An Autochthonous Mouse Model of Myd88- and BCL2-Driven Diffuse Large B-cell Lymphoma Reveals Actionable Molecular Vulnerabilities

Ruth Flümann1,2,3,4, Tim Rehkämper1,2,3,4, Pascal Nieper1,2,3,4, Pauline Pfeiffer1,2,3,4, Alessandra Holzem1,2,3,4, Sebastian Klein5, Sanil Bhatia6, Moritz Kochanek1,2,3,4, Ilmars Kisis1,2,3,4, Benedikt W. Pelzer1,2,3,4, Heinz Ahlert6, Julia Hauer7,8, Alexandra da Palma Guerreiro1,2,4, Jeremy A. Ryan9, Maurice Reimann10, Arina Riabinska1,2,3,4, Janica Wiederstein4, Marcus Krüger4, Martina Deckert2,11, Janine Altmüller12, Andreas R. Klatt13, Lukas P. Frenzel1,2,4, Laura Pasqualucci14, Wendy Béguelin15, Ari M. Melnick15, Sandrine Sander16, Manuel Montesinos-Rongen2,11, Anna Brunn2,11, Philipp Lohneis2,3,5, Reinhard Büttner2,3,5, Hamid Kashkar3,4,17, Arndt Borkhardt6, Anthony Letal9, Thorsten Persigehl2,18, Martin Peifer2,3,19, Clemens A. Schmitt10,20, Hans Christian Reinhardt21, and Gero Knittel1,2,3,4
ABSTRACT

Based on gene expression profiles, diffuse large B-cell lymphoma (DLBCL) is subdivided into germinal center B-cell-like (GCB) and activated B-cell-like (ABC) DLBCL. Two of the most common genomic aberrations in ABC-DLBCL are mutations in MYD88 as well as BCL2 copy-number gains. Here, we employ immune phenotyping, RNA sequencing, and whole-exome sequencing to characterize a Myd88- and Bcl2-driven mouse model of ABC-DLBCL. We show that this model resembles features of human ABC-DLBCL. We further demonstrate an actionable dependence of our murine ABC-DLBCL model on BCL2. This BCL2 dependence was also detectable in human ABC-DLBCL cell lines. Moreover, human ABC-DLBCLs displayed increased PD-L1 expression compared with GCB-DLBCL. In vivo experiments in our ABC-DLBCL model showed that combined venetoclax and PD-1 blockade significantly increased the overall survival of lymphoma-bearing animals, indicating that this combination may be a viable option for selected human ABC-DLBCL cases harboring MYD88 and BCL2 aberrations.

SIGNIFICANCE: Oncogenic Myd88 and Bcl2 cooperate in murine DLBCL lymphomagenesis. The resulting lymphomas display morphologic and transcriptomic features reminiscent of human ABC-DLBCL. Data derived from our Myd88/Bcl2-driven autochthonous model demonstrate that combined BCL2 and PD-1 blockade displays substantial preclinical antilymphoma activity, providing preclinical proof-of-concept data, which pave the way for clinical translation.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid neoplasm in adults and accounts for approximately 35% of all B-cell non-Hodgkin lymphomas (1). DLBCL is a morphologically, biologically, and clinically heterogeneous disease that has historically been subdivided into germinal center B-cell-like (GCB) and activated B-cell-like (ABC) DLBCL using gene expression profiling (1–3). This cell of origin (COO)-based classifier separates subentities with distinct biology, pathogenesis, and clinical response to frontline chemoimmunotherapy (3–5). GCB-DLBCL has been proposed to originate from light-zone GCBs (6), whereas ABC-DLBCL likely derives from postgerminal center plasmablasts (3, 6–8). To capture additional molecular heterogeneity in DLBCL, two independent comprehensive genomic analyses of human DLBCL cases were recently completed and led to the discovery of partially overlapping genetically defined DLBCL categories (9, 10). One group classified approximately 50% of the primary cases in a supervised approach to four genetically defined DLBCL subtypes (10). These were based on COO-associated alterations and identified tumors with co-occurring MYD88- and CD79B mutations (MCD), BCL6 rearrangements and NOTCH2 mutations (BN2), EZH2 mutations and BCL2 rearrangements (EZB), as well as NOTCH1 mutations (N1; ref. 10). An independent analysis first defined

1University of Cologne, Faculty of Medicine and University Hospital Cologne, Clinic I of Internal Medicine, Cologne, Germany. 2Center for Integrated Oncology, University of Cologne, Cologne, Germany. 3Center for Molecular Medicine, University of Cologne, Cologne, Germany. 4Cologne Excellence Cluster on Cellular Stress Response in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany. 5Faculty of Medicine and University Hospital Cologne, Institute of Pathology, University of Cologne, Cologne, Germany. 6Medical Faculty, Department of Pediatric Oncology, Hematology and Clinical Immunology, Heinrich Heine University Düsseldorf, Düsseldorf, Germany. 7Department of Pediatrics, Pediatric Hematology and Oncology, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany. 8National Center for Tumor Diseases (NCT), Dresden, Germany. 9Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts. 10Charité Universitätsmedizin Berlin, Medical Department of Hematology, Oncology and Tumor Immunology, and Molekulares Krebsforschungszentrum - MKF2, Virchow Campus, Berlin, Germany. 11Faculty of Medicine and University Hospital Cologne, Institute of Neuropathology, University of Cologne, Cologne, Germany. 12Cologne Center for Genomics (CCG), University of Cologne, Cologne, Germany. 13Faculty of Medicine and University Hospital Cologne, Institute of Clinical Chemistry, University of Cologne, Cologne, Germany. 14Department of Pathology and Cell Biology, Institute for Cancer Genetics and the Herbert Irving Comprehensive Cancer Center, Columbia University, New York, New York. 15Division of Hematology/Oncology, Department of Medicine, Weill Cornell Medicine, Cornell University, New York, New York. 16Adaptive Immunity and Lymphoma Group, German Cancer Research Center/National Center for Tumor Diseases Heidelberg, Heidelberg, Germany. 17Faculty of Medicine and University Hospital Cologne, Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, Cologne, Germany. 18Faculty of Medicine and University Hospital Cologne, Department of Radiology and Interventional Radiology, University of Cologne, Cologne, Germany. 19Department of Translational Genomics, University of Cologne, Cologne, Germany. 20Kepler Universitätsklinikum, Medical Department of Hematology and Oncology, Johannes Kepler University, Linz, Austria. 21Department of Hematology and Stem Cell Transplantation, University Hospital Essen, University Duisburg-Essen, German Cancer Consortium (DKTK Partner Site Essen), Essen, Germany.

Note: Supplementary data for this article are available at Blood Cancer Discovery Online (https://bloodcancerdiscov.aacrjournals.org/).

R. Flümann, T. Rehkämper, and P Nieper contributed equally to this article.

H.C. Reinhardt and G. Knittel contributed equally to this article.

Corresponding Author: Hans Christian Reinhardt, University Hospital Essen, Hufelandstr. 55, 45147 Essen, Germany; Phone: 49-201-723-3136; Fax: 49-201-723-5961; E-mail: christian.reinhardt@uk-essen.de

Blood Cancer Discov 2021;2:70–91
doi: 10.1158/2643-3230.BCD-19-0059
©2020 American Association for Cancer Research.
recurrent genetic drivers in DLBCL and used a non-negative matrix factorization consensus clustering approach, allowing classification of 98% of cases into five clusters with specific coordinate genetic signatures (9). These clusters were defined by: (i) BCL6 structural variants in combination with NOTCH2 aberrations (C1 DLBCL); (ii) biallelic TP53 inactivation (TP53 mutations and 17p copy-number losses) in combination with haploinsufficiencies of p21.13/CDKN2A and 13q14.2/RB1 (C2 DLBCL); (iii) BCL2 mutations with concordant BCL2 structural variants in combination with EZH2, CREBBP, and KMT2D mutations and additional activating alterations of the PI3K pathway (C3 DLBCL); and (iv) mutations in linker and core histone genes in combination with aberrations in immune evasion molecules, NF-kB, and RAS/JAK/STAT signaling molecules (C4 DLBCL; ref. 9). An additional cluster was defined by 18q gains in combination with MYD88 and CD79B mutations (C5 DLBCL; ref. 9). These large datasets, together with the recently published whole-exome sequencing results of the 1,001 DLBCL cases, have established a framework for the identification of potentially druggable genomic aberrations in human DLBCL (11). In this context, it is important to note that frontline chemoimmunotherapy using R-CHOP, or R-CHOP-like regimens, achieves cure rates of more than 60% (9, 12, 13). However, relapsed or refractory disease represents a major clinical challenge, as these patients are often difficult to salvage, and even high-dose chemotherapy regimens with autologous stem cell support frequently do not provide long-term disease control (14–17). Thus, there is a pressing need for the development and preclinical validation of therapeutic strategies for the treatment of relapsed/refractory disease, as well as strategies to treat elderly and frail patients that do not qualify for intensive chemoimmunotherapy.

A powerful tool to assess the biological effects of targeted therapeutic agents are autochthonous mouse models, which are genetically engineered to carry genomic aberrations that precisely match those observed in the corresponding human disease. The advent of next-generation sequencing technologies has enabled the fine-grained cross-validation of mouse models and human disease. Here, we report the detailed molecular characterization and cross-species comparison of an autochthonous mouse model of Myd88-driven ABC-DLBCL. Note that 29% of human ABC-DLBCLs harbor the p.L265P mutation in B-cell lymphomagenesis, we recently identified a potentially druggable genomic aberration at the orthologous position of human MYD88 and CD79B mutations (20). When the Myd88-p.L252P allele is crossed with an additional mutant strain that conditionally overexpresses BCL2 from the Rosa26 locus upon Cre-mediated deletion of a STOP cassette, the resulting Myd88-p.L252P/WT, Rosa26SL.BCL2.ires.GFP/WT, Cd19-Gtv/WT (MBC) animals develop an aggressive lymphoma (20).

Here, we demonstrate that this MBC model resembles human ABC-DLBCL with respect to morphology, as well as on the transcriptomic and genomic level. In contrast, a Kmt2d/Bcl2-driven lymphoma model displayed more similarity with human GCB-DLBCL. Moreover, our analyses revealed a druggable dependence on BCL2 in murine and human ABC-DLBCL cell lines and tumors, which was not detected in human GCB-DLBCL cell lines. We further demonstrate that Myd88-driven lymphomas display a single-agent response to PD-1 blockade, which is synergistic with concurrent BCL2 inhibition, in vivo. Altogether, these data provide a detailed molecular and functional description of our Myd88-driven ABC-DLBCL model and provide a biological rationale for the use of combined BCL2 and PD-1 inhibition for the treatment of ABC-DLBCL.

RESULTS

**Myd88-p.L252P and BCL2 Cooperate to Induce Splenomegaly and Germinal Center Formation In Vivo**

Two of the most common genomic aberrations in human ABC-DLBCL are oncogenic MYD88 mutations and BCL2 copy-number gains (7, 9–11). Moreover, copy-number gains of 18q21.33, where the BCL2 gene is localized, are significantly enriched in MYD88 mutant DLBCL cases compared with MYD88 wild-type (WT) cases (Supplementary Fig. S1A; ref. 9). To assess the in vivo effects of these aberrations, we performed longitudinal monitoring of WT, Myd88-p.L252P/WT, Cd19-Gtv/WT (MC), Rosa26SL.BCL2.ires.GFP/WT, Cd19-Gtv/WT (BC), and Myd88-p.L252P/WT, Rosa26SL.BCL2.ires.GFP/WT, Cd19-Gtv/WT (MBC) animals using MRI scanning to monitor splenomegaly (Fig. 1A–C). Amplifications involving 18q21.33, the chromosomal location of BCL2, are associated with an overexpression of BCL2 (Supplementary Fig. S1B), which is modeled by the Rosa26SL.BCL2.ires.GFP allele employed in this study. As shown in Fig. 1B and C, MBC animals displayed a significantly increased spleen volume, compared with WT, BC, and MC animals at 10, 20, and 30 weeks. Moreover, at the 20-, 30-, and 50-week time points, the spleen volume of BC animals was significantly larger than that of WT and MC mice (Fig. 1C; 0.001; Welch unpaired two-tailed t test.

![Figure 1](https://example.com/figure1.png)  
**Figure 1.** Germinal center hyperplasia and increased plasma cell pools in MBC animals. **A,** Schematic illustrations of the employed alleles. Exons 2 to 6 of the endogenous Myd88 locus were flanked by loxP sites (triangles). Downstream of the second loxP site, a second set of the exons 2 to 6 was inserted, harboring the L252P point mutation (asterisk). Read-through is prevented by a strong polyadenylation signal (“pA”). Human BCL2 DNA expression is controlled by a CAGGS promoter and prevented by a lox-stop-lox cassette. GFP expression is coupled to BCL2 expression by an internal ribosomal entry site (IRES). The construct is a knock-in into the Rosa26 locus. Both alleles have previously been published (20). The Cd19-Gtv allele is a knock-in into the Cd19 locus and has been previously published (85). **B,** Exemplary axial MR images of 30-week-old animals. Spleens are outlined. **C,** Spleen volumes of WT (n = 5), MC (n = 5), BC (n = 7), and MBC (n = 7) mice were quantified from MR images. **D,** IHC stainings for B220, PNA, and CD3 of splenic sections of 30-week-old WT, MC, BC, and MBC animals. **E,** The germinal center (GC) structures stained by PNA in splenic sections of 30-week-old WT, MC, BC, and MBC animals (≥ n = 4 per genotype). *, P ≤ 0.05; **, P ≤ 0.01; and ***, P ≤ 0.001. Welch unpaired two-tailed t test.
Vulnerabilities in a Myd88/BCL2-Driven DLBCL Mouse Model

A

Myd88 conditional knock-in allele
Rosa26 conditional BCL2 knock-in allele
Cd19 constitutive Cre knock-in allele

B

WT MC BC MBC

C

Spleen volume (µL)

Age (weeks)

10 20 30

D

B220 PNA CD3

WT MC BC MBC

E

GCs per mm² spleen GC area (%) Mean GC size (×10⁴ µm²)

F

% of CD45⁺ B cells Follicular Marginal zone Transitional Early PB Late PB/PC

G

WT MC BC MBC

H

Ig titer (µg/mL)

IgM IgA IgG1 IgG2b IgG2c IgG3

Association for Cancer Research.
Supplementary Fig. S1C). We next performed a histologic assessment of spleen sections derived from WT, MC, BC, and MBC animals (Fig. 1D; Supplementary Fig. S1D). For that purpose, 30-week-old WT, MC, BC, and MBC animals were sacrificed, and spleens were stained with antibodies detecting CD3 (labeling the splenic T-cell zone) and B220 (labeling the splenic B-cell zone), as well as PNA (labeling the germinal center) on serial sections. Neither the number of germinal centers per spleen area nor the germinal center area per spleen area or the average size of the germinal centers differed significantly between WT and MC animals (Fig. 1D and E; Supplementary Fig. S1D). However, BC animals displayed significantly more and larger germinal centers than WT and MC animals (Fig. 1D and E). Similarly, MBC animals showed significantly more and larger germinal centers than MC mice and WT controls (Fig. 1D and E). Of note, we did not detect clonal lymphoma infiltrates in MBC spleens at this time point. Furthermore, the global splenic architecture was not disrupted in MBC mice. In a different set of experiments, we employed a BCL6 antibody to stain splenic germinal centers (Supplementary Fig. S1E). In these experiments, BC animals displayed slightly more and significantly larger germinal centers than WT animals (Supplementary Fig. S1E). Similarly, MBC animals showed significantly more and larger germinal centers than WT and MC controls (Supplementary Fig. S1E). Altogether, these data indicate that oncogenic Myd88 and BCL2 cooperate in enhancing reactive splenomegaly and germinal center formation in vivo.

**Myd88<sup>p.L252P</sup> and BCL2 Cooperate to Drive an Expansion of CD138-Positive Cells In Vivo**

To gain further insight into the cellular composition underlying the germinal center hyperplasia that we observed in BC and MBC animals (Fig. 1D and E; Supplementary Fig. S1D and S1E), we next performed flow cytometry–based immune phenotyping (Fig. 1F; Supplementary Fig. S1F–S1H). We particularly assessed the representation of total B cells (B220<sup>+</sup>) of CD45<sup>+</sup>), follicular B cells (B220<sup>+</sup>/CD93<sup>-</sup>/CD21<sup>low</sup>/CD23<sup>+</sup> of CD45<sup>+</sup>), marginal zone B cells (B220<sup>+</sup>/CD93<sup>-</sup>/CD21<sup>high</sup>/CD23<sup>-</sup> of CD45<sup>+</sup>), transitional B cells (B220<sup>+</sup>/CD93<sup>-</sup>/CD45<sup>+</sup>), early plasmablasts (CD138<sup>+</sup>/B220<sup>+</sup>/MHCII<sup>-</sup> of CD45<sup>+</sup>), and late plasmablasts/plasma cells (CD138<sup>+</sup>/B220<sup>+</sup>/MHCII<sup>-</sup> of CD45<sup>+</sup>) from spleens and bone marrow (BM) of 30-week-old WT, MC, BC, and MBC animals (Fig. 1F; Supplementary Fig. S1F), as well as 50-week-old WT, MC, and BC mice (Supplementary Fig. S1G and S1H). As shown in Fig. 1F, the relative percentage of splenic B cells was significantly increased in BC and MBC mice compared with WT animals. A further subclassification of these B cells revealed that BC animals displayed significantly more follicular B cells compared with WT, MC, and MBC mice (Fig. 1F). Marginal zone B cells were significantly less prevalent in MC, BC, and MBC mice compared with WT animals (Fig. 1F). MBC mice displayed a significantly increased percentage of transitional B cells compared with WT, MC, and BC mice (Fig. 1F). The most striking differences were observed when the percentages of CD138<sup>+</sup> cells were analyzed (Fig. 1F). MC animals displayed significantly more CD138<sup>+</sup> cells compared with WT and BC mice, possibly indicating that oncogenic Myd88 enhances germinal center transition (Fig. 1F). Moreover, oncogenic Myd88 and BCL2 appear to cooperate in the accumulation of CD138<sup>+</sup> early plasmablasts (Fig. 1F). Similarly, MBC mice harbor a significantly higher percentage of late plasmablasts/plasma cells in the spleen and BM compared with WT, MC, and BC mice (Fig. 1F; Supplementary Fig. S1F). The levels of CD138<sup>+</sup> cells found in MBC animals at 30 weeks of age were not reached even by 50-week-old MC and BC mice (Supplementary Fig. S1G and S1H). To investigate whether these plasma cells passed through the germinal center reaction, or whether the engineered mutations preferentially drive the development of extrafollicular plasma cells, we performed full-length B-cell receptor (BCR) sequencing on CD138<sup>+</sup> cells isolated from the spleens of 10-week-old WT and MBC animals (n = 4 per genotype; ref. 21). In short, each individual Ig cDNA molecule is uniquely labeled with a barcode (unique molecular identifier, UMI) during reverse transcription. This UMI allows the assignment of each sequencing read to a cDNA molecule of origin. Reads with identical UMI are grouped into a “molecular identifier group” (MIG), and a nearly error-free sequence is derived for each MIG by consensus assembly of the assigned reads. Analysis of the somatic hypermutation frequency of the derived V(D)J region sequences revealed a minor but significant shift toward mutated Ighm sequences (51.6% and 39.4% of Ighm sequences with more than one mutation for MBC and WT CD138<sup>+</sup> cells, respectively; Supplementary Fig. S2A and S2B). No significant differences in the mutation rates of transcripts with Ighg1, Ighg2c, Ighg3, and Igmu constant regions were observed (Supplementary Fig. S2A and S2B). Ig sequences recovered from CD138<sup>+</sup> MBC cells showed a significant reduction of Ighm transcripts and a significant increase in Ighg2c and Ighg3 isotypes compared with CD138<sup>+</sup> WT cells (Supplementary Fig. S2C and S2D), in line with a role of Toll-like receptor (TLR) signaling in promoting class switch recombination (22–24). Altogether, these data indicate that the CD138<sup>+</sup> cells, which accumulate in MBC mice, pass through the germinal center reaction with at least the same frequency as CD138<sup>+</sup> WT cells. These observations further suggest a role of oncogenic Myd88 in promoting the transition through the germinal center reaction, which may be augmented by the apoptosis-repressing effect of an increased BCL2 gene dosage, ultimately leading to a substantial expansion of postgerminal center B-cell stages in non-lymphoma–bearing animals.

**B-cell–Specific Myd88<sup>p.L252P</sup> and BCL2 Expression Disrupts Self-tolerance In Vivo**

Given the robust expansion of postgerminal center B-cell populations in MBC mice, we next asked whether MBC animals displayed increased serum immunoglobulin levels compared with WT, MC, and BC mice. To address this question, we initially performed electrophoreses with serum samples isolated from 30-week-old animals (Fig. 1G). Consistent with the massive expansion of early and late plasmablasts, as well as plasma cells in MBC mice, we observed a substantially increased gamma-globulin fraction in sera derived from MBC animals compared with WT, MC, and BC samples (Fig. 1G). The gamma-globulin levels of MC and BC animals were only mildly increased compared with WT mice (Fig. 1G). Of note, we detected a monoclonal gamma-globulin band in 1 out of 6 MC animals, whereas no monoclonal band could be detected in WT, BC, and MBC mice at this 30-week time point.
Vulnerabilities in a Myd88/BCL2-Driven DLBCL Mouse Model

(n = 6 each; Fig. 1G). To further dissect this gammopathy, we next performed ELISA experiments to assess the contribution of IgM and the IgG subclasses IgG1, IgG2b, IgG2c, and IgG3 to the gamma-globulin fraction. As shown in Fig. 1H, MBC animals displayed significantly increased serum IgM, IgG1, IgG2b, and IgG2c concentrations compared with WT, MC, and BC animals, whereas there was no significant difference in IgG3 concentrations (Fig. 1H). These data are consistent with the reduced percentage of splenic marginal zone cells, which are a major source of IgG3 in response to T-cell-independent antigens (25). These results demonstrate that 30-week-old MBC animals display a marked polyclonal gammopathy, consisting of class-switched and nonclass-switched immunoglobulins.

Recent reports have provided evidence indicating that chromatin/DNA-associated as well as RNA and RNA-associated antigens can potently activate autoactive B cells through sequential dual engagement of BCR and TLR/MYD88 signaling cascades (26–29). In brief, AM14 RF B cells that bind autologous IgG2a with low affinity can be driven into proliferation by in vitro exposure to affinity-purified IgG2a monoclonal antibodies specific for nucleosomes (30). Using this system, it was shown that activation of RF B cells was driven by IgG2a-chromatin immune complexes, which was DNAase sensitive (26). Moreover, activation was dependent on the synergistic engagement of the BCR- and MYD88-dependent TLR signaling (26). The same BCR/TLR paradigm was shown to hold true for RNA-associated autoantigens, as well as CpG dsDNA antigens (27, 29). It is important to note that full activation of these autoactive B cells was abolished in the absence of Myd88 (26, 27, 29). Moreover, the constitutive B-cell–specific overexpression of BCL2 was recently shown to impair tolerance induction in a series of model systems and to induce a lupus-like serologic phenotype with antinuclear reactivity (31–37). Given the critical role of Myd88 in promoting the activation of autoactive B cells, as well as the impact of BCL2 on tolerance induction, we next asked whether B-cell–specific expression of the Myd88 p.L252P gain-of-function mutation and/or BCL2 overexpression may promote the loss of self-tolerance. For that purpose, we assessed the presence or absence of autoactive serum antibodies using the HEp-2 indirect immunofluorescence staining assay. As shown in Fig. 2A, WT and BC sera produced only a dim anti-IgM fluorescence signal in the HEp-2 assay, whereas MC and MBC animals displayed significantly higher levels of autoactive IgM antibodies. The staining pattern obtained with MC and MBC sera was largely cytoplasmic, indicating that these antibodies may not recognize DNA or RNA-associated antigens (Fig. 2B). We next assessed the presence of IgG autoactive antibodies. As shown in Fig. 2C, we observed a robust staining with sera derived from MC, BC, and MBC animals, whereas WT serum only produced faint signal. Of note, BC serum primarily reacted with nuclear antigens (Fig. 2D). Altogether, these data indicate that B-cell–specific expression of Myd88p.L252P overcomes self-tolerance. Of note, Myd88p.L252P expression primarily results in an increased presence of autoantibodies against cytoplasmic structures, mainly of the IgM isotype, whereas BCL2 overexpression promotes the generation of predominantly antinuclear IgG immunoglobulins. MBC animals showed a mixed phenotype.

B-cell–Specific Myd88p.L252P and BCL2 Expression Leads to an Enhanced B-cell Reactivity In Vivo

B-cell responses are subdivided into T-cell–dependent (TD) and T-cell–independent (TI) responses (38). TD antigens are proteins that are processed and presented on MHC-II surface molecules for detection by CD4+ T helper cells (38). Two types of TI antigens exist (38). TI-I antigens, such as lipopolysaccharide (LPS), CpG, and poly-IC, mediate polyclonal B-cell activation by engaging TLRs (38). TI-II antigens are typically polysaccharides that induce antigen-specific B-cell responses through BCR clustering (38). To ask whether B-cell–specific Myd88p.L252P expression and/or BCL2 overexpression may affect the magnitude or persistence of the humoral immune response induced by TD or TI-II antigens, we immunized WT, MC, BC, and MBC mice with NP-CGG (TD) or NP-Ficoll (TI-II) and quantified the NP-specific IgM and IgG response 4, 7, 10, 21, and 40 days after the vaccination. The maximal IgM response to NP-Ficoll was increased 7.5- and 6.6-fold in MC and BC animals compared with WT, respectively (Fig. 2E). This effect was even more pronounced in MBC animals, where we observed a 14.8-fold increase compared with WT (Fig. 2E). The NP-Ficoll–induced IgG response was similarly enhanced in MC and MBC animals (23.0- and 36.3-fold, respectively), compared with WT (Fig. 2E). However, in contrast to the IgM response, the IgG response was only marginally (4.5-fold) increased in BC compared with WT animals, suggesting BCL2 overexpression may not suffice in promoting class-switch recombination following TI-II–mediated B-cell activation (Fig. 2E). The humoral anti-NP response following exposure to the TD antigen NP-CGG differed from that observed for NP-Ficoll. Both BC and MBC animals displayed a massively enhanced maximal IgM (7.3- and 8.3-fold, respectively) and IgG response (67.7- and 80.6-fold) compared with WT controls (Fig. 2F). In contrast, MC animals displayed only a marginally enhanced IgM and IgG response (2.8- and 27.6-fold) compared with WT mice (Fig. 2F). These data, which are in line with previous reports on the analysis of an EpcBcl2 allele (35), indicate that BCL2 overexpression particularly promotes the humoral response against TD antigens. The expression of Myd88p.L252P promotes class switch in response to TI-II antigen, in agreement with previous studies demonstrating the importance of TLR signaling for class switch to IgG in response to thymus-independent antigen (22, 39).

Expression of Oncogenic Myd88 Drives the Formation of a Protein Supercomplex Containing BCR and Myddosome Components

Recent data from human lymphoma cell lines indicate that oncogenic BCR signaling in a subset of ABC-DLBCL cases is coordinated by a protein complex involving MYD88, TLR9, and IgM (40). This so-called My-T–BCR complex colocalizes with mTOR on endolysosomes, where it promotes NF-κB–mediated prosurvival signaling (40). This report prompted us to ask whether protein complexes involving BCR signaling components, as well as MYD88 and Myddosome components, also assemble in nontransformed Myd88-mutant cells. To this end, we performed proximity ligation assays (PLA), which label proteins that colocalize within a nanometer distance (41). We isolated naïve B cells from WT and MC animals to explore the
Figure 2. MBC animals show exaggerated immune responses to self- and foreign antigen. A, Self-reactive antibodies of the IgM isotype were visualized by a Kallestad HEp-2 assay adapted to the murine system, and mean fluorescence intensities (MFI) were quantified (n = 6 per genotype, two exemplary cases per genotype are shown). B, Quantification of the observed staining patterns. C, Autoreactive IgG immunoglobulins were visualized by an adapted Kallestad HEp-2 assay, and mean fluorescence intensity (MFI) values were quantified (WT, n = 7; MC, n = 8; BC, n = 8; MBC, n = 9; two exemplary cases per genotype are shown). D, Quantification of the observed staining patterns. E and F, WT, MC, BC, and MBC animals (n = 3) were immunized i.p. with either NP-Ficoll (50 μg) or NP-CGG (50 μg) at day 0, and the NP-specific IgM and IgG levels in the sera of animals were measured at days 0, 4, 7, 10, 21, and 40 after immunization by ELISA. Envelopes represent SEM. *, P ≤ 0.05 and **, P ≤ 0.01; Welch unpaired two-tailed t test.
interactome of WT and mutant MYD88. In these experiments, we detected significantly more complexes involving MYD88 and IRAK1, as well as the Myddosome component IRAK4, in unstimulated MC B cells compared with WT controls (Fig. 3A and B). Moreover, naïve B cells from MC animals also displayed significantly more protein complexes consisting of MYD88 and the BCR signaling components IgM and BTK compared with WT controls (Fig. 3A and B). To further substantiate the functional relevance of the increased MYD88-centered complex formation in MC-derived B cells, we next performed immunoblots probing the Myddosome formation–induced autophosphorylation site Thr-345 in IRAK4, the phospho-Tyr-223 residue in BTK, and the IKK2-dependent phospho-Ser-536 site in the NF-κB subunit p65 in unstimulated MC and WT B cells (Fig. 3C). In these experiments, we observed significantly increased p65 phosphorylation on Ser-536 in MC compared with WT B cells (Fig. 3C). Although there was a trend toward increased IRAK4 autophosphorylation on Thr-345 in MC B cells, this did not reach statistical significance (Fig. 3C). Similarly, there was no differential BTK Tyr-223 phosphorylation in MC versus WT B cells (Fig. 3C). Altogether, these observations suggest that mutant MYD88 may constitutively nucleate a signaling hub, which links BCR and TLR signaling components, even in nontransformed B cells. This constitutive protein complex formation is further associated with increased p65 Ser-536 phosphorylation in MC B cells.

**Myd88^p.L252P and BCL2 Cooperate in ABC-DLBCL Lymphomagenesis In Vivo**

We next aimed to determine the effect of B-cell–specific Myd88^p.L252P and/or Rosa26^BCL2.IRES.GFP expression on overall survival and to determine the cause of death in these mice. We previously reported that MC animals display a significantly
reduced overall survival, compared with WT mice and that MBC animals live significantly shorter than MC mice (20). However, it remained unclear whether BC animals display a different overall survival than MC mice. Thus, we performed a direct head-to-head comparison of MC, BC, and MBC animals. As shown in Fig. 4A, BC mice show a significantly reduced overall survival compared with MC animals. Furthermore, and in line with previous results, MBC mice died significantly earlier than MC and BC animals (Fig. 4A). We further determined the cause of death in MC, BC, and MBC mice. Although MBC mice developed life-limiting lymphoma with 83% penetrance, MC and BC mice developed lymphoma in only 20% and 50%, respectively (Fig. 4B). The malignant lesions observed in MBC animals were largely located subdiaphragmatically in mesenteric lymph nodes, but rarely in mediastinal and submandibular lymph nodes (Supplementary Fig. S3A and S3B). We did not detect any obvious central nervous system manifestations. Although spleens were frequently enlarged, we failed to detect evidence for infiltration by clonal lymphoma. Instead, we observed the germinal center expansion detailed in Fig. 1D and E. Moreover, spleen weights did not exceed 450 mg, further indicating a reactive nature of the observed splenomegaly (Supplementary Fig. S3B). The average number of distinct lymph node groups involved was 1.7 (Supplementary Fig. S3C). Immunophenotyping revealed that BC- and MBC-derived lymphomas were almost exclusively B220+ and largely CD138+ (Fig. 4B and C). We note that the CD138 staining pattern displayed some intertumoral heterogeneity, which is illustrated in Supplementary Fig. S3D, where six distinct lymph node manifestations derived from six distinct animals are displayed. The CD138 staining intensity varied between different areas of the same lymphoma manifestation. Moreover, when analyzed at higher magnification, we observed clearly distinct CD138 staining intensity in adjacent cells. It is important to note that the same lymphoma areas stained uniformly positive for GFP, which is only expressed in cells that have expressed the Cd19 Cre allele during their lifetime and therefore belong to the B-cell lineage (Supplementary Fig. S3D). Altogether, these data suggest a postgerminal center plasmablastic differentiation pattern of MBC lymphomas, whereas lymphomas developing in MC mice retained expression of the B-cell marker B220 and were CD138+ (Fig. 4B and C). The proliferation index in MBC lymphomas was 42.7% (±17.3%, n = 19). MC and BC lymphomas displayed similar proliferation indices of 39.1 (+14.5%, n = 4) and 35.9% (±14.3%, n = 5), respectively (Fig. 4C; Supplementary Fig. S3E).

Next to lymphomas, MC, BC, and MBC mice displayed the indicated percentage of cases that had to be sacrificed, due to nonclonal B-cell lymphoproliferation or scratch wounds (Fig. 4B). Particularly, the latter may represent the manifestation of an exacerbated autoimmune phenotype. To determine whether lesions histologically classified as lymphoma truly represent clonal expansions, we next performed RNA-based BCR sequencing (adapted from ref. 42). Although the BCR repertoire in the spleens of WT animals was highly polyclonal, as expected, MBC lesions showed a strongly reduced variation, with the majority of cases being dominated by a single or only a few parallel clones (Fig. 4D and E). Perhaps not surprisingly, the BCR sequence of isolated stable cell lines corresponded to a dominant clone found in the primary lesion (Fig. 4D).

Cell lines derived from MBC lymphomas could be established in vitro and were transplantable into Rag1−/− recipient animals. Upon transplantation, these MBC-derived cell lines formed lymphomas that were indistinguishable from the original lymphomas with respect to morphology, immune phenotype, and proliferation index (Fig. 4F).

To further benchmark our mouse model against human ABC-DLBCL lymphomas, we next used 3′-RNA sequencing (RNA-seq) to assess the transcriptomes derived from MBC lymphomas. Lymphomas derived from Kmo2d−/−VavP-Bcl2; IgklcExi/WT (KBC) mice, which develop follicular lymphoma and GCB-DLBCL-like disease, served as a reference control (43). To specifically ask whether the transcriptome data derived from MBC and KBC lymphomas cocluster with transcriptome data derived from human GCB- or ABC-DLBCL, we performed a gene set enrichment analysis (GSEA) for gene signatures that have previously been shown to effectively distinguish human GCB- from ABC-DLBCL (11) and found a significant enrichment of the GCB and ABC signatures in KBC and MBC tumors, respectively (Fig. 4G). In addition to KBC lesions, we also benchmarked our MBC tumors against the MYC/Pi3K-driven R26G;LSL/Myc/LSL.P110δ; IgklcExi/WT (MPC) Burkitt lymphoma model (44), which constitutes an additional germinal center–derived aggressive lymphoma model (Supplementary Fig. S3F). We observed that MPC lymphomas displayed a significant enrichment of GCB gene expression signatures, whereas MBC lymphomas were enriched, albeit not significantly in this analysis, for ABC signatures (Supplementary Fig. S3F).

Next to transcriptome profiling, we also performed whole-exome sequencing of 17 MBC cases to ask whether the pattern of spontaneous mutations in these lymphomas also resembles the genomic aberrations detected in human DLBCL cases. As benchmarks, we employed two recently

Figure 4. MBC animals develop ABC-DLBCL–like tumors. A, Survival curves of WT (n = 10), MC (n = 104; median 101.9 weeks), BC (n = 74; median 68.7 weeks), and MBC animals (n = 107, median 42.3 weeks). B, Quantification of the terminal phenotype of MC (n = 15), BC (n = 12) and MBC animals (n = 30). C, Exemplary illustration of hematoxylin and eosin (H/E), B220, CD138, and Ki67 stainings of MC, BC, and MBC tumors. D, BCR sequencing–based clonality analysis of WT spleens and two MBC primary tumors (“M552 tumor” and “M108 tumor”) and derived cell lines. Each circle represents a unique BCR sequence, with the circle area representing the clone size. Clones differing in one base are connected by lines to clusters. Clusters consisting of an exacerbated autoimmunity phenotype. To determine whether lesions histologically classified as lymphoma truly represent clonal expansions, we next performed RNA-based BCR sequencing (adapted from ref. 42). Although the BCR repertoire in the spleens of WT animals was highly polyclonal, as expected, MBC lesions showed a strongly reduced variation, with the majority of cases being dominated by a single or only a few parallel clones (Fig. 4D and E). Perhaps not surprisingly, the BCR sequence of isolated stable cell lines corresponded to a dominant clone found in the primary lesion (Fig. 4D).

Cell lines derived from MBC lymphomas could be established in vitro and were transplantable into Rag1−/− recipient animals. Upon transplantation, these MBC-derived cell lines formed lymphomas that were indistinguishable from the original lymphomas with respect to morphology, immune phenotype, and proliferation index (Fig. 4F).

To further benchmark our mouse model against human ABC-DLBCL lymphomas, we next used 3′-RNA sequencing (RNA-seq) to assess the transcriptomes derived from MBC lymphomas. Lymphomas derived from Kmo2d−/−VavP-Bcl2; IgklcExi/WT (KBC) mice, which develop follicular lymphoma and GCB-DLBCL–like disease, served as a reference control (43). To specifically ask whether the transcriptome data derived from MBC and KBC lymphomas cocluster with transcriptome data derived from human GCB- or ABC-DLBCL, we performed a gene set enrichment analysis (GSEA) for gene signatures that have previously been shown to effectively distinguish human GCB- from ABC-DLBCL (11) and found a significant enrichment of the GCB and ABC signatures in KBC and MBC tumors, respectively (Fig. 4G). In addition to KBC lesions, we also benchmarked our MBC tumors against the MYC/Pi3K-driven R26G;LSL/Myc/LSL.P110δ; IgklcExi/WT (MPC) Burkitt lymphoma model (44), which constitutes an additional germinal center–derived aggressive lymphoma model (Supplementary Fig. S3F). We observed that MPC lymphomas displayed a significant enrichment of GCB gene expression signatures, whereas MBC lymphomas were enriched, albeit not significantly in this analysis, for ABC signatures (Supplementary Fig. S3F).

Next to transcriptome profiling, we also performed whole-exome sequencing of 17 MBC cases to ask whether the pattern of spontaneous mutations in these lymphomas also resembles the genomic aberrations detected in human DLBCL cases. As benchmarks, we employed two recently
Vulnerabilities in a Myd88/BCL2-Driven DLBCL Mouse Model

A

Percent survival

Time (weeks)

B

Lymphoma

Nonmalignant

Reactive

Scratch wounds

MC

n = 15

BC

n = 12

MBC

n = 30

C

D

WT 1 spleen

WT 2 spleen

M552 tumor

C1: 20.2%

C2: 44.0%

C3: 13.1%

C4: 12.0%

C5: 11.1%

M552 cell line

C1: 99.4%

M108 tumor

C1: 89.1%

M108 cell line

C1: 99.9%

E

F

G

H

I

Enrichment score

Rank

Cutaneous squamous cell carcinoma

Myeloid leukemia

Non-Hodgkin lymphoma

Primary liver cancer

Squamous cell carcinoma

Thyroid cancer

Uveal melanoma

Vulvar squamous cell carcinoma

Reedy ABC Up  P = 0.001

Reedy GCB Up  P = 0.003

Schmitz et al. (% mutated)

Chapuy et al. (% mutated)

n = 12

n = 15

n = 30

Percent survival

B220+/CD138−

B220−/CD138+

Largest clone (% of reads)

aut. aut. transp. transp.

M108 M108 M552 M552

Enrichment score

Rank

Schmitz et al. (% mutated)

Chapuy et al. (% mutated)

MBC

WT

AACR

American Association for Cancer Research

JANUARY 2021  BLOOD CANCER DISCOVERY  |  79

Downloaded from https://bloodcancerdiscov.aacrjournals.org by guest on September 19, 2021. Copyright 2020 American Association for Cancer Research.
published datasets reporting the mutational profiles of 878 human DBLCL cases (9, 10). We detected a number of mutations in our MBC lymphomas, including abruptions in Pim1, Myc, Kmt2d, Nfkbia, Stat3, Pou2f2, and Hist1h1e (Fig. 4H; Supplementary Table S1), which were also frequently mutated in both human DBLCL cases (9, 11).

We also assessed the serum abundance of 32 distinct cytokines in lymphoma-bearing MBC mice and age-matched WT controls using multiplexed cytokine arrays. We observed significantly higher levels of IFNγ and TNFα in sera of lymphoma-bearing MBC mice, compared with controls (Fig. 4; Supplementary Fig. S4A). In line with these observations, increased TNFα and IL10 plasma levels were recently shown to correlate with poor prognosis in DBLCL (45). Moreover, the TNFα single-nucleotide polymorphism 308G→A was shown to be associated with increased constitutive and inducible TNFα expression and increased risk of DBLCL development (46–49). Furthermore, TNFα was shown to induce an inhibitory gene expression signature in CD4+ T cells during chronic viral infection, indicating that TNFα may affect the ratio of CD4+:CD8+ T cells (50). Conversely, IFNγ was shown to act on CD8+ T cells to enhance their abundance, mobility, and cytotoxicity during viral infection and experimental graft rejection (51, 52). Thus, our cytokine profiling data may indicate that lymphoma-bearing MBC mice display signs of a proinflammatory environment favoring the activity of CD8+ T cells.

To further gauge whether cytokines were produced by the lymphoma cells themselves or rather the nonmalignant components of the lymphoma microenvironment, we compared gene expression profiles of bulk primary MBC lymphoma samples and stable cell lines derived from MBC lymphomas. The relative expression levels of Ccl11, Il1a, Il6, Il12b, Cxcl1, Cxcl5, Ccl2, Cif1, Cif9, Ces1, and Ifng were significantly lower in the three analyzed cell lines compared with the primary lymphoma material, suggesting that these cytokines are, at least partially, synthesized by nonmalignant cells within the microenvironment. In contrast, we found that several cytokines, including Il2, Il10, and Il15 (out of which particularly IL2 and IL15 were previously shown to promote B-cell proliferation; refs. 53, 54), were expressed at high levels by the lymphoma cell lines. Moreover, Tnf was expressed at high levels by two of the three cell lines (Supplementary Fig. S4B). However, it is important to note that mRNA expression levels do not always correlate with protein expression (55). Thus, the data presented here have to be interpreted with care.

**MBC-Derived Lymphomas Display an Actionable BCL2 Inhibitor Sensitivity**

Our data indicate that the MBC model accurately mimics human ABC-DBLCL biology with regard to morphology, as well as genomic and transcriptomic profiles. Thus, we next aimed to employ this model as a preclinical tool. To this end, we initially performed cellular viability assays to determine drug sensitivity. We specifically assessed the sensitivity of four distinct MBC-derived lymphoma cell lines (M191, M190, M108, and M552), as well as two human ABC-DBLCL (U2932 and R11) and two human GCB-DBLCL cell lines (SUDHL10 and OCILY7) toward the IRAK4 inhibitor ND-2158, the IKK2 inhibitor LY-2409881, and the BCL2 inhibitor venetoclax. These experiments revealed that murine MBC-derived cells, as well as the human ABC-DBLCL cell lines included in our screen were sensitive against IKK2, IRAK4, and BCL2 inhibition (Fig. 5A–C). Reminiscent of previously published data (56, 57), we found the two GCB cell lines investigated here to be resistant against BCL2 blockade (Fig. 5A). However, it is important to note that while several human GCB-DBLCL cell lines, such as Hf, Rc, Mea, and OCI-LY19 cells, have been reported to be highly sensitive to BCL2 inhibition (58). Thus, our observations, which are limited to two human GCB cell lines, should not be generalized to all GCB-DBLCL cases. Of note, BH3 profiling (59) confirmed that human ABC-DBLCL cell lines and murine MBC-derived cell lines were BCL2 dependent, whereas the limited number of human GCB-DBLCL cell lines displayed a more prominent sensitivity against MCL1 inhibition (Fig. 5D).

As cell viability assays do not distinguish between cell death and growth arrest, we next performed flow cytometry-based apoptosis measurements, using Annexin V/propidium iodide (PI) costaining. Venetoclax and LY-2409881 effectively induced apoptosis in the MBC lines (Fig. 5E and F). Moreover, venetoclax induced massive apoptosis in both human ABC-DBLCL cell lines, whereas LY-2409881–induced apoptosis was prominent in R11 and relatively mild in U2932 ABC-DBLCL cells (Fig. 5E and F). ND-2158 treatment did not lead to a significant induction of apoptosis in any of the investigated cell lines. Altogether, these data indicate that venetoclax, ND-2158, and LY-2409881 display therapeutic efficacy in the human and murine ABC-DBLCL cell line models investigated in this study. However, only venetoclax and LY-2409881 appear to induce apoptosis, whereas ND-2158 likely reduces proliferation (Fig. 5G; Supplementary Fig. S5A).

Of note, in an extension of this focused candidate approach to discover potentially actionable aberrations in GCB and ABC cell lines, we also conducted a larger discovery screen on human GCB (OCILY7, SUDHL10) and ABC cell lines (R11, U2932), as well as three MBC-derived murine lymphoma cell lines. We specifically determined the IC50 values of 167 additional distinct drug compounds (Supplementary Fig. S5B; Supplementary Table S2). Fitting with our transcriptome data, R11 and U2932 cells clustered with the MBC cell lines.
To ask whether the therapeutic efficacy of venetoclax, ND-2158, and LY-2409881 was reproducible in vivo, we next performed preclinical drug sensitivity studies in a transplant model. To this end, we transplanted 10^7 murine M108 cells i.p. into Rag1^-/- recipient animals. Treatment was initiated 14 days after transplantation. Of note, in a series of preparatory experiments, we had verified that 4 of 4 transplanted animals developed necropsy-verified clonal lymphoma 14 days following transplantation. Animals were treated with venetoclax (200 mg/kg, daily, orally), ND-2158 (150 mg/kg, daily, i.p.), or LY-2409881 (100 mg/kg, daily, i.p.) or were left untreated. As shown in Kaplan-Meier format in Fig. 5H, venetoclax induced a significantly increased overall survival (50.0 ± 4.5 days after transplantation following completion of a 3-week treatment course) compared with vehicle control (32.5 ± 5.9 days). Somewhat surprisingly, ND-2158 did not produce a significant survival gain in these experiments. All ND-2158-treated animals succumbed to lymphoma, indicating a lack of in vivo activity of this compound at the chosen dose in our model. Similarly, LY-2409881 did not lead to significant survival gains in this model. We note, however, that LY-2409881–treated animals uniformly reached predefined termination criteria (weight loss >10%, hunched posture, ragged fur, apathy). Importantly, we did not detect lymphoma in any of the LY-2409881–treated animals at the time of sacrifice, indicating that LY-2409881 may display preclinical activity, which is masked by life-limiting toxicity. This proinflammatory adverse effect of LY-2409881 is in line with previously reported toxicities of IKK inhibitors (60). Collectively, our preclinical data indicate that venetoclax may display activity against a subset of BCL2-altered ABC-DLBCL lymphomas, whereas our data suggest that ND-2158 and LY-2409881 may not be candidates for further development in this entity due to lack of preclinical efficacy or toxicity.

Human ABC-DLBCL and Murine MBC-Derived Lymphomas Display an Actionable PD-L1 Expression

Immune checkpoint blockade is emerging as a potential route for therapeutic intervention in relapsed/refractory DLBCL (61). For instance, the anti–PD-1 monoclonal antibody nivolumab achieved an overall response rate of 36% in patients with DLBCL in a phase I, open-label, dose-escalation, cohort-expansion study (62). Moreover, NF-kB has been shown to be a potent inducer of PD-L1 expression (63). As increased NF-kB activity is a hallmark of ABC-DLBCL, we next assessed the CD274 mRNA (encoding for PD-L1) expression levels in a publicly available dataset comprised of 775 (while 1,001 samples were exome sequenced in this study, only 775 were RNA sequenced) human DLBCL cases (11). This analysis revealed that CD274 and PDCD1 (encoding for PD-1) were significantly overexpressed in ABC-compared with GCB-DLBCL, whereas CD86 was expressed at significantly higher levels in GCB-compared with ABC-DLBCL (Fig. 6A). No entity-specific differences were observed in the expression levels of PDCD1LG2 (encoding for PD-L2) and CTLA4 (Fig. 6A). These data are in line with recently reported analyses, which demonstrate that cytotoxic effector lymphocytes and CD8+ T cells are proportionally more present in ABC-DLBCL samples compared with GCB-DLBCL. Moreover, we found that 27% of ABC-DLBCL cases display a higher than average combined expression of CD274 and CTLA4, and that of these cases, 71% are of the ABC subtype (Supplementary Fig. S6A). In contrast, only 29% of cases with a lower than average expression of both CD274 and CTLA4 were ABC tumors. Building on the observation that human ABC-DLBCL cases frequently coexpressed CD274 and CTLA4, we next asked whether combined BCL2 and PD-1 inhibition may display synergistic cytotoxic activity in this model. For that purpose, we generated a cohort of MBC animals in which lymphoma development was surveilled by longitudinal MRI scans. Upon lymphoma detection, defined by a lesion larger than 75 μL with detectable volume increase in two consecutive

Figure 6. α-PD-1 treatment is an effective strategy for MBC tumors. A, Expression levels of the indicated genes were compared between ABC (n = 310) and GCB (n = 228) DLBCL cases in a previously published RNA-seq dataset (11). B, Thirty-eight human DLBCL samples were categorized into the ABC and GCB subtypes employing the Hans algorithm (101), and PD-L1 was stained immunohistochemically. Grades 0 and 1 were classified as negative/low expression and 2 to 5 as medium/high expression. C, Expression of PD-L1 in primary central nervous system lymphoma samples was analyzed by IHC. MBC animals were monitored for lymphoma development by MRI, and upon tumor detection, treatment was initiated with either ABT-199 (200 mg/kg daily by oral gavage for 3 weeks), α-PD-1 antibody (250 μg twice weekly for 8 weeks), or a combination of both. Exemplary MRI scans 3 weeks after treatment initiation illustrated in D and E show best tumor volume change within 8 weeks of untreated (n = 4). ABT-199–treated (n = 6), α-PD-1–treated (n = 5), or combination-treated (n = 7) MBC. F, Survival after tumor detection of untreated (n = 4), ABT-199–treated (n = 6), α-PD-1–treated (n = 5), or combination-treated (n = 7) MBC animals. G, Timescale of sample collection from α-PD-1–treated MBC tumors. H, Mass cytometric analysis of untreated and α-PD-1–treated tumors. Cells were gated for CD45^+DNA^- (not illustrated) and CD3^+CD4^- as well as CD3^+CD8^- events were selected for further analysis. The adjusted P value and log fold change between α-PD-1 (n = 4) and untreated samples (n = 14) for each marker (individually for the CD4^+ and CD8^+ populations) are depicted in I, and significant markers are highlighted. J, CD3^+CD4^+ and CD3^+CD8^+ population sizes are given as percentages of the DNA^+CD45^- population. K, Differentially expressed markers in the CD3^+CD4^- and CD3^+CD8^- populations. *, P ≤ 0.05; **, P ≤ 0.01; and ***, P ≤ 0.001. A, I, and K, Welch unpaired two-tailed t test; B, Fisher exact test; F, Log-rank test. Scale bars, 50 μm.
scans, animals were randomized in a 1:1:1:1 fashion to receive either vehicle solution (control), venetoclax (200 mg/kg, orally, daily, days 1–21), the anti-PD-1 antibody RMP1-14 (10 mg/kg, i.p., twice weekly, until death), or combined venetoclax plus RMP1-14. Therapy response was longitudinally assessed through weekly MRI scans, which enabled us to gauge depth of remission and duration of response (Fig. 6D and E; Supplementary Fig. S6B). Although both single-agent RMP1-14 and venetoclax produced objective responses in the MBC model (Fig. 6E), these responses were not durable (Supplementary Fig. S6B). The median survival of RMP1-14– or venetoclax-treated mice was 11.5 and 10.1 weeks, respectively, compared with 5.1 weeks in untreated animals. Although the survival difference between untreated and venetoclax-treated mice failed to reach statistical significance, RMP1-14 induced a significant survival gain, compared with untreated controls (Fig. 6F).

The significant single-agent activity of RMP1-14 prompted us to further explore the effects of PD-1 blockade on the composition and activation status of the cells within the lymphoma microenvironment. For that purpose, we employed mass cytometry from lymphoma tissue derived from RMP1-14–treated (250 μg/doe, days 1, 4, and 7, i.p.) or untreated animals (Fig. 6G–K). Our analysis revealed that PD-1 blockade in MBC lymphoma–bearing animals induced distinct phenotypic changes in lymphoma-infiltrating CD4+ and CD8+ T cells, which are in line with reversal of an exhausted phenotype of lymphoma-infiltrating T cells upon treatment (Fig. 6H and I). Although the CD4+ and CD8+ T-cell population sizes did not show significant changes (Fig. 6J), we particularly detected a significant reduction in IRF4 and TIM3 expression in both CD4+ and CD8+ T cells upon RMP1-14 exposure (Fig. 6I and K). Moreover, CD8+ T cells displayed significantly reduced 4-1BB and LAG3 expression following PD-1 blockade. Further, CD4+ T cells displayed reduced CD69 expression upon RMP1-14 exposure (Fig. 6I and K). These data are in line with a reinstated antilymphoma phenotype of lymphoma-infiltrating T cells (72, 73). Lastly, the p53 target gene Cdkn1a (p21) is a potent CDK inhibitor involved in p53-mediated cell-cycle arrest (74). Reduced expression of Cdkn1a in CD4+ T cells following PD-1 blockade thus might be in line with restored proliferation potential. Altogether, these data suggest that PD-1 blockade in lymphoma-bearing MBC mice promotes a phenotypic switch away from an exhausted CD4+ and CD8+ T-cell state in treatment-naive lymphomas. We note that a parallel assessment of serum cytokine levels (n = 32 distinct cytokines) in RMP1-14– and vehicle-treated animals did not reveal any significant differences (Supplementary Fig. S7).

In addition to our assessment of single-agent activities, we also analyzed the effect of combined venetoclax plus RMP1-14, which resulted in a significant overall survival gain (median overall survival 20 weeks) compared with untreated animals or mice exposed to the single agents (Fig. 6D–F; Supplementary Fig. S6B). Altogether, these functional in vivo experiments indicate that combined BCL2 and PD-1 blockade may represent a viable treatment strategy for a molecularly defined subset of ABC-DLBCL cases.

**DISCUSSION**

Here, we characterized a mouse model of Myd88- and BCL2-driven DLBCL. In essence, we show that Myd88 p.L252P and BCL2 cooperate in DLBCL lymphomagenesis. The resulting lymphomas display gene expression profiles that are strikingly similar to human ABC-DLBCL (Fig. 4G; Supplementary Fig. S3F). Moreover, in addition to the engineered aberrations in Myd88 and BCL2, these lymphomas spontaneously acquire single-nucleotide variants that are also detectable in human DLBCL, including mutations in Pim1, Myc, POU2F2, Nfkb1, and KMT2D (Fig. 4H; Supplementary Table S1).

We also assessed the effects of Myd88 p.L252P expression in nontransformed B cells. We specifically analyzed spontaneous, MyD88-centered protein complex formation in naïve B cells and found significantly more complexes involving MYD88 together with IRAK1, IRAK4, IgM, and BTK compared with WT controls (Fig. 3). These ex vivo experiments suggest that MYD88p.L252P constitutively nucleates a signaling complex, physically linking BCR and TLR signaling molecules in nontransformed B cells. These data are in line with the recently reported presence of the so-called My-T–BCR complex in ABC-DLBCL lymphoma cell lines (40). Moreover, these data are supported by a recent report indicating that BTK localizes in a protein complex with MYD88 in p.L265P-expressing OCI-Ly3 DLBCL cells (75).

Further investigation into the impact of Myd88 p.L252P expression in nontransformed B cells revealed the presence of autoreactive antibodies in MC, BC, and MBC animals (Fig. 2A–D). Particularly the robust detection of autoreactive antibodies in lymphoma-prone MBC mice (Fig. 2A–D) is intriguing, as this might suggest a role for autoreactivity in promoting DLBCL lymphomagenesis. A role for BCR stimulation in lymphomagenesis has long been postulated (77). For instance, there is ample evidence indicating that chronic infections, such as hepatitis C (HCV) or Helicobacter pylori, are associated with the development of splenic marginal zone lymphoma (SMZL) and mucosa-associated lymphoid tissue lymphoma, respectively (78). Moreover, in...
HCV-associated SMZL, a single BCR specific to a glycoprotein in the viral envelope has been identified, strongly suggesting that HCV itself contributes to driving SMZL BCR signaling (78). Next to infection-driven BCR signaling, our detection of autoimmune antibodies in MBC animals is in line with the hypothesis that the constant engagement of the BCR by a self-antigen might account for the sustained nature of chronic active BCR signaling (79). In fact, BCR stimulation by self-antigens has been correlated with lymphomagenesis. Epidemiologic analyses revealed associations between B-cell–activating autoimmune diseases, such as Sjögren’s syndrome and systemic lupus erythematosus, with increased DLBCL risk after controlling for all other risk factors (80). Furthermore, analyses of V_{H} gene segment in DLBCL revealed that segment V_{H}4-34 is utilized in approximately 30% of ABC-DLBCL cases (79). Experiments in cell line models of ABC-DLBCL with chronic active BCR signaling provided further evidence for autoreactivity of the V_{H}4-34 segment. The viability of the V_{H}4-34–positive ABC cell line HBL1 depended on the V region–mediated ability of its BCR to bind to self-glycoproteins on its own cell surface (79).

Moreover, chronic active BCR signaling in the ABC-DLBCL cell line OCI-Ly10 is driven by BCR recognition of an antigen in the debris of apoptotic cells (79). OCI-Ly10 was shown to be dependent on BCR specificity mediated by charged amino acids within CDR2 and CDR3 of the V_{H}3-7 region (79). Lastly, the survival of the ABC-DLBCL cell line TMD8 was shown to depend on homotypic interactions with its own FR2 domain, which sustained chronic active BCR signaling (79). These data indicate that a diverse array of self-antigens is responsible for maintaining the survival of ABC-DLBCL cells. Building on these data, it is intriguing to speculate that in our MBC model, the enlarged pool of B cells that are activated by self-antigen and in which anergy is repressed by MYD88-driven TLR signaling might increase the pool of cells that are prone for malignant transformation.

We also employed our MBC model as a preclinical tool that mimics central features of ABC-DLBCL (surface marker profile, expression profile, driver mutations, and spontaneously developing mutational profile). Particularly the analysis of murine and human tissue specimens and transcription profiles revealed that ABC-DLBCL cases display higher CD274 levels than GCB-DLBCL cases. These observations are in line with a previous report in which the authors demonstrated an increased CD274 expression in the murine Cyp{γ CW/WT}Ikk2CAGFP^{LSL/LSL};Prdm1{fl/fl} and Cyp{γ CW/WT}Ikk2CAGFP^{LSL/LSL}; Prdm1^f/f_f/fl–derived lymphoma cells, compared with Cyp{γ CW/WT};YFts^{LSL/LSL}GCB cells (81). There is further substantial evidence that mechanistically supports the high PD-L1 expression levels in our MBC model: IFNγ and Myd88-dependent TLR signaling was recently shown to drive PD-L1 expression in multiple myeloma cells (82). Intriguingly, we found significantly higher IFNγ levels in the serum of MBC mice compared with WT serum (Fig. 4I). Moreover, an in silico analysis revealed that a sizeable fraction of human DLBCL cases (22%) harbor high BCL2 and CD274 expression levels. To particularly target this population, we assessed the preclinical efficacy of combined BCL2 and PD-1 blockade. Although both single agents displayed only mild activity in the MBC model, combined venetoclax and RMP1-14 led to significantly increased tumor volume control and overall survival benefit compared with the single agents or vehicle. These results are in line with previous reports demonstrating that the anti–PD-1 antibody RMP1-14 as a single agent did not lead to a significant overall survival benefit in Cyp{γ CW/WT};Ikk2CAGFP^{LSL/LSL};Prdm1^f/f_fl–/fl lymphoma–bearing mice compared with untreated controls, whereas combined anti–PD-1/anti-CD20 blockade synergistically increased the overall survival in these animals (81).

Our observations provide further evidence for the clinical development of BCL2 and PD-1/PD-L1 inhibitors in the clinical arena. In this context, it is important to reiterate that single-agent venetoclax was recently shown to achieve an overall response rate (ORR) of 18% in relapsed/refractory DLBCL (65). Similarly, single-agent nivolumab achieved an ORR of 36% in DLBCL (62). Our data now indicate that patients with DLBCL displaying high-level PD-L1 and BCL2 exist and that these patients may be particularly well suited to receive combined BCL2 and PD-1 blockade. This strategy may be particularly useful in relapsed/refractory ABC-DLBCL, or those patients who are not eligible for intensive consolidation regimens, involving autologous stem cell support. In our experiments, venetoclax was used at 200 mg/kg daily, which is a dose that is typically used for in vivo experiments in mice (83, 84). Although venetoclax at 200 mg/kg daily did not induce any obvious toxicity in our experiments, it is important to note that this dose exceeds the doses that are clinically applied to human patients (typically 5–20 mg/kg, depending on venetoclax dosing regimen and body weight). Altogether, we provide a detailed molecular analysis of the MBC model, including the comparison with Kmt2d/BCL2-driven lymphomas and a large series of human DLBCL cases. These experiments indicate that the MBC model reflects key aspects of human ABC-DLBCL. Moreover, we employ the MBC model to derive a combination strategy involving PD-1 and BCL2 blockade for the treatment of MYD88- and BCL2-altered aggressive lymphomas.

**METHODS**

**Experimental Mice**

The generation of the Cyp{γ CW}, Myd88^{+/−};Ikk2CAGFP, and Rosa26^{LSL/LSL};CRE-GFP alleles has been described previously (20, 85). For survival analyses, animals that succumbed to disease or had to be killed due to satisfied termination criteria were recorded as events. Animals that died from genotype-unrelated criteria in rare cases (appendicitis, abnormal teeth, injuries inflicted by cage mates) were censored. For transplantation experiments, 10^7 cells were transplanted i.p. into Rag1^{−/−} recipients. In autochthonous treatment studies, onset of lymphoma was defined by a lesion larger than 75 μL detectable by MRI, with a detectable volume increase in two consecutive scans. In transplantation studies, onset of clonal lymphoma was determined in a preceding experiment. ABT-199 was administered as a suspension in 60% Phosal 50PG, 30% polyethylene glycol 400, and 10% ethanol by oral gavage at 200 mg/kg daily. ND-2158 was dissolved in 10% β-cyclodextrin at 15 mg/mL and administered i.p. at 150 mg/kg. The anti–PD-1 antibody RMP1-14 (BioXcell) was administered i.p. 3 days per week (250 μg/admistration).

All animals were housed in a specific pathogen-free facility, and animal breedings and experiments were approved by the local animal care committee and the relevant authorities (Landesamt für...

MR Imaging

MR imaging was performed as described previously (20). In brief, mice were anesthetized with 2.5% isoflurane and scanned on a 3.0T MRI system (Igeia, Philips) with a small rodent solenoid coil (diameter 40 mm, Philips Research Europe). Axial T2-weighted images of the abdomen were acquired (TSE factor: 10, TR: 2674 ms, TE: 65 ms, slice thickness: 1.0 mm). Images were exported in Dicom format, and spleen and tumor volumes were measured by segmentation using the Horos software.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues from mice were cut into 4 μm sections and stained for BCL6 (Santa Cruz Biotechnology, clone C-19), B220 (BD, clone RA3-6B2), CD138 (BD, Cat. No. 553712), Ki67 (Cell Marque), CD3 (Thermo Fisher, clone RM-9107), CD4 (Abcam, Cat. No. ab183685), CD8 (Abcam, Cat. No. ab203035), and biotinylated PNA (Vector Laboratories, B-1075-5). Germinal center positivity, clone C-19), B220 (BD, clone RA3-6B2), CD138 (BD, Cat. No. 553712), Ki67 (Cell Marque), CD3 (Thermo Fisher, clone RM-9107), CD4 (Abcam, Cat. No. ab183685), CD8 (Abcam, Cat. No. ab203035), and biotinylated PNA (Vector Laboratories, B-1075-5). Germinal centers were quantified from BCL6 and PNA stainings using the software ImageJ. Ki67 and CD3 positivity was quantified using the ImmunoRatio plugin for ImageJ (86).

Human samples were cut into 4 μm sections and stained for CD10 (NCL-L-CD10-279, Novocastra), IRF4 (Dako, Cat. No. M7259), BCL6 (Dako, Cat. No. M7211), BCL2 (Dako, Cat. No. M0877), PD-L1 (Dako; clone 28-8), CD3 (Bio-Rad, clone 145-2C11), CD4 (Abcam, clone EPR19514), and CD8 (Abcam, Cat. No. ab203035). Human PD-L1 stainings were graded according to the Cologne Score (87).

Flow Cytometry

For immunotypisation, splenocytes were stained with fluorophore-coupled primary antibodies against CD23 PE, (Ebioscience, clone B3B4), CD93 (PE-Cy7, Biologend, clone AA4.1), CD21/35 (APC-Cy7, BD, clone M5-1142), MHCII (AF700, Biologend, clone 7G6), CD45 (APC-Cy7, BD, clone 30-F11), B220 (Pacific-Blue, BD, clone RA3-6B2), CD138 (PE-Cy7, Biolegend, clone 7G6), CD45 (APC-Cy7, BD, clone 30-F11), CD4 (Abcam, clone EPR19514), and CD8 (Abcam, Cat. No. ab203035). Human MHCII, CD3, and CD4 positivity was quantified using the ImmunoRatio plugin for ImageJ (86).

Human samples were cut into 4 μm sections and stained for CD10 (NCL-L-CD10-279, Novocastra), IRF4 (Dako, Cat. No. M7259), BCL6 (Dako, Cat. No. M7211), BCL2 (Dako, Cat. No. M0877), PD-L1 (Dako; clone 28-8), CD3 (Bio-Rad, clone 145-2C11), CD4 (Abcam, clone EPR19514), and CD8 (Abcam, Cat. No. ab203035). Human PD-L1 stainings were graded according to the Cologne Score (87).

Clonality Analysis

RNA was isolated from cryopreserved tumor tissue using a commercial kit (Qiagen, Cat. No. 74104), and clonality analysis was performed using an adaption of a published BCR clonality analysis approach (42). The cDNA was then synthesized using a reverse transcriptase generating poly-dC overlaps (SMARTScribe, Takara, Cat. No. 639537), allowing for template switching and adapter ligation, which contains a unique barcode (UMI). For first-strand synthesis, primers specific for the constant regions of Igk, Igm, and Igh were used. Two rounds of nested PCR were performed, resulting in an amplification product containing the V(D)J junctions, which was then sequenced. Purified PCR amplicons were end-repaired, A-tailed, and adapter ligated with unique dual indices using the Illumina TruSeq nano kit and protocol but without further PCR amplification. After validation (2200 TapeStation; Agilent Technologies) and quantification (Qubit System; Invitrogen), amplicon libraries were individually quantified using the KAPA Library Quantification kit (Peqlab) and the 7900HT Sequence Detection System (Applied Biosystems) and subsequently spiked-in in larger pools of libraries. The pools were sequenced on an Illumina NovaSeq6000 sequencing instrument using a paired-end 2 x 100 bp protocol. The samples were then error corrected using the published MIGEC algorithm (42). UMI clusters present with less than three reads were discarded. To quantify clone sizes, we first assessed the abundance of individual UMI clusters. Clones differing by one base were connected to clusters, and the size of these clusters was quantified as the sum of the individual clone sizes. BCR

Immunization Experiments

Animals were injected i.p. with 50 μg of either NP-Ficol (Biocat, Cat. No. F-1420-10-BS) dissolved in 200 μL PBS or NP-CCG (Biocat, Cat. No. N-5055E-1-BS) dissolved in 100 μL PBS+10 μL Imject Alum (Thermo Scientific, Cat. No. 77161). Note that 30 μL of blood was drawn from the tail vein at the indicated days. Antibody levels against NP in the collected sera were measured by ELISA. High-protein-binding plates were coated with NP-BSA (5 μg/mL) overnight. Plates were washed with 0.05% PBS-T and blocked with 1% BSA. The samples were prediluted with 1% BSA in PBS at 1:10,000 or higher and incubated on the plate for 2 hours at room temperature. Plates were washed with PBS-T and secondary antibody against murine IgG (Antibodies-online, Cat. No. ABIN376241) or IgM (Novus, Cat. No. NB7497). After 20 minutes, the reaction was stopped by 1 mol/L phosphoric acid, and the plates were read on a plate reader (Tecan).

Immunoblotting

Single-cell suspensions were generated by pressing spleens through a cell strainer (70 μm), and naïve B cells were purified using a CD43-depletion kit (Miltenyi, 130-049-801). Cells were lysed in 4% SDS containing phosphatase and protease inhibitors (Merek, 4906845001 and 05892970001). Protein concentration was measured by BCA assay (Thermo Fisher, 23225), and concentration was adjusted to 800 μg/mL before the addition of Laemmli buffer. Note that 20 μL per sample were loaded onto 10% polyacrylamide gels and blotted onto a PVDF membrane. Membranes were blocked (5% BSA in TBST) and stained overnight with primary antibody (pp65, Cell Signaling Technology, 3033; pIRAK4, Abnova, MAB2538; pBTK, Cell Signaling Technology, 50822; GAPDH, Cell Signaling Technology, 5174). Membranes were washed and incubated with horseradish peroxidase-coupled secondary antibody for 1 hour at room temperature. After washing, membranes were incubated with ECL solution (Amersham) and imaged on a ChemiDoc (Bio-Rad). Densitometric analysis was performed using ImageJ.

Clonality Analysis

Serum Gel Electrophoresis

Serum protein electrophoresis was performed with HYDRAGEL IF 2/4 agarose gels (Sebia) on the HYDRASYS electrophoresis system (Sebia). Therefore, 4 μL serum was diluted 8 μL HYDRAGEL IF diluted (Sebia), electrophoretically separated, and stained with acid violet according to the manufacturer’s instructions.

Hep-2 Assay

Serum was diluted 1:40 in PBS, and 30 μL per sample were added on a Kallestad Hep-2 slide (Bio-Rad, Cat. No. 26101), incubated for 20 minutes in a wet chamber, and washed with PBS for 10 minutes. The slides were then stained with Alexa Fluor 488-coupled secondary antibodies against either murine IgM (Thermo Fisher, Cat. No. A-21042) or IgG (Thermo Fisher, Cat. No. A-11001) for 20 minutes, washed, and covered. Fluorescence intensities were quantified from stainings produced in the same run by analyzing images generated with identical exposure times by ImageJ.
repetitorie were visualized using the software Gephi (https://gephi.org/). Primer sequences and barcode-sample mapping are given in Supplementary Table S3.

**Determination of SHM Frequency in CD138 Cells**

CD138-positive cells were selected using a magnetic antibody-based cell separation kit (Miltenyi, 130-098-257). RNA was isolated from these cells using a commercial kit (Qiagen). Full-length B-cell receptor sequencing was performed as published by Turchaninova and colleagues (21). Primer sequences and barcode-sample mapping are given in Supplementary Table S3. Note that 600 ng of RNA were used for cDNA synthesis, and a cDNA equivalent of 500 plasma cells was used for further PCR amplification steps. Amplicon sequencing was then performed on a MiSeq (asymmetric 400–100-nt paired-end sequencing), with eight samples on a full single lane. Processing of the sequencing data was performed according to the protocol using the MiGEAC software (21, 42). Mapping of the identified BCR sequences was done using the MiXCR (for isotype information) and MiGMAP (for determination of differing bases from germline sequence; ref. 88).

**3′-RNA-Seq**

RNA was isolated from cryopreserved tumor tissue using a commercial kit (Qiagen, Cat. No. 74104). Note that 3′mRNA libraries were generated from total RNA using the Lexogen QuantSeq kit according to the standard protocol. After validation (2200 TapeStation; Agilent Technologies) and quantification (Qubit System; Invitrogen), pools of cDNA libraries were generated. The pools were quantified using the KAPA Library Quantification Kit (Peqlab) and the 7900HT Sequence Detection System (Applied Biosystems) and subsequently sequenced on an Illumina NovaSeq6000 sequencing instrument using a paired-end 2 × 100 bp protocol. We aligned raw sequencing reads to the mouse reference genome (mm10) by using the BWA mem aligner (version 0.7.13-r1126). Concordant read pairs that represent possible PCR duplicates were masked out after alignment. Furthermore, all overlapping regions between the read pairs are considered only once in the analysis. Due to a lack of matched normals for all tumor specimens, we generated a representative nontumor sample by combining normals matching to two tumor samples. This combined normal is used for mutation calling with the latest version of our in-house cancer genome analysis pipeline (94). To correct for genotypes that are not captured by representative normal, we filtered out called mutations that were exactly the same in two or more tumor samples.

**Multiplex Cytokine Assay**

Mouse serum was collected by retro-orbital or tail-vein bleeding. Serum levels of cytokines and chemokines were determined using the Mouse 31-Plex Cytokine/Chemokine Array (Eve Technologies).

**Cell Lines**

Human cell lines were purchased from DSMZ. Cell line identity was verified by short tandem repeat (STR) analysis. Cell lines were cultivated in RPMI with 20% FCS and 1% penicillin/streptomycin. Murine cell lines were established from primary tumors and cultivated in DMSO containing 4.5 g/L glucose, with the following supplements: 10% FCS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, nonessential amino acids, 1% penicillin/streptomycin, and 50 μmol/L beta-mercaptoethanol. No Mycoplasma testing was performed.

**Cell Viability Assays**

Five thousand cells per well were treated with the indicated drugs and doses (dissolved in DMSO, DMSO concentrations were adjusted). After 96 hours of incubation, viability was measured by CellTitre-Glo assay (Promega, Cat. No. G7572) diluted 1:6 in PBS and the luminescence was measured (Infinite M1000 pro, Tecan). The values were normalized to untreated controls on each plate.

**High-Throughput Cell Line Screening**

High-throughput screening was performed on the mouse and human lymphomas cells, as described earlier with minor modifications (95–97). Briefly, the DMSO-dissolved compound library (MedChemExpress) was dispensed with increasing concentrations of the inhibitors in six dilution steps (0.008–25 μmol/L) on a white 1,536-well plate (Corning) using digital dispenser (D300e, Tecan), which ensures precise and robotic compound application in randomized fashion. The compound selection consisted of FDA/European Medicines Agency–approved classical chemotherapeutic drugs and targeted agents, as well as molecules that are currently in clinical trials at various stages, and several investigational compounds (see Supplementary Table S2 for the detailed list). The printed plates were sealed with parafilm, followed by packing in vacuum-sealed bags to avoid evaporation during storage at ~80°C. The cells (~90% viability) were seeded on the thawed predispensed inhibitor plates using an automated Multidrop Combi.
Reagent Dispenser (Thermo Fisher Scientific). Differential responses were monitored with ATP-dependent CellTiter-Glo Luminescent cell viability kit (Promega) after 72 hours of inhibitor exposure using Microplate reader (Spark 10M, Tecan). The outer three wells of the plate were excluded from analysis to circumvent the evaporation effect on the plate edges. Dose–response curves were fitted by non-linear regression [log(inhibitor)] vs. normalized response] variable slope function (GraphPad Prism Inc.). Average drug response values (n = 3) normalized to the mean IC50 for each individual compound over all lines were plotted for the Heatmap visualization, followed by unsupervised hierarchical clustering (R package ggplot).

**PLA**

B cells were isolated from spleens of 15- to 20-week-old WT and MC mice by MACS sorting according to protocol [CD43 (Ly-48) MicroBeads, Miltenyi Biotec, Cat. No. 130-049-801]. PLA was performed with the Duolink-In-Situ-Orange kit (Sigma, Cat. No. DUO92007), as previously published (98). In brief, cells were plated on a 10-well polylysine-coated, microplate (Thermo Scientific, three wells per genotype, condition, and experiment), allowed to adhere for 30 minutes at 37°C, and subsequently fixed with 1% PFA at room temperature for 20 minutes. Cells were then blocked and permeabilized with 0.5% Saponin in Duolink Blocking buffer (Sigma). For PLA probes against specific targets, the following primary antibodies were used: anti-Myd88 (Abcam), anti-BTK (Novus Biologicals, Cat. No. NB1-7829SSS), anti-IRAK1 (Novus Biologicals, Cat. No. NB1-77068SSS), anti-IRAK4 (Invitrogen, Cat. No. 700026), and anti-IgM (Jackson Laboratories, Cat. No. 115-007-020). Primary antibodies were labeled with Duolink In-Situ Probes: Plus (Sigma, DUO92009) or Minus (Sigma, Cat. No. DUO92010) according to protocol. Fixed cells were incubated with Plus and Minus probes overnight at 4°C. Ligation, rolling circle amplification, and mounting of cells were performed according to protocol. Three images per well, each capturing at least 120 cells, were acquired on a Leica SP8 confocal microscope using the Leica LAS X software. Nuclei and PLA signals were counted using BlobFinder version 3.0.0 (99).

**BH3 Profiling**

BH3 profiling was performed using the iBH3 plate-based method as previously published (56). In brief, cell lines were seeded at a density of 1 x 10^4 cells/mL 24 hours before profiling. Four million cells of each cell line were pelleted at 300 x g for 5 minutes and resuspended in 2 mL MEM-P25 [150 mmol/l Mannitol, 10 mmol/L HEPES-KOH, pH 7.5, 150 mmol/L KC1, 1 mmol/L EGTA, 1 mmol/L EDTA, 0.1% BSA, 5 mmol/L Sucinate, 0.25% Poloxamer 188 (Fisher, MT61161RM)]. Cells were permeabilized with digitonin (Sigma, D5628) exposed to BH3 peptides for 60 minutes at 25°C, and mitochondrial Cytochrome C release was measured by flow cytometry using a BD LSRII (Becton, Dickinson and Company). Mitochondrial CYTOchrome C release in the presence of 50 μg/mL (25 μmol/L) Abemaciclib (Enzo, BML-A150-0005) was >90%. Figure 5D represents the mean of three independent experiments.

**Data Accessibility**

Murine exome and 3′-RNA-seq data are available at the Sequence Read Archive under the accession number PRJNA685334. The BCR repertoire sequencing data are available under PRJNA672930. A dataset consisting of DNA and RNA-seq data generated by the lab of Sandeep S. Dave (11) was reanalyzed for this work and is accessible at the European Genome-phenome Archive (accession number EGAD00001003600). Some analyses conducted in this work are based on previously published supplementary data by the labs of Margaret A. Shipp and Louis M. Staudt (9, 10).

**Authors’ Disclosures**

B.W. Pelzer reports grants from German Cancer Aid outside the submitted work. J.A. Ryan reports patent 10761086 issued and patent 10739333 issued. L.P. Frenzel reports other from AbbVie, and grants from Roche and Gilead outside the submitted work. A. Letai reports other from Flash Therapeutics (scientific advisory board) and Dialectic (scientific advisory board), personal fees and other from Zentalis, grants and personal fees from AstraZeneca, and personal fees from Chugai during the conduct of the study, and his employer, Dana-Farber Cancer Institute, holds patents for BH3 profiling. H.C. Reinhardt reports grants from German-Israeli Foundation, Deutsche Forschungsgemeinschaft, Deutsche Jose Carreras Leukämie Stiftung, Else Kröner-Fresenius Stiftung, Deutsche Krebshilfe, German Ministry of Education and Research, and Deutsches Konsortium für Translationale Krebsforschung during the conduct of the study, as well as personal fees from AbbVie, AstraZeneca, Vertex, and Merck, grants from Gilead, and other from CDL Therapeutics GmbH outside the submitted work. No disclosures were reported by the other authors.

**Authors’ Contributions**


**Acknowledgments**

The authors are indebted to their patients, who provided primary material. They thank Alexandra Florin, Marion Müller, and Ursula Rommerscheidt-Fuß from the Institute of Pathology, University Hospital Cologne, for their outstanding technical support. The authors acknowledge the Institute of Forensic Medicine, University of Cologne, for help with STR-based cell line authentication. They thank the CECAD Imaging Facility and Christian Jungel for their support in microscopy. This work was funded through the German-Israeli Foundation for Research and Development (16-5-412.20-2016 to...
H.C. Reinhardt), the Deutsche Forschungsgemeinschaft (KFO-286-RP2 and RE 2246/13-1 to H.C. Reinhardt), the Deutsche Jose Carreras Leukämie Stiftung (R12/08 to H.C. Reinhardt and Promotionsstipendium to P. Nieper), the Else Kröner-Fresenius Stiftung (EKFS-2014-A06 to H.C. Reinhardt and 2016_Kolleg.19 to H.C. Reinhardt and S. Klein), the Deutsche Krebsforschung (Translational Oncology Program 70112951 and Deutsches Konsortium für Translationale Krebsforschung (DKTK), joint funding (Targeting MYC L*10), the Deutsche Krebshilfe (1117240 and 70113041 to H.C. Reinhardt), and the German Ministry of Education and Research (BMBF e:Med 01ZX1303A to H.C. Reinhardt). J. Hauer and A. Borkhardt have been supported by the Deutsche Krebsforschung (Translational Oncology Program 70112951 and Deutsches Konsortium für Translationale Krebsforschung (DKTK), joint funding (Targeting MYC L*10). L. Pasqualetti has been supported by the NIH/NCI (2R01CA172942) and the Leukemia & Lymphoma Society (TRP Grant #6575-19).

Received December 5, 2019; revised October 6, 2020; accepted October 28, 2020; published first November 2, 2020.

REFERENCES


Vulnerabilities in a Myd88/BCL2-Driven DLBCL Mouse Model


An Autochthonous Mouse Model of *Myd88-* and *BCL2*-Driven Diffuse Large B-cell Lymphoma Reveals Actionable Molecular Vulnerabilities

Ruth Flümann, Tim Rehkämper, Pascal Nieper, et al.


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi: 10.1158/2643-3230.BCD-19-0059</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://bloodcancerdiscov.aacrjournals.org/content/suppl/2020/10/31/2643-3230.BCD-19-0059.DC1">http://bloodcancerdiscov.aacrjournals.org/content/suppl/2020/10/31/2643-3230.BCD-19-0059.DC1</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 100 articles, 32 of which you can access for free at: <a href="http://bloodcancerdiscov.aacrjournals.org/content/2/1/70.full#ref-list-1">http://bloodcancerdiscov.aacrjournals.org/content/2/1/70.full#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citing articles</td>
<td>This article has been cited by 1 HighWire-hosted articles. Access the articles at: <a href="http://bloodcancerdiscov.aacrjournals.org/content/2/1/70.full#related-urls">http://bloodcancerdiscov.aacrjournals.org/content/2/1/70.full#related-urls</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, use this link <a href="http://bloodcancerdiscov.aacrjournals.org/content/2/1/70">http://bloodcancerdiscov.aacrjournals.org/content/2/1/70</a>. Click on &quot;Request Permissions&quot; which will take you to the Copyright Clearance Center's (CCC) Rightslink site.</td>
</tr>
</tbody>
</table>