Potential role of hypoxia in early stages of Hodgkin lymphoma pathogenesis

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Supporting Online Material for

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Methods

Cell culture and B cell isolation

The Burkitt lymphoma cell lines BL41, Raji, and Daudi, the GC B cell-type diffuse large B cell lymphoma (GCB-DLBCL) cell lines SUDHL4 and SUDHL6, the cHL cell lines L428, KMH2, UHO1, L1236, and HDLM2, the HEPG2 cells and primary human B lymphocytes were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS, Biochrom AG, Berlin, Germany). 293T cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Aidenbach, Germany) with 10% FCS. All media were supplemented with 1% penicillin-streptomycin (Invitrogen Carlsbad, USA). Cells were incubated at 37°C in a 5% CO_2 incubator. Peripheral blood (PB) B cells were isolated from full blood donations or buffy coats (termed PB B cells, if not labelled otherwise) by Ficoll density gradient centrifugation (Amersham, Freiburg, Germany) and subsequent CD19-MACS strategy (Miltenyi Biotech, Bergisch-Gladbach, Germany) to a purity of >98%.

Hypoxic cell culture

Twentyfour hours prior exposure to hypoxia (1% O_2), DLBCL and HL cell lines underwent a standard cell expansion procedure to maintain a uniform cell density and growth rate. Primary B cells were incubated in culture medium to rest for at least 3 h after isolation. Equal cell amounts were distributed to cell culture plates using the same culture medium. While the control cells remained in a regular incubator, one cell plate was placed in an hypoxic chamber (Invivo 400 Workstation in combination with Gasmixer Q for indicated time points; Baker and Ruskinn, Bridgend, South Wales) whose temperature (37°C) and O_2 concentration was set to adjust several hours before the experiment. An initial O_2 calibration was set according to the atmospheric O_2 concentration and adjustment took place every minute by replacing oxygen with nitrogen, thereby maintaining almost identical conditions for hypoxic and control cells. Unpublished data indicate no measurable pH-changes for the time points that were used in our experimental set ups. Successful and reproducible HIF activation under hypoxic culture conditions was controlled in all experiments by measurement of VEGF mRNA levels.

DUSP1/6 inhibition and quantification of cell viability

Cell lines were exposed to increasing concentrations of the dual specificity protein phosphatase (DUSP) 1/6 small molecule inhibitor (“Dual Specificity Protein Phosphatase 1/6 Inhibitor”; Empirical Formular $C_{22}H_{23}NO$; Merck Millipore, Darmstadt, Germany), a cell-permeable cyclohexylamino-indenone compound that acts as an allosteric inhibitor against substrate binding-induced MAPK phosphatase activity of DUSP1 and DUSP6 (57% and 30%

reduction, respectively), for 72 h. The inhibitor is hereafter called DUSP1/6i. The number of living cells was determined according to their metabolic activity with a 20% CellTiter 96 Aqueous One Solution (Promega Corp., Madison, WI, USA), which contains 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTS-tetrazolium). After incubation at 37°C for 90 min, the absorbance of the solutions was determined at 490 nm using a 96-well plate reader (SAFIRE, Tecan, Austria).

Western blot

Protein extract preparation and Western immunoblotting were performed as described.[28] The lysis buffer contained IGEPAL instead of NP-40 and the primary antibody against HIF-1α (BD Transduction Laboratories, San Jose, USA) was diluted 1:1 000. Additionally, we detected JUN by using the c-Jun antibody clone sc-74543 (Santa Cruz, Dallas, USA; 1:2 000), and β-actin (Sigma-Aldrich, St. Louis, USA; 1:2 000) as a loading control. As secondary antibodies peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Sigma-Aldrich) were used at a 1:10 000 dilution. Immunoreactive proteins were visualized using the Luminol Coumaric acid H_2O_2 system followed by digital detection (Fusion FX7, Peqlab, Erlangen, Germany).

Flow cytometry and survival assay

To analyse the expression of B cell surface markers, their mean fluorescence intensity (MFI) was determined by flow cytometry using a FACS-Canto cytometer (Becton Dickinson, Heidelberg, Germany). Cells were stained with anti-CD19-PE (BD Biosciences, Becton Dickinson) and anti-CD79b-FITC (SN8, DakoCytomation, Hamburg, Germany) antibodies. Cell survival was analysed using the BD Biosciences Annexin V Apoptosis Detection KIT and propidium iodide (PI) (Invitrogen, Carlsbad, USA). For combined analyses only annexin V–APC negative (living) cells were gated to determine the MFI of anti-CD19-PE and anti-CD79b-FITC staining.

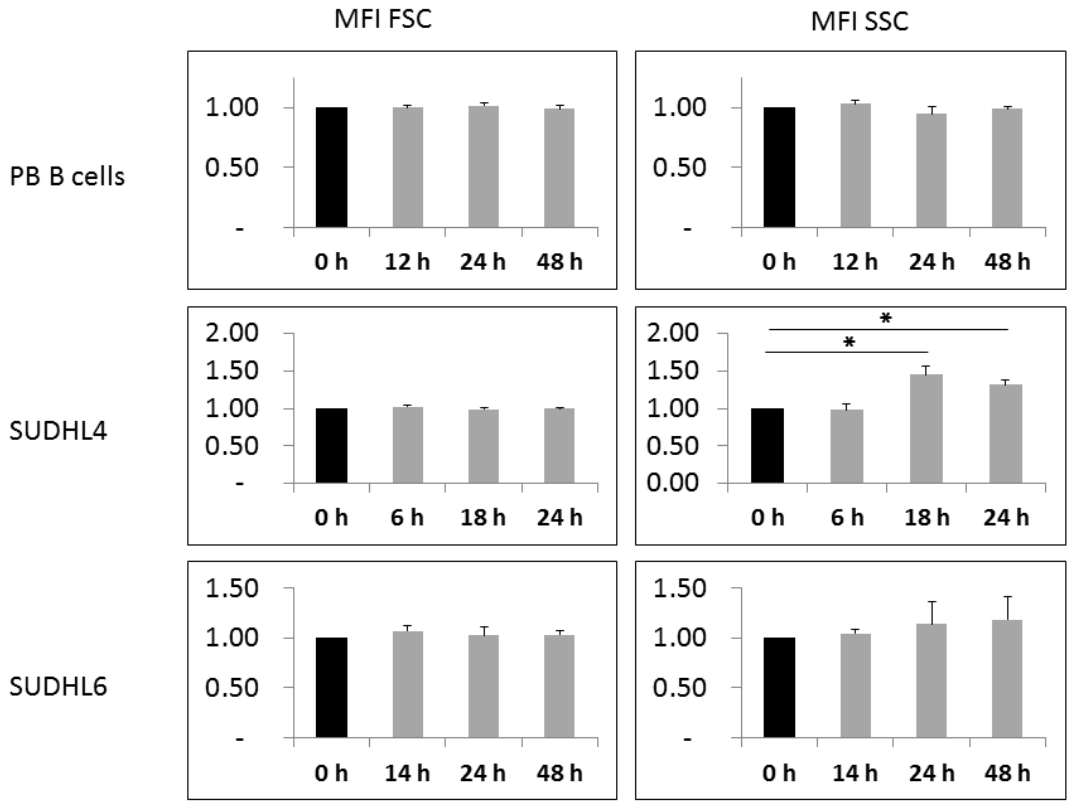
RNA isolation, cDNA synthesis and real-time reverse transcription (RT)-PCR

Cells were lysed and disrupted with Qiashredder™ (Qiagen, Hilden, Germany). Total RNA was prepared from 0.5-1x 10^6 cells using the peqGOLD MicroSpin Total RNA Kit (Peqlab). Ten µL of total RNA were reverse transcribed using the High capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time RT (qRT)-PCR analysis was performed on an ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems) using predesigned Taqman assays (Applied Biosystems) or specific primers and the SYBR Green PCR Master Mix (Sigma) (Suppl. Table 1).

Supplementary Table 1**. Assays and primer sequences used for quantitative real-time PCR***

Gene	Taqman assay	Forward primer (5' to 3')	Reverse primer (5' to 3')
ACTB	Hs99999903_m1		
CD79B	Hs00236881_m1		
ID2	Hs00747379_m1		
CD19	Hs00174333_m1		
PAX5	Hs00277134_m1		
NOTCH1	Hs01062014_m1		
DTX1	Hs00269995		
HES	Hs0017287		
HEY1	Hs00232618		
EBF1	Hs01092694		
LCK	Hs00178427		
MYC	Hs00153408		
VEGFA	Hs00900055		
ACTB		AGCCTCGCCTTTGCCGATC	AGCGCGGCGATATCATCATCC
BCL2A1		GATTGTGGCCTTCTTTGAG	GTTCCACAAAGGCATCC
IL6		GCAGAAAAAGGCAAAGAATC	CTACATTTGCCGAAGAGC
IRF4		CAATGACTTTGAGGAACTGG	CATCATGTAGTTGTGAACCTG
DUSP1		ACTACCAGTACAAGAGCATC	GATTAGTCCTCATAAGGTAAGC
KDM4C		CAGGTTGAGTTTGAAGATGG	TTCACTCTCTTGGGTAACTC
POU2AF1/ BOB1		CCAATGTCACGACAAGAAG	GGCTCTGGGATAGAGATG
PCGF2		CATCGAATTCTACGAAGGTG	GTTTTCTCTTTGTCCCCATC

*For Taqman assays, the ordering number is given, for genes analyzed with the SYBER green method, the primer sequences are given.



Supplemental Figure 1. **The effect of hypoxia on cyto-morphological features as determined by forward and side scatter analysis**

For each experiment aliquots of cells was cultured under normal atmospheric or under hypoxic (1% O_2) conditions for time periods as indicated. Given is the quotient of hypoxia MFI / normoxia MFI values. For PB B cells, SUDHL4 cells and SUDHL6 cells 13, 3 and 9 independent experiments were performed, respectively. *p < 0.05. MFI: mean fluorescent intensity, FSC, forward scatter, SSC, side scatter.