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Induction of STAP-1 promotes neurotoxic activation of microglia

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ABSTRACT

Activated microglia contribute to neurodegenerative processes in the brain and the retina. Via DNA-microarray analysis, we have previously identified up-regulation of several immune-related genes in the dystrophic retina of retinoschisin-deficient ($Rs1h^{-/Y}$) mice. Here we report a strong overexpression of transcripts for the signal-transducing adaptor protein-1 (STAP-1) in isolated $Rs1h^{-/Y}$ microglia. Furthermore, STAP-1 expression was induced in activated bone marrow-derived macrophages as well as LPS-, interferon-γ-, and CpG-stimulated myeloid cell lines. Ectopic expression of STAP-1 in BV-2 microglia changed the morphology and cytoskeletal organization of the cells and transformed ramified cells to an activated state. STAP-1 overexpression also leads to an interaction with the M-CSF receptor/c-Fms diminishing its ligand-dependent phosphorylation. Finally, STAP-1 expressing cells showed strongly reduced migration with increased cytotoxicity against 661W photoreceptor like cells. Taken together, our study implicates a previously unknown role of STAP-1 in pro-inflammatory microglia activation potentially contributing to neuronal apoptosis and degeneration.

Microglia, the resident macrophages of the nervous system, have an important role in immune surveillance [1] and neuronal homeostasis [2]. Microglia activation is a protective mechanism regulating tissue repair and recovery in the early phase of neurodegeneration [3]. However, excessive or sustained activation contributes to acute and chronic of the brain and the retina [4–6]. Activated microglia comprise a distinct group of subpopulations with specific activation markers and diverse functional phenotypes [7]. DNA-microarrays have recently been employed to identify gene signatures characteristic for immuno-regulatory cells [8] or pro-inflammatory microglia in the retina of retinoschisin-deficient ($Rs1h^{-/Y}$) mice [9], a murine model of human X-linked juvenile retinoschisis [10]. Several novel transcripts previously not linked to microglia activation have been identified in these genome-wide expression studies, including a strong induction of the adaptor molecule STAP-1 (signal-transducing adaptor protein-1) [9].

The murine STAP-1 gene was initially identified to encode an adaptor involved in downstream signaling of stem-cell factor and was therefore termed *stem-cell adaptor protein-1* [11]. The 297 amino acid STAP-1 protein strongly binds to c-kit in a yeast two-hybrid screening using a hematopoietic stem-cell library [11]. Another report described STAP-1 as the ortholog of human BRDG1 (BCR downstream signaling 1) mediating Tec-kinase signaling after cross-linking of the B-cell receptor [12]. STAP-1 solely contains Peckstrin homology (PH) and Src homology 2 (SH2) domains and several tyrosine phosphorylation sites. This unique and universal molecular structure opens the possibility that STAP-1 controls the recruitment of signaling kinases to receptor tyrosine kinases in different cell types.

In the present study we report increased STAP-1 levels in pro-inflammatory stimulated microglia and macrophages and provide first evidence that STAP-1 is functionally involved in microglia activation. Heterologous STAP-1 expression impairs M-CSF-receptor signaling, leading to decreased motility and enhanced neurotoxic activity. This suggests that STAP-1 is a molecular target for limiting microglia activation in neurodegenerative disorders.

Materials and methods

Reagents and antibodies. Recombinant human M-CSF was purchased from R&D Systems (Minneapolis, MN, USA) and phalloidin-TRITC was from Sigma–Aldrich (Steinheim, Germany). A hybridoma clone expressing anti-mouse c-Fms/M-CSF-receptor monoclonal antibodies (AFS98) was a gift from Prof. David Hume (Rosslin Institute, Edinburgh, UK). Antibodies against GFP, phospho-tyrosine, phospho-c-FMS, and actin were purchased from Abcam (Cambridge, MA, USA), Calbiochem (Bad-Soden, Germany), Cell Signaling Technology (Beverly, MA, USA), and AbD Serotec (Düsseldorf, Germany), respectively. LPS from *Escherichia coli* 026:B6 and interferon-γ were from Sigma–Aldrich and phosphotioate-stabilized CpG oligonucleotides were from Metabion (Martinsried, Germany). All other chemicals were obtained from Sigma–Aldrich.

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Animals. Retinoschisin-deficient ($Rs1h^{-/Y}$) mice [10] and C57BL/6 mice were maintained in an air-conditioned environment on a 12-h light–dark schedule at 20–22 °C, and had free access to food and water. The health of the animals was regularly monitored, and all procedures strictly adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation of primary retinal microglia and cell culture. Retinal tissue from wild-type and $Rs1h^{-/Y}$ mice was isolated from eye bulbs and purified from contaminating vitreous body and retinal pigment epithelium and choriocapillaris. Retinae were cut into small pieces and incubated for 40 min at 37 °C in 1 ml of PBS with 1 mg/ml collagenase type I (Sigma–Aldrich), 0.3 mg/ml DNase I (Roche Applied Science, Mannheim, Germany) and 0.2 mg/ml hyaluronidase (Sigma–Aldrich). The cell suspension was filtered through a 70 μm cell strainer (Becton Dickinson, Heidelberg, Germany) and subjected to Ficoll density gradient centrifugation for 20 min at 2000 rpm (690 g, without brake). The cells were cultured for 7–11 days in DMEM/10% FCS supplemented with 50 ng/ml recombinant human M-CSF (R&D Systems). The culture conditions for BV-2 microglia cells, RAW264.7 macrophages, and 661W photoreceptor cells have been described previously [8,9].

RNA isolation and reverse transcription. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Purity and integrity of the RNA was assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip® reagent set (Agilent Technologies, Santa Clara, CA, USA). The RNA was quantified spectrophotometrically and then stored at −80 °C. cDNAs were generated using the Reverse Transcription System (Promega, Mannheim, Germany).

Quantitative real-time RT-PCR (qRT-PCR). Amplifications of 50 ng cDNA were performed with the Taqman 7900HT real-time PCR detection system (Applied Biosystems, Foster City, CA, USA) in 20 μl reaction mixtures containing 1× TaqMan Gene Expression Master Mix (Applied Biosystems), 200 nM primers, and 0.25 μl dual-labeled probe (Roche Universal Probe Library). Measurements were performed in triplicates and the results were analyzed with the $\Delta\Delta C_t$ method for relative quantitation. Normalization to three stable reference genes was performed as described earlier [8]. The primers for amplification of STAP-1 were F, 5′-GGA GGG GCT TCA TTC TTA CA-3′, and R, 5′-TTG CCC AGG TAG AAG TGA CA-3′. Inducible NO-synthase (iNOS) mRNA was quantified with primers F, 5′-TGA ACT TGA GCG AGG AGC A-3′, and R, 5′-TTC ATG ATA ACG TTT CTG GCT CT-3′.

Transient and stable overexpression of STAP-1 in BV-2 cells. A FLAG-tagged expression construct for STAP-1 was created by RT-PCR cloning of the full-length protein (amino acids 3–297) into the pFLAG-CMV4 vector using the forward primer 5′-CCC GAA TTC GGC GAA AAA GCC CCC GAA AC-3′ and reverse primer 5′-CCC GGA TCC TTA CTT CAT GCA TTG TGT GG-3′ containing restriction sites for EcoRI and BamHI, respectively. A GFP-STAP-1 fusion protein was created by cloning of the STAP-1 ORF into the vector pcDNA/NT-GFP-TOPO using KpnI and EcoRV sites. Transient transfection of pFLAG or pFLAG-STAP-1 into BV-2 cells was performed with the FUGENE 6 reagent (Roche Applied Science) as recommended by the manufacturer. Stable BV-2 cell transformants expressing GFP or GFP-STAP-1 were created by neomycin selection and expansion of single clones. Successful expression of GFP and GFP-STAP-1 was monitored by GFP fluorescence.

Immunocytochemistry. BV-2 cells were plated overnight on coverslips, fixed with 3.7% paraformaldehyde for 10 min at 37 °C, permeabilized with 0.2% Triton X-100 for 5 min, blocked with 5% non-fat milk, 0.2% Triton X-100, and stained with the anti-GFP antibody. The cells were rinsed and the nuclei counter-stained with DAPI for 10 min at room temperature (0.1 μg/ml in PBS, 4′,6-diamidino-2-phenylindol, Molecular Probes). Filamentous actin was stained by addition of 1.5 μM TRITC-conjugated phalloidin (Sigma). The coverslips were mounted on microscopic glass slides and viewed with a Axioskop 2 fluorescence microscope equipped with an Eclipse digital analyzer (Carl Zeiss).

Immunoprecipitation and immunoblotting. Cells were grown in 10 cm dishes and lysed in 1 ml of precipitation buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors. Cell lysates were incubated over night at 4 °C with 20 μl of protein A-Sepharose beads with 1 μg of anti-c-FMS antibody and then washed with buffer. For immunoblotting, equal amounts of protein samples were separated with PAGE and thereafter transferred to Hybond ECL membranes (Amersham Biosciences) as described previously [13]. The blots were analyzed with the indicated antibodies.

Cell migration assays. Wound-healing assay: BV-2 cells were cultured in 6-well plates and wounded with a sterile plastic tip producing scars. Cell migrating into the open space was monitored by microscopy. Photomicrographs were taken immediately and at 4 h, 8 h, and 24 h after wounding. Transwell migration: 1×10^6 BV-2 cells were cultured on Costar transwell filters with 8 μm pore size (Corning Costar, Bodenheim, Germany). Cells were allowed to migrate into the lower chamber containing 500 μl complete medium with or without 50 ng/ml M-CSF. After 8 h and 24 h of incubation, the number cells in the lower compartment was counted with phase-contrast microscopy.

Phagocytosis assay. One micrometer of blue Latex beads from polystyrene (Sigma–Aldrich) were added to the cells at a concentration of 1 μl beads/ml, and cells were washed with PBS after 24 h incubation. For quantitation, the phagocytosis potential expressed as% positive cells was monitored by counting the optical bead density in up to 100 cells from six individual wells using light microscopy.

NO measurement. NO concentrations were determined by measuring the amount of nitrite secreted by BV-2 cells into the culture medium using the Griess reagent system (Promega). Fifty microliters of cell culture supernatant was collected and an equal volume of Griess reagent was added to each well. After incubation for 15 min at room temperature, the absorbance was read at 540 nm on a BMG FluoStar Optima plate reader (Labtech, Offenburg, Germany). The concentration of nitrite for each sample was calculated from a sodium nitrite standard curve.

Cytotoxicity and apoptosis assay. Microglia cytotoxicity was tested using a culture system of 661W photoreceptor cells with conditioned medium from BV-2 cells. 661W cells were incubated for 24 h either in with 10% culture supernatants from GFP-expressing or GFP-STAP-1 expressing BV-2 cells. Cell viability was studied using the Cytotox96 non-radioactive cytotoxicity assay measuring LDH release (Promega), and apoptotic cell death was determined with the caspase 3/7 glow assay (Promega).

Results

STAP-1 expression is induced in pro-inflammatory activated microglia and macrophages

We have previously detected reactive microglia in the degenerating retina of retinoschisin-deficient mice [14]. DNA-microarray analysis of *ex vivo* isolated activated $RS1h^{-/Y}$ retinal microglia identified several differentially expressed transcripts [9], including a more than 10-fold induction of the expressed sequence tag AI586015 (data not shown). Database search revealed that this sequence was identical to the previously cloned STAP-1 gene [11]. To confirm these findings, we performed quantitative real-time RT-PCR experiments with RNA samples from independently isolated retinal microglia from wild-type and $RS1h^{-/Y}$ mice. STAP-1 mRNA levels were significantly increased in $RS1h^{-/Y}$ microglia compared to non-activated microglia (Fig. 1A), validating our initial expression profiling experiments.

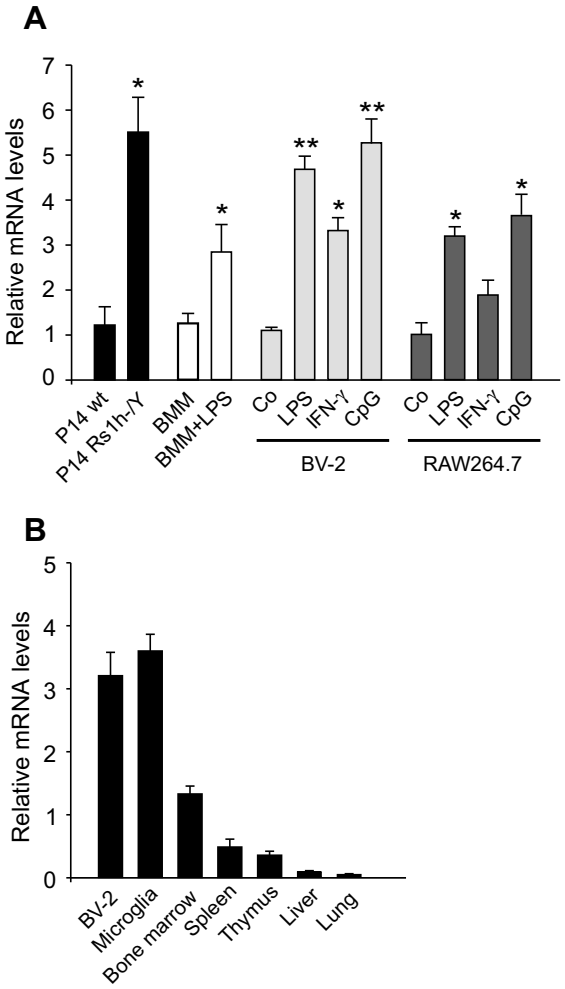

Fig. 1. Expression of STAP-1 mRNA in primary microglia, activated myeloid cell lines and mouse tissues. (A) Real-time qRT-PCR analysis of primary retinal microglia from postnatal day 14 (P14) wild-type (wt) or retinoschisin-deficient ($Rs1h^{-/Y}$) mice, bone marrow-derived macrophages (BMM), BV-2 microglia, and RAW264.7 macrophages stimulated with 50 ng/ml LPS, 20 ng/ml IFN-γ, or 25 μM CpG oligonucleotides. (B) Real-time qRT-PCR analysis of BV-2 cells and primary microglia in comparison to different murine tissues. Data represent means ± SEM of three independent cell cultures or tissue preparations analyzed in triplicates. $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.

We were next interested whether STAP-1 is also expressed and regulated in activated mouse bone-marrow-derived macrophages (BMM), BV-2 microglial cells and RAW264.7 macrophages. STAP-1 mRNA was detected in all three myeloid cell types and pro-inflammatory stimulation with lipopolysaccharide (LPS), interferon-γ (IFN-γ), or synthetic cytosine-guanosine dinucleotide-containing oligonucleotides (CpG) lead to a prominent increase in STAP-1 transcript levels (Fig. 1A). This indicated that STAP-1 gene expression is sensitive to the pro-inflammatory activation state of phagocytes. Examination of STAP-1 mRNA expression in various mouse tissues detected transcripts in bone marrow, spleen and thymus, and a very week expression in liver and lung (Fig. 1B). No PCR signals could be obtained for heart, testis, kidney, and brain (data not shown).

STAP-1 overexpression changes the cell morphology and activation state of microglia

We then asked the question whether induction of STAP-1 has direct functional consequences on microglia activation. Transient ectopic expression of a FLAG-STAP-1 fusion protein had a prominent impact on microglia morphology. The ramified phenotype of mock-transfected BV-2 microglia (Fig. 2A) was completely changed to cluster forming round cell bodies, reminiscent of strongly activated cells (Fig. 2B). To verify these findings and to visualize STAP-1 within cells, stable BV-2 transformants expressing GFP-STAP-1 or GFP as control were generated. GFP-expressing BV-2 cells were phenotypically not different from wild-type cells with long protrusions (Fig. 2C). In contrast, expression of the GFP-STAP-1 fusion protein was detected in the cytoplasm of densely packed cell clumps (Fig. 2D). The cell shape of phagocytes is directly connected to the cytoskeleton. Therefore, we performed a specific staining for filamentous (F)-actin with phalloidin-TRITC in the stable cell lines. Typical for non-activated microglia, F-actin staining in GFP-cells was localized to numerous membrane spikes and ruffles (Fig. 2E). In contrast, GFP-STAP-1-cells showed different cytoskeletal morphology, with an equal distribution of F-actin on the cell surface of rounded cells lacking motile protrusions (Fig. 2F).

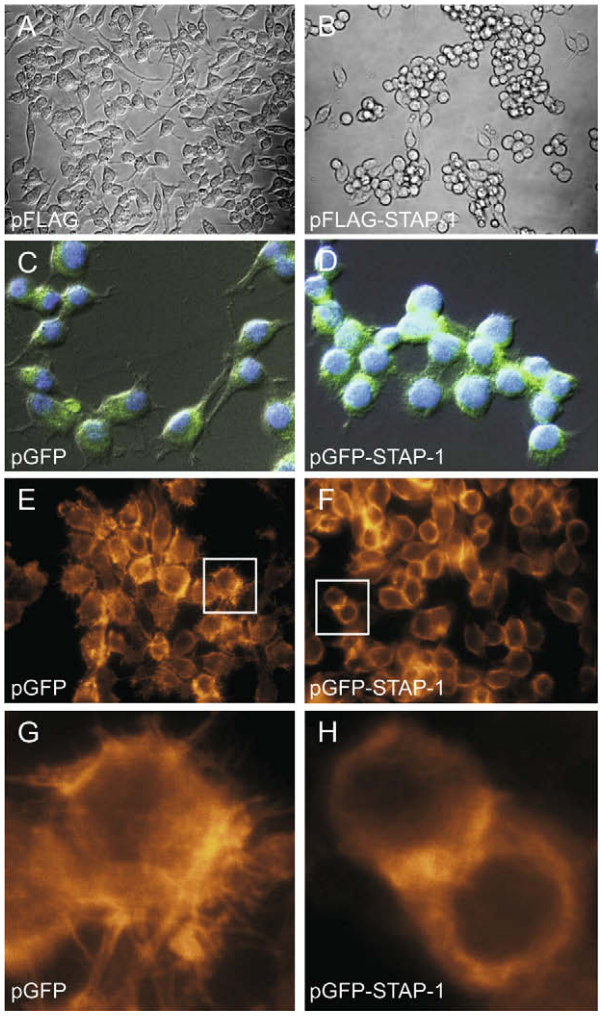

Fig. 2. Ectopic STAP-1 expression changes cell morphology and F-actin cytoskeleton of microglia. BV-2 cells were transiently transfected with pFLAG-CMV4 vector (A) or pFLAG-STAP-1 expression vector (B) and images were taken by phase-contrast microscopy. (C–H) Stable transfection of BV-2 cells with GFP or GFP-STAP-1 expression constructs. Simultaneous GFP immunofluorescence and DAPI-staining of GFP-expressing (C) or GFP-STAP-1-expressing cells (D). (E–H) Phallodin-TRITC-staining of F-actin bundles of GFP or GFP-STAP-1 overexpressing BV-2 microglia. (G,H) 5× magnification of selected cells from (E,F).

STAP-1 blocks migration and M-CSF-induced chemotaxis

To assess whether STAP-1 modulates the motility of microglia, two different *in vitro* assays were performed. In the wound-healing assay, a cell-free zone was created in confluent monolayers of stable transfected BV-2 cells by scratching cells off with a pipette tip. The repopulation of the cell-free zone was then monitored by time-laps microscopy. In control cells, migration into the wounded area started after 4 h, increased at 8 h and was completed by 24 h (Fig. 3A). In contrast, GFP-STAP-1-expressing cells showed a strongly reduced capacity to repopulate the cell-free area (Fig. 3A), indicating a diminished motility.

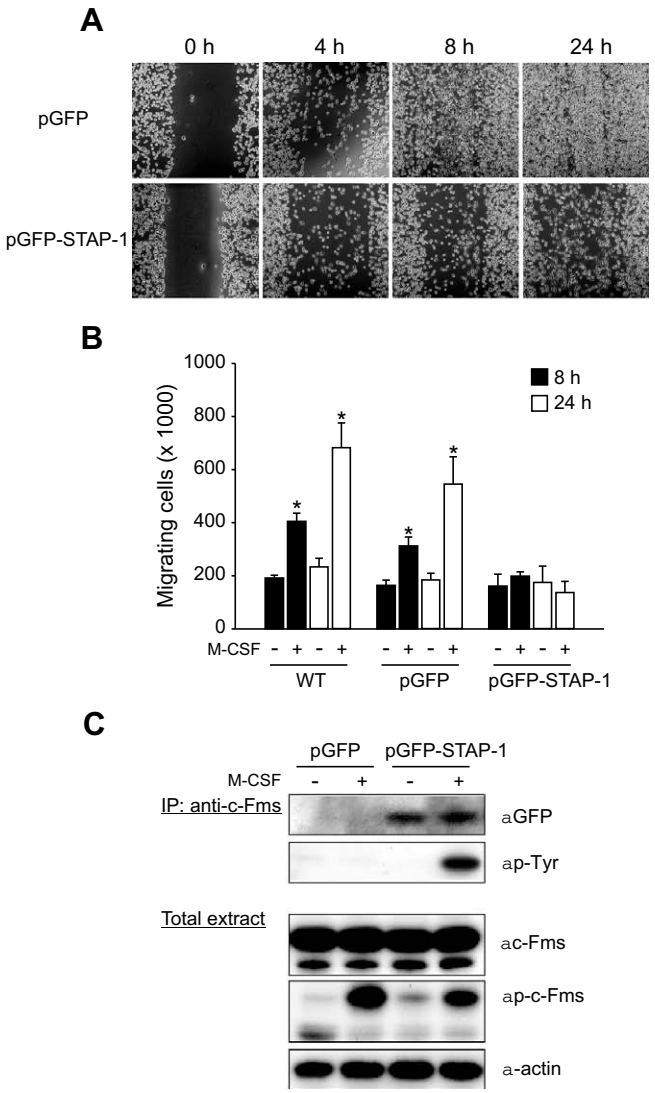

Fig. 3. STAP-1 blocks migration and M-CSF-induced chemotaxis in microglia and interacts with c-Fms. (A) Stable GFP or GFP-STAP-1 expressing BV-2 cells were analyzed in a wound-healing assay. Photomicrographs were taken at the indicated time points after scratching the cell layers. One representative experiment is shown and similar results were obtained in three independent cultures. (B) *In vitro* chemotaxis assay with 8 μm transwell filter cultures. Migration assays were performed in the absence or presence of 50 ng/ml M-CSF in the lower culture chamber. The average number of migrating cells was counted after 8 h and 24 h. Data represent means ± SEM of three independent cell cultures analyzed in triplicate wells. $^{*}P < 0.05$, Student's *t*-test. (C) Stable GFP or GFP-STAP-1 expressing BV-2 cells were left untreated or stimulated with 50 ng/ml M-CSF for 30 min and anti-c-Fms immunoprecipitates (upper panel) or total cell extracts (lower panel) were blotted with the indicated antibodies.

To confirm these effects, chemotaxis assays in transwell microchambers were performed in the absence or presence of M-CSF, a major chemoattractant for phagocytes. The amount of control cells that passed through the filters after 8 h and 24 h was significantly increased by 50 ng/ml M-CSF (Fig. 3B). When GFP-STAP-1 cells were treated with M-CSF, the number of migrated cells did not increase above the basal level (Fig. 3B). Therefore, STAP-1 has a direct effect on microglia motility and chemotaxis.

Since STAP-1 contains a PH domain and regulates M-CSF dependent cell migration, we studied a potential interaction of STAP-1 with c-Fms. Immunoprecipitation with anti-c-Fms antibody resulted in a pull-down of the GFP-STAP-1 fusion protein (Fig. 3C). This receptor association was independent of the M-CSF ligand stimulus, but the subsequent tyrosine phosphorylation of STAP-1 required ligand activation of c-Fms (Fig. 3C). By analyzing total cell extracts from GFP or GFP-STAP-1 expressing cells, we could show that STAP-1 reduced the phosphorylation status of c-Fms (Fig. 3C). Thus, STAP-1 interferes with M-CSF-regulated signaling leading to impaired migration of BV-2 microglia.

STAP-1 increases microglial phagocytosis, NO secretion and neurotoxicity

Given the strong induction of STAP-1 mRNA by pro-inflammatory agents we next studied the phagocytosis of STAP-1 expressing cells in the absence or presence of 50 ng/ml LPS. The basal engulfment of Latex beads was stronger in GFP-STAP-1 cells than controls, albeit not statistically different (Fig. 4A and B). When the cells were activated by LPS, the phagocytic bead uptake in GFP-STAP-1 cells was significantly higher (65%) compared to GFP-expressing cells (48%) (Fig. 4B).

NO secretion is a measure of the cytotoxic potential of phagocytes. The concentration of NO in the cell culture medium was increased by LPS in both stable cell lines, with a tendency to higher NO secretion in GFP-STAP-1 cells (Fig. 4C). NO release is mainly controlled by inducible NO-synthase (iNOS) expression. We therefore measured iNOS mRNA levels and detected significantly more transcripts in LPS-stimulated GFP-STAP-1 cells than controls (Fig. 4D).

We finally analyzed the cytotoxic and pro-apoptotic effects of STAP-1 overexpressing BV-2 cells on neuronal cells. Since we have first identified strongly increased STAP-1 expression in retinal microglia, we used 661W photoreceptor like cells as a neuronal cell culture model for our studies. 661W cells were seeded in the presence of 10% culture supernatant from unstimulated or LPS-activated BV-2 cells. A significant decrease in cell viability of 661W cells was noticed with supernatant from unstimulated or LPS-activated GFP-STAP-1 cells compared to control cells as determined by lactate dehydrogenase (LDH) release (Fig. 4E). Supernatants from GFP-STAP-1 cells also caused significantly higher caspase 3/7 activity in 661W cells than GFP-expressing cells. These results indicate that STAP-1 overexpression leads to cytotoxic activation of microglia with remarkable pro-apoptotic effects on neuronal cells.

Discussion

This is the first study to demonstrate an expression of STAP-1 in activated microglia and macrophages. We show that STAP-1 interacts with c-Fms and controls microglia morphology, migration, and phagocytosis. Ectopic STAP-1 expression leads to increased cytotoxicity and secretion of pro-apoptotic compounds along with a diminished survival of neuronal cells.

Literature reports on STAP-1 are scarce with only three studies published so far [11,12,15]. Masuhara et al. demonstrated that murine STAP-1 is an adaptor protein for the receptor c-kit in

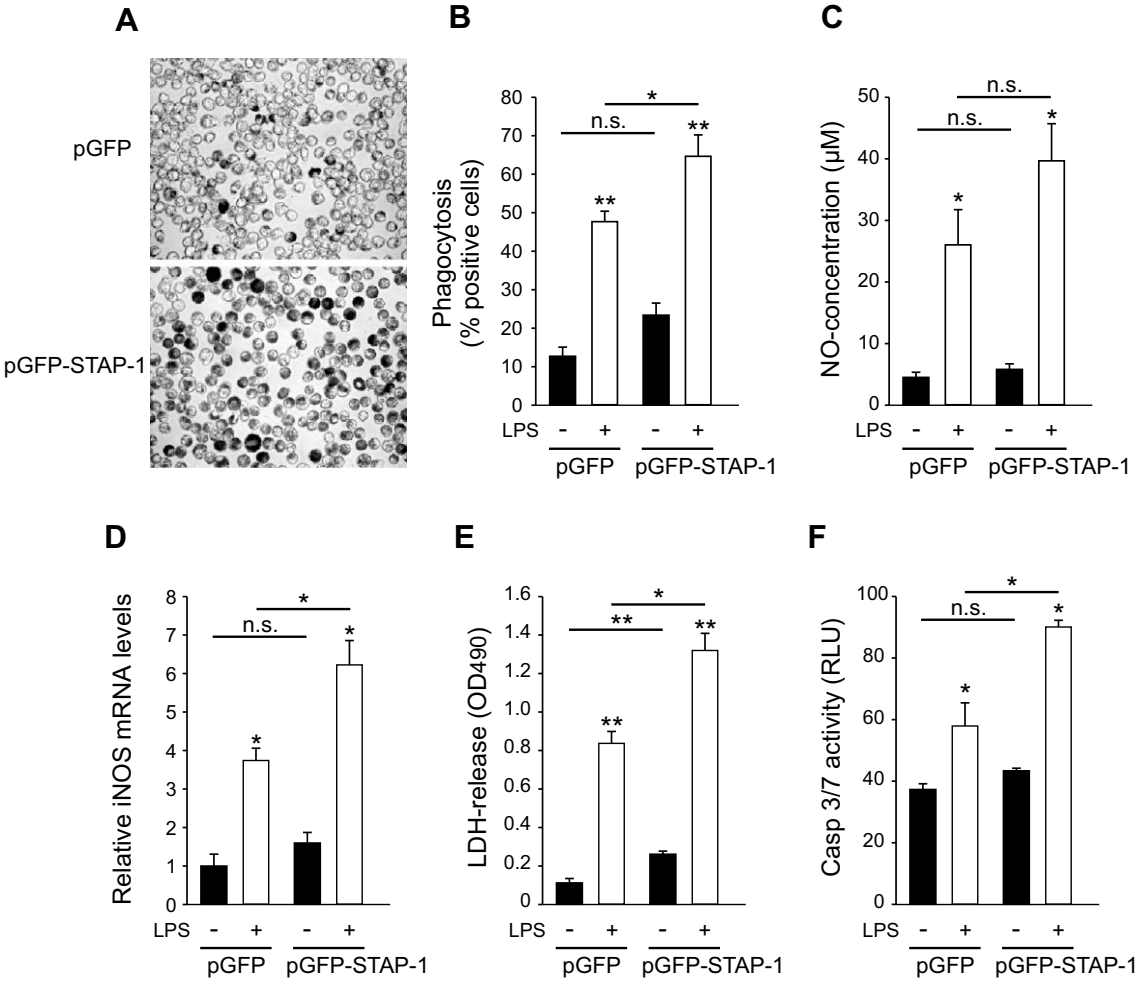

Fig. 4. STAP-1 expression increases phagocytosis, NO secretion and neurotoxicity. (A) BV-2 cells were incubated with Latex blue beads and analyzed for uptake by phase-contrast microscopy. Representative micrographs are shown for GFP and GFP-STAP-1 expressing cells. (B) The optical bead density was counted for up to 100 cells in six fields of view in the absence or presence of 50 ng/ml LPS. Three independent experiments were performed and the mean ± SEM of each phagocytosis index given as% positive cells is displayed. (C) NO release and (D) iNOS mRNA levels of control cells and GFP-STAP-1 cells in the absence or presence of 50 ng/ml LPS. (E) LDH release and (F) caspase 3/7 activation in 661W photoreceptor cells incubated with 10% cell culture supernatant of GFP and GFP-STAP-1 expressing cells treated without or with 50 ng/ml LPS. $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.

hematopoietic stem cells [11]. A high expression in immature bone marrow precursor cells and low expression of STAP-1 in non-hematopoietic tissues was detected, revealing that STAP-1 is a signaling molecule regulating stem-cell physiology [11]. Ohya et al. [12] and Yokohari et al. [15] showed that transcripts of the human STAP-1 ortholog BRDG1 are abundant in human B cells, and that the endogenous protein underwent tyrosine phosphorylation in response to B-cell receptor stimulation. BRDG1 likely functions as a docking protein acting downstream of Tec kinase in BCR signaling. Although these studies do not report expression of STAP-1/BRDG1 in myeloid cells such as microglia or macrophages, they also show a preferred expression of STAP-1 in hematopoietic cells. In agreement with these findings, we detected high levels of STAP-1 in microglia, macrophages and bone marrow, with minor amounts in spleen and thymus and spurious transcripts in liver and lung. We thus speculate that STAP-1 may have signaling functions in different cells of hematopoietic origin.

STAP-2, a protein closely related to STAP-1, was cloned as a c-Fms interacting protein in the liver using a yeast two-hybrid screening [16]. STAP-2 was strongly induced by LPS and interleukin 6 in isolated hepatocytes and STAP-2$^{-/-}$ hepatocytes displayed a diminished acute phase reaction [16]. Interestingly, STAP-2 enhances pro-inflammatory cytokine expression and NF-κB activation in peritoneal macrophages [17] and suppressed M-CSF-induced tyrosine phosphorylation of c-Fms in RAW264.7 cells [16]. Overexpression of STAP-2 in macrophage cell lines also reduced migration of the cells [16]. Ikeda et al. speculated that induction of STAP-2 by pro-inflammatory stimuli and the decreased migratory capacity might act to keep macrophages activated at inflammatory sites [16]. Interestingly, our data on the phenotype of STAP-1 expressing microglia coincide with these findings. We could not detect STAP-2 mRNA in primary retinal microglia or BV-2 cells (data not shown) and therefore suggest that STAP-1 may have similar functions as STAP-2 in the liver, constituting an adaptor protein-dependent activation pathway in microglia. We hypothesize that STAP-1 induction is an important event in microglia function by sustaining the rounded low-migratory cell morphology and activation state. STAP-1 expressing cells are highly phagocytic and cytotoxic. Thus, prolonged STAP-1 activation may be potentially harmful by contributing to neurotoxicity and apoptotic degeneration. Therefore, STAP-1 could be a potential candidate for therapeutic modulation of microglia activity. STAP-1-deficient mice will be a valuable tool to further study the *in vivo* role of this adaptor protein and its involvement in molecular mechanisms of microglia homeostasis.

Acknowledgments

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