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Anti-LRP/LR specific antibody IgG1-iS18 and knock-down of LRP/LR by shRNAs rescue cells from $A\beta_{42}$ induced cytotoxicity

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Alzheimer's disease (AD) is characterized by neurofibrillary tangles, senile plaques and neuronal loss. Amyloid beta (Aβ) is proposed to elicit neuronal loss through cell surface receptors. As Aβ shares common binding partners with the 37 kDa/67 kDa laminin receptor (LRP/LR), we investigated whether these proteins interact and the pathological significance of this association. An LRP/LR-$A\beta_{42}$ interaction was assessed by immunofluorescence microscopy and pull down assays. The cell biological effects were investigated by 3-(4,5-Dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide and Bromodeoxyuridine assays. LRP/LR and $A\beta_{42}$ co-localised on the cell surface and formed immobilized complexes suggesting an interaction. Antibody blockade by IgG1-iS18 and shRNA mediated down regulation of LRP/LR significantly enhanced cell viability and proliferation in cells co-treated with $A\beta_{42}$ when compared to cells incubated with $A\beta_{42}$ only. Results suggest that LRP/LR is implicated in $A\beta_{42}$ mediated cytotoxicity and that anti-LRP/LR specific antibodies and shRNAs may serve as potential therapeutic tools for AD.

Neurodegenerative diseases represent the fourth major cause of global mortality after ischaemic heart disease, cerebrovascular disease and trachea, bronchus and lung cancers. Alzheimer's Disease (AD) is the predominant progressive dementing neurodegenerative disorder afflicting the elderly[1] and is characterized by "positive" and "negative" lesions including amyloid beta plaques, neurofibrillary tangles and neuronal, neuropil and synaptic loss respectively[2,3]. Many of the neuronal perturbations in AD are attributable to and probably induced by the amyloid beta (Aβ) peptide[2]. The Aβ fragment is derived from the transmembrane region of the Amyloid Precursor Protein (APP). Although Aβ is a normal physiological peptide, elevated concentrations of the peptide, which consequently results in the onslaught of AD, are generated either through the misappropriate favouring of the amyloidogenic processing of APP or a decline in Aβ clearance or degradation[4]. The amyloid plaques are predominantly composed of the $A\beta_{42}$ isoform which has a higher aggregation propensity[5] and neural toxicity[6] than the 40 amino acid isoform ($A\beta_{40}$) which predominates in non-diseased brains. However, the prevailing sentiment is that the plaques themselves are not the pathological agents but rather contribute to neural dysfunction through the distortion of neuronal morphology (within a 50 μm radius[7,8]) and by hampering neurotransmission[9]. Rather, it is the soluble Aβ oligomers which are deemed neurotoxic.

The proposed mechanisms whereby Aβ has been reported to impair neuronal function are numerous. A common thread in Aβ induced cytotoxicity and neuronal dysfunction is the requirement for an interaction between the neurotoxic peptide and cellular components, of greatest importance are the lipid membranes and cellular receptors[10].

Owing to the hydrophobic nature of the peptide, Aβ may readily associate with and be subsequently incorporated into plasma[11,12], nucleosomal and lysosomal membranes. This may result in membrane structure distortion and the formation of ion-permissible (of particular concern is Ca^{2+}) channels, the resultant ion influx may induce cytotoxicity[13,14].

Several of the factors thought to contribute to AD, namely oxidative stress, protein degradation, lipid oxidation and slowed signal transmission may be attributed to Aβ interaction with cell surface receptors[15–17]. These include, but are not limited to, N-methyl-D-aspartate receptors (NMDAR), integrins (particularly $\alpha_5\beta_1$), insulin receptors, α-7 nicotinic acetylcholine receptors (α7nAChR), the receptor for advanced glycation end products (RAGE), Ephrin-type B2 receptor (EphB2) and the cellular prion protein (PrP^c)[1,10]. Aβ may thwart NMAR activation and the resultant induction of long term potentiation (LTP) by desensitizing the receptor to synaptic glutamate[10,18] or by prompting receptor internalization[10]. This in turn results in aberrant signaling cascades and ultimately results in synaptic dysfunction and neuronal death.

Although the association between Aβ and PrP^c has been one of mounting interest over the past decade, its biological influence remains to be definitively characterized. It has been suggested that PrP^c plays a role in mediating the devastating effects of Aβ oligomers particularly neuronal and synaptic toxicity and LTP impedance[19] as well as stimulating pro-apoptotic signal transduction cascades[20]. On the contrary a neuroprotective role for PrP^c has been proposed as the protein was reported to hinder β-secretase cleavage of APP[21].

A receptor of noted physiological importance which binds to PrP^c and is implicated in PrP^c internalization is the 37 kDa/67 kDa laminin receptor (LRP/LR)[22]. This multifunctional protein is located in multiple cellular compartments namely the nucleus, cytosol and within the lipid raft domains of the plasma membrane[23,24]. LRP/LR exhibits binding affinities for a multitude of cellular components including: extracellular matrix (ECM) molecules, laminin-1 being of greatest physiological relevance with regard to cellular adhesion, survival and migration as well as cytoskeletal, ribosomal and histone proteins and PrP^c [23,24]. LRP/LR is also of pathological importance as the receptor has been shown to be central in prion protein uptake, propagation and progression of prion disorders[25–27]. Furthermore, LRP/LR plays a central role in metastatic cancer and antibodies targeting the receptor have been reported to significantly impede adhesion and invasion of numerous cancer types, namely fibrosarcoma[28], lung, cervical, colon, prostate[29], breast and oesophageal cancer[30] as well as inhibit *in vitro* angiogenesis[31].

As Aβ toxicity has been posited to be mediated through its association with the lipid raft region of the plasma membrane and its interactions with plasma membrane anchored proteins, and LRP/LR shares mutual binding partners with Aβ (laminin[32] and PrP^c), we aimed to examine whether LRP/LR and Aβ interact on the cell surface and to investigate whether LRP/LR plays a central role in Aβ induced cytotoxicity.

Results

LRP/LR co-localises with Aβ on the cell surface. Indirect immunofluorescence is regularly employed to provide a preliminarily indication of potential interactions at the cell surface[33,34]. Here too this methodology was employed to investigate whether endogenous LRP/LR and Aβ are located in close proximity on the cell surface, which would thereby indicate that an association between these proteins is conceivable. Co-localization of LRP/LR and Aβ was observed on the surface of non-permeabilized HEK293 and N2a cells (Fig. 1c and Fig. 1o, respectively). 2D cytofluorograms represent both green and red fluorescence and the resultant yellow diagonal (Fig. 1d and Fig. 1p) reveals that the fluorescence from both proteins is jointly distributed. These images, in addition to the highly positive Pearson's correlation coefficient (Table 1), verify that LRP/LR and Aβ co-localize on the cell surface. LRP/LR did not co-localize with the Very Late Antigen 6 (VLA6), a laminin binding integrin, (Fig. 1g and Fig. 1s). This was indicated by the 2D-cytofluorogram (Fig. 1h and Fig. 1t) as well as the very low Pearson's correlation coefficient (Table 1).VLA6 thereby served as the negative control[25]. Therefore, owing to the cell surface proximity of LRP/LR and Aβ, an association between these proteins is feasible.

Interaction of Aβ with LRP/LR. Although co-localisation studies between LRP/LR and Aβ proved the proximity of the proteins on the cell surface, this finding merely indicates that an interaction between these proteins is feasible. Therefore a pull down assay was performed to investigate definitively whether a stable interaction exists. Recombinantly expressed LRP::FLAG was immobilized on the anti-FLAG® M2 agarose beads, as highlighted by the red arrow (Fig. 2a and 2e) as it is present in the eluted sample. The identity of the band was further authenticated by immunoblotting (Fig. 2b). Co-incubation of anti-FLAG® M2 beads with LRP::FLAG containing cell lysate to which 100 ng/ml of synthetic $A\beta_{42}$ was applied resulted in the immobilization of both proteins. The presence of $A\beta_{42}$ in the eluted sample (Fig. 2a) was confirmed by equivalent polypeptide position in Fig. 2a lane 6 containing pure, synthetic $A\beta_{42}$ (2 μg). The presence of both proteins in eluted samples (Fig. 2a - lane 5) implies that an association exists. The relevant controls are shown in Fig. 2c–f.

IgG1-iS18 rescues cells from Aβ mediated cytotoxicity. A MTT cell viability assay was employed to assess the cytotoxicity of synthetic amyloid beta ($A\beta_{42}$) at various concentrations on HEK293FT, N2a and SHSY5Y cells (Fig. 3 a–c). Exogenous application of 200 nM and 500 nM $A\beta_{42}$ significantly reduced cell viability in HEK293 cells (Fig. 3a). Co-incubation of cells with 50 μg/ml anti-LRP/LR specific antibody IgG1-iS18 and 500 nM $A\beta_{42}$ significantly enhanced cell viability (Fig. 3a). Similar results, albeit at different $A\beta_{42}$ concentrations were observed for SH-SY5Y (Fig. 3b) and N2A (Fig. 3c) cells. The decrease in cell viability observed in N2a cells (Fig. 3c) was shown to be as a result of hampered cellular proliferation (Fig. 3d). Protocatechuic acid (PCA) an apoptosis inducing agent was employed, at a concentration of 8 mM, as the positive control. Antibody, IgG1-iS18, treatment alone in the absence of Aβ does not significantly enhance cellular viability in all the model cell lines employed (Fig. S1), thereby negating the possibility that IgG1-iS18 non-specifically enhances cellular viability.

To confirm that LRP/LR plays a role in Aβ toxicity, and that the IgG1-iS18 effects observed are not owing to the possible lack of antibody specificity, RNA interference technology and more specifically short hairpin RNAs (shRNAs) were employed to down regulate LRP/LR. When compared to the shRNAscr control, shRNA1.1 transfection resulted in a 20.52% reduction in LRP/LR expression, whilst shRNA7.6 transfection produced a significant 67.46% reduction in LRP/LR expression levels (Fig. 4a and 4b). LRP/LR down regulation (mediated by the aforementioned shRNAs), in the presence of varying concentrations of exogenously administered $A\beta_{42}$, resulted in a significant enhancement in cell viability (Fig. 4c) and cellular proliferation (Fig. 4d). These results are analogous to those obtained employing IgG1-iS18. No significant difference amongst untreated, mock transfected and shRNAscr transfected cells HEK293 cells was observed with regards to both cellular viability (Fig. S2a) and proliferation (Fig. S2b).

Discussion

LRP/LR and Aβ were demonstrated to share close cell surface proximity by indirect immunofluorescence microscopy (Fig. 1c and Fig. 1o) and these results were considered as a primary indication of a potential interaction between these proteins on the cell surface. However, supplementary systems are commonly required to verify the interaction proposed by immunofluorescence data.

In an attempt to confirm the proposed interaction between LRP/LR and the neurotoxic $A\beta_{42}$ peptide, as revealed by co-localization results, pull down assays were performed. The presence of both proteins in the eluted sample suggests that an association between

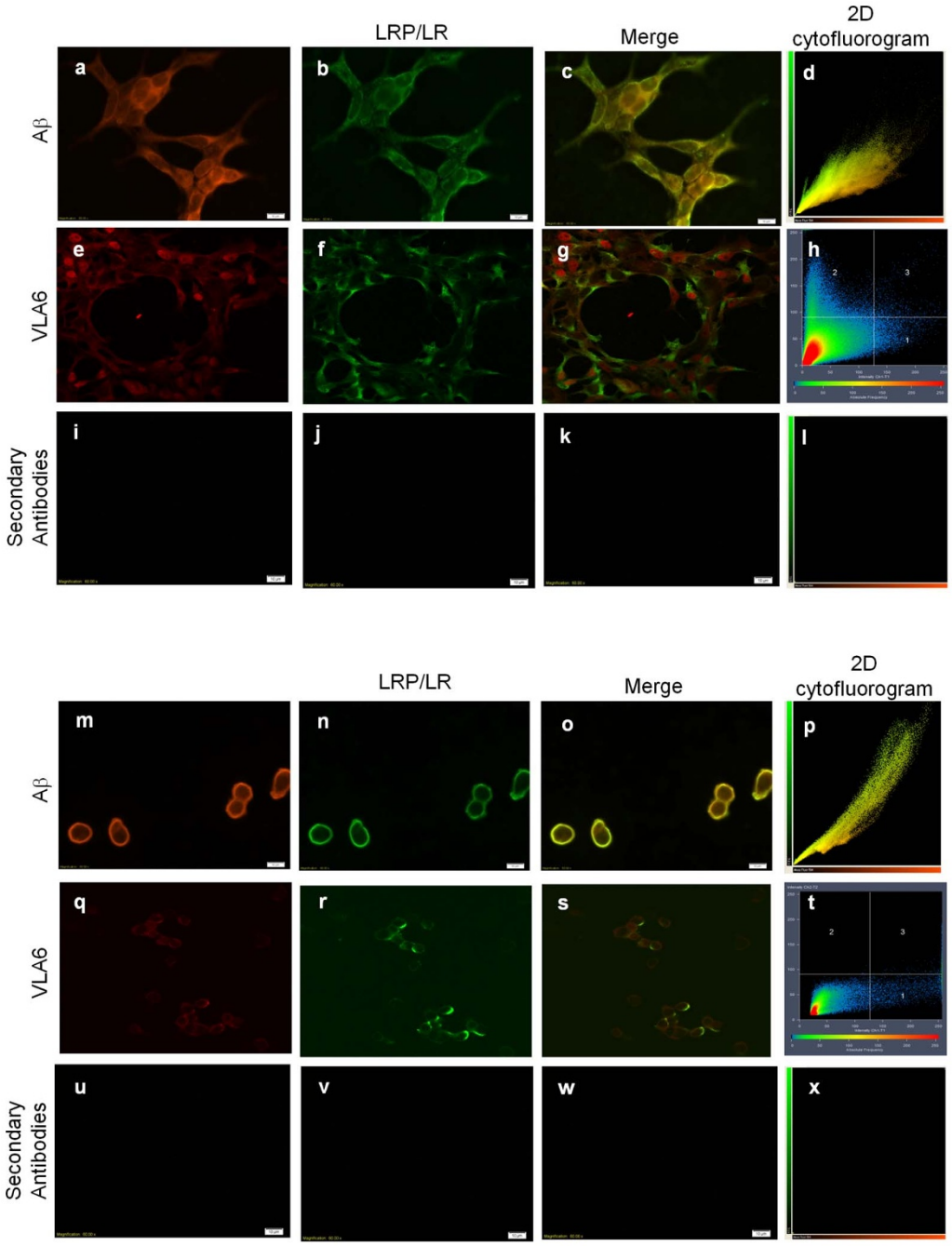

Figure 1 | Cell surface co-localisation between LRP/LR with Aβ. (a) Endogenous cell surface LRP/LR and Aβ on HEK293 (upper panel) and N2a (lower panel) cells were indirectly immunolabelled. Aβ was indirectly detected using anti-β-amyloid (22–35) (Sigma) and anti-rabbit Alexafluor 633 antibodies (Fig. 1a, m). LRP/LR was detected employing anti-IgG1-iS18 (human) and anti-human-FITC (Cell lab) antibodies (Fig. 1b, f and Fig. 1n, r). Merged images (Fig. 1c, o) and 2D-cytofluorograms (Fig. 1d, p) (acquired using CellSens Software) verified the co-localization. The negative control, Very Late Antigen 6 (VLA6) was detected employing anti-VLA6 and anti-rabbit Alexaflour 633 antibodies (Fig. 1e, q). The merged images (Fig. 1g, s) and 2D-cytofluorograms (Fig. 1h, t) demonstrated that VLA6 and LRP/LR do not co-localize on the cell surface. Secondary antibody controls are shown in Fig. 1i-l and Fig. 1u-x. Fluorescence was detected and resultant images acquired using the Olympus IX71 Immunofluorescence Microscope and Analysis Get It Research Software. Scale bars are 10 μm.

Table 1 | Pearson's Correlation Co-efficient for LRP/LR, Aβ and VLA6 cell surface co-localization

	HEK293	N2a
LRP/LR + Aβ	0.926	0.969
LRP/LR + VLA6	0.30	0.12

LRP and $A\beta_{42}$ exists. However, the exclusivity of this interaction could not be verified owing to the presence of contaminant bands present within the eluted sample lane (Fig. 2a, lane 5). These polypeptides may represent numerous LRP ligands, possibly including laminin, PrP^{c}, actin, tubulin[35], heparin sulphate proteoglycans as well as ribosomal and histone components. The control shall be briefly discussed. Anti-FLAG® M2 agarose beads were subjected to incubation in the presence of lysis buffer (Fig. 2c) as well as cell-lysates

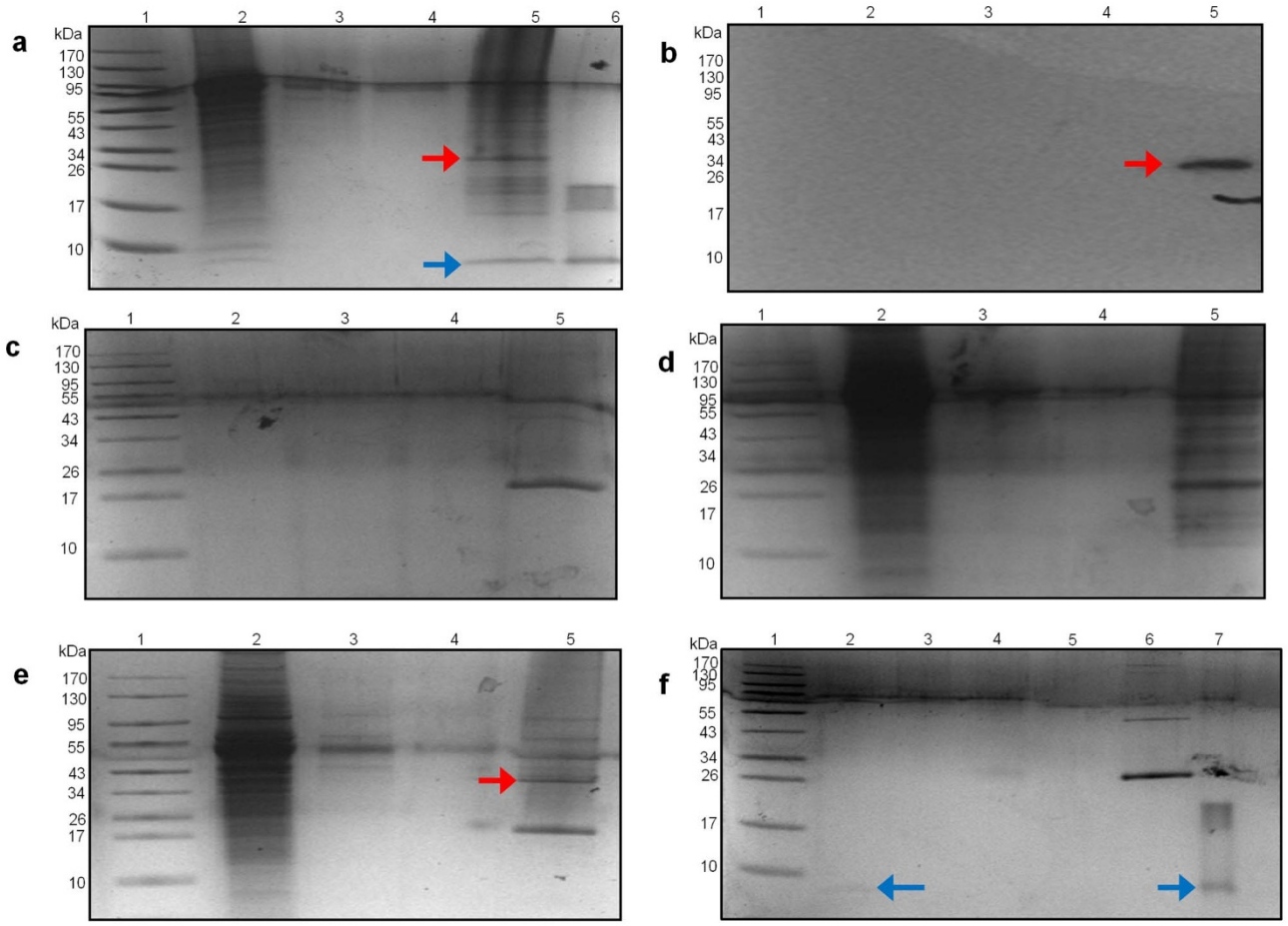

Figure 2 | LRP/LR as a potential Aβ-interacting protein. Pull down assays were employed using FLAG® Immunoprecipitation kit (Sigma Aldrich), to investigate the proteins detectable in unbound samples (lane 2), wash steps (lanes 3 and 4) and eluted samples (Fig. 2a–e: lane 5 and Fig. 2f: lane 6) and 2 μg of synthetic $A\beta_{42}$ (positive control) (Fig. 2a: lane 6 and Fig. 2f: lane 7). (a) Cell lysates containing recombinantly expressed LRP/LR::FLAG were co-incubated with exogenous Aβ. (b) Immunoblot employed to validate the position of LRP::FLAG (~38 kDa). Figures represent anti-FLAG® M2 beads incubated with (c) lysis buffer, (d) non-transfected HE293 cell lysates, (e) HEK293 cell lysates of cells transfected with pCIneo::FLAG as well as (f) pure synthetic $A\beta_{42}$ in the absence of cell lysate. Samples were resolved on 16% Tris-tricine SDS PAGE gels and stained with Coomassie Brilliant Blue. Blue and red arrows are indicative of $A\beta_{42}$ and LRP::FLAG respectively.

lacking recombinant LRP::FLAG expression (Fig. 2d). Furthermore, cell lysates in which LRP::FLAG was recombinantly expressed were analysed and column immobilization was confirmed (Fig. 2E). In addition, this control served to demonstrate the number of cellular components which were able to bind to LRP::FLAG (Fig. 2e). Fig. 2f, served to assess whether the "sticky" nature of $A\beta_{42}$ allowed it to bind to the affinity column in the absence of the tagged protein. Upon analysis of 10 μg of synthetic $A\beta_{42}$, the peptide was present in the unbound soluble fraction (Fig. 2f, lane 2) thereby illustrating that the presence of $A\beta_{42}$ in the eluted sample of the Fig. 2a, lane 5, was owing to an immobilizing interaction with LRP.

As an interaction between LRP/LR and $A\beta_{42}$ has been proposed an investigation into the influence of such an interaction on AD pathogenesis, specifically cellular survival, was justifiable. Significant reductions in cellular viability across all three cell lines were observed at varying concentrations of exogenously administered synthetic $A\beta_{42}$ (Fig. 3a–c). More notably, upon co-incubation of cells with the $A\beta_{42}$ peptide and anti-LRP/LR specific antibody IgG1-iS18, a significant enhancement in cell viability was observed (Fig. 3a–c). These results were further confirmed by shRNA mediated down regulation of LRP/LR (Fig. 4c), thereby demonstrating that the cell rescuing abilities of IgG1-iS18 are not owing to a lack of antibody specificity. Thus, it may be suggested that LRP/LR may be implicated in Aβ mediated cytotoxicity and the association between these proteins may be pathological in nature. It is plausible that this association may be pathological in nature as PrP^c has been reported to be important in mediating the synapotoxic effects of $A\beta$[19] and the neuroprotective role of PrP^c may be inhibited upon its binding to Aβ. Thus both PrP^c and its cell surface receptor LRP/LR[25] may be implicated in mediating this pathological role.

Furthermore, to assess whether the impediment of cellular proliferation contributed to reduced cell viability (Fig. 3a–c), the proliferative potential of N2a cells incubated with varying $A\beta_{42}$ concentrations was evaluated. Cellular proliferation was similarly hampered in the presence of $A\beta_{42}$ and IgG1-iS18 (Fig. 3d) rescued cells from this effect. This result was further corroborated by enhancement in cellular proliferation observed when Aβ was administered to cells in which LRP/LR was down regulated by shRNAs (Fig. 4d). Therefore, it may be proposed that the LRP/LR-$A\beta_{42}$ interaction may possibly result in aberrant proliferative cell signaling pathways. Under physiological conditions, LRP/LR promotes cellular survival, reported through the activation of the Mitogen activated protein (MAP) kinase signal transduction pathway[36]. It is plausible that an interaction between LRP/LR and $A\beta_{42}$ may foil the receptor mediated initiation of proliferative pathways.

In conclusion, it has been demonstrated that an LRP/LR-$A\beta_{42}$ interaction occurred on the cell surface and antibody blockade of LRP/LR by IgG1-iS18 or shRNA mediated down regulation of LRP/

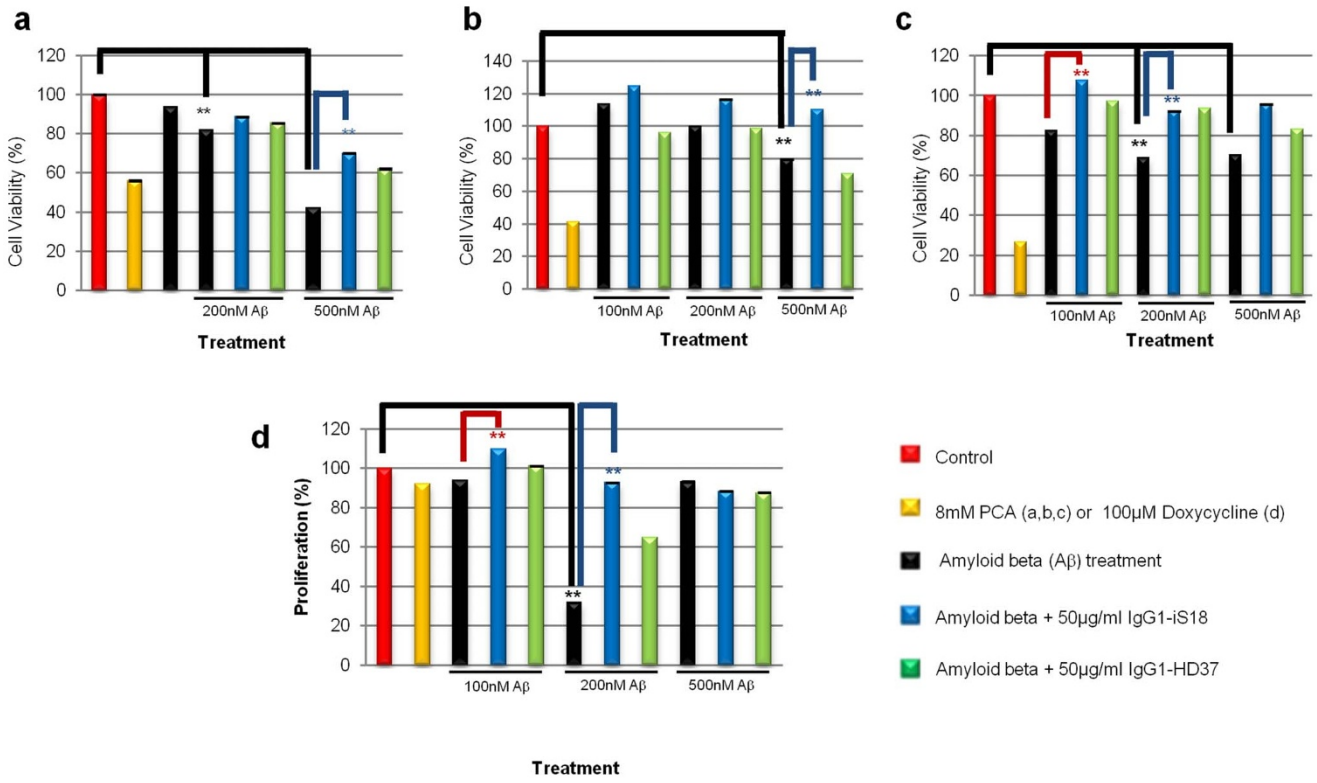

Figure 3 | Cell rescuing effects of anti-LRP/LR antibody IgG1-iS18. (a) Cellular viability of HEK293 cells, as determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (1 mg/ml) assay, post exogenous treatment with synthetic $A\beta_{42}$ and upon co-incubation with anti-LRP/LR IgG1-iS18 or IgG1-HD37 (negative control). The cell viability was assessed 48 h post treatment and the no antibody control was set to 100%. SH-SY5Y (b) and N2a cells (c) were exposed to similar treatments. (d) Cellular proliferation of N2a cells as determined by colorimetric 5-bromo-2′-deoxyuridine (BrdU) non-isotopic immunoassay (Calbiochem®), allowing 4 h for BrdU incorporation into cultured cells. Error bars represent sd. **$p < 0.01$; Student's *t*-test.

LR rescued cells from $A\beta_{42}$ induced cytotoxicity and impedance of proliferation. These results suggest that LRP/LR may contribute to $A\beta_{42}$ mediated pathogenesis in AD and that anti-LRP/LR specific antibodies and shRNAs directed against the receptor mRNA may show promise in the quest for effective AD disease-modulating therapeutics.

Methods

Immunofluorescence Microscopy. HEK239FT and N2a cells were seeded onto microscope coverslips and incubated until a confluency of 50–70% was attained. The cells were subsequently fixed with 4% Paraformaldehyde (10 minutes, room temperature), rinsed thrice with 1xPBS and blocked in 0.5%PBS-BSA (5–10 minutes). Post blocking, coverslips were additionally washed in PBS and placed such that the cell-free side came into contact with the microscope slide. 100 μl of primary antibody solution (diluted in 0.05%PBS-BSA) containing 1 : 150 IgG1-iS18 (human), 1 : 150 anti-VLA6 or 1 : 100 anti-β-amyloid (22–35) (rabbit (Sigma) was administered to the cells. Post an overnight incubation at 4°C in moist containers, coverslips were again washed thrice in 0.5% PBS-BSA and placed on clean slides. A 100 μl volume of a secondary antibody solution containing1 : 300 goat anti-human FITC (Cell Lab) and 1 : 300 goat anti-rabbit IgG conjugated to Alexa Fluor® 633 (Invitrogen) were administered to cells and incubated for an hour in the dark. Post incubation, coverslips were washed twice in 0.5% PBS-BSA and once in PBS and mounted onto clean microscope slides using 50 μl Fluoromount (Sigma Aldrich). The Olympus IX71 Immunofluorescence Microscope and Analysis Get It Research Software were employed to detect fluorescence and acquire images, respectively. Images were analysed and 2D cytofluorograms were constructed using Cell Sens Software.

Pull down assay. HEK293 cells were transfected via calcium phosphate methodology with a pCIneo-LRP::FLAG plasmid for 72 hours at 37°C, 5% CO_2. Pull down experimental samples were composed of 200 μl of HEK293 whole cell lysates in which LRP::FLAG was recombinantly expressed and 10–20 μl of synthetic $A\beta_{42}$ (Sigma-Aldrich) was exogenously administered. Assays were performed using FLAG® Immunopercipitation Kit (Sigma-Aldrich) according to manufacturer's instructions. Samples were subsequently electrophorectically analysed and gels were stained with Coomassie Blue. LRP::FLAG was detected via immunoblotting using murine anti-FLAG antibody (1 : 4000) (Sigma-Aldrich) and goat anti-mouse HRP (1 : 10 000) (Beckman Coulter).

3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. HEK293, N2a and SH-SY5Y cells were seeded in a 96 well plate as to attain 50–70% confluency within 24 hours and incubated in a 5% CO_2 humidified atmosphere at 37°C. Post incubation, synthetic neurotoxic Amyloid beta (Aβ) peptide (Sigma Aldrich) was administered to the cells in varying concentrations (100 nM, 200 nM and 500 nM respectively) to determine the affect thereof on cell viability. In addition, untreated controls (cells incubated in DMEM) as well as positive controls (cells incubated with 8 mM protocatechuic acid(PCA)-an apoptosis inducing agent) were included. Furthermore, cells were additionally co-incubated with Aβ (at the concentrations listed above) as well as either 50 μg/ml IgG1-iS18 antibody or 50 μg/ml IgG1-HD37 antibody (Affimed Therapeutics). Treated cells were incubated (37°C, 5% $C0_2$) for 48 hours, following which 20 μl of 1 mg/ml MTT was added to each well and the cells subsequently incubated (37°C, 5%CO_2) for 2 hours. After incubation, culture media was aspirated and 180 μl of DMSO added to each well to lyse the cells and dissolve the formazan crystals formed within the cells. The absorbance was recorded at 570 nm using an ELISA microtiter plate reader and the percentage survival of the cells, relative to the non-treated controls, calculated. Three separate experiments were performed, each in triplicate.

Bromodeoxyuridine (BrdU) proliferation assay. HEK293, N2a and SH-SY5Y cells were seeded in a 96 well plate as to attain 50–70% confluency within 24 hours and incubated in a 5% CO_2 humidified atmosphere at 37°C. Post incubation, synthetic neurotoxic Amyloid beta (Aβ) peptide (Sigma Aldrich) was administered to the cells in varying concentrations (100 nM, 200 nM and 500 nM respectively) in the presence or absence of IgG1-iS18 or IgG1-HD37. Proliferative potential of treated cells was assessed as per manufacturer's instructions for BrdU Proliferation Assay Kit (Calbiochem®). Three separate experiments were performed, each in triplicate.

Production of shRNA directed against LRP/LR mRNA. shRNAs were designed to be expressed from the H1 RNA Pol III Promoter. shRNA1.1 was designed to be homologous to murine sequences reported in previous studies and shRNA7.6 was designed using The RNAi Consortium. The expression cassettes comprised of a full H1 RNA Pol III promoter sequence, a poly T termination signal and the guide strand on the 3′ arm. The shRNA expression cassettes were generated using nested PCR in

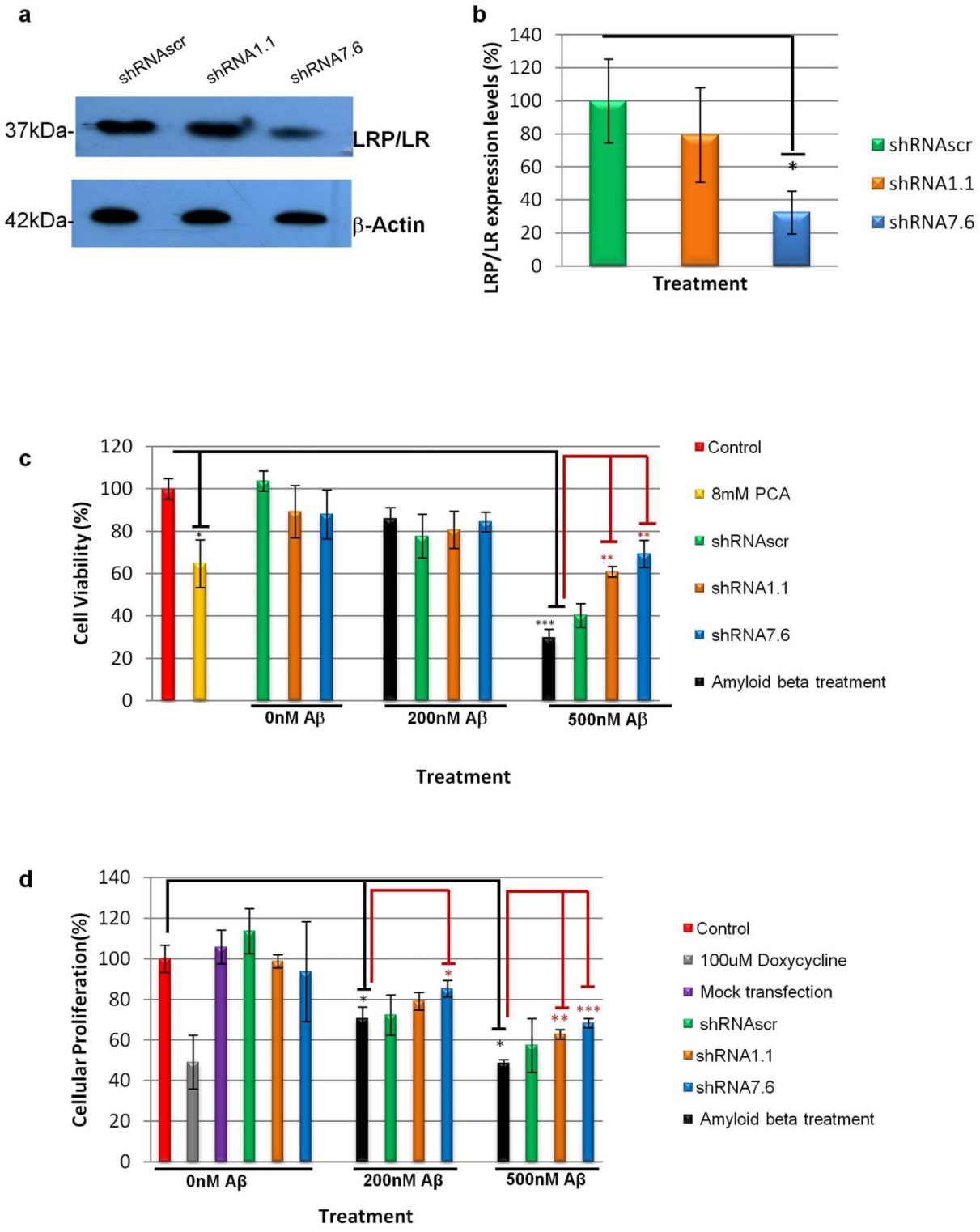

Figure 4 | shRNA-mediated downregulation of LRP/LR and the effects thereof. (a) HEK293FT cells were transfected with shRNAscr, shRNA1.1 and shRNA7.6 using the *Trans*IT®-LT1 Transfection reagent. 72 h post transfection total LRP/LR levels were assessed by Western blotting. β-actin was employed as a loading control. Gels have been cropped for clarity and conciseness purposes and have been run under the same experimental conditions. (b) Bar graph depicting percentage LRP/LR down regulation was generated by quantifying the Western blot band intensities of three independent experiments employing Quantity One 4.6 Software. To assess the role of LRP/LR in Aβ, toxicity 24 h post transfection, varying concentrations of synthetic Aβ was exogenously administered to cells. 72 h post transfection (48 h post Aβ incubation) cellular viability was assessed by MTT assay (c) and cellular proliferation was assessed by BrdU assay (d) Error bars represent sd. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ Student's *t*-test.

which the H1 RNA Pol III promoter served as the template. The forward primer was complementary to that of the H1 RNA Pol III promoter and the shRNA sequences were incorporated into the reverse primers. The resultant PCR products, which coded for the shRNA expression constructs were subsequently cloned into the pTZ57R/T vector (Fermentas).An shRNA that does not target any gene, herein termed scrambled shRNA (shRNAscr), served as the negative control. The LRP/LR target sequence as well as the structure of shRNA1.1 and shRNA7.6 are described in Jovanovic et al., (2013)[37].

Cellular transfection with shRNA directed against LRP/LR mRNA. The *Trans*IT®–LT1 Transfection reagent (Mirus) was employed to transfect LRP/LR shRNA 1 and 7 into HEK293 cells as per the manufacturer's instructions.

Western blotting. Post 72 h transfection of HEK293 cells with shRNAs, cells were lysed and total LRP/LR levels were determined by Western blotting employing IgG1-iS18 (1 : 10 000) and goat anti-human horseradish peroxidase (HRP) (1 : 10 000) (Cell Lab) antibodies, respectively. Western blot band intensities were quantified using Quantity One 4.6 Software.

Assessing cell viability and proliferation post cellular transfection with shRNA. Synthetic $A\beta_{42}$, at varying concentrations, was exogenously administered to transfected cells, 24 h post transfection. Thereafter cells were incubated in the presence of Aβ for an additional 48 h prior to analysis by MTT and BrdU assay respectively.

Statistical evaluation. Student's *t*-tests were used to analyse the data and obtain *p* values. All statistical evaluations were performed using GraphPad Prism (version 5.03) software.

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Author contributions

Conceived and designed the experiments: S.F.T.W. Design of shRNA: M.W. Performed experiments: B.D.C.D., D.G. and K.M. Assisted with the immunofluorescence microscopy: C.P. Antibody (IgG1-HD37) production: U.R., S.K. and M.L. Analysed data: B.D.C.D. Wrote the manuscript: B.D.C.D., K.J. and D.G. Edited the manuscript: B.D.C.D. and S.F.T.W.

Additional information

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