bjh short report

The molecular ontogeny of follicular lymphoma: gene mutations succeeding the *BCL2* translocation define common precursor cells

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Summary

Relapsed follicular lymphoma (FL) can arise from common progenitor cells (CPCs). Conceptually, CPC-defining mutations are somatic alterations shared by the initial and relapsed tumours, mostly B-cell leukaemia/lymphoma 2 (*BCL2*)/immunoglobulin heavy locus (*IGH*) translocations and other recurrent gene mutations. Through complementary approaches for highly sensitive mutation detection, we do not find CPC-defining mutations in highly purified *BCL2*/*IGH*-negative haematopoietic progenitor cells in clinical remission samples from three patients with relapsed FL. Instead, we find cells harbouring the same *BCL2*/*IGH* translocation but lacking *CREB* binding protein (*CREBBP*), lysine methyltransferase 2D (*KMT2D*) and other recurrent gene mutations. Thus, (i) the *BCL2*/*IGH* translocation can precede CPC-defining mutations in human FL, and (ii) *BCL2*/*IGH*-translocated cells can persist in clinical remission.

Keywords: lymphoma, molecular ontogeny, common progenitor cells, *BCL2*/*IGH* translocation, minimal residual disease.

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Introduction

Patients with advanced follicular lymphoma (FL) are considered incurable with standard therapies and typically have chronically relapsing clinical course.[1] Most relapses are thought to originate from common progenitor cells (CPCs) through divergent evolution.[2,3] These CPCs have not yet been directly identified or functionally characterised. Conceptually, CPCs are precursors that harbour some, but not all of the necessary mutations to confer a fully malignant phenotype. CPC-defining mutations are somatic alterations that are detectable in the initial and relapsed tumours. These shared alterations frequently include the FL hallmark translocation B-cell leukaemia/lymphoma 2 (*BCL2*)/immunoglobulin heavy locus (*IGH*) [t(14;18)] and mutations affecting epigenetic modifying genes such as lysine methyltransferase 2D (*KMT2D*), *CREB* binding protein *CREBBP*, E1A binding protein p300 (*EP300*) and enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2*).[2,3]

The *BCL2*/*IGH* translocation is acquired in early B-lineage committed precursor cells through off-target variable, diversity, and joining (VDJ) recombination.[1] With increasing age, more than half of healthy individuals have *BCL2*/*IGH*-translocated cells detectable in peripheral blood.[4] The risk of FL increases with higher levels of circulating *BCL2*/*IGH*-translocated cells, but only a minority of these individuals will ultimately develop FL with varying latencies.[5] The timing and sequence of acquisition of additional mutations that render *BCL2*/*IGH*-translocated cells CPCs and subsequently FL cells, i.e. the mutational ontogeny, is unknown. Two models have been proposed.

In the 'first-hit model' (Fig 1A, left), the *BCL2*/*IGH* translocation is considered the first transformative event in the pathogenesis of FL, mainly supported by its occurrence during early B-cell development and universally truncal nature.[2,3,6] In fact, an elegant mouse model showed that BCL2-overexpressing B cells are capable of multiple re-entries into the germinal centre, supporting the concept of gradual multi-hit lymphomagenesis.[7]

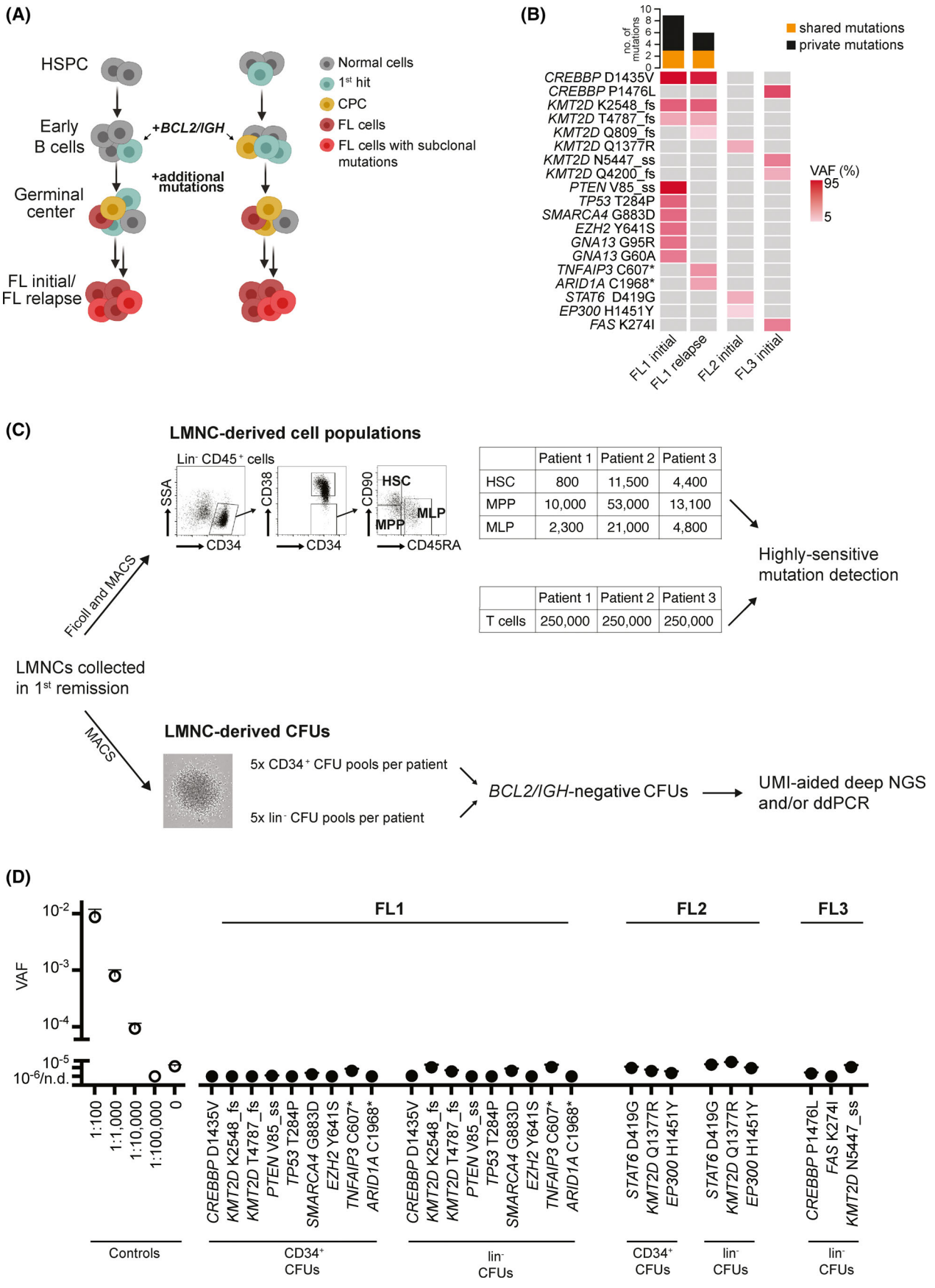
Alternatively, recurrent gene mutations could be acquired in haematopoietic stem and progenitor cells (HSPCs) and contribute to clonal expansion and potentially lymphoid skewing, as has been described in chronic lymphocytic leukaemia.[8–10] These 'primed' HSPCs could then undergo B-lineage commitment and subsequently acquire the *BCL2*/*IGH* translocation ('subsequent-hit model'; Fig 1A, right), thereby constituting the CPC. Supporting this hypothesis, Horton *et al.*[11] previously showed that loss of *Crebbp* in mouse HSPCs promoted lymphomagenesis with long latency, whereas this phenotype was attenuated when *Crebbp* was disrupted at later stages of lymphopoiesis. We previously reported that $CD34^{+}CD19^{-}CD10^{-}$ cells collected from a patient who developed FL 8 years later harboured detectable mutations, including an *EP300* mutation.[10]

In this study, we aimed to expand on that evidence and evaluated both models of FL development in additional human patient samples.

Methods

A detailed methods section is provided in the Supplementary Information.

Fig 1. Mapping of the molecular ontogeny in human FL. (A) Schematic models of oncogenic evolution of FL: 'First-hit model' (left), and 'subsequent-hit model' (right). (B) Oncoprint of non-silent gene mutations in initial and relapse tumour biopsies from three patients with FL. VAF of each mutation are indicated by colour intensity. (C) Experimental workflow illustrating two approaches for highly sensitive mutation detection in HSPC fractions obtained in first remission. (top) Representative flow cytometry sorting strategy. Absolute numbers of sorted cells for each cell fraction and each patient are shown. (bottom) Representative picture of a LMNC-derived CFUs. For every patient, five distinct pools of $CD34^{+}$ and lin^{-} HSPC-derived CFUs were analysed. Each pool was tested for the presence of the specific *BCL2*/*IGH* translocation to ensure that only non-translocated precursor cells were assayed. (D) VAF of indicated mutations in $CD34^{+}$ and lin^{-} LMNC-derived CFUs from all three patients. Controls include *CREBBP* P1476L, *FAS* K274I, *KMT2D* K2548_fs, *KMT2D* T4787_fs, *KMT2D* Q1377R, and *KMT2D* N5447_ss. Error bars indicate the standard error of the mean. BCL2, B-cell leukaemia/lymphoma 2; CFUs, colony forming units; CPC, common progenitor cell; CREBBP, *CREB* binding protein; ddPCR, digital droplet polymerase chain reaction; FAS, Fas cell surface death receptor; FL, follicular lymphoma; HSC, haematopoietic stem cells; HSPC, haematopoietic stem and progenitor cells; IGH, immunoglobulin heavy locus; KMT2D, lysine methyltransferase 2D; LMNCs, leukapheresis-derived mononuclear cells; MACS, magnetic-activated cell sorting; MLP, multilymphoid progenitor cells; MPP, multipotent progenitor cells, n.d., not detectable; NGS, next-generation sequencing; UMI, unique molecular identifier; VAF, variant allele frequency. [Colour figure can be viewed at wileyonlinelibrary.com]

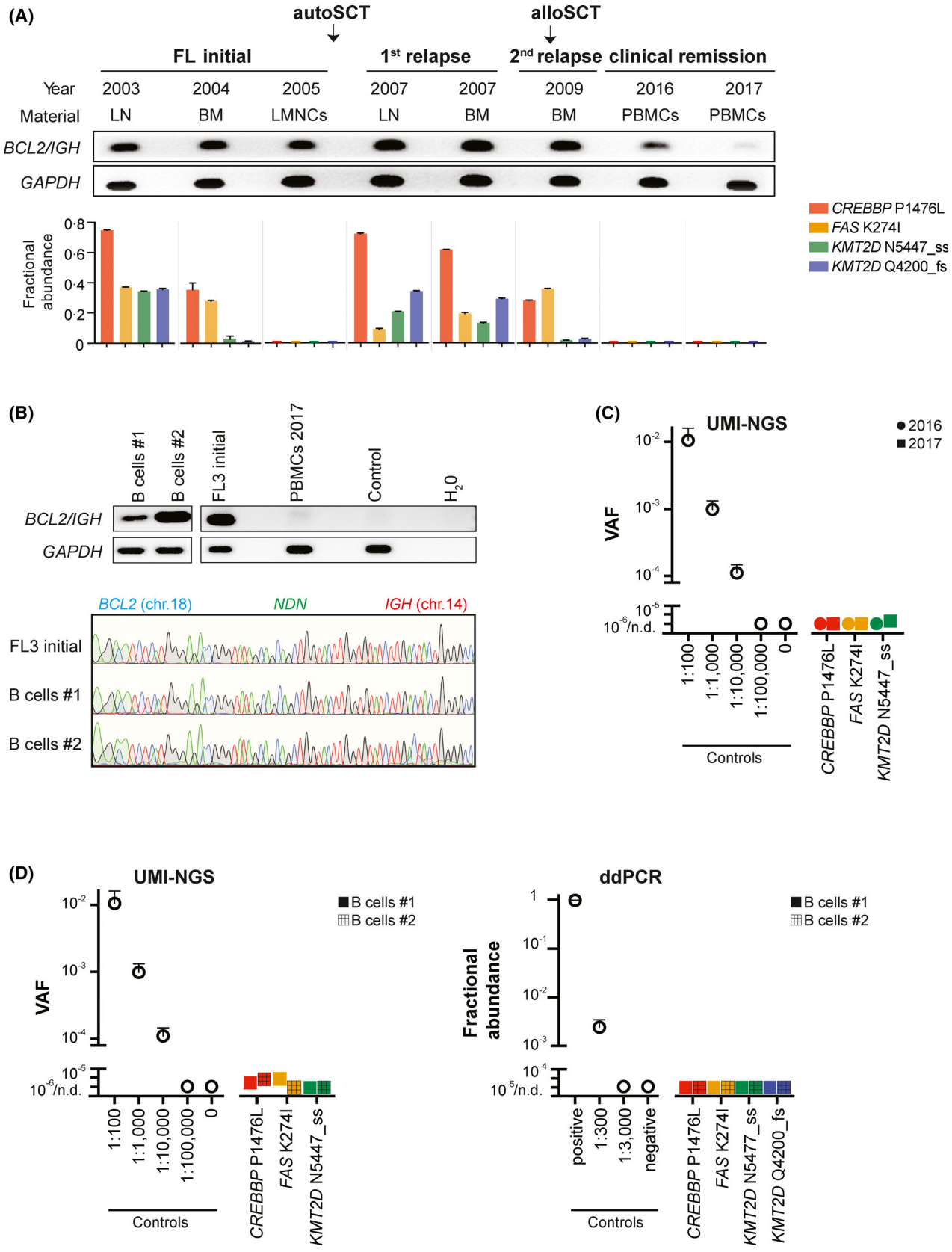


Results and discussion

Direct verification of the 'subsequent-hit model' (Fig 1A) requires the detection of recurrent FL-associated gene mutations in highly purified HSPCs that lack the *BCL2*/*IGH* translocation. We identified three patients who had diagnostic biopsies of their FLs and HSPC-enriched leukapheresis-derived mononuclear cells (LMNC) from autologous stem cell transplantation products collected in first remission after either R-CHOP (rituximab, cyclophosphamide, vincristine, and prednisone; FL1 and FL2) or CHOP (FL3). First, we determined the mutation profiles of the initial FL biopsies by hybrid-capture DNA targeted sequencing, which demonstrated typical mutational landscapes (Fig 1B). Sequencing of the initial FL and an available relapse biopsy from FL1 revealed shared mutations in *KMT2D* and *CREBBP* (Fig 1B). Next, we sorted the LMNCs into haematopoietic stem cell (HSC), multipotent progenitor cell (MPP), and multilymphoid progenitor cell (MLP) fractions (Fig 1C) and performed highly sensitive mutation detection as previously described.[12] However, the low cell numbers in some HSPC fractions (e.g. HSC and MLP of FL1, Fig 1C) precluded testing of all mutations. Eventually, we did not detect any of the evaluable FL-associated mutations, including the CPC-defining *KMT2D* and *CREBBP* mutations, to be significantly enriched in any of the HSPC fractions, i.e. recovered at frequencies significantly above the background signal in matched LMNC-derived T cells and in a peripheral blood sample from a healthy individual (Table SI). To overcome the limitation of limited cell numbers in HSPC fractions, we expanded and functionally enriched precursors with self-renewal capacity by sorting either $CD34^+$ or lineage negative (lin^-) HSPCs and culturing them for 14 days on methylcellulose-based medium that supports the growth of single cell-derived colony forming units (CFUs; Fig 1C). For every patient, we analysed five distinct pools of $CD34^+$ and lin^- HSPC-derived CFUs. The pools contained a median of 1315 (344–1670) and 779 (566–3115) CFUs respectively. As pre-/pro-B cells can still express CD34,[13] we tested all CFU pools for the presence of the FL-specific *BCL2*/*IGH* translocation by real-time quantitative polymerase chain reaction (PCR) to ensure that we really assayed only non-translocated precursor cells. The $CD34^+$ HSPC-derived CFU pools from FL3 were indeed found to be positive for the *BCL2*/*IGH* translocation, demonstrating that *BCL2*-translocated cells can persist/survive on methylcellulose for up to 14 days, and therefore excluded from further analysis (Figure S1). All other CFU pools were tested negative for the *BCL2*/*IGH* translocation and analysed further using unique molecular identifier-aided deep DNA next-generation sequencing (UMI-NGS) for highly sensitive mutation detection, assaying the equivalent of 10 000 cells (500 ng genomic DNA, corresponding to ~500 distinct HSPCs) for each mutation in five replicates. At a limit of detection (LOD) of 10^{-4}, we did not identify any of the tested somatic FL-associated mutations in highly purified HSPC-derived CFUs, including mutations in *KMT2D* and *CREBBP* (Fig 1D; Table SII). As an orthogonal approach and because we could not design suitable primers for one *KMT2D* mutation (Q4200_fs), the lin^- HSPC-derived CFUs from FL3 were also analysed by digital droplet PCR (ddPCR). Again, we did not detect any of the FL-specific mutations in these HSPC-derived CFUs. Thus, our results do not support the hypothesis that CPC-defining mutations, including highly recurrent mutations in *KMT2D* and *CREBBP*, are acquired before the *BCL2*/*IGH* translocation in human FL.

To provide direct evidence of the 'first-hit model', we aimed to identify the FL-specific *BCL2*/*IGH* translocation in cells that have not yet acquired CPC-defining mutations. For this, we further investigated FL3, the only patient in our series who had not received B-cell depleting anti-CD20 antibodies prior to LMNC collection in first remission (see Supplementary Information for detailed clinical course). The FL-specific *BCL2*/*IGH* translocation was detectable in the initial FL and the subsequent relapses, as well as in the LMNCs in first remission and in the post allogeneic transplantation bone marrow (BM) sample (Fig 2A) with a sensitivity down to 10^{-4} (Figure S2). Furthermore, *BCL2*/*IGH* remained detectable in peripheral blood mononuclear cells (PBMCs) obtained in ongoing clinical remission 7 and 8 years after

Fig 2. Informative case of relapsing FL (FL3). (A) Serial analysis of the FL-specific *BCL2*/*IGH* translocation by PCR and the CPC-defining gene mutations by ddPCR. PCR for *GAPDH* was used as a loading control. (B) (top) PCR amplification of the FL-specific *BCL2*/*IGH* translocation from *ex vivo* expanded B cells from the PBMCs obtained in 2017 in two separate attempts (B cells #1 and #2). Genomic DNA from the initial tumour (FL3 initial), the PBMCs collected in 2017 and PBMCs from a healthy donor were used as positive/negative controls. PCR for *GAPDH* was used as a loading control. *NDN* indicates non-templated nucleotides. (bottom) Sanger sequencing traces of the FL-specific *BCL2*/*IGH* translocations from FL3 initial and *ex vivo* expanded B cells. (C) VAF of indicated mutations from PBMCs, collected in 2016 and 2017, by UMI-NGS. Error bars indicate the standard error of the mean. (D) VAF and fractional abundance of indicated mutations from the two *ex vivo* expanded B cells from (B) by UMI-NGS and ddPCR. Error bars indicate the standard error of the mean. alloSCT, allogeneic stem cell transplantation; autoSCT, autologous stem cell transplantation. BCL2, B-cell leukaemia/lymphoma 2; BM, bone marrow; CPC, common progenitor cell; CREBBP, CREB binding protein; ddPCR, digital droplet polymerase chain reaction; FAS, Fas cell surface death receptor; FL, follicular lymphoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGH, immunoglobulin heavy locus; KMT2D, lysine methyltransferase 2D; LMNCs, leukapheresis-derived mononuclear cells; LN, lymph node; n.d., not detectable; NDN, non-templated nucleotides; NGS, next-generation sequencing; PBMCs, peripheral mononuclear cells; UMI, unique molecular identifier; VAF, variant allele frequency. [Colour figure can be viewed at wileyonlinelibrary.com]



the allogeneic transplant respectively, yet with decreasing intensity (Fig 2A). To rule false positive results, we *ex vivo* expanded B cells from the PBMCs obtained in 2017 in two separate attempts (B cells #1 and #2) as previously described,[14] and confirmed the presence of the FL-specific *BCL2*/*IGH* translocation by PCR and Sanger sequencing (Fig 2B). In contrast, the CPC-defining gene mutations affecting *CREBBP*, Fas cell surface death receptor (*FAS*), and *KMT2D*, all of which were detectable in the initial FL and the relapse samples, could not be identified in the bulk LMNCs (Fig 2A) or the lin^- HSPC CFUs derived from these LMNCs (Table S2), nor in the PBMCs (Fig 2A), all of which harboured the FL-specific *BCL2*/*IGH* (Fig 2A). We confirmed that these mutations were also undetectable in the PBMCs by UMI-NGS at a LOD of 10^{-4} (Fig 2C). Finally, we demonstrated absence of these FL-associated mutations in the two pools of *ex vivo* expanded B cells by UMI-NGS and ddPCR (Fig 2D).

In summary, our data provide direct evidence that the *BCL2*/*IGH* translocation can precede the acquisition of CPC-defining gene mutations in human FL. Vice versa, we did not find CPC-defining gene mutations, including *KMT2D* and *CREBBP* mutations, in highly purified *BCL2*/*IGH*-negative HSPCs, suggesting that these mutations are in fact acquired after the *BCL2* translocation. This model is further supported by elegant mouse experiments showing that conditional mutation/inactivation of these genes in B lineage committed cells co-operate with *Bcl2* and promote lymphomagenesis.[15,16]

Two aspects are of particular clinical relevance

First, our data illustrate that B cells in remission samples can harbour the FL-specific *BCL2*/*IGH* translocation but lack additional FL-associated mutations. The lack of CPC-defining mutations indicates that these cells did not contribute to FL relapse. This implies that assessment of minimal residual disease (MRD) by detection of the *BCL2*/*IGH* translocation should be interpreted with caution. Instead, we propose that detection of FL-specific, truncal mutations will further improve the predictive precision of MRD testing.

Second, the lack of detectable CPC-defining mutations in peripheral blood from remission time-points could suggest that CPCs do not circulate to a sizeable extent but are more likely to be tissue-resident within a tumour-supportive microenvironment (e.g. in lymphoid tissues or the BM). This supports the concept of analysing circulating tumour DNA, i.e. cell-free DNA that is released from apoptotic or necrotic tumour cells into the circulation, rather than circulating tumour cells for MRD purposes in FL.[17] Unfortunately, the paucity of circulating CPCs in remission samples of patients with FL also limits our ability to directly identify and functionally characterise these cells. Yet, this is a clinical priority that should be addressed in future studies, as therapeutic targeting of residual CPCs holds promise to ultimately eradicate the disease and cure patients. Finally, tissue-resident CPCs may explain our difficulties in curing patients with FL, as tissue penetration of some highly active anti-lymphoma drugs, including monoclonal antibodies, may be a critical obstacle.[18]

We are fully aware of the shortcomings of our study, including the small cohort size and our limited ability to detect very rare mutations in finite patient material. Importantly, as absence of evidence is not evidence of absence, we cannot exclude the possibility that particular mutations or other FL-priming alterations could precede the *BCL2*/*IGH* translocation in some patients.[10,11] However, in carefully controlled experiments we provide direct evidence that *BCL2*/*IGH* translocations can be acquired before CPC-defining mutations in human FL. Furthermore, we show that *BCL2*/*IGH*-translocated B cells lacking CPC-defining mutations can persist in clinical remission samples.

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Author contributions

Sarah Haebe, William Keay, Deepak Bararia and Oliver Weigert designed the study. William Keay, Stefan Alig, Michael Heide, Abner Louissaint Jr, David M. Weinstock and Oliver Weigert designed, performed and analysed the sequencing experiments. Anne-Wiebe Mohr, Larissa K. Martin, William Keay and Andreas Moosmann designed, performed and interpreted the cell culture experiments. Stefan Krebs and Helmut Blum oversaw the sequencing experiments. Deepak Bararia designed, performed and analysed the flow cytometry experiments. Ramona Secci and Silvia Thoene designed, performed and analysed the ddPCR experiments. Sarah Haebe, William Keay, David M. Weinstock, Michael von Bergwelt-Baildon, Jürgen Ruland, Deepak Bararia and Oliver Weigert interpreted clinical and experimental data. Sarah Haebe, William Keay and Oliver Weigert wrote the manuscript with

input from all authors. Oliver Weigert oversaw all experiments and was involved in all aspects of analysing and interpretation of experimental and clinical data.

Conflict of interest

The authors report no relevant conflicts of interest.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Material. Supplementary Methods.

Table SI. Summary of somatic mutations identified by hybrid-capture DNA target sequencing and subjected to highly sensitive sequencing.

Table SII. Summary of CFU-derived HSPC sequencing.

Figure S1. Detection of *BCL2*/*IGH* MBR and mcr in LMNC-derived $CD34^+$ CFUs.

Figure S2. Sensitivity of nested PCR assay for *BCL2*/*IGH* mcr.

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