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"Human Remyelination Promoting Antibody Inhibits Apoptotic Signaling and Differentiation Through Lyn Kinase in Primary Rat Oligodendrocytes"

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Abstract

Purpose—Human remyelination promoting IgM mAbs target oligodendrocytes (OLs) and function in animal models of multiple sclerosis (MS). However, their mechanism of action is unknown. This study seeks to identify the cellular mechanism of action of a recombinant human IgM on OL survival.

Methods—Binding of rHIgM22 to the surface of rat OLs was studied by co-localization with various markers. RHIgM22-mediated effects on apoptotic signaling in OLs, differentiation markers and signaling molecules were detected by Western blotting and immunoprecipitation.

Results—RHIgM22 co-localized with integrin β3 but not other integrin β-chains in OLs. Downstream of integrin β3 we identified Src family kinase (SFK) Lyn as a key player of rHIgM22-mediated actions in OLs. Lyn immunoprecipitated in a complex together with integrin αvβ3 and PDGFαR. Lyn expression was 9 fold up-regulated and Lyn activation was 3 fold higher in rHIgM22-treated OL cultures compared to controls. RHIgM22 inhibited apoptotic signaling by greater than 10 fold reduction of caspase-3 and capsase-9 cleavage and reduced by 4 fold expression of differentiation markers MBP and MOG in OLs. SFK inhibitors PP2 and SU6656 inhibited Lyn activity and restored caspase-cleavage in OLs. A human IgM that did not promote remyelination and medium were used as controls.

Conclusions—rHIgM22 prevented apoptotic signaling and inhibited OL differentiation by Lyn implying that IgM-mediated remyelination is due to protection of OPC and OLs rather than promotion of OPC differentiation.

Keywords

Demyelination; multiple sclerosis; fibronectin; Src family kinase

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Introduction

Demyelination in the central nervous system (CNS) is the hallmark in multiple sclerosis (MS), which is a primary inflammatory process leading to CNS damage and neurological deficits (Compston and Coles 2002; Noseworthy et al. 2000). Failure of MS lesions to remyelinate is a feature of the disease. Remyelination is mediated by OL precursor cells (OPCs), which are widely distributed throughout the adult CNS. However, it is still unclear whether 1) OPCs detectable in MS lesions survive the inflammatory response but fail to differentiate or 2) OPC and OL fail to survive and detectable OPCs enter demyelinated areas from adjacent tissue. Those OPCs not activated by the inflammatory milieu, remain quiescent and do not differentiate into myelinating OLs. Viable treatments may stimulate OPC differentiation or prevent OPC and OL apoptosis.

Promotion of remyelination by remyelination promoting human IgMs is an encouraging approach that stimulates an increase of remyelinated axons in animal models of MS (Asakura et al. 1996a; Asakura et al. 1996b; Miller et al. 1994; Pavelko et al. 1998; Warrington et al. 2000; Warrington et al. 2007). A recombinant form of a human IgM (rHIgM22) identified from a patient with Waldenström macroglobulinemia will soon enter Phase I clinical trials. The molecular mechanism by which remyelination promoting IgMs induce remyelination is unknown. A previous study showed anti-apoptotic signaling by rHIgM22 in CG4 cells, an OPC cell line, where lipid raft integrity had to be sustained for rHIgM22-mediated effects to occur (Howe et al. 2004).

Here we show that rHIgM22 strongly inhibits apoptotic signaling via reduction of caspase-3 and caspase-9 cleavage and reduces expression of differentiation markers MBP and MOG in OL cultures. We identified Lyn kinase as a key player in rHIgM22-mediated effects in OLs. rHIgM22 induced Lyn expression and activation and reduced c-Src and Fyn expression levels compared to an isotype control human IgM antibody. Src family kinase (SFK) inhibitors PP2 and SU6656 reduced SFK activity, including Lyn activity and restored apoptotic signaling in OLs, linking Lyn activation with rHIgM22-mediated inhibition of caspase-3 and caspase-9 activation. We also isolated integrin αvβ3 and PDGFαR in a complex together with Lyn kinase, suggesting that rHIgM22 acts through a signaling complex containing Lyn, integrin αvβ3 and PDGFαR in OLs.

Material and Methods

Antibodies

Recombinant monoclonal human IgM22 antibody (rHIgM22) (Warrington et al. 2000; Warrington et al. 2007) was produced and purified in our lab. ChromPure human IgM (isotype control) (#009-000-012, human monoclonal) was obtained from Jackson ImmunoResearch (West Grove, PA, USA). Anti-Lyn (sc-7274, mouse monoclonal), anti-PDGFαR (sc-338, rabbit polyclonal) and anti-integrin β8 (sc-25714, rabbit polyclonal) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); antibodies against integrin β3 (AB1932, rabbit polyclonal), integrin β5 (AB1926, rabbit polyclonal), Fyn (MAB8900, mouse monoclonal), myelin basic protein (MBP) (AB980, rabbit polyclonal) and secondary horseradish peroxidase-conjugated antibodies were supplied by Millipore (Temecula, CA, USA). Antibodies against integrin β3 (#4702, rabbit polyclonal), integrin αv (#4711, rabbit polyclonal), Src (#2108, rabbit polyclonal), pSrc (Tyr416) (#2101, rabbit polyclonal), Lyn (#2732, rabbit polyclonal), p44/42 MAP Kinase antibody (#9102, rabbit polyclonal), phospho-p44/42 MAP Kinase antibody (Thr202/Tyr204) (#9101, rabbit polyclonal), phospho-Akt (Ser473) (#9271, rabbit polyclonal), β-actin (#4967, rabbit polyclonal), cleaved caspase-8 (#9429, rabbit polyclonal), cleaved caspase-3 (#9661, rabbit polyclonal), cleaved caspase-9 (#9507, rabbit polyclonal) and

PARP (#9542, rabbit polyclonal) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Akt antibody (BD #610837, mouse monoclonal) was from BD Biosciences Pharmingen (San Diego, CA, USA) and directly labeled FITC anti-mouse/rat CD29 antibody (#102205, Armenian hamster monoclonal) was purchased from BioLegend (San Diego, CA, USA).

Chemicals

Growth factor reduced Matrigel (# 354230) and human plasma fibronectin (# 354008) were purchased from BD Biosciences Discovery Labware (Bedford, MA, USA). DMEM (# 10-017-CV), DMEM/F12 50:50 (# 10-090-CV), HBSS (# 21-022-CV), 0.25 % Trypsin (# 25-050-CV) and sodium pyruvate (# 25-000-Cl) were from Mediatech (Manassas, VA, USA); penicillin/streptomycin (# 15140) and N2-supplement (# 17502-048) were from Invitrogen (Carlsbad, CA, USA); fetal bovine serum (# SH30070.03) was from Hyclone (Waltham, MA, USA); sterile water (# 2F7113) was from Baxter (Deerfield, IL, USA); bovine serum albumin fraction V (# A-3294), Poly-D-lysine hydrochloride (average mol wt 30,000-70,000) (# P7280) and D-(+) glucose (# G5767) were from Sigma (St. Louis, MO, USA); bFGF (# 01-106) and AA-PDGF (# 01-309) were from Millipore (Temecula, CA, USA). SU6656 (#572636) and PP2 (#529576) were purchased from Calbiochem/EMD4Biosciences (San Diego, CA, USA). Enbrel (etanercept) was purchased from Amgen and Wyeth Pharmaceuticals (Thousand Oaks, CA, USA).

Animals

Pregnant Sprague dawley rats were purchased from Harlan Laboratories (Madison, WI, USA).

Cell Culture

Primary mixed glial cultures were prepared as described (Asakura et al. 1996b). In brief, brains were removed from newborn P0 to P1 Holtzman Sprague-Dawley rats, cerebral hemispheres were detached, meninges were removed, hemispheres were minced and transferred to Ca^{2+} - and Mg^{2+} -free HBSS containing 0.025 % trypsin. The tissue was incubated in trypsin solution for 10 min at 37 °C in a rotary shaking incubator at 200 rpm. The tissue was further dissociated by trituration through a sterile 10 ml pipette. The cell suspension was washed by centrifugation and plated on poly-D-lysine-coated flasks (25 μg/ml poly-D-lysine for 1 h at 37 °C and subsequently washed twice with water). These cultures were maintained for 10 days in DMEM supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate. Semi-purified OLs were prepared by shaking, using a version of the original protocol from *McCarthy and de Vellis* (McCarthy and de Vellis 1980). Cells were shaken initially for 1 h at 140 rpm to remove microglia, refed, and shaken again for 20 h at 37 °C at 200 rpm. Microglial and astrocytic contaminants were removed by plating the supernatant cell mixture twice onto non-tissue culture Petri dishes for 30 minutes. The cell suspension was centrifuged for 8 minutes at 850 rpm at 20 °C, resuspended and cultured for 1-9 days in DMEM:F12 (50:50) culture medium containing 0.1 % BSA, 10 ng/ml biotin, 10 ng/ml PDGF, 5 ng/ml FGF, 1 × N2, 100 U/ml penicillin, and 100 μg/ml streptomycin (proliferation medium). Acid washed glass coverslips or 60 mm cell culture dishes were pre-coated with 25 μg/ml poly-D-lysine for 3 h at 37 °C, washed twice with water and treated with a 1:50 dilution of growth factor reduced Matrigel (BD Biosciences) in DMEM overnight at 37 °C or alternatively only with 10 μg/ml human serum fibronectin (BD Biosciences) in sterile PBS overnight at 4 °C. Before use dishes or glass coverslips were rinsed once with PBS. Proliferation medium was changed every second day. After 9 days in culture dishes contained highly enriched populations of OLs (~90 %) with 8-10 % GFAP-positive astrocytes and 1-2 % CD11b-positive (Ox42) microglia.

Microglia was isolated by a low intensity shake off (1h, 120 rpm) of mixed glia. Highly enriched CD11b-positive (Ox42) microglial fractions (98 % pure) were plated on fibronectin coated 60 mm cell culture dishes and cultured for 1-7 days in proliferation medium. Purified GFAP-positive astrocytes were obtained by shaking off mixed glia cultures twice (20 h, 200 rpm). Astrocytic cell layers were trypsinized and re-plated on fibronectin coated 60 mm culture dishes and cultured for 1-7 days in proliferation medium (~95 % purity).

Fluorescence Microscopy Studies

Epifluorescence microscopy used an Olympus IX70 microscope equipped with a PE 94 coldstage (Linkam Scientific Instruments, Tadworth, Surrey, UK), a QuantEM 512SC CCD camera and a 60x 1.4 NA lens. Quantitation of images was performed using the MetaMorph image processing program (Molecular Devices, Sunnyvale, CA) as described (Sharma et al. 2005). All photomicrographs were exposed and processed identically for a given fluorophore. Primary antibodies were used at the following concentration: 10 μg/ml for rHIgM22, 10 μg/ml for isotype control HIgM, 20-30 μg/ml of anti-integrin β3 antibody (chemicon, AB 1932, 1:100 dilution), 20-30 μg/ml of anti-integrin β5 antibody (1:100 dilution), 2 μg/ml of anti-integrin β8 antibody (1:100 dilution) and 5 μg/ml of directly labeled FITC-integrin β1 antibody (1:10 dilution). For colocalization studies, no spillover-fluorescence between fluorophores Cy3 (rHIgM22, ChromPure isotype control IgM) and AF488 (anti-integrin β3, anti-integrin β5, anti-integrin β8) or FITC (anti-integrin β1) was observed.

Western blotting

Isolated OLs were incubated for 1-9 days with 5 μg/ml rHIgM22, 5 μg/ml isotype control HIgM or control medium in 60 mm dishes on fibronectin. Fresh IgM antibodies were added every second day and triplicates were used for each condition. Cells were washed three times with ice-cold Ca^{2+} - and Mg^{2+} -free HBSS and lysed on ice with RIPA buffer supplemented with 1 mM Na_3VO_4, 10 mM NaF, and a protease inhibitor mixture. Cells were scraped and transferred to microfuge tubes and incubated on ice. Cell lysates were homogenized in RIPA buffer by trituration through a 21-gauge needle and centrifuged at 14,000 rpm for 5 minutes at 4 °C to separate from detergent insoluble material. For Western blotting 10-20 μg protein per lane were analyzed as described (Asakura et al. 1996a). For quantitation 3-5 experiments each from independent OL cultures were used with subsequent densitometric analysis of Western blots. For statistical analysis the t-test function of the analysis software *Sigma Plot 9.0* was used.

Immunoprecipitation

Isolated OLs were incubated for 9 days with 5 μg/ml rHIgM22, 5 μg/ml isotype control HIgM or control medium in 60 mm dishes on fibronectin. Fresh IgM antibodies were added every second day and triplicates were used for each condition. For immunoprecipitation cells were washed with ice-cold Ca^{2+} - and Mg^{2+} -free HBSS and lysed in 0.15 M NaCl, 0.5 mM EDTA, 1 % Triton X-100, 0.05 % SDS, 10 mM NaF, and 1 mM Na_3VO_4, protease inhibitor mixture, 0.05 M Tris, pH 7.5 and 0.2 % bovine serum albumin on ice. Cells were scraped and transferred to microfuge tubes and incubated at 37 °C for 5 minutes. Lysates were centrifuged at 14,000 rpm for 5 minutes at 4 °C to separate detergent-insoluble and -soluble material. Supernatant was incubated with Protein G-Agarose (Sigma)/antibody complexes at 4 °C overnight with gentle mixing. Antibody-antigen complexes were washed two times with IP-buffer without bovine serum albumin and washed twice with tris-buffered saline, pH 7.5. Immunoprecipitates were eluted by boiling in SDS-sample buffer and analyzed by Western blotting. IP-results using anti-Lyn antibody (mouse) were normalized against Lyn levels detected with an anti-Lyn antibody from another species (rabbit). For

quantitation 3 experiments each from independent OL cultures were used with subsequent densitometric analysis of Western blots.

Results

RHIgM22 precisely co-localizes with integrin β3, but not with integrin β5 or β8 in OLs

The first goal was to identify the specific target for rHIgM22 on the cell surface of OLs that could be linked to intracellular signaling molecules important for remyelination. It has not been determined whether rHIgM22 binds to a lipid, a protein, a carbohydrate or to multiple different antigens at the plasma membrane (PM) of OLs. However, a previous study showed that rHIgM22 signaling was disrupted by cholesterol depletion, suggesting that the target of rHIgM22 may be associated with cholesterol-enriched lipid rafts (Howe et al. 2004). The fluorescence label intensity of rHIgM22 surface-staining increases with OL maturation (Warrington and Rodriguez 2008). Therefore co-localization studies of rHIgM22 and candidates were performed on cultures of mature (MBP-positive) rat OLs. RHIgM22 and other IgMs were added to live cells at 10 °C to prevent IgM endocytosis. Cells were then fixed and secondary antibodies were added. OLs, grown on laminin (Matrigel), were labeled with rHIgM22 and exhibited distinct punctate structures associated with cell processes observed by phase contrast microscopy (Fig. 1A, A'). Integrins are cell surface proteins that regulate migration, proliferation, differentiation, survival and myelination in OLs (Baron et al. 2005; Baron et al. 2003; Baron et al. 2002; Benninger et al. 2006; Chudakova et al. 2008; Colognato et al. 2002; Colognato et al. 2004; Decker et al. 2004). Many integrins have been shown to be associated with lipid rafts (Baron et al. 2003; Baron et al. 2002; Decker et al. 2004; Decker and ffrench-Constant 2004). We considered the possibility that rHIgM22 may bind to integrins on OLs. OLs express only a limited set of integrin receptors that bind to extracellular matrix (ECM) proteins: the αv integrins αvβ1, αvβ3, αvβ5 and αvβ8; and the laminin receptor α6β1 (Milner and Ffrench-Constant 1994; Milner et al. 1997).

We found a precise co-localization between rHIgM22 binding and integrin β3 binding in OLs (Fig. 1A-D: low magnification; **A'-D'**: high magnification). The co-localization between rHIgM22 and integrin β3 was observed on both live (not shown) and fixed cells (Fig. 1). Integrin β5 (Fig. 1E-H) or β8 (Fig. 1I-L) did not co-localize with rHIgM22. rHIgM22 partially (~60 %) co-localized with integrin β1 (Fig. 1M-P) in OLs. Although OLs at earlier stages of differentiation, microglia and astrocytes express the integrin β3, they were negative for rHIgM22 staining by immunofluorescence (data not shown).

In summary, we identified the vitronectin/fibronectin receptor αvβ3 as a potential target for rHIgM22 in OLs, but determined that this molecule alone is not sufficient for rHIgM22 binding.

RHIgM22 elevates phosphorylated Src family kinase (SFK) and phosphorylated ERK 1/2 levels and increases total Lyn-Kinase, but not c-Src or Fyn levels in OPCs/OLs

The immunocytochemistry data presented suggests that integrin αvβ3 may be a key player in rHIgM22 mediated signaling in OLs. The integrin αvβ3 ligands vitronectin and fibronectin modulate proliferation in a PDGF-integrin cooperative mechanism (Baron et al. 2002). We used fibronectin as a substrate for OLs and examined whether signaling pathways downstream of integrin αvβ3 are affected by rHIgM22. The Src family kinases (SFKs) are non-receptor tyrosine kinases that integrate external signals received through both integrin and growth factor receptors and were good potential candidates to transduce rHIgM22-mediated signals that modulate integrin and growth factor–driven phases of OL development. Fyn associates with integrin α6β1 in OLs and is required to amplify platelet-derived growth factor survival signaling and changes the response from proliferation to

differentiation (Colognato et al. 2004), whereas Lyn associates with integrin αvβ3 in OLs and mediates PDGF-integrin αvβ3-driven proliferation and promotes cell survival (Baron et al. 2002; Chudakova et al. 2008; Colognato et al. 2004).

We analyzed the phosphorylation status of SFKs and other integrin-associated signaling pathways (Colognato et al. 2004; Gu et al. 2003) and in a second step we analyzed expression levels of SFKs c-Src, Fyn and Lyn.

Isolated OLs grown on fibronectin were treated for 1-9 days with rHIgM22 or isotype control HIgM. At day 9 cultures contained ~90 % OPCs/OLs, 8-10 % GFAP-positive astrocytes and 1-2 % CD11b-positive (Ox42) microglia. Approximately 90 % of cells from the OL-lineage were positive for OPC marker A2B5, 40-50 % were O4-positive and ~10 % were positive for differentiation marker myelin basic protein (MBP) (suppl. Fig. 1). Regarding the specificity of our findings in OLs it is important that rHIgM22 binds specifically to OLs and myelin, but not to microglia, astrocytes, neurons, nor any other tested cell type or tissue. To rule out the possibility that contaminating microglia or astrocytes were responsible for rHIgM22-mediated effects in highly enriched OL cultures, all experiments were performed in isolated microglia and astrocytes (suppl. Fig. 2).

Treatment of OL cultures with RHIgM22 for 24 h at day 1 in culture was sufficient to induce a 2.3 fold increase in phospho-SFK(pY416) levels, a 2.0 fold increase in phospho-ERK1/2(pT202/pY204) levels and a 1.5 fold increase in Lyn expression levels compared to isotype control HIgM treated OLs (Fig. 2 A+B) (t-test, p-value = 0.01 for pERK1/2/ rHIgM22 vs pERK/isotype control HIgM; p-value = 0.006 for Lyn/rHIgM22 vs Lyn/isotype control HIgM). However, apoptotic signaling and expression of differentiation marker MBP was barely detectable before day 7 in culture. To strengthen rHIgM22-mediated effects we cultured OLs for 9 days with human IgMs. This ruled out potential toxic side effects of rHIgM22 on OLs when treated over many days.

At day 9 in culture RHIgM22 induced a 2.3 fold up-regulation of phosphorylated SFKs (pY416) and a 2.1 fold up-regulation of phosphorylated ERK1/2 (pT202/pY204) in OLs compared to isotype control HIgM (Fig. 2 C+D). Both changes were statistically significant (t-test, p-value = 0.04 for pSFKs and p-value = 0.04 for pERK1/2). Phosphorylation of Akt was not affected by the addition of rHIgM22 compared to control HIgM. Lyn expression was increased 9 fold in rHIgM22-treated OLs vs control HIgM treated cells, whereas c-Src and Fyn expression were reduced 2.8 fold (c-Src) and 3.2 fold (Fyn) (Fig. 2 C+D). These changes were statistically significant (t-test, p-value = 4.4e-4 for c-Src, p-value = 0.02 for Lyn, and p-value = 8.8e-3 for Fyn). These data suggested that Lyn is a key player in rHIgM22-mediated signaling in OLs. The isotype control HIgM had no significant effect on Lyn expression or SFK activation. We concluded that the IgM-mediated effects on Lyn expression and SFK phosphorylation were not Fc receptor-mediated and specific for the antigen binding part of the rHIgM22 molecule. RHIgM22 did not induce SFK(pY416) activation, Lyn expression or down-regulate c-Src expression levels in isolated microglia or isolated astrocytes (see also **Material and Methods**) (suppl. Fig. 2). We conclude that rHIgM22-mediated effects seen are specific for OPCs/OLs.

RHIgM22 elevates phospho-Lyn levels

RHIgM22 induced Lyn kinase expression, but reduced c-Src and Fyn expression (Fig. 2). It is possible that the observed total increase of phosphorylated SFKs in OLs is due to an increased amount of phosphorylated Lyn. Since no phosphorylation specific antibodies were available for Lyn (pY397), we immunoprecipitated Lyn kinase with an anti-Lyn antibody and blotted against anti-phospho-Src antibody (pY416). This antibody detects phosphorylated Y416 in c-Src as well as the corresponding phosphorylated epitope at Y397

in Lyn kinase. Immunoprecipitations (IPs) were performed from OLs treated for 9 days with rHIgM22, isotype control HIgM or medium.

When identical amounts of total protein from lysates were used for IPs with anti-Lyn antibody (mouse) we detected 3 fold higher Lyn levels in immunoprecipitates from samples pre-treated with rHIgM22 compared to control HIgM or medium after normalization to Lyn (rabbit) antibody. This was statistically significant (t-test, p-value = 0.001 for rHIgM22 vs control IgM; p-value = 0.001 for rHIgM22 vs medium) (Fig. 3). Phosphorylated Lyn (pY397) levels were 3 fold higher after normalization to total Lyn protein in rHIgM22-treated OLs compared to control human IgM or medium treated cells (t-test, p-value = 0.004 for rHIgM22 vs control IgM and p-value = 0.005 for rHIgM22 vs medium) (Fig. 3). Without normalization to the amount of immunoprecipitated Lyn, but normalized to the total amount of protein used for each IP, rHIgM22 increased pLyn (pY397) levels 11 fold compared to control HIgM and 14 fold compared to medium treated OLs (t-test, p-value = 0.001 for pLyn (pY397) in rHIgM22 vs control HIgM; p-value = 0.0008 for rHIgM22 vs medium). This is the most appropriate way to compare phosphorylated Lyn levels, because it reflects the total number of phosphorylated Lyn molecules compared to the protein amount used for the IPs, which was identical for all analyzed samples. In contrast to Lyn the SFK Fyn was not activated by rHIgM22 compared to the isotype control HIgM (suppl. Fig. 3). This indicates that Lyn phosphorylation is the cause for elevated pSFK levels (Fig. 2).

It was shown previously in OLs that Lyn associates specifically with integrin αvβ3, but not αvβ5 (Chudakova et al. 2008) or integrin α6β1 (Colognato et al. 2004). The integrin αvβ3 ligands vitronectin and fibronectin modulate proliferation in a PDGF-integrin cooperative mechanism (Baron et al. 2005; Baron et al. 2003; Baron et al. 2002), suggesting that cell surface molecules integrin αvβ3 and PDGFαR linked to Lyn kinase (Colognato et al. 2004), may play a role in rHIgM22-mediated signaling in OLs. Given the observed co-localization between rHIgM22 and integrin αvβ3 (Fig. 1) and the effects of rHIgM22 on Lyn expression and activation, we investigated the association between integrin αvβ3, PDGFαR and Lyn by immunoprecipitation. We found integrin αv, integrin β3 and PDGFαR co-immunoprecipitated in a complex together with Lyn kinase (Fig. 3), indicating at least a subfraction of total Lyn kinase is associated with integrin αvβ3 and PDGFαR. However, there were no increases in the association between integrin αvβ3 or PDGFαR with Lyn in cells treated with rHIgM22.

RHIgM22 strongly inhibits apoptotic signaling and differentiation in OL cultures

We have shown that rHIgM22 reduces apoptotic signaling in CG4 cells via caspase-3 activation and that lipid raft integrity was essential (Howe et al. 2004). However, it is unknown whether rHIgM22 modulates apoptotic signaling or differentiation in cultured primary OLs. When OLs were cultured on fibronectin with growth factors PDGF-AA and FGF-2 we detected a substantial amount of cleaved caspases (caspase-3 and caspase-9) at day 7 or later in culture. The molecular trigger for the apoptotic signaling was unclear. To exclude effector cells (e.g. activated microglia/macrophages) including death receptor signaling via TNFα as a cause of apoptotic signaling we analyzed levels of activated initiator caspase-8 and treated OLs from day 7-9 in culture with 10 μg of TNF-α blocker etanercept (Enbrel (R)) (suppl. Fig. 4). No substantial amounts of activated caspase-8 were detected and TNF-α blocker etanercept (Enbrel (R) had no effect on cleaved caspase-3 levels in OL cultures (suppl. Fig. 4). Together with low detectable levels of CD11b-positive cells it was unlikely that cell-extrinsic factors caused activation of caspase-3 and caspase-9 in OLs. We suggest that apoptotic signaling in OLs is a result of genotoxic apoptosis through aging of OL cultures under mitochondrial control with a central role of caspase-9, where Lyn plays an important role as a negative regulator when overexpressed in mammalian cells (Grishin et al. 2001; Kabuyama et al. 2002).

We focussed on day 9 in culture when levels of cleaved caspases as well as levels of differentiation markers MOG and MBP were highest compared to earlier time points. When grown with isotype control HIgM for 9 days, apoptotic signaling was induced in cultures as indicated by the extent of cleaved caspase-3, cleaved caspase-9 and PARP, a downstream target of many ICE-like proteases *in vitro* (Cohen 1997; Lazebnik et al. 1994) and one of the main downstream targets of caspase-3 *in vivo* (Nicholson et al. 1995; Tewari et al. 1995) (Fig. 4).

Compared to isotype control HIgM-treated OLs rHIgM22 reduced the amount of the cleaved caspase-3 fragment (19 kDa) 10 fold, the amount of cleaved PARP fragment (24 kDa) 3 fold and the amount of cleaved caspase-9 fragment (17 kDa) 14 fold (Fig.4). All differences were statistically significant (cleaved caspase-3: t-test, p-value = 0.001 for rHIgM22 vs control HIgM; cleaved PARP: t-test, p-value = 0.03 for rHIgM22 vs control HIgM; cleaved caspase-9: t-test, p-value = 0.002 for rHIgM22 vs control HIgM). Cleavage of capase-9 implies involvement of mitochondrial regulation of apoptosis in the culture system used.

To rule out that rHIgM22 rescues dying microglia or astrocytes we cultured isolated microglia and astrocytes on fibronectin under identical conditions as OLs. Although fibronectin activates microglia in culture (Milner et al., 2007), many isolated microglia started to die approximately 2-3 days after plating. RHIgM22 had no beneficial effect on survival of microglia in regards to caspase-3- and caspase-9-activation, but induced activation of both caspases in microglia compared to isotype control HIgM-treated cells (suppl. Fig. 2). No rHIgM22-mediated effects on caspase-3 and caspase-9 activation were observed in astrocytes compared to control HIgM (suppl. Fig. 2). This indicated that apoptotic signaling is mediated via cell-intrinsic pathways involving the mitochondrial system and that microglia are not involved in caspase-3 and caspase-9 activation in OLs.

For the detection of rHIgM22-mediated changes in OL differentiation we measured levels of the late differentiation markers myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). MOG and MBP were reduced 4.5 fold (MOG) and 3.8 fold (MBP) in rHIgM22-treated OLs compared to controls (MOG: t-test, p-value = 0.001 for rHIgM22 vs control HIgM; MBP: t-test, p-value = 0.002 for rHIgM22 vs control HIgM) (Fig. 4), indicating that rHIgM22 inhibits or delays OL differentiation.

In summary, rHIgM22 strongly inhibits apoptotic signaling in OLs and inhibits the expression of mature OL markers MBP and MOG.

Addition of the Lyn inhibitors PP2 and SU6656 to OL cell cultures restores cell apoptosis

To gain information about the involvement of SFKs in rHIgM22-mediated inhibition of apoptosis, OLs were cultured for 8 days in the presence of rHIgM22, isotype control HIgM or medium on fibronectin and then treated with Src family inhibitors, PP2 or SU6656, for 24 h. We show the effects of SFK inhibitors PP2 and SU6656 not only for the rHIgM22-treatment group but also for the isotype control IgM-treated OLs (IC) and the medium-treated OLs (MC) via Western blots (Fig. 5A+C) as well as their corresponding quantitative analysis (Fig. 5B+D). This confirmed previous results (Fig. 2-4) and demonstrated that effects of SFK inhibitors PP2 and SU6656 on OLs are independent of rHIgM22. PP2 and SU6656 are widely used as selective SFK inhibitors (Bain et al. 2003; Bain et al. 2007; Blake et al. 2000) and have been shown to be effective in many cell types including OLs (Chudakova et al. 2008; Contri et al. 2005; Golubovskaya et al. 2003; Griffiths et al. 2004; Park et al. 2004). PP2 (10 μM) and SU6656 (20 μM) significantly reduced rHIgM22-mediated phospho-SFK elevation 44 fold (PP2) and 7 fold (SU6656) compared to DMSO within 24 h (**for statistical significances see** table 1) (Fig. 5A+B). Lyn kinase phosphorylation (pY397) was reduced 18 fold by PP2 and 6 fold by SU6656 compared to

DMSO after normalization to total Lyn protein within the rHIgM22-treatment group (Fig. 5C+D, table 1). C-Src expression levels were increased 3-4 fold in PP2 and SU6656-treated samples compared to DMSO controls (Fig. 5A+B, table 1). Lyn expression levels in rHIgM22-treatment group were not affected by PP2 but significantly reduced by SU6656 (Fig. 5A+B). Therefore, reduction of phosphorylated SFK levels has no effect on Lyn expression. However, reduction of Lyn expression by SU6656 may be due to inhibition/ stimulation of signaling pathways different from SFKs in OLs.

The protective effects of rHIgM22 against apoptosis (i.e. caspase-3 and caspase-9 cleavage) were abolished in cells treated with PP2 or SU6656. Cleaved caspase-3 levels were up-regulated 15 fold (PP2) and 29 fold (SU6656) in rHIgM22-treated OLs compared to DMSO/ rHIgM22-treated cultures (Fig. 5A+B, table 1). PP2-treatment of control cultures increased cleaved caspase-3 levels 4.4 fold (control human IgM) and 4.2 fold (medium) compared to DMSO controls. SU6656-treatment increased cleaved caspase-3 levels 6.4 fold (control IgM) and 6.3 fold (medium) compared to DMSO controls (Fig. 5A+B, table 1).

PP2-and SU6656-treatment increased cleaved caspase-9 levels 12 fold (PP2) and 57 fold (SU6656) in rHIgM22-treated OLs compared to DMSO/rHIgM22-treated cultures (Fig. 5A +B, table 1). PP2-treatment in controls increased cleaved caspase-9 levels 7.5 fold (control IgM) and 5.1 fold (medium) compared to DMSO controls and SU6656-treatment increased cleaved caspase-9 levels 12.5 fold (control HIgM) and 9.6 fold (medium) compared to DMSO controls (Fig. 5A+B, table 1).

RHIgM22 protects OLs from apoptosis independent from Lyn kinase in the presence of SFK-inhibitors. RHIgM22 reduced cleaved caspase-3 levels in PP2-treated OLs 2.3 fold compared to controls and reduced caspase-3 levels 1.7 fold in SU6656-treated OLs compared to controls (Fig. 5A+B, table 1). RHIgM22 reduced cleaved caspase-9 levels in PP2-treated OLs 3.1-3.7 fold compared to controls, but did not reduce cleaved caspase-9 levels in SU6656-treated OLs compared to controls (Fig. 5A+B, table 1), suggesting that other survival pathways independent from Lyn are activated by rHIgM22-treatment (e.g. ERK1/2 activation). Apart from inactivating SFKs we observed different effects of PP2 and SU6656 on ERK1/2 phosphorylation and caspase cleavage. PP2 stimulated ERK1/2 activation compared to the SU6656 and DMSO treatment groups which correlates with a significant reduction in caspase-3 and caspase-9 cleavage compared to the SU6656 treatment group (Fig. 5A+B, table 1).

Confirming our previous data rHIgM22-treatment of OLs increased phosphorylated SFK levels 3.7 fold in DMSO controls compared to control human IgM and 4.0 fold compared to medium; rHIgM22-treatment increased Lyn expression 9 fold compared to control human IgM and 8.0 fold compared to medium and rHIgM22-treatment increased phosphorylated ERK1/2 expression 1.3 fold compared to control human IgM and 1.5 fold compared to medium. Cleaved caspase-3 levels were 8.0 fold reduced by rHIgM22-treatement compared to controls and cleaved caspase-9 levels were 5.5-6.0 fold reduced by rHIgM22-treatement compared to controls (Fig. 5A+B, table 1). All changes in expression or phosphorylation levels were statistically significant (table 1).

In summary, exposure of OLs to PP2 and SU6656 caused both the expected inhibition of rHIgM22-mediated elevated SFK activity as well as the inhibition of Lyn activity and restored OL apoptosis as measured by caspase-3 and caspase-9 cleavage (Fig. 5). These results emphasize the importance of SFK phosphorylation and in particular Lyn phosphorylation for rHIgM22-mediated reduction of apoptosis in OL cultures, where a reduction of Lyn activity correlates with an increase in apoptosis.

Discussion

Promotion of remyelination is a primary target in the treatment of demyelinating diseases such as multiple sclerosis. Remyelination promoting IgMs may be a novel class of therapeutics to enhance OL driven repair. The molecular mechanisms by which the IgM molecules promote remyelination are not understood. RHIgM22 has great potential to be a new therapeutic approach, because a single small dose of antibody was sufficient to induce extensive remyelination in vivo (Warrington et al. 2007; Warrington and Rodriguez 2008). The first aim of this study was to identify potential signaling pathways, including cell surface molecules, involved in rHIgM22-mediated signaling in OLs. The second aim was to determine whether apoptotic signaling in OLs and/or differentiation is affected by rHIgM22-treatment.

We show a precise co-localization between rHIgM22 and the fibronectin receptor in OLs, integrin αvβ3. This co-localization was detected for integrin β3, but not for integrin β5 or β8. We identified Lyn kinase, a downstream effector of integrin αvβ3, as a key player in rHIgM22-mediated signaling in OLs. RHIgM22 induced Lyn expression and Lyn activation in OLs. By immunoprecipitation, Lyn was “pulled down” in a complex together with integrin β3, integrin αv and PDGFαR, suggesting that rHIgM22 acts through a complex containing Lyn and the cell surface molecules integrin αvβ3 and PDGFαR. This supports the co-localization between rHIgM22 and integrin β3. Independent from Lyn activation or up-stream from Lyn activation is the rHIgM22-mediated activation of the mitogen-activated protein (MAP) kinases p44 and p42 (ERK1 and ERK2). ERK1 and ERK2 have been shown to bind directly or indirectly to PDGFα receptor (Heldin and Westermark 1999) and the ERK pathway is involved in the initial survival responses in OLs (Mronga et al. 2004).

RHIgM22 inhibits apoptotic signaling in OLs measured by caspase-3-, caspase-9-and PARP-cleavage and reduces expression of OL differentiation markers MOG and MBP. Inhibition of Lyn activation reduced the anti-apoptotic signaling of rHIgM22, linking rHIgM22-mediated Lyn activation to rHIgM22-induced inhibition of apoptotic signaling. RHIgM22 reduced caspase-3- and caspase-9-cleavage independent from Lyn in the presence of SFK inhibitors PP2 and SU6656, suggesting that ERK1 and ERK2 may be responsible for this effect. PP2 and SU6656 had different effects on ERK1 and ERK2 activation and increase in ERK1 and ERK2 phosphorylation by PP2 correlates with significant lower caspase-3 and caspase-9 cleavage compared to SU6656. Similar results on SFK inhibitors on Lyn kinase activity were obtained in B cells from patients with chronic lymphocytic leukemia (B-CLL) with strongly up-regulated Lyn levels, which were responsible for uncontrolled growth and resistance to apoptosis (Contri et al. 2005).

The link between Lyn activation and inhibition of caspase-3 and caspase-9 activation is still undetermined. Previous studies in OLs showed a correlation between Lyn activity and inactivation of acidic sphingomyelinase (ASMase) at the plasma membrane, suggesting an involvement of PKC δ as mediator between Lyn and ASMase (Chudakova et al. 2008). Inactivated ASMase in turn reduced levels of certain ceramide species responsible for apoptosis (Chudakova et al. 2008).

Growth factors PDGF-AA and FGF-2 can keep OPCs in a constant state of proliferation. OPC proliferation is intrinsically tied to OL differentiation, because OLs that are still in a proliferative state are unable to differentiate into myelinating OLs (Bogler et al. 1990; Canoll et al. 1996). Production of growth factors PDGF-AA and FGF-2 in mixed glia cultures is stimulated by different inflammatory cytokines (Lisak et al. 2006). The proliferative response of spinal cord OPCs to their major mitogen PDGF is enhanced by the astrocytic chemokine growth-regulated oncogene-α (GRO-α) (Robinson et al. 1998). We

speculate that rHIgM22 modulates growth factor PDGF-AA- and FGF-2-mediated inhibition of differentiation by keeping OPCs in a proliferative state rather than directly inhibiting OL maturation.

RHIgM22 may help OPCs and potentially mature OLs to survive the inflammatory burst in acute MS lesions with high levels of cytotoxic mediators (e.g. TNFα, interferon γ) as part of the immune response, which may last many hours till days *in vivo*. In the *in vivo* experiments to enhance remyelination, rHIgM22 was given as a single bolus injection (Warrington et al. 2007). The half-life of rHIgM22 in mice is approximately 15 h (Warrington et al., 2007). The human IgM serum concentration was close to zero 48 h after rHIgM22 administration in mice (Warrington et al., 2007). Within this 48 h rHIgM22 may increase OPC/OL survival and at the same time increase PDGF-mediated inhibition of OPC/OL maturation. Lack of OPC/OL maturation into myelinating OLs is one possible explanation for failure of remyelination in MS lesions (Franklin and Ffrench-Constant 2008). However, the inflammatory milieu is important to activate silent OPCs and therefore trigger OPC maturation (Blakemore et al., 2008), which is possible only when OPCs survive the inflammatory burst. Forty eight h after injection rHIgM22 is degraded and surviving OPCs can start to differentiate into myelinating OLs.

There is an ongoing debate in the MS field about the mechanism for failure of remyelination in MS lesions. It is unclear whether OPC death due to a hostile, non-supporting environment is the primary reason why MS lesions fail to remyelinate (Blakemore 2008) or whether the inhibition of OPC differentiation into myelinating OLs is the major reason for remyelination failure (Franklin and Ffrench-Constant 2008). Our observation using remyelination promoting IgMs on OLs support the importance of apoptosis prevention, but does not support the stimulation of OL differentiation to promote remyelination. Therefore, we cautiously suggest that the failure of remyelination is due to OPC and OL death precipitated by the destructive inflammatory response from activated microglia and macrophages, rather than an inhibition of OPC differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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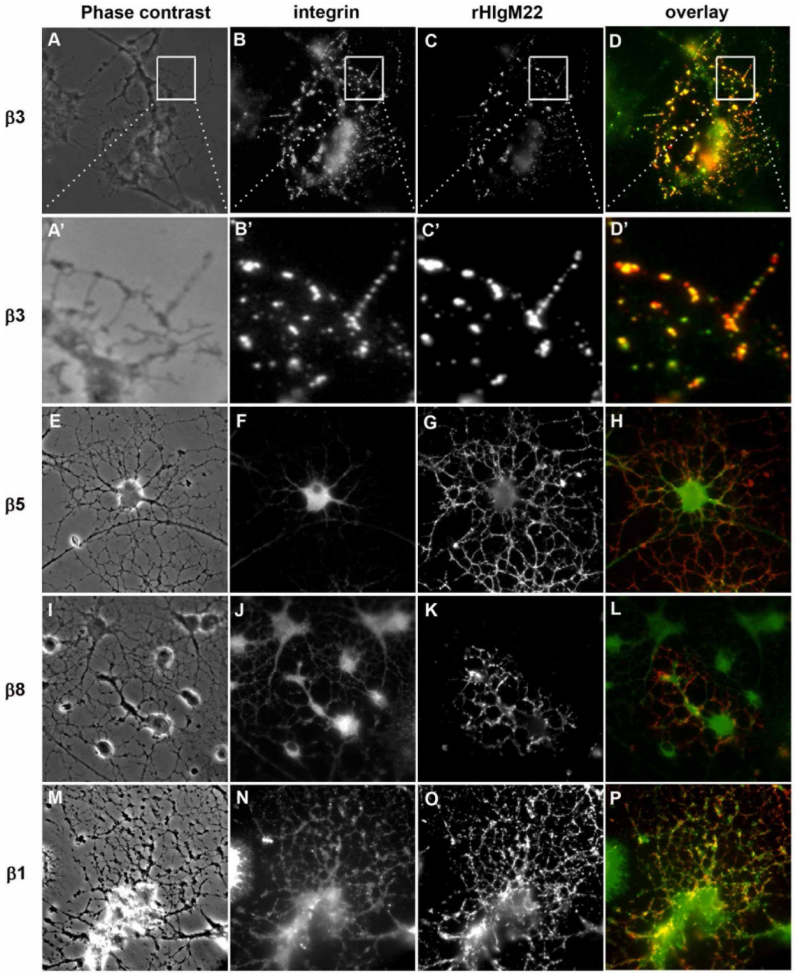
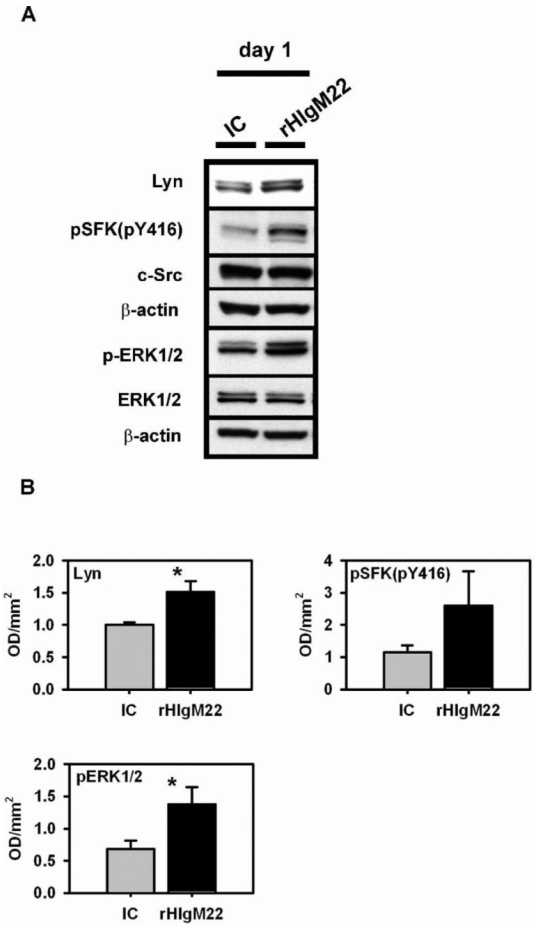


Figure 1. RHIgM22 co-localizes with integrin β3 but not β5 or β8 in OLs

OLs grown on laminin (Matrigel) were cultured for 9 days in defined media, pre-labeled with rHIgM22 (10μg/ml) for 20 minutes in HMEM at 10 °C and then stained with anti-integrin antibodies for another 30 minutes at 10 °C. Subsequently cells were fixed and processed for immunofluorescence. **A-D**: Representative images of co-staining of rHIgM22 and integrin β3; **A'-D'**: magnification of the boxed area in A-D; **E-H:** RHIgM22 and integrin β5; **I-L**: RHIgM22 and integrin β8; **M-P**: RHIgM22 and integrin β1. Phase contrast images are presented in **A, A', E, I, M**. Overlay images display integrin staining in green and rHIgM22 in red. Results were repeated 5 times leading to identical results.



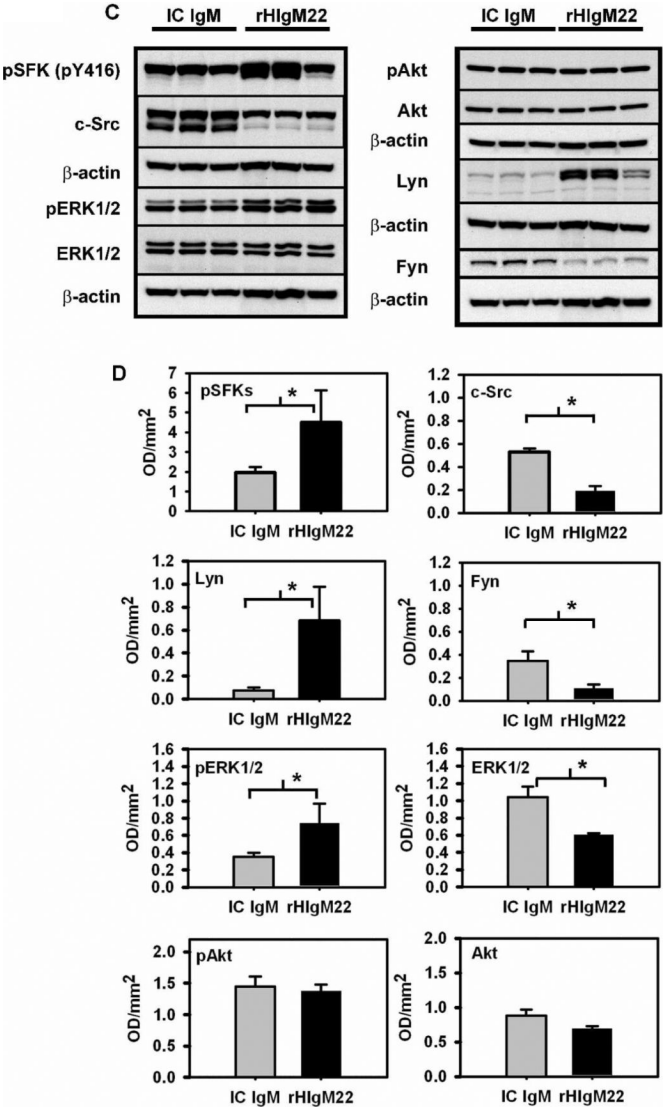


Figure 2. RHIgM22 stimulates SFK and ERK 1/2 activation and strongly induces Lyn but not c-Src or Fyn expression

Western blot analysis of OLs grown on fibronectin for 1 day (**A+B**) or 9 days (**C+D**) in the presence of rHIgM22 (5 μg/ml) or isotype control HIgM (5 μg/ml). **A**. Representative blots showing the levels of phospho-SFKs (pY416), phospho-p44/42 MAP Kinase (pT202/pY204) (pERK1/2), Lyn, Src, p44/42 MAP Kinase (ERK1/2) and β-actin from OLs cultured for 24 h in the presence of IgMs. **B:** Quantitative analysis of 3 independent experiments as in A. Background is subtracted from each value, normalized against β-actin levels or against the non-phosphorylated isoform of the protein. Data are presented as mean ± S.D. (n = 3-5). * $p < 0.05$ compared to controls. IC: isotype control HIgM. **C, D**: 3 separate samples from independent OL preparations are shown for each condition. **C:** Representative blots showing the levels of phospho-SFKs (pY416), phospho-Akt (pS473), phospho-p44/42 MAP Kinase (pT202/pY204) (pERK1/2), Lyn, Fyn, Src, Akt, p44/42 MAP Kinase (ERK1/2) and β-actin. **D:** Quantitative analysis of 3 independent experiments as in C. Background is subtracted from each value, normalized against β-actin levels or against the non-phosphorylated isoform of the protein. Data are presented as mean ± S.D. (n = 3-5). * $p < 0.05$ compared to controls. IC IgM: isotype control HIgM.

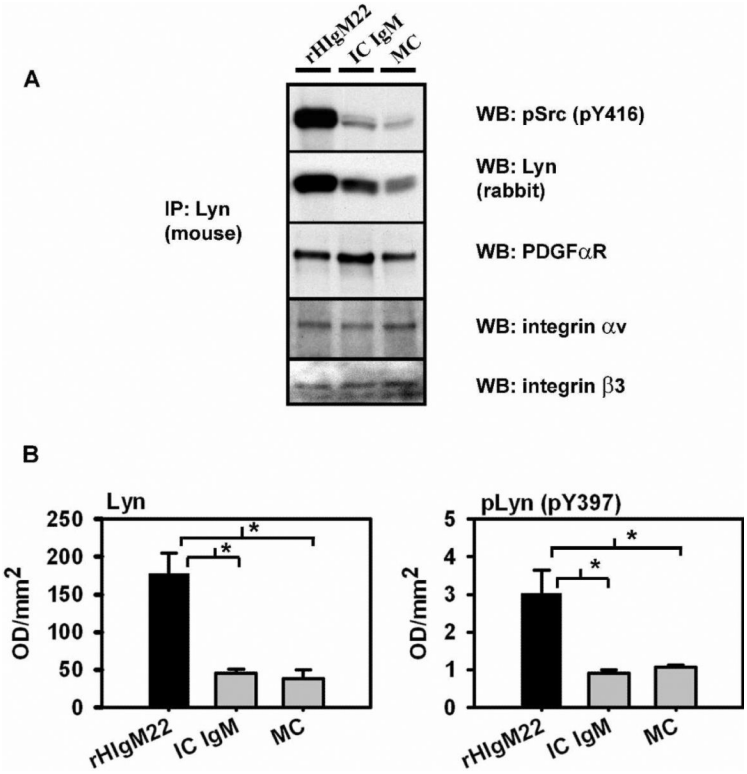


Figure 3. RHIgM22 induces Lyn phosphorylation in OLs

Activation of Lyn was detected by immunoprecipitation (*IP*) using anti-Lyn (mouse) antibody and immunoblotting with a phospho-SFK (pY416) antibody recognizing phosphorylation of Y397 in Lyn. **A:** Representative blots showing Lyn phosphorylation, total Lyn (rabbit) levels, co-precipitated integrin αv and co-precipitated integrin β3 in a complex with Lyn from OLs grown on fibronectin for 9 days with rHIgM22 (5 μg/ml), isotype control HIgM (IC IgM) (5 μg/ml) or medium (MC). **B:** Quantitative analysis of 3 independent experiments as described above. Background is subtracted from each value, normalized against total Lyn (rabbit) (pLyn) or against total protein used for each IP (Lyn). Data are presented as mean ± S.D. ($n = 3$). * $p < 0.05$ compared to controls.

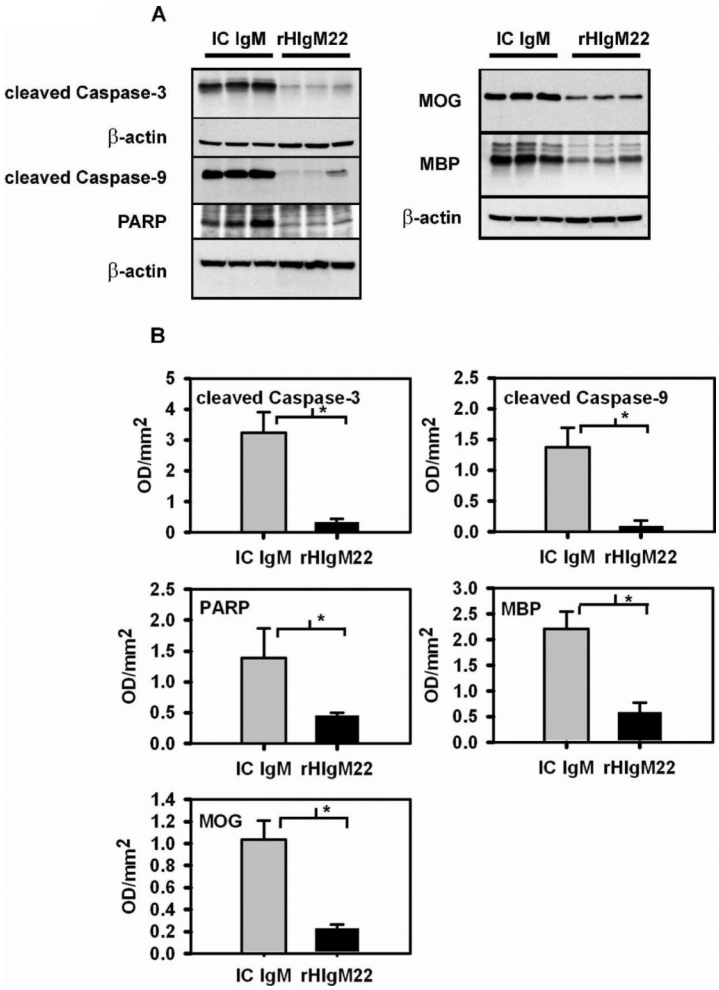
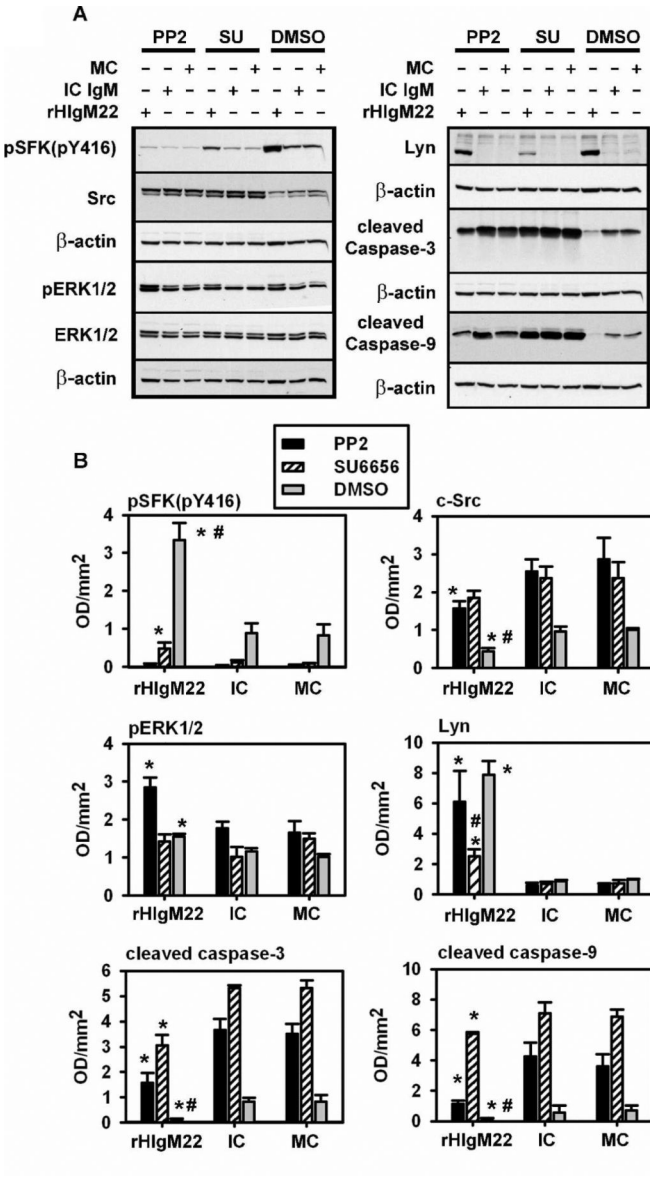


Figure 4. RHIgM22 inhibits apoptotic signaling and differentiation in OLs

A: Representative Western blots of cleaved caspase-3 (19 kDa fragment), cleaved caspase-9 (17 kDa fragment), cleaved PARP (24 kDa fragment), MOG, MBP and β-actin from OLs cultured for 9 days on fibronectin with rHIgM22 (5 μg/ml) or isotype control HIgM (IC IgM) (5 μg/ml). 3 separate samples from independent OL preparations are shown for each condition. **B:** Quantitative analysis from 3 independent experiments under the conditions described above. Background is subtracted from each value and normalized against β-actin. Data are presented as mean ± S.D. ($n = 3$). * $p < 0.05$ compared to controls.



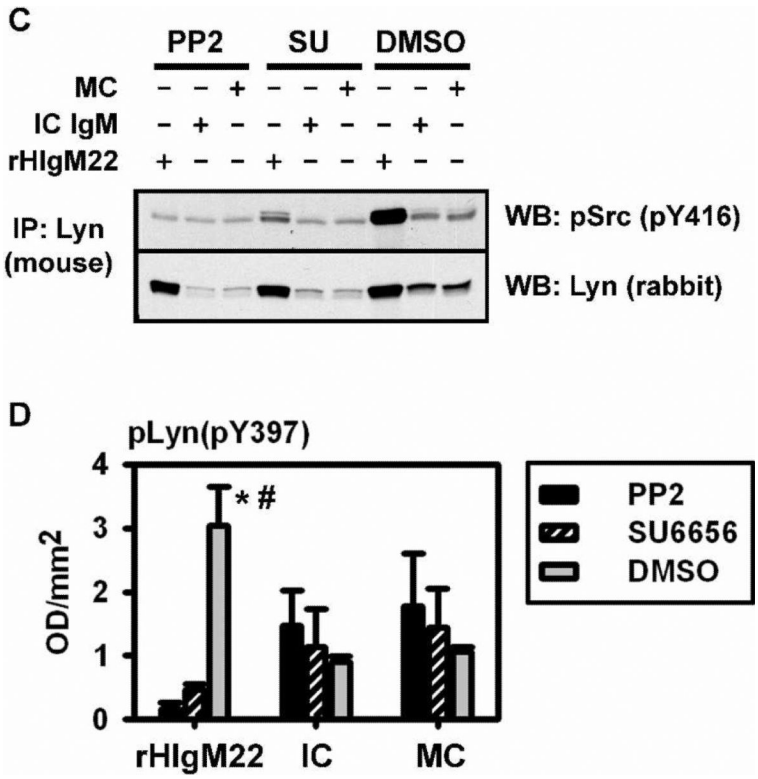


Figure 5. SFK inhibitors PP2 and SU6656 inhibit Lyn and SFK activation and restore apoptosis in rHIgM22-treated OLs

A. Western blot analysis of OLs grown on fibronectin for 9 days in the presence of rHIgM22 (5 μg/ml), isotype control human IgM (IC IgM) (5 μg/ml) or medium (MC) treated for 24 h with PP2 (10 μM), SU6656 (20 μM) or DMSO. **A:** Representative blots showing the levels of phospho-SFKs (pY416), phospho-p44/42 MAP Kinase (pT202/pY204) (pERK1/2), Lyn, Src, p44/42 MAP Kinase (ERK1/2), cleaved caspase-3 (19 kDa fragment), cleaved caspase-9 (17 kDa fragment) and β-actin. **B:** Quantitative analysis from 3 independent experiments as in **A**. Background is subtracted from each value, normalized against β-actin levels or against the non-phosphorylated isoform of the protein. **C:** Immunoprecipitations from samples in **A** with anti-Lyn antibody (mouse) and subsequent Western blots showing the levels of phosphorylated Lyn (pY397) by using anti-pSrc (pY416) antibody and total Lyn levels by using anti-Lyn (rabbit) antibody. **D:** Quantitative analysis from 3 independent experiments. Background is subtracted from each value and normalized against total Lyn protein. Data are presented as mean ± S.D. ($n = 3$). * $p < 0.05$ compared to controls in the same treatment group (e.g. PP2/rHIgM22 vs PP2/IC IgM). # $p < 0.05$ compared to rHIgM22-treated samples in different treatment groups (e.g. DMSO/rHIgM22 vs PP2/rHIgM22). IC IgM: isotype control HIgM.

Table 1

(for Figure 5 only)

Molecules of interest	Treatment groups	Detectable change (x-fold versus control)	Statistical significance (t-test, p-value)
pSFK(pY416)	PP2/rHIgM22 vs DMSO/rHIgM22	44 fold decrease (PP2)	0.0002
pSFK(pY416)	SU6656/rHIgM22 vs DMSO/rHIgM22	7 fold decrease (SU6656)	0.0005
pSFK(pY416)	DMSO/rHIgM22 vs DMSO/isotype control IgM	3.7 fold increase (rHIgM22)	0.001
pSFK(pY416)	DMSO/rHIgM22 vs DMSO/medium control	4 fold increase (rHIgM22)	0.001
pLyn(pY397)	PP2/rHIgM22 vs DMSO/rHIgM22	18 fold decrease (PP2)	0.001
pLyn(pY397)	SU6656/rHIgM22 vs DMSO/rHIgM22	6 fold decrease (PP2)	0.002
Lyn	DMSO/rHIgM22 vs DMSO/isotype control IgM	9 fold increase (rHIgM22)	0.0002
Lyn	DMSO/rHIgM22 vs DMSO/medium control	8 fold increase (rHIgM22)	0.0002
pERK1/2	DMSO/rHIgM22 vs DMSO/isotype control IgM	1.3 fold increase (rHIgM22)	0.004
pERK1/2	DMSO/rHIgM22 vs DMSO/medium control	1.5 fold increase (rHIgM22)	0.001
c-Src	PP2/rHIgM22 vs DMSO/rHIgM22	3.5 fold increase (PP2)	0.0007
c-Src	SU6656/rHIgM22 vs DMSO/rHIgM22	4.2 fold increase (SU6656)	0.0003
cleaved caspase-3	PP2/rHIgM22 vs DMSO/rHIgM22	15 fold increase (PP2)	0.003
cleaved caspase-3	SU6656/rHIgM22 vs DMSO/rHIgM22	29 fold increase (SU6656)	0.0002
cleaved caspase-3	PP2/isotype control IgM vs DMSO/isotype control IgM	4.4 fold increase (PP2)	0.0005
cleaved caspase-3	Su6656/isotype control IgM vs DMSO/isotype control IgM	4.2 fold increase (SU6656)	0.000002
cleaved caspase-3	PP2/medium control vs DMSO/medium control	4.2 fold increase (PP2)	0.0007
cleaved caspase-3	SU6656/medium control vs DMSO/medium control	6.3 fold increase (SU6656)	0.00004
cleaved caspase-3	DMSO/rHIgM22 vs DMSO/isotype control IgM	8 fold decrease (rHIgM22)	0.001
cleaved caspase-3	DMSO/rHIgM22 vs DMSO/medium control	8 fold decrease (rHIgM22)	0.008
cleaved caspase-3	PP2/rHIgM22 vs PP2/ isotype control IgM	2.3 fold decrease (rHIgM22)	0.004
cleaved caspase-3	PP2/rHIgM22 vs PP2/medium control	2.2 fold decrease (rHIgM22)	0.004
cleaved caspase-3	SU6656/rHIgM22 vs SU6656/isotype control IgM	1.7 fold decrease (rHIgM22)	0.001
cleaved caspase-3	SU6656/rHIgM22 vs SU6656/medium control	1.7 fold decrease (rHIgM22)	0.001
cleaved caspase-9	PP2/rHIgM22 vs DMSO/rHIgM22	12 fold increase (PP2)	0.001
cleaved caspase-9	SU6656/rHIgM22 vs DMSO/rHIgM22	57 fold increase (SU6656)	0.0000002
cleaved caspase-9	PP2/isotype control IgM vs DMSO/isotype control IgM	7.5 fold increase (PP2)	0.003
cleaved caspase-9	Su6656/isotype control IgM vs DMSO/isotype control IgM	12.5 fold increase (SU6656)	0.0002
cleaved caspase-9	PP2/medium control vs DMSO/medium control	5.1 fold increase (PP2)	0.005
cleaved caspase-9	SU6656/medium control vs DMSO/medium control	9.6 fold increase (SU6656)	0.00005
cleaved caspase-9	DMSO/rHIgM22 vs DMSO/isotype control IgM	5.5 fold decrease (rHIgM22)	0.003
cleaved caspase-9	DMSO/rHIgM22 vs DMSO/medium control	6 fold decrease (rHIgM22)	0.0002
cleaved caspase-9	PP2/rHIgM22 vs PP2/ isotype control IgM	3.7 fold decrease (rHIgM22)	0.005
cleaved caspase-9	PP2/rHIgM22 vs PP2/ medium control	3.1 fold decrease (rHIgM22)	0.007
cleaved caspase-9	SU6656/rHIgM22 vs SU6656/isotype control IgM	1.2 fold decrease (rHIgM22)	0.03
cleaved caspase-9	SU6656/rHIgM22 vs SU6656/medium control	1.2 fold decrease (rHIgM22)	0.02