

## CD150-MEDIATED AKT SIGNALLING PATHWAY IN NORMAL AND MALIGNANT B CELLS

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**Aim:** To study upstream and downstream events in CD150-mediated Akt signaling pathway in normal human B cells, EBV-transformed lymphoblastoid (LCL) and malignant Hodgkin's lymphoma (HL) B cell lines. **Methods:** To access protein-protein interaction we applied immunoprecipitation, Western blot analysis and surface plasmon resonance (SPR) technique. A novel modification of SPR technique using reduced glutathione bound to golden surface was proposed. Immunostaining and isolation of cytoplasmic fractions and nuclear extracts were performed to detect proteins' localization in cells. Western blot analysis was performed to follow up the phosphorylation of proteins on specific sites and proteins' expression level. **Results:** It was shown that CD150 ligation induced Akt activation in normal tonsillar B cells (TBC), SH2D1A positive LCL and HL B cell lines. The p85 $\alpha$  subunit of PI3K co-precipitated with CD150 cytoplasmic tail. This direct association depends on tyrosine phosphorylation and is mediated by N terminal SH2 domain of p85 $\alpha$ . CD150 initiated phosphorylation of FoxO1 transcription factor in normal B cells as well as in LCL MP-1 and HL cell line L1236. At the same time, CD150 ligation triggered GSK-3 $\beta$  kinase phosphorylation only in immortalized LCL MP-1 and HL cell line L1236. **Conclusions:** We have demonstrated that CD150 receptor could trigger PI3K-mediated Akt signaling pathway in normal, EBV-transformed and malignant B cells. CD150-mediated phosphorylation of Akt downstream targets GSK-3 $\beta$  and FoxO1 in EBV-transformed and HL cells could be one of the mechanisms to avoid apoptosis and support survival program in these immortalized B cells.

**Key Words:** CD150 receptor, PI3 kinase, Akt, GSK-3 $\beta$ , FoxO1, immortalized B cells, Hodgkin's lymphoma.

CD150 (IPO-3/SLAM) is a member of CD2 family of the immunoglobulin (Ig) superfamily of surface receptors [1–5]. It is expressed on activated T and B lymphocytes, dendritic cells and monocytes [3, 6]. Low level of CD150 expression was also found on natural killer T cells, platelets, basophiles and mature dendritic cells [7, 8]. This receptor is upregulated via antigen receptors, CD40, Toll-like receptors and by mitogenes or cytokines (reviewed in [9]). There are experimental evidences that CD150 and SH2D1A adaptor protein are co-expressed during a narrow window of B cell maturation, and SH2D1A may be involved in regulation of B cell differentiation via switching of CD150-mediated signaling pathways [10, 11]. CD150 is co-expressed with SH2D1A adaptor protein in some B-lymphoblastoid cell lines and lymphoma cells with activated B cell phenotype, such as Hodgkin's lymphoma (HL) and ABC-type diffuse large B cell lymphoma [10, 12].

Tumor cells of classical HL are known as Hodgkin and Reed-Sternberg cells (HRS), and originate from preapoptotic germinal center B cells that do not express functional B cell receptor (BCR) [13, 14]. HRS cells are able to escape the regulation mechanisms aimed to eliminate B cells lacking functional BCR. Several aberrantly activated signaling pathways have been identified that contribute to the rescue of HRS cells from apoptosis: JAK-STAT [15, 16], MAPK/ERK initiated by CD30-, CD40-, RANK [17] and CD150 [18] signaling, PI3K/AKT linked to CD40 [19], LMP1 and LMP2a in EBV-positive cases [20–22]; NF- $\kappa$ B due to aberrant expression, mutations, CD40, CD30 stimulation (reviewed in [13, 14, 23]).

Activation of the phosphatidylinositol 3-kinase (PI3K) pathway has been linked with tumor cell growth, survival and resistance to therapy in several cancer types [24]. The main downstream PI3K effector, which controls the survival of normal and malignant cells, is Akt/PKB [25]. Ligation of tumor necrosis factor (TNF) family receptors could induce Akt phosphorylation/activation in normal [26] and HRS cells [19]. Previously, we have demonstrated that Akt could be activated via CD150 in DT40 model system, lymphoblastoid cell line MP-1 [10, 27] and HL cells L1236 [28]. The hypothesis was proposed that CD150-SH2D1A association could play decisive role in CD150-mediated Akt signaling. In current study we further explored the upstream and downstream events in CD150-mediated Akt pathway in normal and malignant B cells.

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**Abbreviations used:** BCR – B cell receptor; CD150ct – CD150 cytoplasmic tail; Cyt – cytoplasmic fraction; HL – Hodgkin's lymphoma; HRS – Hodgkin's and Reed-Sternberg; ITSM – immunotyrosine based switch motif; LCL - lymphoblastoid cell line; NE – nuclear extract; NSH2 – N-terminal SH2 domain; PI3K - phosphatidylinositol 3-kinase; SH2D1A – SH2 domain containing protein 1A; SPR – surface plasmon resonance; TBC – tonsillar B cells; TCL – total cell lysate; TNF – tumor necrosis factor.

## MATERIALS AND METHODS

**Cell lines.** The B lymphoblastoid cell lines (B-LCL) MP-1, CESS, T5-1, freshly infected LCLs from peripheral blood B cells and HL cell line of B cell origin L1236 were maintained in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, and antibiotics.

**Human tissue specimens.** After receiving informed consent in accordance with the Declaration of Helsinki, fresh tonsils were obtained from 23 patients undergoing tonsillectomy. Tonsillar B cells were isolated by density fractionation on discontinuous Lymphoprep (Axis-Shield PoC AS, Norway) and Percoll (Sigma, USA) gradients as described [29]. 96.5–99.6% of cells in CD2<sup>-</sup> populations were of B cell lineage since expressed CD19 and/or CD138 antigens.

**GST proteins preparation.** GST-fusion constructs of CD150 cytoplasmic tail (CD150ct) were prepared earlier and described in [10, 27]. cDNA of p85 $\alpha$  regulatory subunit was a kind gift of Dr. I. Gout (University College, London, UK). N-terminal SH2 domain of p85 $\alpha$  regulatory subunit was cloned into pGEX-2T vector using specific primers, and sequenced (we thank for the help Dr. Vladimir Kashuba, MTC, Karolinska institutet, Sweden). The plasmid encoding GST-SH2D1A was kindly provided by Dr. Kim Nichols (The Children's Hospital of Philadelphia, Philadelphia, PA, USA). Plasmids containing GST-CD150ct, GST-SH2D1A and GST-NSH2-p85 $\alpha$  were transformed into the *Escherichia coli* strains BL21DE3 (Invitrogen, USA) to produce non-phosphorylated fusion proteins, and GST-CD150ct with and without point mutations were transformed to TKX1 strain (Stratagene, USA) for production of tyrosine-phosphorylated fusion proteins.

**Surface plasmon resonance (SPR) instrumentation and SPR analysis.** The substrates and cell design of scanning SPR spectrometer "BioSuplar" ("PLASMON-005") (V. Lashkarev Institute of Semiconductor Physics, NAS of Ukraine, Kyiv, Ukraine) as well as its other characteristics are described in [30]. This spectrometer, which has open measurement architecture, was used to study protein–protein interactions. The peristaltic pump provided a constant solution flow (> 50  $\mu$ l/min) through the experimental cell. SPR kinetics was analyzed with a model that takes into account the heterogeneous processes at the interface using a stretched exponential function [31].

**Surface modification and protein immobilization.** The surface cleaning and activation procedure (for planar metal electrodes of SPR chips, each with a metal layer 500 Å thick consisting of a 450-Å layer of gold on a 50-Å chromium adhesive layer) was carried out at room temperature as follows. To remove organic contamination, the chips were washed in a freshly prepared mixture of aqueous (NH<sub>4</sub>)<sub>2</sub>S<sub>2</sub>O<sub>8</sub> (0.12 M) and H<sub>2</sub>O<sub>2</sub> (30%) (15:6 vol:vol) for 10 min. Traces of heavy metal ions were removed with a freshly prepared mixture H<sub>2</sub>O<sub>2</sub> (30%) and HCl (37%) in water (1:1:3, vol:vol:vol) for 10 min. The surface was modified by thiols COOH-(CH<sub>2</sub>)<sub>11</sub>-SH (3.3  $\mu$ M) and CH<sub>3</sub>-(CH<sub>2</sub>)<sub>8</sub>-SH (1  $\mu$ M), (Sigma, USA) in ratio 1:300 (12 h, 37 °C) [32]. Chips were washed

by ethanol. Cadmium acetate (10 mM solution, room temperature) was used for activation of carboxyl groups at least for 2 h prior experiment. Cadmium acetate was used as an immobilization bridge, which has selectivity to thiogroup of glutathione (as ligand) and interacts with carboxyl groups on charged thiols. Glutathione immobilization: 1 mM solution of reduced glutathione (Sigma, USA) in TBE buffer (pH 8.0) was applied on chips' surface for 1 h at room temperature. Proteins were immobilized in PBS buffer.

**Biochemical methods and antibodies.** Cell lysis, SDS-PAGE, Western blotting, stimulation of cells by anti-CD40 (G28-5, kind gift of Prof. Edward Clark, University of Washington, Seattle, WA, USA), anti-IgM (AffiniPure F(ab')<sub>2</sub> fragment goat anti-human IgM Fc<sub>5b1</sub> fragment specific, Jackson ImmunoResearch laboratories, West Grove, PA, USA), anti-CD150 mAb (IPO-3, produced in IEPOR NASU, Kiev, Ukraine), CD150 immunoprecipitations were performed as described earlier [10, 27]. Western blot results were visualized using a 20x LumiGLO<sup>®</sup> Reagent and 20x Peroxide (Cell Signaling Technology, Beverly, MA, USA). Goat antisera against actin, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-SH2D1A serum was a kind gift of Dr. Kim Nichols (The Children's Hospital of Philadelphia, Philadelphia, PA, USA). Rabbit anti-pAkt (Ser473) and (Thr308), anti-Akt, anti-pFoxO1(Thr24)/FoxO3a(Thr32), anti-FoxO1, anti-FoxO3a, anti-pGSK-3 $\beta$  (Ser9), anti-PI3K p85 $\alpha$  antibody, anti-PARP, anti-Bcl-2 were purchased from Cell Signaling Technology (Beverly, MA, USA).

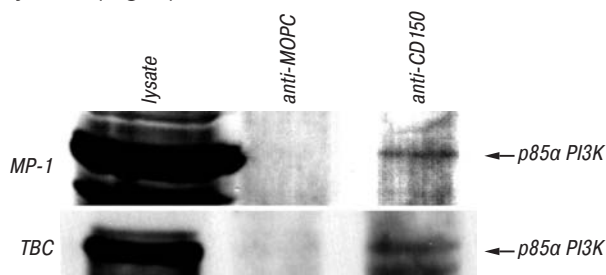
**Immunostaining.** The double staining for CD150 receptor and SH2D1A adaptor protein was done as follows: cells were spun on glass slides (10 x 10<sup>4</sup> per slide); fixed in cold methanol-acetone (1:1) at -20 °C for 1 h; rehydrated with PBS for 1 h; stained with anti-CD150 (IPO-3) mAb (5  $\mu$ g/ml) for 1 h, followed by 3 washes in PBS; incubated with horse anti-mouse Texas Red-conjugated antibodies (VectorLab, CA, USA), and washed 3 times. Cells were stained with anti-SH2D1A antibodies (dilution 1:200) for 1 h, three washes in PBS, incubated with mouse anti-rabbit FITC-conjugated antibodies (DAKO, Denmark), and mounted with 80% glycerol solution in PBS that contained 2.5% 1,4-diazabicyclo-(2.2.2)octane (Sigma). Bisbenzimidazole (Hoechst 33258) was added at a concentration of 0.4  $\mu$ g/ml to the last secondary antibody for DNA staining. The images were recorded on a DAS microscope Leitz DM RB (Leica Inc., Deerfield, IL, USA) with a Hamamatsu dual mode cooled CCD camera C4880 (Hamamatsu City, Japan).

**Isolation of cytoplasmic fraction and nuclear extracts.** Total cell lysates (TCL) were prepared from 5 x 10<sup>6</sup> of cells in RIPA lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EGTA, 1 mM EDTA (pH 8.0), 1% Triton X-100, protease inhibitors' cocktail (Sigma, USA), 1 mM PMSF), and sonicated on ice for 15 s (Sonicator MSE Soniprep 150 Plus, MSE (UK) Limited, UK), spun for 15 min, 17000 g, +4 °C, supernatants transferred to fresh tubes. For subcellular

fractionation, control and stimulated cells ( $20 \times 10^6$ ) were lysed in buffer A (50 mM NaCl, 10 mM HEPES (pH 8.0), 500 mM sucrose, 1 mM EDTA (pH 8.0), 0.2% Tryton X-100, protease inhibitors' cocktail (Sigma, USA), 1 mM PMSF), vortexed and spun for 2 min (875 g, +4 °C). Cytoplasmic fractions (Cyt) of supernatant were collected to fresh tubes. Pellets (nuclei) were washed twice by 1.5 ml of cold buffer B (50 mM NaCl, 10 mM HEPES (pH 8.0), 25% glycerol, 0.1 mM EDTA (pH 8.0)) and spun for 2 min (875 g, +4 °C). Pellets were resuspended in 200  $\mu$ l of buffer C (350 mM NaCl, 10 mM HEPES (pH 8.0), 25% glycerol, 0.1 mM EDTA (pH 8.0), protease inhibitors' cocktail (Sigma, USA), 1 mM PMSF), and sonicated on ice for 15 s, spun for 15 min, 17000 g, +4 °C, and supernatants were transferred to fresh tubes. TCL, NE and Cyt fractions were stored at -80 °C. Protein concentration was measured in all samples (TCL, NE, Cyt), and total amount of 20  $\mu$ g of protein was loaded to each well for Western blot analysis.

## RESULTS

***p85 $\alpha$  subunit of PI3K co-precipitates with CD150 cytoplasmic tail.*** Previously, we have shown that CD150-mediated signals lead to Akt S473 phosphorylation in human B-lymphoblastoid cell line MP-1 [27] and chicken B cell line DT40 [10]. Moreover, using DT40 model system it was shown that CD150-mediated Akt pathway was dependent on SH2D1A binding to CD150, was negatively regulated by Lyn and Btk, and positively - by Syk [10]. Also, another member of CD2 family, CD244 (2B4), upon tyrosine phosphorylation was shown to recruit not only SH2D1A, but also associate with the p85 $\alpha$  regulatory subunit of PI3K [33]. To answer the question whether CD150 could form a complex with PI3K in B cells, we performed immunoprecipitations of CD150 by specific mAb (IPO-3) from tonsillar B cell (TBC) and MP-1 lysates. Western blot analysis of these immunoprecipitates demonstrated that p85 $\alpha$  regulatory subunit of PI3K co-precipitated with CD150 from both examined lysates (Fig. 1).



**Fig. 1.** PI3K p85 $\alpha$  regulatory subunit precipitated with CD150 receptor both from MP-1 and tonsillar B cells (TBC). Western blot analysis with anti-p85 $\alpha$  of CD150 immunoprecipitates. Ig isotype matched MOPC21 antibody served as negative control

***CD150ct directly interacts with p85 $\alpha$  subunit N terminal SH2 domain of PI3K.*** To test whether p85 $\alpha$  regulatory subunit of PI3K directly binds CD150 cytoplasmic tail we applied surface plasmon resonance (SPR) approach. For SPR-based analysis of protein-protein interactions we used tyrosine

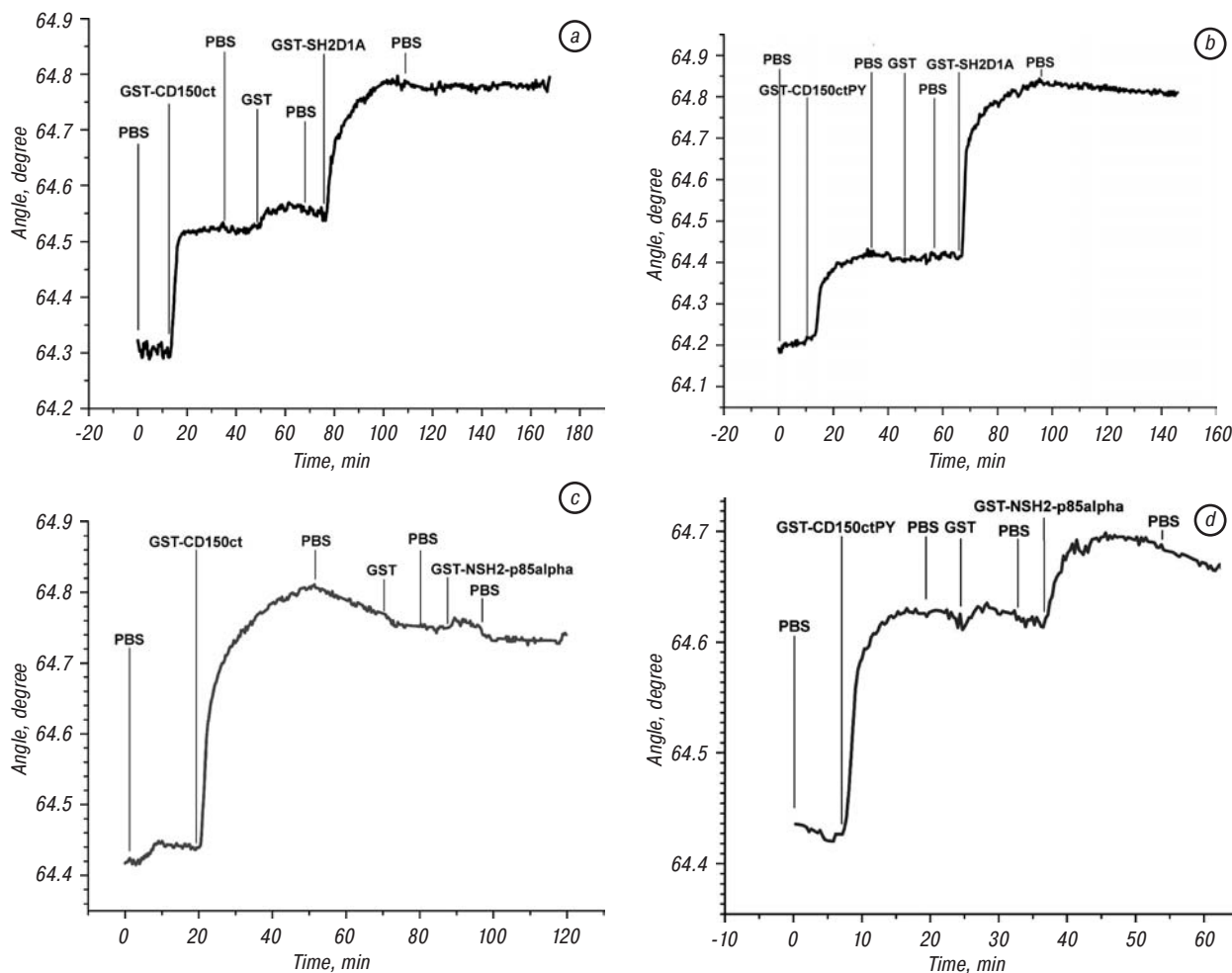
phosphorylated and non-phosphorylated GST-fused cytoplasmic tail of CD150 (GST-CD150ct) with point mutations (Y/F substitution) in ITSM motifs, described earlier [11]. Also, fusion proteins of GST-SH2D1A, GST-fused N-terminal SH2 domain of p85 $\alpha$  regulatory subunit of PI3K (GST-NSH2-p85 $\alpha$ ) were used in this study. GST-NSH2-p85 $\alpha$  was chosen since it was demonstrated for p85-associated receptors EGF and PDGF that the affinity of the N-SH2 domain of p85 $\alpha$  for the receptors correlated with the steady-state level of p85-receptor complex [34]. The quantitative ratio of protein partners was calculated based on molecular weight of interacting proteins.

Interaction between GST-fusion proteins of CD150ct (GST-CD150ct) and SH2D1A (GST-SH2D1A) was shown to be 1:1 for non-phosphorylated GST-CD150ct (Fig. 2, a), and 1:2 for tyrosine phosphorylated GST-CD150ctPY and GST-SH2D1A (Fig. 2, b). This ratio corresponds to the data obtained by biochemical studies, showing that immunotyrosine based switch motif (ITSM) with non-phosphorylated tyrosine Y281 could bind SH2D1A, and that both ITSMs with Y281 and Y327 could bind adaptor protein SH2D1A when phosphorylated [11, 35]. Thus, our results obtained by SPR approach with glutathione immobilized on golden chips, completely match with previously obtained results for CD150-SH2D1A interaction, and, therefore, this approach could be used for studies of GST-CD150ct direct interaction with other SH2-domain containing proteins.

Using this approach we examined GST-NSH2-p85 $\alpha$  interaction with non-phosphorylated (Fig. 2, c) and tyrosine phosphorylated GST-CD150ct (Fig. 2, d). It was found that NSH2 domain of p85 $\alpha$  subunit interacts only with tyrosine phosphorylated receptor (see Fig. 2, d). To determine, which tyrosines (Y281, Y327) in CD150ct are essential for CD150 - NSH2 p85 $\alpha$  interaction we used fusion proteins of CD150ct with point mutations Y/F. It was shown that mutation Y327F did not alter CD150ct-p85 $\alpha$  SH2 interaction. However, double mutant Y281F/Y327 did not bind p85 $\alpha$  SH2 domain (data not shown). Thus, the first ITSM motif containing Y281 should be available and phosphorylated for NSH2-p85 $\alpha$  interaction with CD150ct.

Thus, we demonstrated by SPR that p85 $\alpha$  subunit of PI3K via its N terminal SH2 domain could directly bind CD150ct, and that this interaction takes place in normal TBC and lymphoblastoid cell line MP-1. The direct interaction of CD150 with p85 $\alpha$  subunit of PI3K could be a starting point for regulation of PI3K activation and subsequent Akt activation via CD150.

***CD150-mediated Akt activation in lymphoblastoid cell lines.*** MP-1 and CESS lymphoblastoid cell lines both express CD150 and SH2D1A proteins. Here we demonstrate that CD150 ligation led to phosphorylation of both Akt activation sites (T308 and S473) in MP-1 cells (Fig. 3). In CESS cells CD150 ligation induce only slight Akt phosphorylation on T308 and S473, and CD150 signals had no effect on Akt phosphorylation levels in T5-1 cells and freshly obtained



**Fig. 2.** Representative SPR responses of protein-protein interactions (a-d). Non-tyrosine phosphorylated GST-CD150ct interacted with GST-SH2D1A protein in ratio 1:1 (a). Tyrosine phosphorylated GST-CD150ct (GST-CD150ctPY) interacted with GST-SH2D1A protein in ratio 1:2 (b). Non-tyrosine phosphorylated GST-CD150ct did not bind to GST-NSH2-p85alpha (c). GST-CD150ctPY interacted with GST-NSH2-p85alpha in ratio 1:1 (d)

from peripheral blood B cells LCLs (see Fig. 3). These cell lines differed by adaptor protein SH2D1A expression levels: MP-1 cells expressed SH2D1A protein at high level, CESS cells and LCLs – at much lower level, and T5-1 cells did not express SH2D1A at all ([11] and data not shown).

**CD150 and SH2D1A localization in lymphoblastoid cell lines.** Studies performed on regulation of CD150-mediated Akt activation in DT40 model system [10] and Hodgkin's lymphoma cell lines [10, 28] have shown that CD150-mediated Akt activation could be regulated by SH2D1A expression and its cellular localization. Using immunofluorescent staining here we found that in MP-1 and CESS cells SH2D1A was localized in cytoplasm close to the cellular membrane (Fig. 4). Practically all MP-1 cells in contrary to 10% of CESS cells were SH2D1A positive (see Fig. 4). As CD150-SH2D1A interaction was shown to be crucial for Fyn recruitment and CD150 tyrosine phosphorylation at least in T cells [36, 37] we examined the phosphorylation of receptor in both cell lines. It was shown that CD150 had lower level of tyrosine phosphorylation in CESS cells than in MP-1 cells (data not shown). Thus, the level of SH2D1A expression directly correlated with tyrosine phosphorylation. Since p85 $\alpha$  PI3K

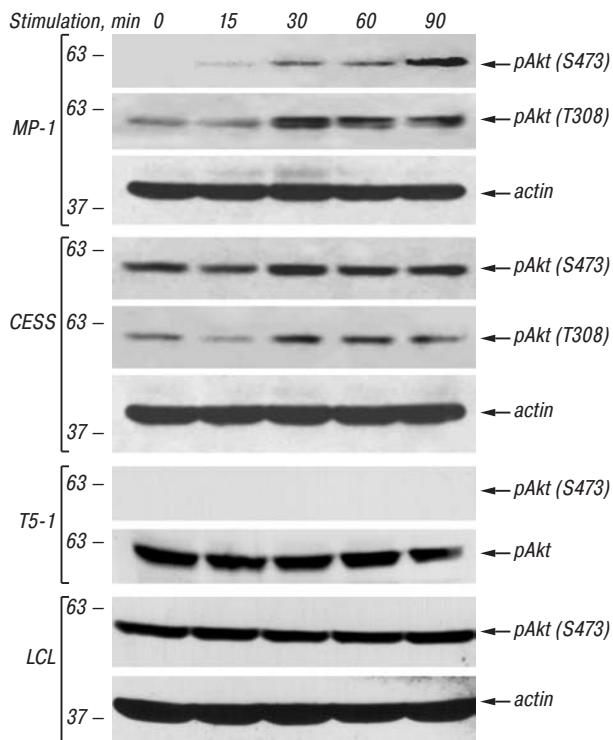
binds only to tyrosine phosphorylated CD150, obtained data could explain the lack of CD150-mediated Akt phosphorylation in cells, which do not express SH2D1A or have low expression level of this adaptor protein.

**Akt activation in primary B cells.** CD150-mediated Akt phosphorylation was studied in dense (naive and memory B cells) and buoyant fractions (activated cells) of TBC. It was shown that Akt kinase could be phosphorylated via CD150 both in naive and activated primary TBC (Fig. 5a, b). As a control, cells were stimulated via B cell receptor. It was found that stimulation of dense TBC via CD150 and IgM had additive effect on Akt phosphorylation (Fig. 5a, c, graph).

**Akt distribution between cytoplasmic and nuclear fractions in LCL and HL cell lines.** Subcellular localization of Akt kinase is very important for its function. In resting unstimulated cells Akt molecules resides both in the cytoplasm and nucleus [38]. Activation of Akt occurs in proximity of plasma membrane and has been shown to be followed by pAkt translocation to cytosol and nucleus [38]. Such translocation allows pAkt to phosphorylate its downstream targets, among which there are cytoplasmic as well as nuclear proteins.

In current study we addressed the question how Akt kinase was distributed between nucleus and cy-

toplasm in EBV-transformed cells MP-1 and HL cells (L1236) before and after stimulation via surface receptors CD40 and CD150. Before ligation of receptors, Akt kinase was mainly localized in cytoplasmic fraction in L1236 HL cells, and partially in the nuclear extract (Fig. 6, a). Upon stimulation of L1236 cells via CD150 and CD40 receptors, Akt was completely exported from the nucleus. In MP-1 cells, Akt kinase was localized exceptionally in cytoplasm (Fig. 6, b). Signals



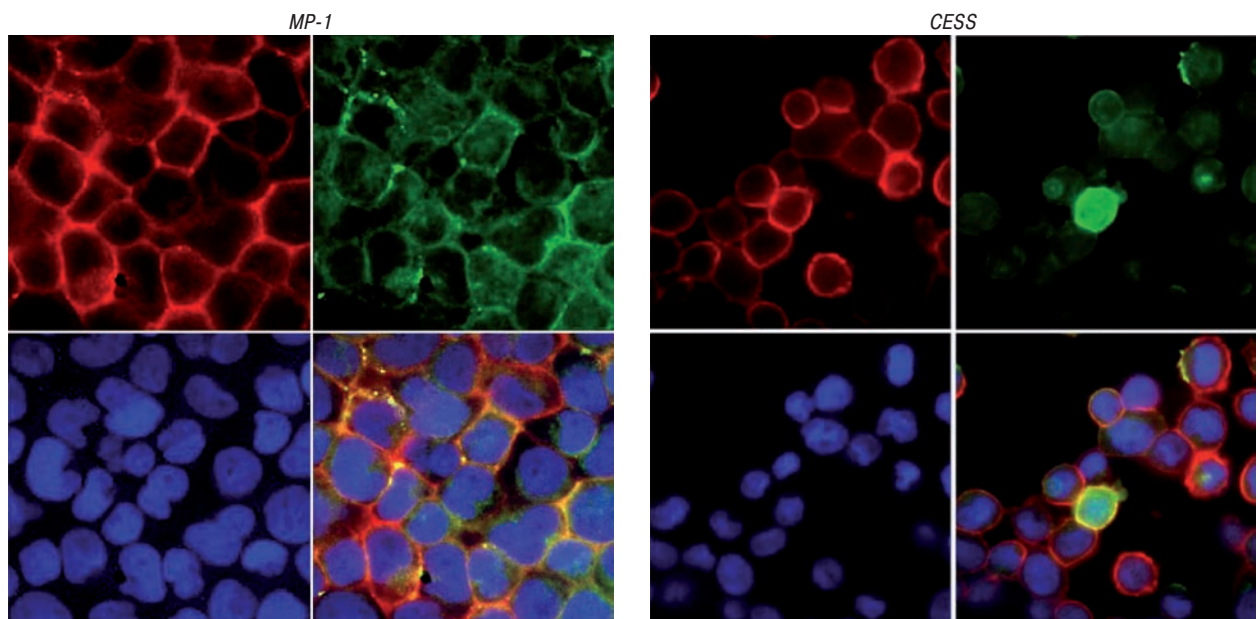
**Fig. 3.** CD150-mediated Akt phosphorylation on serine 473 (S473) and threonine 308 (T308) in lymphoblastoid cell lines (a). Akt kinase was markedly phosphorylated on both sites S473 and T308 in MP-1 cells, only slightly phosphorylated in CESS cells (a, upper panels). The level of Akt phosphorylation was not altered in T5-1 and LCL (b, lower panels). Equal loading was monitored by anti-actin or anti-Akt Western blot

via CD40 or CD150 receptor had no influence on Akt distribution between nucleus and cytoplasm (Fig. 6, b).

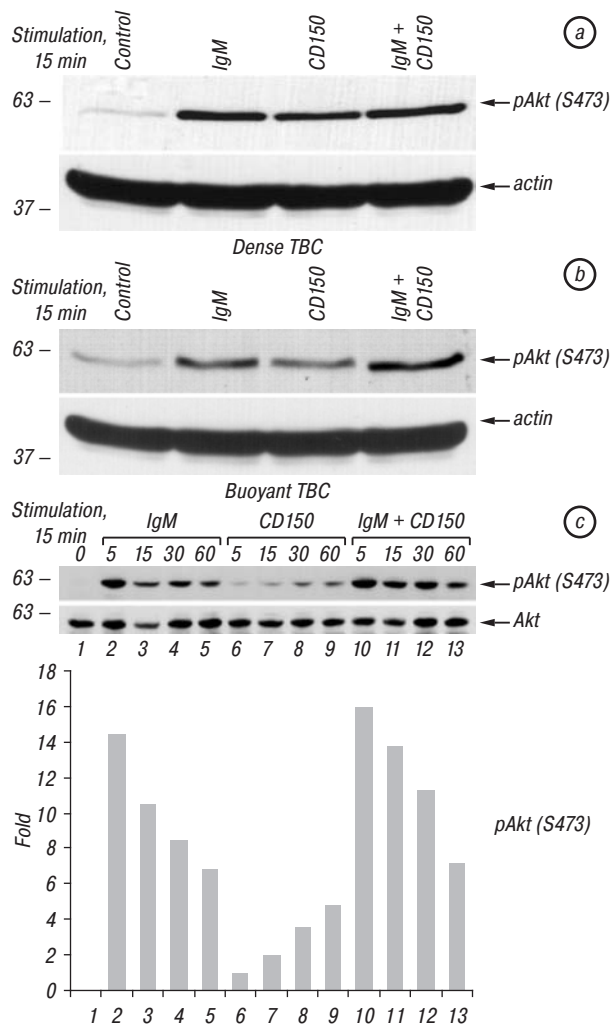
**CD150-mediated phosphorylation of Akt downstream targets in normal and malignant B cells.** Akt kinase regulates the activity of multiple target proteins in cells, which results in promotion of cell survival and inhibition of apoptosis. Among them are stress-activated kinases, BAD, Forkhead family of transcription factors, I $\kappa$ B kinase, GSK-3, mTOR, c-Raf, B-Raf, Nur77, etc (reviewed in [24]).

GSK-3 serine/threonine protein kinase is one of the crucial Akt target proteins. Two homologous mammalian GSK-3 isoforms, encoded by different genes, are known – GSK-3 $\alpha$  and GSK-3 $\beta$ . Akt could phosphorylate GSK-3 $\alpha$  on S21 and GSK-3 $\beta$  on S9, and inactivate these proteins [39]. We examined if CD150-mediated Akt activation could lead to phosphorylation of GSK-3 $\beta$  in primary TBC, LCL MP-1 and HL cell line L1236. It was found that in primary TBC, both in dense and buoyant fractions, the basal level of GSK-3 $\beta$  phosphorylation on S9 was very high, and it was not altered by stimulation via CD150 receptor (Fig. 7, a). At the same time, CD150-mediated Akt activation both in MP-1 and L1236 cells resulted in upregulation of GSK-3 $\beta$  S9 phosphorylation with kinetics similar to Akt S473 phosphorylation (Fig. 7, b, c).

FoxOs transcription factors constitute important downstream targets of the PI3K/Akt signaling pathway. FoxOs are regarded as tumor suppressor genes (reviewed in [40]). FoxOs regulate transcription of proapoptotic genes, while Akt kinase protects cells from apoptosis by phosphorylating FoxOs, which target them to 14-3-3-mediated nuclear export and/or proteasome-mediated degradation [41–43]. The sites for Akt phosphorylation in FoxO1 are T24, S256 and S319, and for FoxO3a are T32, S253 and S315 (reviewed in [40]). Here we explored CD150-mediated phosphorylation of FoxO1/FoxO3a on T24/T32 in comparison



**Fig. 4.** Double immunostaining of CD150 and SH2D1A demonstrated that these two proteins are co-expressed and co-localized close to the cellular membrane in all MP-1 cells. Only 10% of CESS cells were SH2D1A positive, and SH2D1A was also co-localized with CD150, x400

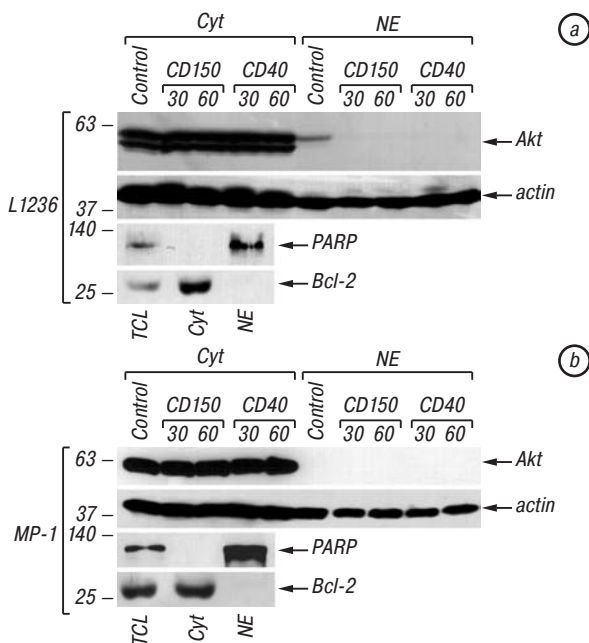


**Fig. 5.** CD150 receptor ligation induced Akt kinase phosphorylation (S473) in TBC. Crosslinking of IgM and CD150 on dense (a,c) and buoyant (b) TBC resulted in Akt phosphorylation on S473. Co-ligation of IgM with CD150 on these cells had additive effect (b and c, graph). Equal loading was monitored by anti-actin or anti-Akt Western blot. The level of pAkt was normalized against the level of Akt using TotalLab program (c, graph).

to stimulation via CD40 and IgM receptors. We found that only FoxO1, but not FoxO3a is expressed in TBC, MP-1 and HL cells L1236 on protein level (data not shown). It was shown that FoxO1 phosphorylation level on T24 was upregulated via CD150 and IgM, but not CD40 in dense fraction (naïve and memory cells) of TBC (Fig. 8, a, upper panel). In buoyant TBC the level of pFoxO1 was not altered by either CD150 or CD40 ligation, but was only enhanced by ligation of IgM (see Fig. 8, a, lower panel).

The basal phosphorylation level of FoxO1 on T24 was quite high in LCLMP-1. Nevertheless signals via CD150 and CD40 upregulated the level of pFoxO1 in 30 and 60 min upon stimulation (Fig. 8, b, graph). The effect of CD150 ligation was more pronounced than of CD40 (see Fig. 8, b, graph).

Similarly, in HL cells L1236 both signals via CD150 or CD40 receptors significantly enhanced the phosphorylation of FoxO1 T24 in 15 and 60 min upon receptors' ligation, however, effect of CD40 crosslinking was more prominent (Fig. 8, c).



**Fig. 6.** Western blot analysis of Akt kinase localization in cytoplasmic fraction (Cyt) and nuclear extracts (NE) of L1236 (a) and MP-1 (b) cells. Akt kinase was localized both in Cyt and NE of L1236 cells, and was completely exported from the nucleus upon L1236 stimulation via CD150 and CD40 receptors (a, upper panel). In MP-1 cells, Akt was localized only in cytoplasmic fraction (Cyt), (b, upper panel). Poly (ADP-ribose) polymerase (PARP) and Bcl-2 expression levels were checked to control the purity of fractions (TCL – total cell lysate). Anti-actin Western blot was performed to control the protein loading to gels

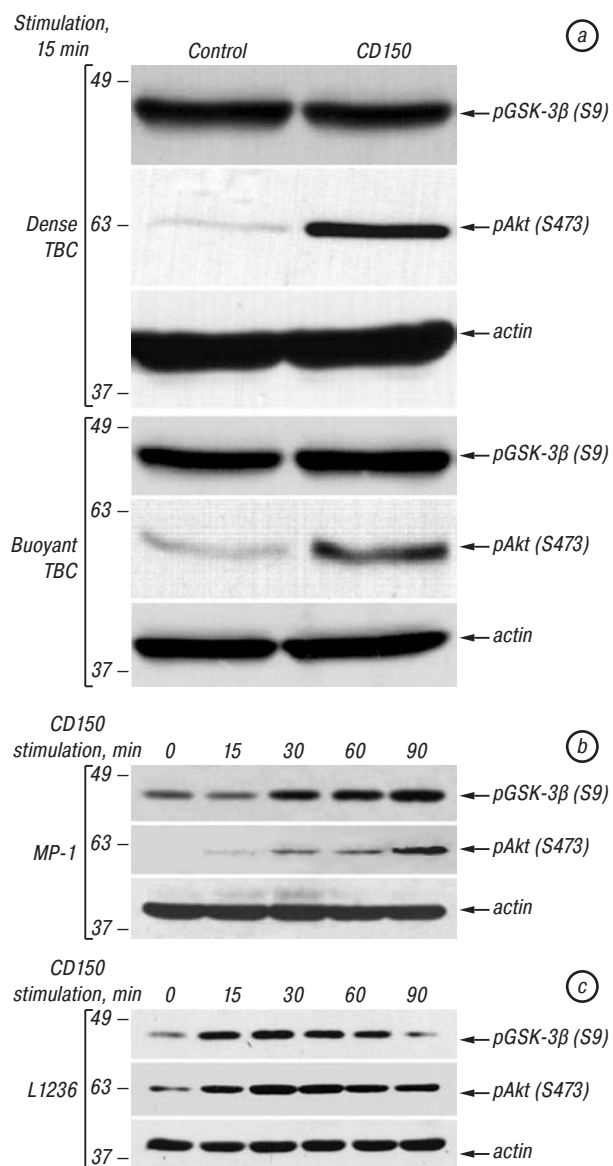
**DISCUSSION**

The serine/threonine kinase Akt/PKB is a crucial regulator of divergent cellular processes, including proliferation, differentiation and apoptosis [24]. Akt signaling is often deregulated in cancer, leading to constitutively active Akt kinase [24].

The phosphorylation of Akt (pAkt S473) was found to be upregulated in HL derived cell lines and in HRS cells in 64% [19] and 100% [44] of primary lymph node sections of HL. In all tested cases, Akt was detected both in HRS cells and the surrounding reactive cells, while active phosphorylated form of Akt was expressed only by the HRS cells [19]. This high level of basal Akt phosphorylation thought to be maintained by signals via tumor necrosis factor (TNF) family receptors CD40, RANK, and CD30 [19]. We have shown previously that CD150 receptor mediated signaling could also contribute to Akt activation in HL cells [10, 28]. Here we demonstrated that this signaling pathway could be activated via CD150 in primary normal B cells, as well as in LCL and HL cell lines.

Previously, it was shown that CD150ct interaction with adaptor protein SH2D1A was necessary for CD150 interaction with protein kinases and ITSMs' phosphorylation [36, 45]. Our data suggested that CD150-SH2D1A association and SH2D1A localization play decisive role in activation of Akt signaling pathway upon CD150 ligation (Fig. 3, 4).

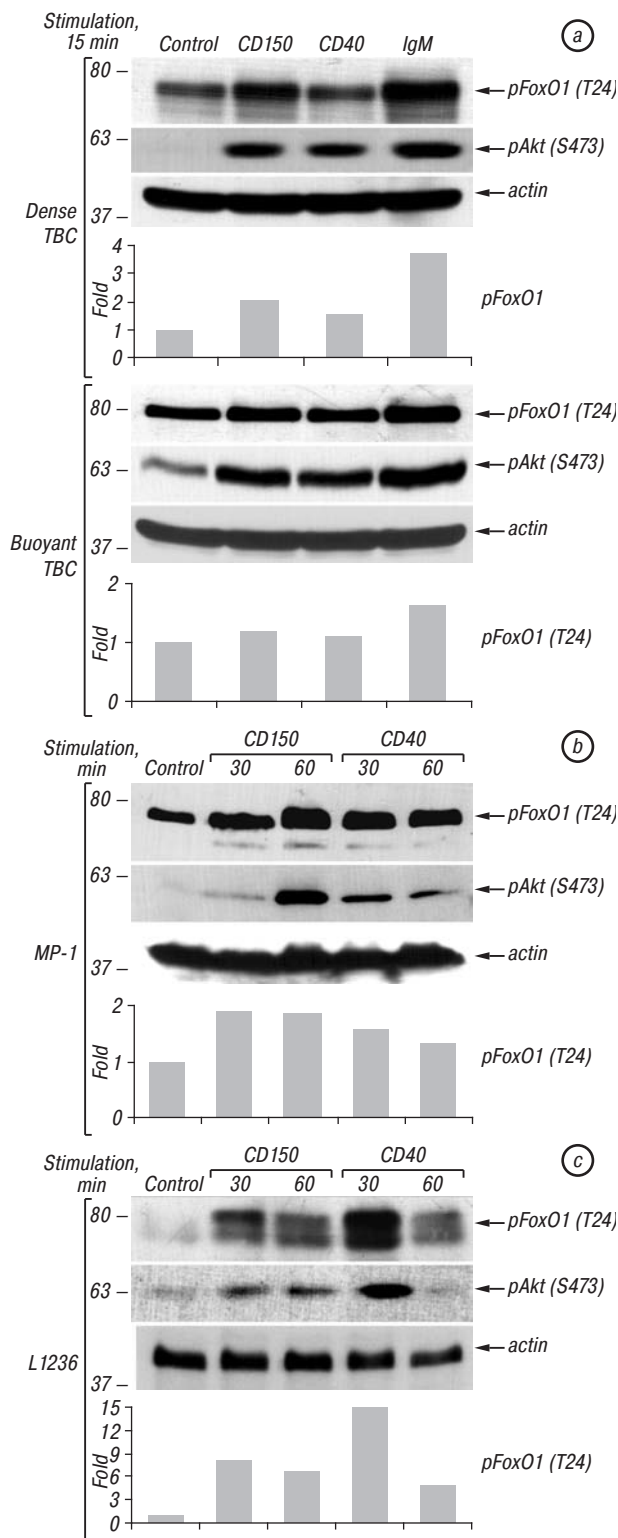
Based on our data it could be suggested that SH2D1A adaptor protein association with CD150ct allows tyrosine phosphorylation of CD150ct by cellular



**Fig. 7.** CD150-mediated phosphorylation of Akt target protein GSK-3 $\beta$  kinase in TBC, MP-1 and L1236 cell lines. Both dense and buoyant TBC had high basal level of GSK-3 $\beta$  phosphorylation, which was not altered by CD150-initiated signaling (a). The level of pGSK-3 $\beta$  was upregulated in MP-1 (b) and L1236 cells (c) upon stimulation via CD150. Anti-pAkt Western blot was performed to monitor Akt phosphorylation upon ligation of CD150. Equal loading was monitored by anti-actin Western blot

tyrosine kinases (Lyn in B cells [10]), which permit direct interaction of CD150ct and p85 $\alpha$  regulatory subunit of PI3K. Immunoprecipitation of CD150 from MP-1 cells and TBC demonstrated that CD150 co-precipitated with p85 $\alpha$  (Fig. 1). By SPR approach, we demonstrated that this interaction was direct, dependent on CD150ct phosphorylation (Fig. 2, c, d), and ITSM motif containing Y281 was necessary for NSH2-p85 $\alpha$  interaction with CD150ct. Thus, CD150-initiated Akt activation in B cells was mediated by direct interaction of CD150ct with p85 $\alpha$  regulatory subunit of PI3K.

For SPR analysis we applied modification of method first proposed by Boltovets *et al.* [32], when glutathione is fixed on the golden surface of sensor chips. This low-cost and effective modification of method for GST-protein immobilization on sensor chips could



**Fig. 8.** CD150-mediated phosphorylation of Akt target protein FoxO1 transcription factor on T24 in TBC, MP-1 and L1236 cell lines. The basal level of pFoxO1 was high both in dense and buoyant TBC fractions (a, control). Ligation of receptors mediated the upregulation of pFoxO1 level in dense cells, and had almost no influence on pFoxO1 level in buoyant cells (a). LCL MP-1 was characterized by high basal level of FoxO1 phosphorylation, which was upregulated by CD150- and CD40-mediated signaling (b). CD150 and CD40 induced strong upregulation of pFoxO1 level in HL cell line L1236 (c). The level of pFoxO1 was normalized against actin or FoxO1 levels using TotalLab program (graphs). Anti-pAkt Western blot was performed to monitor Akt phosphorylation upon ligation of CD150 receptor. Equal loading was monitored by anti-actin or anti-FoxO1 Western blot

be efficiently applied to study other protein-protein interactions using GST fusion proteins as interacting partners without using specific antibodies.

Several downstream effectors of Akt signaling, including GSK-3 $\alpha/\beta$ , and mTOR substrates 4E-BP1 and p70 S6 kinase, were shown to be phosphorylated in primary HL cells [44]. GSK-3 is one of the crucial Akt target protein. GSK-3 phosphorylates a broad range of substrates and is implicated in multiple biological processes apart from its well studied function as regulator of glycogen synthesis. GSK-3 $\beta$  has been shown to regulate cyclin D1 proteolysis and subcellular localization during the cell division cycle, thereby triggering rapid cyclin D1 turnover [46]. Also GSK-3 controls transcription factor c-myc proteolysis and subnuclear localization as phosphorylation of c-Myc T58 facilitates its rapid proteolysis by the ubiquitin pathway [47].

We have demonstrated that GSK-3 $\beta$  was phosphorylated upon CD150 ligation in MP-1 and HL cell line L1236, but not in normal TBC (Fig. 7). It seems like regulation of GSK-3 $\beta$  phosphorylation level/inactivation via CD150 receptor is more critical for immortalized tumor cells (HL) and EBV-transformed cells (LCL), which use all cellular signaling machinery to avoid apoptosis and support their proliferation. In normal TBC GSK-3 $\beta$  is initially switched off (highly phosphorylated on inhibitory site), which is probably needed for further differentiation of these cells.

In mammalian cells, the class O of fork head (fkh) transcription factors is homologous to *Caenorhabditis elegans* transcription factor DAF-16 (abnormal DAuer Formation-16), and consists of four members: FoxO1, FoxO3, FoxO4 and FoxO6 [40]. Several kinases were shown to phosphorylate FoxOs in different sites: Akt, serum and glucocorticoid-inducible kinase (SGK), mammalian sterile 20-like kinase-1 (MST1), cyclin-dependent kinase-2 (CDK2), p90-kDa ribosomal S6 kinase-2 (Rsk-2), dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1a) (reviewed in [40, 48, 49]). As to the other post-translational modifications, FoxOs activity/stability could be regulated by acetylation and ubiquitination [50].

In our study we showed that despite the high basal level of pFoxO1, CD150 mediated FoxO phosphorylation in normal TBC and LCL MP-1 (Fig. 8). Moreover, CD150 as well as CD40 was involved in inactivation of pro-apoptotic FoxO1 transcription factor in HL cell line L1236, which could contribute to the survival of HRS cells. It should be emphasized that all studied B cells expressed FoxO1, but not FoxO3a on protein level. It is an important finding since it was thought that the dominant isoform expressed in lymphocytes, at least on mRNA level, is FoxO3a [49].

Recently, we have shown that CD150 mediates JNK activation in normal and HL cells [18]. JNK kinases could phosphorylate FoxO in transactivating domain [51]. Also, JNK has been shown to phosphorylate 14-3-3 proteins, which result in reduced 14-3-3 binding to partner proteins, including FoxOs [52]. Regulation of multiple posttranslational modifications of FoxOs via

CD150-mediated JNK and other signaling pathways should be further explored to get more clear understanding of the FoxOs activity regulation in normal and malignant B cells.

Subcellular localization of Akt kinase is important for downstream cytoplasmic and nuclear protein targets phosphorylation. Using model cell lines it was shown that activation of Akt results in its nuclear translocation within 20 to 30 min after stimulation [53]. Following BCR stimulation of mouse B cell line pAkt resides both in the cytosol and nucleus [38]. In HL cell lines KM-H2 and L428 pAkt was localized close to the membrane or in cytosol [44]. Here we have shown that in unstimulated L1236 cells Akt kinase was present both in cytoplasmic fraction and nuclear extract. Upon stimulation of cells via CD150 and CD40, Akt was completely exported from the nucleus (Fig. 6). It is consistent with the fact that in most studied cases of primary HL in HRS cells pAkt was shown to be preferentially cytoplasmic [44]. This could be achieved by sustained signals via TNF receptors (including CD40) and CD150. However, the biological effect of this nuclear-cytoplasmic translocation in HL cells should be studied further. We have shown previously that sustained signaling via CD150 and CD40 receptors induced inhibition of cellular proliferation and cell death of L1236 cells [18]. We may hypothesize that retaining of Akt in cytoplasm would prevent the phosphorylation of its nuclear targets in these cells, which could be the reason of inability of Akt survival pathway to inhibit CD150-induced cell death.

Although the PI3K/Akt pathway inhibits apoptosis and promotes cell cycle progression and proliferation [24], the consequences of its activation in HRS cells remain currently unclear. Though Dutton *et al.* [44] showed that HL cell lines and primary HL tumor cases were characterized by high levels of phosphorylated/activated Akt, inhibition of PI3K and mTOR had quite modest effect on cell survival. At the same time, other authors [19] demonstrated that PI3K inhibitor LY294002 had anti-proliferative and pro-apoptotic effects on some studied HL cell lines. Moreover, CD150-stimulation of L1236 HL cell line induced cell death, though this cell line was characterized by simultaneous fast and marked Akt activation via CD150 receptor [18]. Thus, outcome of PI3K/Akt signaling cascade may depend on interaction with other signaling pathways that are initiated by different stimuli from HL microenvironment.

Taken together, in current study we have shown that CD150 ligation induced Akt phosphorylation/activation in normal TBC, LCLs and HL cell lines. It could be assumed that CD150-mediated activation of Akt kinase depends on CD150-SH2D1A interaction followed by phosphorylation of CD150ct and attraction of p85 $\alpha$  regulatory subunit of PI3K. Akt kinase, activated via CD150, could phosphorylate its downstream target FoxO1 transcription factor in normal B cells, as well as in immortalized HL and B-lymphoblastoid cell lines. At the same time Akt phosphorylation of its substrate



GSK-3 $\beta$  kinase was detected only in HL cell line L1236 and LCL MP-1, but not in normal TBC. CD150-mediated Akt signaling, followed by regulation of GSK-3 $\beta$  and FoxO1 activity in EBV-transformed and HL tumor cells could interplay with other CD150-initiated signaling pathways (i.e. MAPK) creating signaling network favorable for maintaining the survival program and escaping apoptosis of transformed B cells.

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