ORIGINAL CONTRIBUTIONS

INTRINSIC DEFECT IN B-LYMPHOBLASTOID CELL LINES FROM PATIENTS WITH X-LINKED LYMPHOPROLIFERATIVE DISEASE TYPE 1. I. CELL SURFACE PHENOTYPE AND FUNCTIONAL STUDIES

*L.M. Shlapatska, L.M. Kovalevska, I.M. Gordiienko, S.P. Sidorenko**
Department of Cell Regulation, R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv 03022, Ukraine

Background: Mutations in *SH2D1A/DSHP/SAP* gene are responsible for the onset of X-linked lymphoproliferative disease type 1 (XLP1) that have increased risk for B-cell lymphoma development. In XLP1 patients SAP deficient NK, NKT and CD8^{+} cytotoxic T cells are inefficient in eliminating EBV-infected proliferating B cells that may partially contribute to the lymphoma development. However, little is known about impairment of B cell characteristics in XLP1. *Aim:* To analyze the cell surface phenotype and functional characteristics of EBV-transformed B-lymphoblastoid cell lines from XLP1 patients (XLP B-LCLs) in comparison with conventional B-lymphoblastoid cell lines (B-LCLs). *Methods:* Studies were performed on SAP-negative B-LCLs T5-1, 6.16, RPMI 1788; SAP-positive B-LCL MP-1 and XLP B-LCLs IARC 739, XLP-D, XLP-8005. Cell surface immunophenotyping was performed using flow cytometry analysis. The level of apoptotic cells (Annexin V-binding), cell viability (MTT assay), and cell proliferation (trypan blue exclusion test) were evaluated in response to ligation of CD40, CD95, CD150 and IgM cell surface receptors. *Results:* A cell surface phenotype and functional features that distinguish XLP B-LCLs from conventional B-LCLs were revealed. XLP B-LCLs showed the upregulated level of CD20, CD38 and CD86 cell surface expression and downregulation of CD40, CD80 and CD150 expression. The major functional differences of XLP B-LCLs from conventional B-LCLs concern the modulation of CD95 apoptosis via CD40 and CD150 receptors and unresponsiveness to proliferative signals triggered by CD40 or colligation of BCR with CD150. *Conclusion:* The data suggest that the B-LCL from XLP1 patients have an intrinsic defect that affects cell activation, apoptosis, and proliferation.
Key Words: B-lymphoblastoid cell lines, X-linked lymphoproliferative disease type 1, CD150, CD40, CD95, apoptosis.

X-linked lymphoproliferative disease (XLP) belongs to X-linked genetically determined primary immunodeficiency syndromes that affect both innate and adaptive immunity. XLP is characterized by severe immune dysregulation, often triggered by Epstein — Barr virus (EBV) infection, with clinical manifestations that include fatal mononucleosis, hemophagocytic lymphohistiocytosis (HLH), B-cell lymphoproliferative disorder, B-cell lymphoma, and dysgammaglobulinemia [1–3]. XLP as rare inherited X-linked immune deficiency (Duncan's disease) was first described by Purtilo et al. in 1975 [4], however the first report should be credited to Bar et al. presenting circumstantial evidence for the association of EBV infection with fulminating lymphoproliferative disease [5]. XLP affects from 1 to 3 million boys in the world, and usually develops in childhood or early adolescence [6]. In response to EBV infection XLP patients develop a fulminant form of infectious mononucleosis with high rate of mortality. Uncontrolled proliferation of EBV infected B cells frequently results in severe lymphoproliferative illness with clinical features of fever, hepatosplenomegaly, lymphadenopathy, and pancytopenia. Clinical manifestations could be acute, like HLH or lymphoma, or less aggressive with recurrent infections and dysgammaglobulinemia. XLP patients often demonstrate more than one phenotype with tendency of progression to more aggressive disease manifestations [6].

The substantial progress in understanding the XLP pathophysiology was made with revealing the molecular basis of this immunodeficiency. First, the XLP gene was mapped to the Xq24–27 of the long arm of the X chromosome, and interstitial deletion of Xq25 was described in a male with XLP [1, 7]. And only in 1998 three groups reported mutations in *SH2D1A/DSHP* gene that are responsible for XLP [8–10]. More than 70 mutations in *SH2D1A* resulting in XLP have been reported to the Human Gene Mutation Database (HGMD), however they were found in 60–70% of XLP cases [1–3]. This stimulated the search for other genetic abnormalities in XLP. In 2006 it was found that mutations of *BIRC4/XIAP*, encoding X-linked inhibitor of apoptosis (XIAP), were associated with XLP phenotypes [11]. Clinical entities linked to SH2D1A and XIAP deficiencies now are referred as XLP type 1 (XLP1) and type 2 (XLP2) respectively [1–3]. In addition, recessive mutations of autosomal genes encoding IL-2-inducible T cell kinase (ITK) [12, 13], CD27 [14], and Coronin 1A [15] are responsible for XLP-like syndromes with EBV-associated lymphoproliferation.

SH2D1A gene encodes the adaptor protein SH2 domain protein 1A (SH2D1A), also called Duncan's disease SH2-protein (DSHP) or SLAM-associated protein (SAP) [8–10]. This small 128 aa protein was first identified as an adaptor protein interacting with signaling lymphocyte activation molecule (SLAM)/CD150 [10]. It was demonstrated that SAP forms complexes with other members of SLAM family receptors by binding to conserved immunoreceptor tyrosine-based switch motif (ITSM) and recruiting Src-family kinases Fyn or Lyn [2, 16–18]. By interacting with SLAM family receptors, SAP is involved in regulation of T-cell help for germinal centers formation, development of NKT cells, cytotoxicity of $CD8^+$ T lymphocytes and NK cells, and T-cell homeostasis mediated by reactivation-induced cell death (RICD) [19–23]. It becomes clear now that function of SH2D1A comprises different biological processes and is much wider than regulation of signaling via SLAM family receptors. Besides ITSM, SAP was shown to bind also other tyrosine-based motifs. By direct interaction with the first immunoreceptor tyrosine-based activation motif (ITAM) of CD3zeta chain, SAP plays a central role in the T cell activation [24]. In B cells SAP is able to bind immunoreceptor tyrosine-based inhibitory motif (ITIM) in FcgRIIb [17] and directly interacts with CD22, possibly also via ITIM [25].

Defects in T, NK and NKT functions and/or development upon SAP deficiency and limited information on expression and function of SAP in B cells contributed to the perception that XLP1 is a disease mainly mediated by defective interactions between lymphocytes. However this cannot account for all clinical manifestations in XLP, especially dysgammaglobulinemia and development of B cell lymphoma [6, 26, 27]. SAP was shown to be expressed in B cells in mouse spleen and germinal centers, subpopulation of human tonsillar B cells, few B-lymphoblastoid cell lines (B-LCLs), EBV-positive type I Burkitt lymphoma cell lines, Hodgkin's lymphoma cell lines, and its expression could be induced by DNA damage in various cell lines of B-cell origin [17, 25, 28–33]. Moreover, it was shown that activation of endogenous wild type p53 in Burkitt lymphoma and B-lymphoblastoid cell lines induce SAP expression [33], and the fate of Burkitt lymphoma precursor cells is decided by the expression in B cells of the proapoptotic SAP and EBV infection [34]. All these indicate that SAP is expressed at the narrow window of B cell differentiation, and SAP deficiency could also affect B-cell maturation. To address the question whether B cells in patients with XLP1 have intrinsic defect, we studied EBV-transformed B-lymphoblastoid cell lines from XLP1 patients (XLP B-LCLs) in comparison with conventional B-LCLs. In the first report we focused on the cell surface phenotype and functional characteristics, and the second report will cover cell signaling pathways and transcription factors expression profile.

MATERIALS AND METHODS

Cell lines and stimulation. Studies were performed on EBV-transformed SAP-negative B-LCLs T5–1, 6.16, RPMI 1788; SAP-positive B-LCL MP-1 (provided by Prof. E.A. Clark, USA); and EBV-transformed B-LCLs from XLP1 patients IARC 739 (interstitial deletion of SH2D1A), XLP-D (C→T mutation at position 462), XLP-8005 (C→T mutation at position 471), kindly provided by Prof. K.E. Nichols (USA). Cell lines were maintained as previously described [30]. For cross-linking of surface receptors, we used the following monoclonal antibodies (mAbs): IPO-3 anti-CD150 (IgG1) (IEPOR NANU); IPO-4 anti-CD95 (IgM) (IEPOR NANU); and G28–5 anti-CD40 (IgG1) (kind gift of Prof. E.A. Clark, USA). F(ab)'$_2$ of goat anti-human IgM (Jackson ImmunoResearch Laboratories, Inc., USA) were used for IgM cross-linking on human B cell lines. Following final concentrations of antibodies were used: 0.5 mg/ml anti-CD40, 1.0 mg/ml anti-CD95, 10.0 mg/ml anti-CD150, and 10.0 mg/ml anti-IgM.

Flow cytometry. Cell lines were immunophenotyped using standard immunofluorescent technique [30] and analyzed with EpicsXL fluorescent flow cytometer (Beckman Coulter, USA). Following directly labeled monoclonal antibodies were used in this study: anti-IgD-PE and anti-CD23-FITC (Becton Dickinson, USA), anti-CD20-PE (Pharmingen, USA); anti-CD38-PE, anti-CD80-FITC and anti-CD86-PE (all from Immunotech, USA). The panel of monoclonal antibodies against CD27 (Becton Dickinson, USA), CD19 (clone CB19) (IEPOR NANU), anti-CD150 (clone IPO-3) (IEPOR NANU), anti-CD95 (clone IPO-4) (IEPOR NANU), anti-CD40 (clone G28–5) (kind gift of Prof. E.A. Clark, USA), and anti-IgM (DAKO, Denmark) were used in indirect immunofluorescent analysis with secondary FITC conjugated goat anti-mouse polyvalent Ig (Sigma, USA). Apoptotic cells were detected using an Annexin V-FITC apoptosis detection kit (Sigma, USA) according to manufacturer's protocol. The data were analyzed using FCS Express 3 software (De Novo Software, USA).

Cell viability assay. The MTT assay was used to assess the cell viability. Cell lines were seeded at $1 \cdot 10^4$ cells per well in 96-well plates and treated with various combinations of monoclonal antibodies for 24 h and 48 h. Subsequently, 20 ml of stock solution (5 mg/ml) of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well. After incubation for 4 h at 37 °C, the multiwell plate was centrifuged to pellet formazan crystals, the medium was discarded, and 100 ml of DMSO was added to each well to dissolve the formazan crystals. The absorbance of formazan at 540 nm was measured by Labsystems Multiskan PLUS (USA). The mean of absorbance for 3 replicates from three independent experiments were used for statistical analysis. The cell viability is presented as a percentage ratio of absorbance of exposed cells to control cells.

Evaluation of cell proliferation. To assay for cell proliferation at 24 h and 48 h time points, each cell line was seeded at a density of $1 \cdot 10^4$ cells per 100 ml in 96-well plate and incubated at 37 °C in triplicates with 100 ml of media or with antibodies against

CD40, CD95, CD150, and IgM alone or in combinations. Proliferation profiles were obtained by direct cell counting using trypan blue exclusion test.

RESULTS AND DISCUSSION

Characterization of cell surface phenotype of EBV-transformed B-lymphoblastoid cell lines from XLP1 patients in comparison with B-LCLs was an initial step of our studies. B-LCLs T5-1, 6.16, RPMI 1788; MP-1 and EBV-transformed B-LCLs from XLP1 patients IARC 739, XLP-D, and XLP-8005 expressed B-lineage specific cell surface markers. While the level of CD19 cell surface expression was similar in all cell lines, XLP-LCLs had higher level of CD20 expression than B-LCLs (Fig. 1). For example, the mean of fluorescent intensity (MFI) for CD20 expression in XLP B-LCL IARC 739 reached 356, in comparison with 89 and 70 in B-LCLs T5.1 and MP-1 respectively. All tested cell lines were IgM positive, however they were heterogeneous by IgD expression (see Fig. 1). XLP B-LCLs and B-LCLs had comparable levels of CD40, CD48, CD95, and CD150 expression; however CD40 and CD150 expression levels in XLP-LCLs were slightly lower than in B-LCLs (see Fig. 1). In IARC 739 the MFI of CD40 and CD150 expression were 115 and 62, whereas in T5-1 they were 150 and 138, and in MP-1 reached 220 and 156, respectively. In general, XLP B-LCLs and B-LCLs had similar levels of CD23 cell surface expression, with exception of B-LCL MP-1, which expresses SAP on protein level (see Fig. 1). XLP B-LCLs, as well as B-LCLs, did not express CD27 (data not shown). XLP B-LCLs were characterized by elevated levels of CD38 and CD86 cell surface expression in comparison with B-LCLs (see Fig. 1). As an illustration, the MFI of CD86 expression in IARC 739, T5-1 and MP-1 were 1007, 403 and 411 respectively, so in XLP B-LCL it was more than twice higher (see Fig. 1). At the same time, in XLP-LCLs the expression level of CD80 was only slightly lower than in B-LCLs (see Fig. 1). Thus, XLP-LCLs have similar to B-LCLs expression levels of CD19, CD40, CD48, CD80, CD95, CD150 and IgM. However, we found elevated levels of CD20, CD38 and CD86 in EBV-transformed B-lymphoblastoid cell lines from XLP1 patients. Since all tested cell lines had comparable level of CD40, CD95, CD150 and IgM cell surface expression, we tested how ligation of these receptor would affect apoptosis, proliferation and viability of XLP B-LCLs in comparison with conventional B-LCLs.

A number of cell surface receptors, especially B-cell receptor (BCR) and members of TNFR family
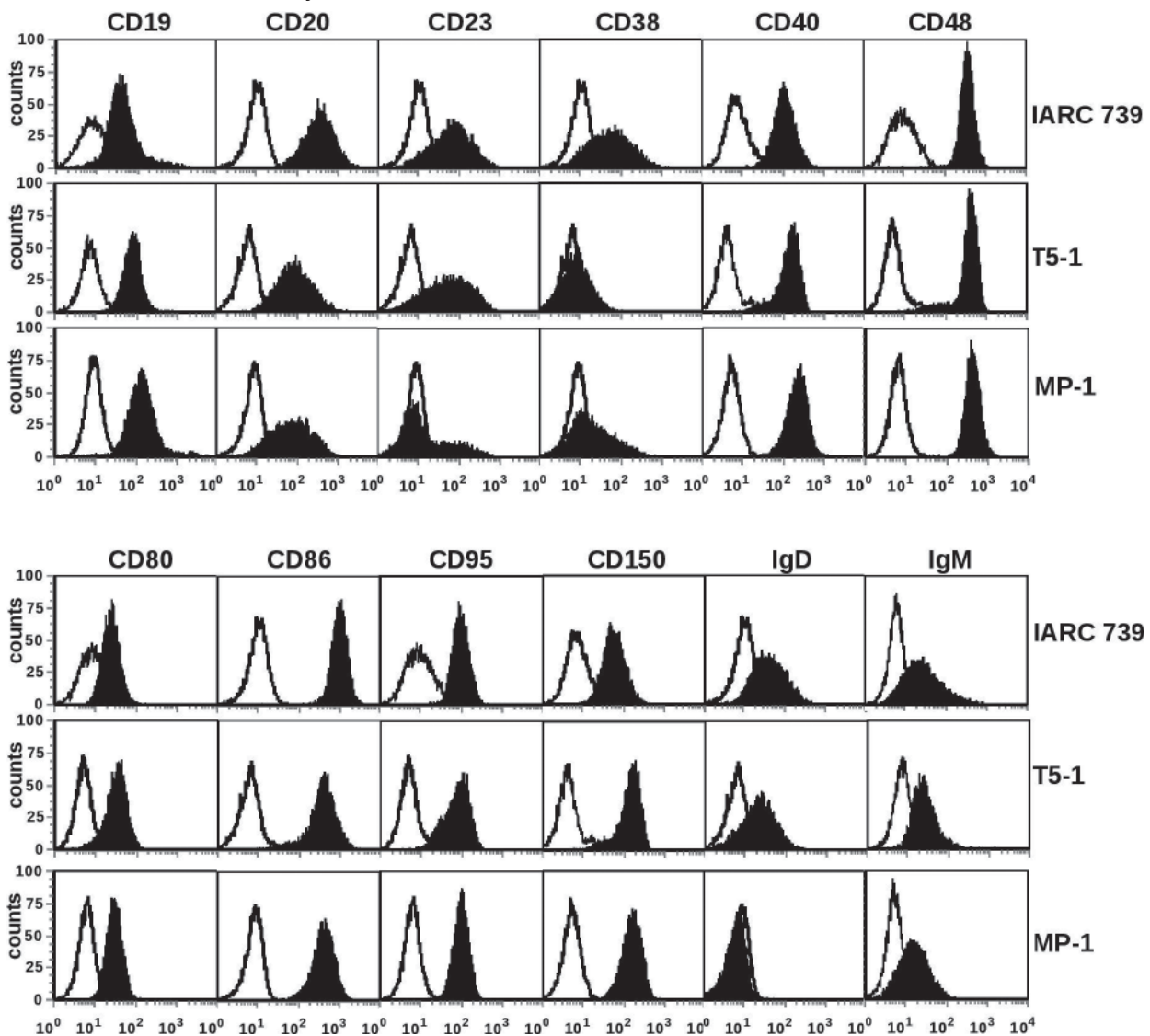
Fig. 1. Cell surface phenotype of XLP B-LCL IARC 739 in comparison with B-LCLs T5–1 (SAP⁻), and MP-1 (SAP⁺). Flow cytometry analysis

are involved in regulation of cell survival during germinal center reactions [35, 36]. Here we tested the level of sensitivity to CD95-mediated apoptosis and its modulation via cell surface receptors in EBV-transformed B-lymphoblastoid cell lines from XLP1 patients. All tested cell lines had comparable levels of sensitivity to CD95-mediated apoptosis induced with anti-CD95 antibody IPO-4 (IC_{50} ranging from 0.75 to 1.5 mg/ml). So, in all experiments the concentration 1 µg/ml of anti-CD95 mAb was used for induction of apoptosis. After 8 hours of incubation with anti-CD95 mAb the levels of Annexin V-binding cells in all tested cell lines were in the range of 20–40% (Fig. 2, a, b).
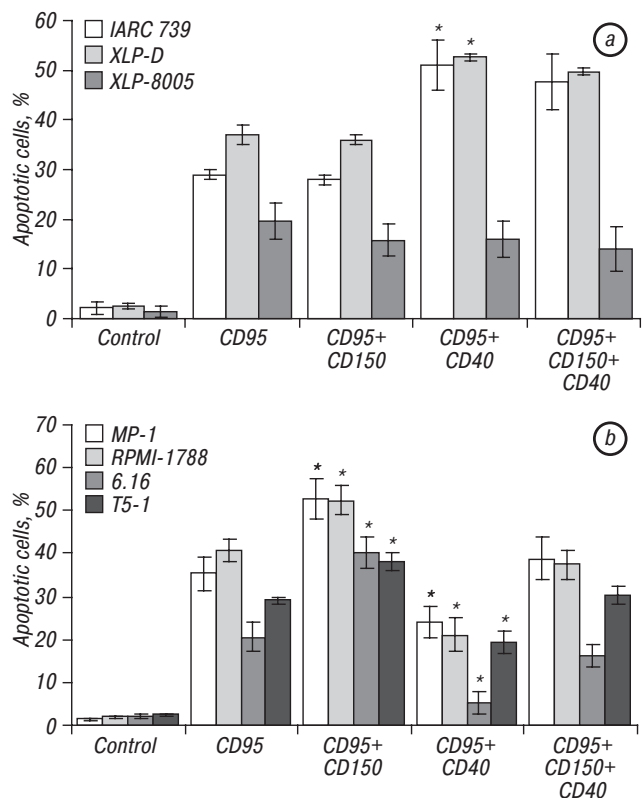
Fig. 2. Modulation of CD95-mediated apoptosis via CD40 and CD150 cell surface receptors. a — XLP B-LCLs IARC 739, XLP-D and XLP-8005. b — B-LCLs MP-1 (SAP+) and SAP- B-LCLs RPMI 1788, 6.16 and T5–1. Percent of Annexin V-FITC binding cells after 8 hours of incubation with antibodies. Flow cytometry analysis. Data of four independent experiments are presented as the mean±SD. *p < 0.05, vs the effect of CD95 alone

It was previously shown that in B-LCLs derived from healthy individuals, as well as in EBV-positive Burkitt's lymphoma cell lines Raji and BJAB, signals via CD40 and CD150 did not induce cell death, but modulated CD95-mediated apoptosis [28]. Indeed, in either SAP+ (MP-1) or SAP- B-LCLs, CD40 ligation before of together with CD95 partially rescued cells from apoptosis (see Fig. 2, b). However, in XLP B-LCLs IARC-739 and XLP-D signals via CD40 were synergistic with CD95-mediated apoptosis (p < 0.05) (see Fig. 2, a). CD150 and CD95 co-ligation augmented the number of apoptotic cells (compared to the effect of CD95 alone) in all tested B-LCLs derived from healthy individuals (p < 0.05) (see Fig. 2, b), but did not alter the level of CD95-induced apoptosis in XLP B-LCLs (see Fig. 2, a). Moreover, while combination of signals via CD40 and CD150 did not affect the level of CD95-mediated apoptosis in B-LCLs (see

Fig. 2, b), in XLP B-LCLs CD150 ligation did not reduce synergistic effect of CD40 and CD95 (see Fig. 2, a). It should be noted that in cell line XLP-8005 neither CD40, nor CD150 cross-linking changed the amplitude of CD95-induced cell death (see Fig. 2, b). These tendencies persisted for 24 and 48 h after cell stimulation, as accessed by cell viability (MTT test) and cell number in the cultures (Fig. 3, a, b). BCR (IgM) ligation practically did not change the number of cells in the culture of three tested cell lines, but reduced their viability (see Fig. 3, a, b). Signals via BCR, which in primary B cells protect from CD95-mediated apoptosis [37, 38], in EBV-transformed B-LCLs were synergistic with CD95 that resulted in significant reduction of cell number and their viability (p < 0.05) (see Fig. 3, a, b). It should be noted that such synergistic effect was observed in XLP B-LCL IARC 739, as well as in SAP+ and SAP- B-LCL MP-1 and T5-1 (see Fig. 3, a, b).
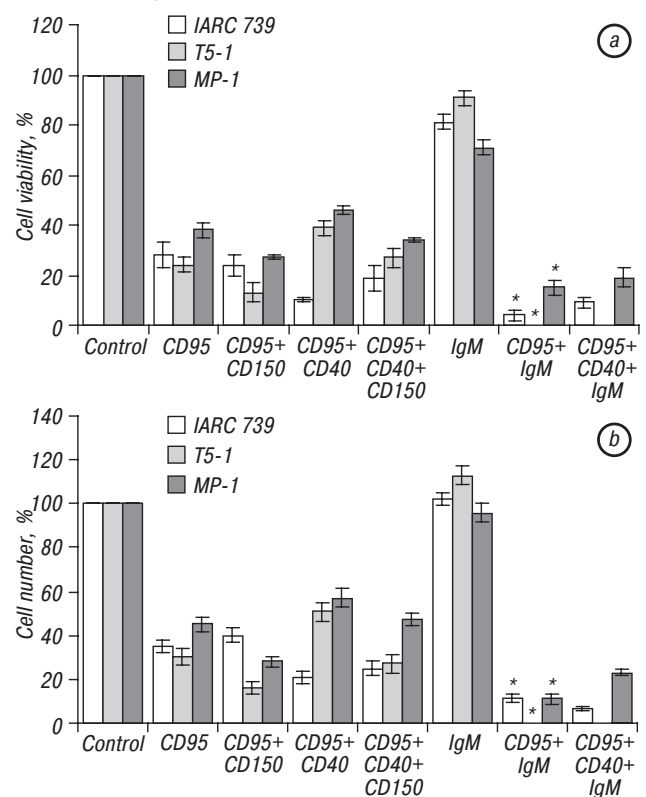
Fig. 3. Modulation of CD95-mediated apoptosis via CD40 and CD150 cell surface receptors in XLP B-LCL IARC 739 in comparison with B-LCLs T5-1 (SAP-), and MP-1 (SAP+) after 48 h of incubation with antibodies. a — cell viability (MTT assay, absorbance at 540 nm); b — cell number (trypan blue exclusion test). The data of three independent experiments are presented as a percent of control value (mean ± SD). *p < 0.05, vs the effect of CD95 alone

Since EBV-infected B-lymphocytes acquire proliferative potential, we addressed the question whether signals via BCR, CD40 and CD150 may contribute to the proliferation of B-LCLs, including XLP1 derived cell line IARC 739. CD40 ligation resulted in proliferative effect in B-LCLs, however it significantly reduced the number of cells in XLP1 IARC 739 cell culture, and colligation with BCR did not change these tendencies (p < 0.05) (Fig. 4). CD150 ligation alone did not affect cell proliferation, but diminished CD40-mediated signals in B-LCLs, but not in IARC 739 (see Fig. 4). At the same time, colligation of CD150 with BCR resulted

in strong proliferative effect in T5-1 cells, but slightly reduced cell numbers in MP-1 and IARC 739 cell cultures ($p < 0.05$) (see Fig. 4). Thus, the major distinct features of XLP B-LCLs are the modulation of CD95 apoptosis via CD40 receptor and unresponsiveness to proliferative signals triggered by CD40 or colligation of BCR with CD150.
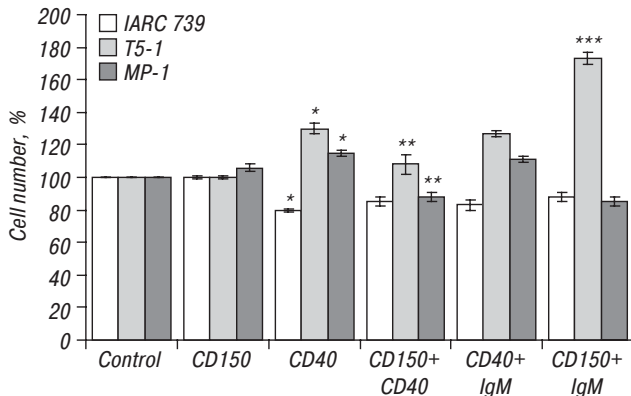
Fig. 4. Effect of CD40, CD150 and IgM cell surface ligation on the number of viable cells. XLP B-LCL IARC 739 is unresponsive to proliferative signals triggered by CD40 or colligation BCR with CD150. The cell number (trypan blue exclusion test) in XLP B-LCL IARC 739 in comparison with B-LCLs T5-1 (SAP⁻), and MP-1 (SAP⁺). The data of three independent experiments are presented as a percent of control value (mean ± SD). *$p < 0.05$, compared to control; **$p < 0.05$, *vs* the effect of CD40 alone; ***$p < 0.01$, as compared to control

EBV is a ubiquitous gamma-herpesvirus that latently infects > 90% of the world's human population. The primary targets of EBV are B lymphocytes, but EBV can also infect other cell types, including epithelial cells, monocytes and DCs [39, 40]. During primary infection, EBV infects resting B lymphocytes and expresses viral gene products driving proliferation of infected B cells. The ability of EBV to restrict expression of its genes allows the virus to persist *in vivo* within resting memory B cells and maintain a lifelong infection. Symptoms of EBV infection are typically mild and often indistinguishable from other mild common illnesses [39, 40]. However, EVB only seems to be an inoffensive virus. It was initially discovered in a Burkitt's lymphoma that had B-cell origin [34]. EBV, classified now by the WHO as a human DNA tumor virus, is associated with various malignancies, such as Hodgkin's and Burkitt's lymphoma, life-threatening post-transplant lymphoproliferative disorders and nasopharyngeal carcinoma [34, 41]. Moreover, deletions or mutations in a small adaptor protein (SH2D1A/DSHP/SAP) diverts EBV infection from benign persistence to the acutely aggressive X-linked lymphoproliferative disease (XLP1) that may rapidly kill the infected individuals. Moreover, the risk for lymphoma development in XLP1 patients is increased 200-fold [34]. What are the challenging factors that so dramatically increased the rate of lymphoma development in XLP1 patients? From one side, in XLP1 patients SAP deficient NK, NKT and CD8⁺ cytotoxic T cells are inefficient in elimination of EBV-infected proliferating B cells that may contribute to the lymphoma development [2, 3, 42].

However, it is clear that several XLP1 clinical features are EBV independent, and lymphoma development is documented both in EBV-positive and EBV-negative XLP1 patients [43–45]. This suggests that the absence of functional SAP has to affect the B cells as well. Our study presents the evidence of intrinsic defect in B-LCLs derived from XLP1 patients. We used the B-LCLs because EBV-transformed B cells are an important model for studying cellular transformation, especially B-LCLs from XLP1 patients, which have increased rate of B-cell lymphoma development. Immunophenotyping of B-LCLs revealed variations in surface expression of B-cell activation markers, especially upregulation of CD38 and CD86 expression in XLP B-LCLs. At the same time, the level of surface expression of IgM, CD40 and CD95 — the main receptor regulators of B cell fate — was comparable in XLP B-LCLs and SAP⁻ as well as SAP⁺ B-LCLs (see Fig. 1).

In vitro immortalization of B cells by EBV, which leads to the establishment of B-LCLs, is accompanied by the expression of a restricted set of viral genes, the so-called latency III program, coding for latent membrane proteins (LMP1, LMP2a, LMP2b), Epstein — Barr nuclear antigens (EBNA1, 2, 3A, 3B, 3C, LP), and small non-coding RNAs (EBERs) [34, 40]. EBV infection, in particular LMP1, was shown to upregulate CD95 expression and to sensitize B cells to CD95-mediated apoptosis [46]. Indeed, all tested B-LCLs, including XLP B-LCLs, were sensitive to CD95-initiated apoptosis (Fig. 2). However, we found the differences in modulation of CD95-mediated apoptosis via cell surface receptors in XLP B-LCLs.

In normal B cell physiology, the CD40 receptor is one of the key regulators of B-cell activation and proliferation [47–49]. CD40 ligation *in vitro* triggers B-cell adhesion, sustained proliferation, and differentiation. The engagement of CD40 on B cells by CD40L/CD154 is essential for initiation and progression of T-cell dependent humoral immune response. CD40 is required for germinal center formation and progression, antibody isotype switching and affinity maturation that are crucial for the generation of memory B cells and long-lived plasma cells. CD40 signaling has been shown to protect immature or mature B cells from BCR-mediated apoptosis [47]. In primary normal B cells CD40 ligation induces rapid and marked upregulation of CD95 expression and enhances sensitivity to CD95-mediated apoptosis, while BCR engagement rescues from apoptosis [48]. However, CD40 may play anti-apoptotic role by rescue from CD95-mediated apoptosis in EBV-transformed or malignant lymphocytes [50–52]. Indeed, in B-LCLs CD40 signals partially inhibited CD95-mediated apoptosis (see Fig. 2, *b*), but in XLP B-LCLs colligation of CD40 and CD95 enhanced apoptosis (Fig. 2, *a*). CD150 alone did not affect cell viability; however, CD150 and CD95 colligation elevated CD95-mediated apoptosis in B-LCLs. We did not reveal the peculiarities of XLP B-LCL in modulation of CD95 mediated signaling via IgM: in all tested B-LCLs IgM colligation with CD95 significantly reduced cell number and viability

(Fig. 3, *a, b*). Signals via CD40 alone exert a low proliferative effect and often are co-stimulatory with other stimuli [49]. We found that, in contrast to B-LCLs, CD40 ligation on IARC 739 XLP cells inhibited cell proliferation; these cells were also unresponsive to proliferative signals triggered by CD150 colligation with IgM (Fig. 4).

Taking together, our studies revealed cell surface phenotype and functional features that distinguish XLP B-LCLs from conventional B-LCLs. These include upregulation of CD20, CD38 and CD86 cell surface expression and slight downregulation of CD40, CD80 and CD150 expression. The major functional differences of XLP B-LCLs from conventional B-LCLs are in the modulation of CD95 apoptosis via CD40 and CD150 receptors and unresponsiveness to proliferative signals triggered by CD40 or colligation of BCR with CD150. Our subsequent paper will embrace some cell signaling studies that underlie functional peculiarities of XLP B-LCLs.

ACKNOWLEDGEMENT

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