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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 stimulation of B-cell receptors (BCR) 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-BCR in the spectrum of autoantigens and antigens of infectious origin. Arsenite resistance protein 2 (Ars2) was identified as the BCR target of three of five activated B-cell type DLBCL cell lines and two of 11 primary DLBCL cases. Compared to controls, Ars2 was hypophosphorylated exclusively in cases and cell lines with Ars2-specific BCR. In a validation cohort, hypophosphorylated Ars2 was found in eight of 31 activated B-cell type DLBCL, but in only one of 20 germinal center B-cell 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 BCR. Ars2 appears to play a role in a subgroup of activated B-cell-type DLBCL. Moreover, transformed DLBCL lines with Ars2-reactive BCR still showed 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 World Health Organization (WHO) classification, DLBCL can be classified based on gene expression profiling (GEP) into an activated B-cell (ABC)-like type, a germinal center B-cell (GCB)-like type and primary mediastinal B-cell lymphoma.[1,2] 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.[3,4] In particular, DLBCL of the ABC-type or the recently specified MCD-type or Cluster 5 harbor recurrent mutations in *MYD88* and *CD79B* genes with dependency on constitutive BCR signaling.[5-8] For primary central nervous system lymphoma, which represents a specific extranodal subtype of DLCBL with molecular similarities to MCD type or C5 with frequent mutations in MYD88 and CD79,[9,10] SAMD14/neurabin-I were recently identified as antigens of BCR, and were hyper-N-glycosylated specifically in patients with SAMD14/neurabin-I-reactive BCR.[11] In systemic DLBCL, a *cis* and *trans* stimulation of the BCR by a so far uncharacterized autoantigen was reported for the HBL1 line. Moreover, an anti-idiotype reactivity of complementarity determining region 3 of the BCR of the 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 BCR of systemic DLBCL using expression cloning of primary cryopreserved specimens and DLBCL lines and subsequent protein array screening.[12-14]

Methods

The study was approved by the local ethics committee (Ärztekammer des Saarlandes 12/13). For expression cloning of DLBCL-BCR, patients' snap-frozen specimens were obtained from the Dr. Senckenberg Institute of Pathology (Frankfurt am Main, Germany). Sera of a second cohort of patients with DLBCL were obtained from the DSHNHL RICOVER-60 trial.

DLBCL cryospecimens, of a third cohort of patients, of which the cell-of-origin had been determined by GEP, were obtained from the Institutes of Pathology of Würzburg and Kiel Universities.

B-cell receptor screening for autoantigens

BCR from nine DLBCL cell lines were prepared by digestion with papain. Moreover, expression cloning of recombinant BCR derived from primary DLBCL cryospecimens was performed, as described in the *Online Supplementary Material*. These DLBCL line-derived BCR and the pooled recombinantly expressed BCR (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.[13,14] To search for further antigens, all recombinant DLBCL-derived antigen-binding fragments (Fab) without reactivity against arsenite resistance protein 2 (Ars2) were screened against variously post-translationally modified UniPEx 1 and 2 protein macroarrays, including sumoylation, ubiquitination, citrullination, and acetylation. Protein macroarrays were sumoylated as described elsewhere[15] and ubiquitination was performed with synchronized HeLa cell extracts.[16] The screening for antigens of infectious origin is described in the *Online Supplementary Material*.

Expression of target antigens and immunotoxins

The expression clone of Ars2 and subsequently the epitope-containing region consisting of amino acids 342-375 of Ars2 were recombinantly expressed with a C-terminal FLAG tag by a pSFI vector in HEK293 cells. Additionally, C-terminally FLAG-tagged full-length Ars2 was transfected by electroporation into U2932 and TMD8 via a pRTS vector.[17] C-terminally FLAG-tagged FamH83 and JmjD4 were recombinantly expressed in HEK293 cells. Site-directed mutagenesis of Ars2 and secondary modifica-

tion of antigens is described in the *Online Supplementary Material*.

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.[18,19] Hence, a truncated form of *Pseudomonas aeuroginosa* exotoxin A (ETA') was used, as the ETA' conjugate can be recombinantly expressed directly. Recombinantly expressed immunotoxins, consisting of Ars2 amino acids 342-375 conjugated to ETA' were either obtained from the Fraunhofer Institute of Experimental Medicine and Immunotherapy (Aachen, Germany) or recombinantly expressed in our laboratory in *E. coli* BL21 and purified by the His-Tag, as described by Nachreiner *et al.*[20]

Enzyme-linked immunosorbent assay (ELISA) for B-cell receptor and serum reactivity against target antigens and competition ELISA with apoptotic debris

Ars2, ubiquitinated FamH83, and sumoylated JmjD4 were confirmed as BCR antigens by ELISA, as previously described.[13] ELISA and competition assays with apoptotic debris are described in detail in the *Online Supplementary Material*.

Western blot and isoelectric focusing

Lysates of DLBCL lines or of whole blood from patients were loaded and separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane using a transblot semidry transfer cell (Bio Rad). After blocking overnight at 4°C in phosphate-buffered saline/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 1 h at room temperature with murine anti-his antibody at a ratio of 1:2,000 (Qiagen), with horseradish peroxidase-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 the *Online Supplementary Material*.

Results

Recombinant BCR in the form of Fab were successfully synthesized from 11 DLBCL cases. Moreover, Fab of "natural" BCR were obtained by papain digestion from nine DLBCL cell lines. From three of these cell lines, recombinant Fab were generated (*Online Supplementary Table S1*).

Screening of protein macroarrays and a library of infective agents

The screening of DLBCL Fab identified an expression clone of Ars2 transcript variant 2 (RZPDp828K0526 from Unipex 2, UnigeneID: Hs.111801) spanning from amino acids 253 to 416 as the candidate antigenic target. The screening of the Fab of DLBCL cases and cell lines on post-translationally modified protein macroarrays 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-BCR.

Confirmation of Ars2, sumoylated JmjD4, and ubiquitinated FamH3 as targets of diffuse large B-cell lymphoma B-cell receptors and determination of the B-cell receptor-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 three of five (60%) ABC-derived cell lines (OCI-Ly3, OCI-Ly10, and

U2932; but not HBL1 and TMD8) and none of four GCB-DLBCL cell lines (Figure 1A). Recombinant BCR from two of 11 DLBCL cases (with unknown cell of origin), but none of nine mantle cell lymphomas and none of 11 primary central nervous system lymphomas were reactive with Ars2 (Figure 1B). Of the two Ars2-reactive DLBCL, one was a non-GCB type and one was unclassified according to immunohistochemistry using the Hans classifier.[21] ELISA with fragments of different lengths of Ars2 as the coat identified a region spanning amino acids 350 to 416 as the BCR-binding epitope (Figure 1C), and all
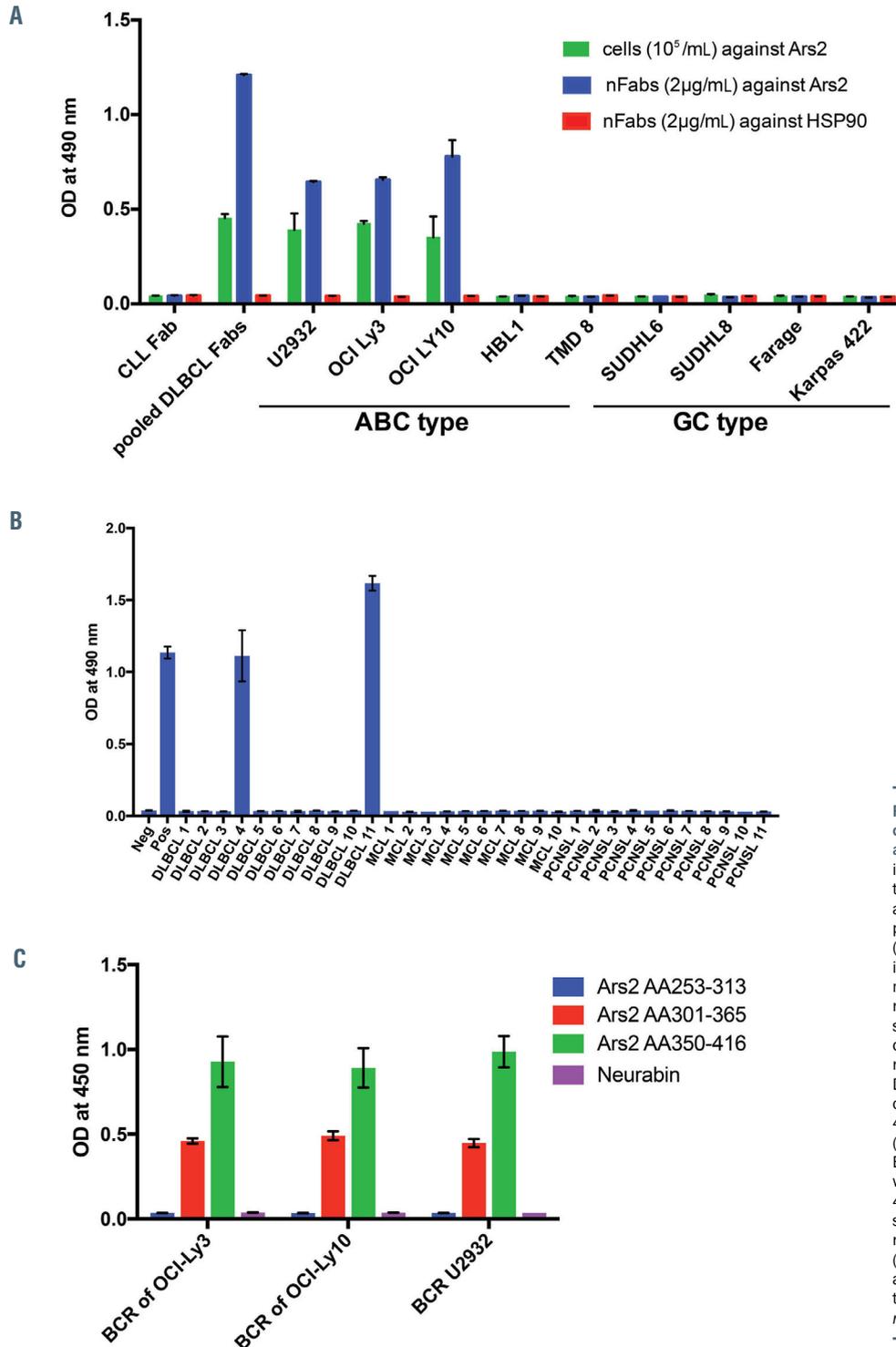

Figure 1. Reactivity of diffuse large B-cell lymphoma-derived B-cell receptors against Ars2. (A) Enzyme-linked immunosorbent assay (ELISA) for reactivity against Ars2 or sumoylated HSP90 as control of diffuse large B-cell lymphoma (DLBCL) cell line-derived natural (Papain-digested) B-cell receptors (BCR) in the Fab format and directly for cell membrane-bound BCR. The columns represent adsorbance at an optical density (OD) of 490 nm (mean and standard deviation). (B) ELISA for Ars2 reactivity of recombinant BCR derived from primary DLBCL cryospecimens and controls. The columns represent adsorbance at OD 490 nm (mean and standard deviation). (C) Determination of the affinity region of BCR against Ars2: The affinity region within Ars2 was amino acids (AA) 301-416 with the highest observed affinity shown for AA 350-416. The columns represent adsorbance at OD 490 nm, (mean and standard deviation). ABC: activated B-cell type; GC: germinal center B-cell type. (*Figure continued on the next pages*)
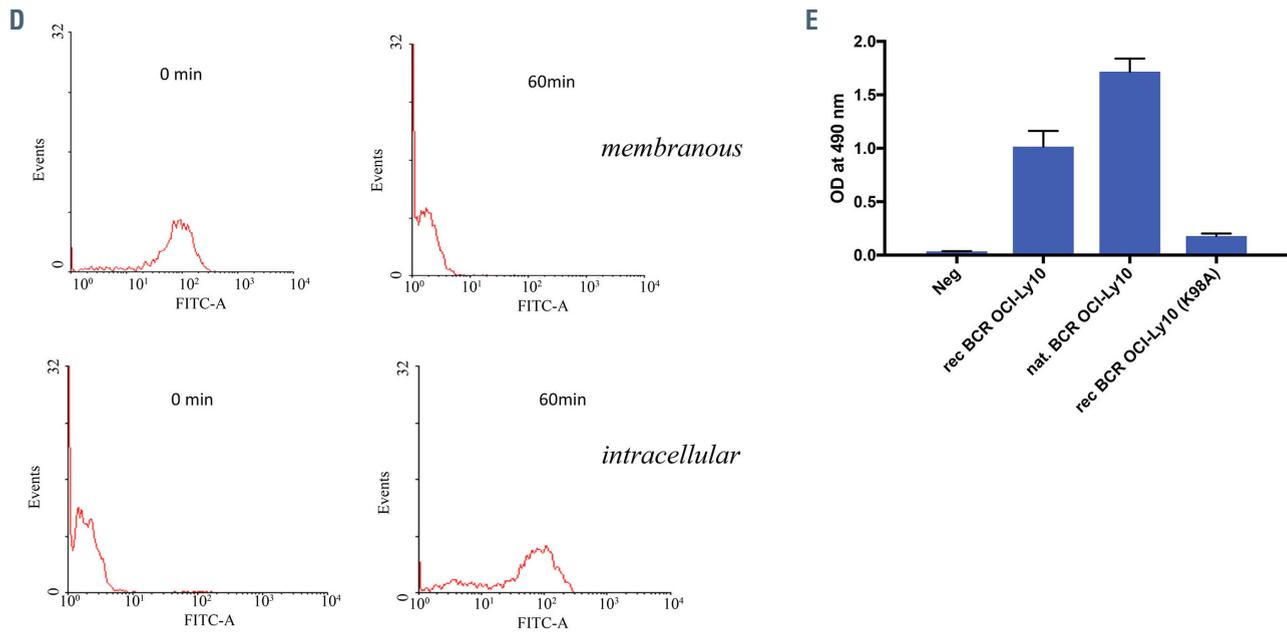
Figure 1. Continued from previous page. (D) Binding of Ars2 and internalization into U2932 cells. At time 0 (left) Ars2 (a fragment of 253 to 416 amino acids) bound to the cell surface of U2932 cells (above) and was not detected intracellularly (below). After 60 min incubation at 37 °C (right) and washing, Ars2 was no longer detected on the cell surface (above), but was found 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 as being responsible for loss of BCR autoreactivity against apoptotic debris. The columns represent adsorbance at OD 490 nm (mean and standard deviation).

Ars2-specific BCR derived from cell lines and cryospecimens 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 Fab and ubiquitinated FamH83 in addition as a target of a recombinant mantle-cell lymphoma–derived Fab (*Online Supplementary Figures S1* and *S2*). Fab did not bind to non-modified JmjD4 and FamH83. Binding of Ars2 to membrane BCR was demonstrated by flow cytometry of DLBCL cell lines with Ars2-reactive BCR, but not for the TMD8 line without Ars2-reactive BCR. Internalization of C-terminally FLAG-tagged Ars2 into U2932 cells was observed after 60 min (Figure 1D). For OCI-Ly10, mutagenesis of K98A of the BCR heavy chain gene had been reported to result in loss of autoreactivity.[8] OCI-Ly10 K98A resulted in loss of affinity against Ars2 (Figure 1E).

Characterization of Ars2 in diffuse large B-cell lymphoma with Ars2-specific B-cell receptors

No obvious differences in molecular weight of Ars2 from DLBCL cases with Ars2-reactive and Ars2-non-reactive BCR and controls were observed in western blots; similarly, Sanger sequencing revealed identical DNA sequences, excluding mutations in the coding sequence as an explanation for the immunogenicity of Ars2. However, isoelectric focusing of cell lines (Figure 2A) and DLBCL cases (Figure 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 DBLCL cases and cell lines without Ars2-specific BCR than in cases with Ars2-reactive BCR and resulted in the disappearance of the differences in electric charge

between both isoforms of Ars2 (Figure 2C), demonstrating that the different isoelectric focusing pattern of Ars2 was due to hypophosphorylation in cases with Ars2-specific BCR. The hypophosphorylated Ars2 isoform was detected in all of the three DLBCL cell lines with Ars2-reactive BCR (i.e., OCI-Ly3, OCI-Ly10, and U2932), but in none of six DLBCL lines without Ars2-reactive BCR. This association in the nine 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 Fab (*Online Supplementary Table S1*) derived from cryospecimens, only the two cases with Ars2-reactive Fab (#3 and #11) showed hypophosphorylation of Ars2. In this cohort of 11 cases of cryospecimens and recombinantly expressed Fab, the association between Ars2 reactivity and presence of hypophosphorylated Ars2 isoform was also statistically significant (Fisher exact *t*-test: two-tailed *P*=0.0182). Looking at the association between BCR reactivity of DLBCL cells and the presence of hypophosphorylated Ars2 isoform overall, considering data from both cell lines and cases with cryospecimen-derived recombinant Fab, the association was statistically highly significant (Fisher exact *t*-test: two-tailed *P*<0.0001).

Hypophosphorylated Ars2 was detected in the biopsies of eight of 31 (26%) ABC-type DLBCL cases characterized by GEP, but in only one of 20 (5%) GCB-type DLBCL and in the peripheral blood from one in 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-termi-

nal FLAG tag in OCI-Ly10, OCI-Ly3 and, as a control, HBL1 (Figure 2D and *Online Supplementary Figure S4*).

Frequency, titers, and IgG subclasses of Ars2 serum antibodies

Ars2 antibodies were detected by ELISA in the sera of four of 98 patients with DLBCL, with titers ranging from 1:800 to 1:1600, and in one of 400 healthy controls. All four patients with Ars2 antibodies in their sera (#22, #27, #41, #73) were carriers of hypophosphorylated Ars2 in the cells of their peripheral blood (Figure 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 diffuse large B-cell lymphoma lines

Western blot analysis of BCR pathway activation after addition of recombinant Ars2 revealed a strong activation in the U2932 cell line with Ars2-reactive BCR, 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 BCR (#4 and #10) did not have MYC-overexpression, as determined by immunohistochemical analysis (*data not shown*). No BCR pathway activation was induced by the control antigen MAZ in the U2932 line or by addition of Ars2 to the HBL1 line (Figure 3A). Addition of recombinant Ars2 induced proliferation of U2932 and OCI-Ly3 cells, but not of DLBCL cell lines without Ars2-reactive BCR, such as TMD8, analyzed by the tetrazolium/formazan EZ4U assay (Figure 3B). This Ars2-induced growth stimulus could be reverted by addition of Ars2-neutralizing recombinant Fab derived from patient #4 (Figure 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 (Figure 3D).

Cytotoxicity of the Ars2/ETA' conjugate

Addition of Ars2-ETA' resulted in inhibition of growth analyzed in proliferation assays. This inhibition could be reverted by preincubation of Ars2/ETA' toxin with the Ars2-reactive recombinant Fab derived from case #4 (Figure 4A). The Ars2-ETA' conjugate exerted a specific and dose-dependent toxicity against the Ars2-reactive
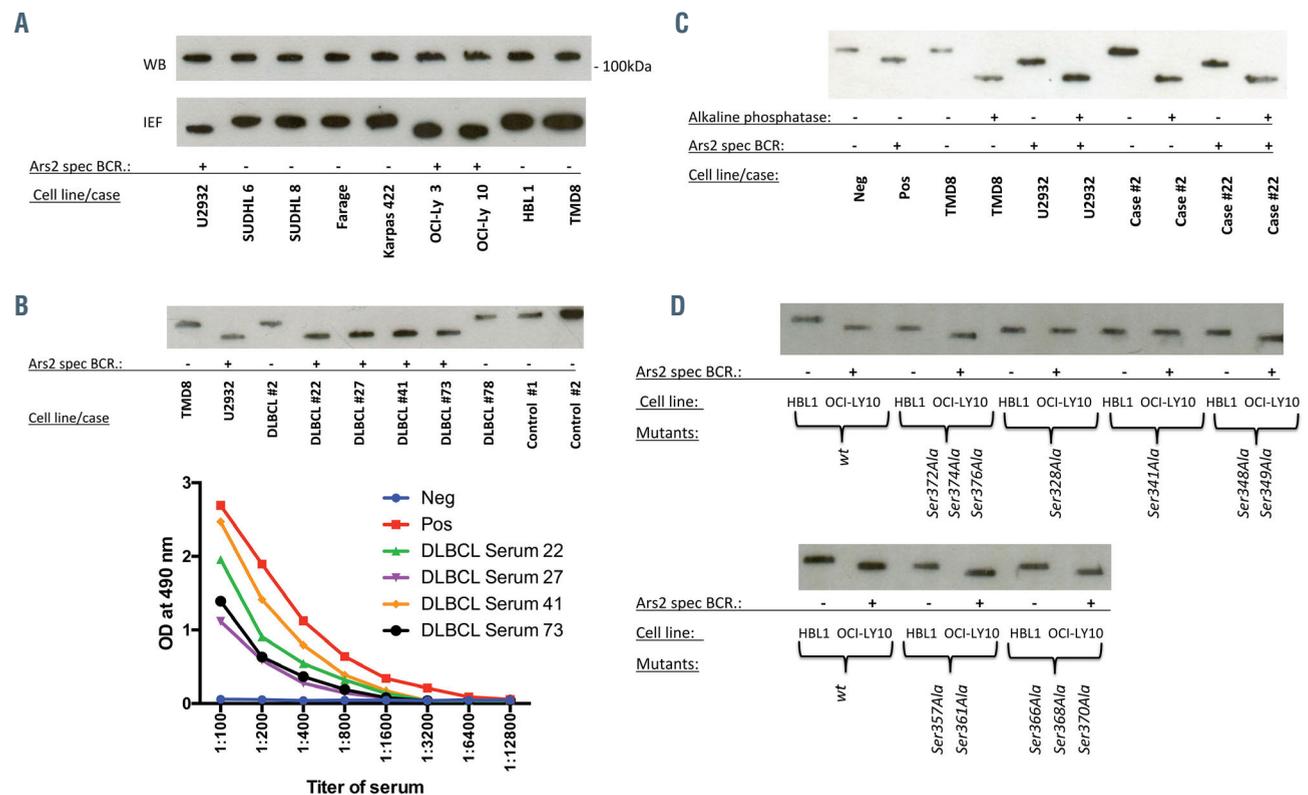

Figure 2. Ars2 is exclusively hypophosphorylated in patients with Ars2-reactivity of lymphoma B-cell receptors. (A) Western blot and isoelectric focusing (IEF) of Ars2 derived from diffuse large B-cell lymphoma (DLBCL) cell lines. Western blot of Ars2 in nine DLBCL cell lines revealed no difference in Ars2 between cell lines with and without Ars2-reactive B-cell receptors (BCR) (above). However, IEF of Ars2 in the DLBCL lines showed a less negative charge of Ars2 in OCI-Ly3, OCI-Ly10 and U2932. These three cell lines had exclusively Ars2-reactive BCR. (B) IEF of Ars2 of whole blood derived from DLBCL patients with Ars2-autoantibodies (#22, #27, #41, #73). The less negatively charged Ars2 isoform was also detected In the peripheral blood of these four patients. Ars2 autoantibody titers ranged between 1:800 and 1:1600 in these four 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 BCR. (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, Ser349 Ala, 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 that of 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 the primary antibody.

BCR-expressing cell lines, but had no observable effect on DLBCL lines lacking Ars2-reactive BCR (Figure 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 35%, 2% and 0% of OCI-Ly3 cells were alive after 24 h, 48 h and 72 h, respectively, contrasting with findings for the wild-type HBL1 cell line without BCR reactivity against Ars2 (97% viable cells at 24 h; 96% at 48 h; and 97% at 72 h) (Figure 4B). In accordance with this, an increase of apoptotic cells was detected after incubation with Ars2-ETA' in U2932 cells expressing Ars2-reactive BCR (Figure 4C), as demonstrated in the annexin V/propidium iodide assay.

Discussion

Beside the two relatively rare target antigens, ubiquitinated FamH83 and sumoylated JmJD4, in the present study, hypophosphorylated Ars2 was identified as a more frequent antigen of BCR from DLBCL lines and recombinant BCR from primary DLBCL cryospecimens. 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 the innate immune response against RNA viruses by
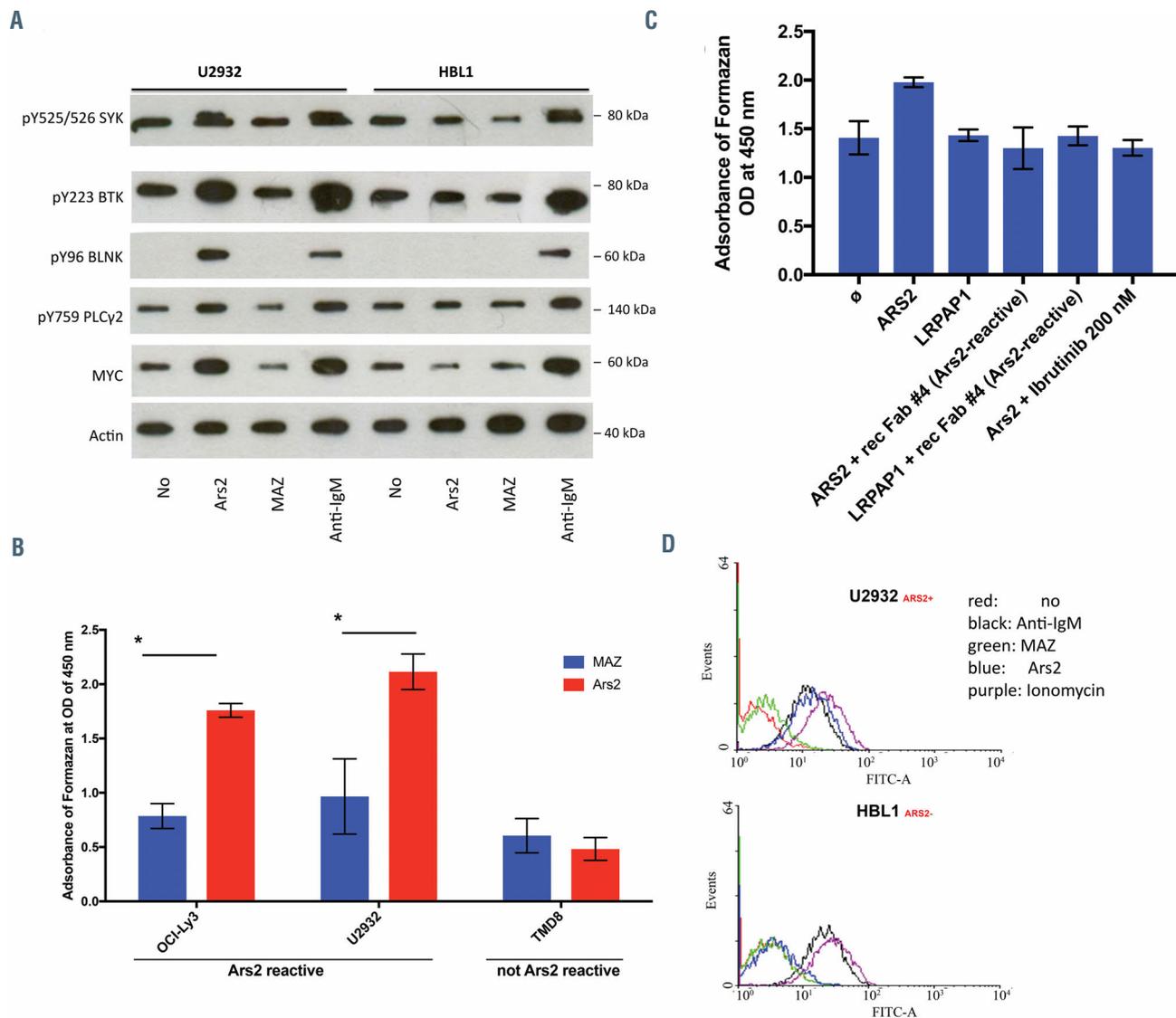

Figure 3. B-cell receptor pathway activation and induction of proliferation by Ars2. (A) B-cell receptor (BCR) pathway analysis by western blot in U2932 and HBL1 after addition of cognate/control antigens Ars2 or MAZ showed strong activation due to the addition of ARS2 to U2932 cells with upregulation 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 statistically significant (*P*<0.01: Student *t*-test) increase of proliferation, as determined by the EZ4U assay (columns represent formazan at an optical density [OD] of 450 nm), while addition of Ars2 had no effect on the TMD8 cell line. Columns and bars represent mean and standard deviation of three experiments. (C) Inhibition of Ars2-induced proliferation by neutralizing Ars2-reactive Fab. Addition of Ars2 together with Ars2-reactive (patient derived, case #4) recombinant Fab 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 adding anti-IgM (black) to U2932 cells, but not after the addition of a control antigen MAZ (green). Addition of Ars2 to control the diffuse large B-cell lymphoma line HBL1 did not result in elevated calcium levels.
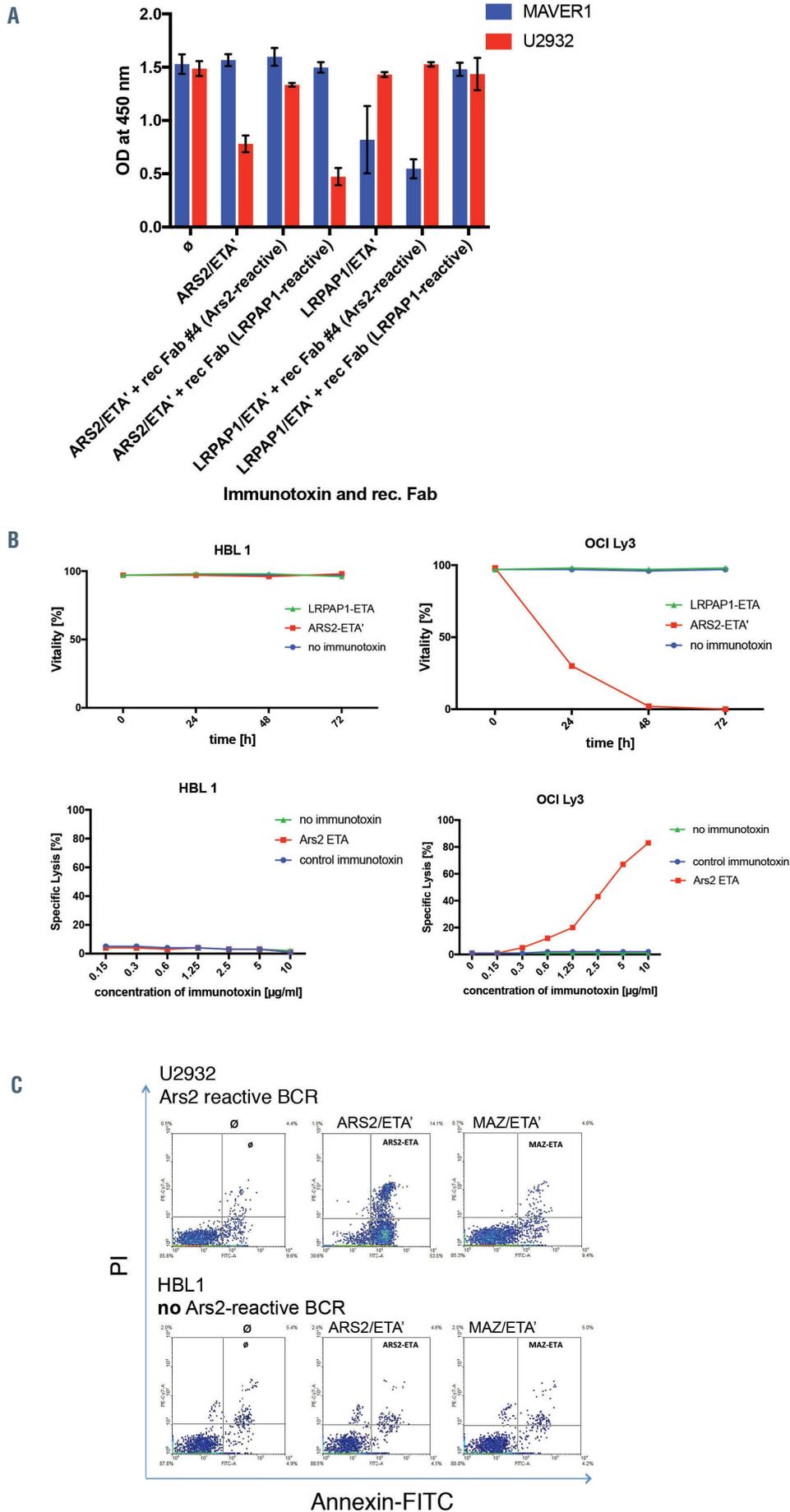
A

B

C

Figure 4. Targeting Ars2-reactive diffuse large B-cell lymphoma with Ars2-containing immunotoxins. (A) Growth inhibition by B-cell receptor (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 co-incubation with neutralizing Ars2-reactive Fab but not by LRPAP1-reactive Fab. Columns (formazan formation detected at an optical density of 450 nm) represent cell proliferation (mean and standard deviation). (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-dependent cytotoxic effect of Ars2/ETA' determined in a lactate dehydrogenase (LDH) release assay. Curves indicate percent specific lysis of the HBL1 line (left) or OCI Ly3 line (right) with and without Ars2-reactive BCR, after incubation with doses from 0 μg/mL to 10 μg/mL Ars2/ETA', LRPAP1/ETA' or phosphate-buffered saline. (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/propidium iodide staining. U2932 cells have Ars2-reactive BCR resulting in a strong increase of early and late apoptotic cells after addition of Ars2/ETA'.

miRNA processing.[23] Ars2 expression was shown to be linked to proliferative states[24] and different roles were described in malignant disesases.[25,26]

All three target antigens of DLBCL-BCR identified in this study share the characteristic of being atypically post-translationally modified, which represents the most likely reason for their immunogenicity. For ubiquitinated FamH83 and sumoylated JmJD4, the lymphoma BCR were specific for the secondary modified isoforms. In contrast, Ars2-reactive BCR of DLBCL bound both the hypophosphorylated and the normally phosphorylated isoforms of Ars2. However, the hypophosphorylated isoform of Ars2 was only observed in cell lines or cryospecimens of DLBCL with BCR reactivity for Ars2 ($P<0.0001$). Similarly, regarding peripheral blood, the hypophosphorylated isoform of Ars2 was 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 post-translational modification is involved in the immunogenicity.

Bringing this into the context of other B-cell neoplasias, in plasma cell dyscrasia SLP2-reactive paraprotein was also not specific for the differentially phosphorylated SLP2 isoform.[27] As for CD4+ T-helper cells specific for hyperphosphorylated SLP2 in plasma cell dyscrasia,[28] one might speculate about a possible role of hypothetical CD4+ T-helper cells specific for hypophosphorylated Ars2 epitopes, which might stimulate Ars2-reactive B cells, which by themselves do not differentiate between normally phosphorylated and hypophosphorylated Ars2. Generally, atypical post-translational modifications represent an accepted mechanism for the breakdown of self-tolerance, with numerous examples in clinical immunology, such as 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,[37-39] and the involvement of atypically modified BCR target antigens in lymphomagenesis including hyperphosphorylated SLP2, ATG13, and sumoylated HSP90 in plasma cell dyscrasia and hyper-N-glycosylated SAMD14/neurabin-I in primary central nervous system lymphoma.[11,15,27,40,41]

Of interest, Ars2 hypophosphorylation and reactivity of DLBCL BCR against Ars2 was nearly exclusively detected in DLBCL of the ABC type. All the cell lines with anti-Ars2 reactivity were of this cell of origin, and in a validation cohort of DLBCL with cell of origin characterized by GEP, hypophosphorylated Ars2 was detected in eight of 31 cases (26%) of the ABC type of DLBCL, but in only one of 20 cases (5%) of GCB-type DLBCL. In a combined analysis of GEP-typed cryospecimens 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 BCR (Figure 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 BCR and expression of hypophosphorylated Ars2, OCI-Ly10 has mutated MYD88 (L265P)[42] and a truncating mutation of CD79A,[7,43] U2932 has a mutated NFκB-pathway by TAK1 mutation,[44] but wild-type CARD11, and wild-type MYD88,[7,45] and OCI-Ly3 has a mutated CARD11 and mutated MYD88 (L265P).[7,42] This demonstrates that, despite pathway-activating mutations, the proliferation of these cell lines might still benefit from an upstream BCR pathway stimulation by a cognate antigen.

From a therapeutic point of view, two hypothetical approaches arise from these data. Firstly, BCR antigens might be used as baits to target lymphoma cells in a specific way (i.e., targeting of a cell-bound antibody by an antigen), similarly to anti-idiotype antibodies or peptibodies,[46] with the advantage of not having to be selected and synthesized individually for each patient, since all Ars2-reactive DLBCL BCR bind the same epitope. Physiologically it is the major task of a surface immunoglobulin to bind its cognate antigen and then internalize it, enabling processing and antigen presentation via MHC class II molecules. Targeting Ars2-reactive BCR 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),[47] or as an additional ectodomain for chimeric antigen receptor T cells.[48] Secondly, regarding the high relative risk of carriers of atypically hypophosphorylated Ars2, investigating ways of modulating this post-translational modification might be worthwhile in the future.

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