Identification of a dysfunctional microglial population in human Alzheimer’s disease cortex using novel single-cell histology image analysis

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Abstract
In Alzheimer’s disease (AD), microglia are affected by disease processes, but may also drive pathogenesis. AD pathology-associated microglial populations have been identified with single-cell RNA-Seq, but have not been validated in human brain tissue with anatomical context. Here, we quantified myeloid cell markers to identify changes in AD pathology-associated microglial populations. We performed fluorescent immunohistochemistry on normal (n = 8) and AD (n = 8) middle temporal gyri, co-labelling the pan-myeloid cell marker, Iba1, with one of 11 markers of interest (MOIs): CD45, HLA-DR, CD14, CD74, CD33, CD206, CD32, CD163, P2RY12, TMEM119, L-Ferritin. Novel image analyses quantified the single-cell abundance of Iba1 and each MOI. Each cell was gated into one Iba1-MOI population (Iba1low MOIhigh, Iba1high MOIhigh, or Iba1high MOIlow) and the abundance of each population was compared between AD and control. Triple-labelling of L-Ferritin and Iba1 with a subset of MOIs was performed to investigate L-Ferritin-MOI co-expression on Iba1low cells. Iba1low MOIhigh myeloid cell populations delineated by MOIs CD45, HLA-DR, CD14, CD74, CD33, CD32, and L-Ferritin were increased in AD. Further investigation of the Iba1low MOIhigh populations revealed that their abundances correlated with tau, but not amyloid beta, load in AD. The Iba1low microglial population highly expressed L-Ferritin, reflecting microglial dysfunction. The L-Ferritinhigh CD74high HLA-DRhigh phenotype of the Iba1low population mirrors that of a human AD pathology-associated microglial subpopulation previously identified using single-cell RNA-Seq. Our high-throughput immunohistochemical data with anatomical context support the microglial dysfunction hypothesis of AD.

Keywords: Microglia, Alzheimer’s disease, Tau, Amyloid beta, Dysfunction, Immunohistochemistry, Single-cell analysis

Background
Alzheimer’s disease (AD) pathology is characterised by the accumulation of amyloid beta plaques and hyperphosphorylated tau tangles [1]. The aggregation of amyloid beta proteins precipitates inflammatory reactions by microglia, the innate immune cells of the central nervous system [2, 3]. Genetic risk factors for AD include loss-of-function mutations in microglia-specific genes, including CD33 and TREM2, which are associated with reduced microglial phagocytic capacity [4–7]. Such microglial-specific genetic risk factors for AD suggest that microglial reactions are not only a result of disease processes, but may drive disease pathogenesis.

Changes in microglial function may impact the development of AD through different processes. The pro-inflammatory microglia hypothesis that posits that deposition of amyloid beta and subsequent phagocytosis by microglia drive chronic microglial activation, making...
them ‘neurotoxic’ [8, 9]. Alternatively, the microglial dys-function hypothesis proposes that AD pathogenesis is modified by loss of normal microglial function, encompassing both dysregulated microglial activation and loss of microglial ‘neurosupportive’ function [10–14]. With two disparate hypotheses, it is unclear whether microglial activity should be enhanced or reduced in order to reduce AD pathology, although it is also possible that both hypotheses are correct but reflect different stages of progression.

Indeed, the traditional dogma of microglia existing in either a pro-inflammatory ‘neurotoxic’ or anti-inflammatory ‘neurosupportive’ state in the human brain oversimplifies the heterogeneity of microglial reactions [15]. Microglial heterogeneity in the normal and diseased brain is now being characterised using novel technologies, including single-cell and single-nuclei RNA sequencing [16–19]. AD pathology-associated microglial subpopulations with unique transcriptomic signatures have been identified, including a population with enriched expression of FTL (L-Ferritin), HLA-DRA, HLA-DRB1, CD14, and CD74 [16, 17] (Additional file 1: Table S1). However, these studies require the dissociation of cells from whole tissue. As such, they are unable to resolve whether changes in microglial populations are driven by human AD pathology or quantify the anatomical location of microglia with unique expression profiles relative to amyloid beta and tau in the brain.

By contrast, immunohistochemical studies in the post-mortem human brain have identified significant changes in the expression of single proteins in microglia associated with amyloid and tau pathology, including HLA-DR, CD45, CD14, CD32, CD163, and L-Ferritin, which are proposed to alter microglial function and influence disease pathogenesis [6, 16, 17, 20–51] (Additional file 1: Table S1). Despite growing evidence linking microglial changes to AD pathogenesis, a recent systematic review revealed marked differences in microglial protein changes in post-mortem human AD tissue between studies (for review see Hopperton et al. [48]). The most consistent changes identified include increases in classical ‘activation’ proteins, like HLA-DR and CD68, while numerous other proteins with more specific functions show high variability between studies or are infrequently assessed [48].

A key contributor to this variability between immunohistochemical studies is the methodology employed to quantify microglial protein abundance. In this study, we developed a novel single-cell image analysis pipeline to investigate multiple myeloid cell markers previously identified as altered in the human AD brain or enriched in an AD pathology-associated microglial population. The markers included proteins more highly expressed by, or specifically expressed by, perivascular macrophages. Because all myeloid cell markers selected for investigation in this study have significance to AD, we have collectively termed them markers of interest (MOI). The advantages of our approach are identification of multiple key microglial proteins, quantification of their abundance at the single cell level, and retaining anatomical context allowing correlation of marker abundance in proximity to amyloid and tau.

Specifically, we investigated the single cell abundance of eleven MOIs (Additional file 1: Table S1) across microglial and perivascular macrophage populations in immunohistochemically stained normal and AD cortex; CD45, HLA-DR, CD14, CD74, CD33, CD206, CD32, CD163, P2RY12, TMEM119, and L-Ferritin. Changes in protein expression were quantified at the single cell level and reflect not only any overall microglial changes in AD, but changes in specific populations associated with the AD pathology markers, amyloid beta, and tau. This work demonstrates a clearer picture of multiple microglial population changes in AD human brain.

**Methods**

**Human tissue selection**

Formalin-fixed middle temporal gyrus (MTG) blocks from eight neurologically normal and eight AD cases from the Neurological Foundation Human Brain Bank were used in this study (Table 1). The AD cases had a history of dementia and were considered at least intermediate AD, based on their National Institute of Aging-Alzheimer’s Association (NIA-AA) ‘ABC’ score. Cases with Lewy body disease or other neurodegenerative pathology were excluded. Normal cases had no previous history of neurological disorders and cause of death was unrelated to any neurological condition. An initial independent pathology assessment reported no other disease pathology aside from normal age-related amyloid presence. The eight normal cases were included in the analysis based on this first pathology assessment. A more comprehensive pathology assessment, including determination of the ‘ABC’ scores, was carried out on all normal cases following our analysis. Of the eight normal cases, seven showed no or low AD neuropathologic changes. However, one normal case (H187) showed an intermediate AD neuropathologic change in spite of the initial pathology report suggesting pathological changes were in the normal range for its age. Given the changes were not high AD changes, the relatively older age of this case, and the lack of neurological...
Table 1  Human cases used for this study

| Case  | Age (years) | Sex | Post-mortem delay (h) | NIA-AA staging (AD neuropathologic change) |
|-------|-------------|-----|-----------------------|-------------------------------------------|
| Normal |             |     |                       |                                           |
| H169  | 81          | Male| 24                    | A2 B0 C1 (none)                           |
| H180  | 73          | Male| 33                    | A2 B0 C1 (none)                           |
| H187  | 98          | Female| 15                   | A2 B2 C1 (intermediate)                   |
| H191  | 77          | Male| 25                    | A0 B0 C0 (none)                           |
| H196  | 85          | Male| 15                    | A1 B0 C0 (none)                           |
| H229  | 88          | Female| 17                   | A2 B1 C2 (low)                            |
| H243  | 77          | Female| 13                   | A0 B0 C0 (none)                           |
| H246  | 89          | Male| 17                    | A0 B1 C0 (none)                           |
| Mean  | 83.5 ± 9.1  | –   | 19.3 ± 6.5             | –                                         |
| AD    |             |     |                       |                                           |
| A299  | 94          | Female| 8.5                  | A3 B3 C2 (high)                           |
| A102  | 84          | Female| 14.5                 | A3 B2 C2 (intermediate)                   |
| A107  | 86          | Male| N/A                   | A2 B3 C1 (intermediate)                   |
| A108  | 94          | Female| 11.5                 | A3 B3 C2 (high)                           |
| A109  | 90          | Female| 31                   | A3 B2 C1 (intermediate)                   |
| A110  | 86          | Female| 15                   | A3 B3 C2 (high)                           |
| A113  | 77          | Male| 3.5                   | A3 B3 C2 (high)                           |
| A119  | 89          | Male| 5                     | A3 B2 C2 (intermediate)                   |
| Mean  | 87.5 ± 5.6  | –   | 12.7 ± 9.2             | –                                         |

Symptoms at time of death, we have maintained the normal classification of this case for our analysis.

**Free-floating immunohistochemistry**

All MOIs investigated in this study were co-labelled with the pan-myeloid cell marker, Iba1, ensuring that functional changes across microglial and perivascular macrophage populations were assessed. As part of the Neurological Foundation Human Brain Bank procedures, the MTG is cut into 4 blocks, of which we used one for this study; typically block 1 or 2 to ensure we were sampling from the middle of the MTG in all cases [52]. For Iba1 co-labelling with MOIs, three 50-μm thick MTG sections approximately 800 μm apart were selected. Free-floating fluorescent immunohistochemistry was performed as previously described [53, 54, 57]. Sections were incubated in Tris–EDTA pH 9.0 for antigen retrieval, and subsequently in primary antibodies against Iba1 and an immunophenotype MOI (Additional file 1: Table S2). Iba1 was visualised using AlexaFluor® 594- or 647-conjugated secondary antibody and immunophenotype MOIs were visualised using AlexaFluor® 488- or 594-conjugated secondary antibody or the AlexaFluor® 488 TSA protocol (Additional file 1: Table S2). For AD pathology load analysis, two MTG sections approximately 800 μm apart were selected per pathology stain. Formic acid antigen retrieval was performed to unmask aggregate epitopes. Sections were incubated in anti-amyloid beta and anti-tau antibodies, visualised using species-specific AlexaFluor®-conjugated secondary antibodies. Nuclei were counterstained with Hoechst. Sections were mounted using PBS and coverslipped using ProLong® Diamond Antifade mounting media.

For representative images demonstrating each MOI co-labelling with Iba1, sections were imaged on an Olympus FV1000 confocal microscope (60× oil). Optical z-stacks were taken through the entirety of the cell body and processes. For quantification, MTG grey matter images were acquired for each section using a Nikon Ni-E microscope (20×, 0.5 NA) with Nikon DS-Ri2 and Qi2 cameras and a motorised stage. Regions of interest (ROIs) encompassing grey matter layers I–VI were imaged based on the Hoechst counterstain. These images were used for (1) the tissue-wide integrated intensity analysis, (2) the single-cell Iba1-MOI analysis for both Iba1-MOI and Iba1-L-Ferritin-MOI co-labelling, and (3) the AD pathology load analysis.

**Paraffin immunohistochemistry**

For the amyloid beta spatial analysis, two sequential 10-μm thick MTG sections were selected from AD cases and received two immunohistochemical staining rounds; Iba1-L-Ferritin/HLA-DR, then amyloid beta/tau. Paraffin immunohistochemistry was performed as previously described [58]. Tris–EDTA pH 9.0 antigen retrieval was performed, and sections incubated in primary then AlexaFluor®-conjugated secondary antibody mixtures. Nuclei were counterstained with Hoechst. Sections were mounted using PBS and coverslipped using ProLong® Diamond Antifade mounting media. After this first round of immunohistochemistry, sections were imaged on a Zeiss Z2 Axioimager (20×) using MetaSystems VSlide acquisition software and MetaCyte stitching software. Sections were then de-coverslipped for AD pathology staining as described above. Sections were imaged after this second round of immunohistochemical staining using the same imaging system.

Images from both staining rounds were opened on VSViewer v2.1.112 and individual channel images extracted from three areas of grey matter per section. Hoechst-stained nuclei in each labelling round were used as intrinsic markers for image registration. Hoechst images were first pre-processed (smoothed) in ImageJ (v1.52p) by applying a 50-pixel rolling background subtraction and a 5-pixel median filter. The processed nuclear images were then registered to each other using a custom-designed Python code. Jupyter Notebook was used to implement an AKAZE affine registration, and a transformation matrix was extracted and applied to all
the individual images in the set [55]. These six channel images were used for the amyloid beta spatial analysis.

**Quantification using MetaMorph custom image analysis pipelines**

Four novel custom image analysis journals were developed in MetaMorph software (Molecular Devices) to quantify Iba1-MOI populations with respect to AD pathology in normal and AD MTG. Journals for tissue-wide integrated intensity analysis, single-cell Iba1-MOI analysis, and amyloid beta spatial analysis are detailed in Additional file 1: Figure S1. All image analysis journals, and validation of their accuracy, are described in detail in the supplementary materials.

The tissue-wide integrated intensity analysis measured the integrated intensity of each MOI within all Iba1-MOI cells in the MTG. Binary masks of both the Iba1 staining and the MOI staining were generated using an adaptive threshold tool and combined to create a “master mask” containing all Iba1-MOI cells. MOI integrated intensity within the master mask was measured. MOI integrated intensity data were normalised to the region of interest tissue area and are thus equivalent to the concentration of the MOI in the tissue.

The single cell Iba1-MOI analysis measured the single-cell average intensity of Iba1 and the MOI from each Iba1-MOI co-label. The Iba1-MOI “master mask” described above was used. Each object within the master mask was considered a ‘cell’ and the average intensity of Iba1 and the MOI measured in each cell. Average intensity per Iba1-MOI cell is equivalent to the protein concentration per cell. Iba1 and MOI average intensities were plotted on an XY scatter plot. All Iba1-MOI cells from all AD and normal cases were pooled and Iba1-MOI populations segregated using a freehand tool, generating gates. Three Iba1-MOI populations were gated; Iba1low MOIhigh, Iba1high MOIhigh, and Iba1high MOIlow. The three population gates were transferred from the pooled Iba1-MOI plot to the xy scatter plot for each normal and AD case. The abundance of cells in each Iba1-MOI population (as a proportion of total Iba1-MOI cells per case) and the mean population intensity of each MOI were measured. Changes in the proportions of cells within each of the three Iba1-MOI populations reflected changes in Iba1 and MOI expression relative to one another. There was no difference in the overall number of Iba1-MOI cells between normal and AD cases (Additional file 1: Table S3). The mean intensity of each MOI population, equivalent to mean fluorescence intensities for flow cytometry data, was used to identify changes in MOI expression parallel to changes in Iba1 expression.

The AD pathology load analysis measured the load of amyloid beta and tau in the MTG. Binary masks of amyloid beta and tau staining were generated using the adaptive threshold tool, and the area of each mask was measured. Data are presented as the mean percentage area of amyloid beta or tau staining across the region of interest against the abundance of each Iba1low MOIhigh population.

The amyloid beta spatial analysis was a modified version of the single-cell Iba1-MOI analysis. Iba1 and MOI average intensity, and distance between each Iba1-MOI cell and its nearest amyloid beta plaque, was measured. Within each cell in the master mask, the intensity of a binary mask of amyloid beta binary was measured. If the intensity of the amyloid beta mask within a cell was above 0, the cell was on or interacting with an amyloid beta plaque and assigned as ‘plaque’. The amyloid beta master mask was subsequently dilated circularly in 5 µm increments. After each dilation, the intensity of the dilated amyloid beta mask was measured within each cell in the master mask. The dilations were discontinued at 50 µm. The distances were grouped as “plaque”, “plaque-adjacent” (up to 50 µm from a plaque) or “non-plaque” (greater than 50 µm from a plaque).

**Statistical analysis**

F test of equality of variances and the Shapiro–Wilk normality were used to determine whether the variances between the two groups being compared were equal and if the data were normally distributed, respectively. If the variances were equal and the data were normally distributed, parametric tests were used. The student’s t test was used to compare between two groups, while the Pearson correlation was used for correlations of measured values with disease pathology, age, and post-mortem delay. The effect of sex was tested by one-way ANOVA with Holm–Sidak’s multiple comparisons. For non-parametric data, the Mann–Whitney test was used to compare between two groups, while the Spearman correlation was used for correlations of measured values with disease pathology, age, and post-mortem delay. The effect of sex was tested by the Kruskal–Wallis test with Dunn’s multiple comparisons. There were no significant correlations of any measured values with age, post-mortem delay, or sex in the normal or AD groups. Thus, these data were not discussed further. All data are presented as mean ± standard deviation, with statistical significance set at p ≤ 0.05. Correlations were considered strong if r ≥ 0.8 and moderate if 0.8 ≥ r ≥ 0.7.
Results
An Iba1<sup>low</sup> myeloid cell population is more abundant in the AD temporal cortex.

We co-labelled Iba1 with one of 11 myeloid cell markers using immunohistochemistry on normal and AD MTG: CD45, HLA-DR, CD14, CD74, CD33, CD206, CD32, CD163, P2RY12, TMEM119, or L-Ferritin. All 11 myeloid cell markers were specifically expressed by Iba1-positive cells in both the normal and AD MTG (Fig. 1). Before performing more comprehensive analyses, we...
measured the total integrated intensity across the sections to determine changes in tissue-wide expression in AD (Additional file 1: Figure S2). Of the 11 MOIs, only the expression of CD45 was significantly increased in AD (Additional file 1: Figure S2).

Given that microglial sub-populations with unique transcriptomic signatures have been identified in human AD brain tissue with single-cell RNA sequencing technologies [16–19], we sought to investigate microglial protein expression changes in immunohistochemically stained tissue at a single-cell level. We developed a custom MetaMorph image analysis pipeline which allowed for single-cell measurements of Iba1 and MOI staining intensity in post-mortem human tissue (Fig. 2A). The individual cell expression data was treated like FACS data (Fig. 2): for each Iba1-MOI co-label, we plotted all Iba1-MOI cells on an XY scatter plot based on their Iba1 and MOI average intensities and manually gated Iba1-MOI populations. For 10 of the 11 MOIs (CD45, HLA-DR, CD14, CD74, CD33, CD206, CD32, CD163, P2RY12, and TMEM119), three Iba1-MOI populations were identified: 1. Iba1low MOIhigh, 2. Iba1high MOIhigh, and 3. Iba1high MOIlow (Fig. 2B, D, F, H, J, L, M, O, Q, and S). In contrast, for L-Ferritin only two Iba1-L-Ferritin populations were identified (labelled as 1 and 3): 1. Iba1low L-Ferritinhigh and 3. Iba1high L-Ferritinlow (Fig. 2U). This finding indicates that the Iba1low population was best delineated by high L-Ferritin expression: if a cell expressed Iba1 at a low level it always expressed L-Ferritin at a high level, and vice versa. Therefore, the Iba1low population was not uniquely identified by high expression of one or a subset of the myeloid cell markers investigated.

For all myeloid cell markers, the intensity distributions of cells positive for Iba1 and/or the MOIs changed in AD, as reflected in the net difference plot for each marker (Fig. 2, Additional file 1: Table S4). When quantified, the most notable change in the abundances of the Iba1-MOI populations identified was a significant increase in the proportion of the Iba1low MOIhigh population for seven of the 11 MOIs in AD (Fig. 3): CD45, HLA-DR, CD14, CD74, CD33, CD32, and L-Ferritin. The increase in these seven Iba1low MOIhigh populations was typically accompanied by a reduction in the respective Iba1high MOIlow populations, albeit this reduction did not reach significance for all seven of these MOIs (Fig. 3A–E, G, K). This suggests a model for the emergence of an Iba1low population or populations in AD, at the expense of an Iba1high population or populations. No significant changes in the Iba1-CD206, Iba1-CD163 and Iba1-P2RY12 populations were identified in AD (Fig. 3H, I). In addition, a significant increase in the Iba1high TMEM119glow population was observed in AD, although this change was not accompanied by significant changes in the Iba1high TMEM119ghigh or Iba1low TMEM119ghigh populations (Fig. 3K).

**Iba1low myeloid cells highly express activation markers**

Next, we sought to further characterise the Iba1low MOIhigh population, the most changed population in AD, to understand how they differed from their respective Iba1high MOIhigh population (Fig. 4). We calculated the mean population intensity for each MOI by determining where the centre of each Iba1-MOI population was on the contour plots presented in Fig. 2. We used the mean population intensities for each marker to determine differences in marker expression between the Iba1low MOIhigh and Iba1high MOIhigh populations in normal and AD MTG. It is important to note that the mean single-cell L-Ferritin average intensity was not compared between the Iba1-L-Ferritin populations as we did not identify an Iba1high L-Ferritinhigh population.

Of the seven MOIs with an increase in the Iba1low population in AD, the mean population intensity of CD45, HLA-DR, CD14, CD74, and CD32 was significantly higher in the Iba1low MOIhigh population relative to the Iba1high MOIhigh population in both normal and AD, suggesting that cells with the highest MOI expression were those with low Iba1 (Fig. 4A–D, G). These MOIs are generally associated with microglial activation functions. While we identified no increase in the Iba1low CD206glow population in AD, the mean population intensity of CD206 was higher in the Iba1low CD206ghigh population relative to the Iba1high CD206ghigh population in both normal and AD (Fig. 4F).

There was no increase in the abundance of the Iba1low P2RY12high and Iba1low TMEM119ghigh populations in AD, distinguishing P2RY12 and TMEM119 from the...
other MOIs. However, unexpectedly, in both normal and AD cases the mean population intensity of P2RY12 was increased in the Iba1<sup>low</sup> P2RY12<sup>high</sup> population relative to the Iba1<sup>high</sup> P2RY12<sup>high</sup> population in both normal and AD cases (Fig. 4I). In this way, P2RY12 aligns with the MOIs generally associated with microglial activation.
functions. In contrast, there was no change in the mean single-cell TMEM119 average intensity between the Iba1low TMEM119high and Iba1high TMEM119high populations in normal and AD cases (Fig. 4).

The changes in the proportions of Iba1-MOI populations demonstrate an increase in the expression of MOIs and a reduction in Iba1. The mean MOI average intensities per cell also demonstrate that the reduced Iba1 expression observed in AD is accompanied by an increase in the expression of a subset of MOIs. The subset of MOIs that is increased in this Iba1low population were CD45, HLA-DR, CD14, CD74, CD206 and CD32, which have previously been associated with microglial activation (Additional file 1: Table S1).

The abundance of the Iba1low myeloid cell populations correlated with tau load but not amyloid beta

Next we correlated the abundance of the Iba1low MOIhigh populations with amyloid beta and tau load in AD cases (Table 2). Aside from CD74 and CD33, all the MOIs that showed an increase in the abundance of the Iba1low MOIhigh population in AD cases showed significant strong linear correlations with tau pathology load (Table 2). CD206, exclusively expressed by perivascular macrophages in the human brain, did not show an increase in the Iba1low CD206high population in AD. Despite this, the abundance of the Iba1low CD206high population significantly correlated strongly with tau pathology load (Table 2).

For all MOIs, the abundance of the Iba1low MOIhigh population did not correlate with amyloid beta load in AD (Table 2). We hypothesised that rather than correlating with overall amyloid beta load, the Iba1low population might be spatially distributed around amyloid beta plaques. To test this hypothesis, we developed a spatial analysis pipeline to quantify the abundance of Iba1low cells at different distances from amyloid beta plaques in

![Fig. 3 Iba1low MOIhigh populations are increased in AD. Iba1-MOI populations were identified by immunofluorescent co-labelling of Iba1 with markers CD45 (a), HLA-DR (b), CD14 (c), CD74 (d), CD33 (e), CD206 (f), CD32 (g), CD163 (h), P2RY12 (i), TMEM119 (j), and L-Ferritin (k). Following the single cell Iba1-MOI analysis, the abundance of the Iba1low MOIhigh, Iba1high MOIhigh, and Iba1high MOIlow populations were quantified as a percentage of the total Iba1-MOI population identified in each normal and AD case. The abundance of each Iba1-MOI population was compared between control and AD using a Mann-Whitney test. Data are presented as mean abundance ± SD (n= 7 or 8). Significance of differences between normal and AD: ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05](image)
To identify Iba1\textsubscript{low} cells, Iba1 was co-labelled with two MOIs: L-Ferritin and HLA-DR. We chose these MOIs because high L-Ferritin expression best delineated the Iba1\textsubscript{low} population, and HLA-DR is a MOI previously shown to be highly expressed by microglia around amyloid beta plaques [17, 49, 56]. Iba1-L-Ferritin-HLA-DR cells were identified as being plaque (on plaques), plaque-adjacent (5-50 µm from plaques), or non-plaque (> 50 µm from plaques).

We first quantified the proportion of total myeloid cells at each spatial location relative to amyloid beta plaques (Fig. 5B). Only 18.1 ± 3.22% of total Iba1-L-Ferritin-HLA-DR cells were located on plaques (Fig. 5C). We subsequently analysed the relative proportions of Iba1\textsubscript{low} and Iba1\textsubscript{high} cells at each spatial location relative to plaques (Fig. 5D, E). With no difference observed at different spatial locations (Fig. 5D, E). Therefore, the abundance of the Iba1\textsubscript{low} population did not correlate with amyloid beta load and Iba1\textsubscript{low} cells were not spatially distributed around amyloid beta plaques.

Iba1\textsubscript{low} microglia, but not perivascular macrophages, co-express L-Ferritin and activation MOIs

While the Iba1\textsubscript{low} population in AD was best delineated by high expression of L-Ferritin, a protein specifically expressed by dystrophic microglia, an Iba1\textsubscript{low} CD206\textsuperscript{high} perivascular macrophage population was also identified [45, 57]. Thus, the Iba1\textsubscript{low} population consists of both microglia and perivascular macrophages and is not fully identified by co-labelling of a single MOI with Iba1. We sought to determine the overlap of different Iba1\textsubscript{low} MOI\textsuperscript{high} populations and their myeloid cell identity by triple-labelling Iba1, L-Ferritin, and one of a subset of MOIs: HLA-DR (Fig. 6A–D), CD74 (Fig. 6E–H), CD32 (Fig. 6I–L), CD206 (Fig. 6M–P), and TMEM119 (Fig. 6Q–T). This subset of MOIs was chosen because they span different functional states and have different expression patterns.
Table 2 Correlations for the abundance of the Iba1low MOIhigh populations and amyloid beta and tau load in AD cases

| Marker      | Amyloid beta | Tau |
|-------------|--------------|-----|
|             | R value      | p value | R value | p value |
| CD45        | −0.143       | 0.752  | 0.810   | 0.022   |
| HLA-DR      | 0.0952       | 0.840  | 0.905   | 0.005   |
| CD14        | −0.262       | 0.536  | 0.833   | 0.015   |
| CD74        | −0.548       | 0.171  | 0.571   | 0.151   |
| CD33        | −0.452       | 0.268  | 0.524   | 0.200   |
| CD206       | 0.167        | 0.703  | 0.833   | 0.015   |
| CD32        | 0.0476       | 0.935  | 0.952   | 0.001   |
| CD163       | 0.238        | 0.582  | 0.619   | 0.115   |
| F2RY12      | −0.214       | 0.619  | 0.667   | 0.096   |
| TMEM119     | −0.0952      | 0.840  | 0.714   | 0.429   |
| L-Ferritin  | 0.214        | 0.662  | 0.896   | 0.003   |

Italicised values were considered statistically significant (p ≤ 0.05).

Iba1low cells were somewhat evenly distributed in the L-Ferritinlow CD32low L-Ferritinhigh CD32high and L-Ferritinhigh CD32low populations (Fig. 6M–P); a high percentage of Iba1low cells were identified as either L-Ferritinhigh CD32low or L-Ferritinlow CD32high with only a small percentage of Iba1low cells highly expressing both L-Ferritin and CD32 (L-Ferritinhigh CD32low = 42.18 ± 17.52% and L-Ferritinlow CD32high = 32.01 ± 13.60%, Fig. 6P; L-Ferritinhigh CD32high = 22.53 ± 12.57%, Fig. 6P). This may be attributed to the high CD32 expression by both perivascular macrophages and microglia in the AD brain [57]. Microglial-specific marker, TMEM119, also showed little to no co-expression with L-Ferritin, with only 21.41 ± 18.64% of Iba1low cells highly expressing TMEM119 and L-Ferritin (Fig. 6Q–T). Therefore, while both TMEM119 and L-Ferritin are both specifically expressed by microglia, they are not expressed by the same Iba1low microglial population.

Discussion

In this study, we used novel quantification methods to determine the expression of 11 microglial proteins in post-mortem human AD cortex. Using a novel single-cell image analysis pipeline, we identified Iba1low MOIhigh microglial populations that were increased in the AD cortex. Further investigation of the Iba1low MOIhigh populations revealed that their abundances correlate with tau pathology load. Iba1low myeloid cells were best delineated by high expression of L-Ferritin, and highly co-expressed L-Ferritin, CD74, and HLA-DR, a phenotype that reflects dysfunction. The results presented in this study are summarised in Additional file 1: Figure S3.

While the Iba1low population was best delineated by high L-Ferritin expression, Iba1low L-Ferritinhigh cells also highly expressed both HLA-DR and CD74. This protein expression signature parallels the transcriptomic signature of the human AD microglial subpopulation identified using single-cell transcriptomic technologies [17]. The AD pathology-associated microglial population was one of four microglial subpopulations identified in normal and AD human prefrontal cortex using single nuclear RNA sequencing technology. This microglial subpopulation was overrepresented in high pathology load AD cases and was enriched with 77 transcripts relative to the other three microglial subpopulations, including MOIs L-Ferritin, HLA-DR, CD74, and CD14 that we investigated in this study [17]. Therefore, our results provide immunohistochemical validation of this AD pathology-associated population, although our spatial analysis shows these microglia are not specifically clustered near plaques.
Iba1low cells are not spatially distributed around Aβ plaques in AD cortex. To characterise the spatial distribution of Iba1low cells relative to Aβ plaques, AD middle temporal gyrus sections were subjected to two rounds of immunofluorescent staining; (1) Iba1 was immunofluorescently co-labelled with L-Ferritin and HLA-DR, and (2) amyloid beta and tau. The images from the two rounds of staining were aligned, allowing us to determine the spatial localisation of Iba1-L-Ferritin-HLA-DR cells relative to amyloid beta plaques and tau tangles. Iba1-L-Ferritin-HLA-DR cells were identified near amyloid beta plaques but showed no spatial distribution around tau. A master mask of total Iba1-L-Ferritin-HLA-DR cells was generated. The location of Iba1-L-Ferritin-HLA-DR cells was classified as plaque, plaque-adjacent, or non-plaque. The proportion of total Iba1-L-Ferritin-HLA-DR cells at plaque, plaque-adjacent, and non-plaque spatial locations was quantified; data shown as mean ± SD (n = 8).

The Iba1low population was gated out from total Iba1-L-Ferritin-HLA-DR cells and no difference in the abundance of Iba1low cells at plaque, each 5 µm plaque-adjacent interval, and non-plaque spatial locations was identified; data shown as median percentage of total Iba1-L-Ferritin-HLA-DR cells at each spatial location (n = 8). When the 5–50 µm intervals were grouped into plaque-adjacent, the abundance of Iba1low cells at plaque, plaque-adjacent, and non-plaque spatial locations remained unchanged; data shown as the percentage of total Iba1-L-Ferritin-HLA-DR cells at each spatial location with a single point per case (n = 8). The abundance of Iba1low cells was compared between amyloid beta spatial locations using a repeated measures two-way ANOVA with the Geisser–Greenhouse correction and Tukey’s multiple comparisons test.

Aβ amyloid beta
In the human brain, L-Ferritin is a specific marker of dystrophic, dysfunctional microglia [10, 45, 57]. The L-Ferritin$^\text{high}$ population identified both here and by Mathys et al. [17] is therefore likely to be dysfunctional. This high expression of L-Ferritin implicates iron dysregulation as a key driver of microglial dysfunction in AD [11, 59–61]. Interestingly, the reduced Iba1 expression in this population also suggests dysfunction. Iba1 is key to microglial membrane ruffling and phagocytosis, and a reduction in expression could suggest perturbed phagocytic capabilities [47]. Therefore, the Iba1$^\text{low}$ L-Ferritin$^\text{high}$ phenotype is a unique immunohistochemical signature to identify dysfunctional microglia in the human AD brain.

We identified strong correlations between the abundance of the Iba1$^\text{low}$ L-Ferritin$^\text{high}$ and Iba1$^\text{low}$ HLA-DR$^\text{high}$ (and other Iba1$^\text{low}$ MOI$^\text{high}$) populations with tau load, but not with amyloid beta load. The correlations of the Iba1$^\text{low}$ populations with tau load is likely indicative of an interaction between microglial function and aggregate pathology, where microglial dysfunction and loss of microglial trophic support is predicted to drive the formation of tau pathology [10]. This relationship has been demonstrated in post-mortem human AD tissue utilising the predictability of tau spread according to Braak staging to investigate the temporal relationship between microglial dysfunction and regional pathology [10]. L-Ferritin-positive dystrophic microglia were identified in Braak Stage I middle temporal gyrus devoid of tau pathology and were more abundant in entorhinal cortex with significant neurodegeneration and tau pathology [10]. Microglial dysfunction and the related loss of trophic support, as demonstrated by high L-Ferritin immunoreactivity and dystrophic morphology, are believed to precede the neurodegeneration and tau pathology in AD. Furthermore, the extent of microglial dystrophy further increases as neurodegeneration advances [12]. Together, with previous temporal relations between microglial dystrophy and tau previously realised, the correlations identified here between the Iba1$^\text{low}$ populations and tau pathology further support the conclusion that the Iba1$^\text{low}$ population is a dysfunctional microglial population in AD.

Given that microglia are known to interact and phagocytose amyloid beta in AD, we expected a correlation between the AD-associated Iba1$^\text{low}$ population and amyloid load [62–68]. We investigated the spatial relationship between the dysfunctional Iba1$^\text{low}$ population and amyloid beta plaques [16, 17]. However, the Iba1$^\text{low}$ population identified by high L-Ferritin and/or high HLA-DR expression was not especially spatially distributed close to amyloid beta plaques. This is in direct contrast to disease-associated microglia in AD mice, including those expressing high L-Ferritin, identified around AD plaques [16, 69]. Human disease-associated microglia may indeed interact with amyloid beta at a certain time point or time points in disease, but these interactions may not persist in end-stage AD human tissue. Alternatively, this difference may reflect species differences. While AD pathology-associated microglia populations highly express phagocytosis-associated genes, high L-Ferritin expression suggests that iron dysregulation in conjunction with amyloid beta phagocytosis drive AD pathology-associated microglial signatures [11, 59–61]. In the human AD brain, iron is heavily concentrated at the centre of amyloid beta plaques and has been implicated in the aggregation of amyloid beta fibrils, demonstrating the likelihood of amyloid beta-iron interactions in AD [70, 71]. Therefore, the interplay between multiple pathogenic processes in AD likely drive the dysfunctional signature identified here and the associated changes in microglial function.

The characteristics of the Iba1$^\text{low}$ population could be interpreted as evidence for the pro-inflammatory hypothesis as well as the dysfunctional hypothesis. While it could be concluded that the Iba1$^\text{low}$ populations we identified provide support for the dysfunctional hypothesis of AD, these Iba1$^\text{low}$ populations could also be considered chronically active and neurotoxic. Indeed, the Iba1$^\text{low}$ populations were best delineated by high L-Ferritin expression, but the Iba1$^\text{low}$ populations also expressed high levels of other MOIs associated with activation functions. The Iba1$^\text{low}$ populations showed higher mean MOI average intensities per cell of CD45, HLA-DR, CD14, CD74, and CD32 relative to their respective Iba1$^\text{high}$ population. Therefore, the increased presence of the Iba1$^\text{low}$ population in the human AD brain could

(See figure on next page.)

**Fig. 6** Iba1$^\text{low}$ population co-expresses of L-Ferritin, HLA-DR, and CD74. To determine the co-expression of dysfunctional marker, L-Ferritin, with other immunophenotype MOIs on the Iba1$^\text{low}$ population, Iba1 was immunofluorescently co-labelled with L-Ferritin and one other MOI, HLA-DR (a–d), CD74 (e–h), CD206 (i–l), CD32 (m–p), and TMEM119 (q–t). All Iba1-L-Ferritin-MOI cells were pooled from all normal and AD cases and plotted for their Iba1 average intensity against L-Ferritin average intensity to identify the Iba1$^\text{low}$ population (a, e, i, m, q). All Iba1$^\text{high}$ cells from all normal and AD cases were subsequently plotted for their MOI average intensity against L-Ferritin average intensity to identify three L-Ferritin-MOI populations; 1. L-Ferritin$^\text{high}$ MOI$^\text{low}$, 2. L-Ferritin$^\text{high}$ MOI$^\text{high}$, and 3. L-Ferritin$^\text{low}$ MOI$^\text{low}$ (b, f, j, n, r). An example of the L-Ferritin-MOI gating is shown on a single AD case for each MOI (c, g, k, o, s). The abundance of each L-Ferritin-MOI population was quantified as a percentage of total Iba1$^\text{low}$ cells identified with each triple label (d, h, l, p, t); data presented as mean (n = 8).
support the contrasting pro-inflammatory hypothesis. However, changes in microglial morphology and phenotype typically associated with chronic activation can also occur as part of cell senescence [13]. Aged microglia express inflammatory proteins not expressed by homeostatic microglia and accumulate lipid molecules. This is a signature that is enhanced in AD pathology-associated microglia [16, 17, 19, 72]. Because the MOIs up-regulated by the Iba1low population span numerous and somewhat polarising functions, these populations are showing signs of exaggerated microglial ageing with impaired or dysfunctional intercellular communications [13]. The exacerbated activation state of the Iba1low population we identified does not necessarily reflect a functional activation state, but instead a dysfunctional one. The characterisation of this dysfunctional microglial population demonstrates the complexity of microglial changes in AD.

By identifying Iba1low populations that expressed L-Ferritin, specific for microglia, or CD206, specific for perivascular macrophages, we confirmed that both microglial cell populations contribute to the Iba1low population in AD. Identifying an Iba1low CD206high population indicates that PVMs comprise a substantial proportion of the Iba1low population. While perivascular macrophages do not express dysfunctional MOI L-Ferritin, the perivascular macrophage Iba1low population exhibits low Iba1 expression, hypothesised to be a sign of dysfunction. The higher expression, by the Iba1low MOIhigh than Iba1high MOIhigh populations, of MOIs associated with activation was also observed with MOIs highly expressed by perivascular macrophages: CD14, CD206, and CD32 [23, 57, 73]. Given that perivascular macrophages phagocytose amyloid beta aggregating around cerebral blood vessels, and the finding of blood–brain barrier breakdown in AD, the maintenance of normal perivascular macrophage function in late AD seems unlikely [74, 75]. Microglia and perivascular macrophages have key phenotypic differences in the normal brain, including their signatures of dysfunction. Overall, our data suggest that both microglia and perivascular macrophages are dysfunctional in AD.

This is one of the first immunohistochemical studies to validate single cell RNA sequencing AD-associated microglial population signatures. The ability to do this is inherently due to the novel single-cell image analysis method we developed to identify the expression of each MOI. Previous immunohistochemical studies have relied on tissue-wide measures, like the tissue-wide integrated intensity measurements presented in this study, which as we demonstrated, can dilute subtle AD-associated cell-by-cell changes observed in heterogeneous populations like microglia [48]. The heterogeneity of microglia in the human brain has been identified with single-cell RNA sequencing, including the complexity of microglial changes in the human AD brain, albeit without extensive spatial context [16–19]. Single-cell measurements in immunohistochemically stained post-mortem human tissue have allowed for the identification of microglial heterogeneity and phenotypic changes in AD with some anatomical context [49, 57]. To provide anatomical context to the disease-associated microglial populations identified, integration of immunohistochemical and single-cell RNA sequencing datasets must be achieved. Although no spatial relationship between the dysfunctional Iba1low microglia and AD pathology was identified here, it establishes context for how image analysis capabilities can be harnessed to generate and analyse single-cell data in immunohistochemically labelled tissue.

Conclusions
Our novel single-cell image analysis pipeline allowed for the identification of an Iba1low microglial population in immunohistochemically labelled human brain. This population was best delineated by high L-Ferritin expression, leading to the hypothesis that this is a dysfunctional microglial population. The phenotype of this population mirrors that of a human AD pathology-associated microglial subpopulation previously identified in a single cell RNA sequencing study. As such, our immunohistochemical data support the microglial dysfunction hypothesis of AD.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s40478-020-01047-9.

Additional file 1. Supplementary file containing supplementary methods, image analysis validation, and supplementary tables and figures.

Abbreviations
AD: Alzheimer’s disease; MTG: Middle temporal gyrus; MOI: Marker of interest; CD: Cluster of differentiation; Iba1: Ionised calcium binding adaptor protein 1; HLA-DR: Human leukocyte antigen, DR isotype; Aβ: Amyloid beta; ROI: Region of interest; TSA: Tyramide signal amplification.

Acknowledgements
We would like to acknowledge Marika Eszes at the Centre for Brain Research (the University of Auckland, New Zealand) for her technical role in the Neurological Foundation of New Zealand’s Human Brain Bank and the imaging team at the Biomedical Imaging Research Unit (the University of Auckland, New Zealand).

Authors’ contributions
MEVS, ELS, BR, MD and MAC conceptualized the study. RLMF and MAC collected and processed the human tissue. CT carried out pathological assessment of all cases. MEVS carried out the experimental procedures, with assistance from HCW. MEVS and ELS developed the custom image analysis pipelines, and LCDS developed out file conversion scripts and cell gating.
methods. MEVS and LCDS carried out all quantification. Data analysis and interpretation were carried out by MEVS, ELS, and BR. Manuscript was prepared by MEVS, and ELS, LCDS, HCM, MAC, and BR carried out the critical revisions. BR, MD, and MAC supervised the study. All authors read and approved the final manuscript.

Funding
MEVS is supported by the Neurological Foundation of New Zealand (W & B Miller scholarship), the University of Auckland, and Brain Research New Zealand. ELS is supported by Marsden FastStart and Rutherford Discovery Fellowship funding from the Royal Society of New Zealand. HCM is supported by the Health Education Trust and Brain Research New Zealand. BR is supported by the Auckland Medical Research Foundation and the Health Research Council of New Zealand. This work was also supported by a programme grant from the Health Research Council of New Zealand. The Neurological Foundation of New Zealand supported the Human Brain Bank.

Availability of data and materials
The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Ethics approval was obtained through the University of Auckland Human Participants Ethics committee (Protocol Number 011654).

Consent for publication
Not applicable.

Competing interest
The authors declare that they have no competing interests.

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Received: 27 July 2020   Accepted: 2 October 2020
Published online: 20 October 2020

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