B-cell receptor reactivity against *Rothia mucilaginosa* in nodular lymphocyte-predominant Hodgkin lymphoma

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Abstract

Nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) is a Hodgkin lymphoma expressing functional B-cell receptors (BCR). Recently, we described a dual stimulation model of IgD$^+$ lymphocyte-predominant cells by *Moraxella catarrhalis antigen* RpoC and its superantigen MID/hag, associated with extralong CDR3 and HLA-DRB1*04 or HLA-DRB1*07 haplotype. The aim of the present study was to extend the antigen screening to further bacteria and viruses. The fragment antibody-binding (Fab) regions of seven new and 15 previously reported cases were analyzed. The reactivity of non-*Moraxella* spp.-reactive Fab regions against lysates of *Rothia mucilaginosa* was observed in 5/22 (22.7%) cases. Galactofuranosyl transferase (Gltf) and 2,3-butanediol dehydrogenase (Bdh) of *R. mucilaginosa* were identified by comparative silver- and immuno-staining in two-dimensional gels, with subsequent mass spectrometry and validation by western blots and enzyme-linked immunosorbent assay. Both *R. mucilaginosa* Gltf and Bdh induced BCR pathway activation and proliferation *in vitro*. Apoptosis was induced by recombinant Gltf/ETA'-immunotoxin conjugates in DEV cells expressing recombinant *R. mucilaginosa*-reactive BCR. Reactivity against *M. catarrhalis* RpoC was confirmed in 3/7 newly expressed BCR (total 10/22 reactive to *Moraxella* spp.), resulting in 15/22 (68.2%) cases with BCR reactivity against defined bacterial antigens. These findings strengthen the hypothesis of bacterial trigger contributing to subsets of NLPHL.

Introduction

Nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) accounts for 5-16% of Hodgkin lymphomas.[1] The disease-defining lymphocyte-predominant (LP) tumor cells have a late germinal center B-cell phenotype. NLPHL variants with a diffuse growth pattern and histiocyte-rich microenvironment are closely related to T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL).[2,3] When NLPHL was first described, a frequently indolent course was noted and

a potential misguided immune response to infection was speculated.[4] In contrast to Hodgkin-Reed-Sternberg cells in classical Hodgkin lymphoma, LP cells are usually negative for Epstein-Barr virus and have a preserved B-cell phenotype, including B-cell receptor (BCR) expression.[5] NLPHL is genotypically distinct from classical Hodgkin lymphoma, with frequent *BCL6* translocations and recurrent mutations in *SGK1, DUSP2* and *JUNB*.[2,6-11] The intraclonal immunoglobulin variable (IgV) region gene diversification,[8] strong expression of BCL6[12] and AID,[13,14] and a histological picture that resembles a germinal center suggest an ongoing immune response.[15] In the recently published classification of the International Consensus Conference NLPHL was renamed to nodular lymphocyte predominant B-cell lymphoma (NLPBL),[16] but in the simultaneously published current 5th version of the World Health Organization classification the term NLPHL was retained.[17] Activation of the nuclear factor (NF)-κB pathway is observed in LP cells;[2] however, NF-κB activity in LP cells is not caused by mutations in *NFKBIA* or T*NFAIP3*[18] or by latent Epstein-Barr virus[5] infection. It may therefore represent a consequence of chronic BCR stimulation. In line with this hypothesis, we demonstrated that dynamic immunological synapses are formed between LP cells and the surrounding rosetting follicular T helper (TFH) cells.[19] IgD+ NLPHL had been reported to have a strong male predominance and to present primarily with cervical manifestations.[20] We reported the reactivities of mostly IgD+ LP cells against the specific antigens of *Moraxella* spp. associated with a specific *IGVH* genotype, a presumably permissive HLA class II haplotype (HLA-DRB01*04 or HLA-DRB01*07), and the presence of light chain-restricted serum antibodies against *M. catarrhalis* RpoC. Moreover, we proposed a BCR co-stimulation model of IgD+ LP cells by *M. catarrhalis* RpoC via the fragment antibody-binding (Fab) region and the IgD-binding superantigen MID/hag via the Fc domain.[21,22] In another study, similar immunoglobulin genes were found in non-IgD+ NLPHL cases.[23] Having identified *M. catarrhalis* as a trigger for a subset of NLPHL, we hypothesized that there may be further, possibly commensal bacteria eliciting NLPHL. The aim of the present study was, therefore, to extend the screening of the recombinant Fab regions of LP cells to other microbial antigens, particularly those that are part of the resident flora of the oral cavity, upper respiratory tract and gut, such as *Rothia* spp, *Enterococcus* spp., and *Lactobacillus* spp. A second aim of the study was to confirm the reactivity against *M. catarrhalis* RpoC by analysis of new cases.

Methods

Study samples

The study was approved by the institutional review boards of the University Cancer Center (UCT), the Ethics Committee at the University Hospital Frankfurt (project number: SHN-06-2018), and the local ethics committee of the Ärztekammer des Saarlandes, Saarbrücken (Ha 147/17). Frozen tissue sections of NLPHL specimens were obtained from the Department of Pathology of the UCT Frankfurt, Tampere University, Tampere, Finland, from the Institute of Pathology, CHUV Lausanne, Switzerland, and from the Department of Pathology, Uppsala University Hospital, Sweden. All NLPHL cases were negative for Epstein-Barr virus. Informed consent was obtained from the patients in accordance with the Declaration of Helsinki. The sera of patients with classical Hodgkin lymphoma or NLPHL were obtained from the HD13 (ISRCTN63474366), HD14 (ISRCTN04761296), HD15 (ISRCTN32443041), HD16 (NCT00736320), HD17 (NCT01356680), and HD18 (NCT00515554) trials of the German Hodgkin Study Group.[24-27] Sera from patients with THRLBCL were obtained from the RICOVER (NCT00052936), Hi-CHOEP (NCT00129090), UNFOLDER (NCT00278408), FLYER (NCT00278421), and CHOP-R-ESC (NCT00290667) trials, and sera from patients with primary mediastinal B-cell lymphoma were obtained from the UNFOLDER (NCT00278408) trial of the German High-Grade Non-Hodgkin Lymphoma Study Group.[28-31]

Fab region screening in bacterial lysates and virome array

Laser microdissection of LP cells from cryospecimens and IgV gene multiplex seminested polymerase chain reactions were performed, as described by Küppers *et al.*[32] This also applies to the prokaryotic expression cloning of the recombinant Fab regions in *Escherichia coli* TG1 (*Online Supplementary Methods*).[33-35]

To screen for the potential reactivity of Fab regions against bacterial antigens, heat-inactivated lysates of different bacterial strains or patients' isolates, including *Rothia aeria* and *Rothia mucilaginosa*, were provided by the Institute of Medical Microbiology and Hygiene of Saarland University, Homburg/Saar, Germany (*Online Supplementary Methods*).

To verify the specificity of the reactivities of NLPHL-derived Fab regions against the components of *R. mucilaginosa*, recombinant Fab regions derived from primary central nervous system lymphoma, diffuse large B-cell lymphoma, mantle cell lymphoma, primary mediastinal B-cell lymphoma and chronic lymphocytic leukemia were screened against these recombinant FLAG-tagged antigens of *R. mucilaginosa* by enzyme-linked immunosorbent assay.[35-37]

In addition, the recombinant NLPHL-Fab regions were screened on a custom-made VIROME microarray representing 2,206 viral genes chosen from 158 viruses (ASU, Biodesign Institute, Arizona State University, AZ, USA) (*Online Supplementary Methods*).

Generation of DEV cells expressing Rothia spp.-reactive B-cell receptors followed by activation, proliferation, and apoptosis assays after stimulation with *Rothia* antigens and immunotoxins

DEV cells expressing *Rothia*-reactive BCR were generated as previously described (*Online Supplementary Methods*).[20,22] DEV cells either expressing a BCR with reactivity against *Rothia mucilaginosa* Gltf or *Moraxella catarrhalis* RpoC, and of IgD or IgG class, were subjected to western blot for activation of the BCR pathway, proliferation and apoptosis assays after stimulation with the respective bacterial antigens or incubation with bacterial antigens conjugated with an immunotoxin (*Online Supplementary Methods*).

HLA genotyping

HLA-DRB1 and HLA-DQ typing was performed for all patients via sequencing (Labor Thiele, Kaiserslautern, Germany).

Statistical analysis

Normality was evaluated using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Statistical significance was calculated using unpaired, two-tailed *t* tests for comparison of parametric distributions, without adaptation for multiple comparisons (Prism 9, GraphPad).

Results

Patient, HLA-DRB1, and IgV gene characteristics

The previous expression-cloned cohort of the Fab regions of 15 NLPHL cases[21,22] was extended by Ig heavy and light chain gene amplification from microdissected LP cells with an additional seven NLPHL cases (Table 1; IgV gene characteristics are presented in Table 2). The median age of the patients with successfully amplified

Table 1. Characteristics of the patients with nodular lymphocyte-predominant Hodgkin lymphoma included in this study

Case	Sex	Age yrs	Primary diagnosis/relapse	Localization	LP cells IgD+	Pattern	Reactivity against *M. cat.* RpoC or *M. osl.* succinate-CoA ligase sub α	Reactivity against *R. mucilaginosa*
1	M	52	Relapse	Axillary	No	A	No	No
2	M	15	Relapse	Supraclavicular	No	A	No	Yes*
3	M	14	2nd relapse	Cervical	Yes	A	Yes*	No
4	M	41	First manifestation	Cervical	No	NA	No	No
5	F	37	First manifestation	Retroperitoneal	No	NA	No	No
6	M	15	Relapse	Cervical	Yes	C	Yes*	No
7	M	51	2nd relapse	Axillary	No	A + D	No	No
8	M	40	Primary progression	Abdominal	No	D + E	No	No
9	M	42	Relapse	Inguinal	Yes	NA	Yes*	No
10	M	12	2nd relapse	Inguinal	Yes	NA	Yes*	No
11	M	15	First manifestation	Cervical	Yes	C	Yes*	No
12	M	31	First manifestation	Cervical	No	A	No	Yes*
13	M	18	First manifestation	Cervical	Yes	D	Yes*	No
14	M	30	First manifestation	Parotid	Yes	E, B + D	Yes*	No
15	F	16	First manifestation	Axillary	Yes	A	No	Yes*
16	M	44	Relapse	Axillary	No	E	No	Yes*
17	M	30	First manifestation	Abdominal	Yes	D	Yes*	No
18	M	26	First manifestation	Cervical	Yes	C	Yes*	No
19	M	11	First manifestation	Inguinal	No	E	No	Yes*
20	F	15	Relapse	Cervical	Yes	C	No	No
21	F	18	Relapse	Infraclavicular	No	A	Yes*	No
22	M	9	First manifestation	Inguinal	No	A	No	No

*IgD+ nodular lymphocyte-predominant (LP) Hodgkin lymphoma; cases 7 and 8: (a) nodular lymphocyte-predominant Hodgkin lymphoma, (b) histological transformation into diffuse large B-cell lymphoma in the same lymph node. M: male; F: female; yrs: years; *M. cat.: Moraxella catarrhalis; M. osl.: Moraxella osloensis; R. mucilaginosa: Rothia mucilaginosa;* NA: not available. Additional information on HLA subtype and stage is available in *Online Supplementary Table S1*.

rearranged IgV genes was 28 (9-65) years, with a male predominance (18/22, 82%). IgD$^+$ LP cells were identified in 11 patients with a median age of 21 (12–42) years (male:female 9:2).

B-cell receptors derived from lymphocyte-predominant cells react with lysates of *M. catarrhalis* and *R. mucilaginosa*

Three of seven of the new recombinant Fab regions bound to lysates of *Moraxella* spp. In combination with seven *Moraxella*-reactive Fab regions from the first 15 cases, 10/22 cases were *Moraxella* spp.-reactive, and nine of them were derived from IgD$^+$ positive cases (Figure 1A).

In contrast, the pooled NLPHL Fab regions reacted in dot-blots against lysates of *R. mucilaginosa*. Following individual testing of these Fab regions, 5/22 NLPHL-derived BCR (#2, #12, #15, #16 and #19, with 1/5 IgD$^+$) reacted against *R. mucilaginosa* lysates (Figure 1A). None of these five Fab regions cross-reacted with *M. catarrhalis* lysates. In western blots of *R. mucilaginosa* lysates using the NLPHL-derived Fab regions as primary antibodies, two bands of approximately 35-40 kDa and 75 kDa were detected (Figure 1B; *Online Supplementary Figures S3* and *S4*).

Following two-dimensional gel-based isolation of single spots (Figure 1C), mass spectrometry was used to identify glycerol-3-phopshate dehydrogenase/oxidase (WP_44150604), Gtf)/Gltf (WP_012903226), type I glyceral-dehyde-3-phosphate dehydrogenase (WP_012903599), 1-deoxy-D-xylulose-5-phosphate synthase (WP_044150996), 2-oxoglutarate dehydrogenase, E2 component, dihydroli-poamide succinyltransferase (WP_044150317), 2,3-butane-diol dehydrogenase (WP_044150456), and iron-sulfur cluster-binding protein (WP_012902908) of *R. mucilaginosa* as probable candidate antigens.

Specific binding of the *R. mucilaginosa*-reactive recombinant BCR in the *R. mucilaginosa* lysate against recombinant Gltf

Table 2. IgV heavy chain gene analysis of lymphocyte-predominant cells and identified antigenic targets.

Case	VH gene	Homology %	DH gene	JH/JL gene	CDR3 length AA	Junction	Identified antigen
1	VH1-3-01	91.3	D3-10*01	JH4*02	20	CAREVRPPRIIMIWGVGLLDFW	Human RPS27a (autoantigen)
2	VH1-46*01	87.5[a]	D3-16*02	JH2*01	15	YYCARDEGDIRRYFDLW	*R. mucilaginosa* Gltf
3	VH3-11*01	95.1	D3-3*01	JH6*03	26	CARVAGAAGRNYNYWSGYWEDYYFMDVW	*M. catarrhalis* RpoC
4	VH4-31*01	99.2	D3-22	JH3*02	19	CARGPPPYDSSGYYSHGLDIW	–
5	VH4-39*01	100	D6-19*01	JH6*04	15	CASMGAVAGMMFGMDVW	Human PC3 (autoantigen)
6	VH3-07*01	95.5	D3-3*01	JH6*04	26	CAREVLRWGGSYDFWSNYYEDYFALDVW	*M. catarrhalis* RpoC
7	VH1-69	86.4	D6-19*01	JH5*02	17	CARDYSRGVCGPRYGMDVW	–
8	VH3-30	89.9	D2-15*01	JH6*02	22	CARKGGDPVLALFVPNFAMDVW	–
9	VH3-48*03	82.0	D3-3*01	JH6*02	30	CAKSVLTAKSGKSYKFWNNYHEDYHYYLMDVW	*M. catarrhalis* RpoC
10	VH4-59*01	86.4[b]	D3-3*01	JH6*03	33	CATVDPTVVEGRVKYYDFWSGYYGTDQRYYYMDVW	*M. catarrhalis* RpoC
11	VH3-11*01	95.5	D3-3*01	JH6*02	30	CARLLTSEGSRKYYDFWSNYWEGYQYYTMDVW	*M. osloensis* SUCLG1
12	VH4-34	100	D3-10*01	JH5*02	16	CARGPYLWFGERGWFDPW	*R. mucilaginosa* Bdh
13	VH3-11*01	83.0	D3-3*01	JH6*03	26	CARLCAAGGRSYDFWSGYYENYFYMEVW	*M. catarrhalis* RpoC
14	VH3-11*01	87.6	D3-3*01	JH6*02	30	CARLIEAGGVGKHYDFWSGYYTVDYYYGMDVW	M. *catarrhalis* RpoC
15	VH3-30*04	89.8	D3-3*01	JH6*02	33	CARTTWVGVVGRIKYYDFWSGYHGTGMEYYTMDVW	*R. mucilaginosa* Gltf
16	VH23*04	90.3	DH6-19*01	JH2*02	19	CAKLPLRPQWLYRGYFDLW	*R. mucilaginosa* Gltf
17	VH3-11*01	87.5	DH6-19*01	JH4*02	28	CARDIHHQWLNPVINPHWVDPVDYFDYW	*M. catarrhalis* RpoC
18	VH1-69*13	99.6	DH5-12*01	JH3*02	18	CASPGRYSGYGYDAFDIW	*M. catarrhalis* RpoC
19	VH3-7*01	94.4	DH3-3*01	JH6*03	35	CATIVLDSIKGSVRYYDFWSGHHGLSYYYYYMDVW	*R. mucilaginosa* Gltf
20	VH2-5*02	96.6	DH3-9*01	JH3*02	18	CAHTHEDILTGSDALDIW	–
21	VH1-2*02	88.9	DH3-22*01	JH4*02	18	CARDRIEDFFDSSGYVYW	*M. catarrhalis* RpoC
22	VH1-18*01	97.6	DH2-15*01	JH3*02	23	CARVPWFGLCSGGSCYEDAFDIW	–
22	VH5-10-1*01	99.6	DH3-10*01	JH5*02	16	CARSLYYGSGPRFDPW	–

Additional information on light chains is available in *Online Supplementary Table S2*. All cases had functional IgV genes. For case 15 two functional light chains were amplified. [a]And insertion in FR3 (AGAAAT). [b]With deletion of 3 nt in CDR2. AA: amine acids; *R. mucilaginosa: Rothia mucilaginosa; M. catarrhalis: Moraxella catarrhalis; M. osloensis: Moraxella osloensis.*

as the antigen or band/spot of 75 kDa and against Bdh as the antigen or band/spot of 39 kDa was verified by enzyme-linked immunosorbent assay (Figure 1D).

In contrast to NLPHL, none of the recombinant BCR derived from primary central nervous system lymphoma, diffuse large B-cell lymphoma, mantle cell lymphoma, or chronic lymphocytic leukemia cases reacted with the *R. mucilaginosa* lysate (*Online Supplementary Figure S3*). Furthermore, no Fab reactivity was detected against the total *R. mucilaginosa* lysate or against any of the other investigated bacteria.

Virome screening of the pooled non-*Moraxella* spp.-reactive Fab regions resulted in 13 candidate antigens with normalized values ≥2: CbCD00959563 of coxsackievirus B1, MsCD00595052 of measles, strain Ichinose WT, CagA_884_2.1, and CagA_FL_2.1 of *Helicobacter pylori* 26695, HrCD00959476 of human rhinovirus A1, HsCD00959820 and HsCD00959751 of H1N1 subtype, CbCD00594880 of coxsackievirus B4 (strain E2), CaCD00959834 of coxsackievirus A9, HrCD00956402 of human rubulavirus 2, HcCD00959896 of human coronavirus NL63, and HsCD00959755 of H3N2 subtype (*Online Supplementary Table S3; Online Supplementary Figure S1*). After expression cloning of the obtained

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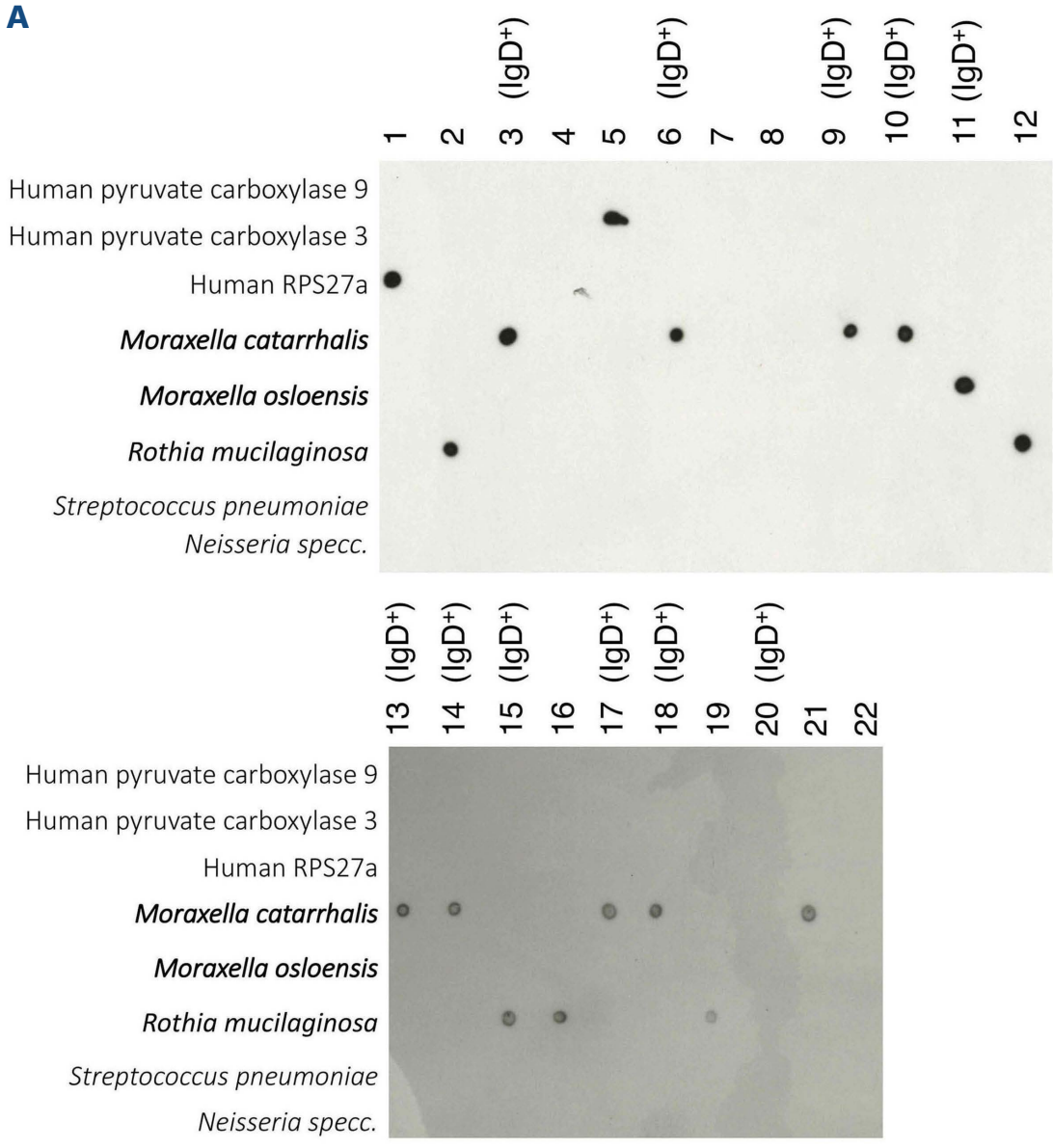

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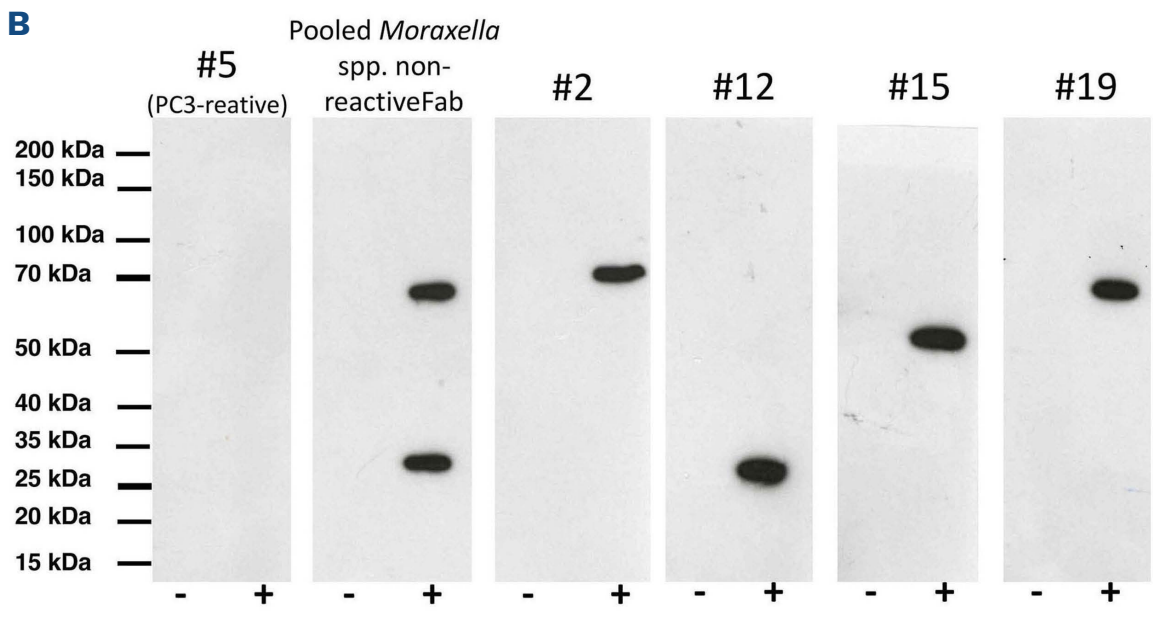

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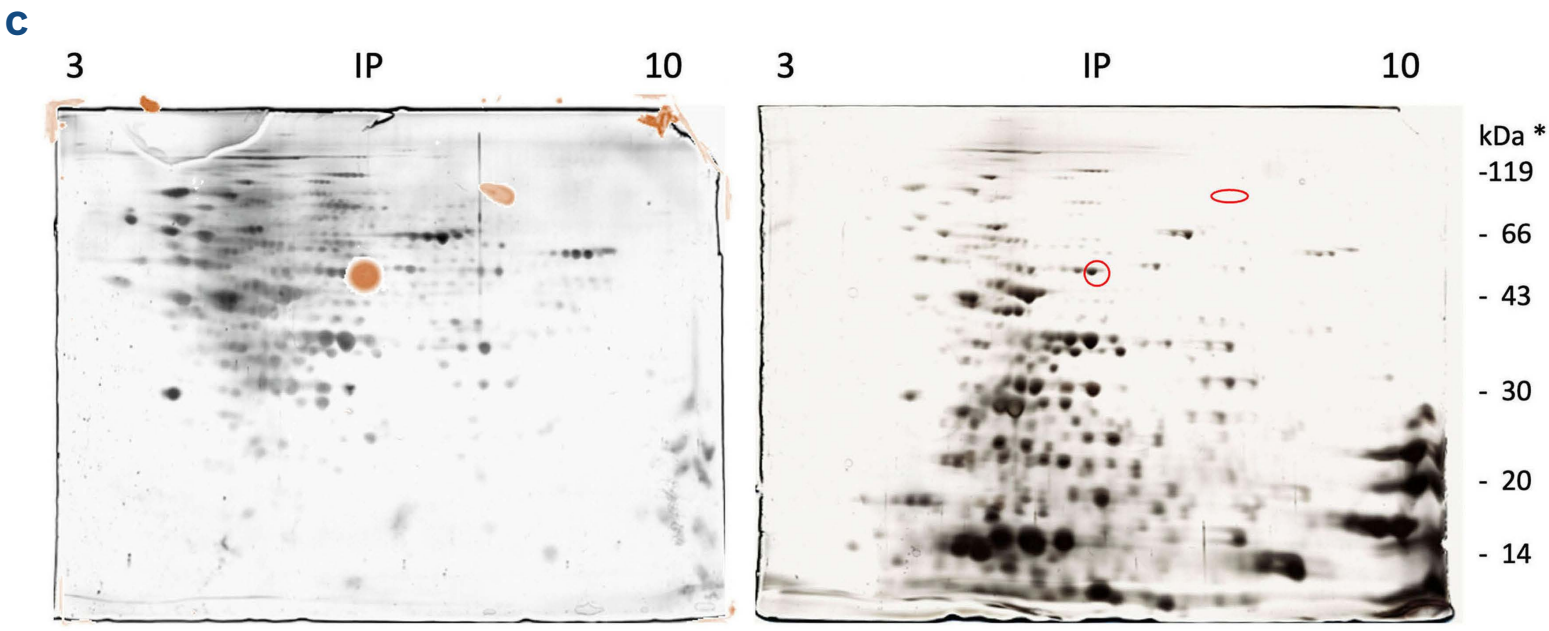

Figure 1. Reactivity of recombinant IgD⁺ lymphocyte-predominant cell-derived Fab regions with bacterial lysates. (A) Representative immuno-dot blots of bacterial lysates, showing reactivity against lysate of *M. catarrhalis* from previously reported Fab regions, and from Fab regions of new cases as validation. In addition there are nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL)-derived recombinant Fab regions of five cases (#2, #12, #15, #16, #19) bound to lysates of *R. mucilaginosa*. (B) Representative western blot of polyacrylamide gel electrophoresis of lysate of *R. mucilaginosa* using pooled and individual recombinant Fab regions of NLPHL, which had shown reactivity against *R. mucilaginosa* as primary antibody. Two bands of approximately 35-40 kDa and 75 kDa were detected. (C) Silver staining of lysate of *R. mucilaginosa* for 45 min of blotting gel (Proteome factory AG, Berlin, Germany), and immunostain of antigens as dots in a two-dimensional gel blot using the recombinant pooled NLPHL Fab region as primary antibody. (D) Enzyme-linked immunosorbent assay with recombinant C-terminally FLAG-tagged potential antigens of *R. mucilaginosa* identified by mass spectrometry. The respective Fab region at a concentration of 10 μg/mL (positive control = rec. the Fab region of patient #11) showed a reactivity against galactofuranosyl transferase and 2,3-butanediol dehydrogenase of *R. mucilaginosa*.

plasmids, non-reactivity against these candidate antigens was confirmed on dot-plots with the pooled NLPHL Fab regions (*data not shown*).

Serum antibodies against galactofuranosyl transferase and 2,3-butanediol dehydrogenase of *R. mucilaginosa* in patients with nodular lymphocyte-predominant Hodgkin lymphoma

Antibodies with relevant titers against *R. mucilaginosa* Gltf were found in 2/98 (2%) and against *R. mucilaginosa* Bdh in 3/98 (3%) of patients with NLPHL enrolled in the clinical trials of the German Hodgkin Study Group (Figure 2A), which is representative of the general population of NLPHL patients. All antibodies against *R. mucilaginosa* Gltf and Bdh were of IgG class and predominantly of the IgG1 subclass, and they were kappa light-chain restricted (Figure 2C, D). The titers ranged from 1:400 to 1:800 (Figure 2B). No patient with classical Hodgkin lymphoma (0/98) (Figure 2A), THRLBCL (0/50), or primary mediastinal B-cell lymphoma (0/80) (*Online Supplementary Figure S4)* pres-

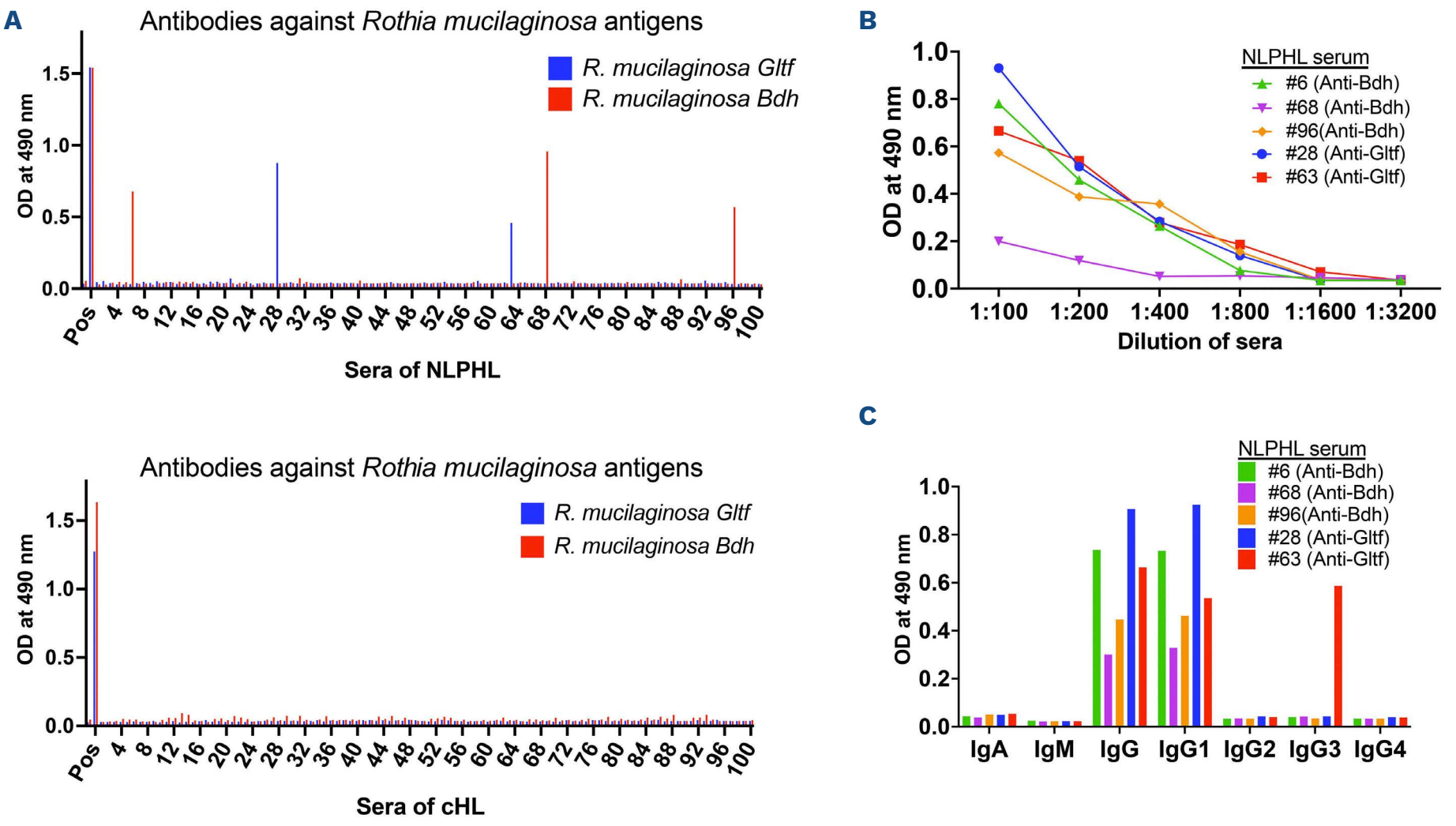

Figure 2. Serological responses against Gltf and Bdh of *R. mucilaginosa*. (A) Sera of patients with nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) and classical Hodgkin lymphoma (cHL) (diluted 1:100) were tested for antibodies against galactofuranosyl transferase (Gltf) and 2,3-butanediol dehydrogenase (Bdh) of *R. mucilaginosa*. (B) Titers of anti-Gltf antibodies and anti-2,3-Bdh antibodies in seropositive patients with NLPHL (left) or cHL (right). The curves represent the optical density (OD) at 490 nm of different serum dilutions. Patients with NLPHL had titers between 1:200 and 1:800. (C) Analysis of Ig classes, and IgG subclasses of serum-antibodies against Gltf and 2,3-Bdh of *R. mucilaginosa* in patients with NLPHL. The anti-Gltf antibodies and anti-2,3-Bdh antibodies were exclusively of the IgG class, mostly IgG1. (D) Light chains of serum-antibodies against Gltf and 2,3-Bdh of *R. mucilaginosa* in patients with NLPHL.

ented serum antibodies against Gltf and Bdh of *R. mucilaginosa*. In summary, antibodies against Gltf and Bdh of *R. mucilaginosa* were found in NLPHL patients but not in patients with related lymphomas.

R. mucilaginosa galactofuranosyl transferase induces stimulation of lymphocyte-predominant cells

Because we hypothesized that specific stimulation of the LP-cell BCR by *R. mucilaginosa* Gltf contributes to lymphomagenesis in a subset of NLPHL, we functionally investigated patient-derived Gltf-specific BCR. For this purpose, cells of the only available NLPHL cell line (DEV) were transfected to express, in a doxycycline-dependent manner, recombinant BCR derived from patients #2 and #3 with constant regions Cγ or Cδ.

Gltf stimulation resulted in strong activation of the BCR signaling pathway in DEV cells that stably expressed Gltf-reactive BCR, as determined by phosphorylation of key BCR signaling factors (pTyr525/526 spleen tyrosine kinase [SYK], pTyr96 B-cell linker [BLNK], pTyr759 phosphoinositide-specific phospholipase C [PLC]γ2, and pTyr223 Bruton tyrosine kinase [BTK]) (Figure 3A). Activation of the BCR pathway was associated with a significant increase in MYC expression (Figure 3A).

Stimulation with recombinant *R. mucilaginosa* Gltf resulted in significantly increased proliferation of DEV cells that stably expressed Gltf-reactive BCR (Figure 3B).

Furthermore, the recombinant immuno-conjugate of *R. mucilaginosa* Gltf conjugated to a truncated form of the exotoxin A of *Pseudomonas aeruginosa* (Gltf/ETA') inhibited proliferation of DEV cells stably expressing Gltf-specific BCR but had no effect on non-transfected DEV cells or on DEV cells that expressed *M. catarrhalis* RpoC-reactive BCR (Figure 3B). An increase in the number of

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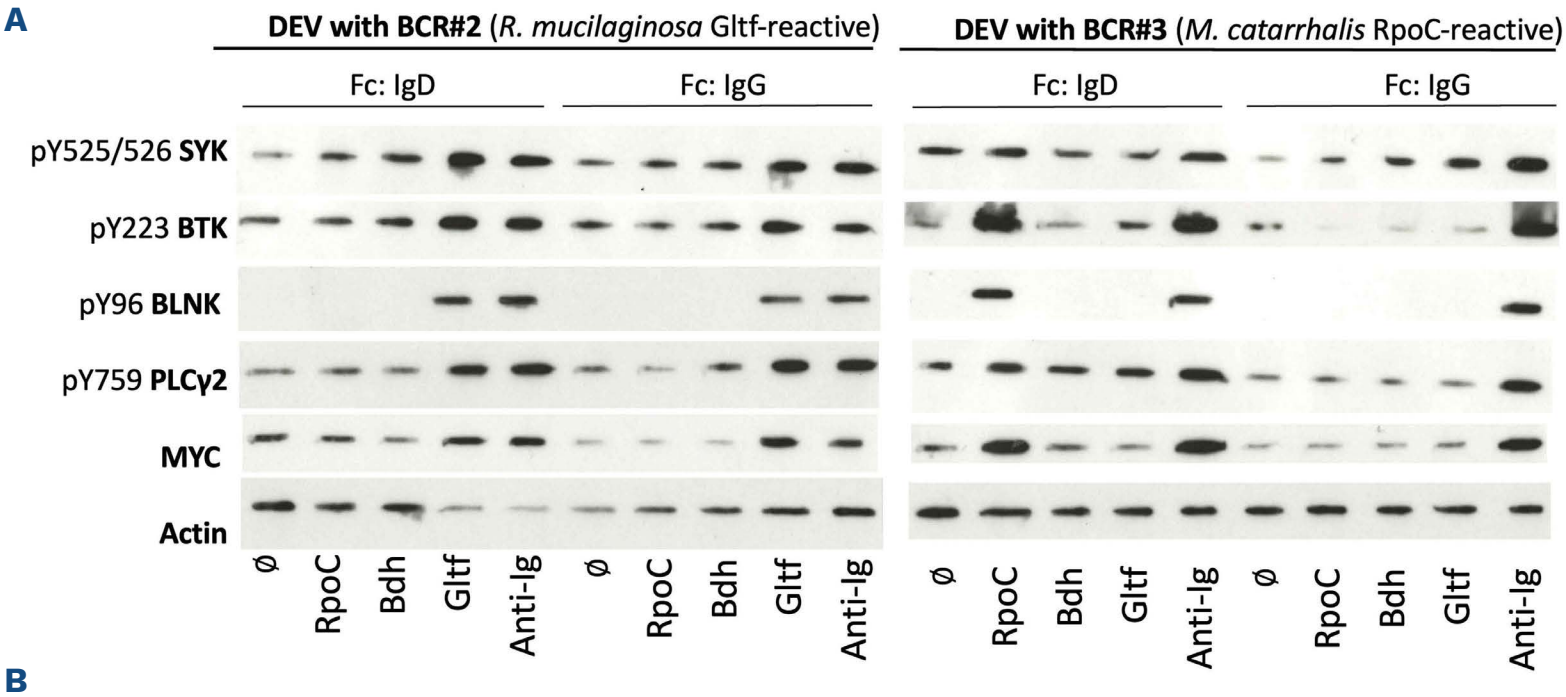

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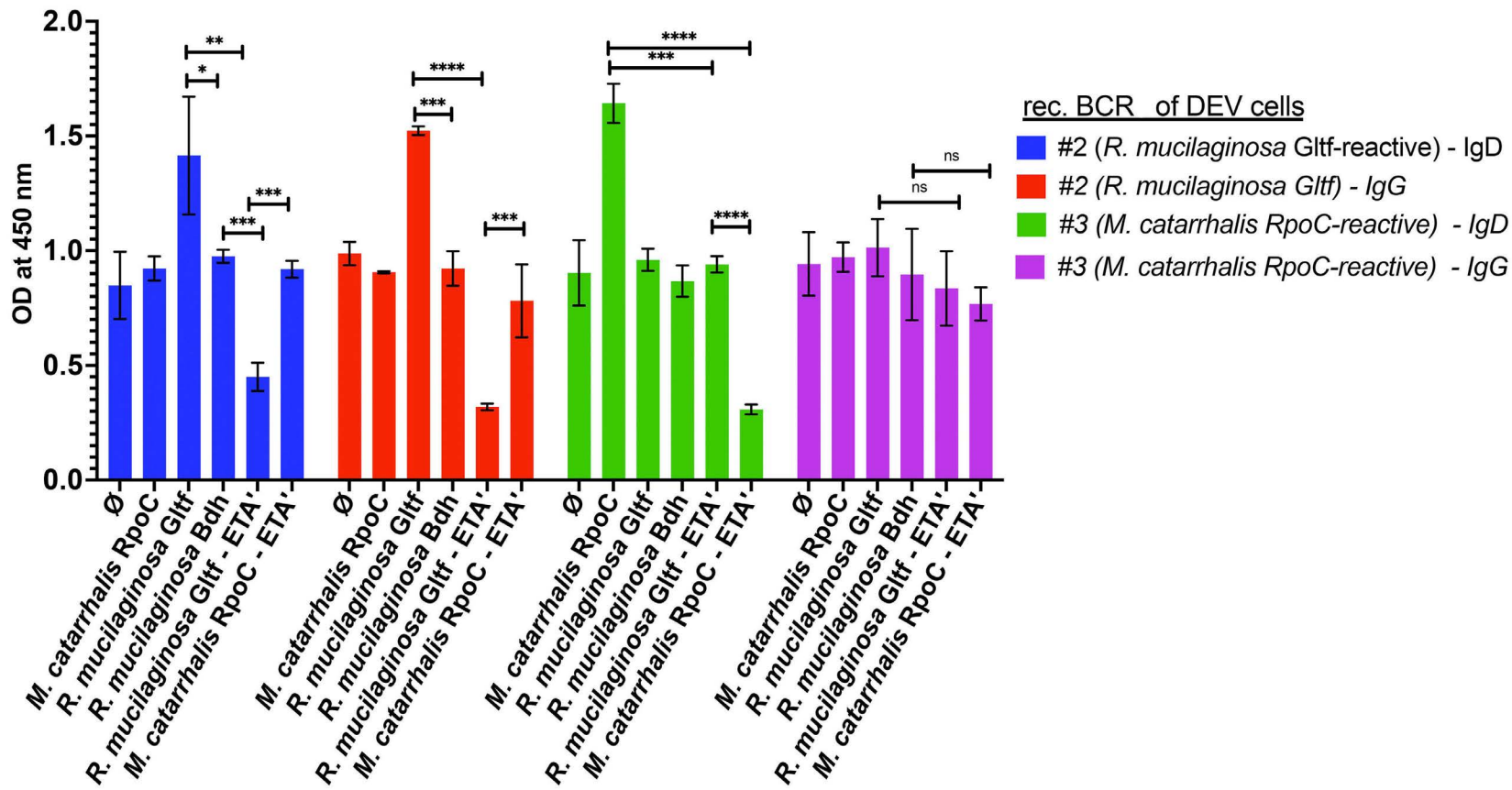

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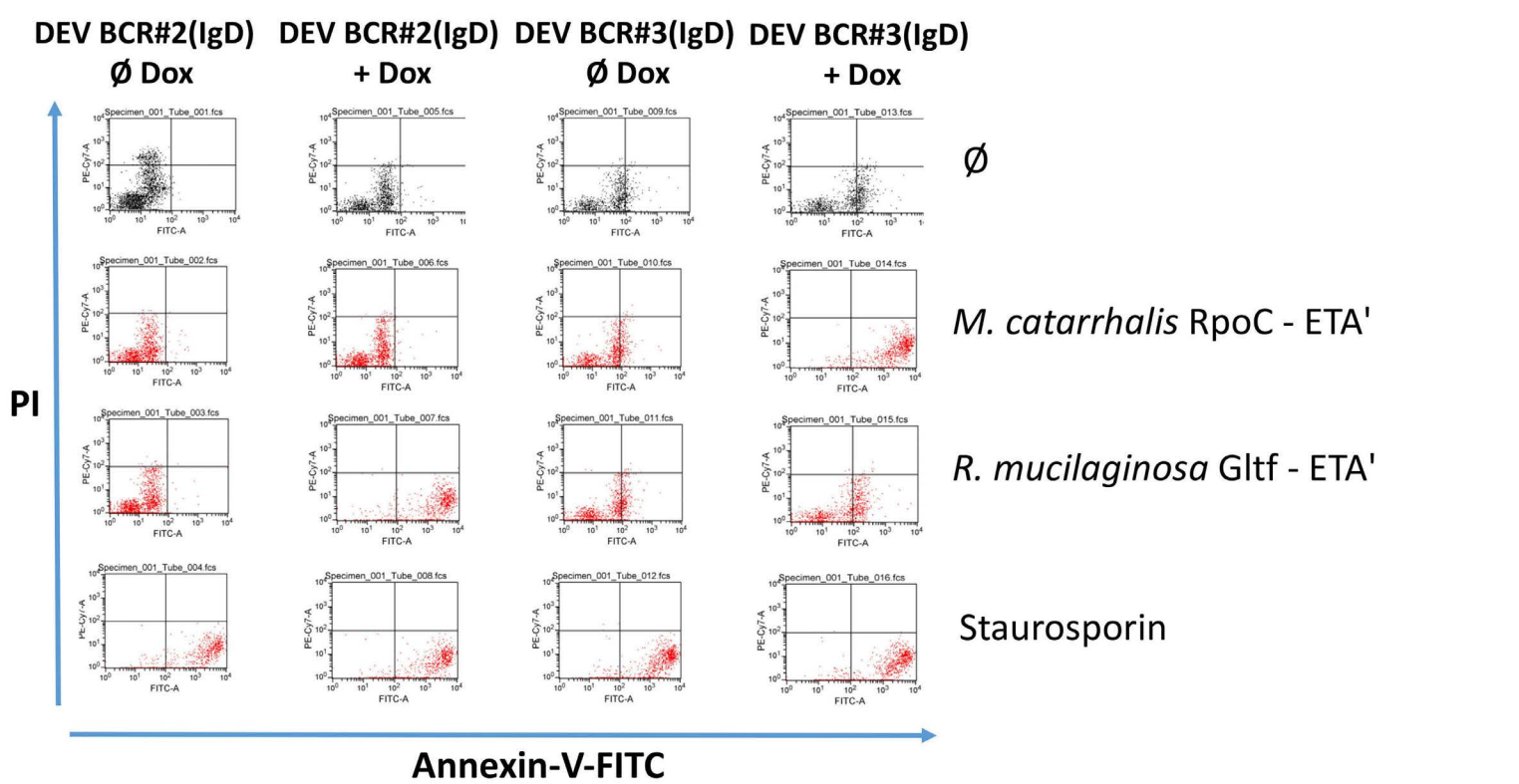

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Figure 3. Stimulation of different types of lymphocyte-predominant cells by Gltf of *R. mucilaginosa*. (A) Activation of the B-cell receptor (BCR) signaling pathway. Western blot analysis of components of the BCR signaling pathway shows activation by *R. mucilaginosa* galactofuranosyl transferase (Gltf) and *by M. catarrhalis* RpoC in DEV cells transfected and expressing *R. mucilaginosa*-Gltf-reactive recombinant BCR of case #2 with stronger bands of the activated isoforms pTyr525/526 SYK, pTyr96 BLNK, pTyr759 PLCγ2 and pTyr223 BTK and of MYC after incubation with *R. mucilaginosa* Gltf. Similarly, reaction to the BCR pathway after incubation with *M. catarrhalis* RpoC to DEV cells with RpoC-reactive BCR of case #3. (B) Tetrazolium proliferation assay with the transfected DEV cells expressing recombinant nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL)-derived BCR with reactivity against Gltf of *R. mucilaginosa* or RpoC of *M. catarrhalis*. Stimulation with Gltf of *R. mucilaginosa* increased proliferation in DEV cells transfected to express Gltf-reactive BCR but not in DEV cells expressing RpoC-reactive BCR. Incubation by ETA' toxin-conjugated to Gltf of *R. mucilaginosa* or to RpoC/ETA' of *M. catarrhalis* on DEV cells expressing either recombinant NLPHL-derived BCR (#2) with reactivity against *R. mucilaginosa* Gltf or against *M. catarrhalis* RpoC-reactive (#3) led to a decrease in proliferation. **P*<0.05; ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001. (C) Apoptosis induced by ETA' toxin-conjugated to Gltf of *R. mucilaginosa* or RpoC of *M. catarrhalis* is dependent on doxycycline-induced expression of Gltf- or RpoC-reactive BCR on lymphocyte-predominant cells. Characterization of DEV cells transfected to express doxycycline-inducible IgD$^+$ recombinant BCR with (from case #2) reactivity against Gltf of *R. mucilaginosa* or RpoC of *M. catarrhalis* (from case #3) by annexin-V/FITC and propidium iodide staining after 24 h culture in the presence of RpoC/ETA', Gltf/ETA' or staurosporin. Dox: doxycycline.

apoptotic cells was detected via annexin V/propidium iodide staining after incubation with *R. mucilaginosa* Gltf/ETA' in the DEV cells expressing a recombinant BCR with the respective reactivity, but not in DEV cells with different reactivity of recombinant BCR - i.e. against *M. catarrhalis* RpoC - or without doxycycline induction of recombinant BCR at all (Figure 3C).

Discussion

The present study strengthens the evidence for the contribution of chronic antigenic simulation by common bacteria to subsets of NLPHL.[38] Two specific antigens of *R. mucilaginosa* were identified as targets for BCR in 5/22 NLPHL cases. In addition, the reactivity against *M. catarrhalis* RpoC antigens was verified for recombinant BCR from 3/7 newly analyzed NLPHL cases, resulting in a total reactivity of 10/22 cases against *Moraxella* spp. In addition to *R. mucilaginosa*, the recombinant lymphoma BCR of most cases (15/22; 68.2%) were reactive against specific bacterial antigens.

As seen before for *M. catarrhalis* RpoC, light chain-restricted antibodies against *R. mucilaginosa* Gltf and 2,3-Bdh, predominantly of the IgG1 subclass, were observed in NLPHL sera. While serum antibodies for *M. catarrhalis* RpoC were relatively frequently identified in NLPHL patients, antibodies against *R. mucilaginosa* were less frequently observed. Both light chain-restricted antibodies against Rpoc and *R. mucilaginosa* are probably the result of a very strong immune reaction against the specific antigen. However, not all patients with such lymphoma BCR reactivity have high titers of serum antibodies; this discrepancy was also seen for lymphoma BCR reactivity against Ars2 in ABC-type diffuse large B-cell lymphoma and for LRPAP1 in mantle cell lymphoma.[39-41] Similar to previous observations of *M. catarrhalis* RpoC,[22] no antibodies against *R. mucilaginosa* Gltf or Bdh were detected in the sera of patients with THRLBCL, the NLPHL-related aggressive B-cell lymphoma subtype,[42,43] suggesting a distinct mechanism in these lymphomas. *R. mucilaginosa* is a commensal, Gram-positive, coagulase-negative coccus and a part of the resident flora of the oral cavity. It typically resides in the anatomical drainage area of cervical lymph nodes.[44] However, in immunocompromised patients, *R. mucilaginosa* can be pathogenic, and bacteremia can occur.[38,39,41-44]

Regarding the previously described association of ultralong CDR3 with reactivity against *M. catarrhalis* RpoC, the extended cohort of NLPHL Fab regions revealed that reactivity against *M. catarrhalis* RpoC was not restricted to BCR with ultralong CDR3, and some cases with ultralong CDR3 were reactive to antigens of *R. mucilaginosa*. Therefore, ultralong CDR3 and skewed IGHD-IGHJ usage are likely not specific to reactivity against a specific antigen but instead represent a relatively frequent structural combination, suggesting that LP cells frequently stem from a B-cell population with ultralong CDR3 that regularly exists in healthy individuals. Recently, in a VH3-23 model of natural antibodies, Wang *et al*. described skewed usage of IGHD2-IGHJ6 and IGHD3-IGHJ6 rearrangements connected to the presence of ultralong CDR3 loops that stabilized the long CDR3 loop rather than contributing to the paratop.[20,45]

The clustering of HLA-DRB1:04 and DRB1:07 haplotypes in *Moraxella*-reactive NLPHL[22] suggests cognate T-cell involvement in the immune reaction that finally leads to lymphoma development. Further studies demonstrated the formation of immunological synapses between TFH and LP cells, which further supports this idea.[19] No HLA restriction was observed in NLPHL cases with *R. mucilaginosa*-directed BCR, with the limitation of a low number of cases. In addition to the lower pathogenicity compared with *M. catarrhalis, R. mucilaginosa* is not known to express a superantigen like MID/hag of *M. catarrhalis*.[46-48] Considering its potential as a chronic antigenic trigger, it should be noted that *R. mucilaginosa* is known to form biofilms. *In vitro*, both *R. mucilaginosa* Gltf and Bdh induced growth by activating the BCR pathway.

A main limitation of the study is that our selection of viral

and bacterial pathogens represents only a fraction of the environmental pathogens; therefore, we likely did not consider all the potential variables. In the current study, we selected *R. mucilaginosa* and *R. aeria* as they occur in a pattern that is comparable to that of *Moraxella* spp. in oropharyngeal samples and other samples of the upper respiratory tract. This necessitates further and extended systematic screening for microbial antigens, including comprehensive microbiome banks. In addition to NLPHL, certain lymphomas, such as duodenal or pediatric follicular lymphoma, different types of marginal zone lymphomas, and more aggressive lymphomas should be studied in this context. The findings lead to the question of why and under which circumstances immune responses against common bacteria - even a component of the oral resident flora - trigger lymphoma.[46-48] From the present study it is difficult to conclude how far bacterial antigens actively support growth of NLPHL once the lymphoma has developed and how far this may be targetable by antibiotic treatment. As eradication of the infectious trigger has been proven successful and has even become standard therapy for some advanced indolent lymphoma entities,[49-53] this might serve as a therapeutic model to be investigated in future clinical trials. However, heterogeneity of LP cells, their active somatic hypermutation of BCR genes, and also transformation into high-grade lymphomas would represent challenges for an antibiotic-based therapeutic approach. On the other hand, the rarity of NLPHL poses a serious challenge to the feasibility of a meaningful clinical trial. Possible proposals for clinical trials might initially investigate the relapsed, advanced setting with an exclusive low-grade manifestation and detection of corresponding antibodies against *M. catarrhalis* or *R. mucilaginosa*.

Given recent reports from retrospective analyses regarding less intensive treatments and active surveillance strategies for NLPHL,[54] the results of the present study should stimulate discussions on clinical trials investigating antibiotic therapy or vaccination strategies against bacteria-associated NLPHL subgroups.

Disclosures

LT has received travel grants from Abbvie, Janssen and EUSA-Pharm, and consultancy fees from Takeda, AstraZeneca, Merck and EUSA-pharm (less than $10,000 each). The other authors declare no competing interests.

Contributions

LT, SLB, and SH designed the study, performed experiments, analyzed data and wrote the manuscript. NF, ER, KL, FN, and NS performed experiments and analyzed data. SR, JB, YJK, RMB, AN, MV, CS, LdL, PB, AE, VP, and GH supplied essential material and interpreted data. RK, ET, KDP, SMH, OC, IAK, ES, MH, MLH, and MB designed the study, interpreted data, and revised the manuscript.

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Data-sharing statement

All relevant data are available in the Article and Online Supplementary Information or from the corresponding author upon reasonable request.

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