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Proinflammatory macrophage activation by the polysialic acid-Siglec-16 axis is linked to increased survival of glioblastoma patients

Supplementary Methods

Antibodies and reagents

The following monoclonal (mAb) or polyclonal (pAb) antibodies were used: PolySia-specific mouse mAb 735 (IgG2a; RRID:AB_2619682, 2 µg/ml for immunofluorescence or 1 µg/ml for immunoblotting), produced in-house as described (1), polySia-specific mouse mAb, clone 2-2B (IgM, Merck, #MAB5324, RRID:AB_95211, 1:400), NCAM-specific mouse mAb 123C3 (IgG1; RRID:AB_2860561, 1:250 for immunofluorescence or 1:1000 for immunoblotting) recognizing the extracellular part of all human NCAM isoforms, provided by Rita Gerardy-Schahn (Hannover Medical School, Hannover, Germany), Siglec-16-specific mouse mAb, clone 706030 (IgG1, R&D systems, Minneapolis, MN, USA, #MAB68192; RRID:AB_2884994, 5 µg/ml), raised against a human Siglec-16 peptide and not cross-reactive with Siglec-11, anti-Siglec-11 mouse mAb 4C4 (IgG2b, BioLegend, San Diego, CA, USA, #681702, RRID:AB_2566496, 1:100), for which cross-reactivity with the extracellular domain of Siglec-16 has been reported (2,3), CD68-specific mouse mAb, clone PG-M1 (IgG3, Agilent, Santa Clara, CA, USA, #IS61330-2, RRID:AB_2892734, 1:100), CD163-specific rabbit mAb, clone EPR19518 (Abcam, Cambridge, UK, #ab 182422, RRID:AB_2753196, 1:400), CD74-specific mouse mAb, clone LN2 (IgG1, Abcam, Cambridge, UK, #ab 9514, RRID:AB_2075504, 1:100), CD11b-specific rat mAb, clone M1/70.15 (Bio-Rad, Hercules, CA, USA, #MCA74GA, RRID:AB_324660, 1:200), CD11c-specific mouse mAb (IgG2a, Leica Biosystems, #NCL-L-CD11c-563, RRID:AB_563490, 1:200), CD3-specific rabbit pAb (Agilent, Santa Clara, CA, USA, #A0452, RRID_AB:_2335677, 1:100), CD8-specific mouse mAb, clone C8/144B (IgG1, Agilent, Santa Clara, CA, USA, #M7103, RRID_AB:_2075537, 1:100), CD45-specific rabbit mAb, clone EP322Y (Abcam, #ab40763, RRID:AB_726545, 1:500), CD34-specific mouse mAb, clone QBEnd/10 (Leica Biosystems, #NCL-END, RRID:AB_442089, 1:300), GFAP-specific rabbit pAb (Merck, Darmstadt, Germany, #G9269, RRID:AB_477035, 1:200), IBA1-specific goat pAb (Novus Biologicals, Centennial, CO, USA, #NB100-1028, RRID:AB_521594, 1:300), IBA1-specific rabbit pAb (IgG, FUJIFILM Wako Shibayagi, Japan, #019-19741, RRID_839504, 1:400, applied for IBA1/CD74 staining), and human mIDH1R132H-specific mouse mAb, clone HMab-1 (IgG1, Merck, #MABC171, RRID:AB_2860562, 1:50) (4).

Secondary antibodies were biotin-conjugated sheep anti-mouse IgG (Boehringer, Mannheim, Germany, #1039285, 1:1000), AlexaFluor-488-conjugated anti-mouse IgM, AlexaFluor-488-conjugated

donkey anti-mouse IgG and goat anti-mouse IgG2a, AlexaFluor-555-conjugated donkey anti-rabbit and anti-goat IgG, AlexaFluor-568-conjugated goat anti-mouse IgG1, AlexaFluor-647-conjugated donkey anti-goat and anti-rabbit IgG (ThermoFisher, Waltham, MA, USA, #A21042, #A21020, #A21131, #A31572, #A21432, #A21124, #A21447, and #A31573, 1:500, each), Cy3-conjugated donkey anti-rat IgG (Merck, #AP189C, 1:500), IRDye-800CW-conjugated goat anti-mouse IgG1, and IRDye-680LT-conjugated goat anti-mouse IgG2a (LI-COR Biosciences, Lincoln, NE, USA, #926-32350 and #926-68051, 1:20,000, each).

Horseradish peroxidase (HRP)-conjugated streptavidin was from Dako/Agilent (Santa Clara CA, USA, #P0397, 1:1000), AlexaFluor-488-conjugated streptavidin from Rockland (Limerick, PA, USA, #S000-41, 1:1000), and the streptavidin/biotin blocking kit from Vector Laboratories (Burlingame, CA, USA, #SP-2002). Antigen retrieval buffers (CC2 #950-123 for CD68/CD163 staining, and CC1 #950-124 for CD3/CD8 and IBA1/CD74 staining), ultraView DAB (3,3'-Diaminobenzidine) Detection Kit (#760-500) and ultraView universal alkaline phosphatase red detection kit (#760-501) were from Ventana/Roche Diagnostics (Rotkreuz, Switzerland), and Q5 polymerase was from New England Biolabs (Ipswich, MA, USA, #M0491). The NucleoSpin RNA kit was from Macherey-Nagel (Düren, Germany, #740955.50). Endosialidase was produced as described (1). All other reagents were from Merck.

Immunostaining

Biotin-tyramide signal amplification. Biotin-tyramide signal amplification was used for immunostaining of Siglec-11 or Siglec-16 in combination with IBA1 on GB sections or with CD11b on spheroid sections, respectively. After antigen-retrieval and permeabilization, endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2/PBS for 15min at RT. After washing, endogenous biotin was blocked with streptavidin and, after further washing, with biotin (1:10 in PBS, 15min at RT, each). Following another washing step, sections were incubated overnight at 4°C with primary antibodies in blocking buffer, consisting of PBS with 0.1% Triton X-100, 2% BSA, and, for staining of spheroids, 5% normal goat serum (Vector Laboratories, #S-1000-20). For the detection of Siglec-specific antibodies, biotin-conjugated anti-mouse IgG was applied for 45min in blocking buffer, followed by washing with PBS, incubation with HRP-conjugated streptavidin in blocking buffer for 30min at RT, washing with PBS and equilibration with TBS, pH 7.6, and incubation with 2.5 µg/ml biotinyl tyramide, 0.1 M imidazole and 0.001% H_2O_2 in TBS, pH 7.6 for 10min at RT. After washing with PBS, enzymatically produced biotin precipitate was labelled by AlexaFluor-488-conjugated streptavidin (1:1000 in blocking buffer), before fluorescent secondary antibodies were applied and sections were embedded as described before (5).

CD68/CD163, CD3/CD8 and IBA1/CD74 IHC on a Ventana autostainer. After antigen retrieval with CC2 buffer (CD68/CD163), or CC1 buffer (CD3/CD8 and IBA1/CD74), sections were incubated with CD68-,

CD3-, or IBA1-specific antibodies and stained with ultraView DAB detection kit, followed by incubation with CD163-, CD8-, or CD74-specific antibodies and staining with ultraView red detection kit, respectively. After three washes in 0.05% Tween/PBS, slides were mounted with Mowiol-4-88 (Merck, #81381).

Specificity controls. Specificity of multi-immunostaining procedures was controlled by omitting one of the primary or secondary antibodies at a time. Specificity of polySia staining was additionally controlled by loss of immunoreactivity after degradation with 10 µg/ml endosialidase applied after antigen retrieval for 3h at 37°C in blocking buffer (1).

Genomic PCR and qPCR

For genotyping PCR, Q5 polymerase was used according to the manufacturer's instructions but with 3 mM $MgCl_2$ and 500 nM of each primer. Template DNA concentrations were 1 ng/µl for tumor samples and 0.05 ng/µl for samples obtained from peripheral blood mononuclear cells (PBMCs) or THP-1 macrophages as described (6). PCR conditions were 2min at 98°C, 26 cycles of 15sec at 98°C, 20sec at decreasing temperatures (65, 64, 63 and 62°C for 6, 5, 11 and 4 cycles, respectively), and 30sec at 72°C, followed by 3min at 72°C. Specific PCR product amplification was confirmed by agarose gel electrophoresis.

For RNA extraction with the NucleoSpin kit, 200 µl supernatant were diluted with buffer RA1 to a volume of 350 µl and processed according to manufacturer's instructions. Total RNA was reversely transcribed and qPCR of *AIF1* and *TNF* was performed by the comparative threshold cycle (ΔC_T) method as described (5) except that 1 µM of each primer and 1.5 ng/µl cDNA template were used for samples from GB specimen. Primers were validated using cDNA obtained from THP-1 macrophages as described previously (6). TNF- and AIF1-specific primers were 5'-GGAGAAGGGTGACCGACTCA-3' (TNF-forward), 5'-CTGCCCAGACTCGGCAA-3' (TNF-reverse), 5'-GACGTTCAGCTACCCTGACT-3' (AIF1-forward), and 5'-GCCTGTTGGCTTTTCCTTTTCT-3' (AIF1-reverse).

Microscope settings and image acquisition

Fluorescent imaging was performed using a 20x Plan-Apochromat objective with a numerical aperture of 0.8 to acquire optical sections of 1.62, 1.88, or 2.09 µm (488, 568, or 647 channel), and five optical sections, each, were merged to maximum intensity projections. For 3D reconstructions, z-stacks of optical sections were obtained using a 63x Plan-Apochromat oil immersion objective with a numerical aperture of 1.4. Light microscopy with Axio Observer.Z1 was performed using a 40x EC Plan-Neofluar objective with a numerical aperture of 0.75. Identical microscope settings were used for all samples within one experimental setting. Images were arranged using Microsoft PowerPoint 2016.

Statistics

Survival curves were compared using the log-rank test (Mantel-Cox method). For multiple comparisons, the False Discovery Rate (FDR) was controlled using the Benjamini, Krieger and Yekutieli procedure and for $P<0.05$ the adjusted P values (q values) are reported. Cox proportional hazard regression models were used for univariate and multivariate analyses of covariates in relation to OS. Two-tailed Fisher's exact tests, Mann-Whitney tests, *t* tests, Kruskal-Wallis tests followed by Dunn's multiple comparisons tests, and two-way ANOVA followed by Tukey's post hoc tests were applied as indicated. Classification and Regression Tree (CART) analysis was performed to determine the best cutoff value to distinguish age groups with regard to OS in both cohorts. Normality and equality of variances were assessed by the Shapiro-Wilk and Bartlett's test, respectively. Statistical significance was assumed at a level of $P<0.05$.

References

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