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ORIGINAL ARTICLE

Chlamydophila psittaci-negative ocular adnexal marginal zone lymphomas express self polyreactive B-cell receptors

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The pathogenesis of *Chlamydophila psittaci*-negative ocular adnexal extranodal marginal zone lymphomas (OAEMZLs) is poorly understood. OAEMZLs are monoclonal tumors expressing a biased repertoire of mutated surface immunoglobulins. Antigenic activation of the B-cell receptor (BCR) may have a role in the pathogenesis of these lymphomas. We have analyzed the reactivity of recombinant OAEMZL immunoglobulins. OAEMZL antibodies reacted with self-human antigens, as demonstrated by enzyme-linked immunosorbent assays, HEp-2 immunofluorescence and human protein microarrays. All the analyzed recombinant antibodies (rAbs) exhibited polyreactivity by comprehensive protein array antibody reactivity and some rAbs also demonstrated rheumatoid factor activity. The identity of several reactive antigens was confirmed by microcapillary reverse-phase high-performance liquid chromatography nano-electrospray tandem mass spectrometry. The tested rAbs frequently reacted with shared intracellular and extracellular self-antigens (for example, galectin-3). Furthermore, these self-antigens induced BCR signaling in B cells expressing cognate surface immunoglobulins derived from OAEMZLs. These findings indicate that interactions between self-antigens and cognate OAEMZL tumor-derived BCRs are functional, inducing intracellular signaling. Overall, our findings suggest that self-antigen-induced BCR stimulation may be implicated in the pathogenesis of *C. psittaci*-negative OAEMZLs.

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INTRODUCTION

Mucosa-associated lymphoid tissue (MALT) lymphomas (also referred as extranodal marginal zone lymphomas (EMZLs)) are the third most common type of B-cell non-Hodgkin lymphomas.[1] MALT lymphomas usually originate in lymphoid tissue acquired in response to infection or persistent inflammation.[2] Consequently, gastric MALT lymphomas are associated with *Helicobacter pylori* infection,[3] intestinal and skin lymphomas are linked to *Campylobacter jejuni* and *Borrelia burgdorferi* infections,[4,5] respectively, and thyroid and salivary glands MALT lymphomas are more frequent in patients with Hashimoto's thyroiditis and Sjögren syndrome.[6,7] Although the pathogenesis of MALT lymphomas in these anatomic locations has been extensively studied,[8,9] the pathogenesis and etiology of ocular adnexal EMZLs (OAEMZLs), the second most common EMZL and the most common tumor occurring in the ocular adnexa, are still controversial.[1,10–12] *Chlamydophila psittaci* infection was implicated in the pathogenesis of OAEMZLs, but this association demonstrated marked differences based on geography.[1,13,14] Most studies originating from North American institutions did not detect *C. psittaci* in OAEMZL tumors.[13,15,16] Further, we have previously reported that *C. psittaci*-negative OAEMZLs usually do not harbor the classical chromosomal translocations t(14;18)(q32;q21) IGH-MALT1 and t(11;18)(q21;q21) API2-MALT1 implicated in the pathogenesis of EMZLs originating in other anatomical locations.[10]

The majority of B-cell lymphomas express unique clonal surface B-cell receptors (BCRs) encoded by rearranged heavy (*IGHV*) and light (*IGKV* or *IGLV*) immunoglobulin (Ig) genes that may transmit survival signals following binding to cognate antigens. BCR activation and signaling is implicated in the pathogenesis of several types of lymphoma.[17–20] We have previously demonstrated a biased *IGHV* (*IGHV4* family and *IGHV4-34* gene) and *IGKV* (*IGKV3-20*) usage and the presence of extensive somatic mutations in *IGHV* genes in OAEMZLs that corresponded to positive and/or negative antigen selection pressures.[11,12] These findings pointed to a potential role for BCR signaling in the pathogenesis of *C. psittaci*-negative OAEMZLs and raised the hypothesis that possible shared tumor antigens may activate and promote the survival of these lymphomas. Detection of intraclonal heterogeneity in these tumors further suggested the existence of continuous antigen stimulation.[11] Previous studies of BCRs from EMZLs originating in the stomach and parotid gland demonstrated polyreactivity, binding to bacterial and self-antigens, including stomach extracts and DNA, and exhibited Ig rheumatoid factor (RF) activity.[21,22] However, the reactivity of BCRs derived from OAEMZLs has not been evaluated. To identify potential antigens that might be

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implicated in the pathogenesis of *C. psittaci*-negative OAEMZLs, we studied the reactivity of recombinant OAEMZL Igs to human proteins.

MATERIALS AND METHODS

Cloning, expression and purification of OAEMZL immunoglobulins

IGHV and *IGKV* genes were cloned from OAEMZL-derived DNA as a part of our previously reported studies characterizing the Ig gene repertoire in these tumors.[11,12] Touchdown enzyme time-release PCR for the detection of *C. psittaci* DNA was performed as previously reported by us.[13] All studies were approved by the University of Miami Institutional Review Board. A total of five OAEMZL specimens, whose *IGHV* and *IGKV* characteristics are summarized in Supplementary Table S1, were selected for the production of recombinant antibodies (rAbs) derived from tumor BCRs using standard protocol described in the Supplementary Materials. All rAbs representing tumor BCRs were expressed on a common IgG3 heavy and light chain backbone, irrespective of the original isotype, to allow comparability in reactivity assays and to ensure that differences in binding activity could be attributed to the specific Ig variable regions.

Human embryonic kidney 293T cells cultured at 37 °C and 5% CO_2 in Iscove-modified Eagle medium supplemented with 5% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin G (GibcoBRL, Grand Island, NY, USA) were simultaneously transfected with the plasmids encoding the tumor *IGHV* and *IGKV* genes using the PolyFect Transfection Reagent (Qiagen, Valencia, CA, USA). The transfected cells were selected in histidinol-containing medium (100 mM) and supernatants were screened by enzyme-linked immunosorbent assay (ELISA) and western blotting to verify rAbs secretion. Identified stable rAb producing clones were grown in Super Low IgG Fetal Bovine Serum (Hyclone, Logan, UT, USA) and adapted for growth in suspension culture. Finally, for production and purification of large quantities of rAbs, the cells were transferred to roller bottles and grown in FreeStyle (Invitrogen, Grand Island, NY, USA) serum-free medium. Supernatants were harvested, and rAbs were purified using protein G beads (Invitrogen) followed by elution with 0.1 M glycine (pH 3.0) and neutralization with 1 M Tris (pH 8.0). Eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions to confirm expression and proper assembly of H and L chains (Supplementary Figures S1A and S1B). As a control, we used IgG3 antibody to receptor tyrosine protein kinase erbB-2 (HER2). This antibody was generated and purified in our laboratory under the same purification conditions that we used to purify the orbital lymphoma-derived BCRs. Concentrations of rAbs were determined by ELISA. Cellular protein lysates derived from HeLa cells infected with *C. psittaci* (kind gift of Dr Ken Fields, UM), which is implicated in the pathogenesis of OAEMZL in some geographic regions, as well as protein lysates from randomly selected bacteria (*Staphylococcus aureus* and *Salmonella enteridis)*, were coated on plates and tested for reactivity with rAbs using ELISAs.

HEp-2 immunofluorescence assay

OAEMZL BCR-derived rAbs were tested for self-reactivity by an indirect immunofluorescence assay using HEp-2 cells (Bion Enterprises, Des planes, IL, USA) in accordance to the manufacturer's instructions. rAbs were diluted in phosphate-buffered saline (PBS) and used at 100–200 μg/ml. Binding was detected with anti-human IgG-fluorescein isothiocyanate antibodies. Evans Blue dye was used as a counterstain. Slides were coated with ProLong Gold Antifade Reagent with 4′, 6′-diamidino-2-phenylindole (Invitrogen) and sealed with a glass cover slip. As suggested by the manufacturer, control staining included PBS alone and antinuclear antigen-negative and antinuclear antigen-positive controls. Samples were examined on a Zeiss ApoTome fluorescence microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA). Pictures were taken using ×60 oil-immersion objective lenses.

Anti i/I tests

Anti i/I reactivity of OAEMZL BCR-derived rAbs was tested as previously reported[23,24] and described in the Supplementary Materials.

Enzyme-linked immunosorbent assays

Triplicate wells of high-binding capacity ELISA plates (Costar, Tewksbury, MA, USA) were coated overnight at 4 °C with 50 μl per well of the following antigens in PBS: human recombinant insulin solution (5 μg/ml; Sigma Aldrich, St Louis, MO, USA), salmon sperm double-stranded (ds) DNA (Sigma Aldrich), salmon sperm single-stranded (ss) DNA (Sigma Aldrich), lipopolysaccharide (LPS) from *Escherichia coli* Serotype 055:B5 (Sigma Aldrich; all at 10 μg/ml) and galectin-3 (R&D Biosciences, Minneapolis, MN, USA; 5 μg/ml, total volume 100 μl/well). Insulin, dsDNA, ssDNA and LPS were selected as antigens to determine polyreactivity and allow comparison with previously published studies investigating lymphoma BCR polyreactivity.[21,25] rAb polyreactivity based on ELISA was defined using the previously proposed criteria of binding to at least two antigens in three independent experiments.[21,25] After washing three times with PBS, the wells were blocked overnight at 4 °C with 200 μl per well of 3% IgG-free bovine serum albumin (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA, USA) in PBS. All rAbs were incubated for 1 h at 0.625, 2.5, 10, 40 and 100 μg/ml at room temperature. Following washing, wells were incubated for 1 h with 50 μl per well of anti-human Kappa-Alkaline Phosphatase (light chain bound; Sigma Aldrich) at 1:5000 dilutions in PBS. After three additional washes with PBS, wells were incubated for 30 min with 50 μl of fresh p-nitrophenylphosphate buffer (Sigma Aldrich). Optical density was measured at 450 nm using Microplate Manager 5.2.1 software (Bio-Rad, Hercules, CA, USA). IgG3 antibody to HER2 (αHER2) and rituximab were used as negative controls.

Immunoreactivity of adsorbed galectin-3 was ascertained using ELISA by overnight coating of plates with galectin-3 recombinant protein at 5 μg/ml. Following three washes with PBS, the wells were blocked for 2 h at room temperature with 200 μl per well of 3% IgG-free bovine serum albumin in PBS. All the rAbs were incubated for 1 h at 2.5, 20 and 100 μg/ml at room temperature. Following washing, wells were incubated for 1 h with 50 μl per well of anti-human Kappa-Alkaline Phosphatase at 1:5000 dilutions in PBS. After three additional washes with PBS, wells were incubated for 1 h with 50 μl of fresh p-nitrophenylphosphate buffer. Optical density was measured at 450 nm using Microplate Manager 5.2.1 software. To exclude nonspecific galectin-3 binding to potential rAbs sugar moieties, ELISA experiments were repeated in the presence of 0.1 M lactose (Sigma Aldrich). To further confirm the specificity of the assay, we carried out competitive inhibition ELISAs for galectin-3. To this end, 20 μg/ml of rAbs were incubated in the plate coated (solid phase) with 5 μg/ml of galectin-3 (100 μl per well) in the presence of a soluble form galectin-3 at 10, 30 and 50 μg/ml. Bovine serum albumin (50 μg/ml) was used as a negative control.

RF activity of OAEMZL-derived rAbs (0, 0.25, 2, 10, 20 and 40 μg/ml) was determined using the RF Ig ELISA kit (DRG Diagnostics, Springfield, NJ, USA) according to the manufacturer's instructions. To determine the specificity of RF activity, ELISAs were repeated following pre-incubation with increasing amounts (10, 20 and 40 μg/ml) of ChromPure human IgG Fc fragment (Jackson ImmunoResearch Laboratories, Inc.) for 30 min followed by 1-h incubation in the presence of OAEMZL antibodies at 20 μg/ml at room temperature.

Protein array experiments

Antibody specificity profiling with four OAEMZL rAbs (Ab4438, Ab4726, Ab5334 and Ab11274) was performed on ProtoArray Human Protein Microarray v.5 (http://www.lifetechnologies.com/us/en/home/life-science/protein-expression-and-analysis/biomarker-discovery/protoarray/resources/protein-microarray-faq.html) containing >9000 human proteins (Life Technologies, Grand Island, NY, USA), as described in the Supplementary Materials. All protein array data can be found at the GEO public database under accession number GSE65443.

Immunoprecipitation and immunoblotting

Protein lysates prepared from HeLa cells, grown in Dulbecco-modified Eagle medium (Invitrogen–Gibco) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin were used for immunoprecipitation and western blotting experiments, performed as previously reported.[26] The following antibodies were used: OAEMZL-derived rAbs (Ab4438, Ab4726, Ab5334 and Ab11274), mouse monoclonal methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2 (MTHFD2) antibody (243.1; Santa Cruz Biotechnology, Dallas, TX, USA), galectin-3 (LGALS3) MaxPab rabbit polyclonal Antibody (D01; Abnova, Taipei City, Taiwan), galectin-1 antibody (ab25138; Abcam, Cambridge, MA, USA), galectin-8 antibody (ab109519; Abcam) and an ICAM4 (intercellular adhesion molecule 4) MaxPab rabbit polyclonal antibody (D01; Abnova). For controls we used rAb1152 and rAb501,

antibodies derived from follicular lymphoma tumors, kindly provided by Dr Ronald Levy (Stanford University).

For mass spectrometry, precipitated proteins were separated on a 10% SDS-polyacrylamide gel electrophoresis gel and detected by silver staining. Bands of the selected size were cut out and submitted for sequence analysis to the Harvard Mass Spectrometry and Proteomics Resource Laboratory (Cambridge, MA, USA). Analysis was performed by micro-capillary reverse-phase high-performance liquid chromatography nano-electrospray tandem mass spectrometry on a Thermo LTQ-Orbitrap (Thermo Scientific, Tewksbury, MA, USA) mass spectrometer.

Immunofluorescence microscopy

Non-manipulated HeLa cells or cells transfected with galectin-3 small interfering RNA (siRNA; ON-TARGETplus SMARTpool—Human LGALS3 siRNA (Thermo Scientific)) or control siRNAs were seeded on glass coverslips in 6-well culture dishes. The cells were fixed with 4% paraformaldehyde in PBS for 10 min and visualized with immunofluorescence microscopy, as described in details in the Supplementary Materials.

Double immunofluorescence labeling

Double immunofluorescence labeling was performed in three cases of OAEMZL lymphomas using paraffin-embedded tissue sections of the tumor samples as previously described.[27] Normal tonsil and colon mucosa sections were used as controls. In brief, all tissue sections underwent heat-induced epitope retrieval in pH 6.0 citrate buffer (Dako, Carpinteria, CA, USA). Endogenous peroxidase was blocked by 3% H_2O_2 solution for 10 min. To avoid nonspecific binding of primary antibodies, serum-free blocking solution (Life Technologies) was applied for 40 min at room temperature. The slides were incubated overnight at 4 °C with anti-galectin-3 antibody (clone 9C4; Leica Biosystems, Buffalo Grove, MA, USA) combined with one of the following antibodies: CD68 (Sigma Aldrich) and CD163 (Santa Cruz Biotechnology) for staining tissue macrophages, CD117 (clone YR145; Cell Marque, Rocklin, CA, USA) for staining mast cells and CD21 (Abcam) for staining follicular dendritic cells. Alexa Fluor 594 (red) goat anti-rabbit IgG conjugate and Alexa Fluor 488 (green) goat anti-mouse IgG conjugate (Molecular Probes-Life Technology, Grand Island, NY, USA) were used as secondary antibodies. The nuclei were counterstained with 4′, 6′-diamidino-2-phenylindole. After aqueous mounting, the slides were viewed using a fluorescence microscope (Olympus BX51, Tokyo, Japan).

Galectin-3 immunohistochemistry

Eight cases of OAEMZL and control formalin-fixed paraffin-embedded tissues were used for immunohistochemistry studies. Tissue sections were cut from formalin-fixed paraffin-embedded tissue blocks at 4 μm and mounted on positively charged slides. Immunohistochemistry was performed using a standard protocol. In brief, sections were baked at 60 °C for 30 min, cooled, deparaffinized, rehydrated, and antigenic retrieval was carried out at a pH of 9 in an automated stainer (Envision Flex Target Retrieval solution high pH in automate PT link, Dako). Galectin-3 staining was performed using galectin-3 antibody (Neo-markers, catalog number MS-1756-S, clone 9C4) using a 1:50 dilution and 20 min antibody exposure time. The galectin-3 antibody was validated using normal B lymphocytes from tonsils as negative and colonic glands as positive controls.

Construction of membrane-anchored OAEMZL immunoglobulin

A plasmid vector (backbone vector (TCAE 5.3)) to express a membrane-bound OAEMZL IgMs was previously described and kindly provided by Dr Shoshana Levy (Stanford University). Flanking restriction sites 5′ SalI, 3′ NheI and 5′ BglII, 3′ BswI were added to the OAEMZL *IGHV* and *IGKV* genes, respectively, via PCR to allow ligation to the TCAE vector.

To generate an A20 BALB/c B-cell lymphoma line expressing OAEMZL IgM, A20 cells obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol (Sigma Aldrich). The A20 cells were transfected with the expression vector described above using the Cell Line Nucleofector Kit V and a Nucleofector II electroporator (Lonza, Walkersville, MD, USA). The transfected cells were cultured in the same media as the parental cell line with the addition of 800 μg/ml of Geneticin (Invitrogen) for stable selection. Expression was confirmed by flow cytometry following cell staining with phycoerythrin-conjugated anti-IgM antibody.

Calcium mobilization assay

A20 cells expressing OAEMZL IgMs and the parental line (6×10^6 cells per sample) were washed twice with complete RPMI 1640 medium and stained with 2 μM of Fluo-4 AM (Invitrogen) for 30 min at 37 °C, as we reported previously.[28] The cells were washed twice again with RPMI 1640 and incubated for 30 min before stimulation. After recording a baseline signal for 30 s, cells were stimulated with goat $F(ab')_2$ anti-human IgM (Invitrogen), recombinant full-length galectin-3 at 40 μg/ml or MTHFD2 at 30 μg/ml (Abcam) and recording was continued for an additional 420 s. Intracellular Ca_2^+ mobilization was measured in real-time on a BD FACS Fortessa (BD Biosciences, San Jose, CA, USA) with a laser tuned at 488 nm. Data were analyzed via FlowJo 7.6.4 software (FlowJo, Ashland, OR, USA).

RESULTS

Self-antigen reactivity of rAbs derived from OAEMZLs

We have generated rAbs corresponding to BCRs expressed by five OAEMZLs (tumors 4726 and 4438 expressing *IGHV4-34* with and without somatic mutations, respectively, both paired with *IGKV3-20*; tumor 4968 expressing *IGHV*3-30 and *IGKV1-33*; tumor 11274 expressing *IGHV*3-23 and *IGKV3-20*; and tumor 5334 expressing *IGHV4-59* and *IGKV3-15*; Supplementary Table S1).

We first examined the reactivity of the generated rAbs with bacterial proteins, as previous studies implicated *C. psittaci* in the pathogenesis of OAEMZLs in some geographic regions. However, the generated rAbs did not react with either cellular lysates prepared from *C. psittaci*-infected cells or with bacterial proteins lysates of *Staphylococcus aureus* and *Salmonella enteridis* (not shown).

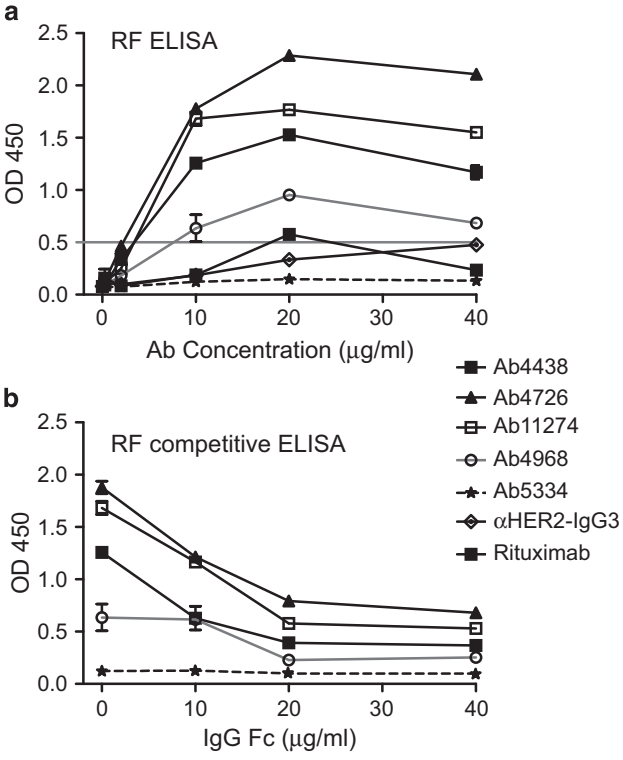

Figure 1. Rheumatoid factor (RF) activity of OAEMZL rAbs. (**a**) OAEMZL antibodies (0–40 μg/ml) cloned from the tumors of five independent patients were tested by ELISA for reactivity towards RF. Rituximab and αHER2-IgG3 were used as negative control. Gray line is indicative of cutoff value as defined by the positive control from the ELISA kit. (**b**) Single concentrations of Abs 4438, 4726, 11274, 4968 and 5334 (10 μg/ml) were incubated with plate-coated (solid phase) IgG in the presence of varied concentration of human IgG Fc (10, 20 and 30 μg/ml) in soluble form. In **a** and **b**, one representative inhibition assays out of three are shown and error bars indicate s.e.m. between triplicate wells.

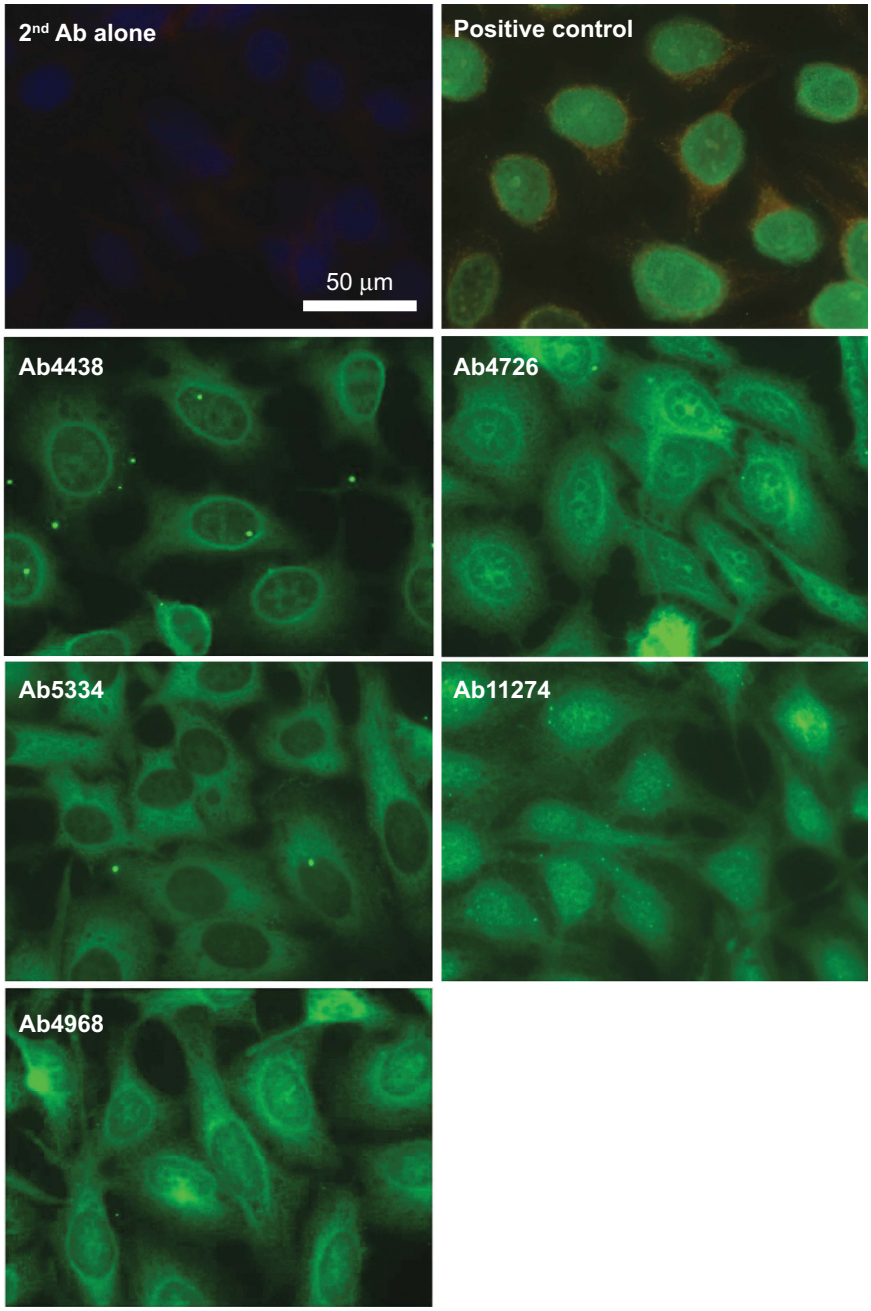

Figure 2. Self-reactivity of OAEMZL rAbs. OAEMZL tumor-derived rAbs were tested for self-reactivity by indirect immunofluorescence assay using HEp-2 cells. rAbs (100–200 mg/ml) were detected using anti-human IgG-fluorescein isothiocyanate. Staining including secondary antibody alone (negative control) and ANA-positive controls are shown. Data are representative of three independent experiments.

As OAEMZLs often use *IGHV* and *IGKV* genes that are frequently implicated in reactivity with I/i antigens and RF activity (for example, *IGHV4-34* and *IGKV3-20*), and BCRs of EMZLs derived from the stomach and parotid gland demonstrate RF activity,[22] we next examined the reactivity of the rAbs with these antigens. The rAbs did not react with I/i antigens (not shown). rAbs derived from OAEMZLs 4726, 4968, 5334 and 11274 exhibited RF activity by ELISA (Figure 1a). This reactivity was blocked in competitive inhibition assays using increasing quantities of purified human IgG Fc fragment (Figure 1b), indicating that the observed RF reactivity was specific.

To test the rAbs derived from BCRs of OAEMZLs for reactivity with a large collection of self-antigens, we performed a screen by an indirect immunofluorescence assay using permeabilized human HEp-2 cells. This is a standard clinical assay for detecting self-reactive antibodies in the serum of patients with autoimmune disorders. All tumor-derived rAbs were reactive with HEp-2 cells. rAb5334 exhibited predominantly cytoplasmic staining, whereas rAbs 4726, 4438, 11274 and 4968 showed combined nuclear and cytoplasmic staining (Figure 2). The diverse staining patterns, as well as the simultaneous nuclear and cytoplasmic staining, suggested three possibilities as follows: (1) reactivity of different OAEMZL BCRs with diverse unique cellular antigens; (2) reactivity with proteins expressed in both nucleus and cytoplasm or (3) that the tested rAbs are polyreactive.

To examine the polyreactivity of the rAbs, a series of classical ELISAs using human recombinant insulin, dsDNA, ssDNA and LPS were performed. PBS, anti-HER2 (αHER2) and rituximab antibodies

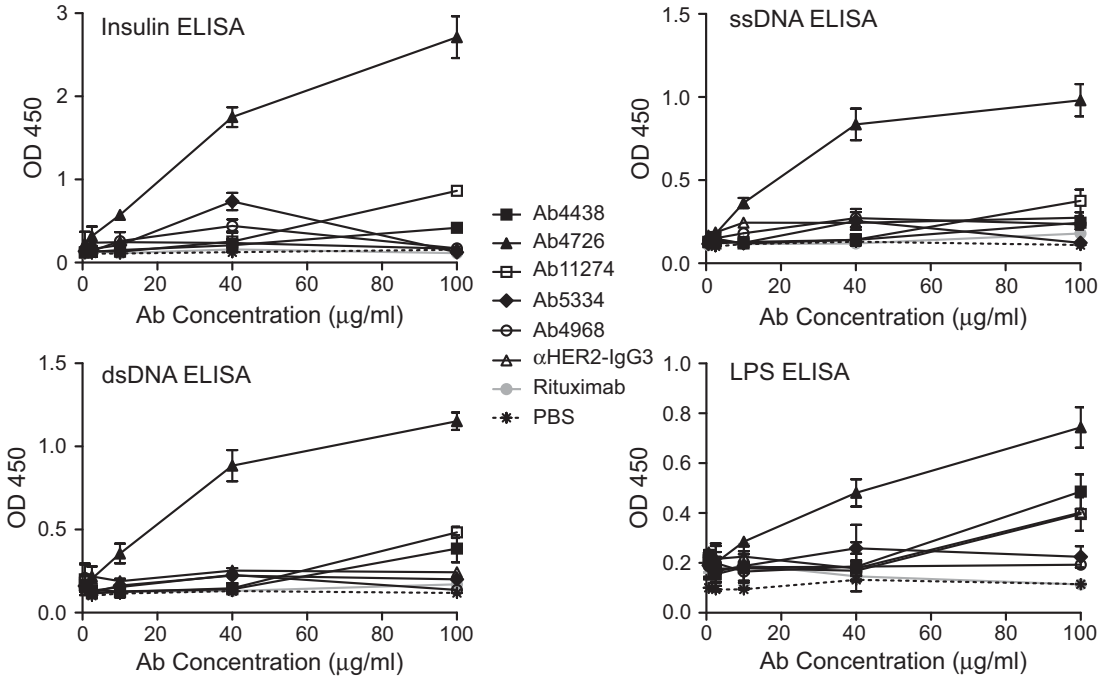

Figure 3. ELISA testing for polyreactivity of OAEMZL rAbs. Five concentrations (0.625, 2.5, 10, 40 and 100 μg/ml) of five Abs cloned from the OAEMZL tumors were tested by ELISA for reactivity towards insulin, ssDNA, dsDNA and LPS. Monoreactive antibodies (αHER2-IgG3 and rituximab) and PBS served as controls. Error bars represent s.e.m. from four independent experiments.

were used as negative controls. The combined results of these ELISAs demonstrated that rAb4726, comprised of IGHV4-34 and IGKV3-20 with somatic mutations, is polyreactive (Figure 3), with dose-dependent increase in binding. The remaining rAbs did not show dose-dependent reactivity to these diverse antigens at low concentrations (0.625, 2.5, 10 and 40 μg/ml), suggesting that they are not polyreactive based on the classical ELISA definition commonly used in previous publications.[21,22] Some of the rAbs demonstrated non-dose-dependent reactivity only at 100 μg/ml, suggesting nonspecific reactivity. Importantly, the αHER2-IgG3 antibody produced and purified under the identical experimental conditions as the rAbs derived from BCRs of OAEMZLs did not exhibit polyreactivity.

Antigen profiling of rAbs derived from OAEMZLs

To identify the self-antigens recognized by the OAEMZL-derived rAbs, we randomly selected four rAbs and performed antibody specificity profiling using ProtoArray Human Protein Microarrays, which contain >9000 proteins. These analyses identified 17, 11, 9 and 25 candidate antigens for rAb4438, rAb4726, rAb5334 and rAb11274, respectively (Table 1 and Supplementary Tables S2–S5). A positive control array using a mouse monoclonal antibody directed to calmodulin kinase IIα printed on every subarray resulted in interaction with calmodulin kinase IIα only. Some of the candidate antigens were unique for specific rAbs (for example, MTHFD2 for rAb4438 and ICAM4 for rAb4726), whereas others were recognized by more than one rAb (for example, galectin-3 (LGALS3) for rAb4438, rAb4726 and rAb11274, galectin-8 for rAb4438, rAb4726 and CAP-GLY domain-containing linker protein, member 4 (CLIP4) for rAb4438 and rAb4726).

To validate these findings, we performed co-immunoprecipitation and co-localization experiments. Reciprocal immunoprecipitation and immunoblotting of Hep2 cellular lysates with a commercial galectin-3 antibody (Figure 4a and Supplementary Figure S2A) or rAb4438, rAb4726 and rAb11274 detected a protein of 30 kDa, corresponding to the galectin-3 protein. Mass spectrometry of the corresponding silver-stained band confirmed the interaction with galectin-3 (Supplementary Figures S3–S6). In contrast, mass spectroscopy using the control antibody immunoprecipitate did not detect galectin-3. Furthermore, these rAbs did not immunoprecipitate galectin-1 (Figures 4a and c), which served as a negative control. Moreover, Hep2 and HeLa cellular lysates immunoprecipitated with follicular lymphoma-derived rAb501 (unknown target) and rAb1152 (directed to the α-myoferlin protein) did not immunoprecipitate either galectin-3 or galectin-1, indicating that the interactions of rAb4438, rAb4726 and rAb11274 with galectin-3 are specific.

The immunoprecipitate of MTHFD2 with a commercial antibody was detected with rAb4438 (Figure 4b). HeLa cellular lysates immunoprecipitated with rAb4438 and blotted with the commercial MTHFD2 antibody detected a protein of size 38 kDa, corresponding to the MTHFD2 protein, whereas rAbs 11274 and 4726 showed no reactivity, corresponding to the antigen profiling array data (Figure 4b and Supplementary Figure S2B). Further, mass spectrometry of the corresponding silver-stained band confirmed that it represented MTHFD2 (Supplementary Figures S7–S8), whereas mass spectroscopy using a control antibody immunoprecipitate did not detect MTHFD2. Additional co-immunoprecipitation experiments using commercially available antibodies confirmed the reactivity of rAb4438, rAb4726 and rAb11274 with galectin-8, CLIP4 and ICAM4 proteins, respectively (Figure 4b and Supplementary Figures S2C and D).

Indirect immunofluorescence assays using a commercial galectin-3 antibody and rAb4438 or rAb4726 confirmed staining co-localization (correlation coefficient $R = 0.725$ and $R = 0.807$, respectively; Figure 4d). Overall, these confirmatory experiments validated the protein array findings.

Galectin-3 is a common self-antigen recognized by rAbs derived from OAEMZLs

We further focused on galectin-3, which was recognized by three different OAEMZL tumor BCRs (rAb4438, rAb4726 and rAb11274)

Table 1. Proteins interacting with rAbs cloned from OAEMZL tumors

OAEMZL antibody	*Interacting proteins*
Ab4438	Keratin 8 (KRT8) Serine/threonine protein kinase 16 Histidine triad nucleotide-binding protein 1 (HINT1) Methylenetetrahydrofolate dehydrogenase (NADP+dependent) 2, methenyltetrahydrofolate cyclohydrolase (MTHFD2) Glycogen phosphorylase, liver form Pyruvate dehydrogenase kinase, isozyme 4 (PDK4) Serine/threonine kinase 16 (STK16) Fc fragment of IgG, low-affinity IIIa, receptor (CD16a) (FCGR3A) Enoyl-CoA hydratase, mitochondrial Copine II (CPNE2) General transcription factor II-I Lectin, galactoside-binding, soluble, 3 (LGALS3) Intercellular adhesion molecule 4 (Landsteiner–Wiener blood group; ICAM4), transcript variant 1 Potassium voltage-gated channel, shaker-related subfamily, beta member 2 (KCNAB2), transcript variant 1 CAP-GLY domain containing linker protein family, member 4 (CLIP4) Cysteine and glycine-rich protein 2 (CSRP2) Lectin, galactoside-binding, soluble, 3 (LGALS3) Lectin, galactoside-binding, soluble, 8 (galectin-8) (LGALS8)
Ab4726	Optineurin Copine-6 Lamin A/C (LMNA) Copine II (CPNE2) Lectin, galactoside-binding, soluble, 3 (LGALS3) Intercellular adhesion molecule 4 (Landsteiner–Wiener blood group) (ICAM4), transcript variant 1 CAP-GLY domain containing linker protein family, member 4(CLIP4) Immunoglobulin kappa constant (IGKC) Lectin, galactoside-binding, soluble, 8 (galectin-8) (LGALS8) EGF-like repeats and discoidin I-like domains 3 (EDIL3) cDNA clone MGC:31944 IMAGE:4878869, complete cds
Ab11274	Catenin (cadherin-associated protein), beta 1, 88 kDa (CTNNB1 Polymerase (RNA) II (DNA directed) polypeptide I, 14.5 kDa (POLR2I) Zinc-finger and SCAN domain-containing protein 2 RAN-binding protein 3 (RANBP3) *N*-glycanase 1 (NGLY1) Breast carcinoma amplified sequence 2 (BCAS2) Sodium channel and clathrin linker 1 Similar to expressed sequence AI593442 (LOC399947) 40S ribosomal protein SA Proteasome (prosome, macropain) 26S subunit, non-ATPase, 4 (PSMD4) v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1) RNA polymerase II-associated protein 3 Myogenin (myogenic factor 4) (MYOG) Neutrophil cytosolic factor 4, 40 kDa (NCF4), transcript variant 1 Annexin A1 (ANXA1) Phosphodiesterase 4A, cAMP-specific (phosphodiesterase E2 dunce homolog, Drosophila) (PDE4A) Myocyte enhancer factor 2A (MEF2A) Ribosomal protein SA (RPSA) Vacuolar protein sorting 37 homolog A (S. cerevisiae) (VPS37A) N-terminal kinase-like protein Zinc-finger protein 410 Protein Dos Chromosome 11 open reading frame 63 (C11orf63), transcript variant 2 Lectin, galactoside-binding, soluble, 3 (LGALS3) General transcription factor II-I
Ab5334	RNA-binding motif, single-stranded interacting protein 2 (RBMS2) SEC24-related gene family, member C (*S. cerevisiae*) (SEC24C) SEC24-related gene family, member C (*S. cerevisiae*) (SEC24C), transcript variant 1 Fc fragment of IgG, low-affinity IIIa, receptor (CD16a) (FCGR3A) Fc fragment of IgG, low-affinity IIIa, receptor (CD16a) (FCGR3A) PCTAIRE protein kinase 3 (PCTK3) Lectin, galactoside-binding, soluble, 1 (galectin-1) (LGALS1) Muscleblind-like (Drosophila) (MBNL1), transcript variant 1 Synaptotagmin IX (SYT9) Similar to FRG1 protein (FSHD region gene 1 protein) (MGC72104) Splicing factor, arginine/serine-rich 10 (transformer 2 homolog, Drosophila) (SFRS10)

Abbreviations: rAb, recombinant antibody; OAEMZL, ocular adnexal extranodal marginal zone lymphoma.

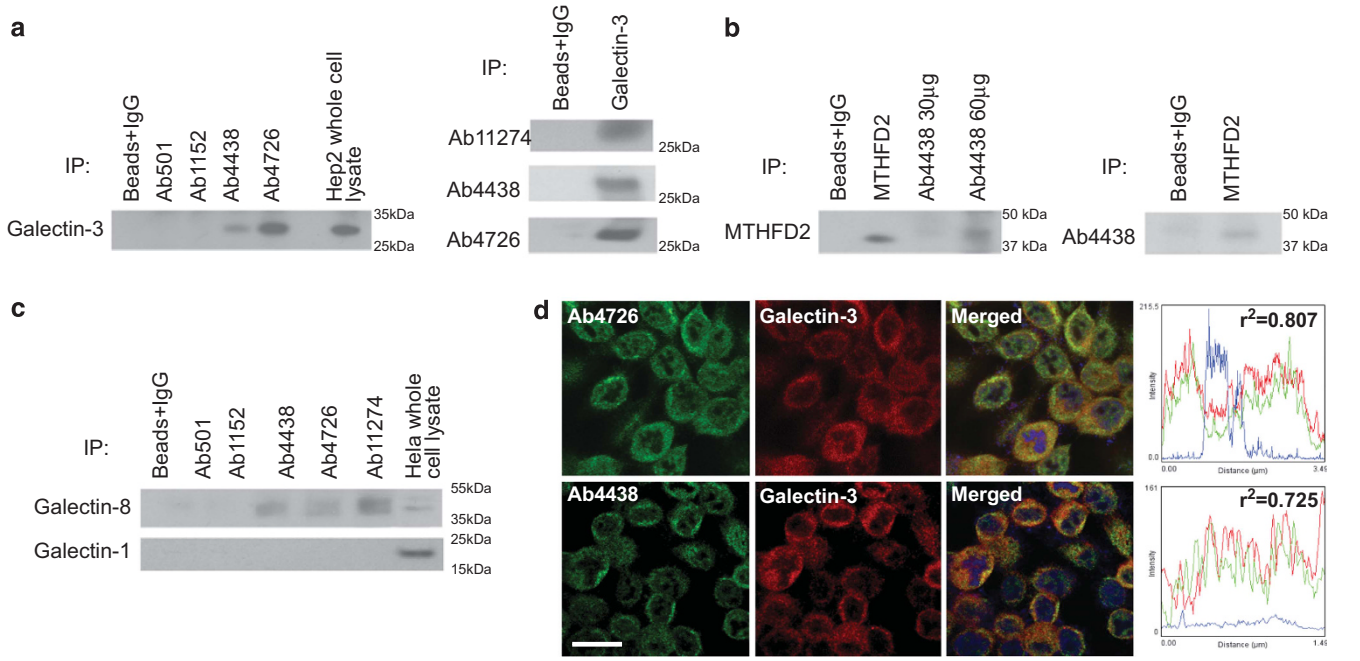

Figure 4. Identification of cognate OAEMZL tumors antigens. (**a**) Left panel: whole-cell lysates of Hep2 cells were immunoprecipitated with rAbs derived from OAEMZL tumors (Ab4438 and Ab4726) or control FL (Ab1152 and Ab501) followed by immunoblotting with galectin-3 antibody. Right panel: whole-cell lysates of Hep2 cells were immunoprecipitated with galectin-3 antibody followed by immunoblotting with Ab4438, Ab4726 and Ab11274. Ab510 and Ab1152 derived from FL tumors served as negative controls. (**b**) Left panel: whole-cell lysates of HeLa cells were immunoprecipitated with Ab4438 followed by immunoblotting with MTHFD2 antibody. Right panel: whole-cell lysates of HeLa cells were immunoprecipitated with MTHFD2 antibody followed by immunoblotting with Ab4438. (**c**) Whole-cell lysates of HeLa cells were immunoprecipitated with rAbs derived from OAEMZL tumors (Ab4438, Ab4726 and Ab11274) followed by immunoblotting with galectin-1 and galectin-8 antibodies. (**d**) HeLa cells were fixed and subjected to IFA using anti-galectin-3 or rAbs derived from OAEMZL (50 µg/ml). Depicted are confocal microscopy images showing binding of rAbs derived from OAEMZL, galectin-3, merged panels and co-localization correlations. FL, follicular lymphoma; IFA, indirect immunofluorescence assay. Bar 50 µm.

and has been implicated in the pathogenesis of multiple cancers.[29–31] Galectin-3 is found in both the nucleus and the cytoplasm and is also secreted into the extracellular microenvironment. Circulating anti-galectin-3 antibodies were previously reported in patients with rheumatoid arthritis, systemic lupus erythematosus and solid tumors.[32,33] All three rAbs interacting with galectin-3 exhibited similar combined nuclear and cytoplasmic staining in HEp-2 cells, as shown in Figure 2. ELISAs performed with and without lactose, a known ligand of galectin-3, eliminated the possibility that glycosylated rAbs reacted with galectin-3 through lectin–carbohydrate interactions (Figure 5a). Competitive inhibition ELISAs performed by blocking binding of immobilized galectin-3 to rAbs via adding excess soluble galectin-3 showed competitive inhibition in a dose-dependent manner (Figure 5b). Bovine serum albumin, which was used as a negative control, only minimally blocked binding, even at 50× excess. Overall, the competitive inhibition results validated the specificity of OAEMZL-derived rAbs to galectin-3. Competitive inhibition ELISAs performed by blocking binding of immobilized galectin-3 to rAbs via adding excess soluble MTHFD2 showed competitive inhibition in a dose-dependent manner (Figure 5c), further demonstrating polyreactivity of rAb4438 to both galectin-3 and MTHFD2 proteins, as suggested by the ProtoArray Human Protein Microarrays. siRNA-induced galectin-3 knockdown markedly decreased staining with both the commercial and tumor-derived rAbs (Figure 5d), further confirming the reactivity with galectin-3.

To examine the expression and origin of galectin-3 in OAEMZL, immunohistochemistry analyses of normal lymphoid and non-lymphoid tissues and primary OAEMZL tumors were performed. Galectin-3 expression was detected in dendritic cell between epithelial cells of the tonsillar crypts and within a subset of macrophages of reactive follicles (Figures 6a and b), colonic mucosal epithelial and endothelial cells (Figures 6c and e) and normal mast cells (Figure 6f). Follicular dendritic cells were negative. In the majority of the analyzed OAEMZL tissues, galectin-3 was expressed in scattered stromal cells with short dendritic projections admixed with the neoplastic cells but not in the neoplastic lymphocytes (Figures 6g and j). To further characterize the stromal cells expressing galectin-3, we performed immunofluorescence studies in colon specimens (used as control) and three OAEMZL tissues (Figure 7 and Supplementary Figure S9). These studies confirmed that galectin-3 was expressed by the same population of stromal cells with short cytoplasmic dendritic processes. Double immunofluorescence studies showed that most of the galectin-3-positive stromal cells coexpressed the monocytic/macrophage marker CD68 but were negative for CD163 and CD117. Using additional slides from reactive tonsils, we found that galectin-3-positive cells did not coexpress the follicular dendritic cell marker CD21 (not shown). These findings indicate that in the majority of OAEMZLs, the BCRs of neoplastic lymphocytes can potentially recognize galectin-3 expressed and/or presented by macrophages present in the tumor microenvironment, suggesting that the tumor microenvironment may serve as a source of continuous antigenic stimulation.

OAEMZL-derived Igs activate BCR signaling upon interaction with cognate antigens

We have demonstrated that BCRs of OAEMZLs recognize an array of self-antigens. Next, we examined whether binding of these cognate self-antigens to OAEMZL BCRs leads to BCR signaling

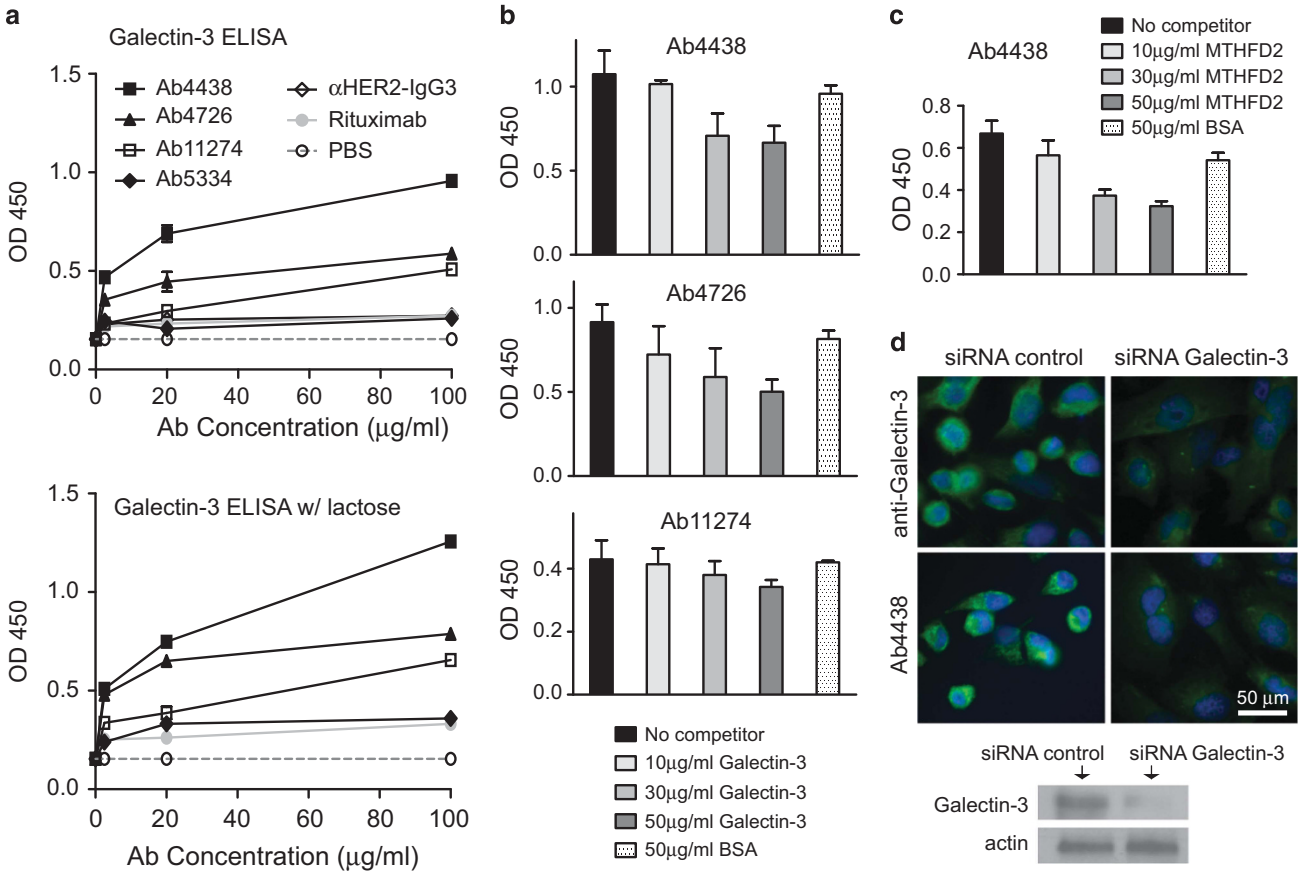

Figure 5. Galectin-3 is a cognate OAEMZL tumor antigen. (**a**) rAbs (0–100 μg/ml) cloned from the OAEMZL tumors were tested by ELISA for reactivity towards galectin-3 in the presence and absence of 0.1 M lactose. Rituximab, αHER2-IgG3 and PBS were used as controls. (**b**, **c**) Abs 4438, 4726 and 11 274 (20 μg/ml) were incubated with plate-coated galectin-3 (solid phase) in the presence of 10, 30, 50 μg/ml galectin-3 in solution (**b**) or MTHFD2 (**c**) in soluble form or 50 μg/ml BSA as a negative control. One representative inhibition assays out of three is shown with error bars indicating s.e.m. between triplicate wells. (**d**) HeLa cells were transfected with galectin-3 siRNA or non-silencing control siRNA. At 48 h post transfection, cells were fixed and subjected to IFA using Ab4438 derived from OAEMZL or anti-galectin-3. Upper panel: microscopic photographs of galectin-3 and Ab4438 staining in HeLa cells. Lower panel: immunoblotting of galectin-3 to confirm galectin-3 knockdown. BSA, bovine serum albumin; IFA, indirect immunofluorescence assay.

activation. To this end, the A20 cell line was transfected to express a membrane-anchored form of OAEMZL IgMs and we measured intracellular Ca_2^+ mobilization as a read out for BCR signaling activation by cognate antigens. Stimulation with goat $F(ab')_2$ anti-human IgM served as a positive control for BCR-induced Ca_2^+ mobilization. We demonstrated that the transfected A20 cells expressed cell surface Ig encoding OAEMZL IgM (4438 and 5334; Figure 8a) and exhibited increased intracellular Ca_2^+ levels following goat $F(ab')_2$ anti-human IgM stimulation (Figure 8b). When stimulated with recombinant galectin-3, only cells expressing surface BCR from OAEMZL (4438) triggered Ca_2^+ release, whereas the parent cell line remained unresponsive (Figure 8b). Similarly, recombinant MTHFD2, which binds to the same BCR, also induced an increase in intracellular Ca_2^+ only in the A20 cells expressing the surface BCR from OAEMZL (4438; Supplementary Figure S10). The MTHFD2-induced kinetic profile of Ca_2^+ release was distinct from galectin-3, likely reflecting a distinct BCR affinity to these antigens, as demonstrated by the protein arrays. A20 cells expressing surface OAEMZL IgM (5334), which did not bind galectin-3 and MTHFD2, did not exhibit Ca_2^+ release upon stimulation with these proteins. Overall, these poofs of function studies demonstrate that OAEMZL-derived BCR signaling is activated following binding to specific antigen.

DISCUSSION

The majority of non-Hodgkin lymphomas express functional surface BCRs that may transmit survival signal via tonic or constitutive activation, with the latter usually associated with somatic mutations in the components of the BCR signaling cascade.[17,34] However, BCR may also transmit survival signals upon binding to a cognate antigen. Indeed, indirect and direct evidence suggest that antigen recognition may have a role in the pathogenesis of chronic lymphocytic leukemia, follicular lymphoma, splenic marginal zone lymphoma and gastric and parotid EMZLs.[18–22,25,35–37] The indirect evidence is mainly based on the immunogenetic data demonstrating a biased *IGHV* gene usage, ongoing somatic mutations and the use of 'stereotyped' BCRs. Additional evidence for the role of antigen stimulation in lymphomagenesis includes the association between certain lymphoma subtypes and specific infections, autoimmune diseases and previous identification of BCR reactivity toward distinct autoantigens. More direct evidence for the role of antigen stimulation and BCR activation in lymphomagenesis are based on intact intracellular signaling of lymphoma-derived BCRs in response to specific antigens in the context of non-tumorous lymphoid cellular models[20] or induction of intracellular BCR signaling in primary lymphoma cells in response to specific antigens.[18,20] Furthermore, the association between chronic active BCR signaling via antigen

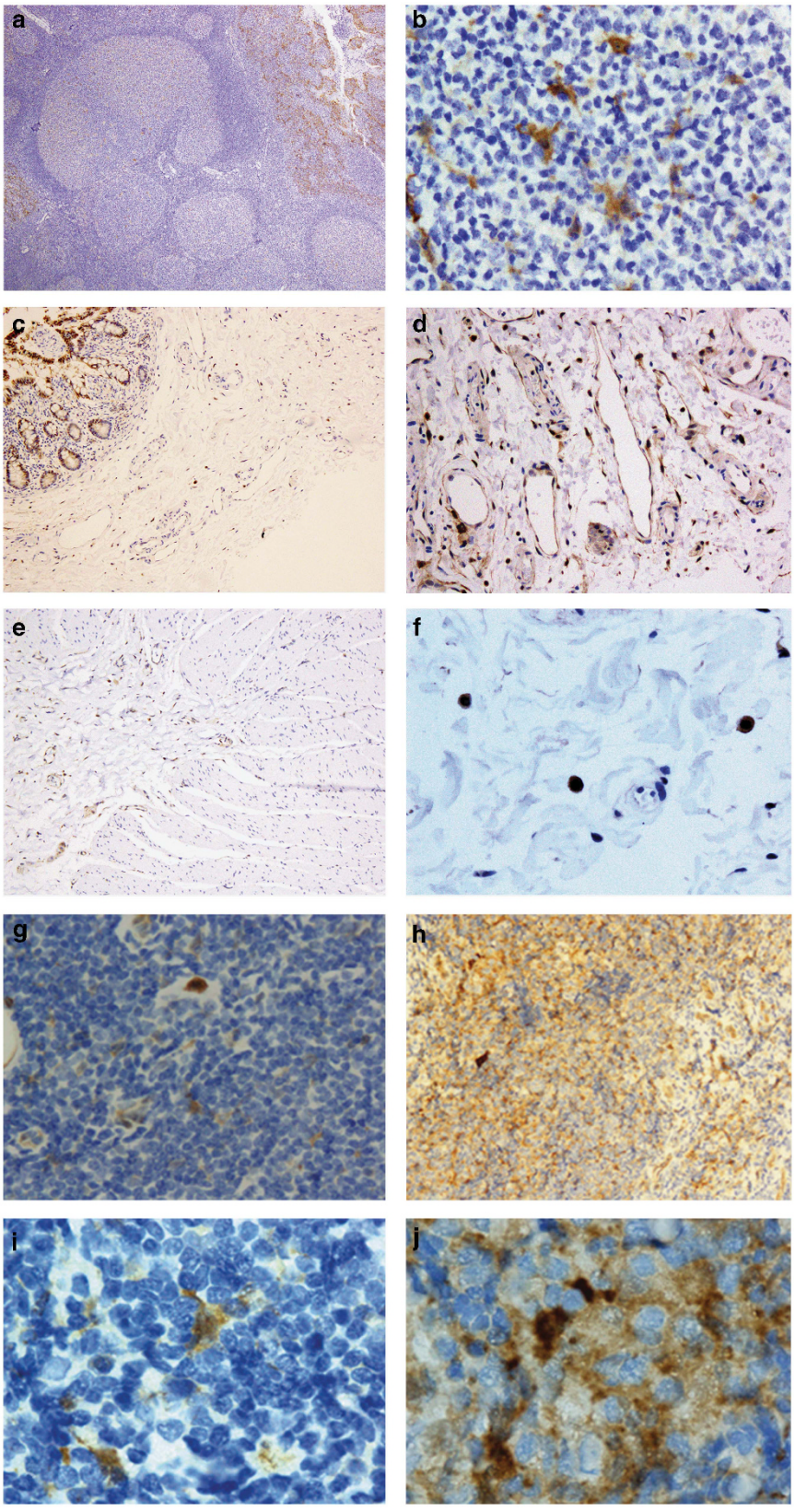

Figure 6. Galectin-3 is expressed in tumor microenvironment of OAEMZL patients. Immunohistochemistry for galectin-3 performed on control formalin-fixed paraffin-embedded tissues and eight cases of OAEMZL. (**a**, **b**) Galectin-3 expression in dendritic cell between epithelial cells of the tonsillar crypts and within a subset of macrophages of reactive follicles. (**c**–**f**) Galectin-3 expression in colonic mucosal epithelial, endothelial cells and (**f**) normal mast cells. (**g**–**j**) In the majority of the analyzed OAEMZL tissues, galectin-3 was expressed in scattered stromal cells with short dendritic projections admixed with the neoplastic cells but not in the neoplastic lymphocytes.

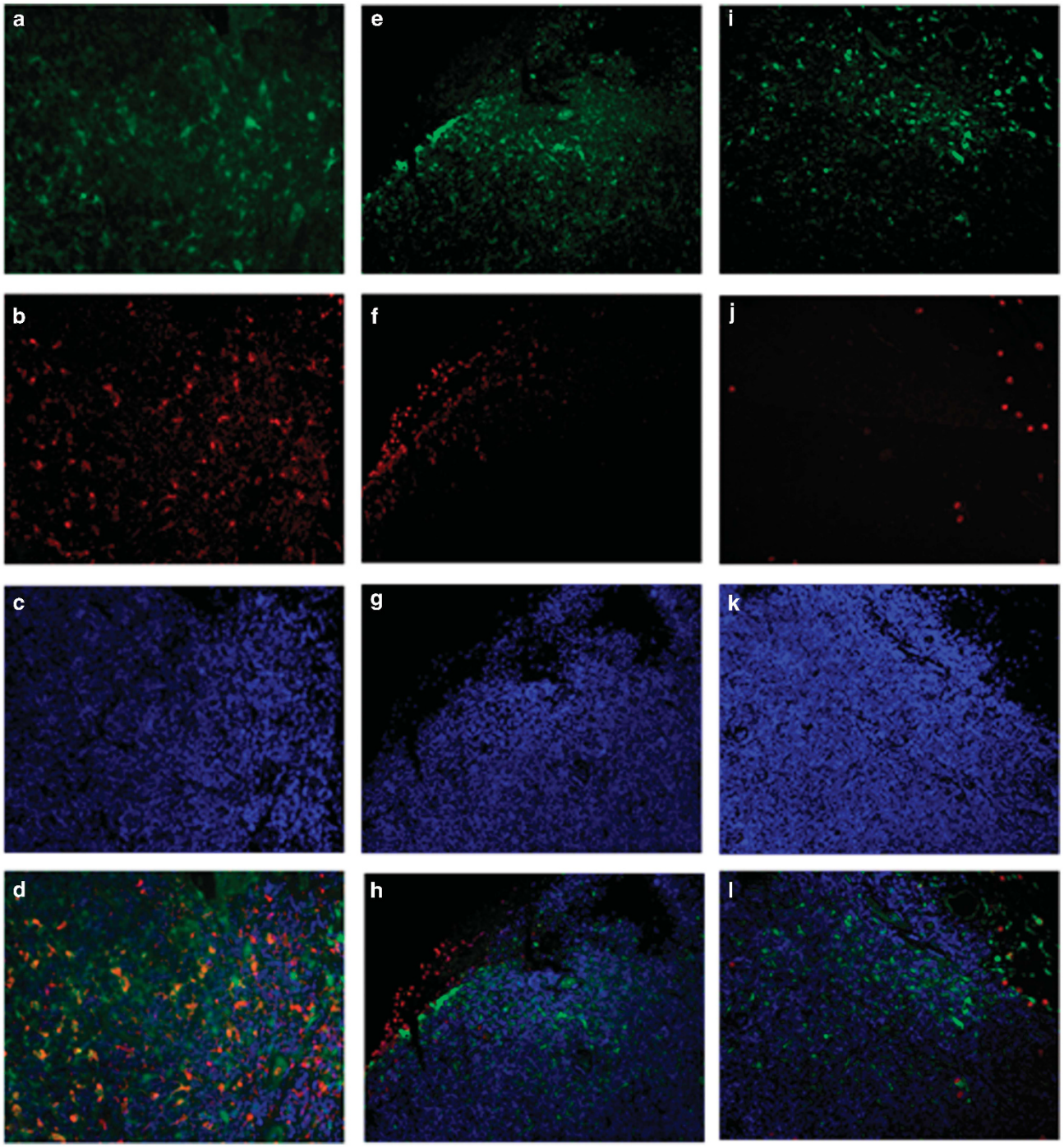

Figure 7. Galectin-3 is expressed by stroma cells double positive for macrophage marker. Double immunofluorescence labeling performed on OAEMZL using galectin-3 in combination with CD68 (**a**–**d**), CD163 (**e**–**h**) and CD117 (**i**–**l**). Galectin-3 (in green, **a**, **e**, **i**) was expressed by a population of stromal cells admixed with the lymphoma cells. These cells exhibit short cytoplasmic projections. A significant number of these cells represent CD68 expressing macrophages (**b**) but are negative for CD163 (**f**) and CD117 (**j**). The merge pictures (**d**, **h**, **l**) include the 4′, 6′-diamidino-2-phenylindole as the counter stain for the nucleus.

stimulation or mutations within the BCR signaling pathway and lymphoma cell survival also point to the potential role of BCR stimulation in lymphomagenesis.[17,38] The clinical efficacy of BCR inhibitors (that is, ibrutinib) in several lymphomas provides further evidence for the role of antigen-induced or autonomous BCR signaling in these tumors.[38–40]

The pathogenesis of OAEMZLs is still unclear. We have previously demonstrated a biased *IGHV* gene use and ongoing *IGHV* gene somatic mutations,[11,12] suggesting a potential role for BCR signaling in the pathogenesis of *C. psittaci*-negative OAEMZLs. Herein we have investigated the identity of potential antigens that can react and stimulate BCRs in these lymphomas.

On the basis of the monoclonal *IGHV* and *IGKV* sequences of OAEMZLs, we generated a comprehensive panel of rAbs expressed as soluble IgG3 and tested their reactivity towards bacterial and self-antigens. The rAbs demonstrate RF activity and

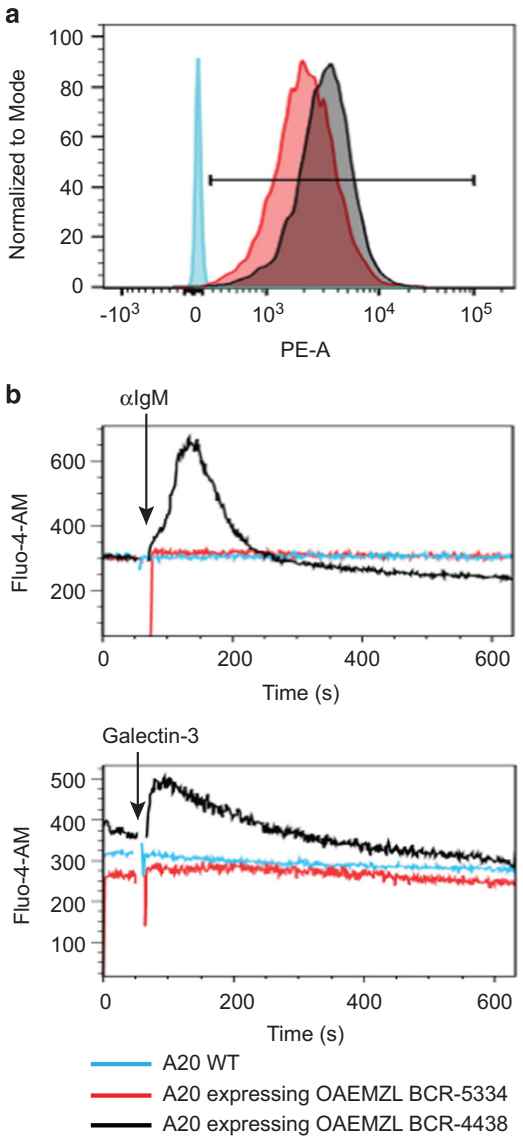

Figure 8. Galectin-3 induces BCR activation in A20 cells expressing Igs derived from the OAEMZL tumor. (**a**) A20 cells were transfected and selected for stable membrane-bound expression of IgM derived from OAEMZL tumor 4438 and 5334. Flow cytometric histograms following cell staining with phycoerythrin-conjugated anti-IgM antibody of OAEMZL expressing A20 cells (red and black) as compared with parent A20 cells (blue). (**b**) Kinetic of calcium mobilization in parent A20 cells (blue) and A20 cells stably expressing IgM derived from OAEMZL tumors 4438 (black) and 5334 (red). Arrow indicates the time point of goat $F(ab')_2$ anti-human IgM (upper panel) or galectin-3 (lower panel) stimulations. Data are representative of two independent experiments. WT, wild type.

reactivity with self-antigens. All the antibodies were polyreactive. BCR polyreactivity magnifies antigen detection power and is a known phenomenon in the context of B-cell lymphomas.[21,35] Polyreactivity to self-antigens may ensure persistent BCR activity and continuous propagation of survival signals in lymphoma cells by eliminating dependence on a single-specific antigen. Furthermore, similar to observations of Ig polyreactivity in HIV infection,[41] lymphoma BCR polyreactivity may also increase downstream signaling by heteroligation of low-affinity antigens to more abundant self-antigens in circumstances in which the specific cognate antigen is not present at concentrations permitting homoligation. Indeed, we observed relatively low expression of galectin-3 in the OAEMZL microenvironment, further supporting the possibility of antigen heteroligation. The majority of the galectin-3 expressing OAEMZL microenvironment stromal cells represented $CD68^+$ macrophages that are normally involved in antigen presentation.[42] It is presently unknown whether the visualized macrophages, localized in close proximity to the lymphoma cells, presented galectin-3 to lymphoma cells or simply expressed this known marker of macrophage activation.[43]

Polyreactive Abs can be germline or affinity matured,[44] in agreement with BCRs derived from OAEMZLs. In healthy individuals, only about 4% of circulating naive B cells express polyreactive Abs.[45] However, up to 23% of memory B cells, from which EMZLs are thought to originate, express polyreactive Abs.[46] Consequently, our finding of polyreactive BCRs in OAEMZLs and other EMZL might have been expected. However, the self-proteins reacting to the rAbs derived from OAEMZLs differ from self-proteins reacting with the BCRs of other lymphoma subtypes. BCRs of EMZLs originating in the stomach demonstrated polyreactivity with self-Ig, DNA, stomach extracts and bacterial antigens.[21] BCRs of splenic marginal zone lymphoma demonstrated polyreactivity with galactosidase, insulin, thyroglobulin, LPS and other uncharacterized cellular proteins.[25] BCRs of follicular lymphoma were reported to react with α-myoferlin, vimentin and other uncharacterized self-cellular proteins.[18,19] This distinct pattern of BCR polyreactivity may reflect diverse mechanisms of lymphomagenesis, leading to a different Ig repertoire used by specific lymphoma subtypes. However, the use of different methodologies to identify self-antigens might also contribute to the observed differences.

Some studies designated lymphoma BCRs as polyreactive or monoreactive following screening of reactivity towards relatively small set of antigens.[18,19,21,22] Only one of our analyzed rAbs showed polyreactivity based on ELISA reactivity with human recombinant insulin, dsDNA, ssDNA or LPS, commonly used in previous studies. Other rAbs derived from OAEMZLs demonstrated polyreactivity with 9–25 self-antigens identified by protein arrays and selectively verified by co-immunoprecipitation experiments and mass spectrometry. The reactivity was 'specific' as binding to a solid-phase target was blocked by the addition of excess soluble target in a concentration-dependent manner. However, the binding could also be inhibited by a cross-reacting antigen, indicating rAb polyreactivity. Notably, many of the tested rAbs reacted with shared intracellular and extracellular self-antigens (for example, galectin-3 and galectin-8). These findings suggest that comprehensive protein array antibody reactivity testing may be necessary to demonstrate the polyreactivity of lymphoma BCRs. This was not performed in many of the previous studies that suggested reactivity with specific antigens in certain subtypes of lymphoma.[18,19,36] Furthermore, most studies focused only on antigen identification and did not analyze the functionality of antigen–BCR binding.[19,21,22,25] Herein we demonstrate that the binding of the identified self-antigens to the BCR of OAEMZLs results in functional intracellular signaling, and thus may be important for lymphoma growth by activating intracellular BCR signaling. Unfortunately, the absence of OAEMZL cell lines and animal models preclude experimental testing of these effects.

To the best of our knowledge, herein we present the first comprehensive analysis of BCR reactivity in lymphoma, specifically OAEMZLs, using ELISA, protein antigen arrays, mass spectrometry and by demonstrating the functionality of the identified antigen–BCR interactions in the context of BCR signaling. We demonstrate that *C. psittaci*-negative OAEMZLs express polyreactive BCRs that react with the same self-antigens. These findings indicate that *C. psittaci*-negative OAEMZLs are likely derived from polyreactive B cells. The previously reported biases in the *IGHV* and *IGKV* repertoire and the presence of intraclonal heterogeneity in

OAEMZLs, coupled with the functional activation of OAEMZL BCRs by cognate antigens, indicate that antigen-driven selection and lymphoma BCR stimulation may have a critical role in the pathogenesis of these tumors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

DZ and SB performed most of the laboratory work, analyzed the data and wrote the manuscript; XL and FG performed the laboratory experiments; HV analyzed the data; DKH and F-TL provided the reagents and useful discussion; and SAC, KK and FV performed the double immunofluorescence experiments. JRC-F performed the laboratory experiments and analyzed the data; ISL conceptualized the idea of the study, supervised the experiments, analyzed the data and wrote the manuscript.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)