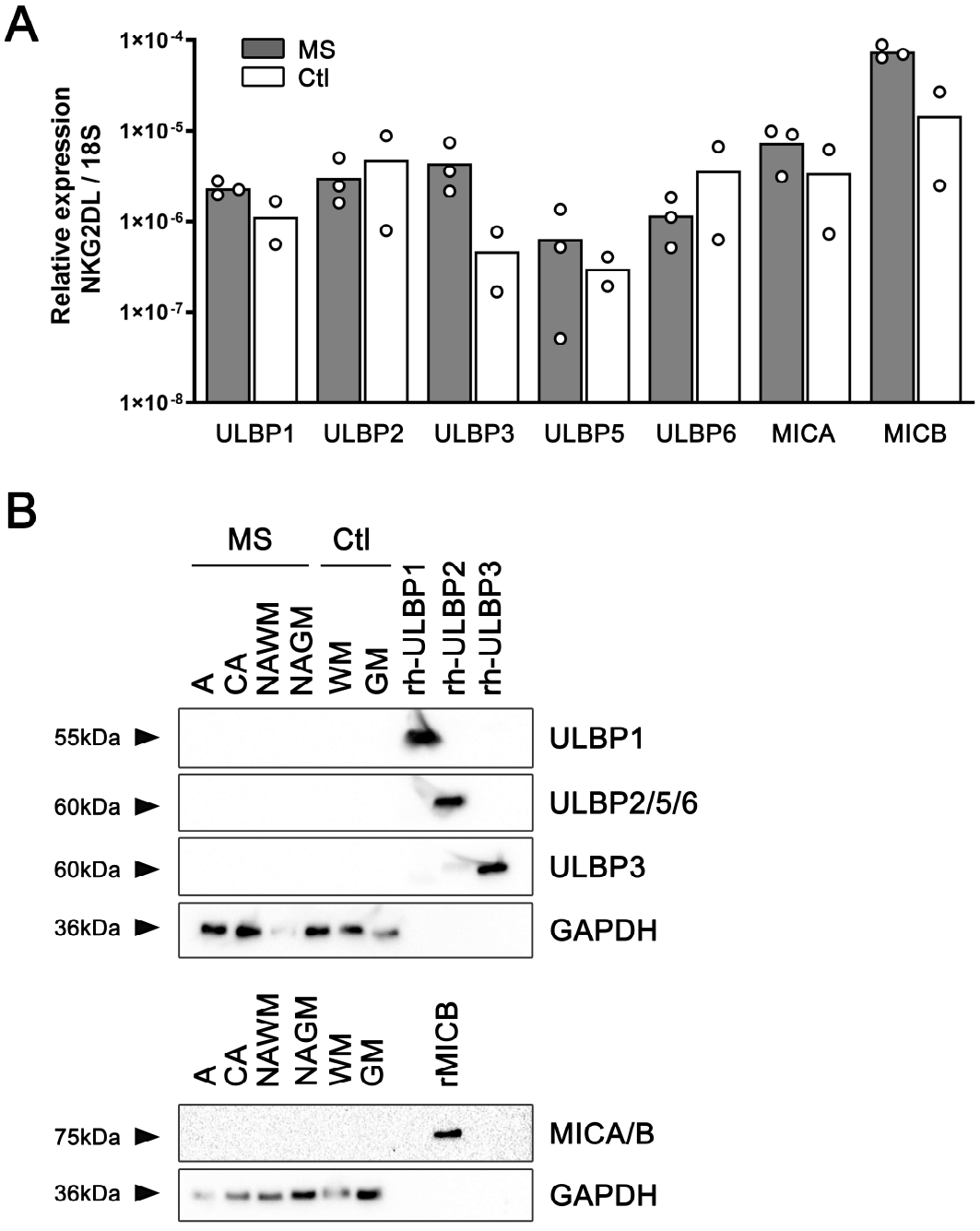
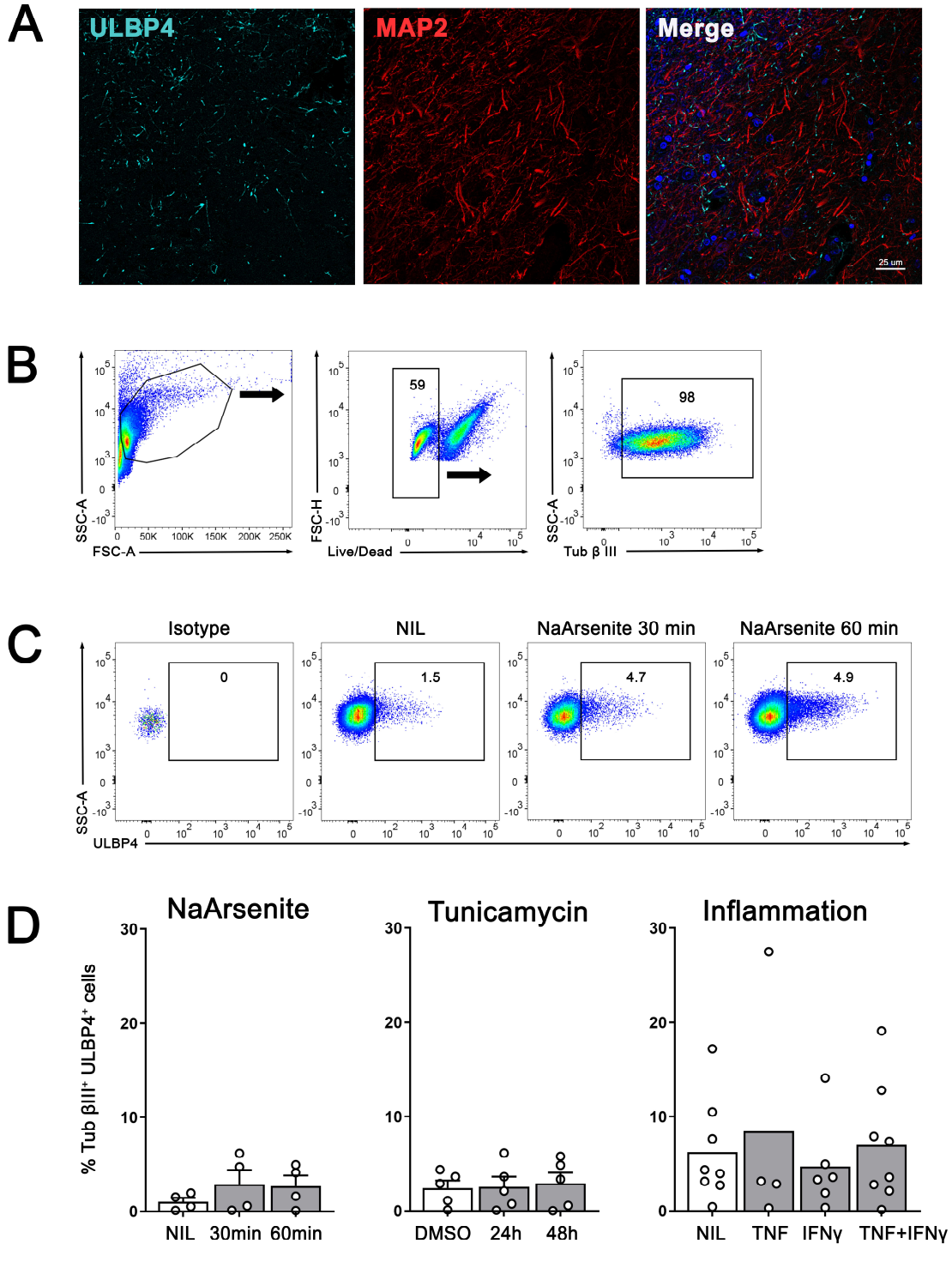


eFigure 1. Other NKG2DLs are detected at mRNA but not at protein levels in post-mortem brain tissues from MS patients and controls.

A) Relative mRNA expression of ULBP1, 2, 3, 5 and 6, MICA and MICB in post-mortem brain samples from MS patients and control patients (epilepsy) (Ctl) expressed as 2-ΔCt compare to 18S. Each dot represents one donor (n=2-3 per group) data are shown as mean. **B)** Western blot analysis of ULBP1, ULBP2/5/6, ULBP3 and MICA/B in post-mortem brain lysates from MS patients and controls (Ctl). MS samples were characterized as Active (A) MS lesion, Chronic active (CA) MS lesion, normal appearing white matter (NAWM), and normal appearing grey matter (NAGM). Control samples were dissected from either white or grey matter (WM; GM). rULBP1, rULBP2, rULBP3 and rMICB were loaded as positive controls. GAPDH detection is used as a loading control.



eFigure 2. ULBP4+ expression by human neurons in post-mortem brain tissue and in vitro. **A)** Representative images for the co-staining of ULBP4 (cyan) and MAP2 (red) in post-mortem MS brain sections tested in four MS donors and as many controls. Nuclei were stained with DAPI (blue). Scale bar = 25 µM. **B-D)** Flow cytometry analysis of ULBP4 expression by human neurons under normal or stress-induced culture conditions. B) Gating strategy from one representative donor. Cell debris, doublets and dead cells were excluded and Tub-βIII+ cells were selected for analysis. **C)** Representative dot plots showing ULBP4 detection on living Tub-βIII+ gated cells. Neurons were either kept under normal culture conditions (NIL) or exposed to sodium arsenite (NaArsenite) for 30 and 60 minutes. **(D)** Quantification of ULBP4 expression on Tub-βIII+ cells after exposure to sodium arsenite (left panel), tunicamycin (middle panel) or pro-inflammatory cytokines (right panel). Each dot represents one donor. Data are shown as mean n= 4-8.

Material and methods

RNA extraction and qPCR

Brain samples were lysed into TRIzol™ Reagent (Thermo Fisher Scientific) and RNA was extracted according to manufacturer's instructions. RNA samples were transcribed into cDNA using Quantitect Reverse transcription kit (Qiagen). Relative gene expression levels were determined by quantitative real-time PCR using primers (see Table 1) as previously published.[1] Relative mRNA expression compared to ribosomal 18S is expressed as $2^{-\Delta Ct}$. The threshold cycle (CT) is defined as the cycle at which the fluorescence level reached a certain amount (the threshold) as calculated by the software *QuantStudio™ 7 Flex Real-Time PCR System*.

For each NKG2DL ligand it was calculated as: $\Delta CT = CT(NKG2DL) - CT(18S)$.

Human post-mortem brain tissues

MS brain sections from blocks used for protein extraction (snap frozen) or immunohistofluorescence (paraffin-embedded) were stained with Luxol Fast blue and hematoxylin/eosin as well as Oil red O to assess the degree of leukocyte infiltration and demyelination and to characterize tissues as active (large leukocyte infiltration and demyelination), chronic active (leukocyte infiltration and more pronounced demyelination) or normal appearing white or grey matter (absence of demyelination and minimal leukocyte infiltration) according to published guidelines.[2]

Protein extraction and western blot

Snap frozen brain sections from six MS patients (five SPMS who were untreated for many years and one transitional RRMS-SPMS who was treated with fingolimod at the time of death) and six controls (four epilepsy patients, one patient with post-operatory complications after nephrectomy and one normal brain) were dissociated in cell lysis buffer. Lysis solutions were sonicated and centrifuged for 10 minutes at 12,000 *g* and supernatants containing protein extracts were stored at -20°C. CSF samples obtained from MS patients and OND patients were centrifuged at 420 *g* for 10 minutes and supernatants were stored at −80°C until used.

Brain protein lysate (2 ug), CSF samples (20-40 uL) and recombinant human proteins (50 ng; R&D Systems) were denatured in loading buffer at 95°C as previously described.[1] Proteins were electrophoresed on a 10% SDS-PAGE gel under reducing conditions, transferred onto PVDF membrane and then blocked as published.[1] Membrane was incubated overnight with primary antibody (Table 1) followed by incubation with HRP-conjugated secondary reagent prior to visualize binding using Western Lightning Plus-ECL (PerkinElmer). Detection of GAPDH and albumin was used as loading and quantification control for brain lysates and CSF respectively. Signal was detected and quantified as previously done.[1]

Immunostaining of post-mortem human brain sections

Paraffin embedded brain sections from MS patients (untreated SPMS) and controls (no brain-related disease) were washed with toluene and decreasing concentrations of ethanol. To retrieve antigen, sections were put for 1h at 90-95°C in sodium citrate buffer. Sections were blocked for 1h with PBS-0.1% Tween 20 containing 5% normal goat serum prior to sequential staining steps for ULBP4, GFAP and DAPI (Table 1). Slides were mounted with gelvatol and observed using a SP5 Leica confocal microscope; images were acquired sequentially in different channels using LASAF software (Leica Microsystems).

Primary cultures of human astrocytes and neurons

Primary cultures of astrocytes and neurons were obtained from human fetal brain as previously described.[3] Astrocytes or neurons were exposed for 48 hours to TNF (2000 U/ml) and/or IFNγ (20 ng/ml) or tunicamycin (2 ug/ml) for 24 or 48 hours. Sodium arsenite was added to astrocytes (0.5 uM) for 2 and 6 hours or neurons (0.1uM) for 30 to 60 minutes, after which cells were washed and left for 24h prior to be collected for flow cytometry staining.

Flow cytometry

Astrocytes and neurons were washed, detached with PBS-EDTA, stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Thermo Fisher Scientific) and then blocked with normal mouse

immunoglobulins (Thermo Fisher Scientific). Cells were stained for ULBP4 or the corresponding isotype control. Cells were fixed and permeabilized with PFA-Saponin[1] and stained for GFAP (astrocyte marker) or Tubulin-βIII (neuron marker) (Table 1). Cells were acquired on a Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

Isolation and culture of $CD8^+$ T lymphocytes and ELISA

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples collected in EDTA-coated tubes (BD Biosciences) using Ficoll density gradient as previously described.[3] $CD8^+$ T lymphocytes were positively isolated using magnetic beads according to manufacturer's instructions (Milteny Biotech). $CD8^+$ T lymphocytes were cultured overnight on anti-CD3 (5 ug/ml) pre-coated plates, with or without recombinant human ULBP4 (rhULBP4, 10 ug/ml) for 18h. Supernatants were harvested, centrifuged and stored at -20°C. IFNγ and GM-CSF levels were assessed by ELISA (DuoSet; R&D Systems) according to manufacturer's instructions.

In vitro live imaging of astrocyte:$CD8^+$ T lymphocyte co-culture

Human astrocytes were stained with orange CMRA and plated as previously described.[3] Isolated $CD8^+$ T lymphocytes were activated overnight on anti-CD3 (5 ug/ml) pre-coated plates in the presence of soluble anti-CD28 (1 ug/ml) and recombinant human IL-15 (1 ng/ml). Activated $CD8^+$ T lymphocytes were collected and stained with CFSE prior to being added to orange-CMRA-stained astrocytes in 4:1 ratio (CD8 T cell: astrocyte) in the presence or absence of rhULBP4 (10 ug/ml). Astrocyte-CD8 T cell co-cultures were imaged using spinning-disc microcopy as published.[3]

Table 1: List of reagents used

Reagent	Application	Catalog number	Source
TaqMan FAM-labeled ULBP1	Real time PCR	Hs00360941_m1	Applied Biosystems
TaqMan FAM-labeled ULBP2	Real time PCR	Hs00607609_mH	Applied Biosystems
TaqMan FAM-labeled ULBP3	Real time PCR	Hs00225909_m1	Applied Biosystems
TaqMan FAM-labeled RAET1E/ULBP4	Real time PCR	Hs01026643_g1	Applied Biosystems
TaqMan FAM-labeled RAET1G/ULBP5	Real time PCR	Hs01584111_mH	Applied Biosystems
TaqMan FAM-labeled RAET1L/ULBP6	Real time PCR	Hs00867544_gH	Applied Biosystems
TaqMan FAM-labeled MICA	Real time PCR	Hs00741286_m1	Applied Biosystems
TaqMan FAM-labeled MICB	Real time PCR	Hs00792952_m1	Applied Biosystems
TaqMan VIC-labeled ribosomal 18S	Real time PCR	4308329	Applied Biosystems
Cell lysis buffer	Western blot	9803	Cell Signaling Technology
recombinant human ULBP1	Western blot	1380-UL-050	R&D Systems
recombinant human ULBP2	Western blot	1298-UL-050	R&D Systems
recombinant human ULBP3	Western blot	1517-UL-050	R&D Systems
recombinant human ULBP4	Western blot, in vitro activation of $CD8^+$ T lymphocytes	6285-UL-50	R&D Systems
recombinant human MICB	Western blot	1599-MB-050	R&D Systems
Goat anti-human ULBP1	Western blot	AF1380	R&D Systems

Goat anti-human ULBP2/5/6	Western blot	AF1298	R&D Systems
Goat anti-human ULBP3	Western blot	AF1517	R&D Systems
Mouse anti-human ULBP4	Western blot , immunohistofluorescence	MAB6285	R&D Systems
Mouse anti-human MICA/B	Western blot	MAB13001	R&D Systems
Rabbit anti-GAPDH	Western blot	5174	Cell Signaling Technology
Rabbit anti-albumin	Western blot	NBP1-32458	Novus Biologicals
HRP-conjugated rabbit anti-mouse	Western blot	P0260	Dako
HRP-conjugated rabbit anti-goat	Western blot	P0449	Dako
HRP-conjugated donkey anti-rabbit	Western blot	711-035-152	Jackson ImmunoReseach
Cy3-conjugated mouse mAb anti-GFAP	immunohistofluorescence	C9205	Sigma-Aldrich
Alexa Fluor™488-conjugated goat anti-mouse	immunohistofluorescence	A11001	Thermo Fisher Scientific
4′,6-diamidino-2-phenylindole (DAPI)	immunohistofluorescence	D1306	Thermo Fisher Scientific
Recombinant human TNF	In vitro assay	PHC 3011	Thermo Fisher Scientific
Recombinant human IFNγ	In vitro assay	RIFNG50	Thermo Fisher Scientific
Tunicamycin	In vitro assay	T7765-1MG	Sigma-Aldrich
Sodium arsenite	In vitro assay	106277	MilliporeSigma

Mouse anti-human CD3	in vitro activation of $CD8^+$ T lymphocytes	16-0037-85	ThermoFisher
Mouse anti-human CD28	in vitro activation of $CD8^+$ T lymphocytes	553294	BD Biosciences
Recombinant human IL-15	in vitro activation of $CD8^+$ T lymphocytes	200-15-10UG	Peprotech
APC conjugated mouse mAb anti-ULBP4	FACS	FAB6285A	R&D Systems
APC conjugated mouse mAb IgG2	FACS	IC0041A	R&D Systems
mouse mAb anti-GFAP-Efluor615	FACS	42-9892-82	eBioscience, Thermo Fisher Scientific
mouse mAb anti-Tubulin-βIII-Alexa Fluor ™488	FACS	53-4510-82	ThermoFisher

References

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3. Lemaitre F, Carmena Moratalla A, Farzam-Kia N, et al. Capturing T Lymphocytes' Dynamic Interactions With Human Neural Cells Using Time-Lapse Microscopy. Front Immunol 2021;12:668483.