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MYC is essential for the formation and maintenance of germinal centers

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Abstract

Germinal centers (GC) are sites of intense B cell proliferation, central for T cell dependent antibody responses. However, the role of MYC, a key cell cycle regulator, in this process has been questioned. Here, we identified MYC positive B cell subpopulations in immature and mature GCs, and show through genetic ablation of *Myc* that they play indispensable roles in GC formation and maintenance. The identification of these functionally critical cellular subsets has important implications for human B cell lymphomagenesis, which mostly originates from GC B cells and

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AUTHOR CONTRIBUTIONS

D.P.C. and K.R. conceived the work and designed experiments, D.P.C., Y.S., S.A.G., A.P., K.K., M.J. and S.R. did experiments and/or analyzed data, B.P.S. and I.M.A. contributed with reagents, D.P.C. and K.R. supervised all aspects of the project, D.P.C. and K.R. wrote the manuscript. All authors discussed results and edited the manuscript.

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frequently involves *MYC* chromosomal translocations. As these translocations are generally dependent on transcription of the recombining partner loci, the *MYC* positive GC subpopulations may be at a particularly high risk for malignant transformation.

In response to T cell dependent antigens, antigen-specific B cells are driven into the germinal center (GC) reaction, which is critical for the generation and selection of memory B and plasma cells expressing somatically mutated high-affinity antibodies¹. GCs are sites of massive B cell proliferation². However, despite extensive research on the GC reaction, the mechanisms driving GC B cell proliferation have remained elusive. An issue of particular importance is the notion that *MYC*, a master regulator of cellular proliferation both in nonhematopoietic and hematopoietic cells including B cells^{3, 4}, does not play a role in this context^{5, 6}.

The *MYC* transcription factor was first identified as the cellular homolog of the transforming determinant carried by the avian myelocytomatosis virus MC29⁷. The conservation of cellular homologs of viral oncogenes across evolutionary time and species suggests important roles for these genes in normal cellular physiology⁷. Indeed, germline ablation of *Myc* leads to early (E9-10) embryonic lethality due to widespread failure in organ and tissue growth⁸. In the hematopoietic compartment *MYC* is required at early developmental stages of both B and T cells in the bone marrow and thymus, respectively^{4, 9}. Experimental evidence demonstrates that *MYC* plays a crucial role in regulating cellular proliferation, apoptosis and differentiation of mammalian cells⁸. During cell cycle progression, *MYC* promotes G₀/G₁-S transition through the activation of genes encoding cyclin-dependent kinase (CDK) complex proteins, including Cyclin D2 (*Ccnd2*), as well as by the repression of CDK inhibitors^{10, 11}.

Although early studies suggested high levels of *MYC* transcripts and protein in human and mouse GC B cells¹²⁻¹⁴, later work refuted these observations, detecting only low *MYC* expression levels in these cells^{5, 6}. According to these studies *MYC* levels were comparable or even lower than the ones observed in quiescent follicular (FO) B cells where *MYC* does not seem to play a major function³, suggesting a dispensable role for *MYC* in GC B cell proliferation. Further support for this hypothesis came from the observation that BCL-6, a master regulator of GC B cell development¹⁵, interacts with the transcription factor MIZ1 (ZBTB17), a known partner of *MYC*, to suppress CDK inhibitors¹⁶, and that BCL-6 itself can repress *MYC* transcription¹⁷. Moreover, in line with the ability of BCL-6 to inhibit *Ccnd2* expression¹⁷, GC B cells predominantly express Cyclin D3 (*Ccnd3*), which, although dispensable for GC initiation^{18, 19}, is required for GC maturation and is not controlled by *MYC*^{10, 11}.

MYC plays a critical role in the proliferation of most, if not all human B cell lymphomas, the vast majority of which originate from GC or post-GC B cells as demonstrated by their somatically mutated immunoglobulin (Ig) genes²⁰⁻²². Indeed, in the GC microenvironments rapidly dividing B cells undergo somatic hypermutation (SHM) and class switch recombination (CSR) in their Ig genes, both of which involve DNA strand breaks²². Infidelity in these processes increases the probability of oncogenic events such as chromosomal rearrangements²². The *MYC* gene itself is frequently involved in chromosomal

translocations in human GC-derived B cell lymphomas²². Such translocations, seen in roughly 10% of diffuse large B cell lymphomas (DLBCLs) and almost all cases of sporadic Burkitt lymphoma, juxtapose *MYC* and enhancers in the Ig loci, leading to deregulated *MYC* expression²². However, clear evidence supports the dependence on gene transcription for the introduction of somatic mutations by activation-induced cytidine deaminase (AICDA)^{23, 24}. Thus currently there is an apparent contradiction between the absence of *MYC* transcription in GC B cells and the recurrent *MYC* translocations observed in the human lymphomas originating from these cells. An attractive hypothesis is that the association of *MYC* deregulated expression with GC B cell lymphomagenesis reflects its role in the regulation of cell proliferation at some stage of the GC reaction.

Here, by performing genetic experiments in the mouse, we found that *MYC* is expressed in subsets of GC B cells in both immature and mature GCs, and that these cells play an essential role in GC formation and maintenance.

RESULTS

MYC target genes are enriched in GC B cells

Conflicting observations were made with respect to *MYC* expression levels between FO and GC B cells^{5, 6, 12-14}. We re-evaluated this question by performing gene expression analysis by quantitative PCR (qPCR) in flow purified splenic FO and GC B cells derived from previously immunized wild-type mice (**Fig. 1a**). First, we compared the transcript levels of genes predominantly expressed in GC versus FO B cells, providing an internal control for our sorting strategy. Consistent with previous findings^{15, 22}, expression of both *Aicda* (*Aid*) and *Bcl6* was significantly increased in GC B cells compared to FO B cells (**Fig. 1b**). In contrast, *Myc* transcripts levels were identical in GC and FO B cells (**Fig. 1c**), in agreement with previous studies and supporting the hypothesis that *MYC* is dispensable in GC B cells^{5, 6}. However, *Myc* mRNA is highly unstable²⁵, therefore transcript levels might not correlate with protein activity²⁵. For that reason we decided to perform gene set enrichment analysis (GSEA)²⁶ on published gene expression profiles of FO and GC B cells²⁷, evaluating *MYC* activity through the enrichment of its target genes, (see Methods for details). Using a *MYC* target gene list derived from mouse B cells²⁸, we observed a highly significant enrichment of *MYC* induced genes in GC B cells, whereas the converse was true for genes downregulated by *MYC*. (**Fig. 1d**). These observations remained statistically significant when genes associated with the GO terms proliferation, cell cycle and growth function were removed from the analysis, arguing against the possibility that the results simply reflected the high proliferative activity of GC B cells (**Fig. 1e**). Similar observations were made using lists of genes containing *MYC* binding motifs in their promoter, and a gene set of *MYC* up-regulated genes obtained through the intersection of *MYC* ChIP-Seq and *MYC* siRNA knockdown data in (GC B cell derived) Burkitt lymphoma lines²⁹ (**Supplementary Fig. 1**). Thus, although on average *Myc* mRNA is not expressed at higher levels in GC than FO B cells, GC B cells display an enrichment of *MYC* target genes.

Some immature and mature GC B cells express MYC

The enrichment of MYC target genes in the absence of increased *Myc* mRNA levels in the overall population of GC B cells led us to hypothesize that MYC activity may be restricted to a fraction of GC B cells. To address this issue, we used a *Myc* reporter allele, (*Myc^{EGFP}*)³⁰, in which a MYC-EGFP fusion protein (MYC^{EGFP}) is expressed from the endogenous *Myc* locus. By flow cytometry, we observed the appearance of MYC positive GC B cells early after immunization, reaching roughly 20% of GC B cells by day 4 (**Fig. 2a,b**), an early time-point of GC development before the onset of strong GC B cell expansion, (**Supplementary Fig. 2**). We also observed a population of GC B cells positive for MYC (~10%) at day 10 after immunization, a time-point that reflects the peak of the GC reaction (**Fig. 2b** and **Supplementary Fig. 2**). Small subsets of non-GC B cells, but not of plasma cells, expressing low amounts of MYC protein were also present in the immunized animals (**Supplementary Fig. 3** and **data not shown**). Using the *Myc^{EGFP}* reporter allele and GC B cell markers, we purified the MYC positive GC B cells as well as their MYC negative counterparts by flow cytometry on day 4 and day 10 after immunization, and performed gene expression analysis by qPCR. MYC positive GC B cells isolated on day 10 displayed increased levels of *Myc* transcripts compared to FO B cells, whereas this was not the case for the MYC positive GC B cells on day 4 after immunization (**Fig. 2c**). Yet, the amount of MYC^{EGFP} in day 4 MYC positive GC B cells were higher than in their day 10 counterparts, likely reflecting the impact of post-transcriptional control on MYC protein⁸ (**Supplementary Fig. 3**). A further analysis of the day 4 MYC positive GC B cells revealed a three-fold decrease of *Bcl6*¹⁵ mRNA compared to FO B cells (**Fig. 2d**), whereas the transcript expression of other GC B cell genes, namely *Mybl1* (A-myb)³¹ and *Aicda*²² was strongly increased (~20 and 50-fold respectively; **Fig. 2d**). Compared to FO B cells there was also an increase in the load of somatic mutations in the Ig heavy chain (IgH) variable (V) region gene rearrangements in day 4 MYC positive GC B cells (**Fig. 2e**). The load of somatic mutations and the fraction of cells carrying these mutations in their IgH V region genes was further increased in day 4 MYC negative GC B cells as well as both MYC positive and negative GC B cells from day 10 immunized mice (**Fig. 2e**). In addition, these GC B cell subpopulations demonstrated markedly elevated transcript expression of *Bcl6*, *Mybl1* and *Aicda* in comparison to FO and day 4 MYC positive GC B cells (**Fig. 2d,e**). Hence, using a *Myc^{EGFP}* reporter allele that allows the visualization of MYC protein in single cells, we identified distinct subpopulations of MYC positive and negative GC B cells in both immature and mature GCs.

Early GC B cells express MYC and BCL-6

Following antigen binding and activation via the B cell receptor, B cells migrate to the T-B cell border where they are co-stimulated through interaction with cognate T helper cells^{2, 32}. Upon productive T-B cell interaction, B cells migrate back to the intra-follicular B cell area (~4 days after initial antigen encounter) where they undergo massive expansion in the environment of follicular dendritic cells (FDCs), forming clusters of proliferating GC B cells³³. BCL-6 is an essential transcription factor for GC B cell formation¹⁵, and is required already at the stage where B cells migrate back into the intra-follicular B cell area³⁴. Given the fact that early in the GC response MYC positive GC B cells display lower transcript

levels of the GC B cell genes *Bcl6*, *Mybl1* and *Aicda* and fewer somatic mutations compared to their MYC negative counterparts (**Fig. 2d,e**), we considered the possibility that these early MYC positive GC B cells represent precursors of the latter. Using immunofluorescence histology we found through the analysis of spleens of mice 4 days after immunization that at the very initial stages of GC B cell cluster formation (\approx 20 cells) the cells in the clusters are positive for both MYC and BCL-6 (**Fig. 3a**, and **Supplementary Fig.4a**), and that as the number of GC B cells in the cluster increases (>20 cells), B cells gradually lose MYC expression (**Fig. 3b,c**, and **Supplementary Fig.4b**). While both MYC positive and negative GC B cells expressed the GC B cell typical *Ccnd3*^{18, 19} (**Fig. 3d**), the MYC positive cells also expressed *Ccnd2*, a direct target of MYC^{10, 11} (**Fig. 3e**). Both *Ccnd2* and *Ccnd3* are known positive regulators of the cell cycle G0/G1-S transition in B cells^{10, 11, 18, 19}. Consistently, we found that more than 40% of the MYC positive GC B cells were at the S-phase of the cell cycle, compared to \sim 20% of MYC negative GC B cells (**Fig. 3f,g**). Taken together these results suggest that early GC B cells are positive for both MYC and BCL-6, display hyperproliferative properties and give rise to GC B cells positive only for BCL-6.

MYC is essential for GC formation

To test whether MYC is critical for GC formation, we genetically deleted *Myc* in GC B cells at an early stage of the GC reaction^{3, 35, 36}. This resulted in a virtually complete ablation of GCs in response to T cell-dependent immunization (**Fig. 4a**, left panel). Analysis of the few remaining GC B cells present in the *C γ 1-cre Myc^{FF} hCD2^{stopFL}* mice for expression of the hCD2 reporter showed that these cells had largely escaped cre-mediated recombination (**Fig. 4a**, right panel, and **Supplementary Fig. 5**). Impairment of GC B cell formation upon *Myc* ablation was further confirmed analyzing additional GC B cell markers by histology and immunohistochemistry (**Fig. 4b**). Ablation of *Myc* in GC B cells interfered with the GC reaction from early on after immunization, consistent with the observed expression of MYC at the initiation of the GC response (**Fig. 4c**). It also prevented B cells from participating in GC responses in Peyer's patches in the absence of intentional immunization, as evident from the strong counter-selection of GC B cells expressing the hCD2 reporter in Peyer's patch GCs, in which the *C γ 1-cre* transgene induces cre-mediated recombination in approximately 30 to 40% of the GC B cells in control animals³⁵ (**Fig. 4d**). We conclude that MYC plays an essential role in both induced and spontaneous GC formation.

BCL-6 fails to rescue GC formation upon MYC ablation

It has been proposed that BCL-6 plays a role in GC B cell proliferation through transcriptional repression of CDK inhibitors via the interaction with MIZ1, a known partner of MYC¹⁶. We therefore decided to test whether prematurely enhanced *Bcl6* expression could rescue the formation of GCs upon *Myc* ablation. To address this issue, we ectopically expressed *Bcl6* using the *I μ BCL6* allele, in which a mouse *Bcl6* cDNA is inserted downstream of the *I μ* promoter in the IgH locus, leading to transgenic *Bcl6* expression from early stages of B cell development onwards³⁷. Although expression of *Bcl6* from the *I μ BCL6* allele was shown to be sufficient to rescue the differentiation of GC B cells in the spleens of *Bcl6* deficient mice³⁷, this was not the case upon conditional ablation of *Myc*

(**Fig. 5a**, and **Supplementary Fig. 6a**). Similarly, ectopic *Bcl6* expression was unable to rescue spontaneous GC formation in Peyer's patches of *Cγ1-cre Myc^{FF} hCD2^{stopFL}* mice (**Supplementary Fig. 6b,c**). The deficiency in induced and spontaneous GC formation upon *Myc* ablation could also not be rescued by ectopic *Bcl2* expression³⁸, emphasizing an essential role for MYC in the development of GC B cells (**Supplementary Fig. 6a,b,d,e**). In contrast, concomitant ablation of the endogenous *Myc* gene and enforced expression of *MYC* from a new *ROSA26* allele, termed *MYC^{stopFL}*, was able to fully rescue GC formation (**Fig. 5b,c** and **Supplementary Fig. 5** and **6a,b**) as well as IgG1 production (**Fig. 5d** and **Supplementary Fig. 6f**). These results demonstrate that MYC plays a non-redundant role in the initiation of GC formation.

Mature MYC⁺ GC B cells localize to the light zone

Early histological observations using light microscopy suggested the compartmentalization of mature GCs in two morphologically distinct areas: a 'dark' zone (DZ), adjacent to the T cell zone, and a 'light' zone (LZ) contiguous to the splenic marginal zone or to the lymph node capsule³⁹. The 'lighter' and 'darker' appearances of the GC LZ and DZ respectively, derive from the fact that B cells in the LZ are scattered amongst a network of FDCs, which are largely absent from the DZ². In an attempt to better characterize the population of mature MYC positive GC B cells, we performed antibody staining for markers that allow the discrimination of B cells in the DZ and LZ of the GC^{2, 40}. Using flow cytometry we found that the vast majority of MYC positive GC B cells had lower levels of CXCR4 and higher levels of CD83, compared to their MYC negative counterparts (**Fig. 6a**). In agreement we found, by histology and immunofluorescence, MYC positive GC B cells to be interspersed amongst FDCs (**Fig. 6b,c** and **Supplementary Fig. 7a-c**). These observations lead us to conclude that the majority of the MYC positive GC B cells in mature GCs localize to the LZ. Consistent with the notion that in the LZ a fraction of GC B cells undergoes T cell-mediated activation⁴⁰, the MYC positive mature GC B cells displayed a phenotype characteristic of recently activated lymphocytes. This phenotype included increased surface expression of the activation markers CD86, CD83, CD40, CD69 and MHCII (**Fig. 6d,e** and **Supplementary Fig. 7d**) and increased transcripts for the 'immediate early' genes *IκBa* (*Nfkbia*, indicating NF-κβ activity) and *Myc* itself (**Fig. 6f** and **2c**). Consistent with increased NF-κβ activity⁴¹, *Irf4* expression was also elevated in the MYC positive GC B cells (**Fig. 6g**), whereas *Bcl6* was downregulated (**Fig. 2d**). This is in agreement with the idea that CD40 activation signals delivered to B cells by T cells in the LZ direct them to class-switching⁴² and inhibit *Bcl6* expression⁴³. The MYC positive GC B cells also displayed an enrichment of MYC target genes compared to their MYC negative counterparts (**Fig. 6h** and **Supplementary Fig. 7e**). Taken together, MYC positive GC B cells in mature GCs mainly localize to the LZ and display an activated phenotype.

MYC positive GC B cells are required for GC maintenance

As a key function of MYC is to promote G₀/G₁-S transition^{10, 11}, we assessed whether mature GC B cells positive for MYC display a proliferative phenotype. Indeed, we found increased mRNA levels for both *Ccnd2* and *Ccnd3* in these cells, compared to FO B cells (**Fig. 7a**). In contrast, MYC negative GC B cells showed increased levels only of *Ccnd3*

(Fig. 7a). DNA labeling and flow cytometry demonstrated that a considerable fraction (~38%) of the MYC positive GC B cells were in the S phase of the cell cycle, while this was the case for ~20% of their MYC negative counterparts (Fig. 7b,c). Given the proliferative phenotype of MYC positive GC B cells in mature GCs we decided to assess whether MYC also plays an essential role in the GC maintenance. To this end we devised an experimental cell transfer system, which allowed us to conditionally ablate *Myc* in mature GC B cells. For that we used CD19 knockout mice (CD19^{KO}), in which GC B cells do not develop⁴⁴, as recipients for mature B cells. Donor cells were derived from mice carrying a *hCD2^{stopFL}* reporter allele either alone or together with a conditional *Myc* allele (*Myc^F*), in combination with the *Mx-cre* transgene⁴⁵. Following cell transplantation, recipient mice were immunized and subsequently treated with Poly(I:C) at the peak of GC B cell formation to achieve cre-mediated recombination in the progeny of the transferred B cells. In control mice we observed a robust GC response following immunization, and cre-mediated recombination was detected in 40 to 50% of mature GC B cells derived from B cells of mice carrying the *hCD2^{stopFL}* allele alone, as measured by hCD2 surface expression (Fig. 7d, upper panel). In contrast, there was a reduction of ~50% in the total fraction of GC B cells when the donor cells were derived from mice carrying the *Myc^F* allele, and only ~3% of the GC B cells were positive for hCD2 (Fig. 7d, lower panel). These results indicate massive counter-selection of MYC deficient mature GC B cells and suggest that *Myc* expression in mature GC B cells is required for the perpetuation of the GC reaction. We conclude that mature GC B cells go through a stage in the LZ at which MYC is essential for their maintenance and that of the GC.

DISCUSSION

In the present work we re-evaluated the role of the transcription factor MYC, a master regulator of cellular proliferation, in GC B cell physiology. This unresolved problem derives from the conflicting observations that GC B cells have one of the fastest rates of cell division known in mammals², while the average level of *MYC* transcripts in these cells is similar to those of quiescent FO B cells^{3, 5, 6}. We found that, whereas the analysis of bulk GC B cells indeed did not show an increase in *Myc* transcript levels versus FO B cells, analysis of MYC protein at the single cell level revealed the presence of hyperproliferative MYC positive subpopulations of GC B cells in both immature and mature GCs, essential for the formation and maintenance of GCs.

Using a conditional knockout approach, we show that ablation of *Myc* at an early stage of GC B cell development leads to a complete absence of GC formation. Furthermore, MYC protein is expressed at the initiation of GC B cell cluster formation, preceding the phase of massive GC B cell expansion in the intra-follicular B cell area. At this stage we did not detect increased *Myc* transcript expression compared to FO B cells. This discrepancy likely reflects mechanisms of post-transcriptional control of MYC protein⁸, and the fact that enhanced *Myc* transcription is an early transitory event peaking at ~2 hours after the activation of B cells, preceding the increase in RNA synthesis and DNA replication²⁵. Repression by BCL-6 may further restrain *Myc* transcription^{17, 46}. This may be desirable, because AICDA, essential in GC B cells for antibody repertoire diversification, can target transcriptionally active genes outside the Ig loci²²⁻²⁴ and promote their translocation²⁴.

In mature GCs, however, we identified another subset of GC B cells displaying increased MYC transcripts and protein levels and localizing predominantly to the LZ. Identical observations were made in human GCs⁴⁶. These cells show features of cellular activation and may represent the fraction of GC B cells in the LZ undergoing T cell mediated activation to reenter the DZ. In agreement, we detected in MYC positive mature GC B cells activation of the NF- κ B pathway and elevated transcript levels of target genes of this pathway, namely *Myc*⁴⁷ itself and *Irf4*⁴², whereas *Bcl6*⁴³ was downregulated. In addition, the hyperproliferative phenotype of the MYC positive mature GC B cells, many of which at the G₂/M stage of the cell cycle which is known to largely segregate to DZ GC B cells⁴⁰, together with the strong impact of acute *Myc* ablation on the total fraction of GC B cells suggests that MYC positive mature GC B cells are poised for DZ re-entry and perpetuation of the GC reaction, an hypothesis further supported by the experiments of Dominguez-Sola et al.⁴⁶.

The transcription factor BCL-6 is essential for the development of GC B cells¹⁵. Detailed examination of the relation between BCL-6 and cellular proliferation led the hypothesis that GC B cells display an alternative, MYC-independent cell cycle program¹⁶. Along these lines, BCL-6 interacts with the transcription factor MIZ1, a MYC partner, to suppress CDK inhibitors, and *MYC* transcription is repressed by BCL-6^{16, 17, 48}. In addition BCL-6 inhibits the expression of *Ccnd2*¹⁷, a MYC target, and *Ccnd3*, which is not controlled by MYC^{10, 11}, is prominently expressed in GC B cells^{18, 19}. Our studies reveal that at early stages of GC formation MYC positive GC B cells express both *Ccnd2* and *Ccnd3*, which possibly contributes to their hyperproliferative phenotype, and that a similar pattern of expression of these genes is observed in mature GC B cells when they upregulate MYC. The cyclic dependence of GC B cell proliferation on MYC activity likely reflects a process termed “cyclic re-entry”, in which step-wise antibody affinity maturation takes place through multiple rounds of selection^{40, 49}. In this setting, the function of MYC in the GC reaction may be to induce ‘competence’²⁵ for GC B cell division to occur, i.e. to decide whether a GC B cell enters a phase of proliferative expansion.

Our work has direct implications for the understanding of the pathogenesis of human GC B cell derived lymphomas carrying *MYC* and *Ig* chromosomal translocations, seen in almost all sporadic Burkitt lymphomas and ~10% of DLBCLs²². B cell lymphomas, the most common type of human lymphoid malignancies overall, mostly originate either from GC B cells or B cells that have passed through the GC reaction²². In the GC microenvironment, B cells undergo processes of DNA mutation (SHM) and recombination (CSR) in their immunoglobulin genes while rapidly dividing, and infidelity in these processes leads to an increased frequency of oncogenic genetic lesions²⁰⁻²². AICDA, the enzyme responsible for both SHM and CSR, participates in the formation of chromosomal breaks in the *MYC* gene locus that lead to *MYC* and *Ig* translocations, and *MYC* transcription is required for this process^{23, 24}. In this study we identified in the mouse subpopulations of GC B cells for which *Myc* transcription and activity is functionally critical. Identical MYC positive GC B cell subsets were also found in the human⁴⁶. Given the mechanism of *MYC* and *Ig* chromosomal translocations, these GC B cell subsets may be prone to undergo the latter

event and thus represent a stage of GC differentiation with a particularly high risk for malignant transformation.

ONLINE METHODS

Ethics statement

All animal care and procedures followed NIH guidelines and were approved by the IACUC (03341) of Harvard University and the Immune Disease Institute, and of the Max Delbrück Center for Molecular Medicine.

Generation of transgenic mice

MYC transgenic mice (*MYC^{stopFL}*), were generated by targeting the *ROSA26* locus using a construct encoding the human *MYC* cDNA, preceded by a *loxP* flanked STOP cassette and marked by a signaling deficient truncated version of human CD2 (*hCD2*) under the control of a internal ribosomal entry site (IRES) downstream of the inserted cDNA. Transgene transcription is controlled by the CAG promoter. *hCD2* reporter transgenic mice (*hCD2^{stopFL}*), were generated following the same procedures as for *MYC^{stopFL}*, whereas *hCD2* cDNA was used and transgene transcription is controlled by the endogenous *ROSA26* promoter. C57BL/6 mouse embryonic stem cells (Artemis pharmaceuticals) were used following described procedures⁵⁰.

Genetic systems to monitor, ablate or induce MYC expression

MYC expression was monitored using the (*Myc^{EGFP}*)³⁰ allele in which the endogenous *Myc* locus encodes a MYC fusion protein with EGFP. For conditional *Myc* ablation and/or enforced *MYC* expression we used a *Myc* conditional loss-of-function allele (*Myc^F*)³ and/or a newly generated *ROSA26* allele, termed *MYC^{stopFL}*. Conditional activation of the *MYC^{stopFL}* allele by cre-mediated recombination is traced by expression of *hCD2*. To report cre-mediated recombination in cells carrying the *Myc^F* allele, we combined it with a novel *ROSA26* reporter allele *hCD2^{stopFL}*. For targeted conditional *Myc* mutagenesis, we used either the *Cγ1-cre* transgene³⁵, or *Mx-cre*, where *cre* expression is under the control of an interferon-responsive promoter element⁴⁵. For enforced *Bcl6* expression, we used an allele that mimics a chromosomal translocation found in DLBCL (*IμBCL6*)³⁷. *EmuBCL-2* mice were used for ectopic *Bcl-2* expression³⁸. Mice carrying either the *Cγ1-cre* or the *Mx-cre* transgenes in combination with the *hCD2^{stopFL}* allele served as controls.

Immunization and acute *Myc* deletion

For T cell dependent immunization, eight- to 10-week-old mice were injected i.v. with 1×10^9 SRBCs (Cedarlane) in PBS. To induce acute *Myc* deletion in mature GC B cells, 10×10^6 CD43^{neg}hCD2^{neg} splenic B cells from either *Mx-cre hCD2^{stopFL}* or *Mx-cre Myc^{FF} hCD2^{stopFL}* mice were transferred into CD19^{KO} mice by i.v. injection. Mice were immunized the next day with SRBCs and given i.p. injections of 300μg of polyinosinic-polycytidylic acid (pIpC; Amersham Biosciences), at day 7 and 10 after immunization. GCs were analyzed at day 13 after immunization.

Flow cytometry

Single-cell suspensions were stained with the antibodies described below. To exclude dead cells we used Topro3 (Invitrogen). Samples were acquired on a FACSCanto II (BD) using FACSDiva software (BD), and analyzed using FlowJo software (Tree Star).

Flow cytometry antibodies		
Antigen	Clone	Company
Mouse_CD19	ID3	BD pharmigen
Mouse_CD95	Jo2	BD pharmigen
Mouse_CXCR4	2B11	BD pharmigen
Mouse_GL7	GL7	BD pharmigen
Mouse_CD40	3/23	BD pharmigen
Mouse_CD38	90	eBioscience
Mouse_CD93	AA4.1	eBioscience
Mouse_MHCII	M5/114.15.2	eBioscience
Mouse_B220	RA3-6B2	eBioscience
Mouse_CD69	H1.2F3	eBioscience
Human_CD2	TS1/8	Biolegend
Mouse_CD83	Michel-19	Biolegend
Mouse_CD21	7E9	Biolegend
Mouse_CD23	B3B4	Biolegend

ELISA and SRBC-specific antibody detection

These analyses were performed following described procedures³⁶.

Quantitative RT-PCR

These analyses were performed following described procedures³⁶. Primer sequences are described below. At least 3 biological samples were assayed in triplicate.

Primers sequences	
<i>Bcl6</i>	5'-GCCACGTTCCCGAGGAGA-3'
	5'-CGTCTGCAGCGTGTGCCTCT-3'
<i>IkBa</i>	5'-TTGGTCAGGTGAAGGGAGAC-3'
	5'-GCTTTCAGAAAGTGCCTCAGC-3'
<i>Irf4</i>	5'-AGGTCTGCTGAAGCCTTGGC-3'
	5'-CTTCAGGGCTCGTCGTGGTC-3'
<i>Mybl1</i>	5'-AGCGCCTGGGAAACCGTTGG-3'
	5'-GCCCTCCTGTTCCACTTTTCTTCGC-3'
<i>Ccnd2</i>	5'-TCGCAAGCTGCCCCAGCAAA-3'

Primers sequences	
	5'-GGCTGCTCCCACGCTTCCAG-3'
<i>Aicda</i>	5'-TAGTGCCACCTCCTGCTCACT-3'
	5'-CAACAATTCCACGTGGCAGCC-3'
<i>Ccnd3</i>	5'-CGGCGGGGTGTGATCACTCG-3'
	5'-TCCTGCGATGGCTCACGGGT-3'
<i>Myc</i>	5'-GCCAGCCCTGAGCCCTAGT-3'
	5'-GGGCTGTGCGGAGGTTTGCT-3'
<i>Hprt</i>	5'-GTCATGCCGACCCGAGTC-3'
	5'-GTCCTGTCCATAATCAGTCCATGAGGAATAAAC-3'

Histology and immunofluorescence

Spleens were fixed with 10% formalin (Sigma), paraffin-embedded and sections stained with hematoxylin (Sigma) and PNA (Vector). For immunofluorescence spleens were embedded in OCT (Sakura) and flash frozen in liquid nitrogen. Tissue sections were cut on a CM3050s cryostat (Leica), fixed in 4% paraformaldehyde (Sigma), and stained with the antibodies described below. Images were acquired with a Nikon Ti-E inverted microscope (Nikon) using Slidebook (3i) and visualized with Photoshop (Adobe). We performed the scoring of MYC and BCL-6 positivity in individual GC B cell clusters using single fluorescence channels and the “Cell Counter” plug-in in ImageJ software (<http://imagej.nih.gov/ij/>).

Immunofluorescence antibodies		
Antigen	Clone	Company
Mouse/Human_BCL-6	sc-C19	Santa Cruz
Mouse/Human_MYC	Y69	Epitomics
Mouse_BCL-6	mG1191 E	eBioscience
Mouse_CD3	145-2C11	BD pharmigen
Mouse_CD35	8C12	BD pharmigen

B cell DNA content and IgH somatic mutation

These analyses were performed following described procedures³⁶.

Microarray

Gene expression profiling was performed for 2 biological replicates of flow sorted MYC^{EGFP} positive and negative GC B cells of 10 day SRBC immunized *Myc*^{EGFP} mice using the mouse WG-6 v2.0 Expression BeadChip array (illumina).

Statistical analysis

Data were analyzed using Wilcoxon-Mann-Whitney test; a p value ≤ 0.05 was considered significant. A single asterisk (*) in the graphs of figures represents a p value ≤ 0.05 , double asterisks (**) a p value ≤ 0.01 , and triple asterisks (***) a p value ≤ 0.001 , and “ns” stands for not statistically significant, i.e., a p value >0.05 . Data in figures are represented as mean \pm standard error of the mean (SEM). Prism (GraphPad) was used for statistical analysis.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA)²⁶ was done using the Broad Institute Java implementation code. MYC associated gene-sets YU_CMyc_UP, YU_CMyc_DN, V\$MYC_Q2 and V\$MYC_MAX_B from the Broad Institute MSigDB were analyzed using the signal-to-noise ratio as ranking measure, and gene labels were permuted 1000 times for NES and FDR estimation. Array data for FO and GC B cells was from GEO:GSE15907 (ImmGen), raw data was processed with RMA background correction and quantile normalization. To create a high-confidence set of MYC up-regulated genes in the Burkitt Lymphoma context we curated the MYC ChIP-Seq peak regions from Seitz et al.²⁹ as follows: only peak regions with mus-musculus orthologous regions and a FDR $<1e-4$ were considered. After gene annotation of the filtered peak regions, only genes showing significant downregulation (adjusted p value ≤ 0.05 and $\text{Log}_2(\text{FC}) \leq -0.5$) in three Burkitt Lymphoma cell lines (via MYC siRNA²⁹) were considered to be functional targets of MYC. This gene set was used for GSEA testing of the FO and GC B cell (GEO:GSE15907) and of MYC^{EGFP} positive and negative GC B cells (GEO:GSE39443) gene expression profiles. The latter was also used for GSEA testing for the MYC gene sets YU_CMyc_UP, YU_CMyc_DN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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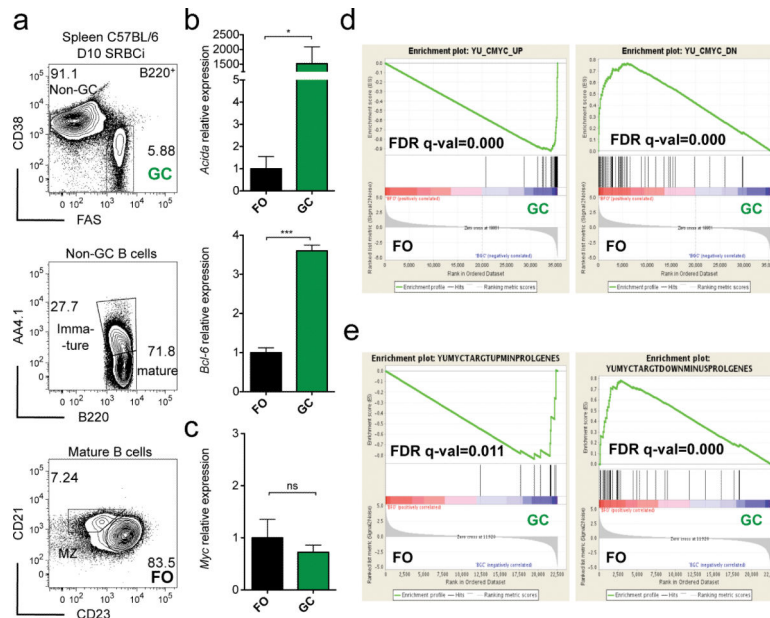


Figure 1. MYC target genes are enriched in GC B cells

(a) Flow cytometry analysis and gating strategy for flow sorting of splenic GC B cells ($B220^{pos}CD38^{low}CD95^{high}$) and FO B cells ($B220^{pos}CD38^{high}AA4.1^{neg}CD21^{pos}CD23^{high}$) of 10 day SRBC immunized (D10 SRBCi) C57BL/6 wild-type mice. (b) Real-time PCR analysis of *Bcl-6* and *Aicda* mRNA of flow sorted GC (green) and FO (black) B cells. Data are presented relative to *Hprt* and normalized to FO B cells, (mean and s.e.m. of triplicates). (c) Real-time PCR analysis of *Myc* mRNA as in (b). Data are presented as in (b). (d) GSEA of FO and GC B cell gene expression profiles for the MYC up-regulated (YU_MYC_TARGETS_UP, left panel) and downregulated (YU_MYC_TARGETS_DOWN, right panel) gene sets. (e) GSEA of FO and GC B cell gene expression profiles for MYC up-regulated (left panel) and downregulated (right panel) gene sets, as in (d) upon removal of genes, from those gene sets, whose GO-terms contain, 'proliferation', 'cell cycle' or 'growth', MINUSPROLGENES. FO and GC B cell gene expression profiles (GEO:GSE15907, ImmGen). See Methods for GSEA analysis details. Data are representative of 3 independent experiments (a-c). ns, not statistically significant. * p 0.05, ** p 0.01, *** p 0.001.

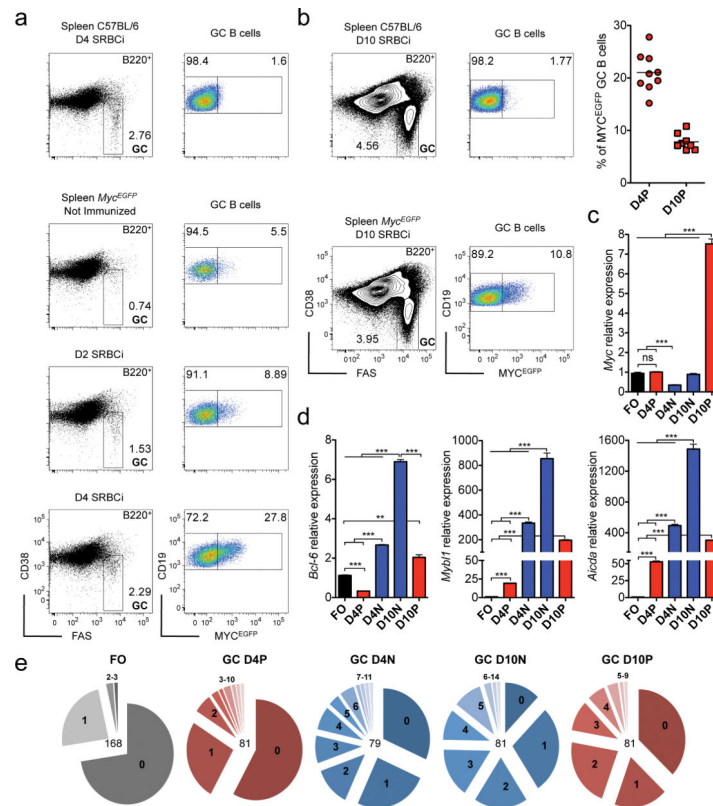


Figure 2. A fraction of both newly formed and mature GC B cells is positive for MYC
(a) Flow cytometry analysis of splenic GC B cells for MYC^{EGFP} expression of 4 day SRBC immunized (D4 SRBCi) C57BL/6 wild-type mice and of *Myc^{EGFP}* mice either not-immunized, 2 day (D2 SRBCi) and 4 day (D4 SRBCi) SRBC immunized. **(b)** Flow cytometry as in (a) of 10 day SRBC immunized (D10 SRBCi) C57BL/6 wild-type and *Myc^{EGFP}* mice. Right panel, graphical representation of the frequency of MYC^{EGFP} positive cells within GC B cells of D4 SRBCi and D10 SRBCi *Myc^{EGFP}* mice **(c)** Real-time PCR analysis of *Myc* mRNA of flow sorted FO and of day 4 MYC^{EGFP} positive (D4P) and negative (D4N) GC B cells, and day 10 MYC^{EGFP} positive (D10P) and negative (D10N) GC B cells. Data are presented relative to *Hprt* and normalized to FO B cells, (mean and s.e.m. of triplicates). **(d)** Real-time PCR analysis of *Bcl6*, *Mybl1* and *Aicda* mRNA as in (c). Data are presented as in (c). **(e)** *Igh* somatic mutation of FO B cells and D4P, D4N, D10N, D10P GC B cells. Numbers in the center of the pie-chart represent sequences analyzed, and in each pie-chart section the number of mutations. Data are representative of at least 3 (a,b), 5 independent experiments (c,d), and of 3 mice (f). ns, not statistically significant. * p 0.05, ** p 0.01, *** p 0.001.

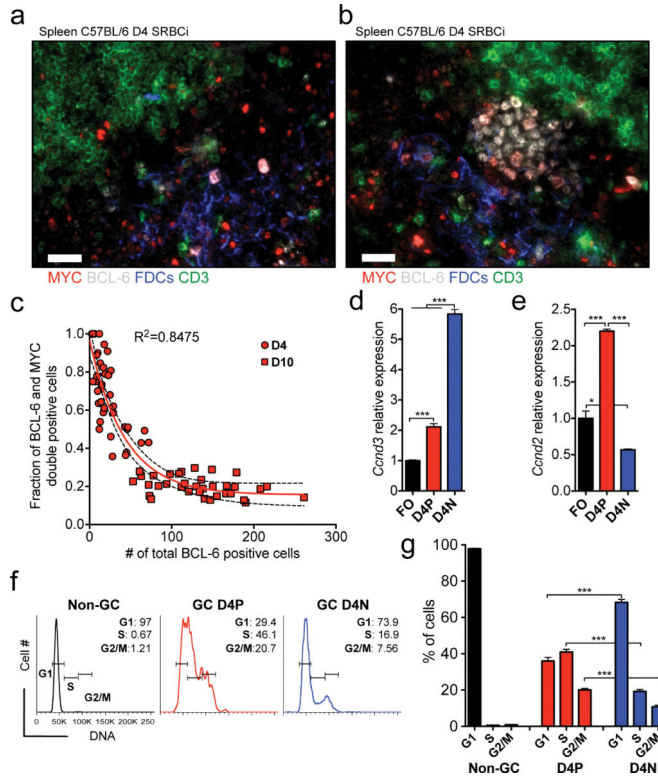


Figure 3. Early GC B cells express both MYC and BCL-6 and are hyperproliferative
(a) Representative image of histological immunofluorescence analysis of spleen of 4 day SRBC immunized (D4 SRBCi) C57BL/6 wild-type mice. T cells (CD3, green), FDCs (CD35, Blue), BCL-6 (white), MYC (red), cut-off of 20 BCL-6 positive cells per FDC area. **(b)** Representative image as in (a), cut-off of >20 BCL-6 positive cells per FDC area **(c)** Graphical representation of the fraction of BCL-6 and MYC double positive cells as function of BCL-6 positive cell count per FDC area. Data of GCs of D4 SRBCi (D4) and D10 SRBCi (D10) C57BL/6 wild-type mice. Each red symbol represents the analysis of one FDC area. Red line represents non-linear regression curve fit assuming an one-phase decay. Dashed line represents 95% confidence interval. **(d)** Real-time PCR analysis of *Ccnd3* mRNA, of flow sorted FO and of day 4 MYC^{EGFP} positive (D4P) and negative (D4N) GC B cells. Data are presented relative to *Hprt* and normalized to FO B cells, (mean and s.e.m. of triplicates). **(e)** Real-time PCR analysis of *Ccnd2* mRNA, as in (d). Data are presented as in (d). **(f)** Flow cytometry analysis of the DNA content of non-GC B cells, and of D4P and D4N GC B cells. **(g)** Graphical representation of the data presented in (f), (mean and s.e.m.). Scale bar 25µm (a, b). Data are representative of at least 3 (a-c,f,g) and 5 (d,e) independent experiments. ns, not statistically significant. * p 0.05, ** p 0.01, *** p 0.001.

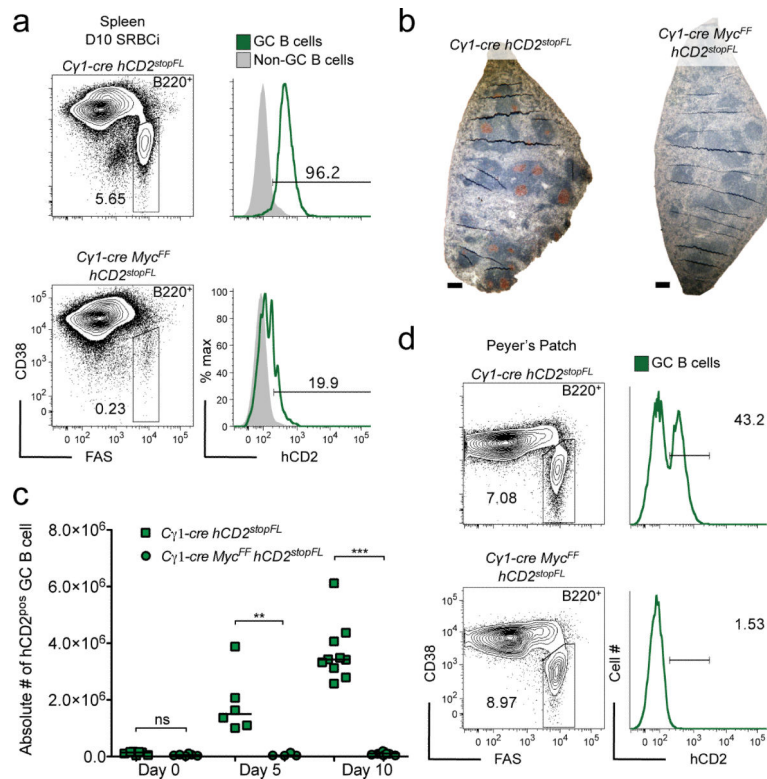


Figure 4. MYC is essential for GC formation

(a) Flow cytometry analysis of splenic GC B cells of 10 day SRBC immunized (D10 SRBCi) mice of the indicated genotypes, right panel analysis of reporter (hCD2) positive GC B cells. (b) Representative images of histological immunohistochemical analysis of spleen of D10 SRBCi mice of the indicated genotypes. PNA (brown) and hematoxylin as counterstain. (c) Graphical representation of flow cytometry data of splenic hCD2 positive GC B cells of mice of the indicated genotypes, either not immunized or 5 and 10 days after SRBC immunization. (d) Flow cytometry analysis of peyer's patch GC B cells of mice of the indicated genotypes, right panel analysis of hCD2 positive GC B cells. Scale bar 25 μ m (b). Data are representative of at least 3 independent experiments (a-d). ns, not statistically significant. * p 0.05, ** p 0.01, *** p 0.001.

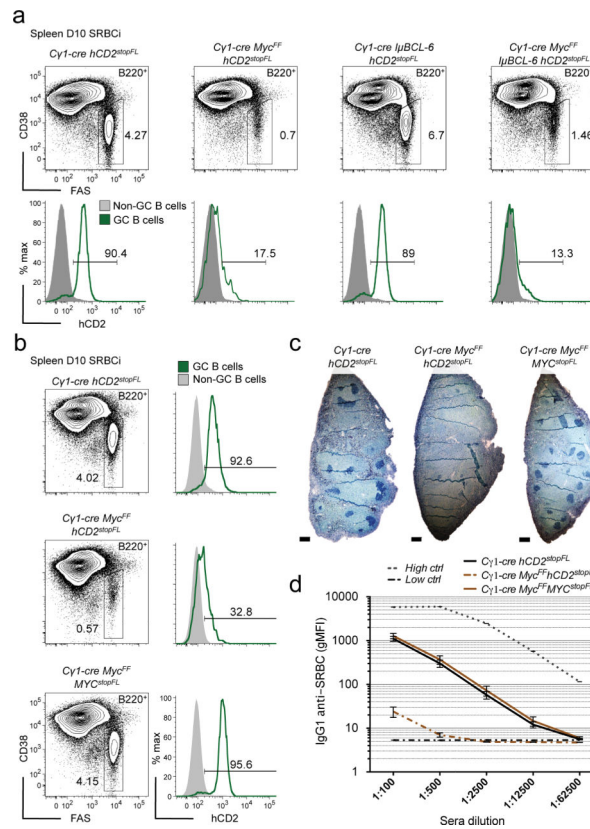


Figure 5. Enforced *Bcl6* expression fails to rescue GC formation upon *Myc* ablation

(a) Flow cytometry analysis of splenic GC B cells of 10 day SRBC immunized (D10 SRBCi) mice of the indicated genotypes, lower panel, analysis of reporter (hCD2) positive GC B cells. (b) Flow cytometry analysis as in (a), of mice of the indicated genotypes, right panel as in (a). (c) Representative images of histological immunohistochemical analysis of spleen of D10 SRBCi mice of the indicated genotypes. BCL-6 (dark blue). (d) Graphical representation of flow cytometry measured anti-SRBC specific IgG1 serum antibodies at D10 SRBCi, (mean and s.e.m.), Low ctrl, unimmunized mice, High ctrl, serum from mice hyperimmunized. Scale bar 25 μ m (c). Data are representative of at least 3 independent experiments (a-c) and of 4 mice (d).

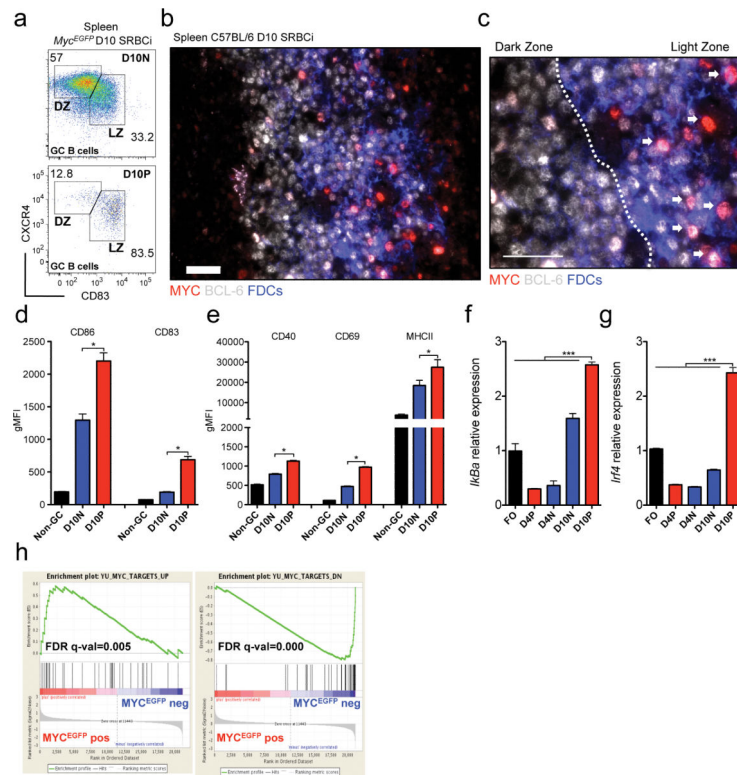


Figure 6. MYC positive GC B cells in mature GCs localize to the light zone and display an activated phenotype

(a) Flow cytometry analysis of splenic MYC^{EGFP} negative (D10N) and positive (D10P) GC B cells of 10 day SRBC immunized (D10 SRBCi) *Myc^{EGFP}* mice. DZ, dark zone; LZ, light zone. (b) Representative image of histological immunofluorescence analysis of spleen of D10 SRBCi C57BL/6 wild-type mice. FDCs (CD35, Blue), BCL-6 (white), MYC (red). (c) Inset, dark zone and light zone boundary, white dashed line. White arrows, MYC positive cells. (d) Graphical representation of flow cytometry analysis of CD86, CD83 expression of non-GC B cells, and of D10N and D10P GC B cells. gMFI, geometrical mean fluorescence intensity, (mean and s.e.m.) (e) Graphical representation as in (d) of CD40, CD69 and MHCII expression. Data are presented as in (d). (f) Real-time PCR analysis of *IγBa* mRNA of flow sorted FO B cells and of D10N and D10P GC B cells. Data are presented relative to *Hprt* and normalized to FO B cells, (mean and s.e.m. of triplicates). (g) Real-time PCR analysis of *Irf4* mRNA as in (f). Data are presented as in (f). (h) GSEA of D10 MYC^{EGFP} positive and negative GC B cell gene expression profiles, for the MYC up-regulated (YU_MYC_TARGETS_UP, left panel) and downregulated (YU_MYC_TARGETS_DOWN, right panel) gene sets. See Methods for GSEA analysis details. Scale bar 25μm (c,d). Data are representative 3 (a-c, d,e) and 5 (f,g) independent experiments. ns, not statistically significant. * p 0.05, ** p 0.01, *** p 0.001.

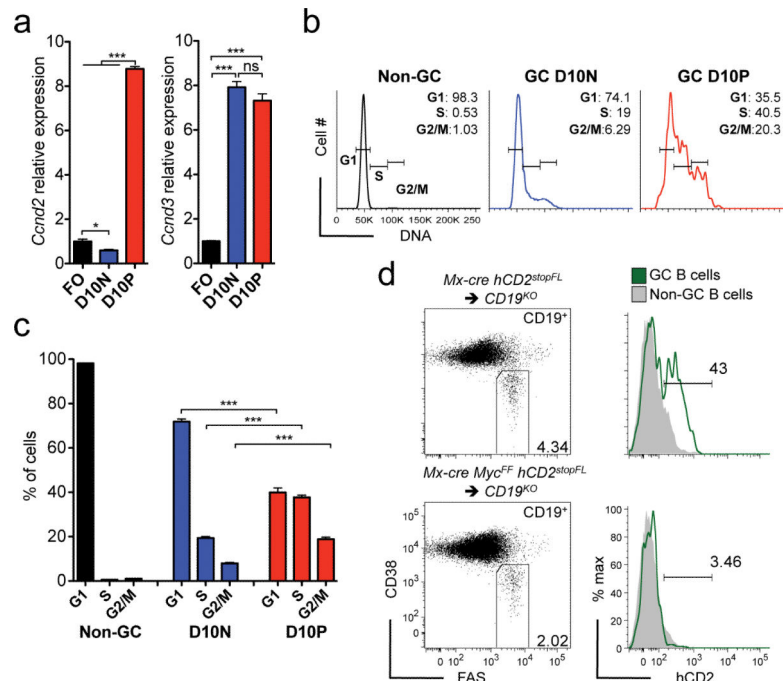


Figure 7. A large fraction of MYC positive mature GC B cells are in cell cycle, and these cells are essential for GC maintenance

(a) Real-time PCR analysis of *Ccdn2* and *Ccdn3* mRNA, of flow sorted FO and of day 10 MYC^{EGFP} negative (D10N) and positive (D10P) GC B cells. Data are presented relative to *Hprt* and normalized to FO B cells, (mean and s.e.m. of triplicates). (b) Representative analysis by flow cytometry of the DNA of non-GC B cells and of D10N and D10P GC B cells. (c) Graphical representation of data presented in (b), (mean and s.e.m.) (d) Flow cytometry analysis of splenic GC B cells 13 days after SRBC immunization in mice of the indicated genotypes. Right panel analysis of reporter (hCD2) positive GC B cells. Data are representative of 5 (a), 3 (b, c) and 2 (d) independent experiments.