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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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Abstract

It has been suggested that B-cell receptor (BCRs) stimulation by specific antigens plays a pathogenic role in diffuse large B-cell lymphoma (DLBCL). Here, it was the aim to screen for specific reactivities of DLBCL-BCRs in the spectrum of autoantigens and antigens of infectious origin. Arsenite resistance protein 2 (Ars2) was identified as the BCR target of 3/5 ABC-type DLBCL cell lines and 2/11 primary DLBCL cases. Compared to controls, Ars2 was hypo-phosphorylated exclusively in cases and cell lines with Ars2-specific BCRs. In a validation cohort, hypo-phosphorylated Ars2 was found in 8/31 ABC-type, but only 1/20 germinal center B cell (GBC)-like type DLBCL. Incubation with Ars2 induced BCR-pathway activation and increased proliferation, while an Ars2/ETA’ toxin conjugate induced killing of cell lines with Ars2-reactive BCRs. Ars2 appears to play a role in a subgroup of ABC-type DLBCLs. Moreover, transformed DLBCL lines with Ars2-reactive BCRs still show growth advantage after incubation with Ars2. These results provide knowledge about the pathogenic role of a specific antigen stimulating the BCR pathway in DLCBL.
**Introduction:**

Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive B-cell Non-Hodgkin lymphoma. According to the WHO classification, DLBCL can be classified based on gene expression profiling (GEP) into activated B cell (ABC)-like type, germinal center B cell (GCB)-like type and primary mediastinal B-cell lymphoma.\(^1\) In contrast to relatively well studied genetic or epigenetic pathway alterations, little is known about specific and complementary external stimuli of different subgroups of DLBCL.\(^2\) In particular, DLBCL of the ABC-type or the recently specified MCD-type or Cluster 5 harbor recurrent mutations in \(\text{MYD88}\) and \(\text{CD79B}\) genes with dependency on constitutive BCR signaling.\(^5,6,7,8\) For primary central nervous system lymphoma (PCNSL), which represents a specific extranodal subtype of DLCBL with molecular similarities to MCD type or C5 with frequent mutations in \(\text{MYD88}\) and \(\text{CD79}\),\(^9,10\) \(\text{SAMD14/neurabin-I}\) were identified as antigen of BCRs recently, and \(\text{SAMD14/neurabin-I}\) were hyper-N-glycosylated specifically in patients with \(\text{SAMD14/neurabin-I-reactive BCRs}^{11}\). For systemic DLBCL a cis and trans stimulation of the BCR by a so far non-characterized autoantigen was reported for HBL1 line. Moreover, an anti-Idiotype reactivity of the CDR3 of the BCR of TMD8 line against an epitope within its own FR2 (\(V^{37}R^{38}\)) was described, and for U2932 and OCI-LY10 lines BCR reactivity against apoptotic cell debris was reported.\(^8\) This prompted us to screen for and characterize possible target antigens of BCRs of systemic DLBCL using expression cloning of primary cryopreserved specimens and DLBCL lines and subsequent protein array screening.\(^12,13,14\)
Methods:

The study had been approved by the local ethics committee (Ärztekammer des Saarlandes 12/13). For expression cloning of DLBCL-BCRs snap-frozen specimens of patients were obtained from the Dr. Senckenberg Institute of Pathology (Frankfurt a. M., Germany). Sera of a second cohort of patients with DLBCL were obtained from the DSHNHL RICOVER-60 trial. DLBCL cryospecimen, of a third cohort of patients, of which the cell of origin (COO) had been determined by GEP, were obtained from the institutes of pathology of Würzburg and Kiel Universities.

BCR screening for autoantigens

BCRs from 9 DLBCL cell lines were prepared by digestion with papain. Moreover, expression cloning of recombinant BCRs derived of primary DLBCL cryospecimen was performed (described in supplementary). These DLBCL line–derived BCRs and the pooled recombinantly expressed BCRs (each at a concentration of 10 µg/mL) were screened on protein macroarrays containing clones of UniPEx 1 and 2 cDNA expression libraries (Bioscience, Dublin, Ireland), as previously described.\textsuperscript{13,14} To search for further antigens, all recombinant DLBCL-derived Fabs without reactivity against arsenite resistance protein 2 (Ars2) were screened against variously posttranslationally modified UniPEx 1 and 2 protein macroarrays, including sumoylation, ubiquitination, citrullination, and acetylation. Sumoylation of protein macroarrays was performed as previously described\textsuperscript{15} and ubiquitination was performed with synchronized HeLa cell extracts.\textsuperscript{16} Screenings for antigens of infectious origin is described in the supplementary.
Expression of target antigens and immunotoxins

The expression clone of Ars2 and subsequently the epitope-containing region consisting of aa 342-375 of Ars2 were recombinantly expressed with C-terminal FLAG tag by pSFI vector in HEK293 cells. Additionally, C-terminally FLAG-tagged full length Ars2 was transfected by electroporation into U2932 and TMD8 via pRTS vector. C-terminally FLAG-tagged FamH83 and JmjD4 were recombinantly expressed in HEK293 cells. Site-directed mutagenesis of Ars2 and secondary modification of antigens is described in the supplementary.

Immunotoxins with monomethyl auristatin E (MMAE) are effective in vivo and established in the clinics, but the synthesis of toxin conjugates with MMAE requires enzyme-cleavable dipeptide linkers and is therefore challenging for academic laboratories. Hence, a truncated form of *Pseudomonas aeruginosa* exotoxin A was used, as ETA' conjugate can be recombinantly expressed directly. Recombinantly expressed immunotoxins, consisting of Ars2 aa 342–375 conjugated to this truncated form of *Pseudomonas* exotoxin A (ETA'), were either obtained from the Fraunhofer Institute of Experimental Medicine and Immunotherapy (Aachen, Germany) or recombinantly expressed in our lab in *E. coli* BL21 and purified by the His-Tag as described by Nachreiner et al.

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 previously described by ELISA. ELISA and competition assays with apoptotic debris are described in detail in supplementary.
**Western blot and isoelectric focusing**

Lysates of DLBCL lines or of whole blood from patients were loaded and separated by a 10% SDS-PAGE and transferred to PVDF membrane using a transblot semidry transfer cell (Bio Rad). After blocking overnight at 4°C in PBS/10% nonfat dry milk, a recombinant Ars2-reactive His-tagged Fab was incubated at a concentration of 2 µg/mL for 1 h at room temperature, followed by incubation for 1h at room temperature with murine anti-his antibody at 1:2,000 (Qiagen), with HRP-labeled anti-mouse IgG antibody (Bio Rad). Chemiluminescence reagent (New England BioLabs) was used for immunoblot detection. Isoelectric focusing was performed as previously described. Proliferation, BCR pathway activation assays, cytotoxicity and apoptosis assays are described in supplementary methods.

**Results:**

Recombinant BCRs in the format of Fabs were successfully synthesized from 11 DLBCL cases. Moreover, antigen-binding fragments of “natural” BCRs were obtained by papain digestion from 9 DLBCL cell lines. From three of these cell lines, recombinant Fabs were generated (Suppl. Tab. 1).

**Screening of protein macroarrays and an infective agents library**

The screening of DLBCL Fabs identified an expression clone of Ars2 transcript variant 2 (RZPDp828K0526 from Unipex 2, UnigeneID: Hs.111801) spanning from aa 253 to aa 416 as the candidate antigenic target. The screening of the Fabs of DLBCL cases and cell lines on posttranslationally modified protein macro arrays revealed sumoylated JmJD4
(RZPDp9027E0216D from Unipex 1; UnigeneID: Hs.555974) and ubiquitinylated FamH83 (RZPDp828G0328 from Unipex 2; UnigeneID: Hs.676336) as candidate antigens. The screening against bacterial lysates of 11 bacterial strains did not reveal any specific reactivity. Screening of an Infectious Disease Epitope Microarray (PEPperCHIP®/Heidelberg, Germany) consisting of 3,760 database-derived B-cell epitopes associated with 196 pathogens, including various bacterial, fungal, parasitic, and viral pathogens, revealed no significant binding of the pooled DLBCL-BCRs.

**Confirmation of Ars2, sumoylated JmjD4, and ubiquitinated FamH3 as targets of DLBCL-BCRs and determination of the BCR-binding epitope**

ELISA with recombinant Ars2 (UnigeneID: Hs.111801) expressed with a C-terminal FLAG-tag in HEK293 confirmed Ars2 as the BCR target antigen from 3/5 (60%) ABC-derived cell lines (OCI-Ly3, OCI-Ly10, and U2932; but not HBL1 and TMD8) and 0/4 GCB-DLBCL cell lines (Fig. 1a). Recombinant BCRs from 2/11 DLBCL cases (with unknown COO), but 0/9 MCLs and 0/11 PCNSLs were reactive with Ars2 (Fig. 1b). Of the two Ars2-reactive DLBCLs, one was a non-GCB and one was unclassified according to immunohistochemistry using the Hans classifier. \(^{21}\) ELISAs with fragments of different lengths of Ars2 as coat identified a region spanning aa 350 to aa 416 as the BCR-binding epitope (Fig. 1c), and all Ars2-specific BCRs derived from cell lines and cryospecimen bound to this epitope. ELISA with recombinant sumoylated JmjD4 or ubiquitinylated FamH83 expressed with a C-terminal FLAG-tag in HEK293 confirmed each as target antigens of one DLBCL-derived recombinant Fabs and ubiquitinated FamH83 in addition as a target of a recombinant MCL-derived Fab (Supplementary Figures 1 and 2). Fabs did not bind to nonmodified JmjD4 and FamH83.
Binding of Ars2 to membrane BCRs was demonstrated by flow cytometry of DLBCL cell lines with Ars2-reactive BCRs, but not for TMD8 line without Ars2-reactive BCRs. Internalization of C-terminally FLAG tagged Ars2 into U2932 cells was observed after 60 min (Fig. 1d). For OCI-Ly10, mutagenesis of K98A of the BCR heavy chain gene had been reported to result in loss of autoreactivity.\textsuperscript{8} OCI-Ly10 K98A resulted in loss of affinity against Ars2 (Fig. 1e).

**Characterization of Ars2 in DLBCL with Ars2-specific BCR**

No obvious differences in molecular weight of Ars2 from DLBCL cases with Ars2-reactive and Ars2-non-reactive BCRs and controls were observed in Western blot; similarly, Sanger sequencing revealed identical DNA sequences, excluding mutations in the coding sequence as an explanation for the immunogenicity of Ars2. However, iso-electric focusing (IEF) of cell lines (Fig. 2a) and DLBCL cases (Fig. 2b) with an Ars2-reactive BCR revealed a less negatively charged Ars2 isoform. Dephosphorylation with alkaline phosphatase treatment led to a stronger reduction of the negative charges of Ars2 from DLBCL cases and cell lines without Ars2-specific BCRs than in cases with Ars2-reactive BCRs and resulted in the disappearance of the differences in electric charge between both isoforms of Ars2 (Fig. 2c), demonstrating that the different IEF pattern of Ars2 was due to hypophosphorylation in cases with Ars2-specific BCR. The hypophosphorylated Ars2 isoform was detected in all of the 3 DLBCL cell lines with Ars2-reactive BCRs (i.e., OCI-Ly3, OCI-Ly10, and U2932), but in none of 6 DLBCL lines without Ars2-reactive BCRs. This association in the 9 analyzed DLBCL cell lines between BCR reactivity against Ars2 and the presence of the hypophosphorylated isoform of Ars2 was statistically significant (Fisher exact t-test: two tailed $p=0.0119$). Regarding the 11 DLBCL cases with recombinantly expressed rec. Fabs (Supplementary table 1) derived from
cryospecimen, only the 2 cases with Ars2-reactive Fabs (#3 and #11) showed hypophosphorylation of Ars2. In this cohort of 11 cases with cryospecimen and expressed rec. Fabs the association between Ars2-reactivity and presence of hypophosphorylated Ars2 isoform was also statistically significant (Fisher exact t-test: two tailed p=0.0182). If looking combined at the BCR-reactivity of DLBCL cells and presence of hypophosphorylated Ars2 isoform in cell lines and cases with cryospecimen derived rec. Fabs the association is statistically highly significant (Fisher exact t-test: two tailed p<0.0001).

Moreover, hypophosphorylated Ars2 was detected in the biopsies of 8/31 (26%) ABC-type DLBCL cases characterized by GEP, but only in 1/20 (5%) GCB-type DLBCL and in the peripheral blood from 1/100 healthy controls.

The hypophosphorylated sites were identified as serine 328 and serine 341 by site-directed point mutagenesis of various predicted sites in Ars2 transfected with a C-terminal FLAG tag in OCI-Ly10, OCI-Ly3, and as a control HBL1 (Fig. 2d and supplementary Fig. 4).

**Frequency, titers, and IgG-subclasses of Ars2 serum antibodies**

Ars2 antibodies were detected by ELISA in the sera of 4/98 patients with DLBCL with titers ranging from 1:800 to 1:1600 and 1/400 healthy controls. All 4/98 patients with Ars2 antibodies in their sera (#22, #27, #41, #73) were carriers of hypophosphorylated Ars2 in the cells of their peripheral blood (Fig. 2b), but this isoform was not detected in the peripheral blood of any of the 94 other patients; resulting in a statistically significant relationship between serum Ars2-autoantibodies and the presence of the hypophosphorylated Ars2 in peripheral blood (Fisher exact t-test: two tailed p<0.0001).
Effects of Ars2 on DLBCL lines

Western blot analysis of the BCR pathway activation after addition of recombinant Ars2 revealed a strong BCR-pathway activation in the U2932 cell line with Ars2-reactive BCRs, demonstrated by a strong upregulation of pTyr525/526 SYK, pTyr96 BLNK, pTyr759 PLCγ2, and pTyr223 BTK. Moreover, this BCR stimulation by Ars2 led to increases in MYC expression. However, regarding MYC two cases with Ars2-reactive BCRs (#4 and #10) did not have MYC-overexpression in IHC analysis (data not shown). No BCR pathway activation was induced by the control antigens MAZ in U2932 line or by addition of Ars2 to the HBL1 line (Fig. 3a). Addition of recombinant Ars2-induced proliferation of U2932 and OCI-Ly3 cells, but not of DLBCL cell lines without Ars2-reactive BCRs as TMD8 analyzed by tetrazolium/formazan EZ4U assay (Fig. 3b). This Ars2-induced growth stimulus could be reverted by addition of Ars2-neutralizing recombinant Fab derived from case #4 (Fig 3c). Furthermore, flow cytometry analysis of U2932 cells showed a strong increase of cytoplasmic calcium levels after incubation with the Ars2 epitope, but not the control antigen (Fig. 3d).

Cytotoxicity of Ars2/ETA’ conjugate

Addition of Ars2-ETA’ resulted in inhibition of growth analyzed in proliferation assays. This could be reverted by preincubation of Ars2/ETA’ toxin with the Ars2-reactive recombinant Fab derived from case #4 (Fig 4a). The Ars2-ETA’ conjugate exerted a specific and dose-dependent toxicity against the Ars2-reactive BCR-expressing cell lines, but had no observable effect on DLBCL lines lacking Ars2-reactive BCRs (Fig. 4b). No toxic effect was observed with the control toxin LRPAP1-ETA’ against OCI-Ly3. Trypan blue staining after addition of 5
µg/mL Ars2-ETA’ showed that after 24 h 35%, after 48 h 2%, and after 72 h 0% of OCI-Ly3 cells were alive, in contrast to the wild-type HBL1 cell line without BCR reactivity against Ars2 (at 24 h: 97%; at 48 h: 96%; and at 72 h: 97% viable cells) (Fig. 4b). In accordance with this, an increase of apoptotic cells was detected after incubation with Ars2-ETA’ in U2932 cells expressing Ars2-reactive BCRs (Fig. 4c), as demonstrated in the annexin V/PI assay.

**Discussion:**

Beside the two relatively rare target antigens ubiquitinated FamH83 and sumoylated JmJD4, in the present study, hypophosphorylated Ars2 was as identified as a more frequent antigen of BCRs from DLBCL lines and recombinant BCRs from primary DLBCL cryospecimen. Ars2 is also known as serrate RNA effector molecule (SRRT). Its gene is located on chromosome 7q21 and the protein is a zinc finger protein consisting of 875 amino acids with a molecular weight of around 100 kDa. Ars2 was described as being involved in miRNA silencing by interacting with the nuclear cap binding complex,

22 and as being involved in innate immune response against RNA viruses by miRNA processing.23 Ars2 expression was shown to be linked to proliferative states24 and different roles were described malignant diseases25,26

All three target antigens of DLBCL-BCRs identified in this study share the characteristic of being atypically posttranslationally modified, which represents the most likely reason for their immunogenicity. For ubiquitinated FamH83 and sumoylated JmJD4, the lymphoma BCRs were specific for the secondary modified isoforms. In contrast, Ars2-reactive BCRs of DLBCL bound both, the hypophosphorylated and the normally phosphorylated isoforms of Ars2. But, the hypophosphorylated isoform of Ars2 was only observed in cell lines or cryospecimens of DLBCLs with BCR-reactivity for Ars2 (p<0.0001). Similarly, regarding
peripheral blood the hypophosphorylated isoform of Ars2 was also only observed in lysates of peripheral blood cells of patients seropositive for Ars2-autoantibodies (p<0.0001). These statistically significant associations between Ars2-reactivity and the presence of hypophosphorylated Ars2, indicate that this posttranslational modification is involved in the immunogenicity.

Bringing this in context to other B-cell neoplasia, in plasma cell dyscrasia SLP2-reactive paraprotein was also not specific for the differentially phosphorylated SLP2 isoform.27 Similar to CD4+ T-helper cells specific for hyperphosphorylated SLP2 in plasma cell dyscrasia,28 one might speculate about a possible role of hypothetic CD4+ T-helper cells specific for hypophosphorylated Ars2 epitopes, which might stimulate Ars2-reactive B-cells, which by themself do not differentiate between normally and hypophosphorylated Ars2. Generally, atypical PTMs represent an accepted mechanism for the breakdown of self-tolerance with numerous examples in clinical immunology, like modified wheat gliadin in celiac disease,29 N-terminally acetylated myelin basic protein in multiple sclerosis,30 citrullinated fibrin/vimentin in rheumatoid arthritis,31,32 phosphorylated SR proteins during stress-induced apoptosis in systemic lupus erythematosus,33,34 immunogenic pSer81 Progranulin isoform and Progranulin autoantibodies,35,36 phosphorylated enolase in pancreatic carcinoma,37,38,39 and the involvement of atypically modified BCR target antigens in lymphomagenesis like hyperphosphorylated SLP2, ATG13, and sumoylated HSP90 in plasma cell dyscrasia and hyper-N-glycosylated SAMD14/neurabin-I in PCNSL.40,41,27,15,11

Of interest, Ars2 hypophosphorylation and reactivity of DLBCL-BCRs against Ars2 was nearly exclusively detected in DLBCL of the ABC type. All the cell lines with anti-Ars2 reactivity were of this COO, and in a validation cohort of DLBCL with characterized COO by GEP,
hypophosphorylated Ars2 was detected in 8 of 31 cases (26%) of the ABC type of DLBCL, but only in 1 of 20 cases (5%) of GCB-type DLBCL. In a combined analysis of GEP-typed cryospecimen and analyzed cell lines, the hypophosphorylated Ars2 isoform was statistically significantly associated with ABC type (p=0.0188).

Considering possible functional effects of Ars2, we observed that its addition stimulated growth of DLBCL lines with Ars2-specific BCRs (Fig. 3), indicating that these lines still depend to some extent on BCR stimulation by their cognate antigen Ars2. Regarding the mutational background of these cell lines with Ars2-reactive BCRs and expression of hypophosphorylated Ars2, OCI-Ly10 has mutated MYD88 (L265P)\textsuperscript{42} and truncating mutation of CD79A\textsuperscript{7,43} U2932 has mutated NFkB-pathway by TAK1 mutation,\textsuperscript{44} but wild-type CARD11, and wild-type MYD88,\textsuperscript{7,45} and OCI-Ly3 has a mutated CARD11 and mutated MYD88 (L265P).\textsuperscript{7,42} This demonstrates, that despite pathway-activating mutations, these cell lines might still benefit in their proliferation from an upstream BCR pathway stimulation by a cognate antigen.

From a therapeutic point of view, in general two hypothetic therapeutic approaches arise from these data. Firstly, BCR antigens might be used as baits to target lymphoma cells in a specific was (i.e., targeting of a cell-bound antibody by an antigen), similar to anti-idiotype antibodies or peptibodies,\textsuperscript{46} with the advantage of not being selected and synthesized individually for each patient, since all Ars2-reactive DLBCL-BCRs bind the same epitope. Physiologically it is the major task of sIg to bind its cognate antigen followed by internalization, enabling processing and antigen presentation via MHC class II. Targeting Ars2-reactive BCRs of DLBCL cell lines resulted in a specific and efficacious killing. Beside this, the Ars2 epitope could be used for bispecific constructs for T- or NK-cell engaging (e.g.,
CD3/Ars2 or CD16/Ars2,\textsuperscript{47} or as an additional ectodomain for CAR-T cells.\textsuperscript{48} Secondly, regarding the high relative risk for carriers of atypically hypophosphorylated Ars2, investigating ways of modulating this PTM might be worthwhile in the future.

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**Authorship contributions:**

LTh, SH, KDP, BK and MP designed the study. SH, MLH performed microdissection of DLBCL cases and data interpretation. WK, AW YJK, RMB, BK, FvB, LTr, MZ, NM, Dkm VP and GH were of great help in the acquisition of DLBCL samples and clinical data. NF performed the protein array experiments, phosphorylation experiments and proteomic experiments. ER did site directed point mutagenesis of Ars2. MK and ER sequenced. LTh, MK, ER and TB performed seminested IgV gene PCRs and BCR expression cloning. LTh, MB, MK, ER, NF performed expression of Ars2-immunotoxins. LTh and MP are responsible for data analysis, interpretation of results and writing of the manuscript. This article is dedicated to the memory of M. Pfreundschuh, who died during its preparation.

**Disclosure of conflicts of interest:**
References:


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Figure legends:

Figure 1: Reactivity of DLBCL derived BCRs against Ars2. A: ELISA for reactivity against Ars2 or sumoylated HSP90 as control of DLBCL cell line-derived natural (Papain-digested) BCRs in the Fab format and directly for cell membrane-bound BCRs. The columns represent absorbance at OD 490 nm, mean and standard deviation. B: ELISA for Ars2 reactivity of recombinant BCRs derived from primary DLBCL cryospecimen and controls. The columns represent absorbance at OD 490nm, mean and standard deviation. C: Determination of the affinity region of BCRs against Ars2: The affinity region within Ars2 was aa 301-416 with the highest observed affinity shown for aa 350-416. The columns represent absorbance at OD 490nm, mean and standard deviation. D: Binding of Ars2 and internalization into U2932 cells: At t0 (left) Ars2 (a fragment 253 to 416) bound to the cell surface of U2932 cells (above) and was not detected intracellularly (below). After 60 min incubation at 37°C (right) and washing Ars2 was not detected anymore on the cell surface (above), but intracellularly (below). Non-specific binding was determined by incubation with antibodies alone. E: ELISA for reactivity against Ars2 of the BCR of OCI-Ly10 wt and K98A. Recombinant and Papain-digested (natural) Fab of OCI-Ly10 bound to Ars2. K98A mutagenesis of recombinant OCI-Ly10 BCR resulted in the loss of reactivity of the recombinant BCR against Ars2. K98A had previously been described for OCI-Ly10 being responsible for losing BCR-autoreactivity against apoptotic debris. The columns represent absorbance at OD 490nm, mean and standard deviation.

Figure 2: Ars2 is exclusively hypophosphorylated in patients with Ars2-reactivity of lymphoma BCRs. A: Western-blot and isoelectric focusing of Ars2 derived from DLBCL cell lines. Western-blot of Ars2 of 9 DLBCL cell lines revealed no difference in Ars2 between cell lines with and without Ars2-reactive BCRs (above). However, isoelectric focusing (IEF) of Ars2 of the DLBCL lines showed a less negative charge of Ars2 in OCI-Ly3, OCI-Ly10 and U2932. These 3 cell lines had exclusively Ars2-reactive BCRs. B: Isoelectric focusing of Ars2 of whole blood derived from DLBCL patients with Ars2-autoantibodies (#22, #27, #41, #73). In the peripheral blood of these 4 patients also the less negatively charged Ars2 isoform was detected. Ars2-autoantibody titers ranged between 1:800 and 1:1600 in these 4 patients. C: Alkaline phosphatase treatment led to disappearance of differences in IEF of Ars2. A stronger reduction of negative charges of Ars2 by dephosphorylation was observed in cases/cell lines without Ars2-reactive BCRs. D: Identification of the hypophosphorylated sites by site-directed mutagenesis and transfection of C-terminally FLAG-tagged Ars2 into U2932 and HBL1. In contrast to wild-type Ars2, mutations in Ser372Ala, Ser374Ala, Ser376Ala, Ser348Ala, Ser349Ala, Ser357Ala, Ser361Ala, Ser365Ala, Ser368Ala and Ser370Ala resulted in hypophosphorylated Ars2 isoforms in both HBL1 and U2932, however the Ars2 isoform of U2932 was still less negatively charged compared to HBL1. Only the mutants Ser328Ala and
Ser341Ala resulted in the disappearance of this difference in electric charge, identifying both Ser328 and Ser341 as the sites of hypophosphorylation. Murine anti-FLAG-antibody was used as primary antibody.

**Figure 3: BCR pathway activation and induction of proliferation by Ars2.** A: BCR pathway analysis by Western blot in U2932 and HBL1 after addition of cognate/control antigens Ars2 or MAZ showed a strong activation due to addition of ARS2 to U2932 cells with upregulation of pTyr525/526 SYK, pTyr96 BLNK, pTyr759 PLCγ2 and pTyr223 BTK and higher expression of MYC. In contrast, no effect of ARS2 on the BCR pathway was observed in HBL1 cells. B: Induction of proliferation by Ars2. Addition of Ars2 to OCI-Ly3 and U2932 lines resulted in a significantly (p<0.01: student’s t test) increase of proliferation determined in the EZ4U assay (columns represent formazan at OD of 450 nm), while addition of Ars2 had no effect on the TMD8 line. Columns and bars represent mean and standard deviation of 3 experiments. C: Inhibition of Ars2-induced proliferation by neutralizing Ars2-reactive Fabs. Addition of Ars2 together with Ars2-reactive (patient derived of case #4) recombinant Fabs prevented induction of growth in U2932 cells. D: Elevation of cytoplasmatic calcium levels by addition of Ars2. Flow cytometry analysis of cytoplasmic calcium levels using Fluo-4 dye showed an increase after addition of the cognate antigen ARS2 (blue) comparable to the effect of Anti-IgM (black) to U2932 cells, but not after the addition of control antigen MAZ (green). Addition of Ars2 to control DLBCL line HBL1 did not result in elevated calcium levels.

**Figure 4: Targeting Ars2-reactive DLBCL with Ars2-containing immunotoxins.** A: Growth inhibition by BCR-antigen/immunotoxins. Growth of U2932 cells was inhibited by addition of Ars2/ETA’, an immunotoxin of the epitope of the cognate antigen Ars2 fused to a truncated form of *Pseudomonas aeruginosa* exotoxin A. This growth inhibition could be prevented by coincubation with neutralizing Ars2-reactive Fabs but not by LRPAP1-reactive Fabs. Columns (formazan formation at an OD of 450 nm) represent cell proliferation, mean and sd. B: BCR-specific lysis of DLBCL cell lines by Ars2/ETA’ immunotoxin. Above: Cytotoxic effect after 24 h, 48 h and 72 h of incubation with 5 μg/mL of recombinant Ars2/ETA’ or LRPAP1/ETA’ immunotoxins. Cell viability of HBL-1 (left) and OCI-LY3 (right) cell lines determined by trypan blue staining. Below: Dose-dependant cytotoxic effect of Ars2/ETA’ determined in LDH release assay. Curves indicate percent specific lysis as of HBL1 line (left) or OCI Ly3 line (right) with and without Ars2-reactive BCRs, after incubation with doses from 0 μg/mL to 10 μg/mL Ars2/ETA’, LRPAP1/ETA’ or PBS. C: Induction of apoptosis by addition of Ars2/ETA’ immunotoxins. Flow cytometric characterization of apoptotic U2932 or HBL1 cells 24 h after addition of Ars2/ETA’ or MAZ/ETA’ by Annexin-V/PI staining. U2932 cell have Ars2-reactive BCRs resulting in a strong increase of the early and late apoptotic cells after addition of Ars2/ETA’.
Fig 4:

a) 

![Graph showing OD at 450 nm for different treatments.](image)

- MAVER1
- U2932

- ARS2/ETA
- ARS2/ETA + rec Fab
- ARS2/ETA + rec Fab (ARS2-reactive)
- LRPAP1/ETA
- LRPAP1/ETA + rec Fab
- LRPAP1/ETA + rec Fab (LRPAP1-reactive)

Immunotoxin and rec. Fab

b) 

- HBL 1
- OCI Ly3

![Graph showing vitality over time.](image)

- LRPAP1-ETA
- ARS2-ETA
- no immunotoxin

![Graph showing specific lysis over concentration of immunotoxin.](image)

- no immunotoxin
- Ars2 ETA
- control immunotoxin

Concentration of immunotoxin [µg/ml]

C) 

- U2932
- Ars2 reactive BCR

- ARS2/ETA
- MAZ/ETA

Pl

- HBL1
- no Ars2-reactive BCR

![Graph showing PI and Annexin-FITC.](image)

Annexin-FITC
**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. 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.

**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. Posttranslationally modified proteins were washed, eluted 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. 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. 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, 4x10⁴/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 37°C, 20 μL of chromophore substrate were added to each well
and adsorbance of formazan was measured at OD 450 nm with a Wallac Victor².

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. 5x10³ /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, 5x10⁵ 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**

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<td>VK7-40*11</td>
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<td>JX7*01</td>
<td>79.4</td>
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<td>CQFFNHSKCY</td>
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<td>VK6-57*01</td>
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<td>JX7*01</td>
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<td>QGQYFSEIPYV</td>
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<td>11</td>
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<td>VH3-11</td>
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<td>JH4*02</td>
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<td>19</td>
<td>CTRODGLHYYDGLYGDPYW</td>
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* 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

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<th>Bacterial lysate</th>
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<th>#9</th>
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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.
Supplementary Figures 2b) Ars2-autoantibodies in the sera of healthy controls. The columns represent the measured absorbance at an OD of 490 nm. In 1 of 244 healthy controls Ars2-autoantibodies were detected. Healthy controls 245-400 not shown.
**Supplementary 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.
Supplementary Figure 3) Identification of the hypophosphorylated sites. In contrast to wild-type Ars2, mutations in Ser372Ala, Ser374Ala, Ser376Ala, Ser348Ala, Ser349Ala, 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: Competitive 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 (hypophosphorylated 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.

![Graph showingOccurrence of autoantibodies against sumoylated JmjD4.](image)

**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.

![Graph showing Occurrence of autoantibodies against ubiquitinated FamH83.](image)
**Supplementary Figure 9** Images excerpts 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.
References:


7. Thurner L, Hartmann S, Fadle N, et al. LRPAP1 is a frequent proliferation-inducing antigen of BCRs of mantle cell lymphomas and can be used for specific therapeutic targeting. Leukemia [Epub ahead of print].