Astrocytes facilitate melanoma brain metastasis via secretion of IL-23

Anat Klein,1,2 Hila Schwartz,2 Orit Sagi-Assif,1 Tsipi Meshel,1 Sivan Izraely,1 Shlomit Ben Menachem,1 Roman Bengaiiev,1 Amir Ben-Shmuel,2 Clara Nahmias,3 Pierre-Olivier Couraud,3 Isaac P Witz1 and Neta Erez2,*

1 Department of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Avi, Israel
2 Department of Pathology, Sackler School of Medicine, Tel Aviv University, Tel Avi, Israel
3 Department of Cell Biology, Institut Cochin, Paris, France

*Correspondence to: N Erez, Department of Pathology, Sackler School of Medicine, Tel Aviv University, Tel Avi, Israel 69978. E-mail: netaerez@post.tau.ac.il

Abstract

Melanoma is the leading cause of skin cancer mortality. The major cause of melanoma mortality is metastasis to distant organs, frequently to the brain. The microenvironment plays a critical role in tumourigenesis and metastasis. In order to treat or prevent metastasis, the interactions of disseminated tumour cells with the microenvironment at the metastatic organ have to be elucidated. However, the role of brain stromal cells in facilitating metastatic growth is poorly understood. Astrocytes are glial cells that function in repair and scarring of the brain following injury, in part via mediating neuroinflammation, but the role of astrocytes in melanoma brain metastasis is largely unresolved. Here we show that astrocytes can be reprogrammed by human brain-metastasizing melanoma cells to express pro-inflammatory factors, including the cytokine IL-23, which was highly expressed by metastases-associated astrocytes in vivo. Moreover, we show that the interactions between astrocytes and melanoma cells are reciprocal: paracrine signalling from astrocytes up-regulates the secretion of the matrix metalloproteinase MMP2 and enhances the invasiveness of brain-metastasizing melanoma cells. IL-23 was sufficient to increase melanoma cell invasion, and neutralizing antibodies to IL-23 could block this enhanced migration, implying a functional role for astrocyte-derived IL-23 in facilitating the progression of melanoma brain metastasis. Knocking down the expression of MMP2 in melanoma cells resulted in inhibition of IL-23-induced invasiveness. Thus, our study demonstrates that bidirectional signalling between melanoma cells and astrocytes results in the formation of a pro-inflammatory milieu in the brain, and in functional enhancement of the metastatic potential of disseminated melanoma cells.

Keywords: melanoma; brain metastasis; astrocytes; microenvironment; neuroinflammation; IL-23

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Introduction

Cutaneous melanoma is the deadliest of all skin cancers and the major cause of melanoma mortality is metastasis to distant organs [1]. Malignant melanoma has the highest inclination to metastasize to the brain of all primary neoplasms in adults. Brain metastases are incurable and encompass terminal prognosis for most patients, with a dismal median survival of < 1 year [2]. Dissemination of cancer cells to distant organs and establishment of clinically relevant metastases is a multistage process. Each of the steps towards metastasis is affected by cells in the tumour microenvironment and by their soluble products [3–5]. In order to colonize the brain, disseminated melanoma cells have to cross the blood–brain barrier and to communicate with brain stromal cells, including microglia and astrocytes. Despite the clear clinical implications, the mechanisms underlying the survival of metastatic melanoma cells in the brain and their interactions with brain stromal cells are poorly characterized.

Astrocytes play a principal role in maintaining brain homeostasis and participate in the repair and scarring of the brain following injuries. Their dysregulation is thought to contribute to the pathogenesis of several diseases, including brain cancer and metastasis [6]. We and others have previously shown that activated astrocytes infiltrate brain metastases [7,8]. Moreover, astrocytes participate in mediating neuroinflammation; the immune reaction in the central nervous system (CNS) to tissue damage, characterized by release of pro-inflammatory cytokines and chemokines, increased blood–brain barrier permeability and leukocyte invasion [9,10]. Inflammation is now accepted as a hallmark and an enabling characteristic of cancer [11]. Multiple studies have demonstrated the role of inflammation in neurodegenerative and autoimmune diseases in the
Astrocytes facilitate melanoma brain metastasis via IL-23

CNS, and have suggested an active role for astrocytes in producing inflammatory mediators [10]. However, very little is known about the role of astrocyte-mediated neuroinflammation in facilitating the growth of metastatic tumour cells in the brain.

We recently showed that brain-derived soluble factors enhance the migration of brain-metastasizing melanoma cells [12]. However, the cellular source of these soluble factors has not been defined. Here we expand these observations and identify functional interactions between brain-metastasizing melanoma cells and astrocytes. We show that astrocyte-derived soluble factors enhance the migration and invasiveness of melanoma cells, and up-regulate the secretion of matrix metalloproteinase MMP2 in human brain-metastasizing melanoma cells (HBMMs). Moreover, the interactions between astrocytes and melanoma cells are reciprocal: brain-metastasizing melanoma cells induced an up-regulation in the expression of pro-inflammatory genes in astrocytes, including IL-23, a pro-inflammatory heterodimeric cytokine [13]. IL-23 was highly expressed in metastases-associated astrocytes in vivo. In vitro, IL-23 was sufficient to enhance melanoma cell invasion and neutralizing antibodies to IL-23 could attenuate this enhanced migration, implying a functional role for IL-23 in facilitating the progression of melanoma brain metastasis.

Materials and methods

Cell culture

The human melanoma brain-metastatic variant YDFR.CB3 (termed HBMMs) was established in our laboratory as previously described [8]:

- mCherry YDFR.CB3 cells were infected to stably express mCherry (pQCXI–mCherry retroviral vector).
- shMMP2 YDFR.CB3: YDFR.CB3 variants were infected with lentivirus (GIPZ) expressing shRNA for human MMP2 (RHS4531–EG4313; Thermo-Scientific).
- Human brain endothelial cells (hCMECs/D3 cells) were maintained in monolayer cultures as previously described [14].
- Human astrocytes (HA; ScienCell Research Laboratories, USA) were maintained on 0.1% poly-L-lysine (Sigma, Israel) in astrocytes growth medium (ScienCell Research Laboratories).
- Cells were cultured in humidified air with 6.5% CO2 at 37 °C. The cultures were routinely tested and found to be free of mycoplasma.

Mice

Male athymic nude mice (Balb/c background), 7–10 weeks old, were purchased from Harlan Laboratories (Israel). The mice were housed and maintained in the specific pathogen-free animal quarters of Tel Aviv University (TAU). All experiments involving animals were approved by the TAU Institutional Animal Care and Use Committee.

Orthotopic and intracardiac inoculation of tumour cells

- Subdermal inoculation: 1 × 10^6/100 μl cells were inoculated subdermally into the right thigh of each mouse, as described previously [8].
- Intracardiac (IC) inoculation: 0.5 × 10^6/50 μl cells were inoculated using a 29-gauge needle into the left heart ventricle of each mouse, as described [8]. The injections were performed under a small animal ultrasound (Vevo 770 High-Resolution System; VisualSonics Inc.).

Magnetic resonance imaging

OCT-embedded brain sections were cut. The sections were blocked with protein block (Dako) for 10 min. Primary antibodies [GFAP (Dako, Denmark), MECA32 (R&D), IL-23 (R&D) and anti-GLAST1 (Miltenyi Biotech), diluted 1:500, 1:100, 1:100 and 1:20, respectively] were incubated for 1 h at room temperature (RT), followed by fluorescently conjugated secondary antibodies for 30 min at RT. Coverslips were mounted using Vectamount with DAPI (Vector Laboratories). The images were viewed with a x 63/1.4 oil objective, using a Leica SP5 microscope and Leica SP5 software (LAS-AF, Leica, Germany) or a confocal microscope (LSM 510, Carl Zeiss, Germany) and LSM image browser.

Adhesion assay

Plates (96-well) were coated with 100 μg/ml collagen type I for 1 h at 37 °C. Human brain endothelial cells (hCMECs/D3 cells; 5 × 10^4/200 μl) were cultured for 24 h to form a confluent monolayer. Adhesion of CFDA-stained melanoma cells to activated hCMECs/D3 cells was performed as we previously described [15].

Migration and transendothelial migration

Melanoma cells (1 × 10^5) were placed into the upper side of 0.2% gelatin-coated 24 Transwell inserts, with pore
sized of 8 μm, in 100 μl RPMI; in the lower chamber, serum-free medium (SFM) was placed or 5 × 10⁶ human astrocytes were seeded. For transendothelial migration assays, human brain endothelial cells were added to the upper chamber, prior to the addition of melanoma cells. Following 24 h of incubation, the upper side of the apical chamber was scraped gently with cotton swabs to remove non-migrating cells, which were fixed with methanol and stained with DAPI. Migrated melanoma cells were documented under a fluorescence microscope, using ACT software.

Quantitative real-time PCR
Total RNA was extracted using the EZ-RNA Total RNA Isolation Kit (Biological Industries), followed by cDNA synthesis with M-MLV Reverse Transcriptase (Ambion Inc.).

Real-time PCR reactions were run in triplicate. The primers used were human MART1, mouse GFAP and GAPDH (reacts with human and mouse cDNA to yield one product), mouse IL-8, GAPDH, CXCL12, COX-2 and CLDN1. qRT–PCR was conducted with iTaq Universal SYBR Green Supermix (BioRad).

Preparation of human astrocyte (HA) conditioned medium (CM)
Astrocytes (1 × 10⁶) were plated on 10 cm² plates in HA growth medium. After overnight incubation, the growth medium was removed and replaced by serum-free medium (SFM) for 24 h of incubation. After an overnight incubation, CM was collected and filtered using a 0.45 μm filter.

Proteomics analysis
HAs incubated in metastatic melanoma tumour cell CM for 24 h were lysed and analysed for the relative expression of 36 cytokines (Human Cytokine Array Panel A, R&D Systems). 100 ng cell lysates were run on each array.

Gelatin substrate zymography
Melanoma cells (5 × 10⁶) were plated in a 24-well plate in growth medium or with astrocytes conditioned medium (CM) for 24 h. Growth medium was replaced by serum-free medium (SFM; Con) or HA-CM for an additional 24 h. Then the CM and SFM media were removed and replaced by SFM for 24 h. Matrix metalloproteinases activity in the conditioned medium was determined as previously described [17].

Statistical analysis
Data were analysed using Student’s t-test and considered significant at p < 0.05. Bar graphs represent mean and standard deviation (SD) across multiple independent experimental repeats, unless otherwise stated.

Results
A model of human melanoma brain metastasis
We previously established an in vivo model for spontaneous brain micrometastasis of a human melanoma patient-derived cell line, selected for their brain-metastasizing capacity [8]. This model enables the study of early changes in the brain microenvironment. To facilitate the detection and in vivo imaging of micrometastases, we engineered human metastatic melanoma cell line YDFR.CB3 cells to express the fluorescent reporter gene mCherry; mCherry–YDFR.CB3 cells are referred to hereafter as human brain-metastasizing melanoma cells (HBMMs). In order to induce spontaneous brain metastases, we injected orthotopically (by subdermal injections) 1 × 10⁶ mCherry-expressing human melanoma cells into nude mice. Primary tumour growth in the transplanted mice was analysed. Micrometastases in brains were first detected approximately 3 weeks postinoculation (Figure 1A, B); 75% of mice develop spontaneous brain micrometastasis following orthotopic injection [8]. In parallel, injection of 0.5 × 10⁶ mCherry-expressing melanoma cells intracardially (IC) into nude mice resulted in the formation of macrometastases approximately 10 weeks postinjection (Figure 1A, C). Interestingly, immunostaining of brain blood vessels 2 weeks after subdermal inoculation revealed mCherry melanoma cells in brain blood vessels even before metastasizing melanoma cells were detected in the brain parenchyma (Figure 1D, E).
Activated astrocytes surround and infiltrate brain metastases

To study the role of astrocytes in the formation of melanoma brain metastases, we assessed the presence of activated astrocytes by immunostaining brain tissue sections for astrocytes that express glial fibrillary acidic protein (GFAP). Using mCherry-labelled melanoma cells, we confirmed our previous results, that metastatic lesions in the brains of injected mice are surrounded and infiltrated by activated astrocytes [8]. Moreover, we showed that there was an increase in astrocyte number compared to normal mouse brain, suggesting an active role for recruited astrocytes in supporting brain metastasis in vivo (Figure 1F–H).

Astrocyte-derived factors facilitate migration and invasiveness of melanoma cells

Two of the more important steps in blood-borne tumour metastasis are the adhesion of circulating malignant cells to the brain vascular endothelium and their subsequent extravasation [18]. Therefore, we next tested the adherence ability of HBMMs to human brain endothelial cells (hCMECs/D3 cells), as compared with their corresponding melanoma cells from the cutaneous variant, which originated from the same melanoma tumour. To that end, we performed an in vitro adhesion assay. Analysis revealed that HBMMs could adhere significantly better to human brain endothelial cells than melanoma cells from the cutaneous variant (Figure 2A).

Astrocytes are an integral part of the blood–brain barrier [19] and the first stromal cells of the brain encountered by metastasizing tumour cells following extravasation. We next asked whether astrocytes could facilitate melanoma cell migration and invasion, and whether paracrine signalling by astrocytes is operative via a monolayer of endothelial cells. To that end, we utilized melanoma cells isolated previously from the brains of injected mice bearing micro- or macrometastases [8] and performed Transwell assays with human astrocytes and endothelial cells (Figure 2B). Quantification of melanoma cell migration revealed that melanoma cells isolated from micro- or macrometastases migrated significantly better in the presence of astrocyte-derived
soluble factors (Figure 2C). Interestingly, cells isolated from brain micrometastases migrated better than cells isolated from macrometastases in response to paracrine signalling from astrocytes (Figure 2C). Moreover, transendothelial migration of melanoma cells was also enhanced in the presence of astrocytes (Figure 2D), suggesting that paracrine signalling between melanoma cells and astrocytes can be transduced via a monolayer of endothelial cells.

Paracrine signalling by brain-metastasizing melanoma cells activates pro-inflammatory factors in astrocytes

We next found that the interactions between melanoma cells and astrocytes are reciprocal: co-culture of melanoma cells with astrocytes induced an up-regulation in the expression of the pro-inflammatory genes COX-2, IL-8 and CXCL12 in astrocytes, and...
Astrocytes facilitate melanoma brain metastasis via IL-23

IL-23 is up-regulated in astrocytes and facilitates melanoma cell migration

IFNγ, CXCL12 and CD40L are known to be secreted by multiple glial cells in the brain during neuroinflammation, including astrocytes [20–24]. IL-23 was shown to be secreted by myeloid cells and T cells [25,26], but the role of astrocyte-derived IL-23 is largely unresolved. IL-23 is a pro-inflammatory heterodimeric cytokine implicated in tissue inflammation [13], neurodegenerative disorders and also in promoting tumour incidence and growth [27–29]. Macrophage-derived IL-23 was recently implicated in neuroinflammation during ischaemic stroke and in Alzheimer’s disease [28,29]. However, the role of IL-23 in astrocytes and in brain metastasis is unknown, and we therefore decided to focus on deciphering its functional role during melanoma brain metastasis.

In order to test whether the up-regulation of IL-23 at the protein level is also evident at the transcriptional
level, we analysed its expression in astrocytes incubated with HBMMs-conditioned medium by qRT–PCR, and found that the expression of IL-23 in astrocytes is up-regulated by 50% following incubation with melanoma-secreted factors, as compared with control cells (Figure 3B). We next tested whether IL-23 contributes to astrocyte-facilitated melanoma cell migration. To that end, we preformed migration and transendothelial migration assays (as described in Figure 2B); 10 ng/ml recombinant IL-23 (rIL-23) was added to the lower chamber and HBMMs were allowed to migrate through a Transwell membrane or via a monolayer of human brain endothelial cells. Quantification of migrated melanoma cells revealed that IL-23 was sufficient to facilitate migration and transendothelial invasion of HBMMs (Figure 3C, D). Moreover, when we inhibited IL-23 in astrocyte conditioned medium by adding anti-IL-23 neutralizing antibodies, the migration and invasiveness of melanoma cells was significantly attenuated (Figure 3E, F), suggesting a functional role of astrocyte-secreted IL-23 in facilitating melanoma cell migration. Interestingly, when we performed a similar experiment with recombinant IFNγ, expression of which was also strongly up-regulated in astrocytes in vitro and in vivo (Figures 3A, 4G), it had no effect on the migration of melanoma cells in a Transwell assay (see supplementary material, Figure S2), suggesting that IFNγ has a distinct functional role in astrocyte-mediated neuroinflammation.

We next asked whether IL-23 is up-regulated in metastases-associated astrocytes in vivo. To that end, nude mice were injected intracardially with HBMMs (as illustrated in Figure 1A) and the presence of macrometastases was validated by MRI imaging 12 weeks after injection. Macrometastases were visually apparent in the brains of injected mice (Figure 4A), but not in normal brains (Figure 4B). mCherry expressing lesions were apparent even without imaging in brains bearing macrometastases (Figure 4C), but not in normal brains (Figure 4D). In order to isolate pure populations of astrocytes for expression analysis without employing a tissue culture step, we performed magnetic cell separation utilizing the astrocyte-specific anti-GLAST antibody, which recognizes an extracellular epitope of the astrocyte-specific glutamate transporter GLAST. Normal astrocytes or metastases-associated astrocytes were isolated from a pool of control, non-injected mouse brains. Similarly, melanoma cells were isolated, utilizing anti-HLA magnetic beads, which specifically bind human cells (Figure 4E). To validate the purity of the isolated astrocytes, we performed qRT–PCR for the expression of GFAP and for the melanoma expressed gene MART1. The results indicated that the isolated astrocyte population was highly pure and that, as expected, GFAP was up-regulated in metastases-associated astrocytes as compared to normal astrocytes (Figure 4F).

Since the pro-inflammatory cytokines IL-23 and IFNγ were up-regulated in astrocytes in vitro following incubation with melanoma cells, we analysed their expression in metastases-associated astrocytes. Indeed, IL-23 and IFNγ were highly up-regulated in astrocytes isolated from melanoma brain metastases, further supporting their functional role in facilitating brain metastases in vivo (Figure 4G). Moreover, immunostaining of brain tissue sections revealed strong expression of IL-23 by metastases-associated astrocytes as well as by metastatic melanoma cells, but not in normal brain (Figure 4H–M). Thus, IL-23 is strongly up-regulated in astrocytes surrounding melanoma brain metastatic lesions in vivo.

Astrocyte-secreted factors activate MMP2 in HBMMs

Seeking to obtain mechanistic insight into the molecular pathways by which astrocytes facilitate melanoma cell invasion, we next investigated changes in the expression of several matrix metalloproteinases (MMPs), known to play an important role in tumour invasion and angiogenesis by mediating degradation of the extracellular matrix [30]. In particular, we examined MMP2 and MMP9; both were previously implicated in melanoma invasiveness and progression [31]. The levels of MMP9 were not altered in HBMMs (data not shown). However, astrocyte-derived soluble factors induced a significant up-regulation in MMP2 in HBMMs at both the transcriptional and the protein level (Figure 5A–C). Gelatin zymography assays further confirmed an elevated functional activity of MMP2 in melanoma cells incubated with astrocytes CM, as compared with control cells incubated with SFM (Figure 5D, E). Since IL-23 could enhance melanoma cell invasion (Figure 3C–F), we next asked whether the astrocyte-induced up-regulation of MMP2 in melanoma cells could be mediated via IL-23. To that end, we neutralized IL-23 in astrocytes CM and analysed the expression of MMP2 in melanoma cells. Indeed, neutralizing IL-23 attenuated the up-regulation of MMP2 in melanoma cells (Figure 5F). Thus, the enhanced invasive phenotype induced in melanoma cells by paracrine signalling from astrocytes could be mediated via up-regulation of MMP2 by IL-23.

Finally, we tested the functional significance of MMP2 for melanoma cell invasiveness. To that end, we knocked down its expression in HBMMs, utilizing shRNA (Figure 5G, H). HBMMs in which the expression of MMP2 was knocked down (shMMP2) were plated on top of a monolayer of human brain endothelial cells and incubated with SFM, rIL-23 or astrocytes CM (as illustrated in Figure 2B). Transendothelial migration of melanoma cells was assessed after 24h. Analysis of the results revealed that knocking down MMP2 significantly inhibited the transendothelial migration of HBMMs, suggesting a mechanistic link between IL-23-induced invasiveness and MMP2 (Figure 5I, J). Interestingly, there was a stronger inhibition of invasion when cells were incubated with IL-23 as compared to astrocyte CM (52% and 37%, respectively), suggesting that additional astrocyte-secreted factors, other than IL-23, are also operative in inducing
Figure 4. IL-23 is up-regulated in metastasis-associated astrocytes in vivo. (A) MRI imaging of macrometastases-bearing brain; arrows, brain metastatic lesions. (B) MRI imaging of normal brain. (C) Brain of IC-injected mouse; arrows indicate mCherry-labelled macrometastases. (D) Normal brain. (E) Astrocyte isolation from fresh brain tissue: astrocytes were isolated from normal or IC-injected macrometastases-bearing brains, first with an anti-HLA antibody, then by anti-GLAST antibody and magnetic beads. (F) qRT-PCR analysis of GFAP and MART1 expression in isolated astrocytes confirms purity: results are normalized to mouse GAPDH; analysis was performed on pools of five brains/group. (G) qRT-PCR analysis for the expression of IL-23 and IFNγ in GLAST+ metastases-associated astrocytes, isolated as above; results are normalized to mouse GAPDH. (H–M) IL-23 is highly expressed by metastases-associated astrocytes: immunofluorescence of brain sections. (H) Green staining for IL-23 (AlexaFluor 488). (I) Brain-metastasizing melanoma cells; red, mCherry. (J) Astrocytes expressing GLAST-1; magenta, APC. (K) Normal brain section, channel overlay L: macrometastases-bearing brain, channel overlay; magnification = x 40; scale bar = 50 μm. (M) Magnification with zoom 1.5; magnification = x40; scale bar = 50 μm; representative images of multiple fields analysed from four mice.
melanoma invasiveness. Taken together, these results implicate IL-23 in inducing a more invasive phenotype in brain-metastasizing melanoma cells, which is mediated at least partially via up-regulation of MMP2.

**Discussion**

The molecular crosstalk between disseminated tumour cells and stromal cells in the metastatic organ is the rate-limiting step in metastasis [32]. The main goal of our study was to uncover the interactions between disseminated melanoma cells and astrocytes, that facilitate the invasion of melanoma cells through the blood–brain barrier and into the brain parenchyma. Our study identifies the pro-inflammatory cytokine IL-23 as a novel astrocyte-derived pro-metastatic mediator. We show that IL-23 is highly up-regulated in astrocytes by human melanoma brain-metastasizing cells in vitro and in metastases-associated astrocytes in vivo. IL-23 was sufficient to enhance melanoma cell invasiveness, and neutralizing IL-23 in astrocytes CM attenuated the up-regulation of MMP2 as well as the enhanced migration, implying a functional role...
for IL-23 in facilitating the progression of melanoma brain metastasis. Astrocyte-secreted factors enhanced migration and the transendothelial invasion of human brain-metastasizing melanoma cells, and induced the secretion of MMP2 by melanoma cells. Moreover, knocking down the expression of MMP2 in melanoma cells attenuated IL-23-induced invasiveness. Thus, our study demonstrated that bidirectional signalling between melanoma cells and astrocytes resulted in the formation of a pro-inflammatory milieu in the brain, enhancing the metastatic potential of disseminated melanoma cells (Figure 6).

Astrocytes were previously implicated in facilitating brain metastasis: breast and lung cancer cells were shown to recruit glial cells and to induce a brain inflammatory response that correlated with tumour growth and with in vitro expression of pro-inflammatory cytokines by astrocytes [33–35]. Moreover, astrocytes were recently suggested to protect melanoma cells from chemotherapy via the endothelin–endothelin receptors signalling axis [36–38]. In addition, astrocytes can protect melanoma cells from chemotherapy by direct contact through the establishment of functional gap junctions via connexin 43 [39,40], but the role of astrocytes in facilitating brain colonization is unknown. Our results show that human melanoma cells can reprogramme astrocytes to express pro-inflammatory cytokines in vitro and in vivo. Moreover, we show that astrocytes are recruited to melanoma brain-metastatic lesions, and that activated astrocytes isolated from brains with melanoma metastases have up-regulated expression of pro-inflammatory factors. In particular, we found that IL-23 was highly up-regulated in astrocytes exposed to HBMMs-secreted factors, both in vitro and in vivo.

IL-23 promotes the expansion and maintenance of IL-17-producing CD4+ T cells (Th17 cells). The IL-23–IL-17 axis has been implicated in the pathogenesis of many chronic inflammatory and autoimmune disorders [13,41]. Moreover, IL-23 promotes tumour incidence and growth in a skin cancer model via up-regulation of the matrix metalloproteinase MMP9, increased angiogenesis and reduced CD8+ T cell infiltration [27]. In the brain, macrophage-derived IL-23 has been implicated in neuroinflammation during ischaemic stroke and in Alzheimer’s disease [28,29]. In addition to its expression by brain macrophages, IL-23 was shown to be expressed by astrocytes under inflammatory conditions [42,43]. However, the role of IL-23 in brain metastasis is unknown. Our study implicates astrocyte-derived IL-23 in facilitating the invasiveness of brain-metastasizing melanoma cells.

Interestingly, a recent study demonstrated that therapeutic targeting of IL-23 has antimetastatic effects in experimental and spontaneous lung metastasis models, including melanoma lung metastasis. These therapeutic effects were dependent on the presence of T cells, as they were lost in RAG−/− mice, suggesting that neutralizing IL-23 may tilt the balance towards antitumour immunity [44]. Future studies in immunocompetent models of melanoma brain metastasis are required to investigate whether astrocyte-derived IL-23 functions via modifying T cell-mediated immunity in the brain.

The invasiveness of tumour cells is facilitated by their ability to degrade basement membranes and invade through the extracellular matrix. Melanoma invasiveness and progression were previously linked with up-regulation of MMP2, which was found in highly metastatic human melanoma cell lines, as compared with non-metastatic cells [45]. Moreover, retrospective analyses of human melanoma samples from large patient cohorts demonstrated a link between MMP2 expression and metastatic disease [31,46]. We show here that the up-regulation of MMP2 in brain-metastasizing melanoma cells is induced by astrocyte-derived factors. Moreover, functional knockdown of MMP2 in
brain-metastasizing melanoma cells resulted in attenuated invasiveness, suggesting a functional link between IL-23-induced migration and MMP2. Thus, our results not only demonstrate a functional role for astrocytes in facilitating brain metastasis, but also offer a mechanistic insight.

Interestingly, a recent study shows that astrocytes can directly support the invasive capacity of human breast and lung cancer cells via astrocyte-secreted MMP2 and MMP9 [47], in agreement with previous studies showing that MMPs can be provided by the tumour microenvironment [48,49]. Thus, while the role of astrocytes in supporting brain metastasis may be general, the specific molecular mechanisms are likely context- and tumour type-specific. Our data, demonstrating reciprocal interactions between melanoma cells and astrocytes, support such tumour specificity; different tumour cells are predicted to activate astrocytes in distinct manners.

Intriguingly, a recent study showed that astrocytes initially oppose the metastatic capabilities of disseminated breast and lung cancer cells in the brain. In order to colonize the brain, tumour cells must avert these metastasis-inhibiting signals [50]. Our results expand these observations and show that brain-metastasizing cells not only resist anti-metastatic signalling by astrocytes but also reprogramme astrocytes to become culprits that actively facilitate brain colonization.

Thus, melanoma micrometastatic cells are capable of activating astrocytes, which in turn secrete various inflammatory factors endowed with a pro-metastatic activity, thereby driving the progression to overt brain metastasis. Elucidating the functional importance of astrocyte-mediated neuroinflammation will lead to the discovery of key regulators within astrocytes that may serve as novel therapeutic targets.

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Author contributions

AK and HS and OSA conceived and carried out experiments and analysed data; TM and RB carried out experiments; SI, SBM and ABS were involved in data collection and generation of figures; CN and POC contributed essential reagents; NE and IPW designed and supervised the study; and NE, AK and IPW wrote the manuscript. All authors had final approval of the submitted and published versions.

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Astrocytes facilitate melanoma brain metastasis via IL-23


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**SUPPLEMENTARY MATERIAL ON THE INTERNET**

The following supplementary material may be found in the online version of this article:

Figure S1. HBMMs CM modifies the expression of CLDN1 and COX-2 in human astrocytes.

Figure S2. IFNy does not affect HBMMs migration.