SUPPLEMENTARY APPENDIX

Identification of the atypically modified autoantigen Ars2 as the target of B-cell receptors from activated B-cell-type diffuse large B-cell lymphoma

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Supplement:

Supplementary Methods:

Cell lines

DLBCL cell lines included were OCI-Ly3, OCI-Ly10, U2932, Su-DHL-6, Su-DHL-8, Farage, and Karpas 422, obtained from the Dr. Senckenberg Institute of Pathology (Frankfurt a. M., Germany), and HBL-1 and TMD8 obtained from the Department of Hematology of Göttingen Medical School (Göttingen, Germany). OCI-Ly3 was also obtained from DSMZ.

Immunoglobulin variable region gene PCR and expression cloning of BCRs

Pools of 30 DLBCL cells microdissected from cryosections were digested with 2 µL of proteinase K (Roche PCR grade) for 4 h at 55°C followed by inactivation for 10 min at 95°C. Subsequently, semi-nested PCRs for VH, Vκ, and Vλ were performed.[1] Similar variable (V) region gene PCRs were performed with DLBCL cell lines. The resulting V genes were analyzed by IMGT-V-Quest. Corresponding functional heavy- and light-chain genes were cloned into modified pCES-1 vector for expression of BCRs in Fab format in *Escherichia coli* TG1 strain, as previously described.[2]

Screening lymphoma BCR for reactivity against infectious antigens

Heat-inactivated lysates from various bacterial strains were obtained from the Institute of Medical Microbiology of Saarland University Medical School (Supplementary Table 2). Dot-blots of bacterial lysates on PVDF membranes were performed with a Bio dot apparatus (Bio Rad, California) according to the manufacturer's protocol. Finally, the recombinant DLBCL-BCRs were screened as a pool together with recombinant BCRs derived from various B-cell neoplasms at concentrations of 1 µg/mL, 10 µg/mL and 20 µg/mL on an infectious disease epitope microarray (PEPperCHIP®/Heidelberg,Germany). As a secondary antibody goat anti-human IgG (H+L) conj. DyLight680 (1:5000) was used for 45 min at room temperature. As a scanner, LI-COR Odyssey Imaging System was used, with a scanning offset of 0.65 mm, a resolution of 21 µm, and scanning intensities of 7/7 (red = 700 nm/green = 800 nm). Data quantification was followed by removal of spots with a deviation of more than 40%. The screening, scanning, and data analysis were performed by PEPperPRINT GmbH in Heidelberg, Germany.

Expression of secondary modified antigens

For secondary modification, 500 µL of cell lysates were incubated for 10 min at room temperature with 10 µL anti-FLAG-affinity matrix and washed afterwards. Ubiquitination of FamH83 and sumoylation of JmjD4 were performed as previously described.[3,4] Posttranslationally modified proteins were washed, eluated by administration of FLAG peptide (100µg/mL), and buffered in PBS. Ubiquitination and sumoylation were verified by ubiquitin- or sumo-specific antibodies (Novus Biologicals Ubi1 NB300-130 and BZL08843).

ELISA for BCR and serum reactivity against target antigens and competition ELISA with apoptotic debris

Ars2, ubiquitinated FamH83 and sumoylated JmjD4 were confirmed as BCR antigens as described before by ELISA.[5] Recombinant FLAG-tagged proteins were bound to Nunc maxisorb plates pre-coated overnight at 4°C with murine anti-FLAG antibody at a dilution of 1:2,500 (v/v; Sigma, Munich). Non-ubiquitinated FamH83 and non-sumoylated JmjD4 served as controls. Blocking was performed with 1.5% (w/v) gelatin in TBS and washing steps were performed with TBS-Tx [TBS, 0.1% (v/v) Tx100]. Individual recombinant Fabs (10 µg/ml) and sera (1:100) were used. ELISA was performed according to standard protocols with biotinylated goat anti-human IgG (heavy and light chain; Dianova) at a dilution of 1:2,500, followed by biotinylated secondary antibodies to determine the IgG subclasses of the reactive antibodies. Peroxidase-labeled streptavidin (Roche) was used at a dilution of 1:50,000. For the determination of the BCR-binding region of Ars2, recombinant fragments of different length were constructed with C-terminal FLAG-tags and expressed as described above. Binding of Ars2-reactive DLBCL-derived Fabs to apoptotic debris was analyzed by competition ELISA. C-terminally FLAG-tagged Ars2 at a concentration of 10 µg/ml was bound to Nunc maxisorb plates pre-coated overnight at 4°C with murine anti-FLAG antibody at a dilution of 1:2,500 (v/v; F3165 Sigma). After blocking with 1.5 % (w/v) gelatin in TBS and washing steps with TBS-Tx [TBS, 0.1 % (v/v) Tx100]. Patient-derived recombinant Fabs of DLBCL with reactivity against Ars2 (case #4) or MCL with reactivity against LRPAP1 each at concentration of 0.5 µg/ml were pre-incubated with apoptotic debris of different cell lines at concentrations from 0.5 µg/ml, 50 µg/ml to 100 µg/ml. Apoptotic debris was produced from the DLBCL cell lines U2932 or

TMD8 and MCL cell line MAVER-1 by incubation of 1x10E5 cells with 1 µM Staurosporin (S4400, Sigma) for 3 d.

Site-directed mutagenesis of Ars2

Using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, US) and Ars2 cDNA fragment coding for C-terminally FLAG-tagged full length Ars2, 12 mutants of the two amino acids with the highest predicted probability as phospho-sites (http://www.cbs.dtu.dk/services/NetPhos/) were constructed, each with an exchange of a serine to an alanine: Ser328Ala, Ser341Ala, Ser348Ala, Ser349Ala, Ser357Ala, Ser361Ala, Ser366Ala, Ser368Ala, Ser370Ala, Ser372Ala, Ser374Ala and Ser376Ala. All FLAG-tagged fragments were cloned into pRTS vector and transfected and expressed in U2932, OCI-Ly3 and HBL1.

Binding of Ars2 and internalization into Ars2-reactive DLBCL cell lines

To analyze binding to and internalization of Ars2 into Ars2-reactive DLBCL lines, C-terminally FLAG-tagged Ars2 (10µg/ml) was added to U2932 and HBL1 cells for 30min at 4°C. Cells were then immediately, or after 1h incubation at 37°C analyzed. To determine surface binding or internalization of FLAG-tagged Ars2 half of the cells were washed and analyzed directly by flow, and the other half of cells was washed, treated by Azide 2% for 5 min to solve possibly bound surface antigen, washed again followed by permeabilization by 0.2% saponin at 4°C. Subsequently cells were incubated with murine anti-FLAG antibody (1:500) followed by FITC labeled anti-murine antibody (1:200) each for 20 min at 4°C with washing steps in between followed by flow cytometry

Proliferation and BCR pathway activation assays

For Western blot analysis of the BCR pathway activation of U2932 expressing BCRs with reactivity against Ars2 and of HBL1 cells expressing BCRs without reactivity against Ars2, of each cell line 1x10E6 cells were incubated with no antigen, Ars2 or MAZ at 5 µg/mL or anti-IgM at 1 µg/mL for 3d at 37°C.[6] Rabbit antibodies against pTyr525/526 SYK diluted 1:2000, pTyr759 PLCγ2 diluted 1:1000, pTyr223 BTK diluted 1:1000 and pTyr96 BLNK diluted 1:1000 (B-cell signaling sampler kit, 9768, CST, Massachusetts, USA) and rabbit antibody against actin diluted 1:2000 (A5060, Sigma) and murine antibody against MYC at a concentration of 1 µg/mL

(Santa Cruz) were used, followed by POX-conjugated anti- rabbit or anti-mouse antibodies diluted at 1:3000. For analysis of cytoplasmatic calcium changes by flow cytometry a FACS Canto analyzer was used and Fluo-4/AM dye (F14201, Molecular probes Invitrogen). U2932 cells and HBL1 cells were resuspended in calcium- and magnesium-free phosphate-buffered saline and loaded with Fluo-4/AM dye (final concentration 2 µM, Invitrogen, Karlsruhe, Germany) for 30 min at room temperature. Ars2 and MAZ as control antigens were added followed by flow cytometry of the cells. Ionomycin (407952, Sigma-Aldrich) at a concentration of 10 ng/µL was used as a positive control for the release of calcium from internal stores. Intracellular calcium levels were repeatedly analyzed immediately after adding the antigen to the dye-loaded cells and mixing. For the analysis of proliferation, a non-radioactive assay (EZ4U, BI 500, Biozol) was performed according to the manufacturer's instructions. Human DLBCL cell lines OCI-Ly3, U2932, and HBL1 were used. In short, $4x10^4$/mL cells of each cell line were seeded in a 200-µL cell culture medium. Recombinantly expressed Ars2, MAZ, and LRPAP1 were added at concentration of 1 µg/mL.[6,7] To test for reversion of Ars2-induced effects, Ars2-reactive recombinant Fab derived from case #4 was added and, as a control, a MCL-derived recombinant Fab. Additionally, effects of ibrutinib were analyzed at 200 nM. After 24 h of incubation at 37ºC, 20 µL of chromophore substrate were added to each well and adsorbance of formazan was measured at OD 450 nm with a Wallac Victor^2.

Cytotoxicity and apoptosis assays

Analysis of binding and internalization of Ars2 into Ars2-reactive DLBCL lines as a prerequisite of Ars2-immunotoxins is described in detail in the supplementary. For the analysis of cytotoxic effects of the Ars2/ETA' toxin, a lactate dehydrogenase (LDH) release assay was used. $5x10^3$ / well OCI-Ly3 and HBL1 cells were incubated with Ars2-ETA', LRPAP1-/ETA' as control at concentrations ranging from 0 to 10 µg/mL. Percent-specific lysis was determined as follows: (experimental lysis minus spontaneous lysis) / (maximum lysis − spontaneous lysis) × 100. Maximum lysis was determined by adding 10% Triton X-100. LDH was measured according to the protocol of the LDH assay kit (04744926001, Roche). For ELISA read-out, Victor II (PerkinElmer, Rodgau, Germany) was used. For the analysis of apoptosis, $5×10^5$ cells/mL suspension of U2932 or HBL1 were treated by addition of Ars2-ETA' or MAZ/ETA, both at 0.5 µg/mL, or Staurosporin (1 µg/mL) for 24 h at 37°C, 5% CO2. Following incubation, the cells were washed twice with PBS and resuspended in 500 µL binding buffer; 5 µL of AnnexinV-FITC

(APOAF, Sigma) and 10 µL of propidium iodide were added to each cell suspension and incubated for 10 min at room temperature, followed by analysis using FACS Canto. Finally, the effects of Ars2-ETA' conjugates on established DLBCL lines were also measured by trypan blue assays at 0, 24, and 48 h.

Supplementary Results:

Supplement table 1: DLBCL cases with cryospecimens, their V-genes, and identified target antigen of recombinant BCRs

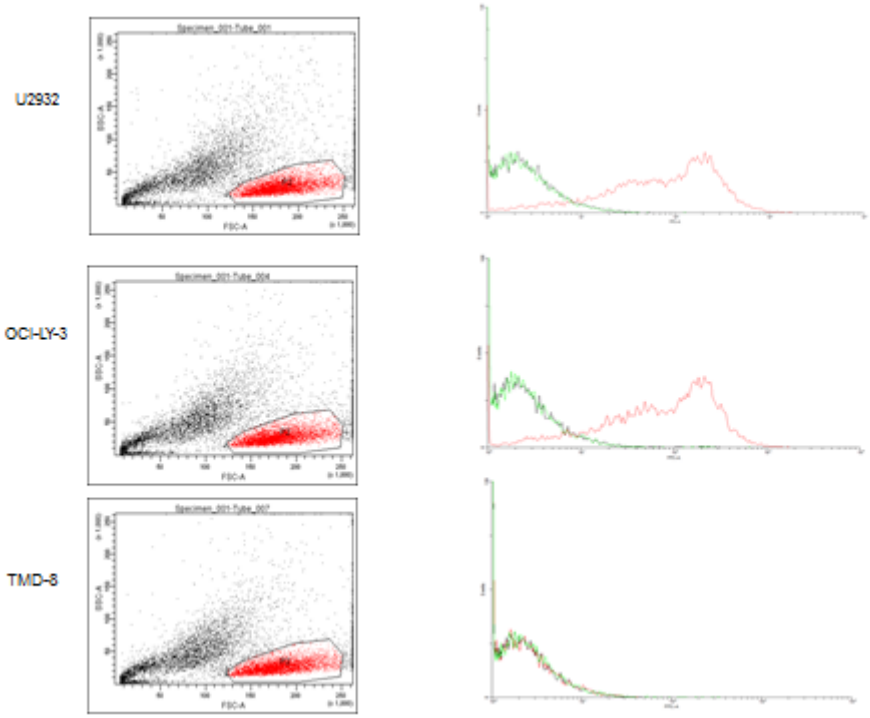
Cell Line / Case #	DLBCL subtype	VH/VL segment Gen	Homology (%)	JH/JL segment Gen	Homology (%)	D segment Gen	CDR 3 length (AA)	Junction	Somatic mutation	Fab production yes / no	Identified antigen	Ars2 Isoform
OCI-Ly3	ABC	VH3-74*02	84.38	JH4*02	87.5	D5-24*01	11	CVRGNGQKCFDYW	yes	yes	ARS2	hypophosphorylated
		VK1-39*01	89.96	JK4*01	92.11		9	CQQNYNFSFTF	yes			
OCI-Ly10	ABC	VH3-7*01	88.89	JH5*01	80.39	D7-27*01	9	CAKGLTGPDSW	yes	yes	ARS2	hypophosphorylated
		Vk2-30*01	94.22	JK1*01	91.67			CLQGTRWPRTF	yes			
U2932	ABC	VH4-39*02	94.16	JH4*02	95,83	D3-22*01	18	CARALSYYDTGGFRYFFDYW	yes	yes	ARS2	hypophosphorylated
		Vk3-20*01	98.58	JK1-01*01	92.11			CQQYRSSPPTWTF	yes			
1	n.d.	VH1-08*01	93.33	J3*01	88.64	D2-15*01	22	CARGDYCSGGICSVAGAPYGMDVW	yes	yes		normal
		Vl7-46*01	97.56	Jl3*02	90.6		9	CLLSYSGARVF	yes			
2	GCB	VH3-7*03	76.5	JH4*02	83.72	D3-9*01	17	CARESGTFRRQNYNVSDFW	yes	yes		normal
		VK3-20*01	91.37	JK4*01	94.74		9	CQQYDSPPLTF	yes			
3	Non-GCB	VH4-34*01	90.32	JH4*02	79.07	DH3-22*01	19	CARELRFYDGSGYVGSLPDSW	yes	yes	ubiquitinylated FAM83H	normal
		Vk3-20	95.32	Jk1*01	89.47		9	CQQYGSSPGTF	yes			
4	Non-GCB	VH1-02*04	93	JH3*02	100	D3-9	25	CARGFDSPGLRYFDWLSTYRGDAFDIW	yes	yes	ARS2	hypophosphorylated
		VL2-23*02	94.29	JL2*01	90.91		11	CCSYAGGSTFVVF	yes			
5	n.d.	VH4-34*01	88.4	JH4*02	79.1	DH3-3*01	15	CARGSQKRFLELIYDYW	yes	yes		normal
		Vl3-21	95.7	JL2*01	92.1		13	CQVWDRDGDHADVIF				
6	GCB	VH3-49*03	89.1	JH3*02	90.9	D3-16*01	16	CSRNGTVFGGLRDDFDIW	yes	yes		normal
		VK3-20	86.5	JK5*01	100		9	CQQYNQSPLTF	yes			
7	n.d.	VH3-21*01	95.6	JH4*02	81	D6-19*01	13	CARGGWAVAGTPDYW	yes	yes	sumoylated JmjD4	normal
		Vk1-17*01	100	Jk2-*01	94.8		10	CLQHNSYLLYTF	no			
8	Non-GCB	VH2-5*01	82.13	JH3*01	90	D3-22	19	CFHTSSDDKIYKSKLDTFDVW	yes	yes		normal
		VK4*01	87.6	Jk4*01	77.14		9	CQQYYISPVTF	yes			
9	n.d.	VH4-30*04	87.3	JH5*02	71.7	D6-19*01	12	CARLRGRIALPSPW	yes	yes		normal
		Vk1-5*03	97.4	Jk1*01	94.1		8	CQQYNNYWTF	yes			
10	n.d.	VH3-15*05	87	JH4*02	91.7	D2-8	13	CGTGGSNRNHYFDFW	yes	yes (2 combinations)		normal
		Vl7-46*01	89.3	JL3*02	79.4		9	CLIFHHGSCVI	yes			
		Vl5-57*01	89.8	JL2*01	90.9		9	CQSYGESSLVF	yes			
11	n.d.	VH3-11	81.43	JH4*02	84.09	DH3-22*01	19	CTRDGALHYYDLSGYYGPQYW	yes	yes	ARS2	hypophosphorylated
		Vk2-28	96.83	Jk1-01	97.14		9	CMQALQTPRTF	yes			

* Cell of origin determined in cases 2, 3, 4, 6 and 8 according to classifier by Hans [8].

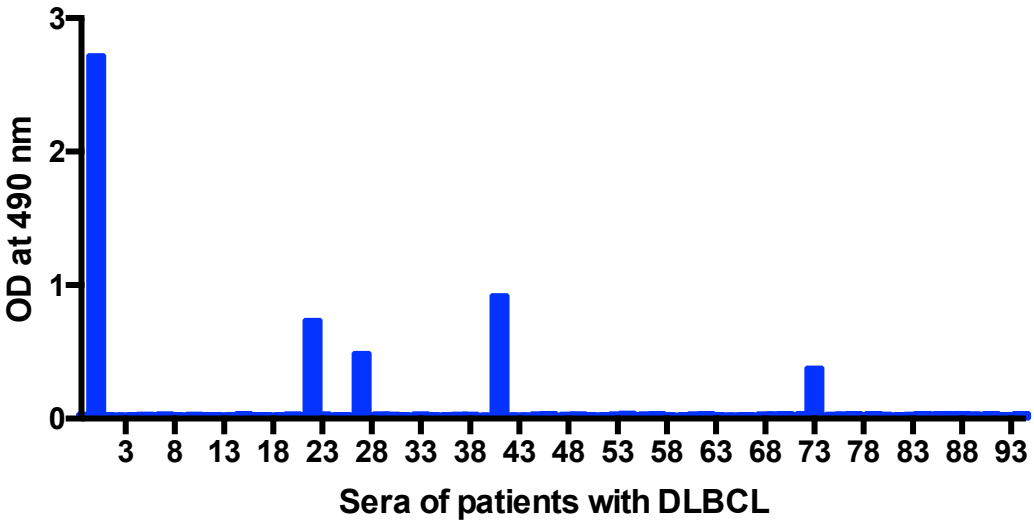
Supplementary Table 2: Reactivity of recombinant DLBCL derived Fabs against bacterial lysates

Bacterial lysate	**Recombinant DLBCL derived BCR in Fab format**													
	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	OCI-Ly3	OCI-Ly10	U2932
M. catarrhalis	no	no	no	No	no	no	no	no	no	no	No	no	no	No
Streptococcus pneumoniae	no	no	no	No	no	no	no	no	no	no	No	no	no	No
Streptococcus pyogenes	no	no	no	No	no	no	no	no	no	no	no	no	no	No
Haemophilus influenzae	no	no	no	No	no	no	no	no	no	no	no	no	no	No
Staphylococcus aureus	no	no	no	No	no	no	no	no	no	no	no	no	no	No
coagulase-negative Staphylococci	no	no	no	No	no	no	no	no	no	no	no	no	no	No
Serratia pneumoniae	no	no	no	No	no	no	no	no	no	no	no	no	no	No
Klebsiella pneumoniae	no	no	no	No	no	no	no	no	no	no	no	no	no	No
Enterobacteriacae	no	no	no	No	no	no	no	no	no	no	no	no	no	No
Escherichia coli	no	no	no	No	no	no	no	no	no	no	no	no	no	no
Neisseria spp.	no	no	no	No	no	no	no	no	no	no	no	no	no	no

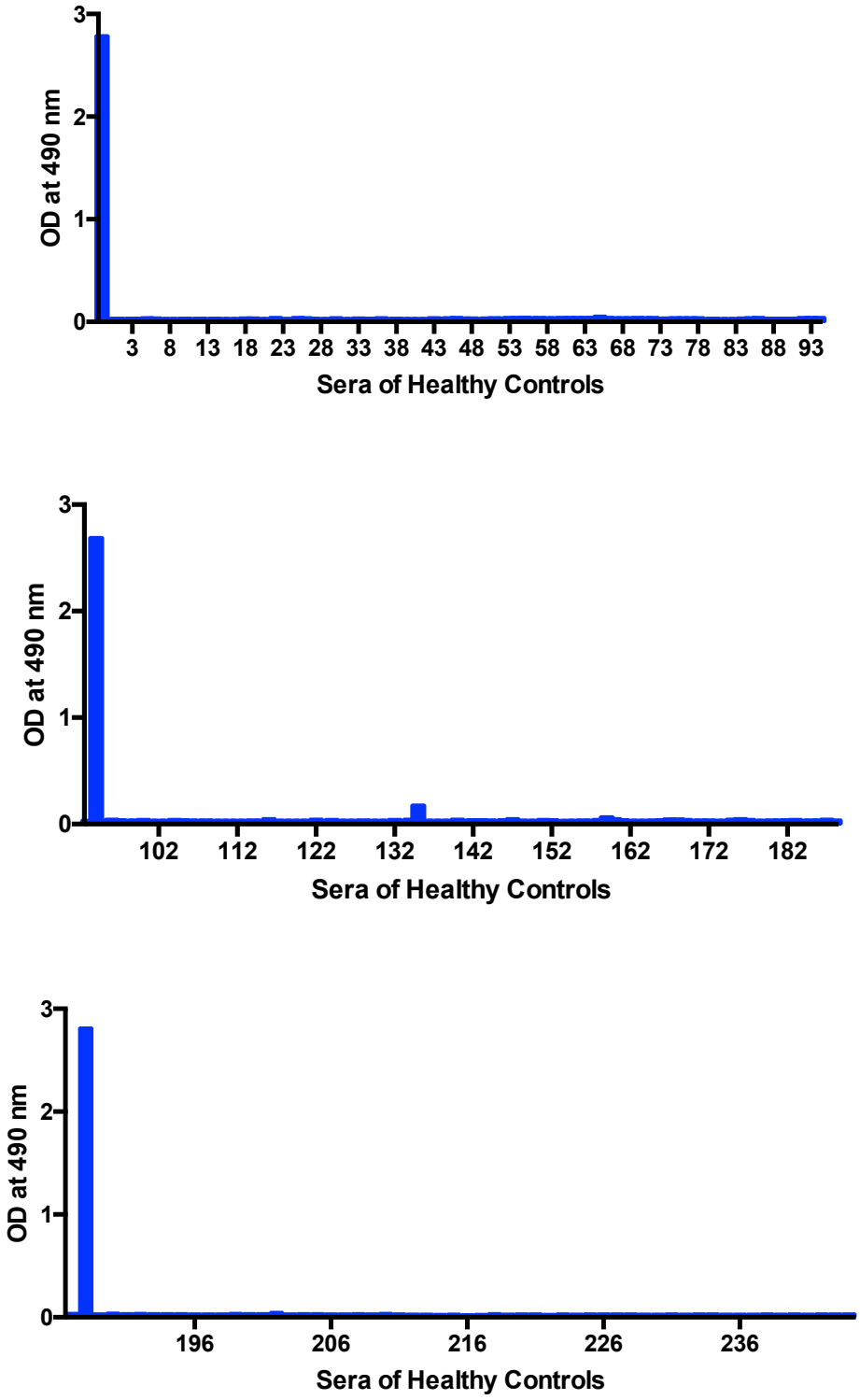
Supplementary Figures:



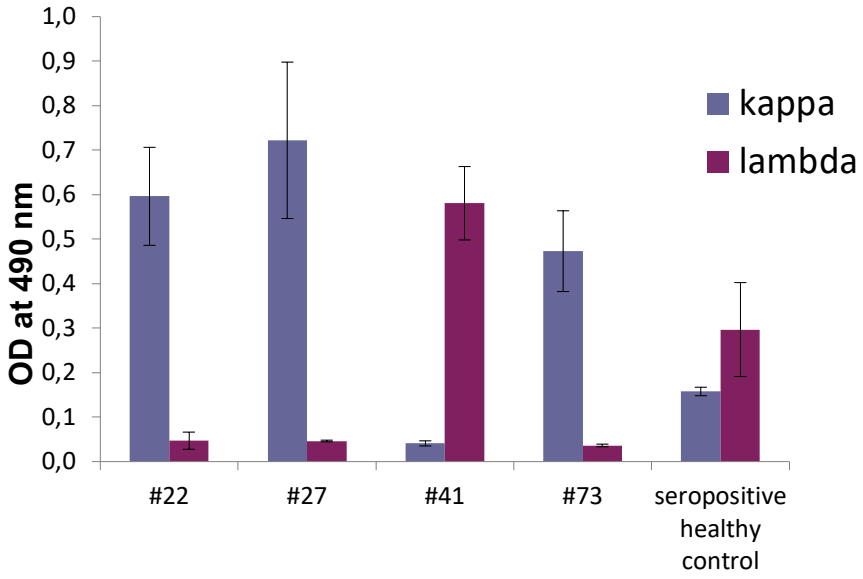
Supplementary Figure 1: Binding of Ars2 to BCRs on lymphoma cell lines. Binding of Ars2 to DLBCL BCRs was analyzed with U2932, OCI-Ly3 and TMD8 cell line. U2932 and OCI-Ly3 cells bound Ars2 (red line), in contrast to MAZ (green line). Both, U2932 and OCI-Ly3 express BCRs with reactivity against Ars2. TMD8, a cell line with a BCR without specificity for Ars2 did not bind Ars2.



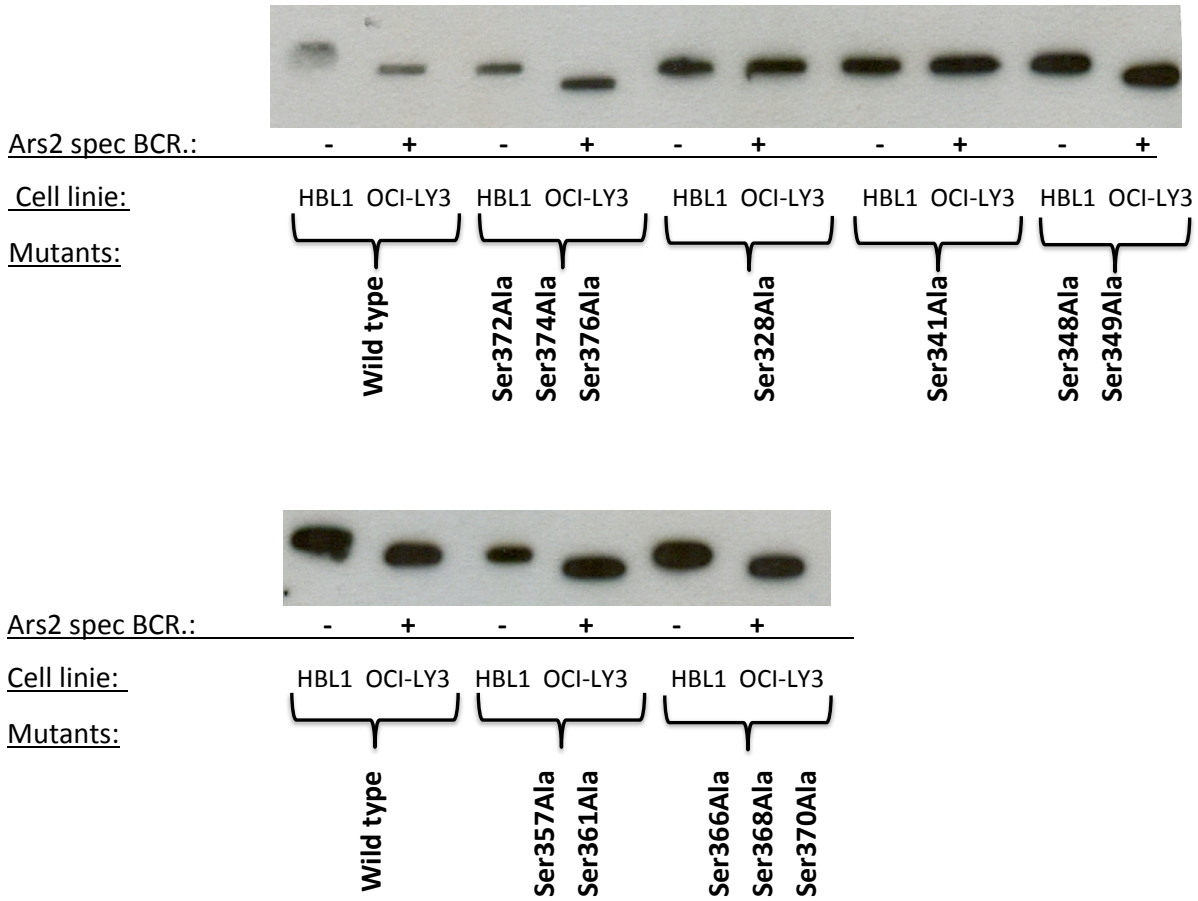
Supplementary Figure 2a) Ars2 antibodies in the sera of patients with DLBCL. The columns represent the measured adsorbance at an OD of 490nm. In 4 of 98 patients Ars2-autoantibodies were detected.



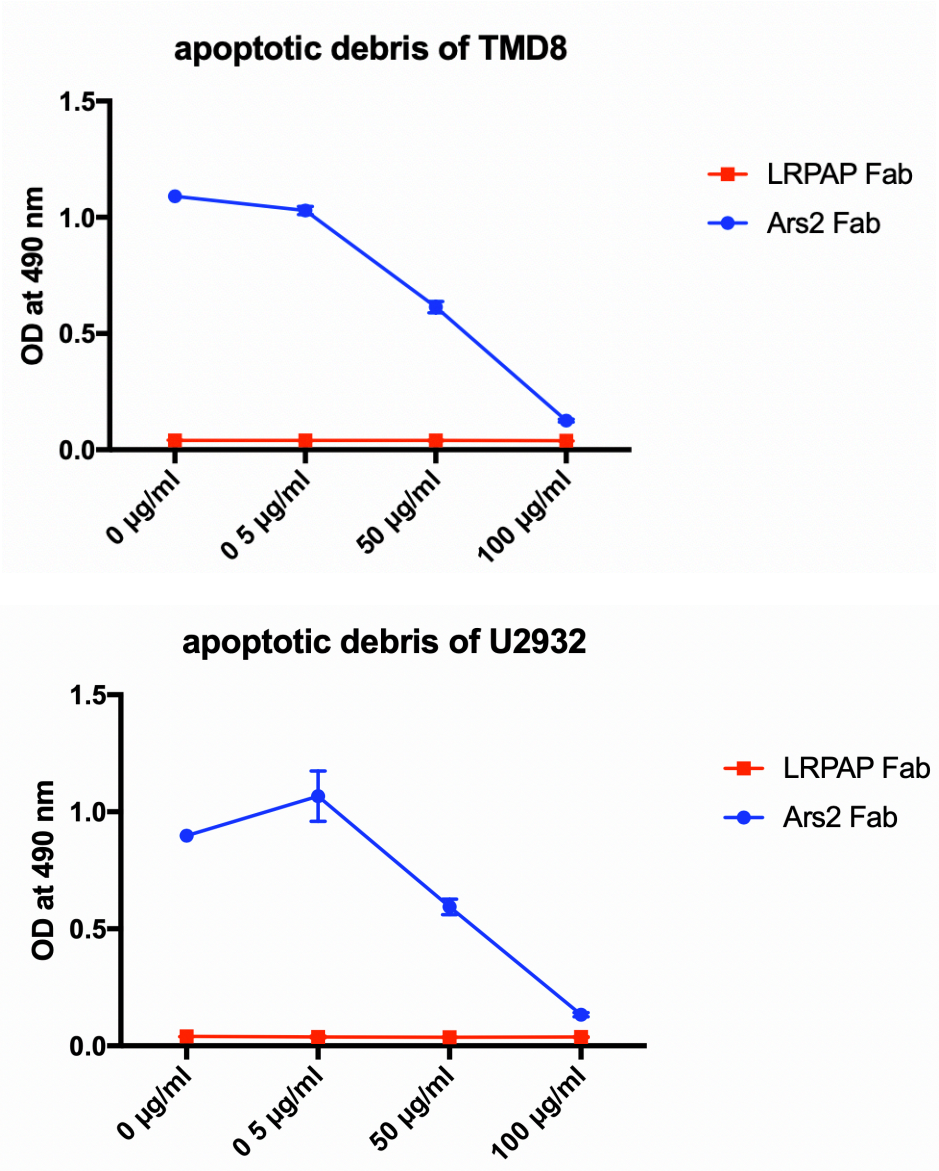
Supplemmentary Figures 2b) Ars2-autoantibodies in the sera of healthy controls. The columns represent the measured adsorbance at an OD of 490nm. In 1 of 244 healthy controls Ars2-autoantibodies were detected. Healthy controls 245-400 not shown.



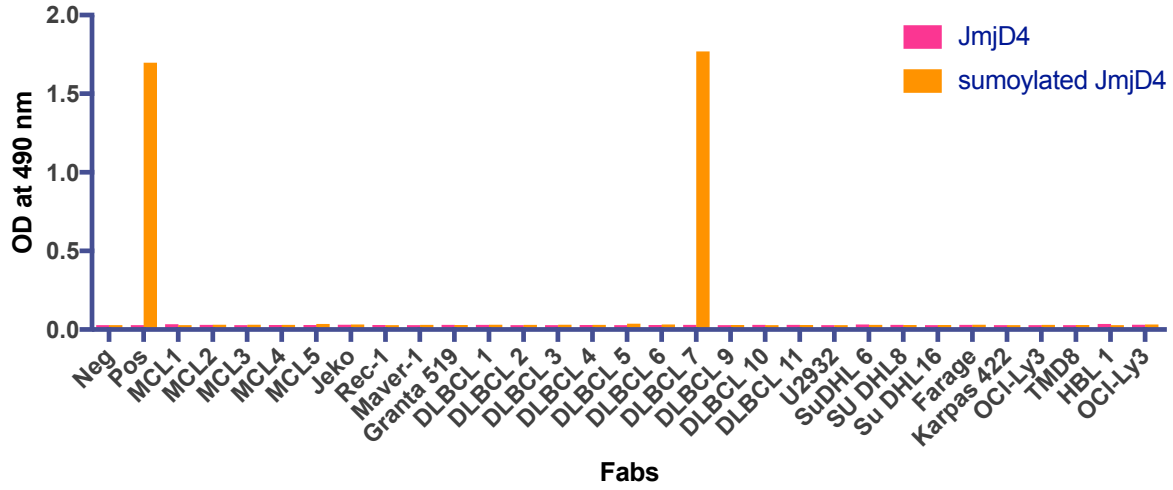
Supplemmentary Figures 2c) Light chains of Ars2-autoantibodies in seropositive patients and healthy control. In the Ars2-autoantibody seropositive patients, either Ars2-autoantibodies with kappa light chain restriction (#22, #27, #73) or with lambda light chain restriction (#41). In contrast the Ars2-autoantibodies of the seropositive healthy control showed no light chain restriction. The columns represent the measured adsorbance at an OD of 490nm.



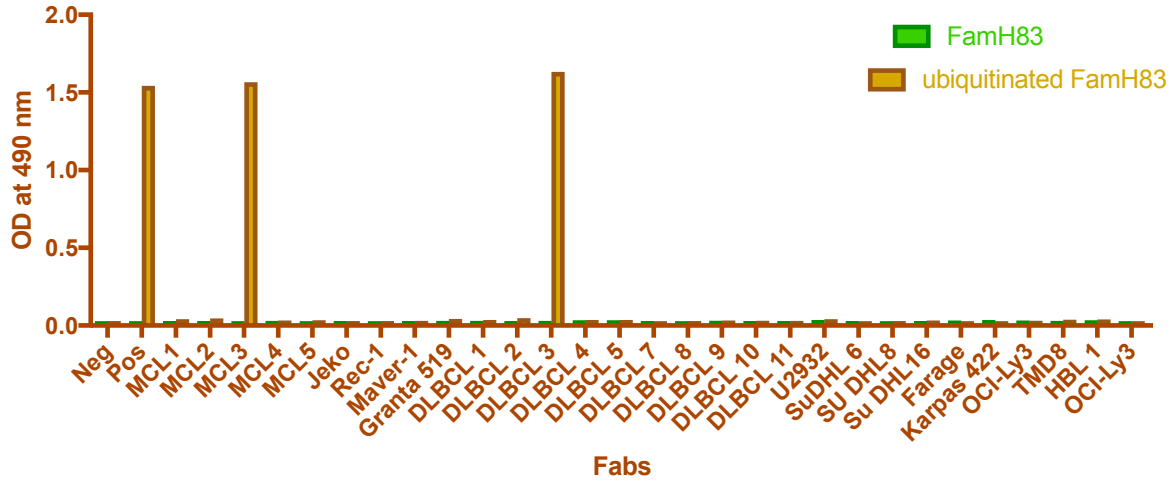
Supplemmentary Figure 3) Identification of the hypophosphorylated sites. In contrast to wild-type Ars2, mutations in Ser372Ala, Ser374Ala, Ser376Ala, Ser348Ala, Ser349 Ala, Ser357Ala, Ser361Ala, Ser365Ala, Ser368Ala and Ser370Ala resulted in less phosphorylated Ars2 isoforms in both HBL1 and OCI-Ly3, however the Ars2 isoform of OCI-Ly3 was still less negatively charged compared to HBL1. The mutants Ser328Ala and Ser341Ala resulted in the disappearance of the difference in electric charge. This implies that Ser328 and Ser341 were phosphorylated in HBL1, but not in OCI-Ly3, identifying both Ser328 and Ser341 as the sites of hypophosphorylation. Transfected wt and mutants of Ars2 were C-terminally FLAG-tagged. Anti-FLAG was used as primary antibody.



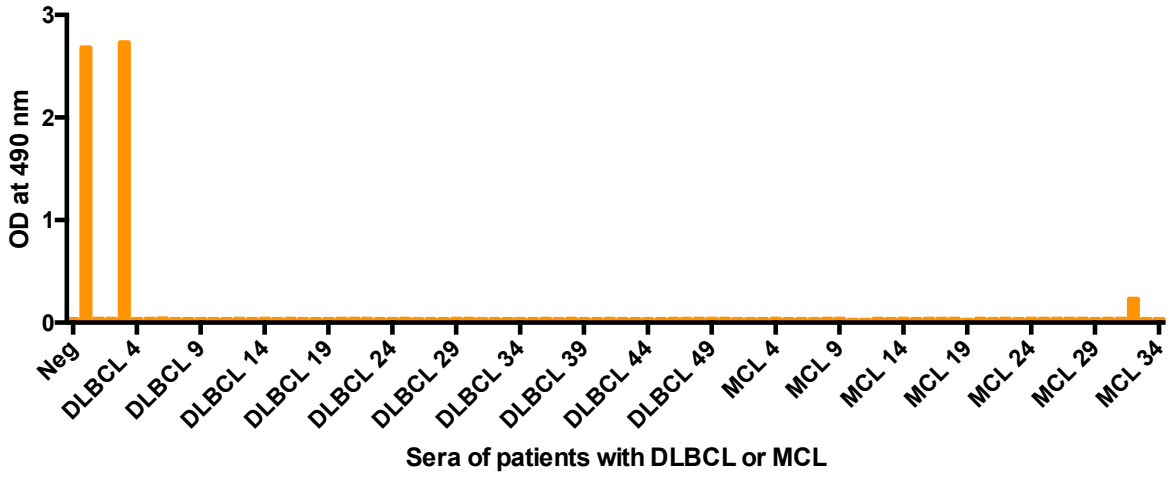
Supplementary Figure 4: Competetive ELISA of lymphoma Fabs between Ars2 and apoptotic cell debris. By the addition of debris of apoptotic cells of TMD8 (wildtype Ars2) or of U2932 (hypophophorylated Ars2), the interaction of coated Ars2 and Ars2-reactive DLBCL BCRs was antagonized in a dose dependent manner. The experiment was performed three times. The concentration of apoptotic debris of TMD8 or U2932 line is indicated at the abscissa.



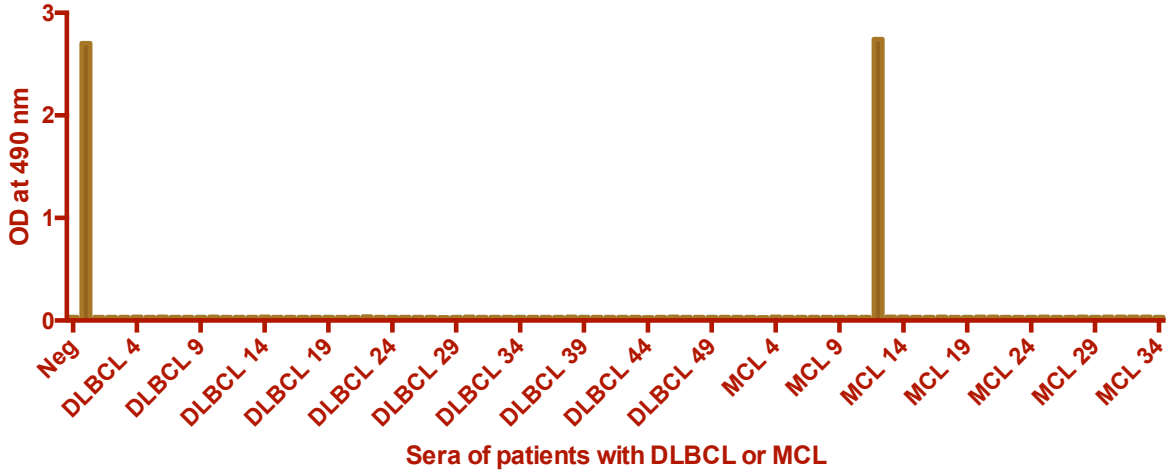
Supplementary Figure 5) ELISA for reactivity against sumoylated JmjD4. The Fabs were derived from DLBCL cases without BCR reactivity against Ars2 and DLBCL cell lines. Recombinant Fabs derived of MCL cases and Fabs of MCL cell lines served as controls. The columns represent adsorbance at OD 490nm consistent with affinity of Fabs against sumoylated JmjD4.The recombinant Fab of DLBCL #7 showed specific reactivity against sumoylated JmjD4.



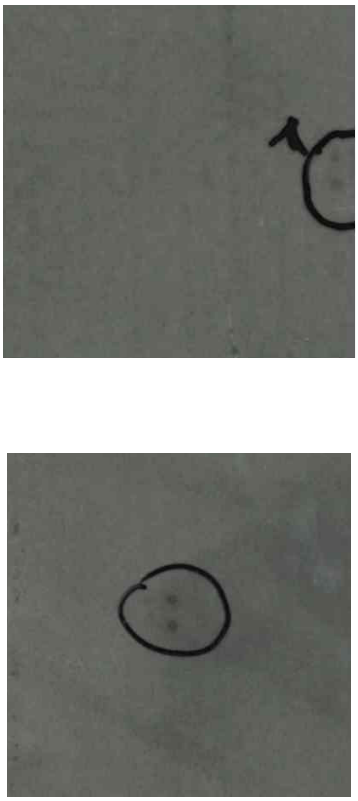
Supplementary Figure 6) ELISA for reactivity against ubiquitinated FamH83. The Fabs were derived from DLBCL cases without BCR reactivity against Ars2 and DLBCL cell lines. Recombinant Fabs derived of MCL cases and Fabs of MCL cell lines served as controls. The columns represent adsorbance at OD 490nm consistent with affinity of Fabs against ubiquitinated FamH83.The recombinant Fabs of MCL #3 and DLBCL #3 showed specific reactivity against ubiquitinated FamH83 but not against FamH83.



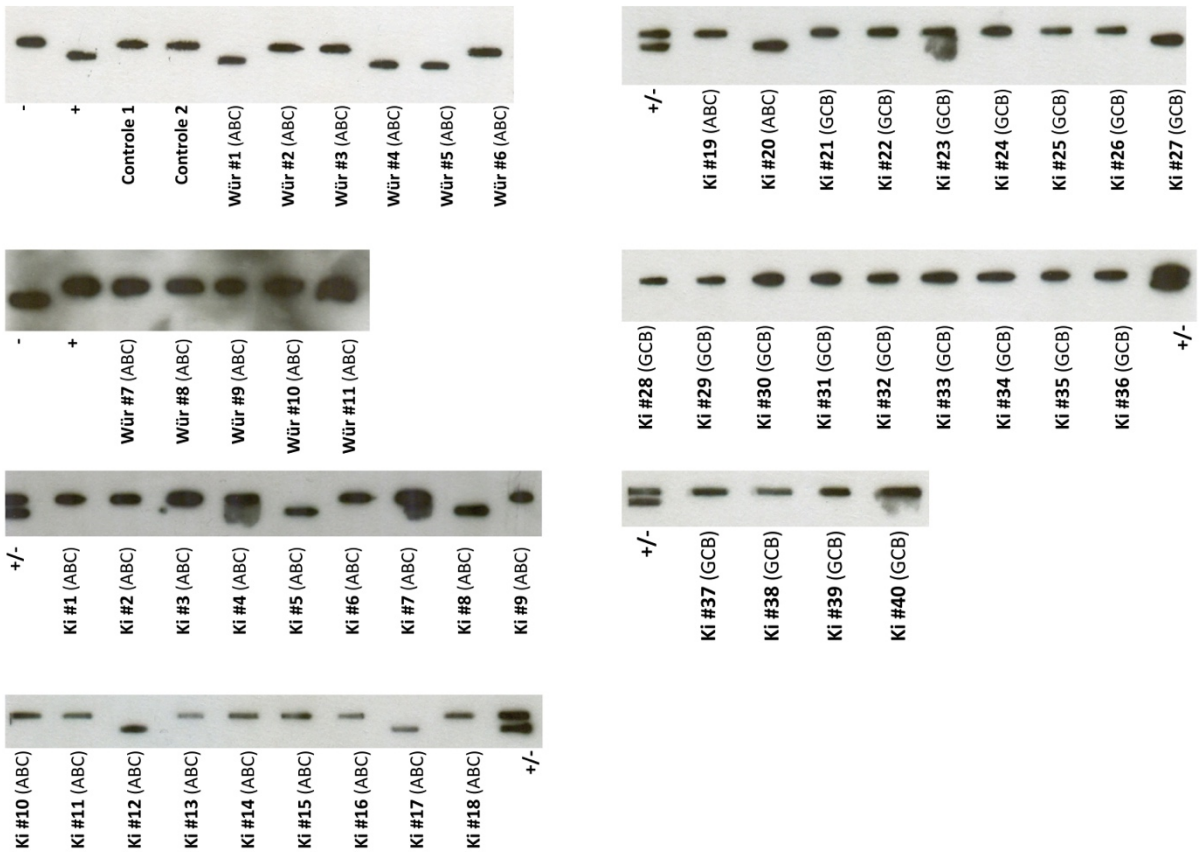
Supplementary Figure 7) Occurrence of autoantibodies against sumoylated JmjD4. The sera of patients with either DLBCL or MCL were screened for autoantibodies against sumoylated JmjD4. In the serum of 1 patient with DLBCL autoantibodies against sumoylated JmjD4 could be detected. The columns represent the measured OD at 490nm.



Supplementary Figure 8) Occurrence of autoantibodies against ubiquitinated FamH83. The sera of patients with either DLBCL or MCL were screened for autoantibodies against ubiquitinated FamH83. In the serum of one patient with MCL autoantibodies against ubiquitinated FamH83 could be detected. The columns represent the measured OD at 490nm.



Supplementary Figure 9) Images exerpts of candidate antigen hits: Ars2 on UNIPEX2 array (above) and of sumoylated JmjD4 on sumoylated and citrullinated UNIPEX2 array (below).



Supplementary Figure 10) Isoelectric focusing of Ars2 of cryospecimen derived of DLBCL cryospecimen, of which the cell of origin (COO) had been previously determined by GEP. These DLBCL samples were obtained from the institutes of Pathology of Würzburg and Kiel Universities.

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