Original Article

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Microglia express GPNMB in the brains of Alzheimer's disease and Nasu-Hakola disease

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Summary **Glycoprotein non-metastatic melanoma protein B (GPNMB) is a type I transmembrane glycoprotein first identified in low-metastatic human melanoma cell lines as a regulator of tumor growth. GPNMB is widely expressed in various tissues, where it is involved in cell differentiation, migration, inflammation/anti-inflammation, tissue regeneration, and neuroprotection. GPNMB is identified in microglia of adult rat brains, neurons and astrocytes of GPNMB transgenic (Tg) mouse brains, and motor neurons of amyotrophic lateral sclerosis (ALS) patients. Nasu-Hakola disease (NHD) is a rare autosomal recessive disorder, characterized by progressive presenile dementia and formation of multifocal bone cysts, caused by genetic mutations of either *TYROBP* (*DAP12*) or *TREM2*. TREM2 and DAP12 constitute a receptor/adaptor signaling complex expressed exclusively on osteoclasts, dendritic cells, macrophages, and microglia. Pathologically, the brains of NHD patients exhibit leukoencephalopathy, astrogliosis, accumulation of axonal spheroids, and remarkable activation of microglia predominantly in the white matter of frontal and temporal lobes and the basal ganglia. At present, molecular mechanisms responsible for development of leukoencephaolpathy in NHD brains remain totally unknown. Recent evidence indicates that disease-associated microglia (DAM) that cluster around amyloid plaques express high levels of GPNMB in Alzheimer's disease (AD) brains. Because microglia act as a key regulator of leukoencephalopathy in NHD brains, it is proposed that GPNMB expressed on microglia might play a protective role in progression of leukoencephalopathy possibly *via* active phagocytosis of myelin debris. In the present study using immunohistochemistry, we have attempted to clarify the expression of GPNMB in NHD brains, compared with AD brains. We found that microglia accumulating in the white matter express an intense GPNMB immunoreactivity in both NHD and AD brains, suggesting that the accumulation of GPNMB-immunoreactive microglia is a general phenomenon in neurodegenerative brains.**

Keywords: Alzheimer's disease, GPNMB, leukoencephalopathy, microglia, Nasu-Hakola disease, osteoactivin

1. Introduction

Glycoprotein non-metastatic melanoma protein B (GPNMB) is a type I transmembrane glycoprotein first identified in low-metastatic human melanoma cell lines as a regulator of tumor growth, alternatively named osteoactivin (*1*). GPNMB is composed of an N-terminal signal peptide, a RGD motif, a polycystic kidney disease (PKD) domain, and a proline-rich repeat domain (PRRD) in its extracellular domain (ECD), a single-pass transmembrane domain, and a short cytoplasmic tail that possesses a half immunoreceptor tyrosine-based activation motif (hemITAM) and a dileucine motif (*2,3*). The transmembrane segment of GPNMB is proteolytically cleaved at a dibasic motif in the juxamembrane region by a disintegrin and

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metalloproteinase (ADAM) family of proteases and matrix metalloproteinases (MMPs) in a process called ectodomain shedding, which results in the release of a soluble form of the GPNMB extracellular domain (*4,5*). The RGD motif serves as an integrin-binding motif, while the PKD domain mediates protein-protein and protein-carbohydrate interactions. The hemITAM motif induces signal transduction following ligand binding. The dileucine motif mediates rapid internalization of GPNMB from the plasma membrane processed for lysosomal/endosomal targeting. GPNMB is a heavily glycosylated protein, possessing 12 putative N-glycosylation sites within its extracellular domain (*2,3*). GPNMB is detected by immunoblot as two glycosylated high molecular weight isoforms (97-kDa, 116-kDa) (*2*). GPNMB is located not only at the plasma membrane but also in the perinuclear cytoplasmic regions, the endoplasmic reticulum (ER), and the Golgi apparatus (*2,6,7*). Extracellular fragments of GPNMB induce the production of matrix metalloproteinase-3 (MMP-3) and activation of ERK1/2 and p38 in mouse fibroblasts (*8*).

GPNMB plays a role in regulation of the homeostasis in various tissues and cells, such as skeletal muscle, bone, the hematopoietic system, the nervous system, epithelial cells, osteoblasts, osteoclasts, macrophages, and dendritic cells (*9,10*). GPNMB is involved in cell differentiation, migration, inflammation/anti-inflammation, tissue regeneration, and neuroprotection (*2,3*). GPNMB acts on osteoblasts to stimulate differentiation, leading to bone mineral deposition (*11*). DBA/2J mice that have a GPNMB mutation resulting in a truncated nonfunctional protein show less bone formation (*12*). On the other hand, GPNMB acts as a negative regulator of osteoclastogenesis (*13*). GPNMB is shown to inhibit osteoclast differentiation through binding to CD44 and inhibiting ERK activation. GPNMB negatively regulates the inflammatory responses of macrophages in a mouse model of experimental colitis (*14*). Using primary mouse astrocytes from CD44 knockout mice, it was indicated that the anti-inflammatory effects of GPNMB require binding to CD44 (*15*).

In the central nervous system (CNS), GPNMB is expressed in the cerebrum, cerebellum, brain stem, and spinal cord of adult rats (*16*). However, at present, the precise cell type expressing GPNMB in the human CNS remains unknown. Purified human oligodendrocytes by panning with anti-galactocerebroside (GalC) antibody express GPNMB (*https://www.brainrnaseq.org*). GPNMB expression is elevated in the substantia nigra of Parkinson's disease (PD) patients compared with age-matched controls (*17*). GPNMB is greatly expressed in motor neurons with extracellular deposits in the spinal cord of amyotrophic lateral sclerosis (ALS) (*18,19*). A subpopulation of Iba1-positive microglia that cluster around amyloid plaques expresses high levels of GPNMB in Alzheimer's disease brains (*20*).

Nasu-Hakola disease (NHD), also designated polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), is a rare autosomal recessive disorder, characterized by progressive presenile dementia and formation of multifocal bone cysts, caused by loss-of-function mutations of either *TYROBP* (*DAP12*) or *TREM2* (*21*). TREM2 and DAP12 constitute a receptor/adaptor signaling complex expressed exclusively on osteoclasts, dendritic cells, macrophages, and microglia. Although NHD patients are clustered in Japan and Finland, approximately 200 NHD cases are presently reported worldwide. Clinically, the patients with NHD show recurrent bone fractures during the third decade of life, and a frontal lobe syndrome during the fourth decade of life, and progressive dementia and death until the fifth decade of life (*22*). Pathologically, the brains of NHD patients exhibit extensive demyelination designated leukoencephalopathy, astrogliosis, accumulation of axonal spheroids, and remarkable activation of microglia predominantly in the white matter of frontal and temporal lobes and the basal ganglia (*23*). At present, molecular mechanisms responsible for development of leukoencephaolpathy in NHD brains remain totally unknown. Because NHD is a pathological entity of microgliopathy where microglia act as a key regulator of leukoencephalopathy, we propose the hypothesis that GPNMB expressed on microglia might play a protective role in progression of leukoencephalopathy possibly *via* active phagocytosis of myelin debris in NHD brains. In the present study, we have attempted to clarify the expression of GPNMB in NHD brains compared with AD brains.

2. Materials and Methods

2.1. *Human brain tissues*

The brain autopsies were performed at the National Center Hospital, National Center of Neurology and Psychiatry (NCNP), Japan, Kohnodai Hospital, National Center for Global Health and Medicine (NCGM), Japan, and affiliated hospitals of Research Resource Network (RRN), Japan. The comprehensive examination by established neuropathologists (YS and TI) validated the pathological diagnosis. Written informed consent was obtained in all cases. The Ethics Committee of the NCNP for Human Brain Research, the Ethics Committee of the NCGM on the Research Use of Human Samples, and the Human Research Ethics Committee (HREC) of the Meiji Pharmaceutical University (MPU) approved the present study.

For immunohistochemical studies, serial sections of the frontal lobe and the hippocampus were prepared from four subjects who died of non-neurological causes (NC), composed of a 63-year-old man who died of prostate cancer and acute myocardial infarction (NC1), a 67-year-

old man who died of dissecting aortic aneurysm (NC2), a 57-year-old man who died of alcoholic liver cirrhosis (NC3), and a 61-year-old man who died of rheumatoid arthritis with interstitial pneumonia (NC4), ten AD patients, composed of a 68-year-old woman (AD1), a 70-year-old woman (AD2), a 68-year-old woman (AD3), a 56-year-old man (AD4), a 59-year-old man (AD5), an 81-year-old man (AD6), a 68-year-old woman (AD7), an 80-year-old man (AD8), a 72 year-old man (AD9), and a 77-year-old woman (AD11), and five NHD patients, composed of a 42-year-old man (NHD1), a 48-year-old woman (NHD2), a 44-year-old man (NHD3), a 32-year-old woman (NHD4), and a 38-year-old man (NHD5). The homozygous mutation of a single base deletion of 141G (c.141delG) in exon 3 of DAP12 was identified in NHD1, NHD2, and NHD5, while the genetic analysis was not performed in NHD3 or NHD4. All AD cases were satisfied with the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria for diagnosis of definite AD (24). They were categorized into stage C of amyloid deposition and stage VI of neurofibrillary degeneration, following Braak's staging (*25*).

2.2. *Immunohistochemistry*

After deparaffination, tissue sections were heated in 10 mM sodium citrate buffer, pH 6.0 by autoclave at 110℃ for 15 min in a temperature-controlled pressure chamber (Biocare Medical, Pacheco, CA, USA). They were treated at room temperature (RT) for 15 min with 3% hydrogen peroxide-containing methanol to block endogenous peroxidase activity. They were then incubated with phosphate-buffered saline (PBS) containing 10% normal rabbit serum at RT for 15 min to block non-specific staining, followed by incubation in a moist chamber at 4℃ overnight with goat polyclonal anti-GPNMB antibody (AF-2550, R&D Systems, Minneapolis, MN, USA). The specificity of anti-GPNMB antibody was validated by Western blot analysis of recombinant human GPNMB protein which was cloned with the pEF6/V5-His TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA) expressed in HEK293 cells.

After washing with PBS, tissue sections were incubated at RT for 30 min with horseradish peroxidase (HRP)-conjugated anti-goat secondary antibody (Nichirei, Tokyo, Japan), followed by incubation with diaminobenzidine tetrahydrochloride (DAB) substrate (Vector, Burlingame, CA, USA). They were processed for a counterstain with hematoxylin. Negative controls underwent all the steps except for exposure to primary antibody. In limited experiments, double immunolabeling was performed using AF-2550 antibody followed by incubation with HRP-conjugated anti-goat secondary antibody and exposure to DAB, and in combination with rabbit polyclonal anti-Iba1 antibody (Wako Pure Chemical, Tokyo, Japan), mouse monoclonal antibody against amyloid-β peptide (12B2; Immunobiological Laboratories, Gunma, Japan), mouse monoclonal antibody against phospho-tau (Ser202, Thr205) (AT8; Thermo Fisher Scientific), mouse monoclonal antibody against apolipoprotein E (APOE) (ab1906; Abcam, Cambridge, UK), mouse monoclonal antibody against GFAP (GA5; Nichirei), or mouse monoclonal antibody against NeuN (ab104224; Abcam), followed by incubation with alkaline phosphatase-conjugated anti-rabbit or anti-mouse secondary antibody (Nichirei) and exposure to Warp Red chromogen (Biocare Medical).

2.3. *Quantification of GPNMB immunoreactivity*

To quantify immunolabeled areas, the images derived from three fields of the frontal cortex or the subcortical white matter per each section were captured at a 200× magnification on the Olympus BX51 universal microscope. They were then processed for quantification by using ImageJ software (National Institute of Health, Bethesda, MD, USA). The differences in the GPNMB-positive areas among AD, NHD and NC subjects were evaluated statistically by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test.

3. Results

First, we validated the specificity of anti-GPNMB antibody AF-2550 by Western blot of a V5-tagged recombinant GPNMB protein expressed in HEK293 cells (~100-kDa, 120-kDa) (Figure 1A). Then, by

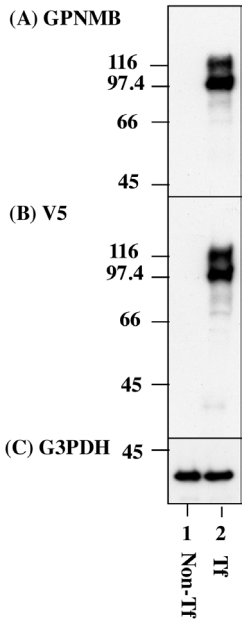

Figure 1. Validation of the specificity of anti-GPNMB antibody. Western blot analysis of a V5-tagged recombinant GPNMB protein with **(A)** anti-GPNMB antibody AF-2550, **(B)** anti-V5 antibody, and **(C)** anti-G3PDH antibody, as a loading control. **(lane 1)** non-transfectant and **(lane 2)** transfectant.

immunohistochemistry, we found that GPNMB is intensely expressed predominantly in amoeboid and hypertrophic microglia located in the subcortical white matter of the frontal lobe and the hippocampus of both AD and NHD brains (Figure 2, panels b, c, d; Figure 3, panels a, b). In contrast, a much smaller area was labelled with GPNMB in NC brains (Figure 2, panel a). In the frontal white matter of NHD, the area of GPNMB-expressing cells showed an 18.9-fold increase compared with NC (p = 0.0035) (Figure 4, panel a). In

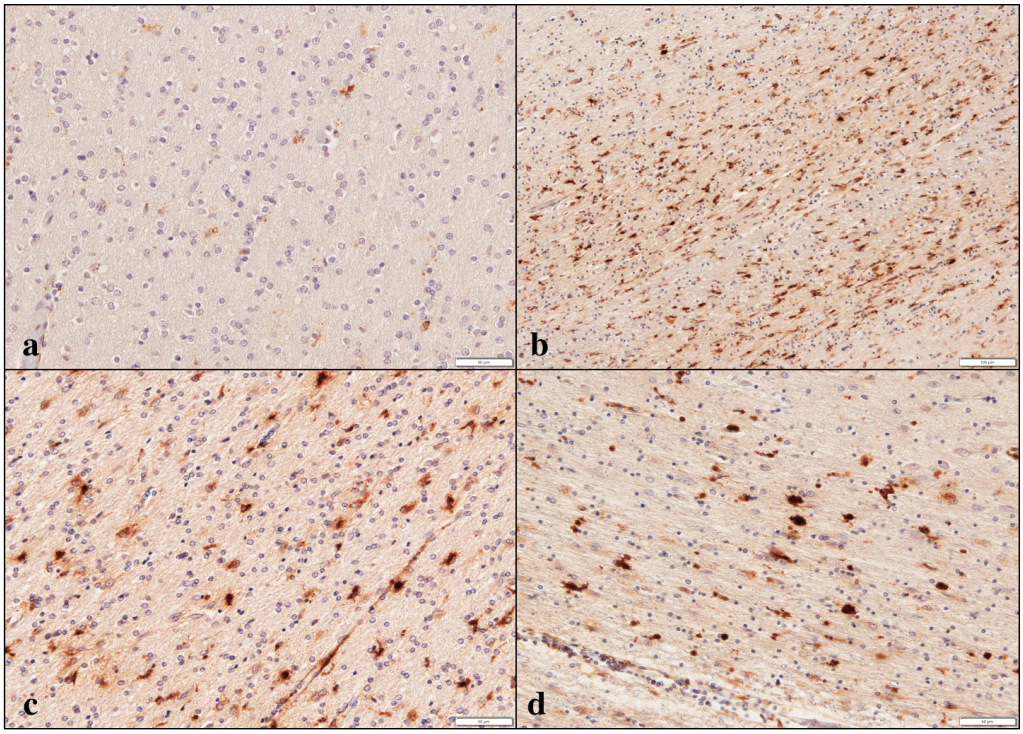

Figure 2. Immunohistochemistry of frontal white matter with anti-GPNMB antibody. (a) frontal white matter, NC, **(b)** hippocampus, AD, **(c)** frontal white matter, AD and **(d)** frontal white matter, NHD. Scale bars indicate **(a, c, d)** 50 µm and **(b)** 100 µm.

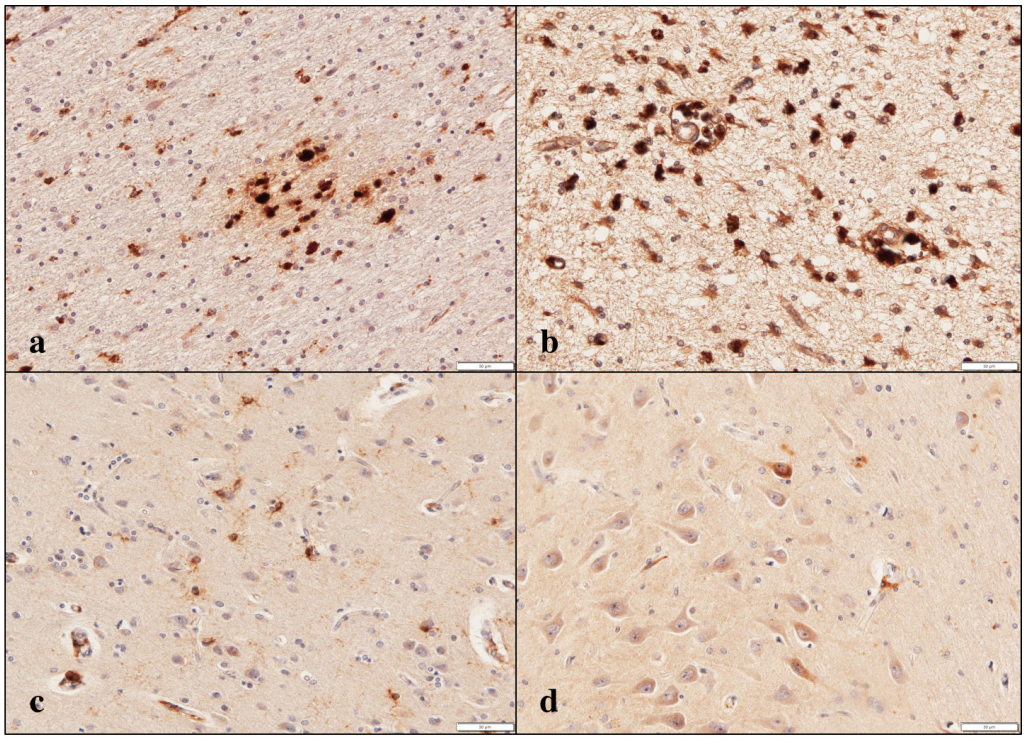

Figure 3. Immunohistochemistry of NHD brains with anti-GPNMB antibody. (a-d) NHD brains. **(a, b)** frontal white matter, **(c)** frontal cortex and **(d)** hippocampus. Scale bars indicate **(a-d)** 50 µm.

the frontal cortex of AD, the area of GPNMB-expressing cells exhibited a 2.4-fold increase compared with NHD (p = 0.0177) and a 5.7-fold increase compared with NC (p = 0.0027) (Figure 4, panel b). Thus, GPNMB-immunoreactive area is greatest in the frontal white matter of NHD and in the frontal cortex of AD. The great majority of GPNMB-expressing cells were labeled with Iba1 but neither with GFAP nor NeuN (Figure 5, panels

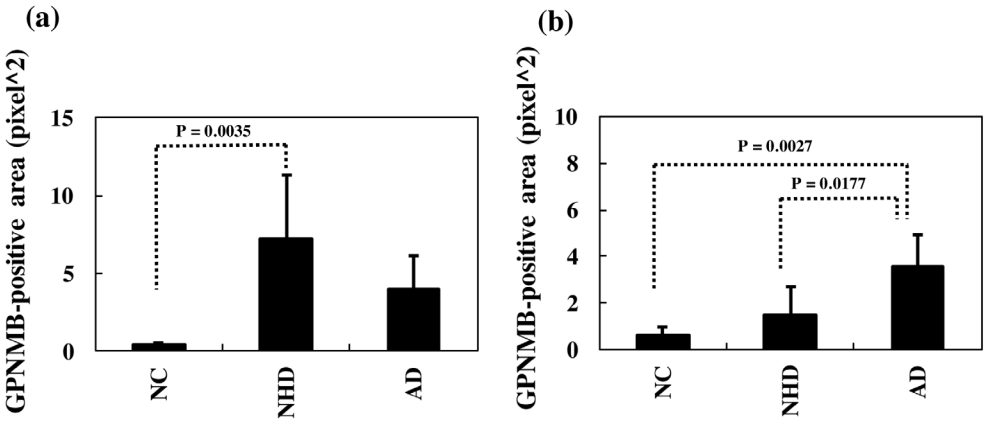

Figure 4. GPNMB-immunolabeled areas of frontal white matter and frontal cortex. The differences in the GPNMB-positive areas among AD, NHD, and NC subjects were evaluated statistically by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test. **(a)** frontal white matter and **(b)** frontal cortex.

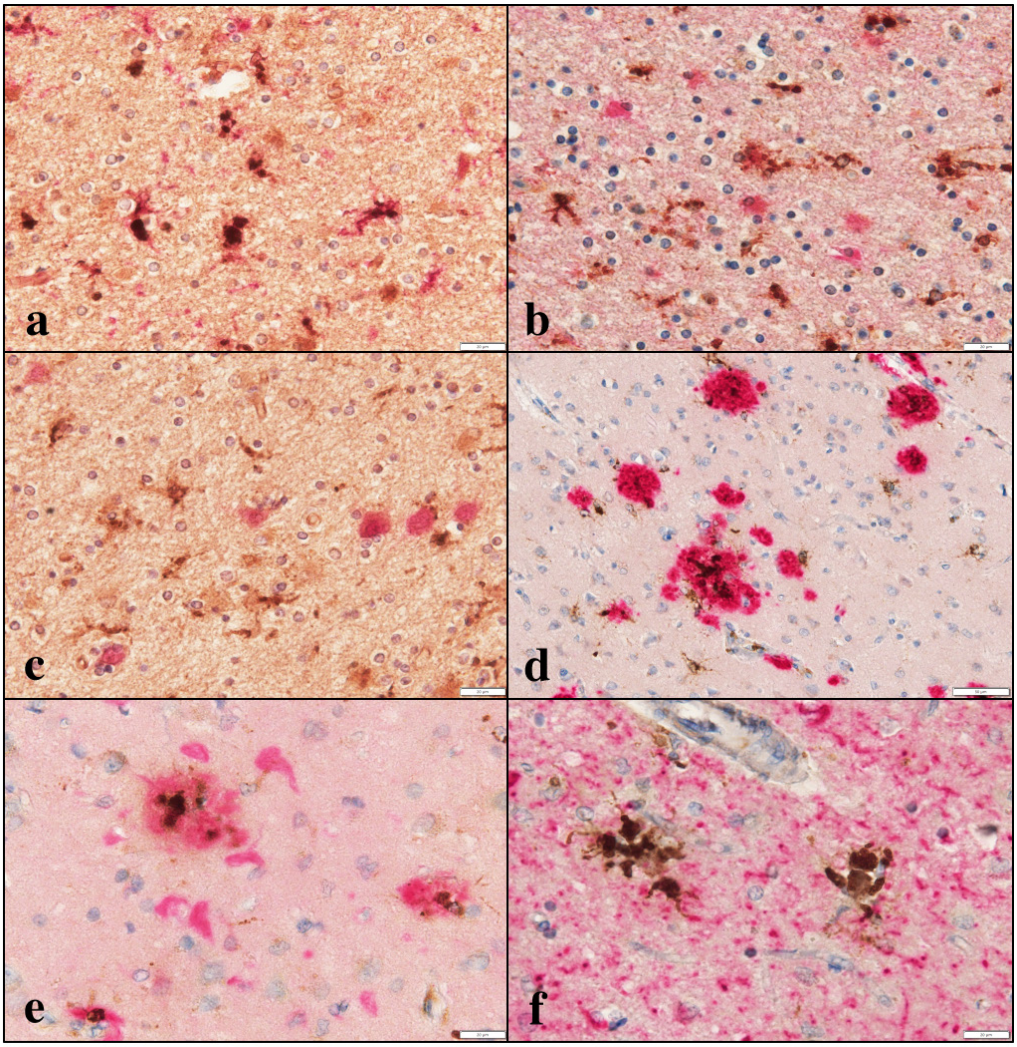

Figure 5. Double immunolabeling with anti-GPNMB antibody and cell type-specific antibodies. (a-f) AD, **(a-c, f)** hippocampus and **(d, e)** frontal cortex. Double immunolabeling of GPNMB (brown) with **(a)** Iba1 (red), **(b)** GFAP (red), **(c)** NeuN (red), **(d)** amyloid-β (red), **(e)** ApoE (red), or **(f)** AT8-tau (red). Scale bars indicate **(a-c, e, f)** 20 μm and (d) 50 μm.

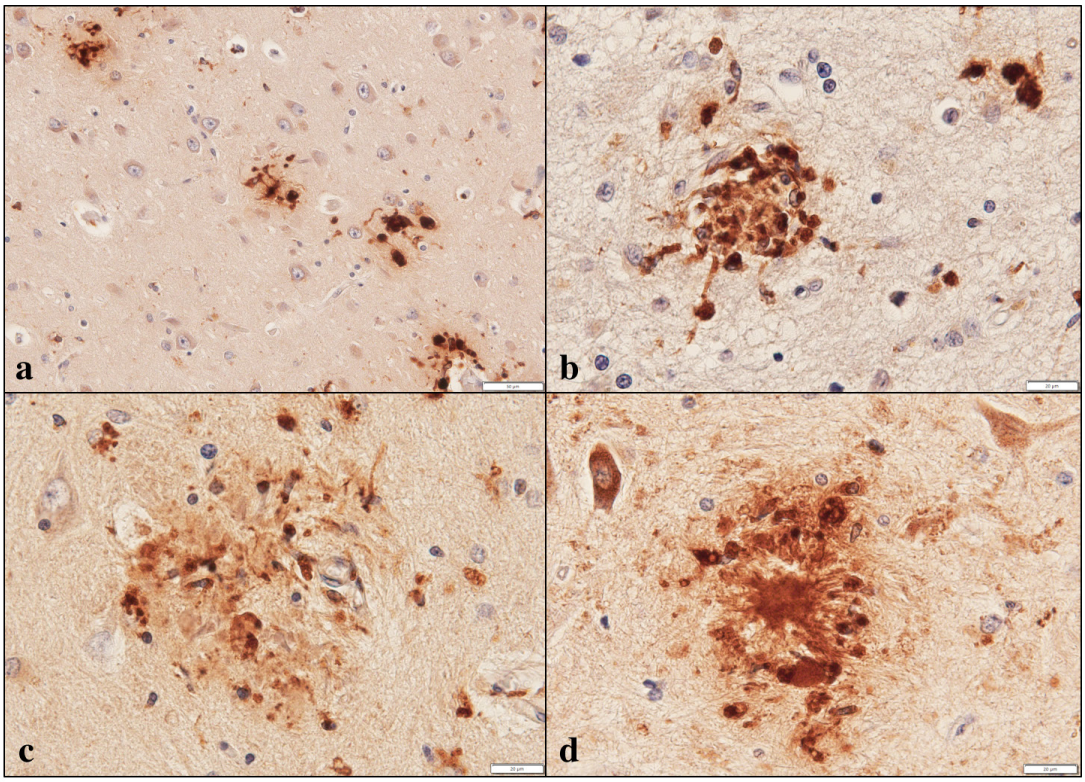

Figure 6. Immunohistochemistry of plaque-forming microglia with anti-GPNMB antibody. (a-d) AD, **(a, c, d)** hippocampus and **(b)** frontal cortex. Scale bars indicate **(a)** 50 μm and (b-d) 20 μm.

a-c). In AD brains, the clusters of GPNMB-expressing microglia accumulated on amyloid-β-positive and APOE-immunolabeled plaques (Figure 5, panels d, e). In AD brains, phosphorylated tau immunoreactivity was often in close contact with GPNMB aggregates (Figure 5, panel f). In AD brains, the clusters of GPNMB-expressing amoeboid and hypertrophic microglia forming plaques were identified frequently in the frontal cortex and the hippocampus (Figure 6, panel a-d). In contrast, only a few clusters of GPNMB-expressing microglia were found in NHD brains (Figure 3, panels a, c). In AD brains, perivascular macrophages and some degenerating neurons occasionally expressed GPNMB, while reactive astrocytes rarely showed GPNMB immunoreactivity. In NHD brains, perivascular macrophages and a few neurons also expressed GPNMB (Figure 3, panels b, d).

4. Discussion

GPNMB is a widely expressed multifunctional protein that regulates cell differentiation, migration, inflammation/anti-inflammation, tissue regeneration, and neuroprotection. Previous studies showed that GPNMB is expressed on astrocytes and neurons in GPNMB Tg mice (*26*). In addition, GPNMB is expressed in motor neurons, but not in astrocytes or microglia in ALS patients (*19*). Recent evidence indicates that microglia express high levels of GPNMB. GPNMB-positive cells express most frequently the microglia/macrophage marker OX42, and occasionally the radial glia marker RC2 or the neuronal marker NeuN in adult rats (*16*). An intraperitoneal injection of lipopolysaccharide (LPS) increases the number of GPNMB and OX42 double-positive cells in the area postrema (*16*). GPNMB is expressed in a subset of $Iba1^+$ microglia in rat brain (*27*). GPNMB is highly expressed in BV2 mouse microglial cells after LPS treatment, significantly upregulates the expression of MMP-3, and GPNMB siRNA dramatically suppresses the expression of TNF-α, IL-1β, iNOS, and NO (*28*). Glioma-associated microglia strongly express GPNMB (*29*). $CD11c^+$ microglia in APP/PS1 mice express high levels of GPNMB (*30*). Importantly, a subpopulation of Iba1-positive microglia that cluster around amyloid plaques expresses high levels of GPNMB in AD brains (*20*), consistent with our results.

Extensive transcriptome analysis of brains of AD mouse models identified a novel type of microglia termed as disease-associated microglia (DAM) or microglial neurodegenerative phenotype (MGnD) showing a unique transcriptional and functional signature (*31,32*). DAM is activated sequentially by TREM2-independent and TREM2-dependent pathways. GPNMB reflects a microglia activation state only present under neurodegenerative conditions, which is characterized by upregulation of a subset of genes, including TREM2, APOE, CLEC7A and CST7 (*20,31,32*). We validated an enhanced expression of

GPNMB on amoeboid and hypertrophic microglia accumulating in the frontal white matter of NHD brains and the frontal cortex of AD brains. GPNMB-expressing clusters of activated microglia resided on amyloid plaques of AD brains, where they were often in contact with APOE-immunoreactive plaques and phosphorylated tau-labeled neurofibrillary tangles. In contrast, neurons and reactive astrocytes did not consistently express GPNMB in NHD and AD brains. Thus, we conclude that the principal cell type expressing GPNMB in NHD and AD brains is microglia previously termed as DAM. However, we could not exclude the possibility that a subpopulation of oligodendrocytes in the white matter expresses GPNMB. These results indicate that the accumulation of GPNMB-immunoreactive microglia is a general phenomenon in neurodegenerative brains.

Several lines of evidence suggest that GPNMB plays a neuroprotective role. Tg mice overexpressing GPNMB show a smaller infarct volume compared with wild-type mice following brain ischemia-reperfusion injury (*33*). GPNMB Tg mice have an improvement in hippocampal memory tasks and long-term potentiation associated with increased levels of the AMPA receptor subunit GluA1 (*26*). GPNMB suppresses motor neuron cell death induced by mutant superoxide dismutase 1 (SOD1) or Tar DNA-binding protein 43 (TDP43), by activating the ERK1/2 and Akt pathways (*18,19*). GPNMB shows a neuroprotective effect on NSC-34 motor neurons by activating the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathways *via* interacting with the alpha subunits of Na^+/K^+-ATPase (NKA) (34). Direct injection of a GPNMB expression plasmid into the gastrocnemius muscle of SOD1G93A mice increases the number of myofibers and prevents myofiber atrophy (*35*). Overexpression of GPNMB protects skeletal muscle from severe degeneration caused by long-term denervation in mice (*36*). All of these observations indicate a protective role of GPNMB against active degenerating processes. Therefore, the enhanced expression of GPNMB on microglia might play a protective role in development of NHD and AD brain lesions.

In conclusion, we identified enhanced GPNMB expression on microglia in the severely affected frontal white matter of NHD brains and the frontal cortex and the hippocampus of AD brains, suggesting that GPNMB-expressing microglia might play a neuroprotective role against ongoing extensive leukoencephalopathy and neurodegeneration.

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