TNF-α respecifies human mesenchymal stem cells to a neural fate and promotes migration toward experimental glioma

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Bone marrow-derived human mesenchymal stem cells (hMSCs) have become valuable candidates for cell-based therapeutical applications including neuroregenerative and anti-tumor strategies. Yet, the molecular mechanisms that control hMSC trans-differentiation to neural cells and hMSC tropism toward glioma remain unclear. Here, we demonstrate that hMSCs incubated with 50 ng/ml tumor necrosis factor alpha (TNF-α) acquired astroglial cell morphology without affecting proliferation, which was increased at 5 ng/ml. TNF-α (50 ng/ml) upregulated expression of numerous genes important for neural cell growth and function including *LIF* (*leukemia inhibitory factor*), *BMP2* (*bone morphogenetic protein 2*), *SOX2* (*SRY box 2*), and *GFAP* (*glial fibrillary acidic protein*), whereas *NES* (*human nestin*) transcription ceased suggesting a premature neural phenotype in TNF-α-differentiated hMSCs. Studies on intracellular mitogen-activated protein kinase (MAPK) signaling revealed that inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) activity abolished the TNF-α-mediated regulation of neural genes in hMSCs. In addition, TNF-α significantly enhanced expression of the chemokine receptor CXCR4 (CXC motive chemokine receptor 4), which facilitated the chemotactic invasiveness of hMSCs toward stromal cell-derived factor 1 (SDF-1) alpha. TNF-α-pretreated hMSCs not only exhibited an increased ability to infiltrate glioma cell spheroids dependent on matrix metalloproteinase activity *in vitro*, but they also showed a potentiated tropism toward intracranial malignant gliomas in an *in vivo* mouse model. Taken together, our results provide evidence that culture-expansion of hMSCs in the presence of TNF-α triggers neural gene expression and functional capacities, which could improve the use of hMSCs in the treatment of neurological disorders including malignant gliomas.

Bone marrow-derived human mesenchymal stem cells (hMSCs) were previously thought to be lineage-restricted and only able to differentiate into mesodermal cell types, such as adipocytes, chondrocytes, and osteocytes.[1] Accumulating data of the past years, however, provide evidence that hMSCs can cross lineage barriers and adopt expression profiles as well as functional phenotypes of neuroectodermal, endo-dermal, and visceral mesodermal cells.[2] One of hMSCs' most striking properties in this context is their apparent potential for transdifferentiation into functional neural cells of both glial and neuronal lineages. These findings suggest hMSCs as valuable candidates for cell-based therapies in patients with developmental and neurodegenerative disorders of the central nervous system (CNS) including multiple sclerosis, Parkinson's disease, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis.[3] The benefit of hMSCs in clinical use is supported by easy accessibility and isolation from bone marrow, *in vitro* expansion in cell culture,

and retransplantation into the same patient without the drawbacks associated with allotransplantation and xeno-transplantation. In contrast, therapeutical concepts based on the application of embryonic and neural stem cells to cure neurological diseases are limited by logistic and ethical problems. *In vivo* studies showed that after peripheral injection MSCs can cross the blood–brain barrier and migrate to damaged areas in the brain, wherein they improve functional recovery in, for example, patients with ischemic stroke.[4] Another interesting aspect in hMSC biology is their tropism for brain tumor tissues,[5] which is similar to that of neural stem cells.[6] High-grade gliomas have the ability for deep infiltration of local structures leading to disease recur-rence despite tumor resection, radiotherapy, and chemo-therapy. The hMSC tropism for gliomas made these cells a promising tool for their use as a gene vector system in the treatment of highly aggressive brain tumors.[5] Despite these multiple potentials of hMSCs, little is known about key

regulators that control commitment of hMSCs to the neural lineage and the molecular mechanisms of hMSC recruitment to gliomas.

Tumor necrosis factor alpha (TNF-α) is a pleiotropic cytokine having important albeit often contradictory roles in numerous physiological and pathological processes including immunity and inflammation.[7] Following injury, TNF-α expression and release is induced within minutes up to a few hours and persists during the following days in damaged tissue. Furthermore, TNF-α is present at elevated levels in various neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sklerosis, and stroke, wherein it is thought to exhibit a proinflammatory function.[8,9] On the other hand, TNF-α is also reported to have a neuroprotective role contributing to repair and recovery after stroke.[10] In the brain, TNF-α was shown to influence growth, survival, and neuronal differentiation of neural stem/progenitor cells, which is mediated via signaling through its cellular receptor tumor necrosis factor receptor (TNF-R)1, thereby modulating tissue regeneration after stroke, inflammation, and status epilepticus.[11–14] At the molecular level, TNF-α-induced effects are mediated via binding to TNF-R1 and/or TNF-R2 with subsequent intracellular signaling through several different pathways including that of NF-κB and the mitogen-activated protein kinases (MAPKs).[9] The three major MAPK cascades comprise ERK1/2 (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), and p38. Phosphorylation of these MAPKs results in their translocation into the nucleus, wherein they modulate gene expression by phosphorylating downstream kinases and transcription factors.[15]

Besides cytokines, the chemokine stromal cell-derived factor 1 (SDF-1 or CXCL12) and its cellular receptor CXCR4 are major modulators of various cell functions such as proliferation and migration with importance in numerous pathological processes including inflammation[16] and tumor progression. Interestingly, SDF-1 expression is strongly upregulated in glioblastoma tissues compared with normal brain.[17] Moreover, SDF-1 production in brain lesions of rats was shown to regulate site-specific trafficking of transplanted bone marrow stromal cells into the damaged areas.[18,19] Additionally, in vitro studies have demonstrated that the migratory potential of hMSCs is not only influenced by SDF-1α but also involves the activity of matrix metalloproteinases (MMPs) and their endogenous inhibitors (tissue inhibitor of metalloproteinases (TIMPs)).[20] The MMPs efficiently degrade components of the extracellular matrix (ECM) thereby enabling together with TIMPs cell migration in a large number of (patho-) physiological processes.[21] As shown in detail recently by our group, short-time treatment with TNF-α enhances the invasive potential of hMSCs by upregulation of MMP-2, MT1-MMP, and TIMP-2 expression and activity.[20]

In this study, we demonstrate that long-term incubation of hMSCs with TNF-α induces a neural phenotype in these cells and potentiates their tropism toward glioma.

Results

The data presented below are representative for measurements accomplished with hMSC samples from three different individuals (Cambrex Lot. # 4F1560, # 8F3520, # 7F3914).

Influence of TNF-α on hMSC morphology and proliferation. Incubation of hMSCs in the presence of TNF-α (50 ng/ml) for 14–28 days induced remarkable changes in cell morphology. After 14 days of treatment, hMSCs had mostly lost their typical flat spindle-shaped morphology and displayed a more elongated and contracted cell contour in comparison with untreated cells. On 28 days of TNF-α exposition, the majority of cells had developed spherical refractile cell bodies, which prevalently showed radial and/or branched cellular extensions (Figure 1a). This morphology is similar to that of neuroglial cells of the astrocyte type.

Examination of hMSC proliferation in the presence of TNF-α within 7 days of cultivation by use of the WST-1 proliferation assay revealed no significant influence at a concentration of 50 ng/ml, whereas lower dosages of the cytokine exhibited a stimulatory effect on cell division with a maximum of twofold augmentation at 5 ng/ml of TNF-α (Figure 1b). Incubation of hMSCs with TNF-α (50 ng/ml) did not increase the regular apoptotic cell proportion (~2%) as determined by annexin V fluorescein labeling (data not shown).

Taken together, these findings indicate that long-term exposition to 50 ng/ml TNF-α triggers differentiation of hMSC into fully mitotic cells with a neuroglial-like morphology.

Microarray analysis of TNF-α-modulated gene expression in hMSCs. To investigate the influence of TNF-α on hMSC gene expression in detail, the GEArray Human Stem Cell Gene Array (SuperArray) was performed that allows to determine the transcription of 266 different marker genes known to be important for identification, growth, and differentiation of various types of stem/progenitor cells. Microarray analysis was carried out in hMSCs after a 14-day incubation period in the absence or presence of TNF-α (50 ng/ml). Our study revealed that TNF-α upregulated the transcription of 40 genes in hMSCs and downregulated 6 genes by at least twofold compared with untreated control cells (Table 1). Many of these genes including GFAP (glial fibrillary acidic protein), MAP2 (microtubule-associated protein 2), LIF (leukemia inhibitory factor), LIFR, BMP2 (bone morphogenetic protein 2), SOX2 (SRY box 2), NES (human nestin), NCAM2, CDH2, S100B, and CXCR4 (CXC motive chemokine receptor 4) are characteristically expressed in neural cells and known for their relevance in the development of these cells. Strikingly, a number of genes found to be modulated in hMSCs by TNF-α are involved in the Wnt signaling pathway, for example, CTNNA1, CTNNB1, FDZ3, FDZ4, WNT3, WNT5A, and WNT7B (Table 1).

Validation of neural gene expression in TNF-α-treated hMSCs. To approve the data obtained by the microarray studies, a time-course mRNA analysis of selected neural marker genes was performed by the use of quantitative real-time polymerase chain reaction (qRT-PCR) in hMSCs cultivated without or with TNF-α (50 ng/ml) for up to 14 days. The transcription of LIF and BMP2 was upregulated about 11-fold and 13-fold, respectively, as compared with untreated hMSCs when determined 1 day after TNF-α stimulation and declined during prolonged cultivation (Figure 2a).
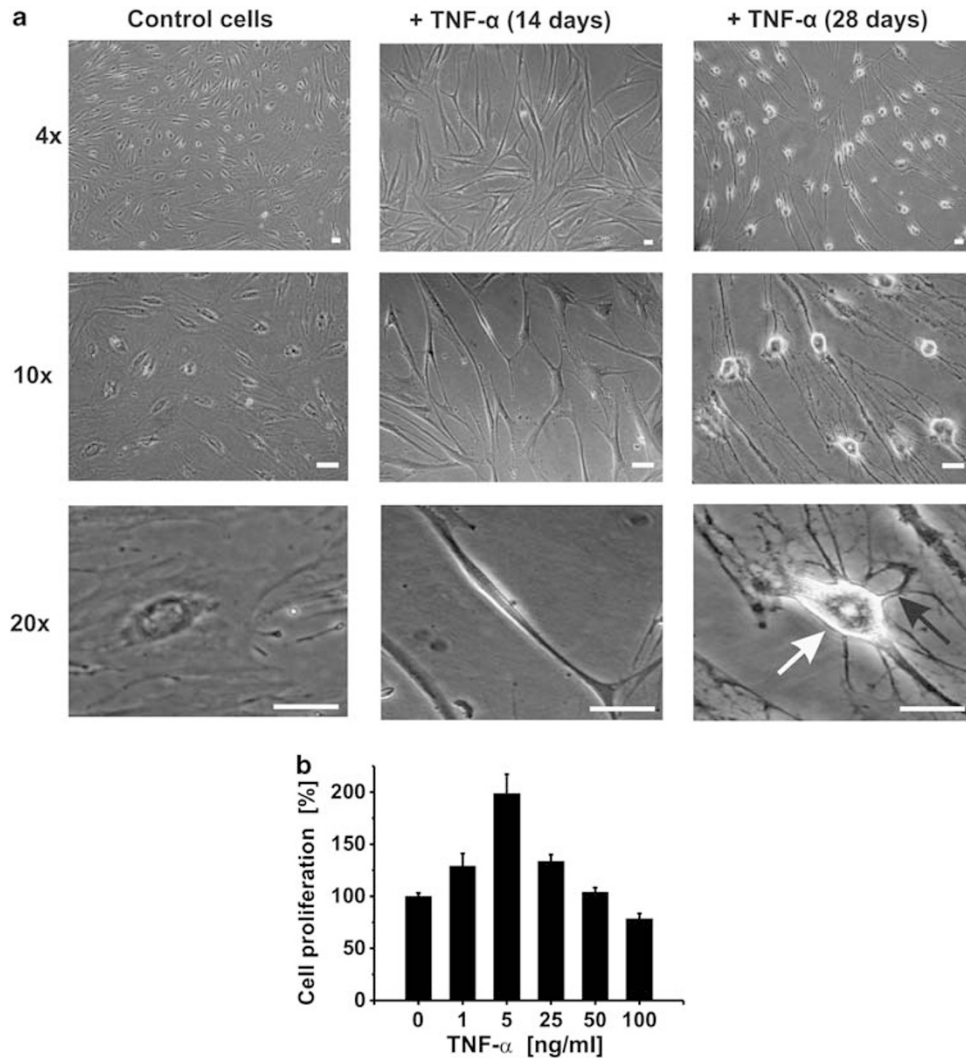
Figure 1 TNF-α-induced changes in hMSC morphology and proliferation. (**a**) Microscopic analysis of hMSCs cultivated in standard growth medium (MSCGM) in the absence and presence of TNF-α (50 ng/ml) for 14 and 28 days. Cell morphology was monitored at different magnifications as indicated. The white arrow denotes the spherical cell body and the black arrow indicates branching cellular extensions. Scale bars, 30 μm. (**b**) hMSCs were incubated without (control) and with increasing concentrations of TNF-α for 14 days and then assayed for cell proliferation using the WST-1 assay. Results are mean values ± S.D. of cell proliferation relative to control (set as 100%) from triplicate cultures and are representative of three independent experiments

Expression of *SOX2* was constantly elevated 2–3-fold throughout the 14-day incubation period, while that of *MAP2* began to increase after 3 days reaching three-fold augmentation after 14 days of exposition to TNF-α. In contrast, basal gene transcription of *NES* encoding for nestin was dramatically diminished 1 day after treatment with TNF-α and further persisted at that low level over 14 days of incubation (Figure 2a). Thus, qRT-PCR analysis in hMSCs confirmed the microarray data, demonstrating that TNF-α stimulates gene expression of *LIF*, *BMP2*, *SOX2*, and *MAP2*, whereas that of *NES* is blocked.

As exemplarily shown on protein level using immunocytochemistry, TNF-α-augmented GFAP production in hMSCs (Figure 2b), which was in agreement with the mRNA data obtained by microarray analysis.

Role of MAPKs in TNF-α-induced hMSC differentiation. Next, we were interested in the intracellular signaling

mechanisms controlling the TNF-α-mediated neural differentiation of hMSCs. To explore the involvement of MAPKs in this process, inhibitors that block the signaling pathways of ERK1/2, p38, and JNK were added to cultures of hMSCs grown in presence of TNF-α (50 ng/ml). After 1 and 7 days of incubation, cells were analyzed for neural marker expression using qRT-PCR. Application of the ERK1/2 inhibitor PD98059 (20 μM) prevented the TNF-α-induced mRNA upregulation of *LIF*, *BMP2*, *SOX2*, and *MAP2*, and also circumvented downregulation of *NES* (Figure 3a). Consistently, protein biosynthesis of GFAP in TNF-α-treated hMSCs was abrogated in presence of PD98059 (Figure 3b). The detailed examination of ERK1/2 phosphorylation by western blotting revealed that TNF-α strongly stimulated intracellular formation of phosphorylated ERK1/2 within 7–14 days of incubation (Figure 3c). TNF-α-induced ERK1/2 phosphorylation was prevented on administration of PD98059 (Figure 3d). Interestingly, TNF-α also increased total ERK1/2 protein

Table 1 Genes modulated in hMSCs on treatment with TNF-α

Accession No.	Symbol	Name	Modulation
NM_001200	BMP2	Bone morphogenetic protein	UP[a]
NM_001202	BMP4	Bone morphogenetic protein 4	DOWN[a]
NM_004329	BMPR1A	Bone morphogenetic receptor, type 1A	UP[a]
NM_001203	BMPR1B	Bone morphogenetic receptor, type 1B	UP[b]
NM_001204	BMPR2	Bone morphogenetic protein receptor, type II	UP[a]
NM_019895	C3orf4	Chromosome 3 open reading frame	UP[a]
NM_001792	CDH2	*N*-cadherin	UP[b]
NM_058197	CDKN2A	Cyclin-dependent kinase inhibitor 2A	UP[a]
NM_001903	CTNNA1	Catenin α 1	UP[a]
NM_001904	CTNNB1	Catenin β 1	UP[a]
NM_003467	CXCR4	Chemokine receptor 4	UP[b]
NM_004412	DNMT2	DNA (cytosine-5-)methyltransferase 2	DOWN[a]
NM_017412	FDZ3	Frizzled homolog 3	UP[a]
NM_012193	FDZ4	Frizzled homolog 4	UP[a]
NM_033136	FGF1	Fibroblast growth factor	UP[a]
NM_002009	FGF7	Fibroblast growth factor 7	UP[a]
NM_001144915	FGFR2	Fibroblast growth factor receptor 2	DOWN[a]
NM_003923	FOXH1	FOXH1 forkhead box H1	UP[a]
NM_002015	FOXO1A	FOXO1A forkhead box O1A	UP[a]
NM_002055	GFAP	Glial fibrillary acidic protein	UP[c]
NM_000876	IGFR2	Insulin-like growth 2 factor receptor	UP[c]
NM_003638	ITGA8	Integrin α 8	UP[c]
NM_000526	KRT14	Keratin 14	DOWN[c]
NM_002309	LIF	Leukemia inhibitory factor	UP[b]
NM_002310	LIFR	Homo sapiens leukemia inhibitory factor receptor	UP[c]
NM_031846	MAP2	Microtubule-associated protein 2	UP[a]
NM_002392	MDM2	P53-binding protein	UP[c]
NM_004540	NCAM2	Neural cell adhesion molecule 2	UP[a]
NM_006617	NES	Human nestin	DOWN[a]
NM_013957	NGR1	Neuregulin 1	UP[a]
NM_005450	NOG	Noggin	UP[a]
NM_002530	NTRK3	Neurotrophic tyrosine kinase receptor type 3	UP[c]
NM_002609	PDGFRB	Platelet-derived growth factor receptor, β-polypeptide	UP[a]
NM_000442	PECAM1	Platelet/endothelial cell adhesion molecule (CD31)	UP[a]
NM_000314	PTEN	Phosphatase and tensin homologue	UP[a]
NM_006272	S100B	S100 calcium-binding protein, β	UP[c]
NM_006942	SOX15	SRY box 15	UP[a]
NM_022454	SOX17	SRY box 17	UP[a]
NM_003106	SOX2	SRY box 2	UP[a]
NM_003107	SOX4	SRY box 4	UP[a]
NM_017489	TERF1	Telomeric repeat-binding factor 1	UP[a]
NM_003242	TGFBR2	Transforming growth factor β-receptor 2	UP[a]
NM_003380	VIM	Vimentin	DOWN[a]
NM_030753	WNT3	Wingless-type MMTV integration site family, member 3	UP[a]
NM_003392	WNT5A	Wingless-type MMTV integration site family, member 5A	UP[c]
NM_058238	WNT7B	Wingless-type MMTV integration site family, member 7B	UP[a]

[a]2–3-fold
[b]> 5-fold change in transcription
[c]3–5-fold
hMSCs were incubated without and with TNF-α (50 ng/ml) for 14 days and subsequently subjected to the stem cell cDNA microarray analysis (Superarray). Results are shown for genes modulated ≥ 2-fold in the TNF-α-treated cells when compared with non-treated cells (set as 1). Expression of genes was normalized to the housekeeping gene cyclophilin B, genes of further interest are marked in grey

detectable in the cell extracts (Figure 3c). In contrast, neither the addition of SB203580, an inhibitor of p38 activity, nor the application of JNK-I2 that blocks JNK activity, had significant influence on mRNA expression of these genes (data not shown). Taken together, these results provide evidence that TNF-α-mediated differentiation of hMSCs is primarily regulated via the ERK1/2 signaling pathway.

CXCR4 expression and function in TNF-α-treated hMSCs. Microarray analysis had indicated that mRNA expression of the chemokine receptor CXCR4 was elevated in hMSCs after stimulation with TNF-α (Table 1). This finding was assured by a time-course study using qRT-PCR, which demonstrated that TNF-α (50 ng/ml) induced a dramatic upregulation of CXCR4 transcription by

more than 20-fold on day 1 and about 35-fold on days 3, 7, and 14 (Figure 4a). Consistently, TNF-α augmented CXCR4 protein present in cell extracts as determined by western blotting (Figure 4b).

Next, we investigated whether or not pretreatment of hMSCs with TNF-α affected their capacity for transmigration through human reconstituted basement membranes (ECM) using the Transwell assay. Applying SDF-1α as a chemo-attractant, hMSCs exhibited an improved potential for directed migration through ECM after 14 days of preincubation with TNF-α (Figure 4c), which may be a consequence of enhanced CXCR4 expression in these cells. In the presence of AMD3100 (10 μg/ml), a specific antagonist for binding of SDF-1 to CXCR4, the capacity for chemotactic invasion toward SDF-1α was efficiently blocked in TNF-α-pretreated
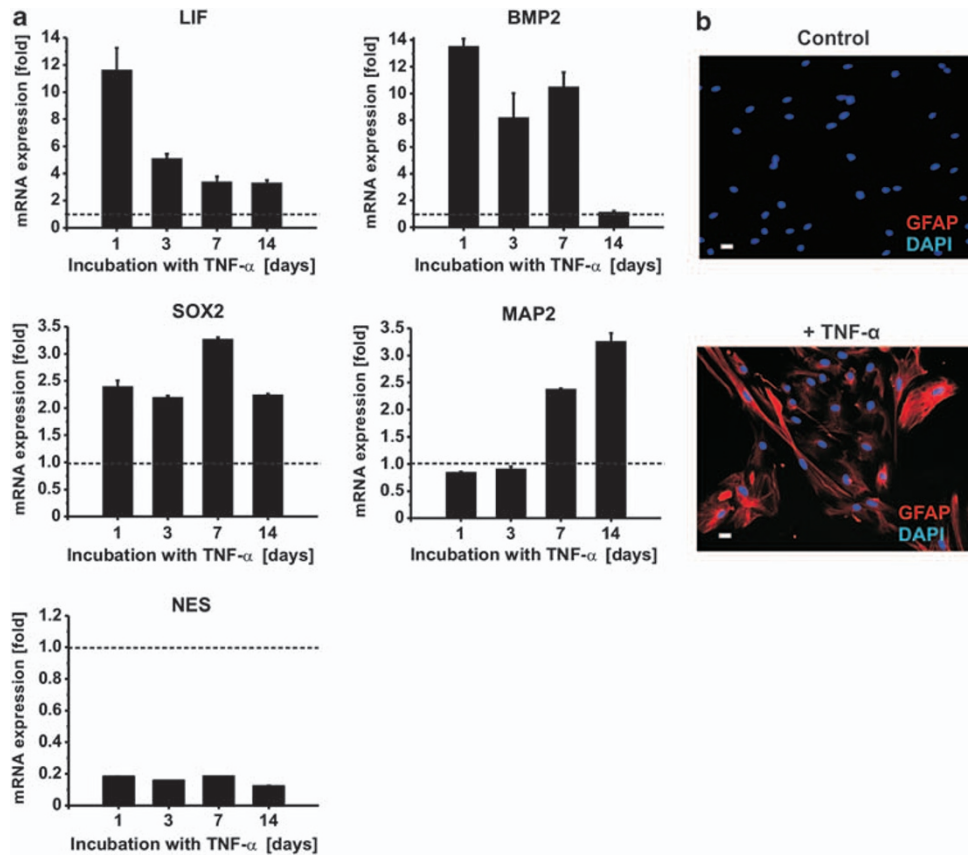
Figure 2 Neural phenotype characterization of TNF-α-treated hMSCs. (**a**) hMSCs were incubated without or with TNF-α (50 ng/ml) for different time intervals. mRNA expression of typical neural genes was examined by qRT-PCR analysis. Results are given as change in mRNA expression normalized against the housekeeping gene CPB relative to untreated cells set as 1. Values represent mean ± S.D. of triplicate measurements and are representative of three independent experiments. (**b**) Immunocytochemistry staining of the neural marker GFAP in hMSCs treated without (control) or with TNF-α (50 ng/ml) for 28 days. GFAP was stained with tetramethyl rhodamine isothiocyanate (TRITC) (red), cellular nuclei were counterstained with DAPI (blue). Magnification × 10. Scale bars, 30 μm

and -untreated hMSCs as well. Similarly, addition of the broad spectrum MMP inhibitor GM6001 (10 μg/ml) caused a dramatic reduction in the potential of hMSCs for trans-migration through ECM (Figure 4c), indicating the requirement of MMPs in this process. Cell viability was not affected by AMD3100 and GM6001 at the concentrations used to achieve maximal inhibition of migration. Summing up, these data suggest that incubation of hMSCs with TNF-α upregulates CXCR4 expression, which results in an increase in cellular susceptibility to SDF-1α-directed invasion facilitated by MMP activity.

Spheroid confrontation assay of hMSCs and glioma cells. Previous studies had revealed that preformed cellular spheroids of hMSCs that were brought into close contact with glioma cell spheroids were able for interpenetration, whereas normal fibroblasts could not.[22] Applying this experimental approach, we investigated whether preincubation of hMSCs with TNF-α had an influence on their capacity to infiltrate U373 glioma cell spheroids. Time-course analysis by fluor-escence microscopy clearly demonstrated a more rapid and extensive infiltration of glioma spheroids by TNF-α-treated hMSCs than by untreated cells after 2 days of co-incubation (Figures 5a and b). In the presence of the MMP inhibitor

GM6001 (10 μg/ml), the mutual infiltration capacity of hMSC and glioma spheroids was lowered in untreated hMSCs and significantly decreased in TNF-α-pretreated hMSCs (Figures 5a and b). Addition of AMD3100 (10 μg/ml) that blocks binding of SDF-1 to CXCR4 did not affect interpenetration of hMSC and U373 cell spheroids, which is probably a consequence of the insignificant SDF-1 secretion from U373 cells as determined by ELISA (data not shown).

Tropism of hMSCs to orthotopic gliomas *in vivo*. Finally, we evaluated whether our *in vitro* findings could be transferred into the *in vivo* situation thus being of potential impact for the application of hMSCs in the treatment of brain tumors. To address this, we compared the extent with which hMSCs and hMSCs preincubated with TNF-α were able to selectively integrate into intracranial human glioma xenografts in nude mice using a chronic cranial window and *in vivo* multiphoton laser scanning microscopy (MPLSM).[23,24] Microscopic images of representative tumor regions acquired 24 h after intravenous injection of quantum dot-labeled hMSCs revealed that hMSCs had infiltrated the growing glioma (Figure 6a). Importantly, preincubation with TNF-α massively increased the number of hMSCs recruited to the tumor (Figure 6b). The elevated recruitment of
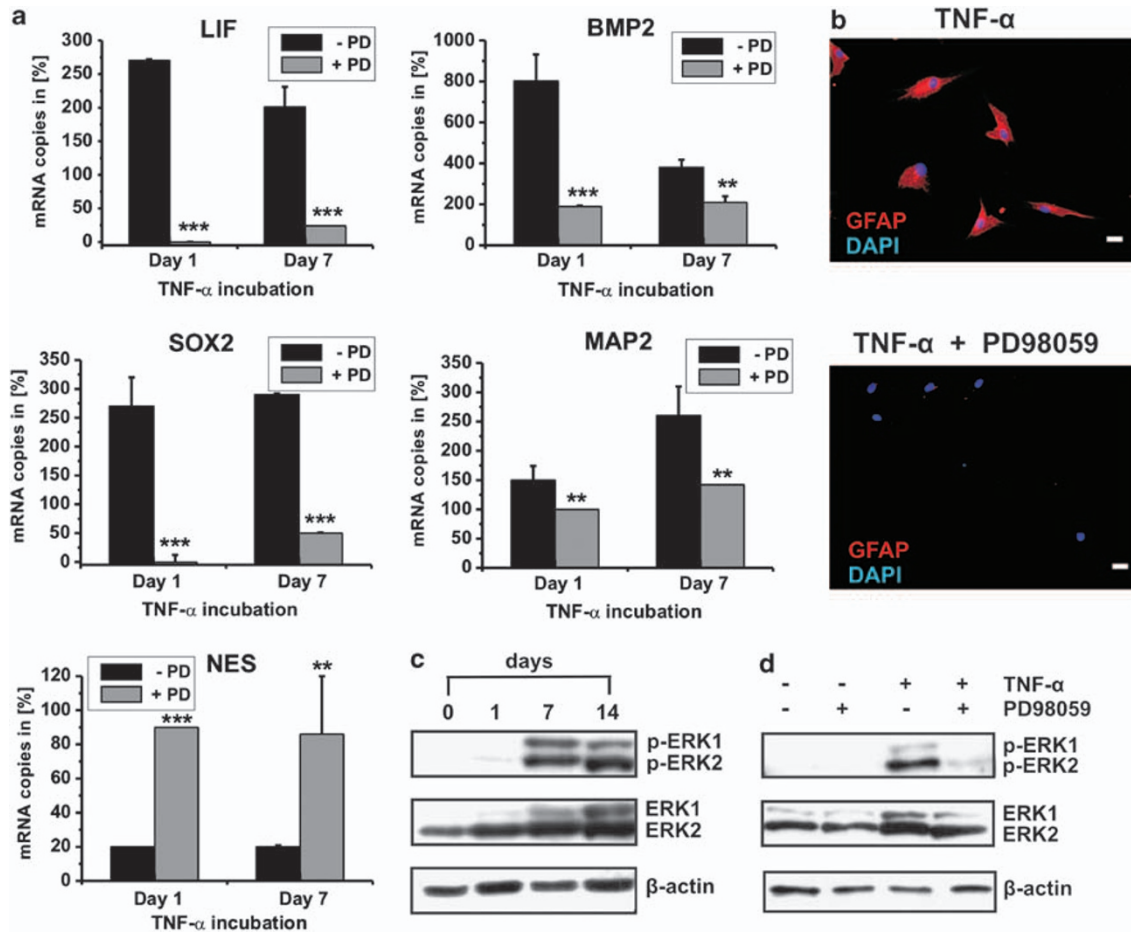
Figure 3 Role of ERK1/2 MAP kinase in TNF-α-induced neural differentiation of hMSCs. (**a**) hMSCs were cultivated in the presence of TNF-α (50 ng/ml) without or with addition of PD98059 (PD) (20 μM), a specific inhibitor of ERK1/2 phosphorylation. mRNA expression of neural marker genes was measured by qRT-PCR at day 1 and 7 of incubation. Results indicate change in mRNA expression relative to untreated hMSCs (set as 100%) after normalization against the housekeeping gene CPB. Values represent mean ± S.D. of one triplicate experiment representative for three independent measurements. (**b**) Immunofluorescence analysis of the GFAP expression in hMSCs incubated for 14 days in presence of TNF-α (50 ng/ml) without or with the addition of PD98059 (20 μM). GFAP was stained with tetramethyl rhodamine isothiocyanate (TRITC) (red) and cell nuclei were counterstained with DAPI (blue). Magnification × 10. Scale bars, 30 μm. (**c**) Western blot analysis of phosphorylated forms (p-ERK1/2) and total amount of ERK1/2 in lysates of hMSCs cultivated in the presence of TNF-α (50 ng/ml) for different time intervals. (**d**) Western blot detection of phosphorylated (p-ERK1/2) and total ERK1/2 after 14 days of co-incubation with or without PD98059 (20 μM). Intracellular β-actin was detected as a loading control

pretreated hMSCs was still evident 72 h days after injection (Figure 6b), indicating a stable integration of these cells into the growing tumor. Quantification of hMSCs detected in the analyzed tumor regions demonstrated that preincubation of the cells with TNF-α led to a significant sixfold increase in the number of hMSCs recruited to the growing glioma that was evident 24 and 72 h after intravenous delivery (Figure 6c). In normal brain regions contralateral to the implantation site, no hMSCs either TNF-α pretreated or not could be detected (data not shown).

Discussion

TNF-α is implicated in a broad spectrum of physiological and pathological processes regulating the function and development of various cell types including neural stem/progenitor cells.[11–14] In hMSCs, TNF-α can act as a chemoattractant and stimulates MMP/TIMP-expression as previously reported by us.[20] Here, we demonstrate that prolonged cultivation of

hMSCs in the presence of TNF-α (50 ng/ml) evokes a remarkable change in cell morphology, which was similar to the characteristic star-shaped appearance of astrocytic glial cells present in brain and spinal cord. One characteristic feature of astrocytes is their ability to synthesize GFAP, a cytoskeletal filament protein, whereas production of nestin and vimentin is abrogated in these cells.[25] After treatment with TNF-α, the hMSCs stained positive for GFAP and showed a reduced transcription level for nestin and vimentin confirming the astroglial nature of these cells. In contrast, the untreated hMSCs constitutively expressed nestin, which is in agreement with previous reports.[26] The intermediate filament nestin is essential in neuroepithelial stem cell function and becomes downregulated during maturation of these cells.[27] The rapid diminishment of nestin in hMSCs may be a consequence of the neural differentiation process initiated by TNF-α in these cells, which is consistent with previous data obtained in hMSCs by other investigators.[28] In contrast, SOX-2 that represents a transcription factor that is typically present in
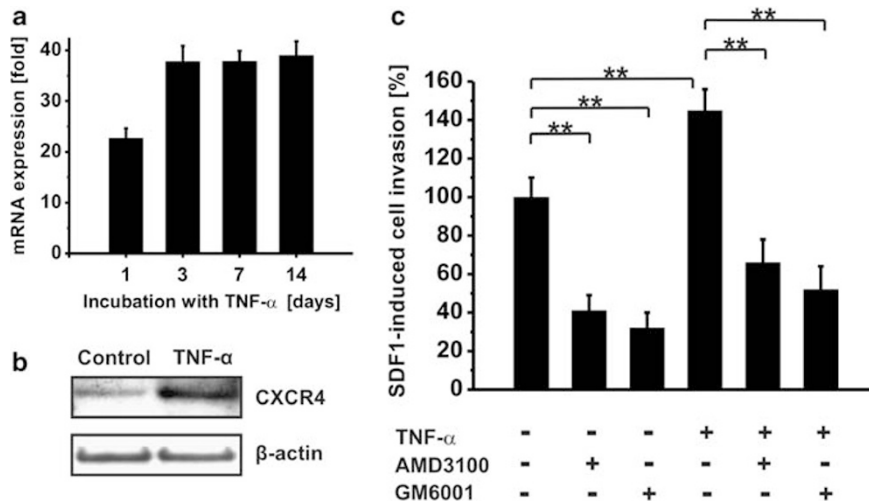
Figure 4 CXCR4 expression and invasion of hMSCs treated with TNF-α. (**a**) hMSCs were incubated without (control) or with TNF-α (50 ng/ml) for different time intervals and subjected to qRT-PCR analysis of CXCR4 mRNA expression. Results are given as the mean ± S.D. of triplicate cultures representative for three independent experiments. Data reflect fold change in expression relative to control cells after normalization against the housekeeping gene CPB. (**b**) Western blot analysis of CXCR4 in protein extracts from hMSCs after 3 days of incubation without (control) and with TNF-α (50 ng/ml) using monoclonal antibodies against 42-kDa CXCR4. Intracellular β-actin was detected on the same blot as a loading control. (**c**) For evaluation of the invasion capacity, hMSCs were pre-incubated in the absence (control) or presence of TNF-α (50 ng/ml) for 14 days and then analyzed in the Transwell invasion assay for their potential to migrate through a barrier of human ECM toward 50 ng/ml SDF-1α as chemoattractant in the lower compartment. The invasion assay was performed without and with the addition of AMD3100 (10 μg/ml), a specific antagonist for binding of SDF-1 to CXCR4, or GM6001 (10 μg/ml), a broad spectrum MMP inhibitor. Cell invasion rate was determined in percent relative to control, which was set as 100%. Results are presented as mean ± S.D. of triplicate measurements and are representative of three independent experiments. **$P < 0.01$

embryonic and neural stem cells being responsible for maintenance of progenitor characteristics[29] was elevated in TNF-α-treated hMSCs suggesting an immature progenitor-like status of these cells. Remarkably, marker proteins characteristically expressed in mature oligodendrocytes and neurons, such as galactocerebroside (Gal-C) and β-tubulin III, respectively, were not synthesized in hMSCs before and after incubation with TNF-α (data not shown), eliminating the possibility of a neuronal phenotype in these cells. Although TNF-α-treated hMSCs showed enhanced transcription of MAP-2, which is typically synthesized in post-mitotic neurons, the absence of MAP-2 protein in these cells again excludes their affiliation to the neuronal lineage.

Previous studies in neuroepithelial progenitor cells have demonstrated that the combined application of the cytokines LIF and BMP-2 induces differentiation toward the astrocytic lineage.[30] In our approach, the expression of BMP-2, LIF, and its receptor were strongly upregulated in TNF-α-treated hMSCs. From these results it can be speculated that the addition of TNF-α to hMSCs triggers secretion of LIF and BMP-2, which may promote astroglial differentiation in these cells by autocrine stimulation.

TNF-α at a concentration of 50 ng/ml as used throughout our experiments had no effect on cell viability and apoptosis in hMSCs. In contrast, similar dosages of TNF-α are reported to induce apoptotic cell death in neural stem/progenitor cells.[13] Lower concentrations of TNF-α, however, consistently stimulated the proliferation of both hMSCs and neural stem/progenitor cells. These findings indicate that the cellular implications initiated by TNF-α are highly dose-dependent and vary between different cell types.

With respect to the underlying mechanisms, TNF-α-induced effects can be mediated by different signal transduction pathways

including that of the MAPKs. Our experiments revealed that ERK1/2 but not p38 and JNK has an essential role in the TNF-α-evoked regulation of neural genes, such as *LIF, BMP2, SOX2,* and *NES,* which may be causative for differentiation of hMSCs toward the neural lineage. These findings are in agreement with recent results obtained in MSCs from mice demonstrating the requirement of ERK in this process.[31]

Recently, the regenerative effect of hMSCs differentiated into neural-like cells has been demonstrated in rat models of Parkinson's disease.[32] Moreover, very novel findings indicate that reactive astrocytes and/or a subset population with stem cell/progenitor features contribute to the healing and regeneration process after brain tissue damage[33] also suggesting the potential application of hMSC-derived astrocytic cells for reparative strategies in the CNS. Remarkably, the neural cells generated by incubation of hMSCs with TNF-α for 14 days were fully mitotic. With regard to their potential clinical application, this feature would favor an *in vitro*-expansion of TNF-α-induced hMSCs before retransplantation into patients.

A prerequisite for stem cells, both endogenous and transplanted, to promote tissue repair and regeneration is their ability for directed migration into the areas of damage, chronic disease, or inflammation. SDF-1 is present at elevated concentrations in CNS tissues during development and injury.[34] This chemotaxin attracts neural stem cells, which typically synthesize high levels of CXCR (Ni *et al.*[35]) allowing the mobilization and recruitment of these cells along SDF-1 gradients. Similarly, we found TNF-α-treated hMSCs to exhibit an increased expression of CXCR4 that promoted the ability for SDF-1α-directed invasion through human ECM *in vitro*, which was facilitated by MMP activity. These findings agree with the recent observation of Ponte *et al.*[36] that incubation with TNF-α facilitates migration of hMSCs toward SDF-1.
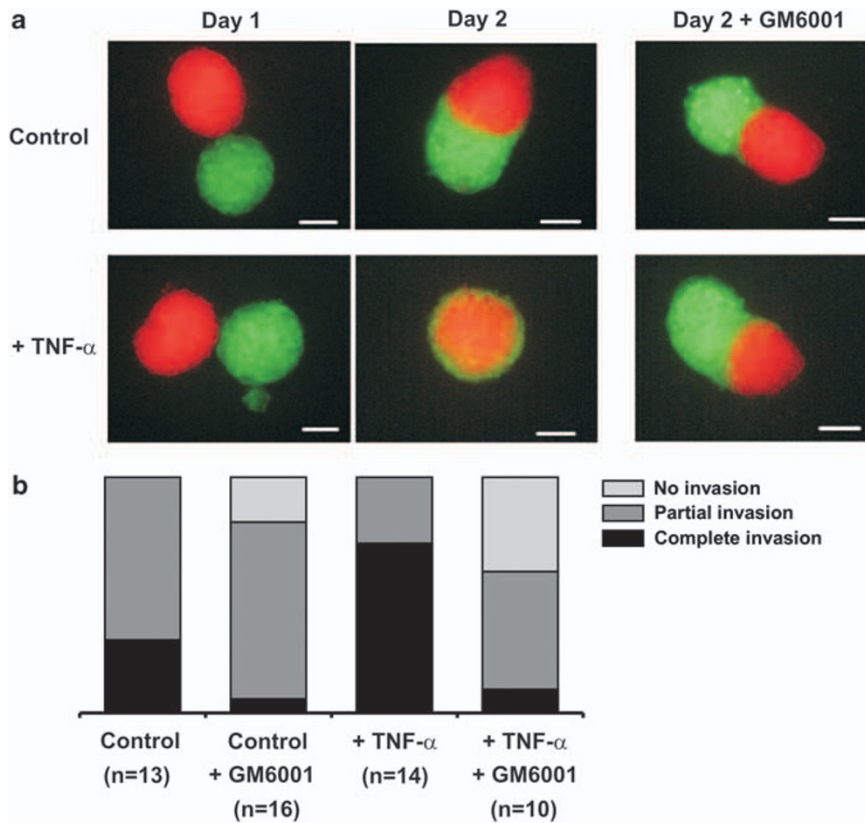
Figure 5 Confrontation assay of hMSCs and glioma cell spheroids. hMSCs were grown in the absence (control) or presence of TNF-α (50 ng/ml) for 14 days. Then the cells were transferred into agar-coated wells of a 96-well plate wherein they formed spheroids, which were applied in a confrontation culture system with spheroids of U373 glioma cells. (**a**) Single spheroids of each cell type were brought into close contact (day 1) and monitored by fluorescence microscopy for cellular interpenetration after 42 h (day 2) in the absence or presence of the MMP inhibitor GM6001 (10 μg/ml). hMSCs were stained with DiI (red) and glioma cells with GFP (green). Each cellular confrontation was done in at least 10 parallel approaches ($n = 10$–16). The images shown are taken from a single approach, which is representative of a group of experiments. Scale bars, 100 μm. (**b**) Semiquantitative visual analysis of spheroid infiltration after 42 h (day 2) of confrontation under the above described conditions

A striking feature of hMSCs is their pronounced tropism for malignant gliomas.[5] After intravascular or local delivery, hMSCs were shown to specifically integrate into gliomas when attracted by the release of cytokines and growth factors from the tumor cells.[22] Glioblastoma multiforme is the most aggressive form of glioma with a median survival time of only 14.6 months for patients after conventional therapy including resection, chemotherapy, and radiation.[37] Thus, innovative therapeutic strategies targeting the invasive tumor cells that have dispersed deeply into surrounding normal tissue are desperately needed. Accumulating evidence from numerous in vitro and in vivo studies has indicated the usefulness and therapeutic efficacy of hMSCs applied as cellular vectors for anti-tumor gene delivery to malignant gliomas.[5] hMSCs engineered into cells, which produce for instance immunotherapeutic cytokines, such as TNF-related apoptosis-inducing ligand or interferon-β, showed profound anti-tumor effects and significantly increased survival after specific engraftment into gliomas as demonstrated in animal models.[38,39] The success of these novel therapeutic strategies critically depends on an effective recruitment of hMSCs to glioma cells and tissues. Thus, increasing the potential of hMSCs to migrate to and within invasive solid cancers might improve the clinical application of these cells as delivery

vector system in the selective treatment of highly aggressive tumors.

Therefore, we investigated the consequences of TNF-α-pretreatment on the glioma tropism of hMSCs. Obviously, local migration to and invasion of hMSCs into glioma spheroids was facilitated in hMSCs that had been preincubated with TNF-α. The enhanced infiltration of TNF-α-differentiated hMSCs into glioma spheroids may be explained by the adoption of an expression profile and properties similar to neural cells such as neural progenitor cells, which are also known for their tropism to brain tumors.[6] Moreover, we showed in our approach that MMP activity seems to be crucial in the interpenetration of hMSCs and glioma cells as demonstrated by use of a broad-spectrum MMP inhibitor. This is in line with our previous findings that MMP production in hMSCs is upregulated by TNF-α[20] and with very recent results from other authors showing MMP-1 to be critically involved in hMSC migration toward human glioma.[40] Although additional chemotactic factors are probably involved in hMSC tropism toward brain tumors, the CXCR4/SDF-1 axis is likely to contribute to this process because SDF-1 is highly expressed in glioblastoma tissues, but almost absent in normal brain.[17]

Just recently, we have demonstrated that MPLSM in combination with a chronic cranial window in nude mice
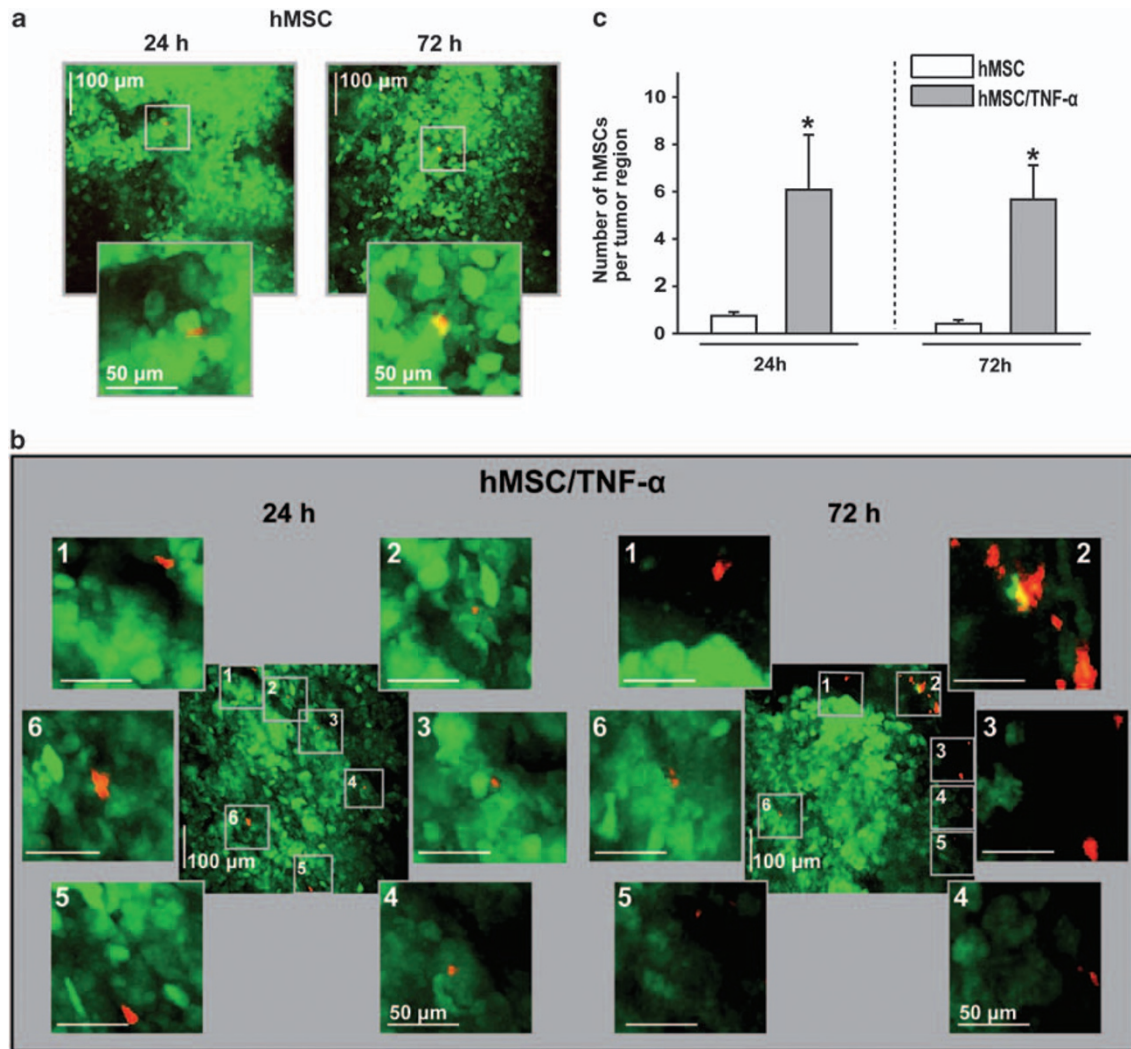
Figure 6 Recruitment of hMSCs to intracranial gliomas *in vivo*. hMSCs either untreated (**a**) or after preincubation *in vitro* with TNF-α (50 ng/ml) for 14 days (**b**) were injected into the tail vein of nude mice bearing U87-gliomas (tumor volume ∼ 1.7 mm^3). After 24 and 72 h, the recruitment of hMSCs into the brain was determined by *in vivo* multiphoton microscopy in combination with a chronic cranial window. Images represent maximum intensity projections of the mouse cortex at 0–200 μm depth. hMSCs were identified by red fluorescence (quantum dot labeling) and U87 glioma cells by green fluorescence (eGFP expression). Insets represent higher magnifications of the areas indicated by the squares. Scale bars represent 100 and 50 μm. (**c**) Quantification of the number of hMSCs per tumor region (650 × 650 × 200 μm = 0.0845 mm^3) as assessed by multiphoton *in vivo* imaging. Values represent mean ± S.D. of three tumor regions per animal (*n* = 4 animals per group). *$P < 0.05$

allows for noninvasive serial detection of brain tumors and metastasis on a cellular resolution *in vivo*.[23,24] Using this experimental approach, we now showed hMSC tropism toward experimental glioma in mice, which is in accordance to previous results.[5] Importantly, pretreatment of hMSCs with TNF-α potentiates the glioma-specific recruitment of hMSCs applied by intravenous injection. So far, local implantation of hMSCs requiring operative access to the tumor is thought to be a suitable strategy to enable therapeutic hMSC tumor infiltration.[5] Yet, enhancing the tumor tropism of intravenously administered hMSCs via TNF-α-pretreatment might become a less invasive alternative in the treatment of malignant gliomas.

In conclusion, our data demonstrate that culture-expansion of hMSCs in the presence of TNF-α generates cells with neuroglial characteristics and enhanced ability for CXCR4/SDF-1-directed migration by changes in the expression level of various neural genes, which is mediated via increased

ERK1/2 activity. These findings improve the development of hMSC-based therapeutical strategies in the treatment of neurodegenerative disorders and malignancies as well.

Materials and Methods

Cultivation and labeling of hMSCs. hMSCs that had been isolated from the bone marrow of healthy persons under informed consent were purchased from Cambrex (Walkersville, MD, USA). The different hMSC lots had been tested by the providing company for purity by flow cytometry and for their ability to differentiate into the osteogenic, chondrogenic, and adipogenic lineage. The cells were positive for CD105, CD166, CD29, and CD44, and negative for CD14, CD34, and CD45.

Cultivation of hMSCs in our laboratory was performed as described previously.[20] Briefly, cells were grown using the mesenchymal-stem-cell-growth medium (MSCGM) BulletKit (Cambrex). For the different experiments, hMSCs were seeded at a density of 1.5×10^3 cells/cm^2 in plastic tissue culture flasks (Nalgen Nunc International, Rochester, NY, USA) and cultured in MSCGM in the absence and presence of up to 100 ng/ml recombinant TNF-α (PeproTech, Rocky Hill, NJ, USA) with a weekly medium change. Analysis of apoptotic cells was performed by

applying the annexin V fluorescein labeling kit (Roche Applied Science, Mannheim, Germany). All studies were carried out with hMSCs of the fifth or sixth passage of cultivation.

Inhibitors used for studies on intracellular MAPK signaling were purchased from Calbiochem (Bad Soden, Germany). PD98059 blocks MEK1/2, which is the upstream activator of ERK1/2, SB203580 inhibits p38 and JNK-inhibitor II (JNK-I2) impedes JNK activity. Concentrations used in the experiments (20 μM for PD98059 and 10 μM for SB203580 and JNK-I2) did not significantly affect cell viability as determined by Trypan blue staining and the WST-1 proliferation assay (Roche Applied Science), which is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. The ability of hMSCs to differentiate into adipogenic and osteogenic cells was confirmed as described.[20]

For *in vivo* studies hMSCs were labeled with red fluorescent quantum dots using the Q-Tracker 605 Cell Labeling Kit (Invitrogen, Carlsbad, CA, USA). Growth medium containing 10 nM Q-Tracker 605 was added to 1×10^6 hMSCs and incubated for 1 h at 37 °C, 5% CO_2 according to the manufacturer's protocol. After incubation with Q-Tracker 605, hMSCs were washed with phosphate-buffered saline (PBS), incubated in growth medium for 1 h, and then used for the *in vivo* experiments. Untreated hMSCs and TNF-α-preincubated hMSCs did not exhibit differences in staining intensity and cell viability after labeling with Q-Tracker 605.

Cultivation and labeling of glioma cells. The human glioblastoma cell line U87MG was obtained from the American Type Culture Collection. Green fluorescent tumor cells were generated by transfection using the plasmid pEGFP-C3 (Clontech Laboratories, Inc., Palo Alto, CA, USA) and lipofectamine. Clones of cancer cells expressing enhanced green fluorescent protein (eGFP) were isolated, cultured in selective medium for 3 weeks, and subsequently enriched by fluorescence-activated cell sorting (Becton Dickinson, Heidelberg, Germany). *In vitro* growth rates of wild-type and eGFP-transfected cancer cell lines were equal.

Quantitative real-time polymerase chain reaction. mRNA expression of neural marker genes was determined by qRT-PCR. RNA isolation from hMSCs, cDNA synthesis, and PCR were performed as described.[20] qRT-PCR was carried out on a LightCycler (Roche Applied Science) applying the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Applied Science). For amplification of specific human neural marker gene transcripts, the LightCycler Primer Sets for *LIF*, *BMP2*, *SOX2*, *MAP2*, and *NES* as well as for *CXCR4* and CPB (cyclophilin B as a housekeeping gene standard) were used following the manufacturer's instructions (Search LC, Heidelberg, Germany).

Microarray analysis. The microarray technology was applied to investigate TNF-α-induced changes in the expression of characteristic stem cell genes in hMSCs. The Stem Cell cDNA GEArray (Superarray, Frederick, MD, USA) was performed using 1 μg of total RNA from hMSCs following the manufacturer's protocol. Briefly, 1 μg of RNA was transcribed into cDNA labeled with Biotin-dUTP and then hybridized to immobilized nucleic acid probes, which specifically encode for a total of 266 different genes expressed by stem cells at various stages of differentiation. Target cDNA that had bound to its specific probe was detected by chemiluminescence. The signal produced at each spot is representative to the amount of specific mRNA present in the original sample. Densitometric quantification was performed using the ArrayVision software (Interfocus, Mering, Germany).

Immunocytochemistry. For immunocytochemistry analysis of the typical neural protein marker GFAP, hMSCs were seeded onto culture slides (BD Biosciences, Franklin Lakes, NJ, USA). The cells were fixed in 4% paraformaldehyde in Dulbecco's PBS (Sigma, Hamburg, Germany). Subsequently, the culture slides were washed in PBS and blocked with 0.5% Triton X-100 and 10% normal goat serum (NGS) during 30 min at room temperature. The culture slides were incubated with mouse antibodies against GFAP (Sigma, Munich, Germany) at a dilution of 1:300 in a solution containing 0.5% Triton X-100 and 10% NGS for 2 h at 37 °C. Incubation was performed for 1 h at room temperature. After several washes in PBS, the culture slides were incubated with the secondary antibody for 45 min at room temperature. Secondary subclass-specific antibodies coupled with red fluorescent tetramethyl rhodamine isothiocyanate (Molecular Probes, Karlsruhe, Germany) were applied at a dilution of 1:50. After three further washes, the culture slides were mounted in ProLong Gold Antifade Reagent with DAPI (Molecular Probes). Analysis was performed at 20 °C using the Olympus IX70 microscope (Olympus Europa GmbH, Hamburg, Germany) with objective lens Olympus × 20 (numerical aperture 0.55), and images were

taken using SensiCam camera (PCO Imaging, Kelheim, Germany) with Image-ProPlus software (Media Cybernetics, Bethesda, MD, USA).

Immunoblotting analysis. Protein extraction from hMSCs, SDS-poly-acrylamide gel electrophoresis, and western blotting was performed as described.[20] The blotted membranes were incubated overnight with monoclonal mouse antibodies against total ERK1/2 (1:2000), phospho-ERK1/2 (1:1500) (both from Cell Signaling Technology, Danvers, MA, USA), β-actin (1:200) and CXCR4 (1:400) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in Tris-buffered saline containing 0.1% Tween-20 (TBS-Tween buffer) with 5% bovine serum albumin. The membranes were washed in TBS-Tween buffer and then incubated with anti-mouse or anti-rabbit IgG (Cell Signaling) conjugated with peroxidase as secondary antibodies at a dilution of 1:8000 in TBS-Tween buffer for 30 min. Detection of bound antibodies was accomplished by the enhanced chemoluminescence system (GE Healthcare Life Sciences, Freiburg, Germany). Recombinant protein standards (Invitrogen, Karlsruhe, Germany) were used for molecular mass determination.

Cell invasion assay. The capacity of hMSCs to transmigrate through a barrier of human ECM toward a gradient of chemoattractants was analyzed using the Costar Transwell chamber system (Costar, Pleasanta, CA, USA) as previously described in detail.[20] hMSCs (5×10^3) were seeded into the upper compartment and serum-free medium containing 50 ng/ml SDF-1α (Peprotech) as chemo-attractant was added to the lower compartment of the Transwell system. Each invasion experiment was performed in triplicate. After 48 h of incubation, cells that had migrated into the lower compartment were counted. The invasion rate was calculated from the number of migrated cells to the total cell number.

For inhibition experiments, hMSCs were pre-incubated for 30 min without or with 10 μg/ml GM6001 (Calbiochem), a synthetic broad-spectrum MMP inhibitor, or with 10 μg/ml AMD3100 (Sigma), a highly specific antagonist for binding of SDF-1 to CXCR4. Thereafter, the cells were transferred into the upper compartment of the Transwell system. The respective inhibitors were also added at the same concentrations to the medium in the upper and lower compartment. Preceding measurements had shown that incubation of hMSCs with the inhibitors (10 μg/ml, 48 h) did not significantly affect cell viability and proliferation.[20]

Confrontation culture invasion assay. hMSCs were pre-incubated in the absence or presence of TNF-α (50 ng/ml) for 2 weeks. Subsequently, cellular spheroids were generated from these hMSCs as well as from U373 glioma cells and their capacity for interpenetration was analyzed as described by Schichor *et al.*[22] hMSC spheroids were stained with red fluorescence Dil and brought into close contact with spheroids of U373 glioma cells, which had been stably transfected with green fluorescent protein (GFP). Cellular infiltration was monitored in the absence and presence of the MMP inhibitor GM6001 (10 μg/ml) or the CXCR4 antagonist AMD3100 (10 μg/ml) at different time intervals. The analysis was carried out at 20 °C with the Zeiss Axiovert 25 CFI fluorescence microscope HB0 (Carl Zeiss MicroImaging GmbH, Jena, Germany) and the objective lens × 10 (numerical aperture 0.25). Images were taken using a Canon Power Shot G2 digital camera (Canon Deutschland GmbH, Krefeld, Germany). The confrontation experiment was performed in at least 10 parallel attempts for each combination of spheroids.

Animal model and dynamic *in vivo* MPLSM. In order to study the *in vivo* recruitment of hMSCs from the peripheral blood to brain tumors, human glioma cells were implanted into the cortex of nude mice. We used *in vivo* MPLSM to sequentially image individual tumor regions of the growing glioma through a cranial window. Cranial window preparation and stereotactic cortical tumor cell injection was performed as previously described.[23,24] Briefly, 8- to 10-week-old male NMRI nu/nu nude mice (Charles River Laboratories, Sulzfeld, Germany) were anesthetized with an intraperitoneal injection of ketamine/xylazin (100 mg/kg ketamine and 10 mg/kg xylazin) before a circular cranial bone flap (diameter 6 mm) was removed. The craniectomy was covered with a custom-made cover glass, which was glued to the skull. Three weeks after cranial window preparation, the cover glass was temporarily removed, and a single-cell suspension of 1×10^5 U87-eGFP cells was injected stereotactically into the mouse cortex. Tumors were allowed to grow for 14–17 days until the tumor diameter reached approximately 1.5 mm (range 1.3–1.7 mm, microscopically confirmed) with a total volume of approximately 1.7 mm^3. In all, 5×10^5 hMSCs either preincubated with 50 ng/ml TNF-α for 14 days or left untreated were suspended in 200 μl PBS and injected into the tail vein of tumor bearing mice ($n = 4$ per group). All animal experiments were

performed according to the Bavarian state regulations for animal experimentation and were approved by the state authorities.

For *in vivo* imaging of hMSC-recruitment to gliomas, MPLSM was performed using a MaiTai Titanium Sapphire laser (710–920 nm, 10 W pump laser; Newport Spectra Physics, Darmstadt, Germany) equipped with a two-photon microscope (LSM 510; Zeiss, Jena, Germany). Mice were anesthetized by a mixture of O_2 and 1% isoflurane. Fixation of the cranial window was performed using a custom made microscopic stage (Narishige, Tokyo, Japan). Images were acquired at an excitation wavelength of 850 nm. For each animal ($n = 4$ per group), three random tumor regions and one region in the hemisphere contralateral to the tumor implantation site were imaged through the cranial window and quantified for the presence of hMSCs. For each region, 66 images spared 3 microns apart were collected from the brain surface producing $650 \times 650 \times 200\,\mu m$ volume stacks (0.0845 mm^3) with a resolution of $0.64 \times 0.64 \times 3\,\mu m$ per pixel.

Data analysis. One-way analysis of variance followed by the Dunnett's test was used for comparison of multiple groups. The Mann–Whitney rank-sum test was applied for analyzing differences between the two treatment groups in the animal model studies. Significance was assumed for $P < 0.05$. Statistical analysis was performed by use of the Origin 8.0 software (OriginLab, Northampton, MA, USA) and the SigmaStat 3.5 software (Systat, Erkrath, Germany).

Conflict of interest

The authors declare no conflict of interest.

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