amyloid β–mediated inflammatory activation of mouse astrocytes

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ABSTRACT
Glial immune activity is a key feature of Alzheimer’s disease (AD). Given that the blood factors heme and hemoglobin (Hb) are both elevated in AD tissues and have immunomodulatory roles, here we sought to interrogate their roles in modulating amyloid β (Aβ)-mediated inflammatory activation of astrocytes. We discovered that heme and Hb suppress immune activity of primary mouse astrocytes by reducing expression of several proinflammatory cytokines (e.g. RANTES) and the scavenger receptor CD36, and reducing internalization of Aβ1-42 by astrocytes. Moreover, we found that certain soluble (> 75 kDa) Aβ1-42 oligomers are primarily responsible for astrocyte activation and that heme or Hb association with these oligomers reverses inflammation. We further found that heme up-regulates phospho-protein signaling in the phosphoinositide 3-kinase (PI3K)/Akt pathway, which regulates a number of immune functions, including cytokine expression and phagocytosis. The findings in this work suggest that dysregulation of Hb and heme levels in AD brains may contribute to impaired amyloid clearance and that targeting heme homeostasis may reduce amyloid pathogenesis. Altogether, we propose heme as a critical molecular link between amyloid pathology and AD risk factors, such as aging, brain injury, and stroke, which increase Hb and heme levels in the brain.

INTRODUCTION
Alzheimer’s disease (AD) affects more than 40 million people worldwide (1). Despite decades of research, we still lack an effective therapy for AD, and have much left to learn about the mechanisms involved in AD pathogenesis. Neuroinflammation, in particular, is increasingly recognized as an important aspect of AD pathology (2), but whether glial activity promotes pathogenesis (3) or is neuroprotective (4,5) remains an open question. Glial activity plays an important role in neuroprotection and maintaining tissue homeostasis by regulating metabolism (6), pruning neurites and synapses (7), and clearing pathogens such as the hallmark AD protein, amyloid-beta (Aβ) (8). However, glial inflammatory activity can also promote a neurotoxic microenvironment via overexpression of neurotoxic cytokines (9-11) and reactive oxygen species (12), among other factors. Moreover, there is increasing evidence that glia efficiently clear Aβ early in AD, but that they become dysfunctional with time (13-15), perhaps due to changes in environmental factors and immunomodulatory signaling during AD progression (11,13). While the role of microglia in AD has long been acknowledged, astrocytes are increasingly emerging as important modulators of AD pathology. Specifically, astrocytes have been reported to migrate towards Aβ plaques and uptake and degrade Aβ (14,16-18), suggesting that deficits
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in astrocyte immune function may contribute to AD pathogenesis.

Increased brain tissue levels of the immunomodulatory blood factors heme and hemoglobin (Hb) are characteristic of AD (19-22) and are associated with a number of AD risk factors, including age, brain injury, and stroke (23,24). Notably, analysis of postmortem human AD tissue has shown increased heme in the temporal lobe (21) and increased Hb mRNA and protein in the inferior temporal gyrus and parietal gray and white matter (22), respectively. In fact, heme has been shown to colocalize with Aβ deposits in AD tissue (25) and Hb has been found within senile plaques and cerebral amyloid angiopathy (22). Furthermore, both heme and Hb have been reported to bind Aβ and alter aggregation state (19,21). Studies in macrophages and endothelial cells indicate heme stimulates the immune response via toll-like receptor 4 (TLR4) signaling (26). Moreover, Hb can promote inflammation independently of heme (27). Despite these prior observations pointing to potential roles for heme and Hb in modulating AD pathogenesis, the effects of heme and Hb on Aβ-mediated inflammatory response and the physiologic consequences of heme and Hb interactions with Aβ remain unknown.

Herein, we sought to elucidate the effects of heme and Hb on astrocyte immune function and delineate how heme and Hb specifically affect astrocyte inflammatory response to Aβ. Surprisingly, our data reveal that heme and Hb are able to reduce astrocyte activation and phagocytic capacity by direct cell signaling and through interactions with Aβ1-42. Given that Hb and heme levels are increased in AD brains (19,20), our findings represent a new paradigm for understanding astrocyte dysfunction and neuroinflammation in AD. They specifically suggest that heme/Hb homeostatic machinery could represent a viable therapeutic strategy for AD. Lastly, the suite of cell biological and biophysical methods that we employed can be easily adapted to probe the effects of a multitude of molecules found to be dysregulated in the AD brain for their effects on neuroinflammation.

RESULTS

Heme and hemoglobin modulate Aβ1-42-induced astrocyte inflammatory cytokine expression

We used primary mouse astrocytes generated from CD-1 pups as a model system to delineate the effects heme and Hb on Aβ1-42-induced inflammatory response. Though neonatal-derived astrocyte cultures are widely used, microglial contamination is a concern in assessing inflammatory response (30). We did not, however, detect expression of the microglia-specific marker, Iba-1, in Aβ1-42-stimulated cultures (Figure S1), suggesting that our cultures were not significantly contaminated with microglia. Since physiologic concentrations of Aβ1-42 are reported to be in the sub-low nM range (31-34), we used 50nM Aβ1-42 for all culture conditions. The concentrations of heme and Hb have not been defined in Alzheimer’s disease, though they are thought to be on the order of 10-100 μM in hemolytic disorders (29,35). Thus, we tested our primary conditions at equimolar 50nM heme or Hb and verified using high 25 μM concentrations. Heme or Hb was added to astrocyte culture medium containing fetal bovine serum, which has ~60 nM heme and < 10 nM Hb (Figure S2). The heme present in serum is not bioavailable to Aβ1-42, as it is almost all exclusively associated with high affinity hemoproteins, Hb or hemopexin, which exhibit tight dissociation constants (KD) for heme, KD < 100 fM (36).

Canonical markers of astrocyte activation, such as glial fibrillary acidic protein (GFAP), are not reliable activation markers in culture due to high baseline expression (37,38). Therefore, we used a multiplexed immunoassay to robustly quantify...
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astrocyte activation in terms of protein expression of 32 cytokines into the culture medium (EMD Millipore, Billerica, MA). As expected, astrocytes conditioned for 24 hr with Aβ1-42 increased expression of numerous pro-inflammatory cytokines, including IL-1β (39), RANTES (40), and GM-CSF (41) (Fig. 1a). Since we measured a total of 32 cytokines, we aimed to create a cytokine profile that could be used to discern differences between groups. To do so, we utilized a discriminant partial least squares regression (DPLSR) analysis (42). We have previously used this approach to identify a cytokine profile distinguishing postmortem human control and AD brain tissues (9). Applying this analysis here identified an axis called a latent variable (LV1) that distinguished Aβ1-42 conditioned wells from all other conditions (Fig. 1b). The LV1 axis consisted of a profile of cytokines that were most different between groups (Fig. 1c), while LV2 defined a second axis of cytokines that were most different in the heme+Aβ1-42 condition (Fig. 1d). By plotting each sample in terms of its score on LV1 (representing a composite indicator of cytokine expression), we found that Aβ1-42-induced cytokine expression was significantly increased compared to controls or Aβ1-42 wells that were co-treated with heme or Hb (Fig. 1e). Plotting of selected individual cytokines revealed a trend where certain pro-inflammatory cytokines, such as RANTES and GM-CSF (43) were downregulated by heme and Hb, while others, such as KC (44) and MCP-1 (45), were not substantially modulated (Fig. 1f), reflecting our multivariate analysis (Fig. 1b-e).

We additionally conditioned astrocytes with a high concentration (25 μM) of heme associated with hemolytic disorders (29,35), and which promotes cytokine expression in the RAW264.7 macrophage cell line (Figure S3a). Analysis of the same panel of 32 cytokines in astrocytes demonstrated that 25 μM heme produced no change in cytokine expression compared to vehicle (Figure S3b). Moreover, when applied together with Aβ1-42, 25 μM heme further reduced cytokine expression compared to treatment with 50 nM heme+Aβ1-42 (Figure S3b). Applying the same concentrations of heme to the SIM-A9 cell line (46), we found that neither low nor high concentrations of heme alone were highly pro-inflammatory (Figure S3c). Moreover, 50 nM heme or Hb suppressed cytokine expression compared to treatment with Aβ1-42 alone. In contrast, 25 μM heme applied together with Aβ1-42 amplified cytokine expression compared to treatment with Aβ1-42 alone (Figure S3c).

Together, these data demonstrate that heme and Hb reduce astrocyte inflammatory response as quantified by cytokine expression. Moreover, in contrast to microglia and macrophages, this anti-inflammatory effect is exerted on astrocytes by both low and high concentrations of heme.

Heme and hemoglobin reduce astrocyte uptake of Aβ1-42 and phagocytic capacity

Since heme and Hb reduced Aβ1-42-induced expression of multiple inflammatory cytokines (Fig. 1), we next investigated their effects on astrocytic capacity to scavenge Aβ1-42 and other substrates. First, we conditioned astrocytes with 50 nM Aβ1-42, either alone or together with heme or Hb. We then used immunocytochemistry (ICC) to stain for Aβ1-42 using the 6E10 antibody (BioLegend, San Diego, CA). Astrocytes treated with Aβ1-42 alone showed Aβ1-42 aggregates within the plane of the cell (Fig. 2a, arrows). In contrast, astrocytes co-conditioned with heme, and to a lesser extent Hb, showed little Aβ1-42 within the plane of the cell and substantial labeling on the cell surface, suggesting that heme and Hb suppress Aβ1-42 internalization (Fig. 2a, arrows).

To determine if heme or Hb affected scavenger activity for other substrates, we next interrogated their effects on astrocyte internalization of killed *E. coli* particles. We treated astrocyte cultures with killed *E. coli* microparticles that were labeled with a trypan-quenched fluorescein (Thermo Fisher). Fluorescent intensity (λex = 480 nm; λem = 520 nm) was quantified on a microplate reader and revealed that Hb, and to a lesser extent Hb, reduced microparticle internalization compared to control (Fig. 2b). To determine if these effects also modulated phagocytosis, we incubated astrocytes with pH-sensitive pHrodo® Zymosan particles, which fluoresce in phagosomes. Quantification of phagocytic cells using fluorescence microscopy (Figure S4) demonstrated a significant reduction of phagocytosis in Hb-treated astrocytes and a non-significant reduction by heme (Fig. 2c), mirroring our observations with killed *E. coli* particles.

Since heme and Hb appeared to have similar effects, though to differing degrees, we next wanted to determine if these effects were associated with
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Heme and hemoglobin modulate Aβ1-42 aggregation state and morphology.

The ability of heme and Hb to interact with and alter the aggregation state of Aβ1-42 (21,22) may be responsible for attenuating Aβ1-42-mediated inflammatory activation of astrocytes and clearance of Aβ1-42. In order to better understand heme and Hb interactions with Aβ1-42 and their consequences on Aβ 2° structure, aggregation state and morphology, we used a combination of UV/vis spectroscopy, circular dichroism (CD) spectroscopy, ThT fluorescence assays, size exclusion chromatography (SEC), and transmission electron microscopy (TEM). First, using UV/vis spectroscopy, we found that titration of Aβ1-42 into aqueous solutions of heme and Hb resulted in changes in heme absorbance spectra consistent with high affinity interactions. In the case of heme, we found that Aβ1-42 interacts with heme in a 2:1 stoichiometry, with apparent dissociation constants of $K_D = 100 \text{ nM}$ and $K_D = 3 \mu\text{M}$ (Fig. 3a-c), consistent with prior studies (51,52). In the case of Hb, we found that each monomer of Aβ1-42 interacts with each monomer of tetrameric Hb (Fig. 3d-f).

Second, using CD spectroscopy, we found that the β-sheet 2° structure of Aβ1-42 remains intact upon heme binding (Fig. 3d). Further, CD spectroscopy revealed that a mixture of Hb and Aβ1-42 yields a CD-derived 2° structure that is distinct from the sum of the individual Hb and Aβ1-42 spectra (Fig. 3g), confirming biophysical association between Hb and Aβ1-42. Third, using ThT fluorescence ($\lambda_{ex} = 450 \text{ nm}; \lambda_{em} = 482 \text{ nm}$) as a probe for Aβ1-42 fibrillization (53), we found that heme and Hb could prevent the formation of ThT-positive Aβ1-42 aggregates (Figure S5a) and heme de-aggregates pre-formed ThT-positive Aβ1-42 fibrils (Figure S5b), consistent with prior studies demonstrating that heme and Hb suppress Aβ1-42 fibrillization (19,54).

We next analyzed the effects of heme and Hb on the morphology and size of insoluble and soluble Aβ1-42 species using SEC and TEM. TEM analysis of insoluble Aβ1-42 revealed the presence of four morphologically distinct species that shift in distribution upon the presence of heme or Hb: extended long fibrils (Type I), tangled fibrils (Type II), amorphous aggregates (Type III), and short fibrils (Type IV) (Fig. 4a). This analysis was conducted by incubating 25 or 100 µM Aβ1-42 alone or with 2x heme or 100 µM Aβ1-42 with 25 µM Hb at 37 °C for 16 hours and pelleting out insoluble Aβ1-42. The predominant species in the Aβ1-42 only samples are the Type I extended fibrils (Fig. 4b), which are greater than 1 µm in length and less than 10 nm in diameter, consistent with previous analysis of Aβ1-42 fibrils (55). The Type IV short fibrils, which are ~30 nm in length and ~8 nm in diameter, are associated only with the presence of Type I and Type II fibrils. We therefore do not consider Type IV species in our analysis of heme and Hb effects on Aβ1-42 aggregation and fibrillization. Incubation with heme results in the conversion of the Type I species to the amorphous Type III species in the insoluble fraction, with little effect on the Type II species. In the case of Hb, while there is greater variation between two independent trials, it is clear that Hb also has a profound effect on fibril morphology, shifting the species distribution from Type I extended fibrils to Type II tangled fibrils (Fig. 4b). Further, it is worth noting that the insoluble species generated from the application of heme and Hb are not enriched with heme (Figure S6), suggesting that heme remains largely associated with soluble Aβ1-42 species. Altogether, our studies with heme and Hb are consistent with prior work demonstrating that it reverses and/or suppresses Aβ1-42 fibril growth (19,21). However, our work shows that heme and Hb also have the capacity to increase the formation of certain HMW soluble oligomers of Aβ1-42 and, with respect to Hb, can alter fibril morphology from extended (Type I) to tangled fibrils (Type II).

In order to analyze the effects of heme and Hb on soluble Aβ1-42 aggregation state, size, and morphology, we subjected the soluble Aβ1-42 fraction, after pelleting out insoluble Aβ1-42, to SEC analysis. SEC analysis of soluble Aβ1-42 reveals the
presence of HMW aggregates, > 75 kDa, that elute in the void volume, 8 mLs, as well as low molecular weight (LMW) species, < 6.5 kDa, that elute at ~19 mLs (Fig. 4c). Both heme and Hb result in the loss of the LMW species eluting at 19 mLs in favor of the HMW species eluting at 8 mLs. Further, UV/vis spectroscopy indicates that the 8 mL species is associated with heme (Fig. 4c-d) and immunoblot analysis of the 8 mL peak with Hb further demonstrates the co-elution of both Hb and Aβ1-42 (Figure S7a). By comparison, free Hb elutes at 12 mLs (Figure S7b). The HMW oligomers that elute in the 8 mL fraction are exclusively Type III amorphous Aβ1-42 aggregates both in the absence and presence of heme or Hb as indicated by TEM analysis (Fig. 4e). The LMW Aβ1-42 species eluting at 19 mLs appears by TEM to be a smaller amorphous aggregate than the Type III HMW species that elutes at 8 mLs (Figure S8).

In order to determine if the Type III species are on-pathway to form amyloid fibrils, we incubated 1 μM of the SEC isolated Type III species, with or without heme/Hb, as well as the unseparated stock mixture of 2 μM Aβ1-42 for 48 hours at 37 °C and analyzed them by TEM. Interestingly, unlike the unseparated stock mixture of Aβ1-42, which readily forms fibrils, the Type III species did not readily form fibrils (Figure S9).

Altogether, these data demonstrate that heme and Hb do not simply de-aggregate or prevent fibril formation, but also act to promote the formation of distinct heme and Hb associated soluble HMW oligomers. The isolated Aβ1-42 species and their inflammatory response is summarized in Table 1.

### Inflammatory activation of astrocytes by soluble Aβ1-42 aggregates is reversed by association with heme or hemoglobin.

A combination of centrifugation and SEC identified a number of soluble and insoluble Aβ1-42 species (Table 1), including ones that associate with and/or are produced as a consequence of heme and Hb. Recent studies of isolated Aβ from postmortem human tissues have revealed that different species have distinct cytotoxicities and ligand affinities (56,57). We therefore sought to identify which Aβ1-42 species were pro-inflammatory and assess the effect heme or Hb association with these species had on inflammatory activation of astrocytes. Toward this end, we conditioned astrocytes with 50 nM preparations of each soluble and insoluble Aβ1-42 species for 24 hr and assayed their ability to stimulate the expression of inflammatory cytokines relative to 50 nM of the un-separated Aβ1-42 stock mixture and vehicle control. Most interestingly, we found that application of the soluble >75 kDa HMW Type III oligomer is highly inflammatory and comparable to that of the application of the un-separated Aβ1-42 stock mixture (Fig. 5a). In marked contrast, preparations of the Aβ1-42 pellet, which primarily consists of the Type I extended fibrils, or the soluble LMW species yielded minimal cytokine expression in astrocytes compared to the un-separated Aβ1-42 stock mixture (Figure S10a).

We next determined what role heme and Hb association play in mediating Aβ1-42 inflammatory activation. Strikingly, heme or Hb association with the highly inflammatory soluble >75 kDa HMW Type III oligomer completely reverses its inflammatory activation of astrocytes (Fig. 5). On the other hand, the Hb-associated insoluble pellet, which has a greater fraction of Type II “tangled fibrils” relative to the non-inflammatory Aβ1-42-only pellet that is primarily composed of Type I “extended fibrils” (Table 1), produced a high inflammatory response in astrocytes (Figure S10b). It is worth noting that the heme associated insoluble Aβ1-42 pellet could not be tested for inflammatory activation of astrocytes due to the very low amount of Aβ1-42 in the insoluble fraction. This is likely due to the fact that the insoluble fraction does not contain fibrils and only consists of the Type III amorphous aggregate, which is present in both soluble and insoluble fractions (Fig. 4). However, given that the heme-associated soluble Type III Aβ1-42 species are non-inflammatory (Fig. 5a), we would predict that the insoluble Type III Aβ1-42 species is likewise non-inflammatory.

Overall, these data paint a complex and nuanced picture of the roles of heme and Hb in modulating the inflammatory activity of Aβ1-42 on astrocytes:

1. Aβ1-42 alone forms a soluble HMW > 75 kDa species that is highly inflammatory. It also forms insoluble extended fibrils (Type 1) and smaller oligomeric species that have minimal inflammatory properties.
2. Direct association of heme or Hb with the soluble HMW > 75kDa Aβ1-42 species reduces astrocyte cytokine expression.
3. The presence of heme and Hb changes the Aβ1-42 species found in the insoluble fraction.
4. The Type II “tangled fibrils” in the insoluble fraction produced by Hb is highly inflammatory.

**Heme and hemoglobin modulate the PI3K/Akt pathway**

We have established that heme and Hb exert their modulatory effects on astrocyte immune activity both by physically associating with Aβ1-42 (Figs. 3-4) and by a second mechanism independent of Aβ1-42 (Fig. 2). The latter suggests that heme and Hb have the capacity to impact Aβ1-42 clearance through its effects on immune signaling. To gain insight into how heme or Hb modulates immune signaling, we quantified phosphorylation of 11 phospho-proteins in the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) /Akt signaling pathway. The PI3K/Akt pathway is of particular interest because of its known role in modulating autophagic clearance of Aβ in neurons (58) and because of its known ability to inhibit M1 macrophage polarization (59), which is linked to expression of scavengers receptors, including CD36 (60). Further, this pathway is important to astrocyte immune function as it is involved in regulating astrocyte viability, migration, autophagy, and production of cytokines and inflammatory mediators (61-65).

Since phospho-protein signaling occurs on a much faster time scale (on the order of minutes) than other phenotypic responses (66), we analyzed phospho-proteins from astrocytes conditioned with combinations of Aβ1-42, 50nM heme, and 50nM Hb at 5 and 15min time points (Figure S11a-b). To simultaneously account for data from both time points, we concatenated the time point data and used D-PLSR analysis to identify signaling differences between conditions. Analyzing the effects of heme alone identified two axes of interest with respect to heme and Aβ1-42 (Fig. 6a). First, LV1 separated heme+Aβ1-42 to the right with all other conditions to the left. Among other signals in the pathway, LV1 consisted of phospho-Akt, phospho-PTEN, and phospho-TCS2 at the 5min time point as top correlates with the heme+Aβ1-42 condition (Fig. 6b). The D-PLSR analysis also determined that both heme and heme+Aβ1-42 were increased along LV2 (Fig. 6a), which consisted of phospho-mTOR at 15min, and phospho-IRS1 at both 5 and 15min time points as top correlates with heme or heme+Aβ1-42 (Fig. 6c). Plotting all condition groups along LV1 revealed that heme+Aβ1-42 was significantly different from heme alone (Fig. 6d), whereas plotting along LV2 revealed that treatment with either heme alone or with heme+Aβ1-42 were significantly different from vehicle controls (Fig. 6e). Thus, these data indicate that heme can significantly shift signaling within the PI3K/Akt signaling pathway, which is modulated by Aβ1-42. Applying the same analysis to Hb revealed that Hb did not significantly modulate signaling within the pathway compared to either control or Aβ1-42 alone (Figure S11c-e).

Since heme more substantially modulated PI3K/Akt signaling (Fig. 6) and CD36 expression (Fig. 2d-3) than Hb, we next hypothesized that inhibition of the pathway would restore astrocyte scavenger activity. To test this, we used rapamycin, which inhibits signaling through mTOR, a central node within the PI3K/Akt pathway (Fig. 6f). Indeed, co-treatment of heme+Aβ1-42 with 10 nM of rapamycin yielded partial recovery of CD36 expression (Figs. 6g and S12). Importantly, these signaling data for the first time reveal that 1) the PI3K/Akt pathway is stimulated by heme and 2) that the PI3K/Akt pathway regulates astrocyte scavenger activity.

**DISCUSSION**

Neuroinflammation is recognized as a key component of AD pathology (2). However, it is unclear if glial activity promotes neurotoxicity (3) or is neuroprotective(4,5). In reality, the consequences of neuroinflammation occupy a continuum between neuroprotective (3) and neurodegenerative (4,5). In terms of protection against AD, glial activation is essential for clearance of cytotoxic Aβ species. In terms of aggravating AD pathogenesis, excessive neuroinflammation contributes to a number of detrimental effects, including “fatigued” glia that are unable to clear Aβ, generation of toxic reactive oxygen species, and hyperactivated microglia that indiscriminately phagocytize neurons (11-15). Further complicating matters is the reality that different species of Aβ may affect neuroinflammation in very different ways. Moreover, the effects of each species may be modulated by other aspects of tissue pathology. Herein, we have identified heme and Hb as key AD-
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relevant immunomodulators and have probed mechanisms that mediate the inflammatory activation of astrocytes. Moreover, we have identified key Aβ1-42 species that are responsible for astrocyte activation and the effects of heme and Hb on the inflammatory potential of these species. Overall, our data indicate that heme and Hb suppress the Aβ1-42-mediated inflammatory activation of astrocytes, suggesting that these factors contribute to AD pathogenesis by impairing Aβ clearance mechanisms.

A pathological hallmark of AD is a weakened blood brain barrier (BBB) and a concomitant increase in blood and serum factors in the brain, including infiltrating red blood cells, heme, Hb, and haptoglobin (19,20,67). Since both heme and Hb have previously been shown to alter Aβ oligomerization (21,22) and macrophage immune activity (28,68), they have the potential to modulate AD pathogenesis. Though heme is known to have neurotoxic properties at high concentrations associated with hemorrhage (69), ours is the first study to evaluate the effects of heme and Hb on astrocyte immune activity at concentrations that are physiologically relevant outside of hemorrhage. Astrocytes are one of three glial cell types that take on varying immune and neuronal support functions (70). In addition to being essential regulators of neuronal metabolism, astrocytes possess vital immune functions, and play essential roles in clearance of Aβ and regulation of microglial activity during AD pathogenesis (71,72). Our integrated analysis of cytokines, signaling, scavenger activity, and biophysical analysis of heme/Hb association with Aβ1-42 reveals for the first time that Hb and heme strongly modulate astrocyte immune activity. Moreover, heme/Hb effects are imparted both by directly modulating astrocyte function and by physically binding to Aβ1-42.

Simultaneous analysis of the relative expression of 32 cytokines provided us with a detailed view of astrocyte inflammatory response to Aβ1-42, heme, Hb, and heme/Hb-bound species of Aβ1-42. We began our study by applying Aβ1-42 and either heme or Hb to astrocyte cultures (Fig. 1a). Our multivariate analysis (Fig. 1b-c) identified a composite cytokine variable that integrated all readings from each condition applied. Scoring each sample on this composite variable demonstrated that both heme and Hb reduced cytokine expression (Fig. 1e). From this analysis, Aβ1-42 conditioning strongly correlates with RANTES, GM-CSF, and IL-1β, which were all downregulated in cultures cotreated with heme or Hb (Fig. 1f). Of these, RANTES is a pro-inflammatory chemokine and involved in microglial recruitment (73,74), GM-CSF promotes microglial proliferation (75), and IL-1β is a highly pro-inflammatory cytokine upregulated early in AD (76) that has been shown to promote Aβ clearance in a mouse model (77). Interestingly, application of heme or Hb at low concentration (50 nM) did not strongly suppress expression of other cytokines, including IP-10, KC, and MCP-1, which are all involved in immune cell recruitment (44,45,78). However, application of a high-dose of heme (25 μM) reduced these cytokines as well (Figure S3a-b), suggesting that multiple mechanisms are associated with heme/Hb suppression of inflammatory response. We note that while all of the cytokines modulated by heme and Hb are well established to modulate immune activity, they have not generally been found to be neurotoxic.

Dual mechanisms of heme and Hb immunomodulatory activity (effects on astrocyte immune signaling and effects produced by binding to Aβ1-42) represent one possible explanation for why low doses of heme/Hb suppresses certain cytokines and not others, while high doses of heme suppress cytokines globally. Evidence for heme and Hb down-regulating astrocyte inflammatory activity through physically modifying Aβ1-42 stems from our finding that heme and Hb bind to a particularly inflammatory species of Aβ1-42. Indeed, our SEC analysis revealed that a soluble HMW oligomeric species of Aβ1-42 (> 75 kDa) produced the principal inflammatory response compared with other fractions (Figs. 4, 5, S10). Moreover, the Aβ1-42 HMW soluble oligomer was the only species that was verified to be associated with heme or Hb and strongly suppressed cytokine expression compared to the unbound oligomer. Further, this result is particularly important since it provides a physiological context to previously described Aβ-heme and Aβ-Hb interactions, as soluble Aβ oligomers are abundant in human AD brain tissue and have high binding affinities and neurotoxic properties (57,79).

On the other hand, while fibrillar Aβ1-42 is non-inflammatory, the effect of Hb on altering Aβ1-42 fibril morphology, contributes to the formation of a highly inflammatory “tangled” fibrillar species
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(Figs. 4a and S10). Thus, while Hb renders a soluble oligomer non-inflammatory, it acts to increase the inflammatory potential of an insoluble fibril. Altogether, the isolation of distinct Aβ1-42 species and the consequence of heme or Hb on their distribution and inflammatory activation of astrocytes highlight the complex and nuanced nature of Aβ-mediated immune signaling. Indeed, the observation of changes in neuroinflammation over the course of AD may reflect the competing effects of multiple Aβ, Aβ-heme, and/or Aβ-Hb species that have differing immunomodulatory activities. Additionally, the peroxidase activity of heme-Aβ complexes may further act to modulate the inflammatory response (54).

A second mechanism of heme/Hb control of inflammatory activity is via direct action on astrocyte signaling, which is supported by our observations that heme and Hb suppressed microparticle internalization, phagocytosis, and CD36 expression (Fig. 2) in experiments free of Aβ1-42. Our results in astrocytes generally point toward heme as having anti-inflammatory effects at both low and high heme concentrations. Moreover, we found that scavenger activity of astrocytes was inhibited by low (50 nM) heme concentrations and that both low and high (25 μM) heme concentrations inhibited scavenger activity of SIM-A9 microglia (Figs 2 and S13). Nevertheless, the canonical role of heme in immune signaling, primarily delineated in macrophages and endothelial cells, is that it stimulates inflammation via TLR4 (26). In contrast, our astrocyte data indicates that heme or Hb reduce the inflammatory response and stimulate phosphorylation of multiple signaling molecules in the PI3K/Akt pathway. In addition to regulating metabolism, this pathway regulates autophagy and is a known modulator of inflammation and phagocytosis (60,80,81). Additionally, the mTOR inhibitor rapamycin partially recovered expression of CD36 (Figs. 6g and S12), defining a novel and causal role for the PI3K/Akt pathway in heme signaling and suggesting that inhibition of this pathway has potential therapeutic efficacy for promoting Aβ clearance.

How are heme signals integrated to control immune activity? Extracellular “free” heme can be internalized by heme transporter HRG-1 and Hb may be internalized by the Hb receptor CD163, though the latter is primarily expressed in the brain by microglia (82-84). Once in the cell, free heme or Hb-derived heme, can be catabolized into CO, biliverdin, and bilirubin, which all possess anti-inflammatory properties (85-88). Importantly, CO is known to activate the Akt pathway, providing a plausible explanation for heme-mediated immune signaling (89). An alternative mode of heme signaling may involve heme binding to a number of heme-regulated transcription factors, including p53, Bach1, and Rev-erb-α/β, which control genes important for immune function (90-92). Given that it is increasingly recognized that heme is a dynamic and mobile molecule important for a number of signaling pathways (36,93-95), future work will involve elucidating the targets of heme signaling during inflammation.

What is the physiologically relevant concentration range and source of bioavailable heme and Hb in human control and AD brains? These unresolved questions would dictate the extent to which heme or Hb association to Aβ or heme-mediated immune signaling would occur in vivo. In the extreme case of hemolysis and hemorrhage, heme and Hb have been estimated to be in the 10-100 μM regime (35). Given estimates of brain [Aβ] span the pM to nM range (96,97) and the relatively tight heme-Aβ and heme-Hb interactions, K_D < 100 nM and K_D = 350 nM (Fig. 3c), it is likely that a significant fraction of Aβ is associated with heme and Hb in the AD microenvironment. An obvious source of heme and Hb during AD pathogenesis is from the vasculature since a weakened BBB is associated with AD. However, given the recent discovery of heme exporters (98,99), an intriguing alternative possibility is that astrocytes and/or other brain cells modulate neuroinflammation via control of extracellular heme export. The recent development of fluorescent (36,100,101) and activity (95) based heme sensors will be critical for elucidating brain heme homeostasis and the absolute concentrations of heme within cells and in the extracellular space in Alzheimer’s disease.

We close by noting that changes in heme and Hb represent only one aspect of a complex mosaic of factors in brain pathophysiology that affects Aβ aggregation and brain immune activity during Alzheimer’s disease. Indeed, many factors with the potential to modulate both Aβ aggregation and glial immune activity have been identified within Aβ plaques, including proteoglycans, cytokines,
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metals, apolipoprotein E, and proteases, among others (102). Nevertheless, there has been limited characterization of the effects of these molecules on Aβ speciation, and astrocyte and microglial immune activity. In this work, we have established an integrated methodology to elucidate the individual effects of particular AD-relevant molecules on astrocyte immune activity via 1) direct effects on astrocyte immune signaling and 2) effects due to physical association with Aβ or modulation of Aβ speciation. Our approach is readily generalizable and may yield broad new insights into the mechanisms promoting immune dysfunction in AD.

Altogether, our data indicate Hb and heme are potent modulators of astrocyte immune activity by dual mechanisms. The first is by direct signaling to astrocytes, mediated at least in part by the PI3K/Akt pathway. The second is by physical association with a highly inflammatory Aβ oligomer, whereby heme or Hb suppressed this inflammatory behavior. Given reports of increased Hb in late stage AD and in transgenic mouse models, Hb and heme signaling and physical activity represent possible mechanisms responsible for astrocyte fatigue in AD tissues, thereby permitting amyloid pathogenesis. Additionally, recent findings of BBB leakage early in AD suggest that Hb concentration may be locally increased at the vascular wall (103). By extension, Hb and heme activity may be responsible for a high prevalence (~90%) of cerebral amyloid angiopathy in AD patients (103). The recent report of heme specific single domain antibodies may represent a new therapeutic strategy to limit heme availability to Aβ (35). Further, our observation that rapamycin was able to partially restore astrocyte immune activity suggests that intervening in Hb/heme signaling represents a promising therapeutic strategy for AD. More broadly, our approach establishes a rigorous methodology to interrogate the immunomodulatory effects of diverse proteins, and other molecules that co-localize or associate with Aβ.

EXPERIMENTAL PROCEDURES

Recombinant Aβ1-42 Preparation

For all experiments, hexafluoroisopropanol (HFIP) pre-treated Aβ1-42 (rPeptide Watkinsville, GA) was diluted from stocks of 50 µM or 500 µM Aβ in 1% NH₄OH that were stored at -80°C. Prior to reconstitution, Aβ1-42 was retreated with 500 µL HFIP per milligram of Aβ1-42 overnight to prevent pre-aggregation. HFIP was evaporated prior to dilution in 1% NH₄OH.

Primary mouse astrocyte cultures

Astrocyte cultures were derived from postnatal day 0-1 CD1 mice (Charles River Laboratories) under a protocol approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee. Cortices were isolated following an existing protocol (104) and triturated in plating medium with a 1 mL sterile pipette tip. Plating medium consisted of MEM (Thermo Fisher) with 10% horse serum (Sigma), 1% antibiotic/antimycotic solution (Sigma), and 0.3% glucose solution (Sigma). Cells were left to attach overnight to T-75 flasks coated in 0.1 mg/mL poly-d-lysine (Sigma). After 24 hours, flasks were knocked to remove debris, rinsed with PBS, and plating medium was replaced with astrocyte medium (ScienCell) with 2% fetal bovine serum (ScienCell), 1% penicillin/streptomycin solution (ScienCell), and 1% astrocyte growth serum (ScienCell), in which cultures were maintained for up to four passages for conditioning. Cultures were maintained in a 37°C, 5% CO₂ humidified incubator.

SIM-A9 microglial cultures

SIM-A9 cells (American Type Culture Collection (ATCC), Manassas, VA) were cultured in DMEM:F12 (ATCC) supplemented with 10% heat inactivated bovine serum (Thermo Fisher) and 5% heat inactivated horse serum (Thermo Fisher). Cultures were maintained in a 37°C, 5% CO₂ humidified incubator.

RAW 264.7 macrophage cultures

RAW 264.7 cells (ATCC) were cultured in DMEM (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (Thermo Fisher) and 1% antibiotic/antimycotic solution (Sigma). Cultures were maintained in a 37°C, 5% CO₂ humidified incubator.

Cell conditioning and lysis

For cytokine expression, phospho-protein signaling, and western blot analyses, primary astrocytes were plated in 6 well plates and conditioned with combinations of hemin chloride (50 nM; EMD Millipore), human hemoglobin (50
nM; Sigma), rapamycin (10 nM; Selleck Chemicals, Houston, TX), and Aβ 1-42 (50 nM; rPeptide) in 1% w/v NH₄OH. For CD 36 analysis, cells co-conditioned with rapamycin were first pre-conditioned with rapamycin for 1 hr. Conditions were applied at 75% confluency, for 24 hours, after which conditioning medium was collected for cytokine analysis and cell lysates were collected for phospho-protein signaling and western blot analyses using the Bio-Plex cell lysis kit (Bio-Rad, Hercules, CA), with the addition of one cOmplete mini protease inhibitor tablet (Roche, Basel, Switzerland) and 20 µL phenylmethylsulfonyl fluoride (Sigma) per 5 mL of lysis buffer. Lysates were placed in microcentrifuge tubes and inverted at 4°C for 10 minutes. Lysates and medium were centrifuged at 4°C for 10 minutes at 13.2 kRPM and supernatant collected and stored at -80°C until analysis.

**Multiplexed phospho-protein and cytokine signaling analysis**

For phospho-protein signaling analysis, cell lysates were thawed on ice and centrifuged at 4°C for 10 minutes at 13.2 kRPM. Protein concentrations were determined using a Pierce BCA Protein Assay (Thermo Fisher) and normalized with Milliplex® MAP Assay Buffer (EMD Millipore) to 2 µg protein per 25 µL for Akt/mTor pathway analysis or 1 µg protein per 25 µL for MAPK pathway analysis. These protein concentrations were selected because they fell within the linear range of bead fluorescent intensity vs. protein concentration for detectable analytes. Multiplexed phospho-protein analysis was conducted for the Akt/mTOR pathway by adapting the protocols provided for the Milliplex® MAP Akt/mTOR 11-Plex (p-Akt, p-GSK3αβ, p-IGF1R, p-IR, p-IRS1, p-mTOR, p-p70S6K, p-PTEN, p-RPS6, and p-TSC2) and phospho-protein magnetic bead kits (EMD Millipore).

For cytokine signaling analysis, conditioned medium was thawed on ice and centrifuged at 4°C for 10 minutes at 13.2 kRPM. All samples were diluted 2:3 (conditioned medium to assay buffer), because this dilution fell within the linear range of bead fluorescent intensity vs. protein concentration for detectable analytes. Multiplex cytokine analysis was conducted by adapting the protocol provided for the Milliplex® MAP Mouse Cytokine/Chemokine 32-Plex Kit, with beads for Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIP-MAP, MIP-2, RANTES, TNF-α, and VEGF. Cytokine and phospho-protein signaling kits were read on a MAGPIX® system (Luminex, Austin, TX).

**Western blot analysis**

Cell lysates, obtained as described above, were thawed on ice then centrifuged for 10 min at 10,000 kRPM and 4°C. Protein concentration was determined using a Pierce BCA Protein Assay and equal amounts of protein were dissolved in reducing sample buffer, boiled, and loaded onto SDS polyacrylamide gels. Follow separation by electrophoresis, proteins were transferred to a Hybond P 0.45 µm polyvinylidene fluoride membrane (GE Healthcare, Piscataway, NJ). Membranes were blocked at room temperature (RT) for 1 hr with 5% milk in Tris buffered saline containing 0.01% Tween 20. Membranes were probed at 4°C overnight with rabbit anti-CD36 (1:500; Novus Biologicals, Littleton, CO), and mouse anti-α-tubulin (1:2000; Sigma). Membranes were then incubated with AlexaFluor conjugated secondary antibodies (1:2000; Thermo Fisher) for 2 hrs at RT. Imaging of blots was performed using an Odyssey CLx imager (LI-COR Biosciences, Lincoln, NE). Protein quantification was performed using Image Studio Lite 5.2 (LI-COR Biosciences).

**Aβ 1-42 internalization assay**

Primary astrocytes were plated in 0.1 mg/mL poly-d-lysine treated half-area 96 well, glass bottom plates at a density of 10,000 cells per well and maintained in a 37°C, 5% CO₂ humidified incubator. At 75% confluence, cells were conditioned with either 50 nM Aβ 1-42, 50 nM Aβ 1-42 plus 50 nM hemin chloride, or 50 nM Aβ 1-42 plus 50 nM human hemoglobin in astrocyte medium for 24 hours. Cells were fixed with 4% PFA, permeabilized for 10 min at RT with 0.1% Triton X-100, and blocked with a 5% BSA, 3% goat serum (Sigma) solution for 1 hr. Primary antibody incubation was performed overnight at 4°C, using the 6E10 antibody (1:200; BioLegend) in 0.5% BSA wash buffer. After washing with wash buffer, fixed cells were incubated with Alexa Flouro 488 goat anti-mouse secondary antibody (1:200; Thermo Fisher) for 2 hours at RT. Cells were co-
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stained with DAPI (100 ng/mL; Thermo Fisher) for nuclei and Alexa Fluor 555 Phalloidin (1:40; Thermo Fisher) for actin.

Confocal microscopy was performed on a Zeiss LSM 700 laser scanning inverted microscope to obtain 15-30 optical sections with 1-µm interval thickness. Orthogonal projections were rendered using Zen 2.3 software (Zeiss, Oberkochen, Germany).

**E.coli particle internalization assay**

Primary astrocytes or SIM-A9 microglia were plated on 96 well plates at a density of 10,000 cells per well and left to adhere overnight in a 37°C, 5% CO₂ humidified incubator. Cells were treated with either control, 50 nM hemin chloride or 50 nM human hemoglobin conditions for 4 hrs. Conditioning medium was aspirated and cells were incubated with the E.coli fluorescent BioParticle suspension from the Vybrant™ Phagocytosis Assay Kit (Thermo Fisher) for 1 hr. Extracellular fluorescence was quenched with trypan blue. Fluorescence was read on a SpectraMax M3 microplate reader (λex = 480 nm; λem = 520 nm) (Molecular Devices, Sunnyvale, CA).

**Phagocytosis assay**

Primary astrocytes or SIM-A9 microglia were plated in 0.1 mg/mL poly-d-lysine treated half-area 96 well, glass bottom plates at a density of 10,000 cells per well and left to adhere overnight in a 37°C, 5% CO₂ humidified incubator. Cells were treated with either control, 50 nM hemin chloride or 50 nM human hemoglobin conditions for 24 hrs. Conditioning medium was aspirated and cells were incubated with a fluorescent pHrodo™ Red Zymosan BioParticle suspension (Thermo Fisher) diluted in astrocyte medium for 2 hrs. After removing BioParticle suspension, cells were fixed with 4% PFA, permeabilized for 10 min at RT with 0.1% Triton X-100, and blocked with a 5% BSA, 3% goat serum (Sigma) solution for 1 hr. Primary antibody incubation was performed overnight at 4°C, with rabbit anti-GFAP (1:1000; Novus Biologicals). After washing with wash buffer, fixed cells were incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (1:200; Thermo Fisher) for 2 hours at RT. Cells were co-stained with DAPI (1 ng/mL; Thermo Fisher) for nuclei. Fluorescence microscopy was performed on a Zeiss Axio Observer Z.1 inverted microscope and quantified using ImageJ.

**Fluorescence assay for heme quantification**

Heme concentration in pellet fractions was determined by a fluorescence-based assay(105). Briefly, samples were boiled in the presence of 1 M oxalic acid to remove iron from the protoporphyrin ring of heme. Fluorescence of protoporphyrin IX was measured with excitation at 400 nm and emission at 662 nm on a Tecan Infinite 200 Pro plate reader. Heme concentration was determined by comparison with serial dilutions of heme standards quantified by UV/vis using the extinction coefficient of aqueous heme at 612 nm of 4431 cm⁻¹ M⁻¹ (36) on a Cary 60 UV/vis spectrophotometer.

**UV/visible spectroscopy**

For heme-Aβ₁₋₄₂ binding, freshly dissolved Aβ₁₋₄₂ was diluted to 100 µM in PBS and titrated into 500 nM heme in PBS. The UV/visible (UV/vis) spectral changes were monitored on a Cary 60 UV/vis spectrophotometer. The heme Soret band at 395 nm was plotted vs. Aβ₁₋₄₂ concentration and fit with the two-site binding model described in the Electronic Supplementary Material to determine apparent dissociation constant. For Hb-Aβ₁₋₄₂ binding, freshly dissolved Aβ₁₋₄₂ was diluted to 200 µM in PBS and titrated into 500 nM Hb. The heme Soret band of Hb at 412 nm was plotted vs. Aβ₁₋₄₂ concentration and fit with the one-site binding model described in the Electronic Supplementary Material to determine apparent dissociation constant.

**Circular dichroism spectroscopy**

Aβ₁₋₄₂ was freshly dissolved from frozen stocks at 20 µM in the presence or absence of 20 µM heme or 5 µM Hb in 1X PBS. Spectra were measured on a Jasco J-815 CD spectropolarimeter. Spectra were prepared from an average of 70 scans from 300 – 190 nm with 1 nm steps at 200 nm/min.

**Size exclusion chromatography**

Aβ solutions were incubated at 37 °C for 16-18 hours without agitation. To separate soluble and insoluble species,
Aβ samples were pelleted at 21,100 x g for 5 minutes to remove insoluble fibrillar material. The pellet was resuspended in 100 µL of 1X PBS for subsequent TEM analysis. The supernatant was collected and chromatographed over a Superdex 10/300 GL size exclusion column on an Agilent 1260 Infinity HPLC with an in-line photodiode array detector. Elution of Aβ was monitored by reading the absorbance at 220 nm and 280 nm, while elution of heme associated species were monitored by reading absorbance at 400 nm. The ratio of heme to Aβ in SEC fractions was determined by a fluorescence-based assay for heme detection (105). Quantification of Aβ in SEC or pellet fractions was accomplished by UV/vis spectroscopy using the peptide aromatic absorbance at 220 nm relative to serial dilutions of an Aβ stock solution. Ratio of Aβ and Hb in mixed Aβ/Hb SEC fractions was determined by immunoblotting for Aβ or Hb using 6E10 (1:5000; BioLegend) and H4890 (1:5000; Sigma) antibodies, respectively. Absorbance at 220 and 408 nm was then used to determine Aβ and Hb concentrations respectively.

Transmission Electron Microscopy
TEM images were taken on a Jeol 100 CXII transmission electron microscope operating at 100 kV. Aβ SEC and pellet samples of 25 µM or 100 µM Aβ1-42 alone or with 2x heme and 100 µM Aβ1-42 with 25 µM Hb were stained using 1% uranyl acetate on 400 mesh continuous Formvar-coated grids (Ted Pella, Redding, CA). Briefly, 2 µL of sample was placed on the grid for 1 minute and excess liquid was blotted away with filter paper. Next, uranyl acetate was added for 45 seconds and excess liquid was again removed by blotting with filter paper before the grid was allowed to air dry. Grids were then stored in a desiccator. Images were collected from across each grid. The distribution in morphology of various Aβ species were scored by counting species types in ~150-200 µm² areas for the pellet and ~100 µm² for the SEC fractions in two independent experimental trials.

Thioflavin T Assays
Aβ was freshly dissolved from frozen 1mg/mL 1% NH₄OH treated aliquots to 110 µM stock solution in 1X PBS for Thioflavin T (ThT) assays. A final concentration of 20 µM ThT and 6 µM AB was used to test for aggregation in the presence of heme or Hb. ThT fluorescence was monitored for 24 hours at 37 °C on a Tecan Infinite 200 Pro with excitation at 450 nm and 9 nm bandwidth and emission at 482 nm with a 20 nm bandwidth. For the fibril de-aggregation assay, Aβ at 110 µM in 1X PBS was allowed to aggregate into fibrils overnight at room temperature without agitation. At each time point, fibrils were then diluted to 6 µM in the presence or absence of heme and placed at 37°C. 10 µL of each sample was added to 200 µLs of 20 µM ThT. ThT fluorescence was monitored at various time points on a Biotek Synergy Mx with 5s shaking before measuring fluorescence at 435 nm excitation and 9 nm bandwidth and 486 nm emission with 9 nm bandwidth, as was reported previously for an Aβ and heme ThT assay (54).

Partial least squares regression
D-PLSR analysis was performed in MATLAB using the partial least squares algorithm by Cleiton Nunes available on the Mathworks File Exchange. All data was z-scored prior to inputting into the algorithm. For all analyses, an orthogonal rotation in the LV1-LV2 plane was performed in order to identify LVs that best separated conditions.

Statistics
All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Values are presented as mean ± standard error of the mean. Statistical significance was determined, as appropriate, using Student’s t-test, ordinary one-way analysis of variance (ANOVA) followed by Dunn’s or Sidak’s post hoc test, or Kruskal-Wallis ANOVA followed by Dunn’s post hoc test. Normality of data was tested using the Shapiro-Wilk test of normality. Levels of significance were set to *p< 0.05, **p< 0.01, ****p< 0.0001.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

L.B.W., A.R.R., S.B.S., R.K.D. conceived and designed the experiments and wrote the paper. S.B.S. conducted all astrocyte cell culture work, all Luminex analysis, and all PLSR analysis. R.K.D. conducted all biophysical analysis of the interactions between Aβ1-42 and heme or Hb. K.J.S. conducted microglial phagocytosis experiments.

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**FIGURES AND TABLES**

| Species          | Method of Isolation | Inflammatory Response     | Cytokine Panel   |
|------------------|---------------------|---------------------------|------------------|
| Insoluble Aβ     | Pelleted at 21,000xg| Moderately Inflammatory   | Figure S10a      |
| HMW Aβ Oligomers | SEC Peak at 8mLs    | Highly Inflammatory       | Figure 5a        |
| LMW Aβ Oligomers | SEC Peak at 19 mLs  | Not Inflammatory          | Figure S10a      |
| Insoluble Aβ + heme | Pelleted at 21,000xg | Not tested               | N/A              |
| Insoluble Aβ + Hb | Pelleted at 21,000xg| Moderately Inflammatory   | Figure S10b      |
| HMW Aβ w/heme or Hb | SEC Peak at 8mLs   | Not Inflammatory          | Figure 5a/5c     |

Table 1. Aβ species isolated and assayed for immunomodulation of astrocytes

Summary of Aβ1-42 species isolated from centrifugation and SEC. Indicated are the isolation method and immunomodulatory effects of each isolated species on astrocytes as determined by Luminex based cytokine profiling in Figs. 5 and Figure S10.
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Fig. 1 Heme and Hb modulate cytokine protein expression

a Quantification of 32 cytokines expressed into the medium of primary mouse astrocyte cultures via Luminex analysis. Each column is z-scored. Compared to vehicle control (0.001% NH4OH), cytokine expression is increased in response to 50 nM Aβ1-42. Moreover, co-conditioning of Aβ1-42 with either 50 nM heme or 50 nM Hb suppressed cytokine expression (n=3 wells).

b A discriminant partial least squares regression (D-PLSR) analysis, generated from the cytokine expression dataset, identified a latent variable (LV1), based on cytokine expression, which separates Aβ1-42-only treated astrocytes from all other conditions along the horizontal axis. LV1 depicts a linear combination of cytokines that correlate with the Aβ1-42-only condition, and identifies RANTES as the top correlate with Aβ1-42 treated astrocytes in the panel of 32 cytokines.

c LV2 depicts a linear combination of cytokines that correlate with the heme+ Aβ1-42 condition.

d Plotting LV1 scores for each group shows that the LV1 profile segregates Aβ1-42 treated astrocytes from all other conditions (n=3, p=0.0087; vehicle vs Aβ1-42).

f Plotting cytokine expression for 6 consistently measured cytokines, RANTES, M-CSF, IL-1β, IP-10, KC, and MCP-1 reveals that heme and Hb suppress expression of some, but not all cytokines. Data are presented as mean ± SEM. **p<0.01; ordinary one-way ANOVA with Dunnett’s post-hoc test.
Fig. 2 Heme and Hb modulate astrocyte internalization of Aβ₁₋₄₂ and phagocytic capacity

**a** Confocal imaging of primary astrocytes incubated with 50 nM Aβ₁₋₄₂ (left) stained with DAPI (blue), Alexa Fluor 555 Phalloidin (red), and anti-Aβ 6E10 (green) reveals Aβ₁₋₄₂ within the plane of the cell. Coincubation with 50 nM heme (center) or 50 nM Hb (right) reduce Aβ₁₋₄₂ internalization. Arrows indicate Aβ₁₋₄₂ localization inside the cell (left) or on the cell surface (center, right).

**b** Primary astrocytes, pre-incubated with 50 nM heme or 50 nM Hb, were incubated with trypan-quenched, fluorescein labeled, killed *E.coli* particles. Particle internalization, measured by fluorescent intensity using a microplate reader, significantly decreased upon incubation with 50 nM Hb (n=28 wells, p=0.0129; vehicle vs. Hb).

**c** Primary astrocytes, pre-incubated with 50 nM heme or 50 nM Hb, were incubated with pH sensitive pHrodo beads to assess phagocytic capacity. The percentage of total cells uptaking beads, quantified by fluorescence microscopy (Figure S4), was significantly reduced upon treatment with Hb (n=33 images; vehicle and Hb, n=33 images; heme, p=0.000025; vehicle vs. Hb).

**d** Primary astrocytes conditioned with vehicle control, 50 nM heme, and 50 nM Hb were lysed and analyzed via western blot for CD36 expression. Quantification, normalized by α-tubulin, reveals that both heme and Hb downregulate expression of CD36 (n=4 wells; p=0.0076; vehicle vs. heme, p=0.0269; vehicle vs. Hb).

**e** Western blot depicting CD36 expression.

Data are presented as mean ± SEM. *p< 0.05, **p< 0.01; ordinary one-way ANOVA with Dunnett’s post hoc test. ****p< 0.0001; Kruskal-Wallis ANOVA with Dunn’s post hoc test. Scale bars: 50 μm.
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Fig. 3 Heme and Hb bind to Aβ1-42

a The change in UV/vis spectrum of 500 nM heme in PBS upon the titration of 0-2 equivalents of Aβ1-42 indicates the formation of a 1:1 Aβ1-42:heme complex.
b The change in UV/vis spectrum of 500 nM heme in PBS upon the titration of 2-10 equivalents of Aβ1-42 indicates the formation of a 2:1 Aβ1-42:heme complex.
c The change in absorbance at 395 nm from the data in panels A and B is plotted as a function of the ratio of monomeric total [Aβ] to total [Heme] and fit to the 2-site binding model described in the Electronic Supplementary Material, revealing a $K_{D1} < 100$ nM and a $K_{D2} = 3 \mu$M.
d CD spectra of 20 µM Aβ (orange) and a 1:1 Aβ + heme mixture (green) after 3 hours of incubation at 37 ºC demonstrates that the β-sheet 2° structure of Aβ1-42 remains intact in the presence of heme.

e The change in UV/vis spectrum of 500 nM Hb in PBS upon the titration of Aβ1-42 indicates that one molecule of monomeric Aβ1-42 interacts with each monomer of tetrameric Hb.
f The change in absorbance at 412 nm from the data in panel E is plotted as a function of the ratio of monomeric [Aβ] to [Hb] monomer and fit to a 1-site binding model described in the Electronic Supplementary Material, revealing a $K_{D} = 380$ nM.
g CD spectra of 20 µM Aβ1-42 (orange), 5 µM Hb (red), a 1:1 mixture of Aβ and Hb (green), and the sum of the individual Aβ1-42 and Hb spectra (black).
Fig. 4 Effects of heme and Hb on Aβ1-42 oligomerization and morphology

a Representative TEM images of the Aβ1-42 species found in soluble and insoluble Aβ1-42 fractions. The particular images displayed are from the Aβ1-42 pelleted fraction (left panel) and Aβ1-42 + Hb pelleted fraction (right panel). The distinct fibril and aggregate types observed are indicated by the arrows and classified as: Type I (black), Type II (blue), Type III (red) and Type IV (green). Scale bars: 100 nm (left), 200 nm (right).

b Distribution of the Aβ1-42 species in two independent trials of the pelleted fractions of 25 µM Aβ1-42 (trial 1, left panel) or 100 µM Aβ1-42 (trial 2, left panel), 100 µM Aβ1-42 with 200 µM heme (trial 1, middle panel) or 25 µM Aβ1-42 with 50 µM heme (trial 2, middle panel), and 100 µM Aβ1-42 with 25 µM Hb (both trials, right panel).

c Overlays of size exclusion chromatograms of 100 µM Aβ1-42 (black) or 100 µM Aβ1-42 with 200 µM heme (red) after incubation for ~16 hours at 37 ºC and centrifugation to remove insoluble material. Elution was monitored at 220 nm (top) and 400 nm (bottom) for Aβ peptide and heme respectively. Black * denotes 8 mL fraction that associates with heme.

d Overlays of size exclusion chromatograms of 100 µM Aβ1-42 (black) or 100 µM Aβ1-42 with 25 µM Hb (red) after incubation for ~16 hours at 37 ºC and centrifugation to remove insoluble material. Elution was monitored at 220 nm (top) and 400 nm (bottom) for Aβ peptide and heme respectively. Black * denotes 8 mL fraction that associated with heme. Red * denotes the free Hb peak (Figure S7).

e Distribution of Aβ1-42 species in two independent trials of the SEC 8 mL fraction (denoted by black * in panels C and D) of 25 µM Aβ1-42 (trial 1, left panel) or 100 µM Aβ1-42 (trial 2, left panel), 25 µM Aβ1-42 with 50 µM heme (trial 1, middle panel) or 100 µM Aβ1-42 with 200 µM heme (trial 2, middle panel) and 100 µM Aβ1-42 with 25 µM Hb (both trials, right panel).
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**Fig. 5 Heme and Hb mediated immunomodulation of astrocytes is Aβ₁₋₄₂ species dependent**

a Heatmap of z-scored cytokine expression show upregulation of expression of 32 cytokines by primary mouse astrocytes upon incubation with high molecular weight (HMW) >75 kDa Aβ₁₋₄₂ aggregates, isolated via size exclusion chromatography (SEC), which is suppressed upon binding with heme (n=3). b Plotting cytokine expression of RANTES, GM-CSF, IL-1β, IP-10, KC, and MCP-1 shows reduction in relative expression of all six cytokines when SEC-isolated Aβ₁₋₄₂ species are bound with heme. c Heatmap of z-scored cytokine expression shows a broad suppression of cytokine expression by primary mouse astrocytes upon incubation with Hb-bound SEC isolated HMW Aβ₁₋₄₂ aggregates. d Plotting cytokine expression of RANTES, GM-CSF, IL-1β, IP-10, KC, and MCP-1 shows reduction in relative expression of all six cytokines when SEC-isolated Aβ₁₋₄₂ species are bound with Hb.
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Fig. 6 PI3K/AKT pathway signaling is modulated by heme

a D-PLSR analysis of astrocyte PI3K/Akt phospho-protein signaling identifies a latent variable (LV1) which separates the heme+Aβ1-42 condition from the heme-only condition along the horizontal axis, and second a latent variable (LV2) which separates all heme conditions from the vehicle condition along the vertical axis. b LV1 depicts a linear combination of phospho-proteins at the 5 and 15 min time points that correlate with the heme+Aβ1-42 or heme-only conditions. LV1 identifies upstream elements of the pathway, including p-PTEN, p-Akt, and p-TSC2 at 5 min as top correlates with the heme+Aβ1-42 condition. c LV2 depicts a linear combination of phospho-proteins at the 5 and 15 min time points that correlate with the heme and heme+Aβ1-42 conditions or the vehicle control. LV2 identifies p-mTOR at 15 min and p-IRS at 15 and 5 min as top correlates with both heme conditions. d Plotting LV1 scores for each group shows that the LV1 profile significantly segregates the heme+Aβ1-42 signaling effects from heme-only signaling effects (n=3, p=0.0022; heme vs. heme+Aβ1-42). e Plotting LV2 scores for each group shows that the LV2 profile significantly segregates all heme conditions from the vehicle control (n=3, p=0.0125; vehicle vs. heme, p=0.0275; vehicle vs heme+Aβ1-42). f Illustration of the PI3K/Akt signaling network, depicting nodes involved in mediating immunomodulatory and phagocytic functions. g CD36 expression in the presence of Aβ1-42 and heme, quantified by western blot (Figure S12), is recovered by treatment with rapamycin (n=4; heme+Aβ1-42, n=3, heme+rapamycin+Aβ1-42, p= 0.0022). Data are represented as mean ± SEM. For panel D: **p<0.01; ordinary one-way ANOVA with Dunnett’s post-hoc test. For panel E: *p<0.05; Kruskal-Wallis ANOVA with Dunn’s post-hoc test. For panel H: **p<0.01; Student’s t-test.
Heme and hemoglobin suppress amyloid β–mediated inflammatory activation of mouse astrocytes

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