Microfluidic Chemotaxis Platform for Differentiating the Roles of Soluble and Bound Amyloid-β on Microglial Accumulation

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Progressive microglial accumulation at amyloid-β (Aβ) plaques is a well-established signature of the pathology of Alzheimer's disease, but how and why microglia accumulate in the vicinity of Aβ plaques is unknown. To understand the distinct roles of Aβ on microglial accumulation, we quantified microglial responses to week-long lasting gradients of soluble Aβ and patterns of surface-bound Aβ in microfluidic chemotaxis platforms. We found that human microglia chemotaxis in gradients of soluble Aβ42 was most effective at two distinct concentrations of 23 pg.mL⁻¹ and 23 ng.mL⁻¹ Aβ42 in monomers and oligomers. We uncovered that while the chemotaxis at higher Aβ concentrations was exclusively due to Aβ gradients, chemotaxis at lower concentrations was enhanced by Aβ-induced microglial production of MCP-1. Microglial migration was inhibited by surface-bound Aβ oligomers and fibrils above 45 pg.mm⁻².

Better understanding of microglial migration can provide insights into the pathophysiology of senile plaques in AD.

Microglial cells are resident macrophages in the central neural system (CNS) and have multiple functions in normal and pathological brains. Ramified microglia are believed to constantly scan the brain and clear the CNS of toxic agents and debris. In the context of Alzheimer’s disease (AD), diminished clearance of Aβ due to impaired microglia is postulated to be one of the mechanisms of Aβ plaque formation. Once Aβ forms plaques, microglia become hyper-activated, accumulate around the plaques, and consequently secrete neurotoxic agents including neuroinflammatory mediators, reactive oxygen species, and free radicals. Understanding the mechanisms that lead to microglial accumulation and inflammatory responses may provide useful insights into developing strategies for AD treatment. However, several studies report different interactions between microglia and other factors. Previous in vivo studies of microglia migration were hampered by both the inability to observe long-term effects of Aβ on microglia and the complexity of senile plaques, which are deposits of oligomeric and fibrillar Aβ, surrounded by a “halo” of soluble oligomeric Aβ, activated glia, and dystrophic neurites. Activated microglial cells take on various morphologies: rounded, ramified shapes, rods to amoeboids, which complicate the visual tracking of these cells individually. Furthermore, previous in vitro attempts to study rat microglia migration in the presence of short lived, damaged axons, could not establish long-lasting gradients and were not conclusive for differentiating slow accumulation of microglia from random navigation.

Here, we have developed a novel microfluidic chemotaxis platform to study the motility of microglia responding to various types of Aβ in a regulated manner. The new platform models soluble and bound Aβ environments in AD brains and enables us to measure the distinct effects of both gradients of soluble Aβ, lasting longer than a week and surface-bound patterns of Aβ on the migration of microglia.

Results
A microfluidic platform provides a gradient of soluble Aβ and allows monitoring of microglial motility. Based on in vivo observation of microglial accumulation on Aβ plaques, we hypothesize that soluble Aβ is an
effective stimulus in recruiting microglia, while insoluble, surface-bound Aβ can capture the attracted microglia at the sites of surface-bound Aβ (Fig. 1a). To understand how microglial migration is regulated by Aβ in AD, we designed a unique microfluidic chemotactic platform composed of a large central reservoir (100 μL) supplying various types of soluble factors and two side reservoirs (100 μL each) containing medium. Human microglia were loaded in the annular (4 μL) compartment and their migration was observed towards the central (1 μL) compartment (Fig. 1b–c). The central and annular compartments were linked by migration channels (10 × 50 × 500 μm² in height, width, and length) forming gradients of soluble factors. Compared to a parallel configuration having equal surface areas for cell loading and cell collection, our axisymmetric configuration has a small central cell collecting compartment, which is smaller than a large annular cell loading compartment by a factor of 6. This enhances detection of recruitment. The long and thin migration channels can screen out unwanted entrance of inactivated microglia through mechanical constraints. Using this platform, we observed the migration of microglial cells, at single cell resolution, in real-time, for extended periods (Fig. 1d). We verified that refreshing media every two days allowed our experimental conditions to have a reasonable degree of physical and chemical stability of soluble Aβ forms: 90 and 95% of original Aβ gradients of monomeric and oligomeric soluble Aβ for up to nine days (Supplementary Fig. S1), 74 and 84% of the total amounts of monomeric and oligomeric Aβ solutions for two days (Supplementary Fig. S2). Thus, for most experiments, we refreshed media including soluble factors every two days. To evaluate the response of microglia to various types of Aβ, we tested 200 independent conditions at the same time, by imaging microglia in 8 plates, each with twenty-five platforms in an array format (Supplementary Fig. S3).

Directional migration of microglia towards soluble Aβ. To characterize the kinetics of microglial migration driven by soluble Aβ, we monitored individual microglial cells exposed to stable gradients of soluble Aβ for four days using time-lapse microscopy. We observed that in the first 24 hours after introducing soluble Aβ into a reservoir, microglia changed shape, became more elongated, and produced filopodia in random directions (Fig. 2a, Supplementary Movies S1). However, most of the cells remained stationary for the first 24 hours. Microglia started migrating on the second day of exposure to soluble Aβ, and migration was directional towards the soluble Aβ reservoir (Fig. 2b). In the absence of soluble Aβ, microglia expanded their bodies but did not migrate during four-day experiments (Supplementary Movies S2). We measured comparable effects of monomeric and oligomeric forms, and measured a maximal migration speed of 5.1 ± 4.2 μm.hr⁻¹ at 23 ng.mL⁻¹ soluble monomeric Aβ (Fig. 2c). We observed that microglia also entered the central compartment, the source of 2.3 ng.mL⁻¹ soluble Aβ monomers (Fig. 2d). Additional quantification of the effect of soluble Aβ on cellular distribution was performed by analyzing the changes in cellular density profile over time (Fig. 2e).

Figure 1 | Replication of AD-Aβ environment in a microfluidic chemotaxis platform. (a) We hypothesize that at early stages microglia are recruited by soluble Aβ (sAβ) (left) and at late stages co-localized with surface-bound Aβ (bAβ) near Aβ plaques and neurons in AD brains (right). (b), (c) A microfluidic chemotaxis platform models the pathological Aβ environments in AD brains and provides surface-bound Aβ inside the central compartment (CC) and gradients of soluble Aβ between the CC and the annular compartment (AC), along migration channels (MC). The axisymmetric configuration enhances the microglia counting during Aβ recruitment and the long and thin migration channels help avoid counting errors due to spontaneous migration. (d) Fluorescent images show human microglia (red) inside the AC and soluble Aβ (light green) and patterned bAβ (dark green) inside the CC. Microglia move in a gradient of sAβ between AC and CC, along the MC (left-lower inset). Microglia and bAβ co-localize in the CC after six days (right-upper inset). Scale bars, 200 μm.
To compare the effect of soluble Aβ in various forms and concentrations on microglial recruitment, we defined a recruitment index, R.I. (as defined in Supplementary Methods, Supplementary Fig. S7), representing the fraction of microglia cells recruited to the central compartment after loading soluble Aβ in the central compartment. We compared the R.I. values under various types of soluble Aβ, for eight days (Fig. 3a, Supplementary Fig. S4) and found that the R.I. value reached 5.0 ± 0.9 after exposure to monomeric synthetic Aβ23, at 23 ng.mL⁻¹ and 4.0 ± 1.0 after exposure to oligomeric forms at 230 ng.mL⁻¹ on ‘day 8’ (Fig. 3b). Measuring the concentration dependence of directional migration speed and R.I. revealed two peaks of activity at two concentrations of soluble Aβ that were three orders of magnitude apart (23 pg.mL⁻¹ and 23 ng.mL⁻¹) (Fig. 2c and Fig. 3c). Interestingly, the low peak concentrations correspond to the levels in normal and early AD brains, while the high peak to late AD brains³⁵.

**Microglial recruitment by human-derived soluble Aβ.** To validate the biological relevance of our platform, we measured the microglial response to gradients of soluble Aβ enriched or depleted samples derived from tris-buffered saline (TBS) extracts of human AD brains as previously described⁴⁴. For this purpose, low (8 to 20 kDa) and high molecular weight fractions (more than 100 kDa) were separated by size exclusion chromatography (Fig. 3d). Both low-molecular-weight soluble Aβ (LMW) and high-molecular-weight soluble Aβ (HMW) fractions were similar in recruiting microglia towards the central compartment on the eighth day (R.I. = 3.7 ± 0.5 for LMW and R.I. = 3.9 ± 0.6 for HMW) (Fig. 3e-f). Removing the soluble Aβ by immune-purification considerably reduced the microglial accumulation (R.I. = 2.7 ± 0.1 for LMW and 2.7 ± 0.7 for HMW), suggesting that a major factor of the TBS extract inducing microglial migration was Aβ.

**Self-promoting microglial recruitment by Aβ-induced MCP-1 secretion.** Because typical chemotactic responses of various cells display only one activity peak, we investigated further the mechanisms behind the double peak chemotactic activities of microglia in response to Aβ. To probe if a second chemoattractant may explain the unusual double chemotactic peak, we utilized a multiplexed cytokine assay to examine 27 cytokines expressed into the media collected from microglia cultured in the presence of soluble Aβ, at 2.3 pg.mL⁻¹ for two days. We identified five cytokines at detectable levels: IL-1ra, IL-6, IL-8, MCP-1, and MIP-1b while the remaining cytokines were either below, or just at the threshold of detectability (Fig. 4a). Arrows indicate saturated measurement of cytokines relative to the standard curve. Because MCP-1, MIP-1a, and MIP-1b are known chemoattractant molecules for macrophages³⁰,³¹, we measured microglial recruitment under gradients of soluble Aβ in addition to a mixture of neutralizing antibodies against MCP-1, MIP-1a, and MIP-1b and observed reduced activity at 23 pg.mL⁻¹ but not at 23 ng.mL⁻¹ of soluble Aβ (Fig. 4c). In control experiments, we validated that MCP-1 alone can promote microglial recruitment and verified that neutralizing antibodies can reduce microglial recruitment towards the single cytokine (Fig. 4d). We further measured the MCP-1 concentration in media from microglia stimulated with various concentrations of both monomeric and oligomeric soluble Aβ. We estimated the maximum secretion of MCP-1 at 1,433.9 ± 816.0 and 1,457.3 ± 109.4 fg.cell⁻¹ in the presence of 2.3 pg.mL⁻¹ of soluble monomeric and oligomeric Aβ, respectively and 554.4 ± 224.3 fg.cell⁻¹ in the absence of soluble Aβ (Fig. 4b). Interestingly, the MCP-1 secretion in the presence of 2.3 pg.mL⁻¹ Aβ was significantly higher than at other concentrations of Aβ oligomers. While the migration of microglia occurs at higher levels of the gradient (23 pg.mL⁻¹) than uniform Aβ (2.3 pg.mL⁻¹), the difference could be explained by the lower effective concentration at the initial region of a gradient. It is possible that MCP-1 plays a role in the peak microglial recruitment activity at a gradient of 23 pg.mL⁻¹ soluble Aβ, in addition to other reported roles in microglial-mediated neurodegeneration³².
Reduced microglial mobility and viability on Aβ fibril-coated surface. In AD brains, microglia presumably migrate towards plaques, and then remain stably associated with the plaques. To examine the mobility of microglia on surface-bound Aβ, we prepared wells uniformly coated with surface-bound Aβ fibrils and tracked individual microglia cells moving on these surfaces (Fig. 5a). We observed that the migration speed of microglia decreased substantially from 26 to 2 μm.hr⁻¹ with an increase of surface-bound Aβ concentration from 9 to 1,125 pg.mm⁻²; consistent with the possibility that surface-bound Aβ inhibited microglial migration (Fig. 5b). We also observed some of the microglia internalizing particles formed on Aβ fibril–coated surfaces, as well as dead cells (Supplementary Movies S3, S4). At the same time, the viability of microglia decreased substantially on highly concentrated surface-bound Aβ fibrils. Only 50% of microglia remained viable at 1,125 pg.mm⁻² after four days, suggesting acute toxicity of surface-bound Aβ fibrils to microglia.

MCP-1 secretion stimulated by surface-bound Aβ. The level of secreted MCP-1 was elevated also by bAβ about two-fold (1,128.0 ± 149.8 and 1,088.7 ± 378.2 fg.cell⁻¹) under Aβ oligomers and Aβ fibrils at 9 ng.mm⁻², respectively) compared to without Aβ (554.4 ± 96.0 fg.cell⁻¹) (Fig. 5c).

Microglial co-localization with patterned Aβ. To further understand how microglia target surface-bound Aβ–rich plaques, we patterned surface-bound Aβ42 patterns by calculating a localization index (L.I., defined in Supplementary Methods, Supplementary Fig. S8), representing the amount of localized cells on the patterned surface-bound Aβ relative to the day of microglia plating. We found that microglia co-localized with surface bound Aβ42 patterns at concentrations higher than 45 pg.mm⁻². We measured a L.I. = 1.4 ± 0.1 with oligomers and L.I. = 1.9 ± 0.5 with fibrils at 90 pg.mm⁻² on the eighth day (Fig. 5e–f).

Microglial recruitment and accumulation in the presence of soluble and patterned Aβ. To reconstruct the Aβ microenvironment analogous to that encountered by microglia near plaques, a core of fibrillary Aβ surrounded by a halo of soluble Aβ likely oligomers in the AD brains, we combined patterned surface-bound Aβ42 by using PDMS stencils of 2 mm holes and the gradient of soluble Aβ42 in the microfluidic platform (Fig. 5g, Supplementary Fig. S6). The combination of a gradient of soluble Aβ42 oligomers at
5 nM and surface-bound Aβ fibrils at 90 pg.mm⁻² achieved the most effective localization by 2.5 times compared to 1.6 times with surface-bound Aβ fibrils at 90 pg.mm⁻² only (Fig. 5h–i).

Discussion
Our study demonstrates that soluble monomeric and oligomeric Aβ serve as a “recruiting signal” and bound fibrillar and oligomeric surface-bound Aβ acts as a “targeting signal” during microglia recruitment and localization. Together, soluble and insoluble Aβ have synergistic effects on microglial accumulation to sites of Aβ deposits, and could explain microglial accumulation in the vicinity of Aβ plaques in the AD cortex.

Moreover, the MCP-1 dependent mechanism of microglia recruitment in response to lower doses of Aβ may be important during physiological neuroinflammation and could be relevant to the early stages of microglial activation in AD. The microfluidic platform can be extended to the study of migration of other cells relevant to the progression of neurodegenerative diseases, and could help quantify the potency of various cytokines and chemokines, which have been detected in the brain alone and in combinations, in modulating microglia recruitment and accumulation. With high-throughput capabilities and regulated microenvironments, our platforms can facilitate systematic monitoring of microglial migration and its modulation by compounds for the treatment of neuroinflammation in various disease conditions, including Alzheimer’s disease.

Methods
Microfluidic platform fabrication. Negative photoresists, SU-8 50 and SU-8 100 (MicroChem, Newton, MA, USA), were sequentially patterned using standard lithography on a 4” silicon wafer to create a mold for cell migration channels of 50 μm in height and chemokine compartments of 100 μm in height. A mixture of a base and a curing agent with a 10:1 weight ratio (SYLGARD 184 A/B, Dowcorning, Midland, MI, USA) was poured onto the SU-8 mold and cured for one hour at room temperature under vacuum and, subsequently, cured for more than 3 hours in an oven at 80 °C. The cured polydimethyl-siloxane (PDMS) replica was peeled off from the mold and holes were punched for fluid reservoirs. Arrayed holes were also laser-cut (Zing 24, Epilog Laser, Golden, CO, USA) into a thin PDMS membrane of 250 μm in thickness (HT 6240, Bisco Silicones, Elk Grove, IL, USA) and an acrylic plate of 6 mm in thickness. The machined membrane and the plate were glued together using uncured PDMS and incubated at 80 °C overnight. This assembly was irreversibly bonded first to the PDMS replica using oxygen plasma at 50 mW, 5 ccm, for 30 seconds (PX-250, March Plasma Systems, Petersburg, FL, USA), and later to a glass-bottomed UniWell plate (MGB001-1-2-LG, Matrical Bioscience, Spokane, WA, USA). Immediately after the bonding, 10 μL of poly (l-lysine) solution (PLL, M.W. 70,000–150,000, 1.0 mg.ml⁻¹ Sigma-Aldrich Co. LLC, St. Louis, MO, USA) was injected into the each platform and incubated for 2 hours at a room temperature to promote cellular adhesion. PLL-treated surface was rinsed with autoclaved and 0.2 μm filtered water (AM9920, Life Technologies, Grand Island, NY, USA) and then

Figure 4 | MCP-1 secretion from microglia under stimulation of soluble Aβ and self-promoted microglial recruitment. (a) Microglial cells are cultured in wells containing sAβ42 and discernibly expressed five cytokines (MCP-1, MIP-1b, IL-1α, IL-6, and IL-8) are measured among tested twenty-seven human cytokines from extracted solutions. (b) Highest levels of a cytokine, MCP-1 are measured when microglia are cultured under sAβ42 in monomers and oligomers at 2.3 pg.ml⁻¹. (c) Microglial cells are cultured in the presence of gradients of cytokine-neutralizing antibody (Ab) combined with sAβ42 or cytokines and reduction of recruitment index is measured in the presence of neutralizing antibody against MCP-1 and sAβ42 at 23 pg.ml⁻¹ but not 23 ng.ml⁻¹. (d) MCP-1 is validated to be a potent chemoattractant for microglia and the recruitment is inhibited by immune-neutralization of MCP-1. However, MIP-1α and MIP-1β have no microglial chemoattractant activity in this assay. (Student’s t-test. *P < 0.01 for oligomers with respect to no sAβ42). n well = 3, n cell = 2,000 in (a), (b) and n platform = 4, n cell = 2,500 in (c), (d) for each condition. Data represent mean ± s.e.m.
the devices were filled with cell culture medium containing 50% DMEM: F-12 supplemented with 5% FBS (Invitrogen, Grand Island, NY, USA), 10 ng.mL⁻¹ of M-CSF (AF-300-25, PeproTech Inc., Rocky Hill, NJ, USA), 25 µg.mL⁻¹ of gentamicin (G1397, Sigma-Aldrich), and 2.5 µg.mL⁻¹ of amphotericin (A2411, Sigma-Aldrich).

Cell preparation. Human microglial cells (HMG 030, Clonexpress, Inc., Gaithersburg, MD, USA) were isolated initially as a free-floating population of cells from fetal brain tissue samples digested with collagenase and grown in a proprietary medium for 1–2 weeks. Before the experiment, cells were washed using medium without serum and the cell membrane was labeled with red fluorescent dye (PKH26PCL, Sigma-Aldrich). After centrifugation (400 g for 5 minutes), the cell pellet was re-suspended in 1 mL of Dulbecco’s phosphate-buffered saline (G8278, Sigma-Aldrich) and immediately mixed with 4 µL of dye solution (P9691, Sigma-Aldrich) in 1 mL of Dulbecco’s medium. The cell dye mixture was incubated at room temperature for 4 minutes and periodically mixed by pipetting in order to achieve a bright, uniform, and reproducible labeling. After the incubation, the staining was stopped by adding an equal volume (2 mL) of 1% BSA in PBS and incubating for 1 minute to remove excess dye. Unbound dye was washed by centrifuging and re-suspending the cells in the culturing medium. Finally, the stained microglia cells were suspended in the culturing medium at the concentration of 1 M cells.mL⁻¹. Ten µL of cell solution was injected into each platform and 100 µL of a culturing medium was added into side and central extra wells. The loaded cells were incubated at 37 °C supplied with 5% CO₂ for at least 12 hours.
two days before adding the various Aβ solutions. In the experiments, the cell culture media and Aβ solutions were replaced every two days while collecting the used solutions for Aβ analysis.

**Sourcing of human brain tissue.** Brains from human subjects with a diagnosis of Alzheimer’s disease were obtained through the Massachusetts Alzheimer’s Disease Research Center. The Massachusetts Alzheimer’s Disease Research Center (ADRC) serves, among other clinical and research activities, as a repository of samples from patients with Alzheimer’s disease. ADRC clinical and research activities are reviewed among others by the Institutional Review Board at the Massachusetts General Hospital. In addition, the ADRC has a Certificate of Confidentiality issued by the US Department of Health & Human Services/National Institutes of Health to protect the privacy of individuals who are enrolled in this research against forced disclosure in any civil, criminal, administrative, legislative or other proceeding, either at the federal, state of local level. The human samples used in this study were anonymised. The Institutional Review Board at the Massachusetts General Hospital viewed the use of anonymized autopsy material for biochemistry assays as exempt from review.

**Aβ extraction from human brain tissues.** Cortical gray matter from frontal lobe of AD patient brains was homogenized in 5 volumes of TBS (Tris-buffered saline with protease inhibitor cocktail (Roche)) with 25 strokes on a mechanical Dounce homogenizer and centrifuged at 260,000 × g for 30 min at 4 °C. The supernatant was used as a TBS-soluble fraction27,28. 750 µl of TBS-soluble fraction of human brains was separated by a size exclusion chromatography on double superdex 75 columns (GE Healthcare Life Sciences, Piscataway, NJ, USA) in 50 mM ammonium acetate pH 8.5 with an AKTA purifier 103,30. The individual fractions separated by SEC were analyzed by immunoblotting and Aβ specific sandwich ELISA and fraction 6 to 9 (>100 kDa) was used as a HMW Aβ fraction and fraction 30 to 33 (20–8 kDa) was used as a LMW Aβ fraction. Immediately after the size-exclusion chromatography, we removed soluble Aβ including soluble alpha Aβ precursor protein (APP) from the fractions by immune-depleting with 6E10 antibody (Signet, Dedham, MA, USA).

**Time-lapse imaging.** For continuous time-lapse imaging to track individual cells, we kept the UniWell plate on an automatically microscope (Eclipse Ti, Nikon Inc., Melville, NY, USA) integrated with a heated incubating stage (LiveCell 05-11-0032 Rev B, Pathology Devices Inc., Westminster, MD, USA), which was set at 37.5 °C, 5% CO2, and 85% humidity. We imaged cells at every 1-hour (NLS Elements, Nikon Inc.) using a bright field and every 6-hour using a TRITC fluorescent microscope for 4 days with a 10× objective lens and a perfect focusing system in a phase contrast mode. For continual time-lapse imaging to count cells, we kept the plate in an incubation chamber set at 37.5 °C and 5% CO2 and then imaged it using 4× objective lens in a large area-mode of 8 × 8 mm2 with a 15× stitching, a phase contrast mode, and a TRITC fluorescent microscope every two–three days for seven days. For continual time-lapse imaging to monitor gradient stability, we kept the plate at room temperature in a dark room, and imaged using 4× objective lens in a large area-mode of 12 × 10 mm2 without stitching, a phase contrast mode, and a TRITC fluorescent microscope every two–three days for nine days.

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**Authors contributions**

H.C., L.Z., B.T.H. and D.I. conceived the microfluidic platforms. H.C. and T.H. performed experiments and analysed data. T.H. prepared and analysed human-derived soluble Aβ. H.C. and E.W. developed the algorithms for analyzing microglial mobility. Y.H. executed ELISA, western blot assays, EM imaging, and analyzed data. L.B.W. and K.M.H. executed a multiple cytokine assays and analyzed data. H.C., L.B.W., B.T.H. and D.I. wrote the manuscript.

**Additional information**

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Competing financial interests: The authors declare no competing financial interests.

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