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Tumour-associated microglia/macrophages predict poor prognosis in high-grade gliomas and correlate with an aggressive tumour subtype

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Aims: Glioblastomas are highly aggressive and treatment resistant. Increasing evidence suggests that tumour-associated macrophages/microglia (TAMs) facilitate tumour progression by acquiring a M2-like phenotype. Our objective was to investigate the prognostic value of TAMs in gliomas using automated quantitative double immunofluorescence. Methods: Samples from 240 patients with primary glioma were stained with antibodies against ionized calcium-binding adaptor molecule-1 (IBA-1) and cluster of differentiation 204 (CD204) to detect TAMs and M2-like TAMs. The expression levels were quantified by software-based classifiers. The associations between TAMs, gemistocytic cells and glioblastoma subtype were examined with immuno- and haematoxylin–eosin stainings. Three tissue arrays containing glioblastoma specimens were included to study IBA-1/CD204 levels in central tumour and tumour periphery and to characterize CD204+ cells. Results: Our data revealed that the amount of especially CD204+ TAMs increases with malignancy grade. In grade III–IV, high CD204 expression was associated with shorter survival, while high IBA-1 intensity correlated with a longer survival. In grade IV, CD204 showed independent prognostic value when adjusting for clinical data and the methylation status of O6-methylguanine–DNA methyltransferase. Our findings were confirmed in two bioinformatics databases. TAMs were more abundant in central tumour tissue, mesenchymal glioblastomas and gliomas with many gemistocytic cells. CD204+ TAMs co-expressed proteins related to tumour aggressiveness including matrix metallopeptidase-14 and hypoxia-inducible factor-1α. Conclusions: This is the first study to use automated quantitative immunofluorescence to determine the prognostic impact of TAMs. Our results suggest that M2-like TAMs hold an unfavourable prognostic value in high-grade gliomas and may contribute to a pro-tumourigenic microenvironment.

Keywords: CD204, glioblastoma, glioma, macrophages, microglia, prognosis

Introduction

Gliomas are the most common primary brain tumours in adults. Patient prognosis differs considerably within the different World Health Organization (WHO) grades being poorest for patients with the grade IV tumour – the glioblastoma multiforme (GBM). In 2005, temozolomide was introduced as supplement to standard treatment of GBM patients [1] resulting in improved survival, especially for patients with methylated O6-methylguanine–DNA methyltransferase (MGMT) promoter [2–4]. Additional therapeutic strategies have not...
been able to further improve prognosis, and the 2- and 5-year survival remain below 25% and 10%, respectively [3,4]. The inefficacy may be explained by several mechanisms of resistance [4,5], and increasing evidence suggests that factors in the tumour microenvironment including hypoxia and nonneoplastic cells may constitute extrinsic resistance mechanisms [6]. The tumour microenvironment could, thus, be a target for anticancer therapy.

In removed GBM tissue, tumour-associated microglia/macrophages (TAMs) have been reported to constitute up to 30% [7–10]. TAMs are recruited from the brain and bone marrow to the site of the tumour through the action of signalling molecules released by glioma cells and other cells in the microenvironment [8,9,11,12]. Reportedly, glioma cells suppress the immune surveillance of TAMs by skewing them towards the pro-tumourigenic M2 phenotype while inhibiting development of the anti-tumourigenic M1 phenotype [8,9,13–16]. However, recent studies indicate that glioma cells induce a mixed population of TAMs expressing both M1- and M2-related molecules [11,17–20] possibly resembling a more undifferentiated M0 phenotype [21]. In return, TAMs support tumour growth and progression [8–10] by stimulating proliferation [22], migration [23,24], invasion [12,22–25] and angiogenesis [8,9,26], overall indicating a complex bidirectional communication between glioma cells and TAMs.

Using immunohistochemistry, the number of TAMs was shown to increase with malignancy grade in gliomas [15,27,28]. In addition, high amounts of TAMs expressing M2-related markers, e.g. cluster of differentiation 204 (CD204) and 163 (CD163), have been associated with increasing WHO grade and poorer prognosis [15,29,30]. However, these studies neither investigated the influence of M2 TAMs on survival in separate WHO grades nor performed multivariate analysis to examine the independent prognostic value. Based on these unresolved issues, our aim was to explore the prognostic impact of TAMs in a large population-based glioma cohort by quantifying the expression of the microglial/macrophage markers ionized calcium-binding adaptor molecule-1 (IBA-1) and CD204 using double immunofluorescence. IBA-1 is considered a specific TAM marker [31] and CD204 a marker of M2-like TAMs [9]. We used an automated quantitative fluorescence approach enabling continuous measurements of area and intensity reducing some of the inter- and intra-observer variability seen in conventional semi-quantitative pathologist-based scoring [32,33]. Early on, we discovered that the distribution of TAMs in especially GBMs was highly heterogeneous. We, therefore, investigated the correlation between TAMs, gemistocytic tumour cells and GBM subtype as tumour aggressiveness may depend on both the specific molecular subtype and the level of gemistocytic tumour cells. In addition, we characterized the phenotype of CD204+ TAMs by double immunofluorescence using a panel of eight markers related to immune activation and tumour aggressiveness.

Materials and methods

Patient tissue

Samples were obtained from the Region of Southern Denmark (RSD) glioma cohort investigated and well-characterized previously [34–40]. Tissue from 240 patients was included for the IBA-1/CD204 analysis. All patients were diagnosed with a primary glioma between 2005 and 2009. Table S1 provides an overview of relevant patient characteristics including clinical and histological data. A tissue array containing eight GBM specimens was included to study the expression of IBA-1/CD204 in central tumour and tumour periphery. In addition, two tissue arrays with 34 GBM specimens were included to characterize CD204+ cells. All samples were evaluated by two pathologists and reclassified according to WHO guidelines 2016 [41]. See Data S1 for information on procedures used for reclassification.

Normal brain tissue was obtained from two adult patients at autopsy. Cause of death was unrelated to any disease in the central nervous system (CNS). Tissue was obtained from 10 patients with primary diffuse large B-cell lymphoma (DLBCL) in the CNS. 10 patients diagnosed with first-time CNS metastasis from malignant melanoma (MM), and 10 patients diagnosed with first-time CNS metastasis from non-small cell lung cancer (NSCLC).

Double immunofluorescence

Immunofluorescence stainings were performed as previously reported [34,35,42,43]. See Data S1 for information on the staining procedure and Table S2 for information on antibodies used.
Automated quantitative fluorescence analysis

Fluorescence image analysis and quantitation were done using the Visiopharm integrated microscope and software module (Visiopharm, Hørsholm, Denmark) consisting of a Leica DM 6000B microscope and Olympus DP72 camera (Olympus, Ballerup, Denmark). Super images were acquired at 1.25× magnification using bright field settings, and sampling regions containing vital tumour tissue were manually outlined. Sample images were collected by systematic uniform random sampling at 20× magnification. Images were reviewed ensuring at least five images per tumour, and only images with at least 20% viable tumour tissue within the picture frame were included for further processing. Images were classified using algorithms developed in the Visiomorph module. Individual pixels were assigned labels based on threshold intensities of the colour bands. To quantify the expressions of IBA-1 and CD204, the tumour area within each picture frame was defined manually as a region of interest (ROI). For the quantification of double-positive expression (i.e. IBA-1⁺ CD204⁺ pixels), IBA-1 was used as an inclusion marker and defined as a ROI, then the expression of CD204 was evaluated within this ROI enabling measurements of double-labelled protein expression (Figure 1). Output variables were area fractions (AF), defined as area of positive expression divided by the area of the respective ROI, and mean intensity (MI) of IBA-1 and CD204 in total tumour tissue as well as of CD204 within the IBA-1⁺ area (i.e. CD204TOTAL, CD204IBA-1). Prior to sampling, calibration was performed to adjust for run variation within the staining. This was accomplished using adjacent sections of a GBM tissue array (Figure S1).

Similar algorithms were generated to characterize the phenotype of the CD204⁺ cells. The area of double-stained area was quantified as a fraction of the total CD204⁺ area resulting in a quantitative estimate of double-positive cells. In addition, the intensity of the marker of interest was measured in the total CD204⁺ area.

MGMT status

O6-methylguanine–DNA methyltransferase promoter status was retrospectively obtained in 161 patients using pyrosequencing (QIAamp DNA FFPE Tissue kit; Qiagen, Hilden, Germany) as previously described [39].

Gemistocytic cell component

Presence of gemistocytic tumour cells (gemistocytes) was evaluated in 11 diffuse astrocytomas (DA), 17 anaplastic astrocytomas (AAs) and 20 GBMs from the RSD glioma cohort using haematoxylin–eosin stainings. The 20 GBMs represented 10 of the patients with the lowest and 10 of the patients with the highest AF CD204TOTAL. All samples were scored blinded and semi-quantitatively. At 40× magnification, five positions per slide were scored from 0 to 4 (0: no gemistocytes; 1:<10%; 2: 10–49%; 3: 50–90%; 4: >90% gemistocytes), and a mean score was calculated for each tumour.

Immunohistochemical subtyping

The association between TAMs and molecular subtype was investigated by staining the 20 GBM samples described above with three proneural markers: delta-like 3, NEUronal Nuclei and oligodendrocyte transcription factor 2 (OLIG-2), as well as three mesenchymal markers: CD44, vascular endothelial growth factor (VEGF) and chitinase 3-like 1 (YKL-40/CHI3L1) [44–47]. Immunohistochemistry was performed as previously described [36,37,39,40]. The tumours were scored and indexed as proneural or mesenchymal as previously reported [44]. See Data S1 for information on the staining procedure and Table S2 for information on antibodies used.

Patient dataset analysis

mRNA expressions of IBA-1 (AIF-1) and CD204 (MSR-1) in gliomas were explored using GlioVis (https://glio.vis.bioinfo.cnio.es, data were exported July 2016). From the Cancer Genome Atlas (TCGA), mRNA data were available for 620 patients for analysing the association with malignancy grade [48] and for 460 GBM patients (the Agilent 4502SA dataset) for survival and subtype analyses [49]. From the Glavendel dataset, mRNA data were obtained for 276 patients including 159 GBM patients [50]. Datasets were exported directly from GlioVis. Survival analyses were carried out using the median as cut-off value.

Statistical analysis

Student’s t-test or Mann–Whitney U-test was used when comparing two groups. One-way ANOVA with Bonferroni’s
correction or Kruskal–Wallis test followed by Dunn’s multiple comparisons test was used for comparison of more than two groups. Correlation analyses were performed using Spearman’s correlation test. Overall survival (OS) was defined as time from primary surgery until death or date of censoring (May 2017). Survival functions were illustrated by Kaplan–Meier plots, and differences were compared by log-rank tests. WHO grade I was excluded in the statistical analysis due to limited numbers \((n = 3)\). CD204 and IBA-1 estimates were investigated as binary variables with the median as a prespecified cut-off value. Exploratory cut-point analyses were performed for grade IV as previously described [34,40], and only results using the optimal cut-points are shown. Multivariate analyses were performed using the Cox proportional hazard model.

All assumptions were tested. Statistical analyses were carried out in STATA (StataCorp LP) or Prism 5.0.

Figure 1. Image recording and classification. (A–D) Each image was recorded as three separate greyscale images for cyanine 5 [ionized calcium-binding adaptor molecule-1 (IBA-1)] (A), fluorescein (CD204) (B) and DAPI (nuclei) (C) and subsequently assigned colours and superimposed to form the complete image (D). Images were then classified by three classifiers based on threshold intensities of the colour bands. (E,F) To quantify the total expression of IBA-1 (IBA-1\(^{\text{TOTAL}}\)) and CD204 (CD204\(^{\text{TOTAL}}\)), the tumour area within each picture frame was defined as region of interest (ROI) with digital image analysis. (G) For the quantification of double-positive expression (i.e. IBA-1\(^+\) CD204\(^+\)), IBA-1 was used as an inclusion marker and defined as a ROI, and then the expression of CD204 was evaluated within this ROI enabling measurements of double-labelled protein expression (CD204\(^{\text{IBA-1}}\)).
had moderate to high levels of IBA-1 + cells with (found for AF CD204 IBA-1 and MI CD204 IBA-1, except 50% of the IBA-1 + area co-localized with CD204 (median = 0.24 (median = 0.10), while the CD204 + area comprised up to 0.24 (median = 0.048)). On average, 50% of the IBA-1+ area co-localized with CD204 (median = 0.45), but up to 90% co-expression was observed (Figure 2J).

To explore whether the vast existence of TAMs was specific to gliomas, expression levels of IBA-1 and CD204 were investigated in normal brain tissue, in tissue from primary brain DLBCL and metastases from NSCLC and MM (Figure S2A-H). AF IBA-1 TOTAL and MI IBA-1 TOTAL were highest in gliomas compared with normal brain, MM and DLBCL (P < 0.001), as well as NSCLC (P < 0.01 and <0.001). AF CD204 TOTAL was lower in normal brain (P < 0.001) and in DLBCL (P < 0.05) compared with gliomas, and gliomas had the highest MI CD204 TOTAL (P < 0.05). Similar was found for AF CD204 IBA-1 and MI CD204 IBA-1, except for NSCLC which did not differ significantly from gliomas (Figure S2G,H).

Results

Expression of CD204 and IBA-1 in gliomas, normal brain, and other brain neoplasms

CD204 and IBA-1 were expressed in the membrane and cytoplasm of TAMs including their ramifications (Figure 2A-I). In pilocytic astrocytomas, the amount of IBA-1+ cells was moderate to high while few cells expressed CD204 (Figure 2A). Diffuse astrocytomas and oligodendrogliomas (OD) showed moderate IBA-1 and sparse CD204 expression (Figure 2B-D). AAs had moderate to high levels of IBA-1+ cells with some CD204+ cells, especially in isocitrate dehydrogenase (IDH) wild-type tumours, (Figure 2E,F), whereas anaplastic OD had lower IBA-1 levels compared with AAs with limited expression of CD204 (Figure 2G). GBM (Figure 2H) and especially gliosarcomas (Figure 2I) exhibited high expression of IBA-1 and/or CD204.

When applying the software-based classifiers, all quantitative estimates except MI IBA-1 TOTAL (P = 0.084) showed overall significant increases with malignancy grade (P < 0.001) being significantly highest in grade IV tumours (Figure 2J,K). In grade IV, the IBA-1+ area contributed with up to 0.30 of the total tumour area (median: 0.10), while the CD204+ area comprised up to 0.24 (median = 0.048). On average, 50% of the IBA-1+ area co-localized with CD204 (median = 0.45), but up to 90% co-expression was observed (Figure 2J).

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TAMs and survival in WHO grade II–IV gliomas

Investigating the association between TAMs and prognosis in grade II-IV gliomas, high AF IBA-1 TOTAL was significantly associated with shorter OS when dichotomized at the median (HR = 1.50; 95% CI: 1.15–1.97; P = 0.003), while MI IBA-1 TOTAL did not correlate with survival (HR = 0.93; 95% CI: 0.71–1.20; P = 0.57). High levels of AF CD204 TOTAL and MI CD204 TOTAL correlated with poorer prognosis when divided at the median (HR = 2.02; 95% CI: 1.54–2.67; P < 0.001 and HR = 1.85; 95% CI: 1.41–2.42; P < 0.001). Similar was found for AF CD204 IBA-1 and MI CD204 IBA-1 (HR = 1.93; 95% CI: 1.48–2.53; P < 0.001 and HR = 1.92; 95% CI: 1.47–2.51; P < 0.001). In the multivariate analyses, high levels of AF CD204 TOTAL (HR = 1.81; 95% CI: 1.35–2.44; P < 0.001) and AF CD204 IBA-1 (HR = 1.25; 95% CI: 1.01–1.81; P = 0.042) correlated with shorter OS (Table S3).

TAMs and survival in WHO grade II–III

In WHO grade II, neither IBA-1 nor CD204 expression levels were associated with prognosis when dichotomized at the median (Figure S3A-D). When adjusting for performance status, IDH status and histology in the multivariate analysis, high AF IBA-1 TOTAL correlated with better prognosis, while CD204 did not impact prognosis (Table S4).

In WHO grade III, no prognostic value was found for AF IBA-1 TOTAL when dichotomized at median (Figure S3E). High levels of MI IBA-1 TOTAL tended to associate with longer OS (Figure S3F), while high CD204 levels correlated significantly with shorter OS when divided at the median (Figure S3G,H). When accounting for performance status, IDH status and histology in the multivariate analysis, CD204 had a negative prognostic value (Table S5).

TAMs and survival in WHO grade IV

AF IBA-1 TOTAL was not associated with prognosis in univariate (HR = 0.99; 95% CI: 0.74–1.32; P = 0.95) (Figure 3A) or multivariate analyses (HR = 1.06; 95% CI: 0.79–1.43; P = 0.68) (Table 1) when dichotomized at the median, and no optimal cut-points were identified. High MI IBA-1 TOTAL tended to associate with
Figure 2. Expression of ionized calcium-binding adaptor molecule-1 (IBA-1) and CD204 in gliomas. (A–I) Examples of the double immunofluorescence staining in the different histological subtypes: PA (A), wtIDH DA (B), mIDH DA (C), OD (D), wtIDH AA (E), mIDH AA (F), AOD (G), GBM (H) and GS (I). Arrows and inserts show double-positive cells. (J) The area fraction of IBA-1 TOTAL, CD204 TOTAL and CD204IBA-1 increased with malignancy grade. (K) Mean intensities of CD204 TOTAL and CD204IBA-1 were higher in high-grade compared with low-grade gliomas, while no difference was observed for MI IBA-1 TOTAL. Horizontal lines indicate the median. *P < 0.05; **P < 0.01; ***P < 0.001. Scale bar 100 μM. AA, anaplastic astrocytoma; AOD, anaplastic oligodendroglioma; DA, diffuse astrocytoma; GBM, glioblastoma multiforme; GS, gliosarcoma; mIDH, mutated isocitrate dehydrogenase; OD, oligodendroglioma; PA, pilocytic astrocytoma; wtIDH, wild-type IDH.
improved survival when divided at the median (data not shown). However, 40 patients (21%) had a MI above 107.0 which was the optimal cut-off, and these patients survived significantly longer than patients with a lower intensity (HR = 0.64; 95% CI: 0.45–0.92; \( P = 0.014 \)) (Figure 3B), and this was significant in the

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multivariate analysis (HR = 0.66; 95% CI: 0.46–0.95; P = 0.026) (Table 1).

High levels of AF CD204\textsubscript{TOTAL} predicted shorter OS (HR = 1.36; 95% CI: 1.02–1.81; P = 0.038) (Figure 3C) when dichotomized at the median, which was the optimal cut-point. This was also significant when adjusting for the clinical parameters (HR = 1.67; 95% CI: 1.23–2.27; P = 0.001) (Table 1). High MI CD204\textsubscript{TOTAL} tended to correlate with shorter OS when dichotomized at the median (data not shown). This association was significant in the univariate (HR = 2.21; 95% CI: 1.48–3.29; P < 0.001) and multivariate analysis (HR = 2.27; 95% CI: 1.50–3.43; P < 0.001) when dividing at the optimal intensity cut-off of 129.1 corresponding to the 84 percentile (Figure 3D and Table 1). Similar hazard ratios were found when examining the prognostic impact of double-positive TAMs (data not shown).

Dichotomizing patients based on AF IBA-1\textsubscript{TOTAL} and AF CD204\textsubscript{TOTAL}, patients with high CD204 and low IBA-1 had the poorest prognosis with a median survival of 5.1 months (P < 0.01) (Figure 3E), suggesting

Table 1. Multivariate Cox regression analyses of IBA-1 and CD204 levels in patients with WHO grade IV tumours including clinical parameters

| No. of patients | HR (95% CI) | P-value | HR (95% CI) | P-value | HR (95% CI) | P-value |
|-----------------|-------------|---------|-------------|---------|-------------|---------|
| Age             |             |         |             |         |             |         |
| Performance status |             |         |             |         |             |         |
| 0–1             | 1.00        |         | 1.00        |         | 1.00        |         |
| 2–4             | 1.39 (1.23–1.58) | <0.001  | 1.39 (1.23–1.58) | <0.001  | 1.38 (1.22–1.57) | <0.001  |
| Tumour crossing midline |             |         |             |         |             |         |
| No              | 1.00        |         | 1.00        |         | 1.00        |         |
| Yes             | 1.24 (0.73–2.09) | 0.43    | 1.23 (0.73–2.08) | 0.43    | 1.24 (0.74–2.09) | 0.42    |
| Postsurgical treatment |             |         |             |         |             |         |
| Stupp\textsuperscript{*} | 1.00        |         | 1.00        |         | 1.00        |         |
| PC\textsuperscript{†} | 1.76 (1.24–2.51) | 0.002   | 1.75 (1.23–2.50) | 0.002   | 1.85 (1.30–2.64) | 0.001   |
| Non\textsuperscript{‡} | 8.90 (5.35–14.8) | <0.001  | 8.83 (5.32–14.7) | <0.001  | 9.33 (5.60–15.5) | <0.001  |
| AF/MI |             |         |             |         |             |         |
| Low             | –           | –       | 1.00        |         | 1.00        |         |
| High            | –           | –       | 1.06 (0.79–1.43) | 0.68    | 0.66 (0.46–0.95) | 0.026   |

IBA-1, ionized calcium-binding adaptor molecule-1; WHO, World Health Organization; AF, area fraction; MI, mean intensity; PC, palliative care.

\textsuperscript{*}Treatment according to the publication by Stupp et al. [1].

\textsuperscript{†}Palliative treatment is radiotherapy alone (60 Gy/30–33 fractions), hypofractionated radiotherapy alone (30–34 Gy/10 fractions), hypofractionated radiotherapy with chemotherapy, or chemotherapy alone.

\textsuperscript{‡}No postsurgical treatment.

Bold values represent the significant P values (P < 0.05)
that TAMs with intense expression of CD204 are the most important prognostic indicator.

**TAMs, postsurgical treatment and survival**

To further investigate the influence of TAMs, we stratified GBM patients based on postsurgical treatment and the level of total IBA-1 and CD204. AF IBA-1\(^1\)TOTAL did not influence OS in the postsurgical-treated patients (HR = 1.00; 95% CI: 0.85–1.17; P = 0.99) or in patients only undergoing surgery (HR = 0.98; 95% CI: 0.70–1.38; P = 0.91) (Figure 4A). In the group who received postsurgical treatment, patients with high MI IBA-1\(^1\)TOTAL had longer OS than patients with low MI (HR = 0.78; 95% CI: 0.64–0.95; P = 0.013), while this correlation was insignificant in patients who only received surgery (HR = 0.95; 95% CI: 0.63–1.42; P = 0.70–1.38; P = 0.91) (Figure 4A). In the group who went surgery (HR = 0.66; 95% CI: 0.66–1.31; P = 0.79) (Figure 4B). In the group receiving postsurgical treatment, high AF CD204\(^1\)TOTAL predicted shorter OS (HR = 1.20; 95% CI: 1.03–1.42; P = 0.019), while no difference was found in the group who only underwent surgery (HR = 0.92; 95% CI: 0.66–1.31; P = 0.66) (Figure 4C). In the group receiving postsurgical treatment, patients with high MI CD204\(^1\)TOTAL had poorer prognosis compared with patients with low MI CD204\(^1\)TOTAL (HR = 1.52; 95% CI: 1.21–1.91; P < 0.001). No difference was found in the group undergoing surgery only (HR = 1.35; 95% CI: 0.88–2.07; P = 0.17) (Figure 4D).

**TAMs, MGMT status and survival**

The association between MGMT and TAMs was evaluated in 161 GBM patients, who were stratified based on MGMT methylation status and total IBA-1 or total CD204. High AF IBA-1\(^1\)TOTAL levels tended to associate with shorter survival in patients with methylated MGMT promoter (m-MGMT) (HR = 1.26; 95% CI: 0.98–1.60; P = 0.065), while no association was found for MI IBA-1\(^1\)TOTAL (HR = 0.83; 95% CI: 0.61–1.13; P = 0.23) (Figure 4E,F). In the patients with unmethylated MGMT promoter (u-MGMT), both high AF IBA-1\(^1\)TOTAL and high MI IBA-1\(^1\)TOTAL levels were associated with better outcome (HR = 0.80; 95% CI: 0.64–0.99; P = 0.036 and HR = 0.72; 95% CI: 0.54–0.95; P = 0.020) (Figure 4E,F). In the m-MGMT group, patients with high AF CD204\(^1\)TOTAL had poorer prognosis than patients with low AF CD204\(^1\)TOTAL (HR = 1.38; 95% CI: 1.07–1.77; P = 0.009), while this was not the case in the u-MGMT group (HR = 1.06; 95% CI: 0.85–1.31; P = 0.61) (Figure 4G). In patients with m-MGMT, high MI CD204\(^1\)TOTAL was associated with shorter survival (HR = 1.67; 95% CI: 1.12–2.51; P = 0.009), whereas the prognostic value was less evident in patients with u-MGMT (HR = 1.34; 95% CI: 1.00–1.78; P = 0.043) (Figure 4H).

When MGMT status was included in the multivariate analysis, AF IBA-1\(^1\)TOTAL and MI IBA-1\(^1\)TOTAL were insignificant regarding prognosis, whereas high levels of both AF CD204\(^1\)TOTAL (HR = 1.66; 95% CI: 1.16–2.37; P = 0.005) and MI CD204\(^1\)TOTAL (HR = 2.11; 95% CI: 1.31–3.40; P = 0.002) predicted poorer OS (Table 2). Similar was found for both high AF CD204\(^1\)TOTAL and MI CD204\(^1\)TOTAL (data not shown).

**TAMs and tumour heterogeneity**

GBMs displayed intertumoural (Figure 5A–C) and intratumoural (Figure 5D) heterogeneity in terms of the expression of IBA-1 and CD204. IBA-1\(^1\) and/or CD204\(^1\) TAMs tended to accumulate in perivascular areas (Figure 5E,F), pseudopalisading necroses (Figure 5G) and perinecrotic areas (Figure 5H). In the tumour periphery, TAMs primarily expressed IBA-1 and appeared more ramified compared with the central tumour where they often had an amoeboid morphology. Interestingly, some double-positive TAMs were seen at the edge between central tumour and tumour periphery (Figure 5I). Quantified, the amount of TAMs was lower in the periphery compared with the central zone (P < 0.01 or P < 0.05) (Figure 5J and Table S6). The IBA-1 intensity was similar in the two areas, while the CD204 intensity decreased from the central to the peripheral tumour area (P < 0.05) (Figure 5K and Table S6).

The relation between TAMs and the presence of gemistocytes was examined by assessing the gemistocytic cell component in 48 astrocytomas (11 DAs, 17 AAs and 20 GBMs). High amounts of CD204 and high AF IBA-1\(^1\)TOTAL positively correlated with gemistocytic score. Contrarily, in GBMs, high MI IBA-1\(^1\)TOTAL inversely correlated with gemistocytic score (Table S7).

To estimate the associations between tumour subtype and TAMs, 20 GBMs were immunohistochemically subtyped using three proneural and three mesenchymal...
markers (Figure 5L). The mesenchymal tumours generally had higher levels of AF IBA-1 TOTAL \((P = 0.022)\) and CD204 \((P < 0.001)\) (Figure 5M,N) and a higher gemistocytic score \((P = 0.020)\) (Table S6), whereas the proneural tumours showed higher levels of MI IBA-1 TOTAL \((P = 0.012)\) (Figure 5N and Table S6). In addition, high AF CD204 TOTAL \((HR = 2.91; 95\% CI: 1.03–8.18; \(P = 0.035)\) and the mesenchymal subtype \((HR = 2.97; 95\% CI: 1.01–8.76; \(P = 0.040)\) showed similar survival curves, and both correlated with decreased OS (Figure 5O).

### TAMs and CD204 phenotype

To explore the phenotype of CD204+ TAMs in patient tissue, we performed double immunofluorescence with CD204 and markers related to inflammation and immune activation, as well as to infiltration, proliferation, angiogenesis and necrosis (Figure 6A–H) \([8,9,11,21,51]\). On average, 44% and 8% of the CD204+ area co-expressed the marker major histocompatibility complex II (HLA-DR) and tumour necrosis factor alpha (TNF-\(\alpha\)), respectively (Figure 6A,B,I). While 10% and 3% co-localization was seen with the anti-inflammatory markers interleukin 10 (IL10) and transforming growth factor beta1 (TGF-\(\beta1\)) (Figure 6C, D,I). Most of the CD204+ area co-expressed matrix metalloproteinase 14 (MMP14). Average co-expression of hypoxia-inducible factor-1alpha (HIF-1\(\alpha\)) was 13%. VEGF and CD204 rarely overlapped, whereas 9% of the CD204+ area co-localized with epidermal growth factor receptor (EGFR) (Figure 6E–I). A comparable scatter plot was found when measuring the intensity of the marker of interest within the CD204+ area (Figure S4).

CD204+ cells did not express glial fibrillary acidic protein, OLIG-2, microtubule-associated protein 2, or smooth muscle actin indicating that tumour cells and vascular smooth muscle cells do not contribute to the CD204+ cell population (Figure S4).

### Patient databases

We evaluated two bioinformatics databases, validating that both IBA-1 and CD204 increased with malignancy grade (Figure S5A,B). In the Glavendeel and TCGA datasets, high CD204 mRNA levels correlated with poorer prognosis in GBM \((HR = 1.41; 95\% CI: 1.01–1.95; \(P = 0.039)\) and \(HR = 1.29; 95\% CI: 1.05–1.57; \(P = 0.013)\) (Figure S5C,D). As expected, the mesenchymal subtype had the highest level of CD204 compared with the proneural and classical subtypes \((P < 0.001)\) (Figure S5E,F).

### Discussion

This study shows that M2-like TAMs predict poorer prognosis in high-grade glioma and are associated with more aggressive tumours as these cells are more frequent in tumours with high gemistocytic cell count and in GBMs of the mesenchymal subtype. Characterizing the M2-like TAMs in patient-derived GBM tissue revealed that M2-like TAMs may contribute to a tumour microenvironment favouring especially tumour infiltration and expansion, but also angiogenesis and tumour resistance (Figure 7).

We found that CD204 and IBA-1 levels increased with malignancy. This is in accordance with other studies on primary gliomas using general TAM markers CD68 \([15,26,28]\) and IBA-1 \([27,28]\), as well as M2 markers CD163 and CD204 \([15]\). Prosniaik et al. \([30]\), too, showed malignancy to correlate with high expression of M2-related molecules including IL-10, TGF-\(\beta\), CD163 and CD204 at mRNA levels and/or immunohistochemically using human glioma specimens. However, these studies primarily used semi-quantitative scoring, and fewer patients (between 32 and 107 patients) were included.

The acquired phenotype of TAMs was found to affect prognosis, while the general amount of TAMs was less important as the fraction of IBA-1+ in the total tumour.
area (AF IBA-1 TOTAL) did not influence OS. The IBA-1 intensity, however, seemed to correlate with better outcome in patients grade II, III and IV gliomas. In contrast, high amounts of CD204 predicted poorer survival in WHO grade III–IV gliomas, but not in grade II tumours. This was confirmed in two bioinformatics databases. Previous studies have shown that high CD163/CD68 [15] and CD163/IBA-1 ratios [29] as well as high CD163 content [30] predict shorter survival in high-grade gliomas. We used CD204 to identify M2 TAMs as CD163 is only reliable as a M2 marker when in combination with the transcription factor

Table 2. Multivariate Cox regression analyses of IBA-1 and CD204 levels in patients with WHO grade IV tumours including clinical parameters and MGMT status

|                     | No. of patients | Baseline model | AF IBA-1 TOTAL | MI IBA-1 TOTAL |
|---------------------|----------------|---------------|----------------|----------------|
| Age                 | 161            | 1.01 (1.00–1.03) | 0.13           | 1.01 (1.00–1.03) | 0.15 | 1.01 (0.99–1.03) | 0.17 |
| Performance status  |                |               |                |                |
| 0–1                 | 100            | 1.00          | 1.00           | 1.00           |
| 2–4                 | 61             | 1.43 (1.25–1.64) | <0.001        | 1.43 (1.25–1.64) | <0.001 | 1.41 (1.23–1.62) | <0.001 |
| Tumour crossing midline |            |               |                |                |
| No                  | 148            | 1.00          | 1.00           | 1.00           |
| Yes                 | 13             | 1.78 (0.97–3.27) | 0.064         | 1.79 (0.97–3.31) | 0.064 | 1.76 (0.95–3.24) | 0.071 |
| Postsurgical treatment |            |               |                |                |
| Stupp*              | 92             | 1.00          | 1.00           | 1.00           |
| PC†                 | 40             | 1.45 (0.95–2.21) | 0.084         | 1.45 (0.95–2.22) | 0.084 | 1.52 (0.99–2.33) | 0.056 |
| None‡               | 29             | 9.50 (5.35–16.9) | <0.001        | 9.55 (5.36–17.0) | <0.001 | 9.83 (5.55–17.4) | <0.001 |
| MGMT methylation status |          |               |                |                |
| Unmet               | 88             | 1.00          | 1.00           | 1.00           |
| Met                 | 73             | 0.85 (0.59–1.23) | 0.40         | 0.85 (0.58–1.24) | 0.40 | 0.86 (0.60–1.24) | 0.42 |
| AF/MI               |                |               |                |                |
| Low                 | –              | –             | –             | 0.98 (0.69–1.38) | 0.89 | 0.75 (0.49–1.15) | 0.18 |
| High                | –              | –             | –             | –             |

|                     | AF CD204 TOTAL | MI CD204 TOTAL |
|---------------------|---------------|----------------|
| Age                 | 161           | 1.02 (1.00–1.03) | 0.086     | 1.01 (1.00–1.03) | 0.099 |
| Performance status  |                |               |            |                |
| 0–1                 | 100           | 1.00          | 1.00        | 1.00           |
| 2–4                 | 61            | 1.47 (1.28–1.69) | <0.001    | 1.40 (1.22–1.61) | <0.001 |
| Tumour crossing midline |          |               |            |                |
| No                  | 148           | 1.00          | 1.00        | 1.00           |
| Yes                 | 13            | 1.74 (0.94–3.22) | 0.076    | 1.85 (1.00–3.41) | 0.051 |
| Postsurgical treatment |            |               |            |                |
| Stupp*              | 92            | 1.00          | 1.00        | 1.00           |
| PC†                 | 40            | 1.67 (1.08–2.58) | 0.022    | 1.47 (0.96–2.25) | 0.078 |
| None‡               | 29            | 8.63 (4.88–15.3) | <0.001    | 10.4 (5.81–18.8) | <0.001 |
| MGMT methylation status |          |               |            |                |
| Unmet               | 88           | 1.00          | 1.00        | 1.00           |
| Met                 | 73            | 0.96 (0.66–1.39) | 0.82     | 0.89 (0.61–1.28) | 0.52 |
| AF/MI               |                |               |            |                |
| Low                 | –            | –             | –          | 1.00           |
| High                | –            | –             | –          | 1.66 (1.16–2.37) | 0.005 | 2.11 (1.31–3.40) | 0.002 |

IBA-1, ionized calcium-binding adaptor molecule-1; WHO, World Health Organization; AF, area fraction; MI, mean intensity; PC, palliative care; MGMT, O6-methylguanine–DNA methyltransferase.
*Treatment according to the publication by Stupp et al. [1].
†Palliative treatment is radiotherapy alone (60 Gy/30–33 fractions), hypofractionated radiotherapy alone (30–34 Gy/10 fractions), hypofractionated radiotherapy with chemotherapy, or chemotherapy alone.
‡No postsurgical treatment.
Bold values represent the significant P values (P < 0.05)
V-maf avian musculoaponeurotic fibrosarcoma oncogene homologue (c-MAF) [51,52]. Further, assessing the general population of TAMs with CD68 may be problematic. Reportedly, M1 macrophages express high levels of CD68 [51], and recently, CD68 expression was found in many cases to be lower compared with CD163 in GBMs [52,53]. In addition, CD68 was shown to be expressed by some human glioma cells [54,55]. Overall, these findings question its suitability as a general marker of TAMs.

In our study, we included more patients compared to previous prognostic studies on TAMs in gliomas [15, 197].

*P < 0.05; **P < 0.01; ***P < 0.001. Scale bar 100 μM.

Figure 5. Tumour-associated microglia/macrophages (TAMs) heterogeneity in glioblastomas. (A–D) Phenotypic differences in ionized calcium-binding adaptor molecule-1 (IBA-1) (red) and CD204 (green) expression were seen between tumours (A–C) and within the individual tumour (D). Arrows indicate double-positive cells. (E–H) Besides expression in viable tumour tissue, TAMs were also present around blood vessels (Asterisk) (E,F) and in perinecrotic areas including pseudopalisading necroses (Plus) (G,H) often in clusters. (I) The density of TAMs, especially CD204⁺ TAMs, was higher in the central tumour (insert 1) compared with the border zone (insert 2) and the tumour periphery (insert 3). (J,K) The amount of CD204 was significantly lower in the tumour periphery compared with central tumour. (L) Tissue from 20 patients with glioblastoma was immunohistochemically subtyped using three proneural markers (PN, shown for oligodendrocyte transcription factor 2 (OLIG-2)) and three mesenchymal markers (MES, shown for CD44). (M,N) Especially CD204 expression was higher in the mesenchymal than in the proneural subtype. (O) Survival curve for the 20 patients with glioblastoma based on the area of CD204 in total tumour area (area fraction CD204TOTAL) and subtype. Horizontal and vertical lines indicate mean ± SEM.
However, our study also had a limited number of patients with grade II–III gliomas and may not be representative for the entire population. The statistical analyses should, thus, be interpreted with caution.

In our multivariate analyses, CD204 was an independent predictor of poor OS in grade III–IV tumours. Similar has been reported in carcinomas including pancreatic [56,57], bladder [58] and lung cancers [59].

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**Figure 6.** Characterization of CD204⁺ tumour-associated microglia/macrophages. (A–H) The phenotype of the CD204⁺ cells (green) was investigated using markers related to M1 polarization: HLA-DR (red) and TNF-α (red) (A, B). M2 polarization: IL10 (red) and TGF-β1 (red) (C, D) as well as to tumour aggressiveness: MMP-14 (red), HIF-1α (red), VEGF (red) and EGFR (red) (E–H). (I) For the marker of interest, the double-positive area was quantified as a percentage of the total CD204⁺ area. Arrows and inserts show double-positive cells. Horizontal and vertical lines indicate mean ± SEM. Scale bar 100 μM. HLA-DR, human leucocyte antigen DR/major histocompatibility complex class II DR; TNF-α, tumour necrosis factor alpha; IL10, interleukin 10; TGF-β, transforming growth factor beta 1; MMP-14, matrix metallopeptidase 14; HIF-1α, hypoxia-inducible factor-1 alpha; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor.

**Figure 7.** Schematic illustration of the presumed cross-talk between glioblastoma cells and M2-like TAMs. In the tumour microenvironment, M2-like tumour-associated microglia/macrophages expressing high amounts of CD204 with lower levels of ionized calcium-binding adaptor molecule-1 and HLA-DR interact with the glioblastoma cells favouring a mesenchymal shift in the tumour cells. The M2-like TAMs produce MMP14, HIF-1α, IL10, EGFR and TNF-α possibly stimulating angiogenesis and especially matrix remodelling. With scavenger functions of CD204, e.g. clearance of myelin, extracellular matrix components and apoptotic cells, M2-like TAMs may ultimately enable tumour infiltration and progression. G, gemistocytes; TAM, tumour-associated microglia; TAMΦ, tumour-associated macrophage; MES, mesenchymal glioblastoma cells; IL10, interleukin 10; TNF-α, tumour necrosis factor alpha; EGFR, epidermal growth factor receptor; MMP14, matrix metallopeptidase 14; HLA-DR, human leucocyte antigen DR/major histocompatibility complex class II DR; HIF-1α, hypoxia-inducible factor-1 alpha.
while no independent prognostic value was found of CD204+ TAMs in oesophageal cancer [60] or in primary CNS lymphoma [61]. Contradictory results have been reported in colorectal cancer [62–64], but in gastric [65] and ovarian cancer [66] high density of M1 TAMs and high M1/M2 ratio were associated with longer survival, whereas M2 TAMs themselves (identified with a CD163 antibody) showed no influence on prognosis. Zhang et al. [64] conducted a meta-analysis on solid tumours analysing primarily the impact of CD68+ TAMs and found that a high density of TAMs in most cancers appears to associate with poorer OS. Summarized, the prognostic value of TAMs may differ among and within the different cancers dependent on the tumour microenvironment, but also on the specific marker used to identify them. Most research including our results indicates that M2 TAMs favour tumour progression.

TAMs were heterogeneously distributed in gliomas at the inter- and intratumoral level. This is possibly explained by the predominant cellular component in the tumours and the molecular subclass of the glioma. For the first time, we report that CD204+ TAMs at the tumours and the molecular subclass of the glioma.

The M2-related marker CD204 belongs to the scavenger receptor family and is capable of binding and internalizing a range of ligands including apoptotic cells and chemically modified or altered molecules, for example, low-density lipoproteins, collagen and myelin [73,74]. Consistent with its function and with previous observations [73], normal brain tissue had low levels of CD204. In GBMs, the amount and intensity of CD204 varied with some tumours expressing predominantly CD204 compared with IBA-1. IBA-1 is a general microglia/macrophage marker [75], but the expression level is upregulated upon cellular activation, and high levels of IBA-1 are associated with increased cell motility [31,76] and pro-inflammatory activity in macrophages [77,78]. Further, in Alzheimer’s pathology, IBA-1 expression correlated with absence of dementia and better cognitive function possibly explained by a higher level of microglial active surveillance, while the opposite correlations were found for CD204 [79]. Analysing the relation between IBA-1 and CD204, we found an inverse correlation between the intensity levels (data not shown), and survival analysis showed that TAMs with IBA-1LOW/CD204HIGH phenotype correlated with worse patient outcome, overall indicating that TAMs with IBA-1HIGH phenotype may represent a population of activated TAMs which have not been polarized in the M2 direction and that the extent of M2 polarization is important. Most research indicates that M2 macrophages are a heterogeneous population that can be divided into M2a, M2b and M2c subsets [80]. Gabrusiewicz et al. [21] showed that CD204 is mainly augmented in M2a and M2c macrophages in vitro. When we investigated the functional properties of the CD204+ TAMs, we found conjunctionally expression of proteins related to M1 (HLA-DR, TNF-α) and M2 (IL-10, TGF-β1) activation. Our data contradict in vitro findings performed on rodent microglia arguing that M2 TAMs produce more TGF-β1 and IL10 compared with TNF-α [16,81,82], as we found little to moderate overlap between CD204 and all three cytokines. Our data indicate that TAMs cannot be delimited into a classical M1 or M2 activation state, but represent a unique phenotype. This is supported by recent microarray and RNA sequencing data on TAMs isolated from murine and human gliomas [17–21] and by results found in pancreatic cancer [83]. Reportedly, M2 TAMs show reduced expression of HLA-DR resulting in poorer antigen presentation [9,51], although the downregulation of the co-stimulatory molecules CD80 and CD86 may be of greater importance [13,14]. We only found 50% co-expression between CD204 and HLA-DR indicating an overall impaired ability to present antigens. However, the range of HLA-DR co-expression was between 3% and
85% suggesting that a subset of M2 TAMs resembled the HLA-DR myeloid-derived suppressor cells (MDSCs), while another subset resembled a more classical HLA-DR\textsuperscript{HIGH} M1 phenotype. This reflects the heterogeneity also among M2 TAMs and indicates that their polarization might depend on the location of the specific TAM within the tumour tissue as well as its specific cellular origin (macrophages vs. microglia vs. MDSCs) and on the stage of disease.

**M2 TAMs may contribute to treatment resistance**

In GBM patients, CD204\textsuperscript{+} TAMs correlated with poorer survival in patients receiving postsurgical treatment. When we included MGMT methylation, high amounts of CD204 also predicted poorer survival in patients with MGMT methylated promoters, while the survival detriment was less evident in patients with unmethylated MGMT promoters. This suggests that M2 TAMs contribute to treatment resistance in GBMs. The importance of myeloid cells including TAMs in the therapeutic response in cancer has recently been reviewed [84].

In GBMs, tumour resistance has also been linked to tumour hypoxia [6,85]. Supported by previous reports [86–88], we observed that TAMs accumulated in areas of hypoxia, and when investigating the phenotype of CD204\textsuperscript{+} cells, we found up to 50% co-expression with HIF-1\textalpha{}. Reportedly, hypoxic conditions induce a mesenchymal shift in GBMs [85,89] and fine-tune the M2 polarization of TAMs [90], leading to an upregulation of HIFs and HIF target genes by TAMs including MMPs and proangiogenic factors, for example, TNF-\textalpha{} [86,87].

In a breast carcinoma model, the tumour volume was reduced, and docetaxel proved more effective when the tumour environment contained HIF-1\textalpha{} knockout macrophages as compared with a microenvironment with wild-type macrophages [91]. In summary, this suggests that TAMs and hypoxia co-ordinate a microenvironment favouring tumour resistance and progression.

Primarily, IBA-1\textsuperscript{+} TAMs were present in the tumour periphery, whereas CD204\textsuperscript{+} TAMs were restricted to the central tumour and the area adjacent to the tumour periphery, suggesting that M2 TAMs may contribute to tumour invasiveness. This is in agreement with a study in which TAMs were found to reside at the invasive front [82]. Our immunofluorescence characterization of the CD204\textsuperscript{+} TAMs revealed a substantial co-expression of MMP-14, which reportedly acts as a co-factor for extracellular matrix breakdown and tumour expansion [8,9,22,24] as well as of EGFR which has been linked to increased tumour invasiveness and proliferation/survival [24,25]. Together with the scavenger function of CD204, this indicates that TAMs co-expressing CD204 and MMP14 may be effective mediators of matrix remodelling facilitating tumour infiltration.

**Conclusions**

Recent genetic profiling of TAMs in human GBM indicates that TAMs can take on several phenotypes along the M1-M2 spectrum including the more undifferentiated M0 state [21]. Our results demonstrate that the prognostic impact of TAMs in gliomas does not depend on the total amount of TAMs, but on their acquired functional phenotype. High levels of the M2-related marker CD204 correlated with increasing malignancy grade and poor patient survival in WHO grade III and IV independently of clinical-pathological parameters. CD204\textsuperscript{+} TAMs were associated with a more aggressive tumour subtype and expressed proteins that could enable tumour progression. These results highlight the significant existence of TAMs in the tumour microenvironment and demonstrate that quantitative assessment of CD204 is feasible and potentially a clinically valuable prognostic biomarker in high-grade gliomas. Deeper insight into the impact of TAMs in gliomas may open new possibilities and expand the field of treatment strategies using TAMs as novel targets.

**Ethics approval and consent to participate**

The study was approved by the Regional Committee on Health Research Ethics for Southern Denmark (Project-ID: S2D090o80) as well as the Danish Data Protection Agency (file number: 2009-41-3070). The use of tissue was not prohibited by any patient according to the Danish Tissue Application Register.

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Author contributions
MDS and BWK conceived and designed the experiments. MDS and RDH collected and assembled the data. MDS, RHD and HBH analysed and interpreted the results. SH and BWK contributed with reagents/materials/analysis tools. MDS drafted and edited the manuscript. All authors have read and approved the final manuscript.

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Conflict of interests
The authors declare that they have no conflicts of interest.

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Supporting information
Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1. Run variation and calibration.
Figure S2. Expression of IBA-1 and CD204 in normal brain tissue and brain neoplasms.
Figure S3. Impact of TAMs on overall survival in WHO grade II and grade III gliomas.
Figure S4. Characterization of CD204+ TAMs.
Figure S5. Bioinformatics databases.
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Table S3. Multivariate Cox regression analyses of IBA-1 and CD204 levels in patients with WHO grade II–IV gliomas.
Table S4. Multivariate Cox regression analyses of IBA-1 and CD204 levels in patients with WHO grade II tumours, including performance status, IDH status and histology (n = 22).
Table S5. Multivariate Cox regression analyses of IBA-1 and CD204 levels in patients with WHO grade III tumours, including performance status, IDH status and histology (n = 22).
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Table S7. Correlation between the gemistocytic cell score and the level of TAMs in astrocytomas.
Data S1. Materials and methods.

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