

## Class-switch recombination occurs infrequently in germinal centers

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5  
6 Class Switch Recombination Occurs Infrequently in Germinal Centers

7  
8 Jonathan A. Roco<sup>1</sup>, Luka Mesin<sup>2</sup>, Sebastian C. Binder<sup>3</sup>, Christian Nefzger<sup>5</sup>, Paula  
9 Gonzalez-Figueroa<sup>1</sup>, Pablo F Canete<sup>1</sup>, Julia Ellyard<sup>1</sup>, Qian Shen<sup>1</sup>, Philippe A. Robert<sup>3</sup>,  
10 Jean Cappello<sup>1</sup>, Harpreet Vohra<sup>1</sup>, Yang Zhang<sup>6</sup>, Carla R. Nowosad<sup>2</sup>, Arien Schiepers<sup>2</sup>,  
11 Lynn M. Corcoran<sup>7,8</sup>, Kai-Michael Toellner<sup>6</sup>, Jose Polo<sup>5</sup>, Michael Meyer-Hermann<sup>3,4</sup>,  
12 Gabriel Victora<sup>2</sup>, and Carola G. Vinuesa<sup>1,9</sup>.

13  
14 *Affiliations:*

15  
16 <sup>1</sup>*Dept of Immunology and Infectious Disease & Centre for Personalised Immunology,*  
17 *The John Curtin School of Medical Research, The Australian National University,*  
18 *Canberra, Australia.*

19 <sup>2</sup>*Laboratory of Lymphocyte Dynamics, Rockefeller University, New York, NY, 10065,*  
20 *USA.*

21 <sup>3</sup>*Department of Systems Immunology and Braunschweig Integrated Centre of Systems*  
22 *Biology, Helmholtz Centre for Infection Research, Rebenring 56, 38106 Braunschweig,*  
23 *Germany.*

24 <sup>4</sup>*Institute for Biochemistry, Biotechnology and Bioinformatics, Technische Universität*  
25 *Braunschweig, Braunschweig, Germany.*

26 <sup>5</sup>*Department of Anatomy and Developmental Biology & Australian Regenerative*  
27 *Medicine Institute, Monash University, Wellington Road, Clayton, VIC 3800, Australia.*

28 <sup>6</sup>*Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham*  
29 *B15 2TT, UK.*

30 <sup>7</sup>*Molecular Immunology Division, The Walter and Eliza Hall Institute of Medical*  
31 *Research, Parkville, Victoria, Australia.*

32 <sup>8</sup>*Department of Medical Biology, The University of Melbourne, Parkville, Victoria,*  
33 *Australia.*

34 <sup>9</sup>*China-Australia Centre for Personalised Immunology, Dept of Rheumatology,*  
35 *Shanghai Renji Hospital, Shanghai JiaoTong University, Shanghai, China.*

36  
37  
38 **Corresponding author:** Carola G Vinuesa ([carola.vinuesa@anu.edu.au](mailto:carola.vinuesa@anu.edu.au)).

39 **Lead author:** Jonathan Roco ([jonathan.roco@anu.edu.au](mailto:jonathan.roco@anu.edu.au))

## 40 **Summary**

41 **Class switch recombination (CSR) is a DNA recombination process that replaces**  
42 **the immunoglobulin (Ig) constant region for the isotype that can best protect**  
43 **against the pathogen. Dysregulation of CSR can cause self-reactive BCRs and**  
44 **B cell lymphomas; understanding the timing and location of CSR is therefore**  
45 **important. Although CSR commences upon T cell priming, it is generally**  
46 **considered a hallmark of germinal centers (GCs). Here we have used multiple**  
47 **approaches to show that CSR is triggered prior to differentiation into GC B cells**  
48 **or plasmablasts and is greatly diminished in GCs. Despite finding a small**  
49 **percentage of GC B cells expressing germline transcripts, phylogenetic trees of**  
50 **GC BCR from secondary lymphoid organs revealed that the vast majority of CSR**  
51 **events occurred prior to the onset of somatic hypermutation. As such, we have**  
52 **demonstrated the existence of IgM-dominated GCs, which are unlikely to occur**  
53 **under the assumption of ongoing switching.**

54

## 55 **Introduction**

56 Class switch recombination (CSR) is an intrachromosomal DNA rearrangement of the  
57 immunoglobulin (Ig) heavy chain locus. As a result, IgM-IgD mature B cells are able to  
58 express antibodies of the IgA, IgG or IgE classes that differ in effector functions,  
59 without altering the specificity for the immunizing antigen ([Stavnezer et al., 2008](#)). CSR  
60 relies primarily on activation of the enzymes Activation-induced cytidine deaminase  
61 (AID), uracil-DNA glycosylase (UNG) and Apurinic-Apyrimidinic Endonuclease 1  
62 (APE1) to specifically target intronic areas called switch (S) regions ([Guikema et al.,](#)  
63 [2007](#); [Muramatsu et al., 2000](#); [Rada et al., 2002](#)). DNA breaks introduced by these  
64 enzymes lead to the recombination of the variable heavy chain (VDJ) segment with a  
65 different constant heavy (C<sub>H</sub>) chain gene (isotype) ([Stavnezer et al., 2008](#)). Selection  
66 of the appropriate isotype during infection is driven by cytokines and T cell help  
67 ([Kawabe et al., 1994](#); [Snapper and Mond, 1993](#)), which induce transcription across the  
68 specific S regions with production of germline transcripts (GLTs) ([Lorenz et al., 1995](#)).  
69 GLTs (also known as switch-transcripts) are spliced, polyadenylated non-coding  
70 mRNAs transcribed from specific promoters located upstream of each set of S regions

71 (except for IgD). Expression of GLTs precedes DNA recombination of the Ig-C<sub>H</sub> genes  
72 in B cells primed to undergo CSR (Stavnezer, 1996). For this reason, GLTs have long  
73 been used as a reliable molecular marker to study the onset of CSR *in vivo* ([Cogné](#)  
74 [and Birshtein, 2004](#); [Lorenz et al., 1995](#)).

75

76 Germinal centers (GCs) are specialized microenvironments in secondary lymphoid  
77 organs, formed upon immunization. GCs are critical for the formation of long-lived  
78 plasma cells and memory B cells. Within these structures, B cells undergo somatic  
79 hypermutation (SHM) and clonal selection based on the affinity of the BCR for the  
80 immunizing antigen. Likewise, GCs are also considered to be the main areas where  
81 CSR takes place ([Klein and Dalla-Favera, 2008](#); [Vinuesa et al., 2009](#)). Although SHM  
82 and CSR are two independent processes, both depend on the activity of AID  
83 ([Muramatsu et al., 2000](#)), which is expressed at highest amounts in GC B cells. These  
84 observations might have reinforced the idea that CSR is predominantly a GC process.

85

86 CSR has been predominantly studied using *in vitro* culture systems or after clonal  
87 expansion of B cells *in vivo*. In these studies, an important role of the GC environment  
88 in CSR has been postulated after finding GLTs are predominantly expressed by human  
89 cells bearing centrocyte-specific markers, including absence of CD77 ([Liu et al., 1996](#)).

90 However these markers have been later shown to also identify activated B cells and  
91 plasmablasts ([HogerCorp and Borrebaeck, 2006](#)). The association between CSR and  
92 GCs also comes from studies in genetically-manipulated mice with impaired formation  
93 of follicular structures that are likely to also perturb early T cell:B cell encounters  
94 ([Shinkura et al., 1996](#)). Nevertheless, extrafollicular (EF) responses are known to  
95 produce switched antibodies and induction of CSR has been detected as early as day  
96 2 during a primary immune response ([Cerutti, 2008](#); [Fagarasan et al., 2001](#); [Jacob et](#)  
97 [al., 1991](#); [Pape et al., 2003](#); [Toellner et al., 1996](#)). Despite this evidence, it is still

98 generally believed that isotype-switching is an ongoing process that continues and is  
99 enhanced within GCs.

100

101 Here, we used transgenic mouse models that allow us to unequivocally distinguish GC  
102 B cells, extrafollicular plasmablasts (EFPBs) and their precursors from the earliest  
103 stages of an immune response *in vivo*. We show that CSR is initiated over the first few  
104 days in a primary response and prior to EF and GC commitment ceasing soon after B  
105 cells become GC cells and SHM commences. We also demonstrate the existence of  
106 IgM-dominated GCs which are unlikely to occur under the assumption of ongoing  
107 switching.

108

## 109 **Results**

110 **GLT expression is triggered at the early stages of B cell activation and rapidly**  
111 **declines within GCs.**

112 In order to identify the cells in which CSR is first triggered we took advantage of SW<sub>HEL</sub>  
113 mice in which ~5-15% of the B cells carry a high-affinity BCR against hen egg  
114 lysozyme (HEL) ([Phan et al., 2003](#)). We adoptively transferred 3-15 x 10<sup>4</sup> SW<sub>HEL</sub> B  
115 cells into C57BL/6 mice along with mutated HEL (HEL<sup>2x</sup>) protein conjugated to sheep  
116 red blood cells (SRBCs) (Fig. 1A). SW<sub>HEL</sub> B cells bind HEL<sup>2x</sup> with moderate affinity and  
117 undergo CSR and SHM normally ([Paus et al., 2006](#)). Practically, all transferred B cells  
118 are known to be recruited into the response ([Chan et al., 2009](#)). As described in these  
119 previous studies, upon HEL-SRBC immunization adoptively-transferred SW<sub>HEL</sub> B cells  
120 first appeared at the T cell:B cell border on day 1.5, at the periphery of the follicles on  
121 day 2.5, and within primary follicles on day 3 (Fig. 1B). On day 3.5, HEL-binding B cells  
122 were found forming nascent GCs and HEL-binding extrafollicular plasmablasts  
123 (EFPBs) were also seen (Fig. 1B). A homogeneous B cell population was observed by  
124 flow cytometry up to day 3 (Fig. 1C). Consistent with the immunofluorescence findings,

125 day 3.5 marked the appearance of GC B cells and EFPBs by flow cytometry. EFPBs  
126 were distinguished by downregulation of the chemokine receptor CXCR5 as well as  
127 B220 (Fig. 1C), shown to occur as B cells express BLIMP1 (Fig. 1D and S1A) and  
128 localize to extrafollicular foci ([Chan et al., 2009](#)). GC B cells seen at day 3.5 remained  
129 CXCR5<sup>hi</sup> B220<sup>hi</sup> (Fig. 1C) and also expressed FAS death receptor (Fig. S1A). These  
130 populations were sorted at 12h-24h intervals from day 1.5 after immunization and  $\gamma$ 1  
131 and  $\gamma$ 2b-GLTs, the most abundant isotypes in the SRBC response ([Phan et al., 2005](#)),  
132 were quantified by qPCR. GLTs were first seen at day 1.5, peaked at day 2.5-3 prior  
133 to GC formation and declined rapidly thereafter to become barely detectable 48 hours  
134 later (Fig. 1E). Of note, total RNA amounts used for PCR amplification and RNA quality  
135 were comparable throughout the time-course (Fig. S2A). *Aicda* mRNA (encoding AID)  
136 was first detected at day 2.5; 12h after production of the first GLT and 24h prior to the  
137 appearance of EFPB or GC (Fig. 1E). Expression of *Bcl6*, the transcription factor  
138 required for GC B cell differentiation, was first detected at day 3.5 (Fig. 1E). Thus, CSR  
139 is triggered prior to EF or GC B cell commitment and GLTs decline prior to GC  
140 formation.

141

142 **Class-switched antibodies are detected prior to GC formation and are**  
143 **comparable within GCs and EFPBs.**

144 We next compared the production of surface IgG in GCs and EF foci (Fig. 2A-D). Class-  
145 switched B cells first appeared at day 2.5, one day after detection of the first GLT (Fig.  
146 2B), coinciding with the earliest detection of *Aicda* mRNA (Fig. 1E). IgG<sup>+</sup> cells  
147 increased exponentially over the following two days, reaching a plateau from days 4.5  
148 to 6.5 with approximately 70% of GC B cells and 90% of EFPBs having switched (Fig.  
149 2C and 2D and Fig. S2B). The paucity of IgM<sup>+</sup> EFPBs at this time point may be due to  
150 the greater proliferative ability of switched cells ([Martin and Goodnow, 2002](#); [Tangye](#)  
151 [et al., 2003](#)).

152

153 The affinity of SW<sub>HEL</sub> B cells for HEL<sup>2x</sup> is still quite high ( $8 \times 10^7 \text{ M}^{-1}$ ) and there is some  
154 evidence that signal strength may play a role in driving early B cell events ([Chan et al.,](#)  
155 [2009](#); [Paus et al., 2006](#)). To exclude the possibility that strong and uniform early-  
156 switching may have been driven by affinity; we repeated the experiments using HEL<sup>3x</sup>,  
157 which carries an additional mutation that further lowers the affinity of the SW<sub>HEL</sub> BCR  
158 interaction to  $1.5 \times 10^6 \text{ M}^{-1}$  ([Paus et al., 2006](#)). We observed a small delay in the  
159 formation of GCs using HEL<sup>3x</sup>; at day 3.5 HEL-binding B cells still appeared as a single  
160 population of activated B cells that haven't yet become GC cells (Fig. S3A). As seen  
161 with HEL<sup>2x</sup>,  $\gamma$ 1-GLT peaked at this pre-GC stage and a dramatic decline in GLTs was  
162 seen at the peak of the GC reaction (Fig. S3B-C). These results confirm that early and  
163 rapidly declining switching is a reproducible finding.

164

#### 165 **Germline transcription is rapidly induced at the T cell:B cell border**

166 We next sought to visualize the precise location in which isotype-switching was  
167 initiated in C57BL/6 mice with a polyclonal BCR repertoire. For this we used C $\gamma$ 1-  
168 Cre:mT/mG mice ([Casola et al., 2006](#); [Muzumdar et al., 2007](#)), in which production of  
169 the  $\gamma$ 1-GLT can be tracked by GFP expression. After SRBC immunization the first  
170 GFP<sup>+</sup> cells were detected on day 2 and were found predominantly at the T cell:B cell  
171 border (Fig. 2E). By day 3, GFP<sup>+</sup> cells had expanded and could be found within primary  
172 follicles that had yet not formed GCs. Within the following 48h GFP<sup>+</sup> cells were seen  
173 filling both GCs and extrafollicular foci. Thus, similar to the SW<sub>HEL</sub> B cell response,  
174 class-switching is induced outside the follicles, 24h prior to GC formation.

175

#### 176 **Single cell profiling of DZ and LZ B cells reveals that germline transcripts are** 177 **greatly diminished within GCs.**

178 To ensure we were not underestimating GLT production in light zone (LZ) cells  
179 (centrocytes), thought to be the B cell subset in which germline transcription is  
180 activated ([Liu et al., 1996](#)), we performed single cell qPCR studies (Fig. 3A-E). Single

181 day 3 IgM<sup>+</sup> and day 6.5 IgM<sup>+</sup> SW<sub>HEL</sub> GC B cells ([Brink et al., 2015](#)), subdivided into LZ  
182 and dark zone (DZ) cells according to CXCR4 and CD86 expression (Fig. 3A-B and  
183 Fig. S4A-B), were flow cytometry purified. Compared to 52% of day 3 activated B cells  
184 expressing  $\gamma$ 1-GLT, less than 3% of DZ or LZ B cells expressed this transcript (Fig.  
185 3C-E). Similar results were observed for  $\gamma$ 2b-GLT, with 45% of day 3 B cells positive  
186 but only 4.8% and 1% of DZ and LZ B cells respectively expressing this GLT (Fig. 3C-  
187 E). Of note, 38% of GLT-positive B cells expressed more than one GLT.

188

189 It has been suggested that CSR requires Foxo1 and c-Myc transcription factor co-  
190 expression ([Sander et al., 2015](#)). Consistent with this, ~70% of day 3 B cells were  
191 Foxo1 and c-Myc double positive, and ~80% of these double positive cells expressed  
192 GLTs (Fig. S4C-D). By contrast, despite 41% of LZ cells expressing *Foxo1*, and 9%  
193 co-expressing *Foxo1* and *c-Myc* (Fig. S4C-D), none of the *Foxo1* and *c-Myc* double  
194 positive LZ cells expressed GLTs (Fig. S4D). This was also true for DZ B cells, with no  
195 double positive cells expressing GLTs (Fig. S4D). These results support the notion that  
196 GLT production leading to activation of CSR is not a feature of mature GCs.

197

### 198 **Germline transcripts remain low in long-lived GCs.**

199 We next sought to investigate responses to a different antigen and adjuvant and  
200 exclude the possibility that short-lived GCs such as those induced by SRBC may not  
201 be favorable to ongoing switching. For this, we transferred NP-reactive B1-8<sup>hi</sup>  
202 tdTomato<sup>+</sup> (tdT<sup>+</sup>) cells together with NP-CGG ([Shih et al., 2002](#)) into C57BL/6 mice  
203 primed with CGG in CFA 3 days earlier (Fig. 4A and Fig. S5A). This priming strategy  
204 makes the kinetics of the first few days comparable to that shown for SW<sub>HEL</sub> responses  
205 (Fig. 1C), but with GCs persisting longer (Fig. 4B). Similar to the HEL<sup>2x</sup>-SRBC  
206 response,  $\gamma$ 1-GLT peaked between day 2-2.5 (Fig. 4C); at this time point, GLTs were  
207 found in cells with an intermediate phenotype (CD38<sup>int</sup> Fas<sup>int</sup>) between EFPB and GC  
208 B cells (Fig. 4B-4C). GLTs had declined considerably by day 6.5 (Fig. 4C). At a later



209 stage of this immune response (day 14-18, Fig. 4D-4E), GC B cells maintained low  
210 amounts of  $\gamma$ 1-GLT (Fig. 4F). Consistent with early induction of CSR, IgG expression  
211 in NP-binding B cells was first seen at day 2 (Fig. 4G). IgG<sup>+</sup> GC B cells peaked and  
212 reached a plateau between day 4 and day 8, remaining at constant numbers through  
213 day 18 (Fig. 4G), a period in which GCs were sustained (Fig. 4H). Thus, there was no  
214 evidence of reactivation or increased rates of Ig-switching in the late stages of the GC  
215 response.

216

217 The product of the episomes looped-out from the IgH locus after C<sub>H</sub> gene  
218 recombination, known as switch circle transcripts, were assessed in all the adoptive  
219 transfer experiments performed. Despite efficient detection of switch circle transcripts  
220 in B cells activated *in vitro* (Fig. S5B-C), these byproducts of CSR could not be  
221 detected at any timepoint during the *in vivo* primary responses, either in pooled or  
222 single antigen-specific B cells (data not shown). This is likely to be due to the transient  
223 nature of these molecules and the low number of copies generated by a fraction of  
224 antigen-specific B cells. Of note, published studies measuring switch circle transcripts  
225 were conducted using *in vitro* culture systems or higher numbers of responding B cells  
226 such as those seen during reactivation of memory B cells ([Kinoshita et al., 2001](#); [Liu  
227 et al., 1996](#); [McHeyzer-Williams et al., 2015](#); [Wesemann et al., 2011](#)).

228

### 229 **Phylogenetic analysis reveals CSR occurs prior to the onset of SHM.**

230 In order to assess CSR in mice with a polyclonal repertoire without the need for  
231 adoptive transfers that might lead to underestimation of ongoing switching, we  
232 determined the timing of CSR in polyclonal GCs through phylogenetic analysis (Fig.  
233 5A-C). For this, we performed *in situ* photoactivation of single GCs within lymph nodes  
234 from GFP photoactivatable (GFP-PA) mice ([Tas et al., 2016](#); [Victoria et al., 2010](#)). This  
235 allows cells within the same GC to be fluorescently tagged, and then flow cytometry-  
236 sorted as single GC B cells. GFP-PA mice were immunized with CGG in alum, and

237 photoactivation followed by flow cytometry-purification performed 15 or 20 days later,  
238 to allow multiple rounds of division and SHM within GCs. The SHM burden in the V-  
239 region and the induction of recombination in the C<sub>H</sub> region was assessed by *Igh* mRNA  
240 sequencing in each cell. With this approach, clonal trees containing both switched and  
241 unswitched B cells can be used to establish the timing of CSR, where the CSR point  
242 can be inferred as the last common ancestor of the switched and unswitched cells.  
243 Thus, the number of somatic mutations at the inferred CSR point serves as a  
244 “molecular time stamp”, which can be compared to the total SHM burden of cells  
245 present in the GC at the time of analysis: CSR points occurring in cells with zero  
246 mutations would indicate CSR precedes SHM and therefore occurs prior to GC onset;  
247 whereas CSR points occurring in cells that have accumulated mutations would suggest  
248 CSR is an ongoing process in GCs.

249

250 IgG1 is the most common isotype found in the CGG-alum response. To maximize the  
251 possibility of identifying IgM to IgG1 CSR events, we screened GCs for expanded IgM<sup>+</sup>  
252 and IgG<sup>+</sup> B cell clones, including in the analysis clones containing ≥4 IgM<sup>+</sup> cells (Fig.  
253 S6A). This approach led to sequencing 13 clones, including all 8 clones that contained  
254 both IgM and IgG cells (Fig. 5A-B). Phylogenetic trees were displayed against the  
255 number of somatic mutations in each cell (x-axis in Fig. 5B), where the inferred CSR  
256 points are depicted as red filled triangles (Fig. 5B). We found that several trees  
257 remained IgM, indicative of not having switched after entering the GC; these were  
258 attributed a CSR point of zero (red open triangles, Fig. 5B). Whereas the overall  
259 mutation burden in GC B cells was substantial (Fig. 5C, mean of 5 mutations per cell),  
260 most clones had switch points at zero mutations, with a few at one mutation, and only  
261 a single clone underwent switching at an inferred branch point bearing four mutations.  
262 Importantly, we found a number of highly expanded and diversified clones (e.g, the top  
263 two clones at days 15 and 20 in Fig. 5B) for which CSR was either not detected or  
264 occurred in a common precursor with 0 or 1 mutations (Fig. 5B-C). Thus, substantial

265 SHM can occur in the absence of detectable CSR. Of note, no sequential switching  
266 events were detected in GCs, with IgG1, IgG2b and IgG3 always arising directly from  
267 IgM<sup>+</sup> cells (Fig. 5B). Similar findings were obtained from analysis of clonal trees within  
268 GCs from Peyer's patches: there were no CSR events within GC regardless of whether  
269 the trees were enriched in IgM<sup>+</sup>, IgA<sup>+</sup> or IgG2b<sup>+</sup> cells (Fig. SX 5D-E). This data supports  
270 previous evidence that IgA switching predominantly occurs outside germinal centers  
271 ([Reboldi et al., 2016](#)). Mutational analysis of polyclonal GCs supports that CSR is  
272 restricted to the pre-GC or early GC periods, and is uncommon after cells have  
273 accumulated several mutations in mature GCs.

274

#### 275 ***In-silico* modelling of CSR predicts lack of ongoing switching in GCs.**

276 We found several IgM-dominated GCs (Fig. 5A, green-dominated columns). Prominent  
277 IgM<sup>+</sup> GC responses have been previously reported in responses to SRBC cells,  
278 although these are relatively short-lived ([Shinall et al., 2000](#)). Our detection of IgM-  
279 dominated GCs late in the response argues against ongoing switching. This prompted  
280 us to analyze *in silico* whether constant versus decaying CSR would differ in the  
281 distribution of isotype-dominated GCs ([Binder and Meyer-Hermann, 2016](#); [Meyer-  
282 Hermann, 2014](#); [Meyer-Hermann et al., 2012](#)). Analysis of the isotype diversity of *in  
283 silico* GCs revealed that with constant switching, the probability of finding IgM-  
284 dominated GCs was negligible (Fig. 5F), even when combined with a preferential  
285 output of switched GC B cells (Fig. 5G) ([Martin and Goodnow, 2002](#)). These results  
286 were generated with constant influx of IgM-dominated B cells until day 6 post  
287 immunization but still hold true with ongoing influx of IgM<sup>+</sup> B cells for longer time  
288 periods (Fig. S7A-B) or with a higher antigen-uptake rate of IgM B cells (Fig. S7C-D).  
289 Thus, given the observed mean IgG dominance of GCs at late time points, no tested  
290 isotype-specific differences allowed induction of both, IgM- and IgG-dominated GCs in  
291 the same setting, as required by our experimental results. In contrast, with a decaying  
292 switching probability using a decay constant derived from the dynamics of  $\gamma$ 1-GLT

293 expression (Fig. 1E), a broad spectrum of IgM or IgG-dominated GCs develops (Fig.  
294 5H). As we have seen IgM-dominated GCs (Fig. 5A-B), this result supports a model  
295 with CSR limited to the first days of the GC reaction. Next, we shifted the time at which  
296 the decay starts from day 3.5 post-immunization (GC onset) to day 19.5 in our *in silico*  
297 model, keeping the integrated switching probability unchanged (Fig. 5I). The resulting  
298 isotype diversity of GC simulations dropped when CSR occurred later than day 6-7  
299 post-immunization (Fig. 5I). This suggests that a determination of the isotype before  
300 the GC phase of intense B cell selection promotes diversification of the GC isotype  
301 dominance at later times while an ongoing switching probability would homogenize the  
302 isotype distribution of GC output.

303

#### 304 **APE1 endonuclease is downregulated in germinal center B cells**

305 Our data so far demonstrated that downregulation of GLT production is a potent  
306 mechanism to dampen CSR in GCs. We considered the possibility of additional  
307 mechanisms that might contribute to limit CSR within GCs operating downstream of  
308 GLT production. Once GLTs have been produced, CSR relies on activation of AID,  
309 uracil-DNA glycosylase (UNG) and APE1 to target switch-regions ([Guikema et al.,  
310 2007](#); [Muramatsu et al., 2000](#); [Rada et al., 2002](#)). APE1 is required in a dose-  
311 dependent manner during CSR ([Masani et al., 2013](#); [Xu et al., 2014](#)), whereas it is  
312 dispensable for SHM ([Stavnezer et al., 2014](#)). By contrast, SHM does not need APE1  
313 but instead requires APE2 ([Masani et al., 2013](#); [Sabouri et al., 2009](#); [Stavnezer et al.,  
314 2014](#)). We compared RNA expression data of these enzymes in human naïve vs GC  
315 B cells. Expression of *AID*, *UNG* and *APEX2* were all increased in GC B cells, whereas  
316 *APEX1* appeared downregulated (Figure 6A). These observations are consistent with  
317 a previous report in bulk mouse GC B cells ([Stavnezer et al., 2014](#)). We next asked  
318 whether APE1 downregulation is a feature of both LZ and DZ B cells (Fig. 6B). Our  
319 data revealed a significant decline in APE1 protein in both DZ and LZ areas of human  
320 GCs (Fig. 6B-E). Thus, in addition to greatly diminished GLT induction in GC B cells,

321 APE1 downregulation emerges as an additional back up mechanism to prevent CSR  
322 in GCs.

323

### 324 **BCL6 binds the promoter region of $\gamma$ 1-GLT and APE1**

325 We next considered whether the BCL6 transcriptional repressor that determines GC B  
326 cell fate could contribute to the downregulation of GLTs, *Apex1* or both. BCL6 has  
327 been reported to bind to the  $\epsilon$ -GLT promoter ([Audzevich et al., 2013](#)). We therefore  
328 tested whether this was also the case for the promoter of  $\gamma$ 1-GLT. Bioinformatic  
329 analysis in the mouse promoter region of  $\gamma$ 1-GLT revealed the presence of putative  
330 binding sites for BCL6 (Fig. S8A-C). We used CHIP-qPCR to validate this observation  
331 using the Nojima coculture system to induce *in vitro* derived GC (iGC) B cells ([Nojima  
332 et al., 2011](#)). We found that indeed, BCL6 binds the promoter region of  $\gamma$ 1-GLT (Fig.  
333 S8D-E). Our analysis of published CHIP-on-chip data ([Ci et al., 2009](#)) revealed BCL6  
334 was bound to the *APEX1*, but not *APEX2* promoter regions in human primary GC B  
335 cells (Fig. 6F) as well as to the promoters of the well-established BCL6 targets, *TLR1*  
336 and *BCL6*. Thus, BCL6 may also act as key regulator of CSR by actively repressing  
337 the transcription of GLTs and APE1 expression in GC B cells.

338

## 339 **Discussion**

340 Here we have shown that, unlike the prevailing idea that CSR is favored in GCs, this  
341 process largely occurs outside GCs. The association between CSR and GCs was first  
342 established when AID was discovered and shown to be expressed mainly in GCs, and  
343 essential for both SHM and CSR ([Muramatsu et al., 2000](#)). Further studies mapped  
344 the cells expressing GLTs in humans to centrocytes on the basis of markers no longer  
345 used ([Liu et al., 1996](#)) since they identify a heterogenous population that also contains  
346 activated B cells ([HogerCorp and Borrebaeck, 2006](#)). Studies describing lack of CSR

347 in mice lacking GCs further cemented the notion that CSR was a GC process; these  
348 mice also had defective T cell:B cell encounters needed for CSR.

349

350 We have provided several lines of evidence that collectively show that isotype  
351 switching is activated before GC formation and rapidly declines as B cells differentiate  
352 in EFPBs or GCs during a primary immune response against TD-antigens. CSR, is  
353 initiated as early as day 1.5 following immunization and before GC formation via the  
354 production of GLTs. Analysis of GLT expression at the single cell level in purified IgM<sup>+</sup>  
355 activated B cells and IgM<sup>+</sup> GC B cells provided evidence that GLTs were produced at  
356 very small amounts in mature GCs. Phylogenetic trees constructed from Igh-mRNA  
357 sequencing of photoactivated GC B cells demonstrated that CSR ceased soon after  
358 the onset of SHM. These trees also revealed the existence of IgM-dominated GCs,  
359 which are unlikely to occur under the assumption of ongoing-switching. GLTs were first  
360 visualized at the T cell:B cell border prior to GC formation in a polyclonal B cell  
361 response using Cγ1-Cre:mT/mG mice and a comparable proportion of class-switched  
362 B cells were found in the EF and follicular areas of the spleen, with no further  
363 enrichment within GCs. This was independent of the immunizing antigen, the longevity  
364 of the GC response or the adjuvant used. Together, our work demonstrates early CSR  
365 with rapid decline in GCs, challenging the general belief that CSR is predominantly a  
366 GC process.

367

368 IgM<sup>+</sup> memory B cells are likely to be necessary to maintain a reservoir of antigen-  
369 experienced B cells ([Reynaud et al., 2012](#)) that can be rapidly engaged upon infection  
370 with either evolving or antigenically-related pathogens ([Bernasconi et al., 2002](#); [Pape](#)  
371 [et al., 2011](#)) but are not functionally committed to any particular effector process, which  
372 includes preserving their capacity to switch to any isotype. This, in turn, would result  
373 in a much broader spectrum of antigen-specific B cells, both in terms of BCR affinities  
374 and functional properties, filling the memory B cell compartment after each

375 immunization. Our *in silico* modeling suggests that with constant CSR, the probability  
376 of IgM<sup>+</sup> GC B cells, and by extrapolation antigen-experienced IgM<sup>+</sup> memory B cells, is  
377 greatly diminished. By contrast, an early down-regulation of switching increases  
378 isotype diversity. It is well known that humoral memory in humans can last for many  
379 years, even decades. IgM<sup>+</sup> memory B cells are considered more stable over time  
380 compared to their class-switched counterpart, and thus represent a reservoir of  
381 lymphoid-memory that can be induced at recall when the titers of primary protective  
382 antibodies have declined ([Pape et al., 2011](#)).

383

384 Lack of ongoing switching in GCs may be an important determinant of GC kinetics  
385 given the evidence that IgM and IgG tails have different influences on B cell behavior  
386 ([Martin and Goodnow, 2002](#)). It is possible that the larger GCs found in GCs of AID-  
387 deficient mice that cannot undergo CSR and are therefore IgM-dominated ([Muramatsu  
388 et al., 2000](#)) may reflect a greater permanence of IgM in GCs compared to their IgG  
389 counterparts. There is some evidence that switched and unswitched memory B cells  
390 have different transcriptional profiles ([Seifert et al., 2015](#)) suggesting important  
391 functional differences. Understanding what these differences are will also help explain  
392 further the biological significance of reducing CSR in GCs. It is likely that low  
393 expression of AID, which is first detected on day 2.5, in conjunction with APE1 are  
394 sufficient for CSR; whereas high expression of AID and APE2 in GCs might be required  
395 for SHM. APE1 protein expression was reduced in GC B cells, including DZ and LZ B  
396 cells. This data was confirmed by RNA-seq and in single cell studies, where fewer GC  
397 B cells expressed *Apex1* mRNA compared to early-activated B cells. Whilst AID is  
398 lowly expressed in recently activated B cells compared to GC B cells, CSR is  
399 considered very efficient in nature ([Stavnezer et al., 2008](#)): AID can attack anywhere  
400 within the S region, which can extend as long as 10-12kb, resulting in a high likelihood  
401 of inducing the DNA breaks required for CSR. We therefore propose that CSR is  
402 induced early during B cell activation, and then it is silenced in GCs due to several

403 mechanisms including a reduction in GLT production and a reduction in APE1 protein.  
404 The latter is likely mediated by the transcriptional repressor BCL6, as suggested by  
405 BCL6 binding to the *Apex1* promoter.

406

407 Our data is compatible with the notion that naïve B cells undergo CSR upon interacting  
408 with antigen and receiving T cell help prior to GC entry. We speculate that memory B  
409 cells may undergo CSR also upon interaction with T cells outside GCs, prior to  
410 differentiation into extrafollicular plasmablasts or re-entry into a GC. Sequential class-  
411 switching of memory B cells to downstream isotypes is well documented at least in the  
412 context of IgE responses ([He et al., 2015](#)). CSR not only appears to be repressed in  
413 GCs; previous studies have suggested that CSR also ceases upon B cell differentiation  
414 into plasmablasts in a BLIMP1-mediated manner ([Shaffer et al., 2002](#)). Lack of ongoing  
415 switching in GCs may be an important determinant of GC kinetics given the evidence  
416 that IgM and IgG tails have different influences on B cell behavior ([Martin and](#)  
417 [Goodnow, 2002](#)). It is possible that the larger GCs found in GCs of AID-deficient mice  
418 that cannot undergo CSR and are therefore IgM-dominated ([Muramatsu et al., 2000](#))  
419 may reflect a greater permanence of IgM in GCs compared to their IgG counterparts.

420

421 Besides being the core apurinic-apyrimidinic endonuclease for CSR, APE1 is probably  
422 best known for its vital role in the BER-pathway, an important arm of the DNA damage  
423 response that repairs with high-fidelity damaged bases ([Fortini and Dogliotti, 2007](#);  
424 [Krokan and Bjoras, 2013](#)). Under normal circumstances, APE1 recruits several BER  
425 components to execute the repair of AP-sites. Considering that APE1 is essential for  
426 cell survival and is ubiquitously expressed in all cells ([Al-Safi et al., 2012](#); [Fung and](#)  
427 [Demple, 2005](#); [Xanthoudakis et al., 1996](#)), the downregulation of APE1 in GC B cells  
428 is surprising, although in line with published data ([Stavnezer et al., 2014](#)). APE1  
429 downregulation may explain how DNA lesions introduced during SHM are spared from  
430 correct repair by the error-free BER machinery ([Stavnezer et al., 2014](#)). Indeed, the



431 accurate repair of dU residues generated by AID and UNG would antagonize the  
432 acquisition of somatic mutations in the IgV region needed for correct affinity maturation.  
433 This idea is further supported by the findings that DNA Pol $\beta$  is not downregulated in  
434 GC B cells ([Schrader et al., 2009](#)). Complete abrogation of the BER-pathway would be  
435 risky in cases where off-target mutations arising outside the Ig locus demand faithful  
436 repair.

437

438 Programmed DNA damage during SHM and CSR is a tightly regulated event, yet off-  
439 target activity of AID causing double-strand DNA breaks outside the IgV and S regions  
440 has also been reported and contributes to genomic instability ([Liu and Schatz, 2009](#)).  
441 Up to 95% of all lymphoid cancers are believed to have a B cell origin as a  
442 consequence of AID-dependent gene translocations and fusions or mutations affecting  
443 *cis*-regulatory elements ([Nussenzweig and Nussenzweig, 2010](#)). CSR itself is an  
444 important contributor to DNA lesions including aberrant gene translocations.  
445 Restricting CSR from taking place in GC B cells would help to reduce the likelihood of  
446 pathogenic double-strand breaks. Another potential advantage of limiting CSR in the  
447 GC comes from the observations of autoimmunity-inducing gene translocations or  
448 insertions occurring in the IgH locus during CSR ([Nussenzweig and Nussenzweig,](#)  
449 [2010](#); [Tan et al., 2016](#)): these occurrences would make this recombination process  
450 particularly risky in GCs, where not only cells are intensely proliferating, but also their  
451 output is destined to become long-lived memory B cells or plasma cells.

452

453

## 454 **STAR Methods**

455 **Lead author: Jonathan Roco**

456

457 **Animals**

458 C57BL/6, SW<sub>HEL</sub> ([Phan et al., 2003](#)), B1-8<sup>hi</sup> tdTomato ([Shih et al., 2002](#)),  
459 Cy1Cre:mT/mG ([Casola et al., 2006](#); [Muzumdar et al., 2007](#)), PA-GFP ([Victoria et al.,](#)  
460 [2010](#)) and AID-Cre-Confetti ([Tas et al., 2016](#)) mice were bred and maintained in  
461 specific-pathogen-free conditions at the Australian National University (ANU),  
462 Canberra, Australia; The University of Birmingham, Birmingham UK; and The  
463 Rockefeller University, New York, USA. All experiments were performed according to  
464 the regulations approved by the local institution ethics committee, including the  
465 Australian National University's Animal and human Experimentation Ethics  
466 Committees.

467

#### 468 **Human samples**

469 Human tonsils were obtained from consenting donors at The Canberra Hospital and  
470 Calvary John James Hospital (Canberra, ACT, Australia), following routine  
471 tonsillectomy. Tonsils were processed by mechanical disruption of the tissue and cells  
472 were isolated using Ficoll Hypaque (GE Healthcare Life Sciences) gradient  
473 centrifugation ([Papa et al., 2017](#)). All experiments with humans were approved by the  
474 Australian National University's Human Experimentation Ethics Committee and the  
475 University Hospitals Institutional Review Board.

476

#### 477 **SW<sub>HEL</sub> B cell adoptive transfers**

478 SW<sub>HEL</sub> mice heterozygous for both light- and heavy-variable chain alleles of the anti-  
479 HEL BCR were sacrificed by cervical dislocation and splenocytes were collected.  
480 Single cell suspensions were obtained by mechanically disrupting the tissue through  
481 70 µm nylon mesh filters (BD Bioscience) using complete RPMI 1640 media (Sigma-  
482 Aldrich). The exact frequency of SW<sub>HEL</sub> B cells was determined by flow cytometry prior  
483 to adoptive transfer using HEL protein conjugated to Alexa Fluor 647 (A647). SW<sub>HEL</sub> B  
484 cells (CD45.1<sup>+</sup>) were resuspended in PBS1x and adoptively transferred (intravenous  
485 injection; *i.v.*) into congenic C57BL/6 recipient mice (CD45.2<sup>+</sup>) along with 2x10<sup>8</sup> sheep

486 red blood cells (SRBCs) conjugated to a mutated form of hen egg lysozyme (HEL<sup>2x</sup>)  
487 ([Paus et al., 2006](#)). For experiments analyzing the early stages of the immune  
488 response (days 1.0 - 2.5) 1.5x10<sup>5</sup> HEL-binding cells were transferred; whereas for  
489 analysis of late phases (days 3 - 8.5) 3x10<sup>4</sup> cells were given.

490

#### 491 **B1-8<sup>hi</sup> tdTomato (tdT) B cell adoptive transfers**

492 C57BL/6 recipient mice (8-10 weeks old) were pre-immunized by intraperitoneal (*i.p.*)  
493 injection of 100 µg of chicken gamma globulin (CGG; #C-1000-10, Biosearch  
494 Technologies) emulsified in Complete Freund's adjuvant (CFA; #F5881, Sigma-  
495 Aldrich). Three days later, mice were intravenously transferred with B1-8<sup>hi</sup> tdT<sup>+</sup> cells  
496 along with 50µg of 4-hydroxy-3-nitrophenyl acetyl (NP) conjugated to CGG (ratio 30-  
497 39, #N-5055D-5, Biosearch Technologies). This mixture was delivered in 200µL of  
498 PBS 1x. Single cell suspensions from B1-8<sup>hi</sup> tdT<sup>+</sup> donor splenocytes were prepared in  
499 a similar fashion compared to SW<sub>HEL</sub> B cell adoptive transfers. For investigation of early  
500 stages (17h - day 1.5) of the immune response 1.8x10<sup>5</sup> B1-8<sup>hi</sup> tdT<sup>+</sup> cells were  
501 transferred. For late stages (days 2 - 18) recipient mice received 6x10<sup>4</sup> B1-8<sup>hi</sup> tdT<sup>+</sup>  
502 cells. The exact frequency of B1-8<sup>hi</sup> tdT cells was determined by flow cytometry prior  
503 to transfer, as measured by binding of the hapten NP conjugated to APC.

504

#### 505 **Flow cytometry analyses and FACS sorting**

506 Single cells suspensions were prepared from mouse spleens, lymph nodes and human  
507 tonsils as previously described ([Papa et al., 2017](#); [Tas et al., 2016](#)). After processing,  
508 cell subsets were examined using flow cytometry using the following antibodies. For  
509 mouse tissues: B220-APCCy7 (#103224, BioLegend), BLIMP1 (#150004, BioLegend),  
510 CD11b-A700 (#101222, BioLegend), CD11b-FITC (#553310, BD Bioscience),  
511 CD16/32 (mouse Fc-block, #553152, BD Bioscience), CD3-A700 (#100216,  
512 BioLegend), CD3-biotin (#100303, BioLegend), CD3-FITC (#553062, BD Bioscience),  
513 CD38-A700 (#56-0381-82, eBioscience), CD38-BV421 (#562768, BD Bioscience),

514 CD38-PE (#120707, BioLegend), CD45.1-PB (#110722, BioLegend), CD45.1-A700  
515 (#110724, BioLegend), CD95-BV421 (#562633, BD Bioscience), CD95-PE (#554258,  
516 BD Bioscience), CXCR5-biotin (#551960, BD Bioscience), IgD-FITC (#11-5993-85,  
517 eBioscience), IgG1-biotin (#553441, BD Bioscience), IgG2a-biotin (#550332, BD  
518 Bioscience), IgG2b-biotin (#406704, BioLegend), IgG2c-biotin (#553504, BD  
519 Bioscience), IgG3-biotin (#553401, BD Bioscience), IgM-FITC (#553437, BD  
520 Bioscience), IgM-PECy7 (#25-5790-82, eBioscience), Streptavidin-APC (#S868,  
521 Thermo Fisher) and Streptavidin-BV605 (#405229, BioLegend). For human tonsils:  
522 CD19-PECy7 (#557835, BD Bioscience), CD27-FITC (#555440, BD Bioscience),  
523 CD38-PE (#347687, BD Bioscience), CD4-APCCy7 (#557871, BD Bioscience), CD86-  
524 A421 (#562432, BD Bioscience), CXCR4-APC (#306510, BioLegend) and Human  
525 TruStain FcX (Fc Receptor Blocking Solution, #422302, BioLegend). Antibody  
526 cocktails were prepared in flow cytometry buffer: PBS1x (Sigma-Aldrich) containing  
527 2% fetal bovine serum (FBS, Gibco) and 2mM EDTA (Sigma-Aldrich). For detection of  
528 HEL-binding cells, HEL protein (Sigma-Aldrich) was conjugated to A647 using a  
529 protein labeling conjugation kit (Thermo Fisher). Dead cells were excluded using either  
530 7-Aminoactinomycin D (7-AAD, Thermo Fisher) or Zombie aqua dye (#423102,  
531 BioLegend). Cells were stained with primary antibodies followed by secondary  
532 reagents for 30 min at 4°C in the dark. Intracellular stain was performed using the  
533 FOXP3 Transcription Factor Staining Buffer Set (#00552300, eBioscience) according  
534 to the manufacturer's instructions. Samples were acquired on a LSRII or Fortessa  
535 cytometer (BD) and analyzed using FlowJo software v10.3 (LLC).

536 **Immunofluorescence**

537 Frozen tissue sections were fixed in cold acetone for 10-20 min. Donor-derived SW<sub>HEL</sub>  
538 B cells were detected in the spleen of recipient mice as previously described ([Paus et](#)  
539 [al., 2006](#)). T-cell areas were identified with anti-CD3-biotin antibody (#100303,  
540 BioLegend) followed by streptavidin conjugated to Alexa 350 (#S11249, Thermo  
541 Fisher). B cell follicles were visualized by staining with anti-IgD FITC (#11-5993-85,  
542 eBioscience). Cy1-Cre:mT/mG spleen sections were stained with anti-CD3 (#550275,  
543 BD Pharmingen) and anti-IgD (#553438, BD Pharmingen) followed by biotin  
544 conjugated goat anti-hamster antibody (Jackson ImmunoResearch), streptavidin  
545 Alexa 405 (#S32351, Thermo Fisher) and donkey anti-rat Alexa-647 antibody (Jackson  
546 ImmunoResearch). For human samples, tonsil sections were blocked and  
547 permeabilized with 3% BSA (Sigma-Aldrich) and 0.5% Triton X-100 (Sigma-Aldrich).  
548 APE1 was detected using anti-APE1 antibody (sc-17774, Santa Cruz Biotechnology)  
549 followed by donkey anti-mouse Alexa 568 antibody (#A10037, Thermo Fisher).  
550 Follicles were identified using anti-human IgD-FITC antibody (#555778, BD  
551 Bioscience). Cell nuclei were counterstained using DAPI (Sigma-Aldrich). Stained  
552 sections were mounted using Vectashield (Vector Laboratories, #H-1200) and  
553 visualized using an Olympus IX71 inverted fluorescence microscope or a Zeiss Axio  
554 ScanZ1. Images were compiled using Photoshop CS6 software.

555

556 ***In vitro* B cell cultures**

557 Flow cytometry purified human tonsil B cells were cultured in complete media: RPMI  
558 1640 media (Sigma-Aldrich) supplemented with 2mM L-Glutamine (Gibco), 100 U  
559 penicillin-streptomycin (Gibco), 0.1mM nonessential amino acids (Gibco), 100mM  
560 HEPES (Gibco), 55mM  $\beta$ -mercaptoethanol (Gibco) and 10% FBS (Gibco). Cells were  
561 maintained for 72h in an incubator at 37°C with 5% CO<sub>2</sub>.

562 **Immunoblot**

563 Naive, DZ and LZ B cells were isolated from human tonsils by flow cytometry. One  
564 fraction of naïve B cells was stimulated *in vitro* for 72h with IL-21 and CD40L (10ng/mL  
565 and 1µg/mL, respectively). Total protein extraction was performed using RIPA buffer  
566 (Thermo Fisher) supplemented with protease inhibitor (Roche). 10µg of whole-cell  
567 extracts from each cell subset were separated by SDS-PAGE (12% w/v), blotted onto  
568 nitro-cellulose membranes and incubated with anti-APE1 antibody (sc-17774, Santa  
569 Cruz Biotechnology). β-actin was used as a loading control (#A5441, Sigma-Aldrich).  
570 Enhanced chemiluminescence (ECL) development was performed after incubation  
571 with secondary antibodies conjugated to horseradish peroxidase (HRP) using Pierce  
572 ECL Western Blotting Substrate reagent (Thermo Fisher) according to manufacturer's  
573 instructions. Images were acquired on an Image Quant LAS 4000 machine (GE  
574 Healthcare Life Sciences). Densitometry analysis was performed using Image Studio  
575 software version 5.2.5 (LI-COR Biosciences).

576

577 **qPCR analysis**

578 Total RNA was extracted from mouse samples with Trizol (#15596026, Thermo  
579 Fisher). RNA quality and concentration were determined with an Agilent 2100  
580 Bioanalyzer instrument. Only samples with a RIN score over 8 were selected for DNA  
581 digestion and cDNA synthesis using RQ1 RNase-Free DNase (#M6101, Promega)  
582 and SuperScript III Reverse Transcriptase (#18080093, Thermo Fisher), respectively.  
583 Duplex qPCR analyses were conducted for each target gene (FAM-labeled probe sets)  
584 using *Ubc* (VIC-labeled, Taqman assay #Mm01198158\_m1, Thermo Fisher) or *Actb*  
585 (HEX-labeled probe set, Biosearch Technologies) as reference genes. Samples were  
586 measured in triplicate using an Applied Biosystems 7900HT Fast Real-Time machine  
587 (Thermo Fisher) with the following thermocycler condition: 50°C for 2 min (1 cycle);  
588 95°C for 10 min (1 cycle); 40 cycles of 95°C for 15 sec and 60°C for 1min. For GLT-  
589 expression studies the following primers and dual-labeled BHQ probes were used:

590  $\gamma$ 1-GLT:  
591 F: 5'-CGAGAAGCCTGAGGAATGTGT-3'  
592 R: 5'-GGAGTTAGTTTGGGCAGCAGAT-3'  
593 P: 5'-FAM-TGGTTCTCTCAACCTGTAGTCCATGCCA-3'

594  $\gamma$ 2b-GLT:  
595 F: 5'-CGCACACCTACAGACAACCAG-3'  
596 R: 5'-GTCACAGAGGAACCAGTTGTATC-3'  
597 P: 5'-FAM-CCAGGGGGCCAGTGGATAGACTGAT-3'

598  $\gamma$ 2c-GLT:  
599 F: 5'-GGACCACTAAAGCTGCTGACACAT-3'  
600 R: 5'-AACCTTGACCAGGCATCCT-3'  
601 P: 5'-FAM-AGCCCCATCGGTCTATCCACTGGC-3'

602  $\gamma$ 3-GLT:  
603 F: 5'-GACCAAATTCGCTGAGTCATCA-3'  
604 R: 5'-ACCGAGGATCCAGATGTGTCA-3'  
605 P: 5'-FAM-CTGTCTATCCCTTGGTCCCTGGCTGC-3'

606  $\mu$ -GLT:  
607 F: 5'-TCTGGACCTCTCCGAAACCA-3'  
608 R: 5'-ATGGCCACCAGATTCTTATCAGA-3'  
609 P: 5'-FAM-ATGTCTTCCCCCTCGTCTCCTGCG-3'

610 Actb:  
611 F: 5'-CGTGAAAAGATGACCCAGATCA-3'  
612 R: 5'-TGGTACGACCAGAGGCATACAG-3'  
613 P: 5'-HEX-TCAACACCCCAGCCATGTACGTAGCC-3'

614 These assays have been previously described ([Marshall et al., 2011](#)) and were  
615 manufactured by Biosearch Technologies. Data is expressed as a fold-change using  
616 the  $\Delta\Delta C_T$  method.

617 **One step qPCR analysis**

618 Total RNA from 2,000-15,000 cells was purified using a PicoPure RNA Isolation Kit  
619 (#KIT0204, Thermo Fisher) according to the manufacturer's instructions. DNase  
620 treatment was performed on-column using RNase-Free DNase Set (#79254, Qiagen).  
621 Quality and concentration were determined as previously described using an Agilent  
622 2100 Bioanalyzer instrument. Real-time one-step RT-PCR quantification was  
623 performed using the QuantiTect Multiplex RT-PCR Kit (#204643, Qiagen) in a final  
624 volume of 6 $\mu$ L. Duplex reactions with limiting primer concentrations were conducted in  
625 the same well for  $\gamma$ 1-GLT (FAM-labeled probe set) and *Actb* (HEX-labeled probe set).  
626 Reactions were run in an Applied Biosystems 7900HT Fast Real-Time machine with  
627 the following thermocycler condition: 50°C for 20 min (1 cycle); 95°C for 15 min (1  
628 cycle); 40 cycles of 94°C for 45 sec and 60°C for 45s. Expression of the target genes  
629 was normalized using the reference gene *Actb* and presented as a fold-change using  
630 the  $\Delta\Delta C_T$  method.

631

632 **Single cell qPCR**

633 Single cell qPCR was performed as previously described ([Nefzger et al., 2016](#)). In  
634 brief, cells were flow cytometry deposited into qPCR 96-well plates filled with 10 $\mu$ L of  
635 lysis buffer and processed with the Single Cell to  $C_T$  kit (Life Technologies). cDNA was  
636 produced from the lysate as per kit's instructions. Samples were submitted to 18 cycles  
637 of pre-amplification using the following TaqMan assays (Thermo Fisher):

638 *Actb* (#Mm00607939\_s1),

639 *Foxo1* (#Mm00490672\_m1),

640 *Apex1* (#Mm01319526\_g1),

641 *Apex2* (#Mm00518685\_m1),

642 *Prdm1* (#Mm00476128\_m1),

643 *Bcl6* (#Mm00477633\_m1),

644 *Aicda* (#Mm01184115\_m1)



645 GLTs were detected using the probe sets previously described (see qPCR analysis  
646 section). Single cell qPCR data collection was performed with a Biomark instrument  
647 (Fluidigm) on pre-amplified templates that were positive screened for the housekeeper  
648 *Actb* (manually tested by qPCR). Reactions were run for 40 cycles. Data was analyzed  
649 using the Biomark software package “Real-Time PCR analysis” (Fluidigm). Data  
650 cleaning and normalization were done using custom R code (R version 3.3.3, R Core  
651 Team). The limit of detection was set to 40 cycles. Undetermined  $C_T$  were given a  
652 value of 40. Heatmaps and violin plots of the resulting data were generated using the  
653 ggplot2 package (version 2.2.1) in the R environment.

654

#### 655 **GC B cell clonal trees from photoactivatable (PA)-GFP-transgenic mice**

656 PA-GFP-transgenic mice ([Victoria et al., 2010](#)) were immunized with CGG-alum and  
657 draining popliteal lymph nodes (pLNs) were harvested 15 and 20 days later. Two  
658 individual GCs per pLN were photoactivated and single-cell sorted independently.  
659 Heavy-chain variable-segment ( $V_H$ ) genes from individual B cells were amplified,  
660 sequenced and analyzed as described previously ([Tas et al., 2016](#)). Briefly, single cells  
661 were lysed in TCL buffer (Qiagen) with 1%  $\beta$ -mercaptoethanol. SPRI bead isolated  
662 RNA was reverse-transcribed into cDNA using an oligo(dT) primer. *Igh* and *Igk*  
663 transcripts were amplified from cDNA by PCR. Single-cell barcoded PCR amplicons  
664 were sequenced using the MiSeq platform (Illumina, MiSeq Reagent Nano Kit v2). Ig  
665 heavy variable (V), diversity (D) and joining (J) gene segments were assigned using  
666 the IMGT ([Lefranc et al., 2009](#)) and VBASE2 ([Retter et al., 2005](#)) databases.  
667 Functional V(D)J sequences were grouped into clones only when sharing *Ighv* and *Ighj*  
668 gene segments and junction regions (identical length and more than 75% amino acid  
669 identity in CDR3). Data for day 15 was re-analyzed from published sequences ([Tas et](#)  
670 [al., 2016](#)) using a similar methodology, and data for day 20 was generated *de novo* for  
671 this study. Only clones containing  $\geq 3$  IgM<sup>+</sup> cells, or at least one IgM<sup>+</sup> and one IgG<sup>+</sup>  
672 cell, were considered informative and are presented in this paper. Clonal phylogenies

673 and trees were constructed by the inference methodology algorithm GCtree ([DeWitt et](#)  
674 [al., 2018](#)).

675

#### 676 **Using AID-Cre-Confetti mice to track Peyer's Patch GCs**

677 AID-Cre-Confetti mice ([Tas et al., 2016](#)) were used to visualize and track single GCs  
678 in the follicles of Peyer's Patches (PPs) of unimmunized mice. In these mice, upon the  
679 administration of tamoxifen, AID-expressing GC B cells are marked with one of ten  
680 fluorescent color combinations. In order to induced Cre-mediated recombination, 10  
681 mg of tamoxifen in corn oil was administered twice by oral gavage two days apart. PP's  
682 were analyzed 18 days after the final administration of tamoxifen. Single PP's were  
683 isolated from the entire length of the small intestine and imaged with two-photon  
684 microscopy to locate single GC follicles. Individual GCs were then isolated by manual  
685 dissection prior to single-cell sorting. Following sorting, single cells were processed for  
686 sequencing as previously described.

687

#### 688 **Nojima cell co-cultures.**

689 Co-cultures using the Nojima feeder cells were conducted as previously described  
690 ([Nojima et al., 2011](#)). Spleens were collected from C57BL/6 wild type mice and naïve  
691 B cells were magnetically purified using a B cell isolation kit (#130-090-862, Miltenyi  
692 Biotec) according to manufacture's instructions. The purified B cells were labelled with  
693 Cell Trace Violet (CTV) (#C34571, Thermo Fisher) according to manufacturer's  
694 instructions, and then co-cultured for 72h with Nojima feeder cells in complete media  
695 supplemented with recombinant murine IL-4 (10ng/μL, PeproTech). These *in vitro*  
696 derived GC (iGC) B cells were flow cytometry-purified to assess binding of Bcl6 to the  
697 γ1-GLT promoter by ChIP.

698 **Chromatin immunoprecipitation (ChIP)-qPCR**

699 ChIP-qPCR experiments were conducted as previously described ([Kagey et al., 2010](#)).  
700 Briefly, *in vitro* derived germinal centre B cells were flow cytometry-purified and then  
701 cross-linked with 1% formaldehyde for 10 min at room temperature, after which glycine  
702 was added to stop the reaction. Cells were washed three times with PBS 1x at 4°C,  
703 lysed with SDS-lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8) on ice for  
704 10 min. Chromatin was sonicated using a Bioruptor instrument (Diagenode) to  
705 generate DNA fragments of 300-1000bp. Anti-Bcl6 (sc-858, Santa Cruz) antibody was  
706 used for ChIP. Amplification of ChIP DNA was performed using 2XSYBR Green PCR  
707 Master Mix (Applied Biosystems) in a 7900HT Fast Real-Time machine (Thermo  
708 Fisher). qPCR reactions were performed in duplicate with the following thermocycler  
709 condition: 50°C for 2 min (1 cycle); 95°C for 10 min (1 cycle); 40 cycles of 95°C for 15  
710 sec and 60°C for 1 min. ChIP-qPCR data was normalized using the percent input  
711 method  $2^{-(\text{average } C_T \text{ input} - \text{average } C_T \text{ IP sample})}$  (ThermoFisher). Primer sets  
712 used for ChIP-qPCR experiments are listed below:

713 Iy1-P1:

714 F: 5'-GCTCCACCTACCTTGTCTTTAT-3'

715 R: 5'-GAGATGGGTTTCAGAGTGTCATAG-3'

716 Iy1-P2:

717 F: 5'-CACTCTCACTCCAGGGTATAGA-3'

718 R: 5'-TGAGACCCAGAACACAGAATTAG-3'

719 Iy1-P3:

720 F: 5'-CTCCCACAACCTGTACCTAAAT-3'

721 R: 5'-GGACATGGAAGTAGAGGATCAAA-3'

722 Iy1-P4:

723 F: 5'-GTCAGGAAAGAGTGGGCATAA-3'

724 R: 5'-CTGGCTGTACTCCTGTTTCTC-3'

725 Iy1-TSS:

726 F: 5'-GGGCAGGACCAAAACAGGAA-3'

727 R: 5'-TTTCCCTGCTGACCCCACTC-3'

728

729 **Bioinformatic analysis of ChIP-on-chip datasets and prediction of BCL6-binding**  
730 **sites in GLTs**

731 Using ChIP-on-chip data ([Ci et al., 2009](#)) of BCL6 gene targets in human GC B cells,  
732 the APEX1 and APEX2 loci were analyzed for BCL6 binding peaks as previously  
733 described ([Ci et al., 2009](#)). The known BCL6 targets *TLR1* and *BCL6* were used as  
734 controls. The data set was obtained from GEO, accession number GSE15179. BCL6  
735 binding sites in the  $\gamma$ 1-GLT promoter were predicted using the JASPAR 2018 database  
736 ([Khan et al., 2018](#)).

737

738 **Statistical Analysis**

739 Datasets were analyzed using Mann-Whitney test (U test, two-tailed), except for  
740 quantification of western blots, in which a paired t-test (two-tailed) was used. To  
741 compare more than two groups or sets of data a Kruskal-Wallis test was performed  
742 followed by Dunn's post-test. The test employed to analyze the different experiments  
743 is indicated in each figure legend. Statistical tests were selected based on the  
744 distribution and the variance characteristics of the data. Normality was assessed with  
745 Shapiro-Wilk test. All statistical analyzes were performed with Prism software version  
746 7 (GraphPad Software) and R software version 3.3.3 (R Core Team). The exact *p*-  
747 values are shown in each figure.

748

749 **Mathematical modeling**

750 All simulations are based on a previously published agent-based model of B and T cell  
751 dynamics within the GC ([Meyer-Hermann et al., 2012](#)), which lacks an isotype  
752 switching model (see below). Briefly, the model describes dynamics of B and T cells  
753 in discrete three-dimensional space including diffusion of chemotactic signals that

754 influence cell motility. The GC reaction starts with founder B cells migrating into the  
755 virtual GC area within the first four days at a rate of 2 cells per hour (of note, the B cell  
756 influx was prolonged in supplementary Fig. S7A). Each B cell divides six times before  
757 it is allowed to differentiate to a LZ phenotype that depends on antigen collection for  
758 survival beyond a critical time period. Antigen is collected by B cells in an affinity-  
759 dependent manner, where affinity of a B cell for an antigen is represented by Hamming  
760 distance in four-dimensional shape space. Furthermore, B cells depend on T cell help  
761 for survival. For competing B cells, T follicular helper ( $T_{FH}$ ) cells polarize towards the  
762 cell with the higher amounts of collected and processed antigen. B cells collect  $T_{FH}$   
763 signals and require a sufficient total amount of collected signal for survival. Each  
764 selected B cell returns to the DZ and divides a number of times that depends on the  
765 amount of collected antigen, a mechanism termed dynamic number of divisions  
766 ([Meyer-Hermann, 2014](#)) and supported by experimental data ([Gitlin et al., 2014](#)).  
767 Differentiation to GC output cells is induced in a probabilistic manner (LEDAX model)  
768 ([Meyer-Hermann et al., 2012](#)). A full description of the modeling framework and its  
769 compatibility with recent experimental data has been recently published ([Binder and](#)  
770 [Meyer-Hermann, 2016](#)).

771

772 For the present context of B cell isotype switching, a new model of GC dynamics  
773 needed to be developed, which explicitly represents the different isotypes and allows  
774 for different models of how isotype switching happens. Newly arrived cells are  
775 assumed to predominantly express IgM. In accordance with early measurements of  
776 the amount of IgG<sup>+</sup> B cells (Fig. 1C and Fig. 2A-D), we assume that 35% of the founder  
777 cells are already expressing IgG. At each division event, the daughter cells switch from  
778 IgM to IgG with a defined probability. This switching probability  $p$  can be either constant  
779 or decrease over time according to an exponential decay model with a half-life  
780 corresponding to the observed decrease in GLTs (Fig. 1E),  $p(t) = p_0 e^{-\gamma t}$ , where  $p_0$   
781 denotes the switching probability at the beginning and  $\gamma$  is the decay of the switching

782 probability over time. The initial switching probability is not a free parameter, this was  
783 determined by data on the relative amount of either isotype at later time points of the  
784 reaction in Fig. 4F and 4G for both the constant ( $p = 0.03$  at each division, unless  
785 stated otherwise) and the dynamic ( $p_0 = 0.15$  at each division) switching model  
786 separately.

787

788 To account for a possible preferential output for the IgG isotype, we introduced a bias  
789 factor,  $\eta$ , that increases the probability for IgG<sup>+</sup> cells to become plasma cells while  
790 decreasing the output probability for IgM<sup>+</sup> cells by the same amount, keeping the total  
791 amount of output cells comparable. The different conditions were simulated in 400 *in*  
792 *silico* GCs and the distribution of the fraction of IgG<sup>+</sup> B cells at day 21 after onset of  
793 the GC reaction was evaluated among these 400 GCs.

794

795 To test for the impact of the timing of CSR, we combined the dynamic switching model  
796 with a delay in switching, leading to a limited time interval for switching at different  
797 times of the GC reaction. We tested a delay of different time intervals,  $t_{\text{switch}}$  (Fig. 5G).  
798 Each delay was simulated in 400 GCs and the diversity of the IgG fraction at day 21  
799 within these 400 simulations was assessed using the difference between the upper  
800 and the lower quartile (interquartile range, IQR).

801

802 All simulations were performed using custom C++ code. Simulation output was  
803 analyzed using the R statistics language; plots of the simulation output were created  
804 using the ggplot2 library.

805

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1026

1027 **Author contributions statement**

1028 J.R. conducted most of the experiments, figure editing and data analysis. P.G., P.C.,  
1029 J.E., Q.S. and J.C. helped with the experiments, human sample processing and  
1030 provided intellectual input. H.V. provided intellectual input and expertise in the analysis  
1031 and acquisition of flow cytometry data. L.M.C provided help with the analysis of RNA-  
1032 seq data. K.M.T. provided intellectual input, helped in the design of single cell studies  
1033 and together with Y.Z. performed the Cy1-Cre:mT/mG mouse experiments. L.M., C.N.,  
1034 A.S. and G.V. performed the experiments using the PA-GFP and the AID-Cre-Confetti  
1035 mouse models, generation of clonal trees, provided intellectual input and contributed  
1036 to data analysis. S.C.B., P.R. and M.M.H. provided intellectual input and performed  
1037 the *in silico* modeling. C.N., J.R. and J.P. conducted the single cell qPCR studies. J.R.  
1038 and C.G.V. wrote the manuscript. C.G.V. was the main supervisor of this project. All  
1039 authors reviewed the manuscript.

1040

1041 **Competing interests**

1042 The authors declare no competing financial interests.

1043

1044 **Data and materials availability**

1045 Data and materials can be made available upon request to the corresponding author.

1046

1047 **Code availability**

1048 Custom code used in this study can be made available upon request to the  
1049 corresponding author.

1050 **Legends to Figures**

1051

1052 **Figure 1. Isotype switching commences prior to germinal center onset.**

1053 **A)** Adoptive transfer protocol of SW<sub>HEL</sub> B cells and HEL<sup>2x</sup>-SRBCs into congenic  
1054 recipient mice (see STAR Methods).

1055 **B)** Immunofluorescence images of spleen sections collected from recipient mice as in  
1056 **(A)**. Sections were stained for SW<sub>HEL</sub> B cells (red), IgD (green), and CD3 (blue). Scale  
1057 bars = 200  $\mu$ m.

1058 **C-D)** Representative flow cytometric plots showing gating strategy to identified donor-  
1059 derived SW<sub>HEL</sub> B cells after adoptive transfer **(C)** and expression **(D)** of BLIMP1 vs  
1060 CXCR5 or B220, and Fas vs CXCR5 in HEL-binding B cells recovered 5 days after  
1061 challenge as shown in **(A)**.

1062 **E)** qPCR gene expression profile of purified donor-derived SW<sub>HEL</sub> B cells for  $\gamma$ 1-GLT,  
1063  $\gamma$ 2b-GLT, *Aicda*, and *Bcl6*. Data was normalized to the reference gene *Ubc* and is  
1064 presented as a fold-change compared to day 3.5 values using the  $\Delta\Delta C_T$  method. Dots  
1065 represent the mean of pooled biological replicates as in **(C)**.

1066 Data is representative of two independent experiments. n = number of recipient mice  
1067 used at each time point. Please also see Figure SX.

1068

1069 **Figure 2. Class switching proceeds at comparable rates in germinal centers and**  
1070 **extrafollicular sites and early visualization of germline transcription in a**  
1071 **polyclonal response.**

1072 **A)** Flow cytometric plots showing gating strategy employed to identify donor-derived  
1073 SW<sub>HEL</sub> B cells after immunization as shown in Figure 1.

1074 **B-C)** Flow cytometric analysis for surface expression of IgG1 and IgM in naïve (day 0),  
1075 activated (day 2.5) **(B)**, EFPB and GC SW<sub>HEL</sub> B cells (day 4.5 - 6.5) **(C)**. Numbers  
1076 indicate the percentage of donor-derived HEL<sup>+</sup> IgG1<sup>+</sup> cells.

1077 **D)** Quantification of IgG1, IgG2b and total IgG in EFPBs or GC B cells as shown in **(C)**.  
1078 Bars represent medians and dots individual mice (n=4). Horizontal grey bars show  
1079 comparisons between EFPB and GC subsets at the same time point (Mann-Whitney  
1080 U test). Horizontal purple and blue bars show comparisons between EFPBs or GC B  
1081 cells (Kruskal-Wallis test), respectively. Numbers on top of bars indicate the respective  
1082 *p*-value. ns = not significant.

1083 **E)** Immunofluorescence images of spleen sections from C $\gamma$ 1-Cre:mT/mG mice after  
1084 SRBC immunization at the indicated time points: CD3 (grey), IgD (blue), C $\gamma$ 1-Cre  
1085 (green) and non-activated B cells (red).

1086 Data is representative of two (A-D) and three (E) independent experiments. Please  
1087 also see Figure SX.

1088

1089 **Figure 3. Single cell analysis of germline transcripts in early activated and GC B**  
1090 **cells.**

1091 **A-B)** Flow cytometry plots showing gating strategy to purify HEL-binding B cells after  
1092 HEL<sup>2x</sup>-SRBC immunization. Donor-derived cells were single cell purified as IgM<sup>+</sup>HEL<sup>+</sup>  
1093 B blasts (day 3) **(A)** and IgM<sup>+</sup>HEL<sup>+</sup> GC B cells (day 6.5) **(B)**. GC B cells were subdivided  
1094 as either DZ or LZ cells based on CXCR4 and CD86 expression as shown.

1095 **C)** Heatmap showing single cell qPCR expression profile of selected targets in B  
1096 blasts, DZ and LZ SW<sub>HEL</sub> B cells purified as described in **(A-B)**.

1097 **D)** Quantification of raw C<sub>T</sub> values for  $\gamma$ 1-GLT,  $\gamma$ 2b-GLT, *Aicda*, *Bcl6* and *Apex1*  
1098 obtained in **(C)**. Violin plots depict data distribution; each dot represents an individual  
1099 cell.

1100 **E)** Pie charts showing quantification of target genes as shown in **(D)**. Numbers indicate  
1101 the percentage of cells expressing the indicated target.

1102 The limit of detection for analysis was set to 40 cycles, cells with a C<sub>T</sub> value < 40 were  
1103 considered positive events. NTC = no template control. Bulk = bulk population control  
1104 of 20 cells. Please also see Figure SX.



1105

1106 **Figure 4. Expression of GLTs remains low in late GC responses**

1107 **A)** Adoptive transfer protocol of B1-8<sup>hi</sup> tdTomato (tdT) B cells to investigate the early  
1108 phases of the immune response to NP-CGG (see STAR methods).

1109 **B)** Flow cytometric plots showing gating strategy to identify B1-8<sup>hi</sup> tdT<sup>+</sup> B cells as shown  
1110 in (A). Top panel shows representative plots of CD38 vs Fas for donor-derived B1-8<sup>hi</sup>  
1111 tdT<sup>+</sup> B cells and bottom panel the profile for recipient cells in the same mouse.

1112 **C)** qPCR gene expression profile for  $\gamma$ 1-GLT in purified donor-derived B1-8<sup>hi</sup> tdT<sup>+</sup> B  
1113 cells as for **(A-B)**. See (F) for details.

1114 **D)** Adoptive transfer protocol of B1-8<sup>hi</sup> tdT<sup>+</sup> B cells to investigate the late phases of the  
1115 immune response to NP-GCC. C57BL/6 mice were immunized as shown in **(A)**.

1116 **E)** Flow cytometric plots showing gating strategy to analyze surface expression of total  
1117 IgG at day 2 (top panel) and days 4 - 18 (bottom panel) in splenocytes harvested from  
1118 mice immunized as in **(D)**.

1119 **F)** qPCR gene expression profile for  $\gamma$ 1-GLT in purified donor-derived B1-8<sup>hi</sup> tdT<sup>+</sup> B  
1120 cells as shown in **(D)**. Duplex qPCR analyses were conducted using *Actb* as reference  
1121 gene. Data is presented as a fold-change compared to day 1.0 B cells (C) or day 4  
1122 GC B cells (F) using the  $\Delta\Delta C_T$  method. Dots represent individual mice and the black  
1123 line connects the group medians.

1124 **G-H)** Flow cytometric quantification of total IgG in donor-derived B1-8<sup>hi</sup> tdT<sup>+</sup> cells **(G)**  
1125 and total numbers of B1-8<sup>hi</sup> tdT<sup>+</sup> GC B cells **(H)** in the spleens of C57BL/6 transferred  
1126 mice identified as in **(D)**. Number of mice used in each time point: day 2 (n=5), day 4  
1127 (n=5), day 8 (n=4), day 14 (n=5) and day 18 (n=5). Data is representative of three  
1128 independent experiments. Total cell numbers were normalized to  $1 \times 10^6$  splenocytes.  
1129 Please also see Figure SX.

1130

1131 **Figure 5. Lack of ongoing switching in IgM<sup>+</sup> B cells from established germinal**  
1132 **centers and *in silico* modelling**

1133 **A)** Schematic representation of the clonal and isotype composition of the GCs obtained  
1134 from the popliteal lymph nodes (pLNs) of PA-GFP mice immunized 15 or 20 days  
1135 earlier with CGG in alum. Each column represents a single GC and the boxes in each  
1136 column represent individual clones determined by phylogenetic analysis of single cell  
1137 mRNA  $V_H$  sequences. The size of each box has been scaled to reflect the number of  
1138 cells in each clone. Grey represents  $IgG^+$  B cells and green represents  $IgM^+$  B cells,  
1139 as determined by *Igh* mRNA sequences. The boxes outlined in black indicate those  
1140 selected for the somatic mutation analysis depicted in **(B)**, based on mixed composition  
1141 by both  $IgG^+$  and  $IgM^+$  cells, and the presence of 4 or more  $IgM^+$  cells.

1142 **B)** Charts showing clonal trees representing the phylogeny of  $V_H$  sequences within B  
1143 cell clones (symbols according to the legend in the bottom panel).

1144 **C)** Summary of the data in **(A-B)** showing the SHM content of individual B cells at the  
1145 time of the inferred switch event (filled red arrowheads). Clones containing only  $IgM^+$   
1146 cells (empty red arrowheads) were pooled with those in which switching occurred at  
1147 the level of the unmutated precursor (zero mutations). For each time point 5 different  
1148 mice in 3 independent experiments were included.

1149 **F-H)** Histograms showing distribution of  $IgG$  fractions at the end of affinity maturation  
1150 *in silico* in **(F)** constant switching probability of  $p = 0.03$ , **(G)** constant switching  
1151 probability combined with an increased probability of  $IgG^+$  cells to leave the GC and  
1152 **(H)** dynamic switching with an initial switching probability of  $p = 0.15$  and a decaying  
1153 switching probability of  $\gamma = 0.035 \text{ h}^{-1}$  (see Fig. 1E). Each distribution shows the fraction  
1154 of  $IgG^+$  B cells at day 21 after the onset of the GC reaction.

1155 **I)** Effect of class switch timing on the diversity of  $Ig$ -isotypes in simulated GCs.  $t_{switch}$   
1156 (horizontal axis) denotes the time post GC onset (time post immunization minus 3.5  
1157 days) at which CSR is started with a decreasing probability. Each point corresponds  
1158 to the interquartile range of the  $IgG$  fraction among B cells at the end of *in silico* GC  
1159 reactions in 400 simulations. Please also see Figure SX.

1160 **Figure 6. APE1 is downregulated in human GC B cells and its expression is**  
1161 **modulated by BCL6**

1162 **A)** Barplot showing expression of human *BCL6*, *AICDA*, *UNG*, *APEX1*, and *APEX2*  
1163 genes from purified tonsillar naïve B cells and GC B cells by RNA-seq. Data is  
1164 presented as the log<sub>2</sub> fold-change between reads per kilobase per million reads  
1165 (RPKMs) of GC B cells relative to those on naïve B cells. The bars represent means  
1166 and error bars ± standard deviations. Dots represent individual donors (n=5).

1167 **B)** Immunofluorescence images of human tonsil samples showing APE1 (red), IgD  
1168 (green), and DAPI (blue). Scale bars = 200 µm.

1169 **C)** Flow cytometric plots showing the gating strategy to purify naïve, DZ and LZ B cells  
1170 from human tonsils. Activated B cells correspond to naïve B cells stimulated *in vitro* for  
1171 72h with IL-21 and CD40L.

1172 **D)** Immunoblot of human APE1 protein in naïve, DZ, LZ and activated B cells. β-actin  
1173 was used as a loading control.

1174 **E)** Quantification of APE1 protein by densitometry as for blot in **(D)**. APE1 expression  
1175 was normalized using β-actin. Data is presented as a fold-change relative to naïve B  
1176 cells. Horizontal black bars represent means and dotted grey lines connect samples  
1177 derived from the same tonsil donor. Numbers on top indicate the respective *p*-value  
1178 from two-tailed paired t-test analysis, n = 3.

1179 **F)** Regions where BCL6 binds to the promoter region of the genes encoding for APE1  
1180 (*APEX1*), APE2 (*APEX2*), BCL6 and TLR1, as determined by ChIP on chip ([Ci et al.,](#)  
1181 [2009](#)).

1182 **Legend to Supplementary Figures**

1183

1184 **Figure S1.**

1185 **A)** Gating strategy employed to identify donor-derived SwHEL B cells after adoptive  
1186 transfer into congenic recipient mice immunized with HEL<sup>2x</sup>-SRBCs or HEL<sup>3x</sup>-SRBCs.

1187 **B)** Flow cytometric plots showing expression of different markers by responding donor-  
1188 derived SW<sub>HEL</sub> GC B cells gated as CXCR5<sup>hi</sup> B220<sup>hi</sup> compared to HEL-binding EFPBs  
1189 identified as CXCR5<sup>lo</sup> B220<sup>lo</sup>.

1190

1191 **Figure S2**

1192 **A)** Dot plot showing raw C<sub>T</sub> values for Figure 1E.  $\gamma$ 1-GLT,  $\gamma$ 2b-GLT, *Bcl6* and *Aicda*  
1193 expression values are shown together with those for the reference gene *Ubc*.  
1194 Horizontal grey lines show the C<sub>T</sub> mean expression value of *Ubc* across all samples.  
1195 Dotted grey lines are  $\pm 2$  C<sub>T</sub> values from *Ubc* C<sub>T</sub> mean.

1196 **B)** Quantification of IgM in EFPBs or GC B cells for samples shown in Fig 2D. Bars  
1197 represent medians and dots individual mice (n=4). Data is representative of two  
1198 independent experiments.

1199

1200 **Figure S3**

1201 **A)** Flow cytometric plots showing gating strategy used to identify donor-derived SwHEL  
1202 B cells after immunization with HEL<sup>3x</sup>. In brief, CD45.2<sup>+</sup> recipient mice were adoptively  
1203 transferred with CD45.1<sup>+</sup> SWHEL B cells ( $3 \times 10^4$  -  $15 \times 10^4$ ) and simultaneously *i.v.*  
1204 challenged with  $2 \times 10^8$  SRBCs conjugated to HEL<sup>3x</sup>. Spleens were harvested at the  
1205 indicated time points to analyze the immune response.

1206 **B)** qPCR gene expression profile for  $\gamma$ 1-GLT. Duplex qPCR analyses were conducted  
1207 using *Actb* as a reference. Data is expressed as a fold-change compared to day 3.5

1208 mean expression value using the  $\Delta\Delta\text{CT}$  method. Dots represent the mean of pooled  
1209 biological replicates (day 0 - 3.5) or individual animals (day 6.5 - 8.5).

1210 **C)** Dot plot showing raw  $C_T$  values for  $\gamma 1$ -GLT and the reference gene *Actb*. Samples  
1211 were measured in triplicate. Horizontal green and blue lines show the  $C_T$  mean value  
1212 of all samples for  $\gamma 1$ -GLT and *Actb*, respectively. Dotted blue lines depict  $\pm 2 C_T$  values  
1213 from *Actb*  $C_T$  mean. NTC = no template control.

1214

#### 1215 **Figure S4**

1216 **A)** Violin plots showing expression levels for the genes *Cxcr4* and *Cd86* by single cell  
1217 qPCR in activated B cells (day 3) and GC B cells (day 6.5), subdivided as LZ and DZ  
1218 cells.

1219 **B)** Pie charts showing quantification of target genes shown in **(A)**. Numbers indicate  
1220 the percentage of cells expressing the indicated target. Cells with a  $C_T$  value  $< 40$  were  
1221 considered positive. Sample were obtained as shown in Figure 2.

1222 **C)** Heatmap showing single cell expression of  $\gamma 1$ ,  $\gamma 2b$ ,  $\gamma 2c$  and  $\gamma 3$ -GLTs by qPCR in  
1223 double positive *Foxo1*<sup>+</sup>*Myc*<sup>+</sup> B blast, DZ and LZ GC B cells. Activated B blasts were  
1224 purified on day 3, whereas GC B cells (both DZ and LZ subsets) were isolated on day  
1225 6.5 (see Figure 3 for details).

1226 **D)** Pie charts showing quantification of  $\gamma 1$ - and  $\gamma 2b$ -GLT in double positive *Foxo1*<sup>+</sup>*Myc*<sup>+</sup>  
1227 cells as for **(C)**. Numbers indicate the percentage of cells expressing the indicated  
1228 target. Cells with a  $C_T$  value  $< 40$  were considered positive events (see Figure 3E for  
1229 details).

1230

#### 1231 **Figure S5**

1232 **A)** Gating strategy used to identify donor-derived B1-8<sup>hi</sup> tdT<sup>+</sup> B cells after adoptive  
1233 transfer into congenic recipient mice immunized with NP-CGG (see Figure 4 for  
1234 details).

1235 **B)** Dot plot showing raw  $C_T$  values for  $\gamma 1$ -switch circle transcript ( $\gamma 1$ -SWCT) and the  
1236 reference gene *Actb*. Purified naïve B cells from C57BL/6 wild type mice were  
1237 stimulated *in vitro* for 24h, 48h and 72h with IL-4 and LPS. Samples were pre-amplified  
1238 for  $\gamma 1$ -SWCT for 22 cycles, and then analyzed by qPCR.

1239 **C)** Analysis of  $\gamma 1$ -SWCT expression as shown in **(B)**. DNA fragments amplified by  
1240 qPCR were resolved by electrophoresis in a 1.5% agarose gel. NTC = no template  
1241 control.

1242

### 1243 **Figure S6**

1244 **A)** Charts showing clonal diversity of GCs isolated from popliteal lymph nodes (pLNs)  
1245 after photoactivation in mice immunized 15 days before with CGG-Alum. Two  
1246 individual GCs per pLN were photoactivated and separately flow cytometry-sorted, as  
1247 described ([Tas et al., 2016](#)). Pie charts show clonal distribution of sequenced Igh  
1248 genes in each GC. In the inner ring, each slice represents one distinct clone  
1249 represented in greyscale, colored slices indicate clones that were found in both GCs  
1250 (top and bottom pie charts) from the same lymph node. In the outer ring the isotype  
1251 IgG (black) or IgM (white) of each clone is indicated. Numbers in the center of each  
1252 chart are the total number of IgM/IgG cells sequenced. Clonal trees represent the  
1253 phylogeny of IgM heavy-chain variable-segment ( $V_H$ ) sequences within each clone  
1254 containing more than 2 cells per clone from pLNs that have more than 5 IgM cells  
1255 (symbols according to the legend in the top panel). Pairs are from 5 different mice in 3  
1256 independent experiments.

1257 **D-E)** GCs were visualized in the Peyer's patches of unimmunized AID-Cre-Confetti  
1258 mice. **(D)** Pies of isotype distribution across clones were assembled for single GCs  
1259 from two separate mice. Inner segments denote clones, outer colored ring denotes  
1260 isotype. n/n = number of clones/ total number of sequenced cells. **(E)** Clonal trees  
1261 representing the phylogeny of  $V_H$ -segment sequences were constructed from the most

1262 heavily expanded clones containing more than 3 members per clone (grey segments  
1263 in pies). Symbols correspond to the legend in the lower left panel.

1264

1265

1266 **Figure S7**

1267 **A-B)** Same simulation as in Figure 5F with ongoing influx of IgM<sup>+</sup> B cells into the GC.

1268 The influx rate decays from 2 cells per hour to zero following a sigmoidal function with  
1269 half value at day 10 post-GC onset and a width of 10 days.

1270 **C-D)** Same analysis as in **(A-B)** but, instead of modulating the B cell influx duration,

1271 with IgM<sup>+</sup> B cells collecting twice as much antigen per interaction with FDC, which  
1272 induces more signaling in the subsequent selection steps. In **(A-C)**, the sensitivity of

1273 GC reactions to ongoing influx and increased antigen uptake, respectively, is shown

1274 with unchanged ( $p=0.03$  per division) switching probability. In **(B-D)** the switching

1275 probability was adapted to  $p=0.05$  and  $p=0.18$  per division, respectively, in order to

1276 make the simulation consistent with the measured mean IgG dominance. Each graph

1277 represents 100 simulation replicates.

1278

1279 **Figure S8**

1280 ChIP-qPCR analysis of BCL6 in primary B cells using the Nojima culture system.

1281 Splenic naïve B cells were magnetically purified and co-cultured for 72 h with Nojima

1282 cells supplemented with IL-4. These *in vitro* derived GC (iGC) B cells were flow

1283 cytometry purified to assess binding of BCL6 to the  $\gamma 1$ -GLT promoter by ChIP-qPCR.

1284 **A)** Diagram depicting the chromosome location of the  $\gamma 1$ -GLT promoter (blue arrow)

1285 2000 bp upstream of the  $I\gamma 1$  coding sequence (grey segment) in the mouse genome.

1286 Green arrows indicate the binding location of the primers used for ChIP-qPCR as

1287 shown in **(E)**.

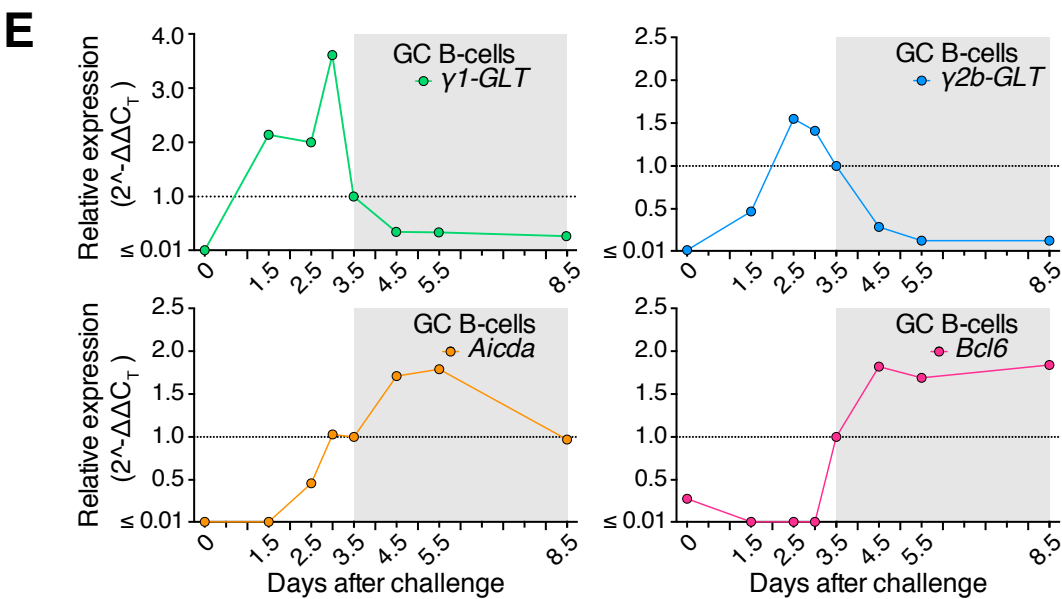
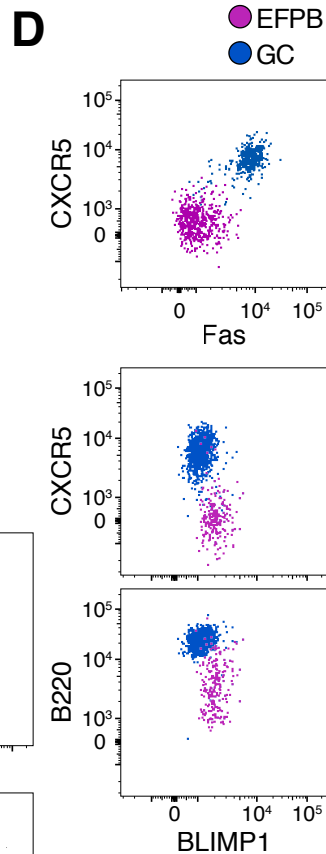
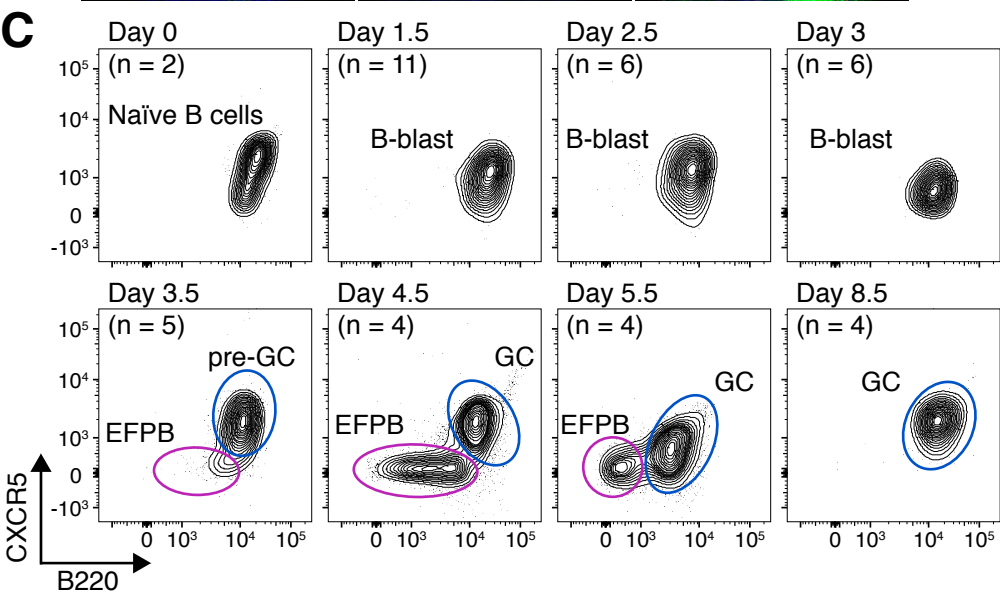
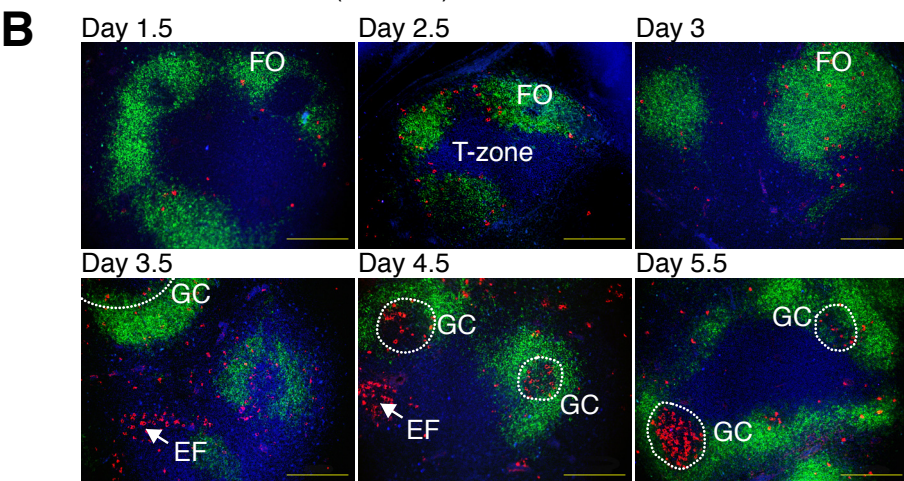
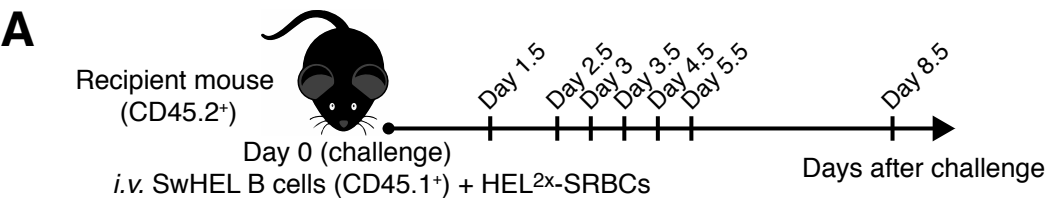
1288 **B)** Consensus binding motif for BCL6 in murine B cells.

1289 **C)** Predicted BCL6-binding sites in the  $\gamma$ 1-GLT promoter scanned with the JASPAR  
1290 2018 database. Yellow and red triangles indicate the location of the predicted sites in  
1291 the  $\gamma$ 1-GLT promoter.

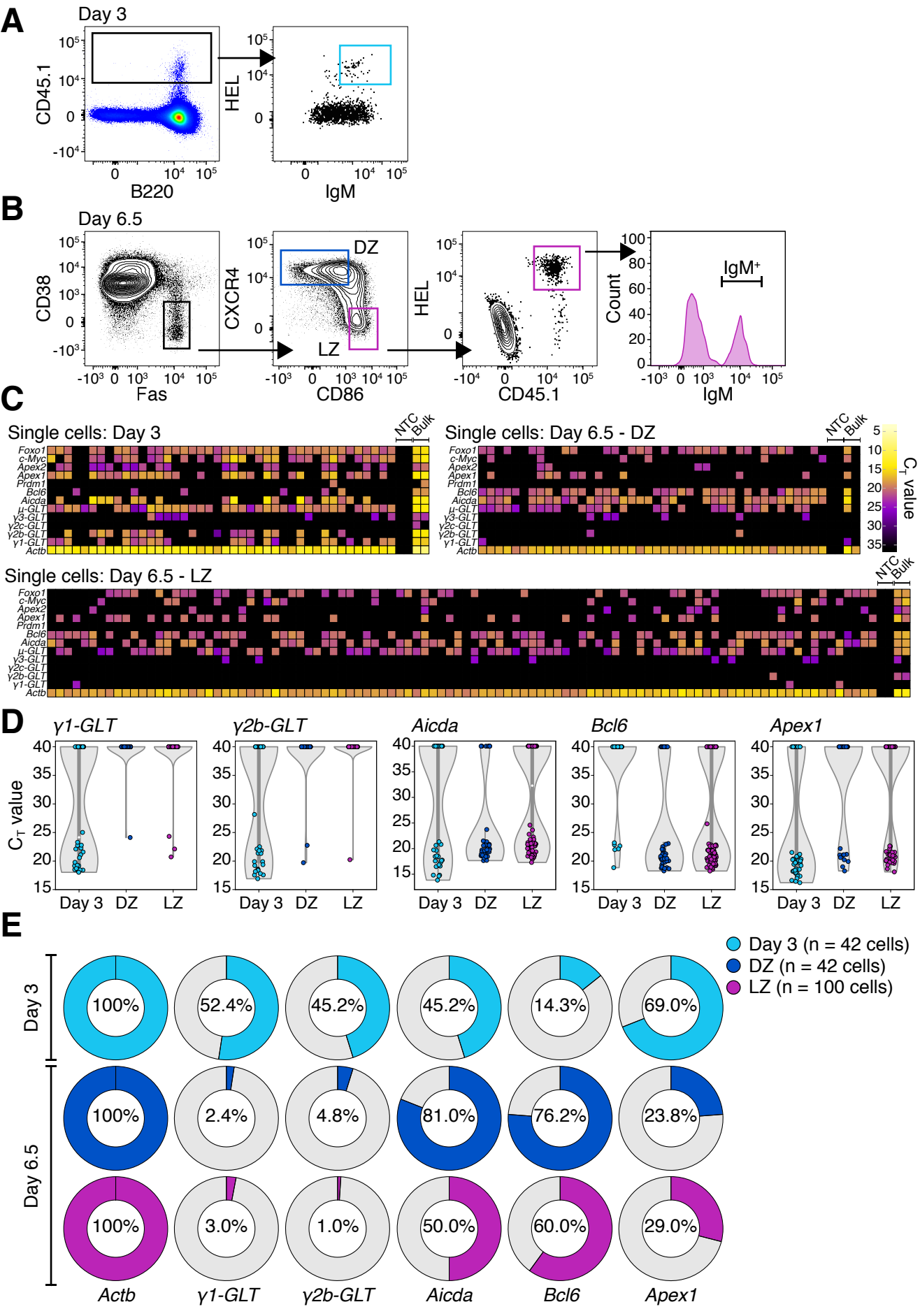
1292 **D)** Flow cytometric analysis of iGC B cells showing induction of BCL6 after 72h of  
1293 culture with Nojima cells and IL-4.

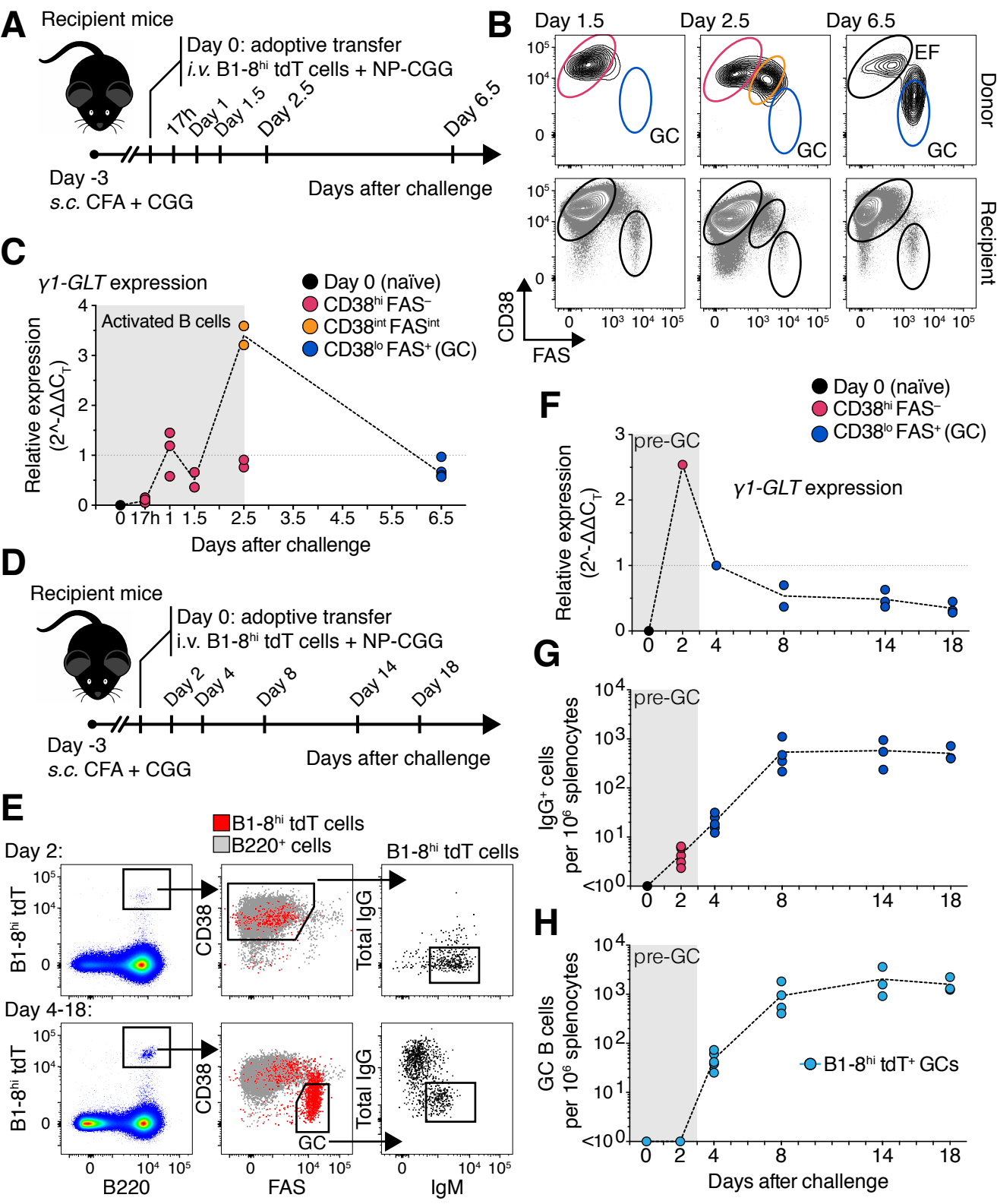
1294 **E)** Bar plot showing enrichment of BCL6 binding across the  $\gamma$ 1-GLT promoter in iGC B  
1295 cells and naïve B cells by CHIP-qPCR. Data was normalized using the percent input  
1296 method.

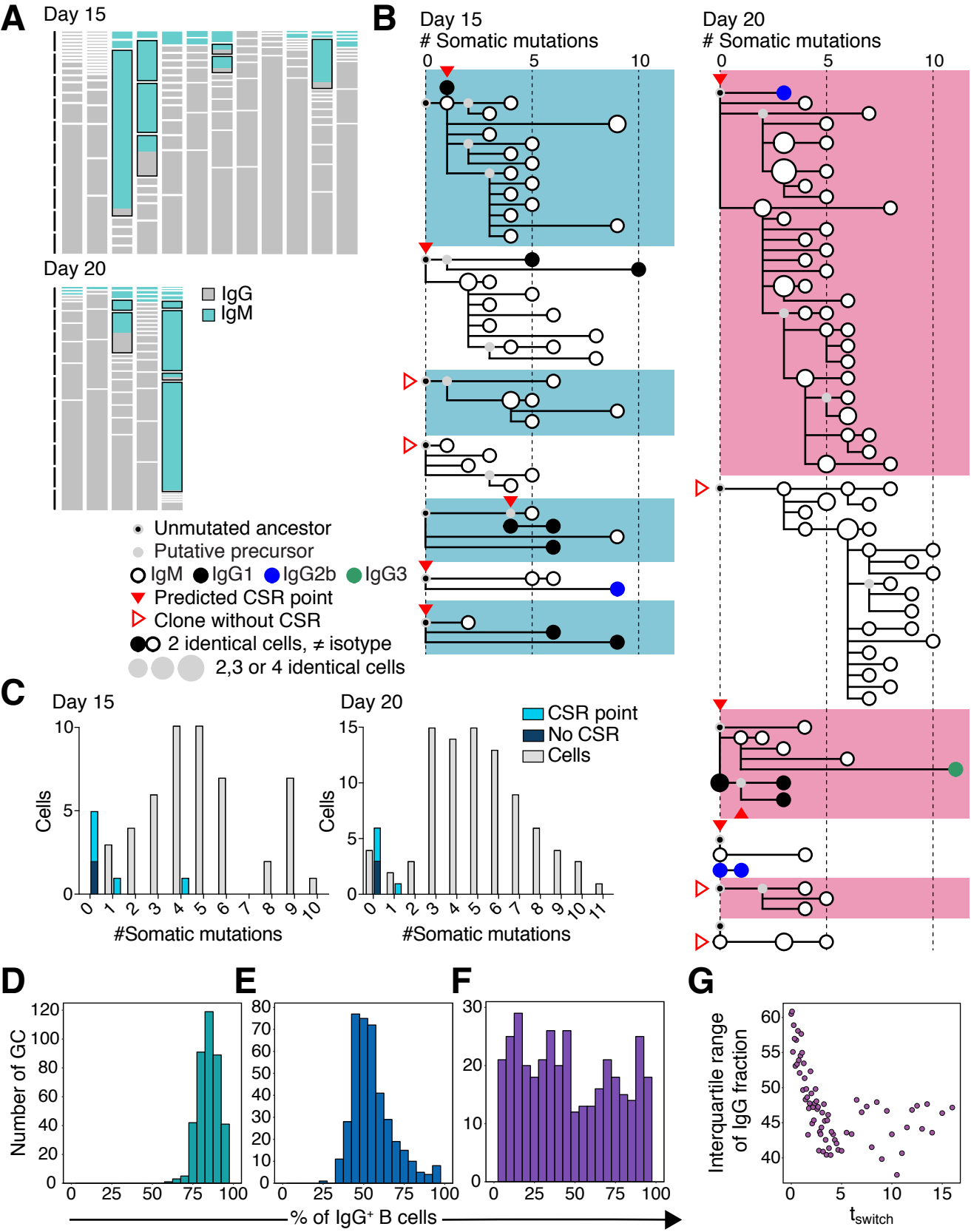


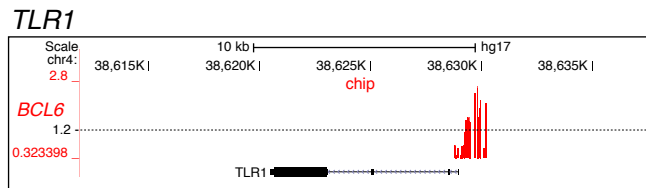
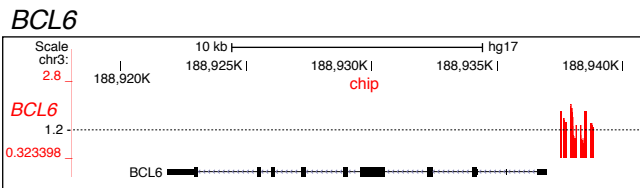
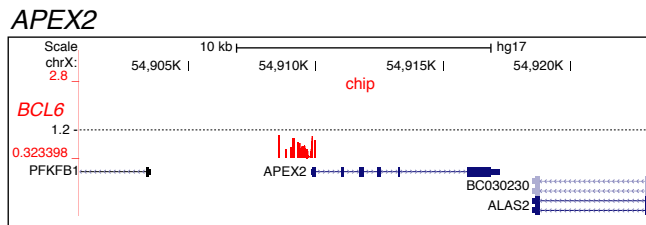
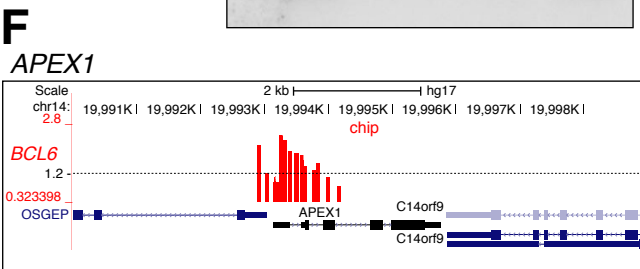
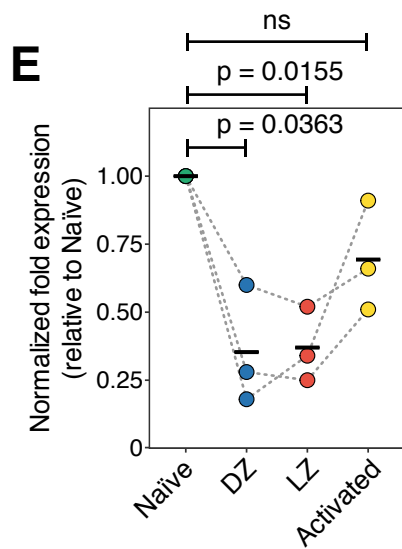
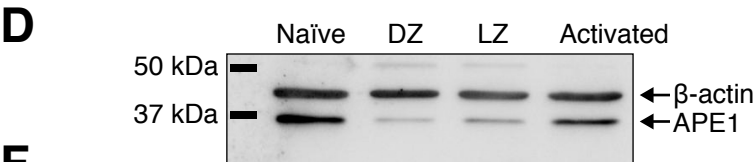
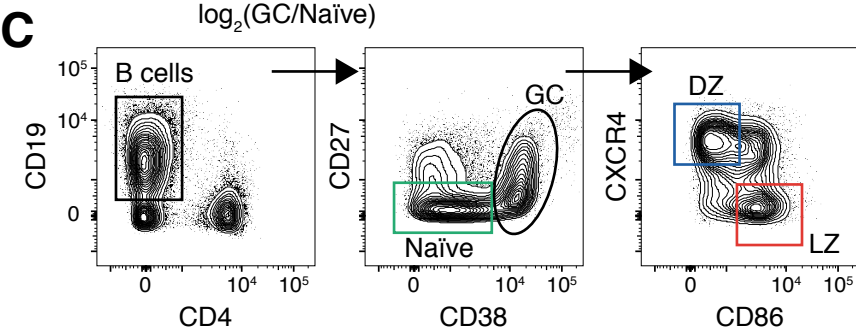
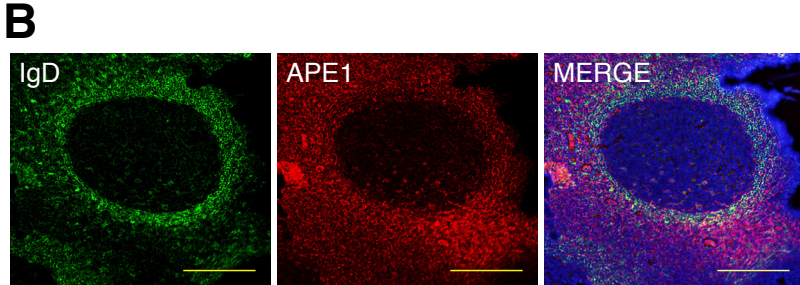
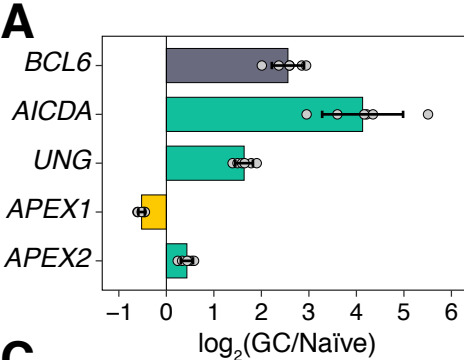






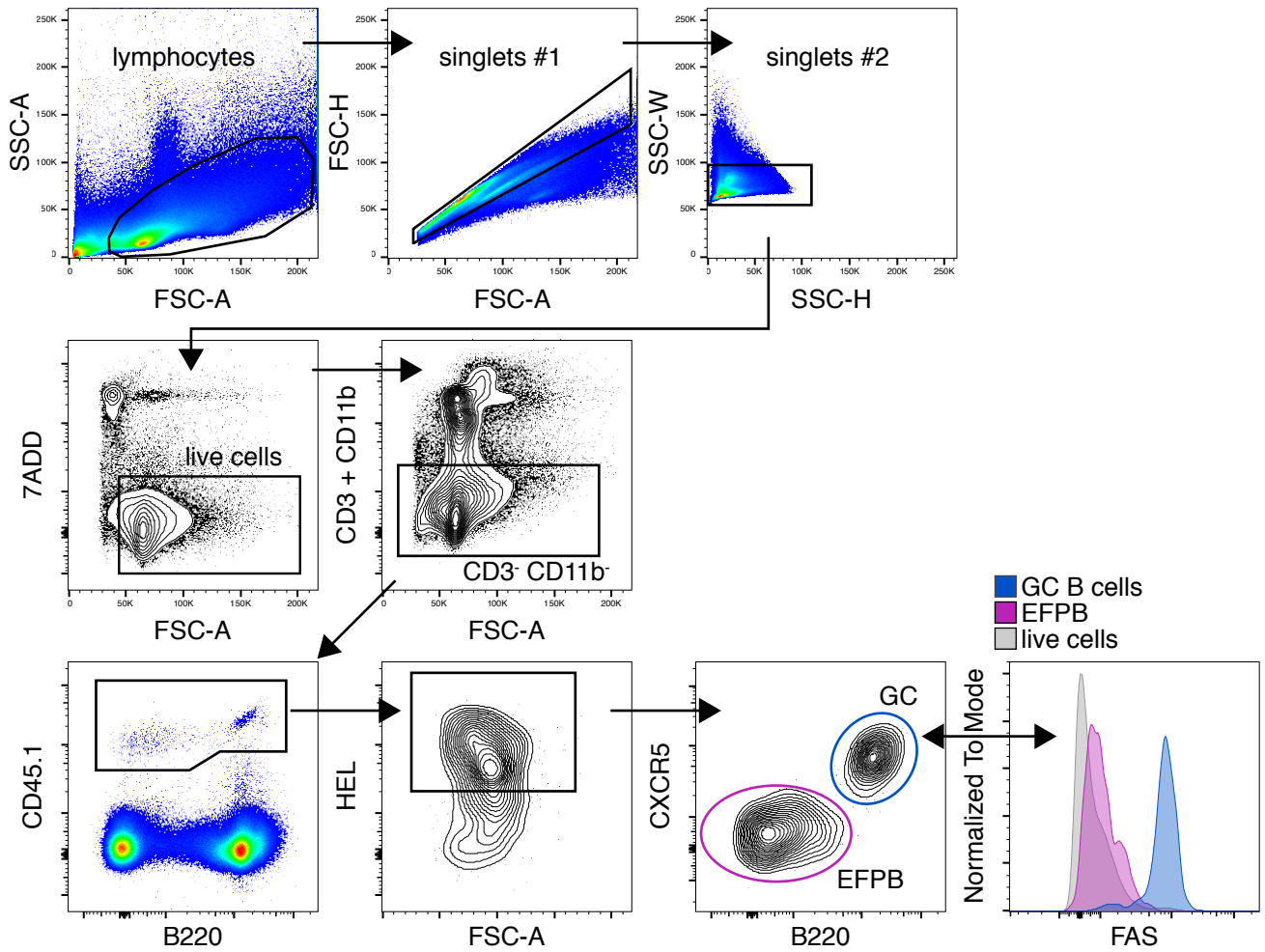




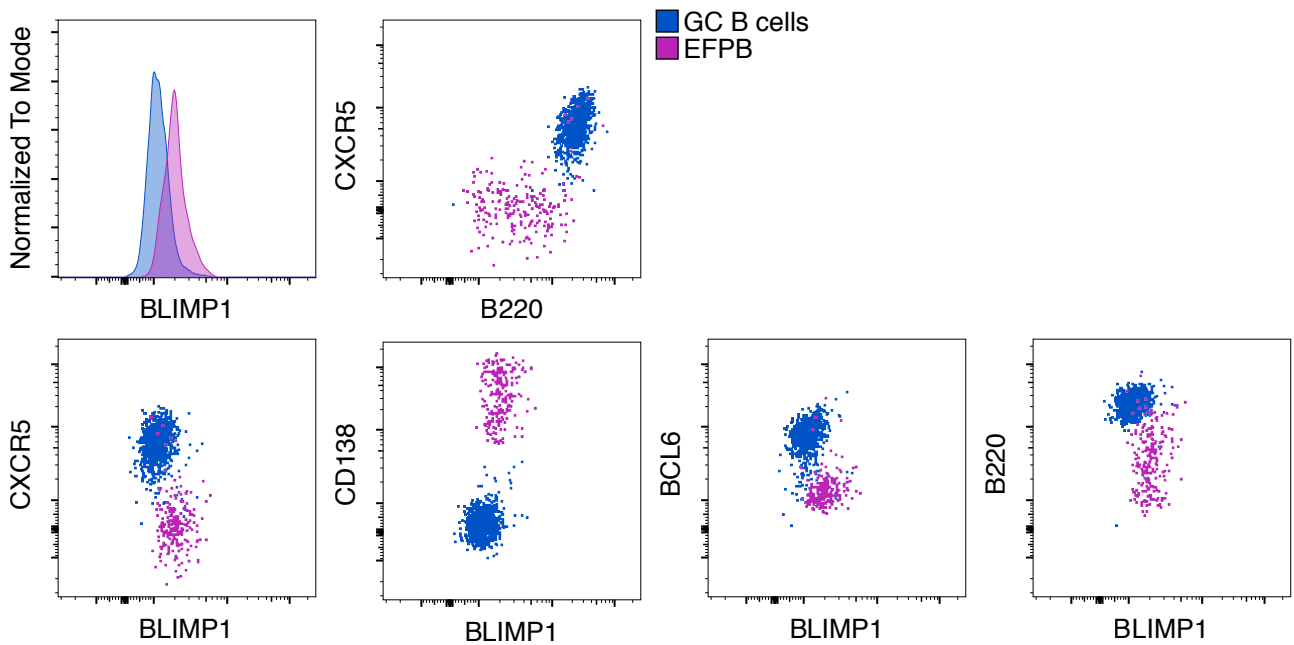


# Figure S1

## A



## B



**Figure S1. Characterization of donor-derived Sw<sub>HEL</sub> B cells by flow cytometry. Related to Figure 1.**

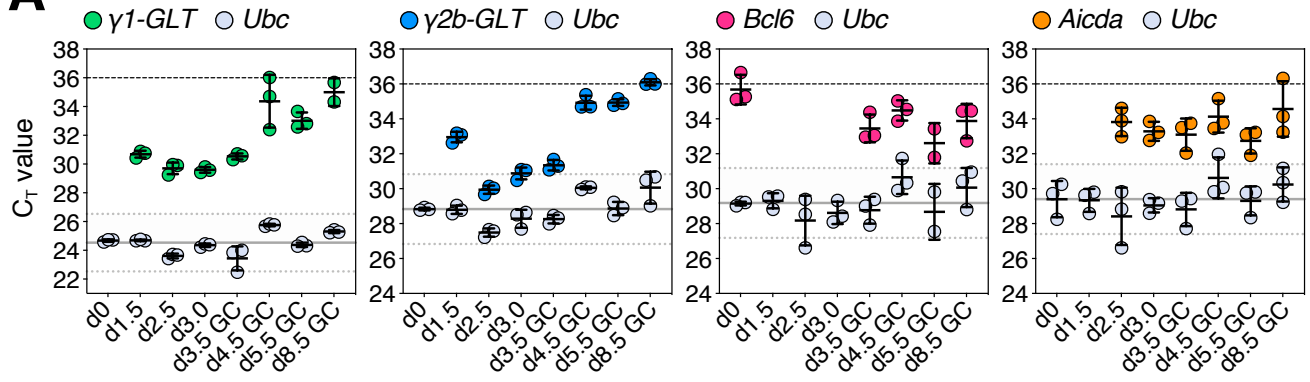
**A)** Gating strategy employed to identify donor-derived Sw<sub>HEL</sub> B cells after adoptive transfer into congenic recipient mice immunized with HEL<sup>2x</sup>-SRBCs or HEL<sup>3x</sup>-SRBCs.

**B)** Flow cytometric plots showing expression of different markers by responding donor-derived Sw<sub>HEL</sub> GC B cells gated as CXCR5<sup>hi</sup> B220<sup>hi</sup> compared to HEL-binding EFPBs identified as CXCR5<sup>lo</sup> B220<sup>lo</sup>.

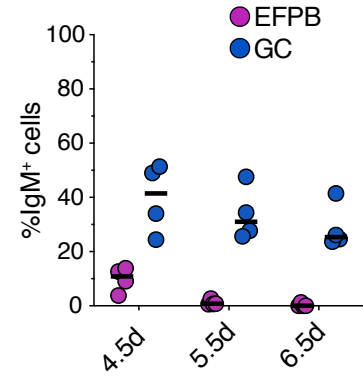


# Figure S2

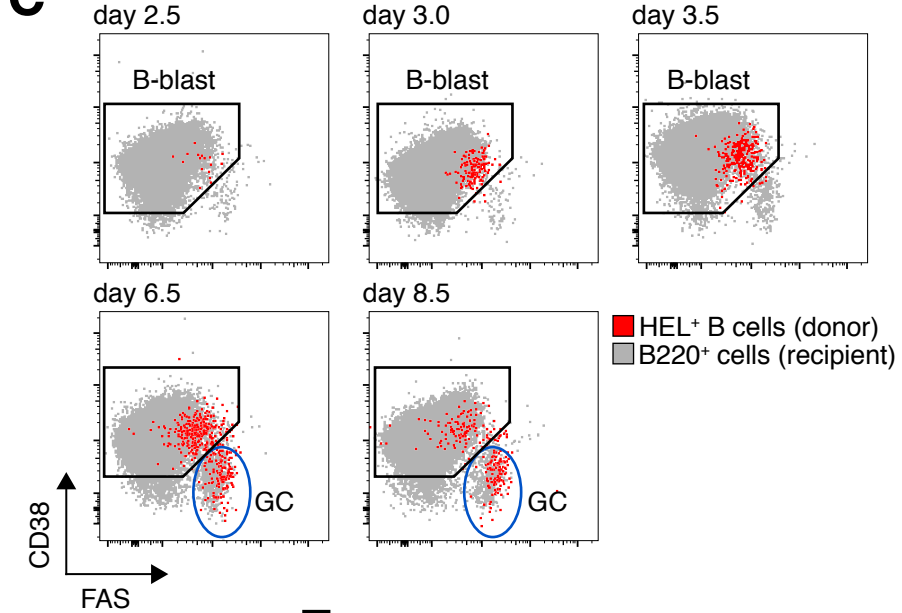
**A**



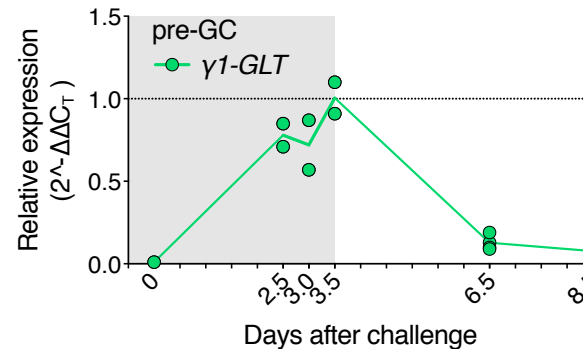
**B**



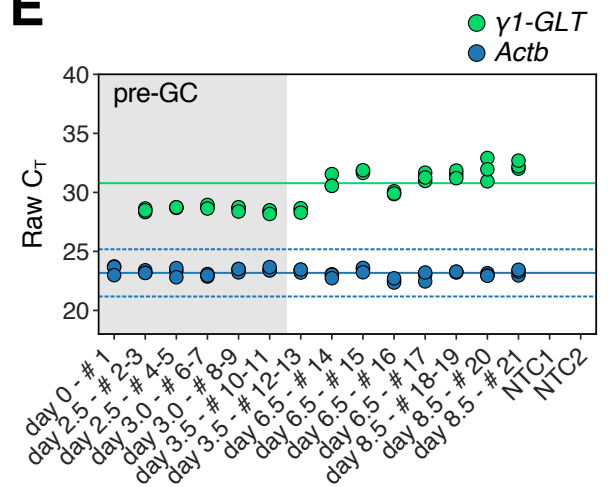
**C**



**D**



**E**



**Figure S2. Raw gene expression profiles and quantification of surface IgM in Sw<sub>HEL</sub> B cells, and analysis of CSR upon immunization with HEL<sup>3x</sup>-SRBCs. Related to Figure 1 and Figure 2.**

**A)** Dot plot showing raw C<sub>T</sub> values for Figure 1E. *γ1-GLT*, *γ2b-GLT*, *Bcl6* and *Aicda* expression values are shown together with those for the reference gene *Ubc*. Horizontal grey lines show the C<sub>T</sub> mean expression value of *Ubc* across all samples. Dotted grey lines are  $\pm 2$  C<sub>T</sub> values from *Ubc* C<sub>T</sub> mean.

**B)** Quantification of IgM in EFPBs or GC B cells for samples shown in Fig 2D. Bars represent medians and dots individual mice (n=4). Data is representative of two independent experiments.

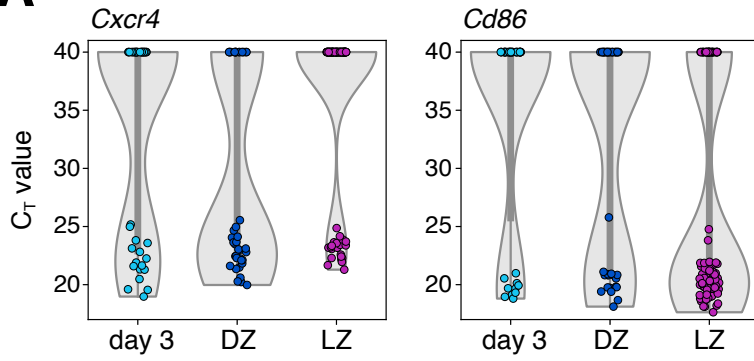
**C)** Flow cytometric plots showing gating strategy used to identify donor-derived Sw<sub>HEL</sub> B cells after immunization with HEL<sup>3x</sup>. In brief, CD45.2<sup>+</sup> recipient mice were adoptively transferred with CD45.1<sup>+</sup> SW<sub>HEL</sub> B cells ( $3 \times 10^4$  -  $15 \times 10^4$ ) and simultaneously *i.v.* challenged with  $2 \times 10^8$  SRBCs conjugated to HEL<sup>3x</sup>. Spleens were harvested at the indicated time points to analyze the immune response.

**D)** qPCR gene expression profile for *γ1-GLT*. Duplex qPCR analyses were conducted using *Actb* as a reference. Data is expressed as a fold-change compared to day 3.5 mean expression value using the  $\Delta\Delta$ CT method. Dots represent the mean of pooled biological replicates (day 0 - 3.5) or individual animals (day 6.5 - 8.5). n = 4 mice per timepoint.

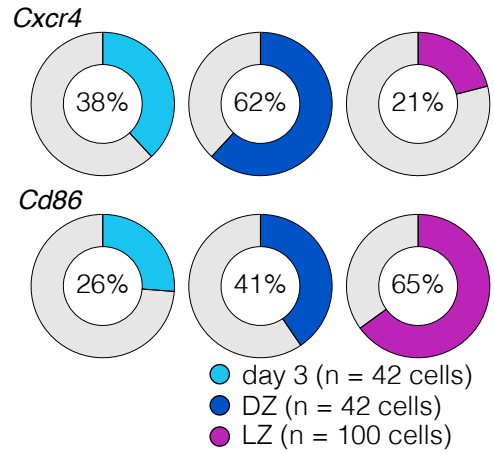
**E)** Dot plot showing raw C<sub>T</sub> values for *γ1-GLT* and the reference gene *Actb*. Samples were measured in triplicate. Horizontal green and blue lines show the C<sub>T</sub> mean value across all samples for *γ1-GLT* and *Actb*, respectively. Dotted blue lines depict  $\pm 2$  C<sub>T</sub> values from *Actb* C<sub>T</sub> mean. NTC = no template control.

# Figure S3

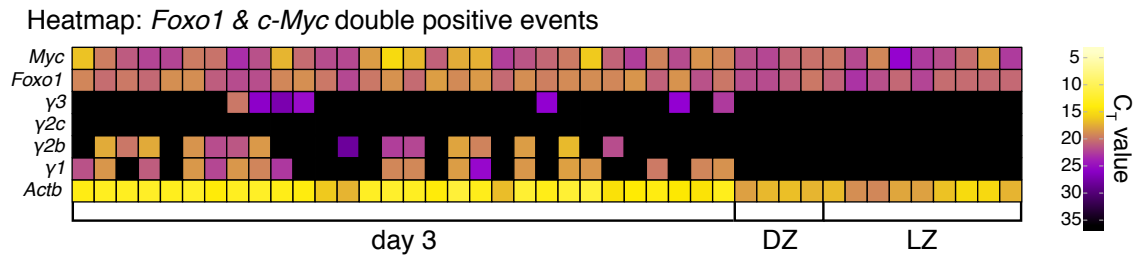
**A**



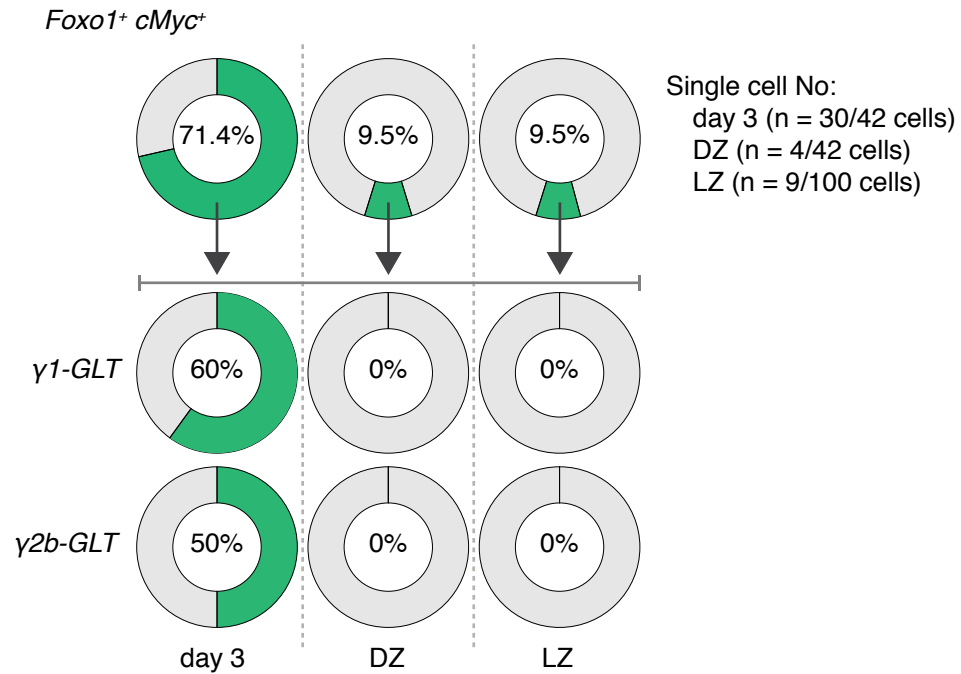
**B**



**C**



**D**



**Figure S3. *Cxcr4* and *Cd86* gene expression in SwHEL B cells and GLT expression analysis in *Foxo1*<sup>+</sup> *c-Myc*<sup>+</sup> SwHEL B cells. Related to Figure 3.**

**A)** Violin plots showing *Cxcr4* and *Cd86* gene expression in the indicated subsets by single cell qPCR.

**B)** Pie charts showing quantification of target genes as shown in **(A)**.

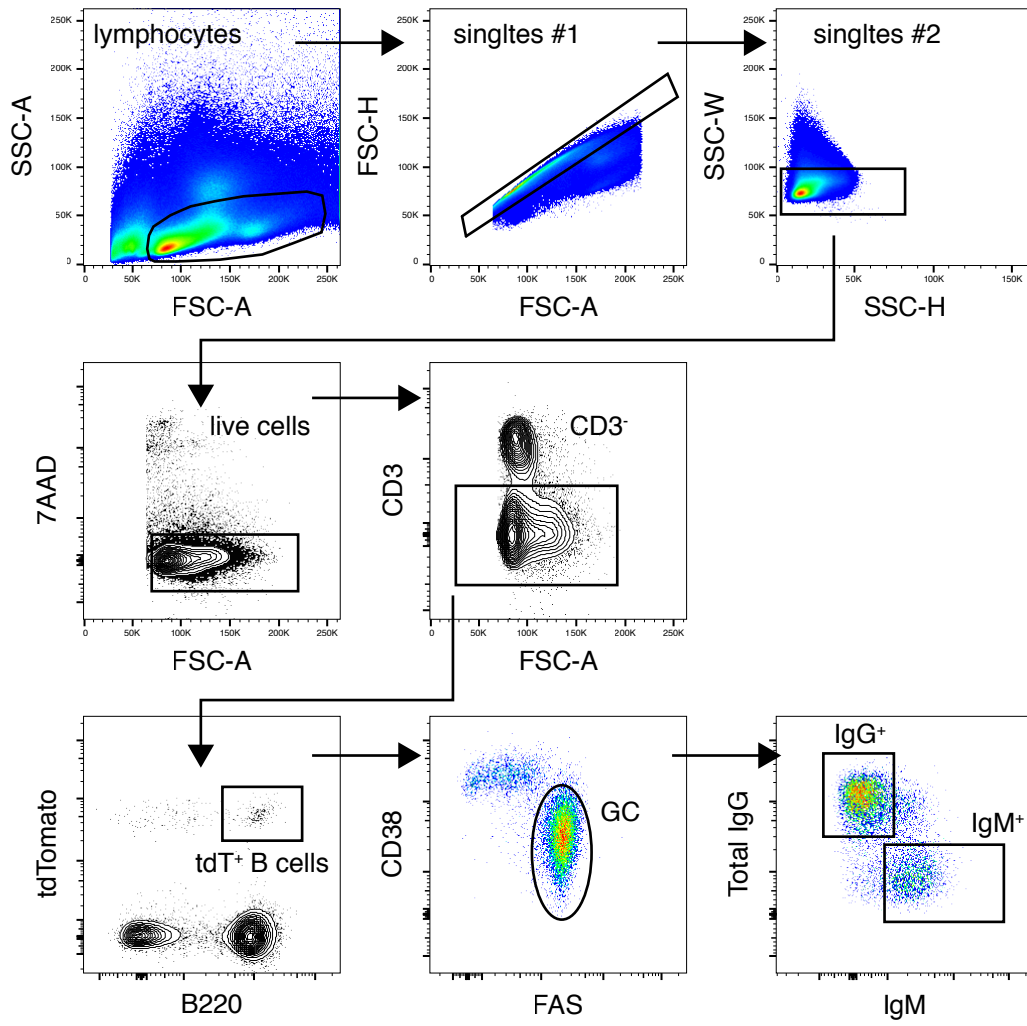
**C)** Heatmap showing single cell expression of  $\gamma$ -GLTs by qPCR in double positive *Foxo1*<sup>+</sup>*Myc*<sup>+</sup> B blast, DZ and LZ GC B cells (see Figure 3 for details).

**D)** Pie charts showing quantification of  $\gamma 1$ - and  $\gamma 2b$ -GLT in double positive *Foxo1*<sup>+</sup>*Myc*<sup>+</sup> cells as for **(C)**.

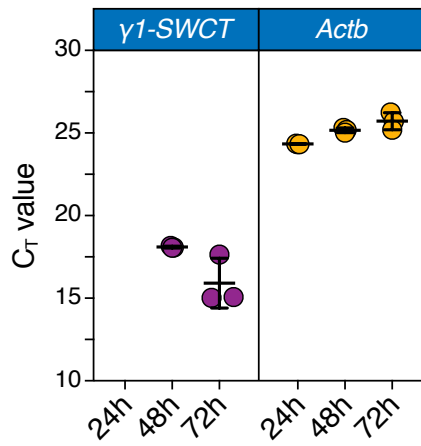
In pie charts (B and D), numbers indicate the percentage of cells expressing the indicated target. Cells with a  $C_T$  value < 40 were considered positive events (see Figure 3 for details).

# Figure S4

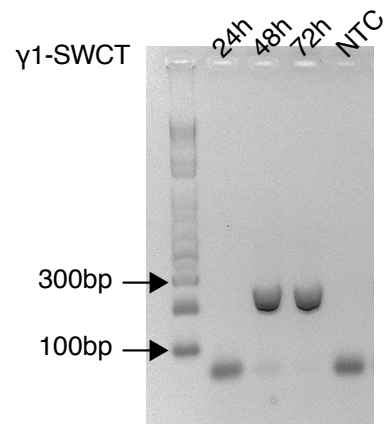
**A**



**B**



**C**



**Figure S4. Characterization of B1-8<sup>hi</sup> tdT<sup>+</sup> B cells and *in-vitro* analysis of switch circle transcript formation. Related to Figure 4.**

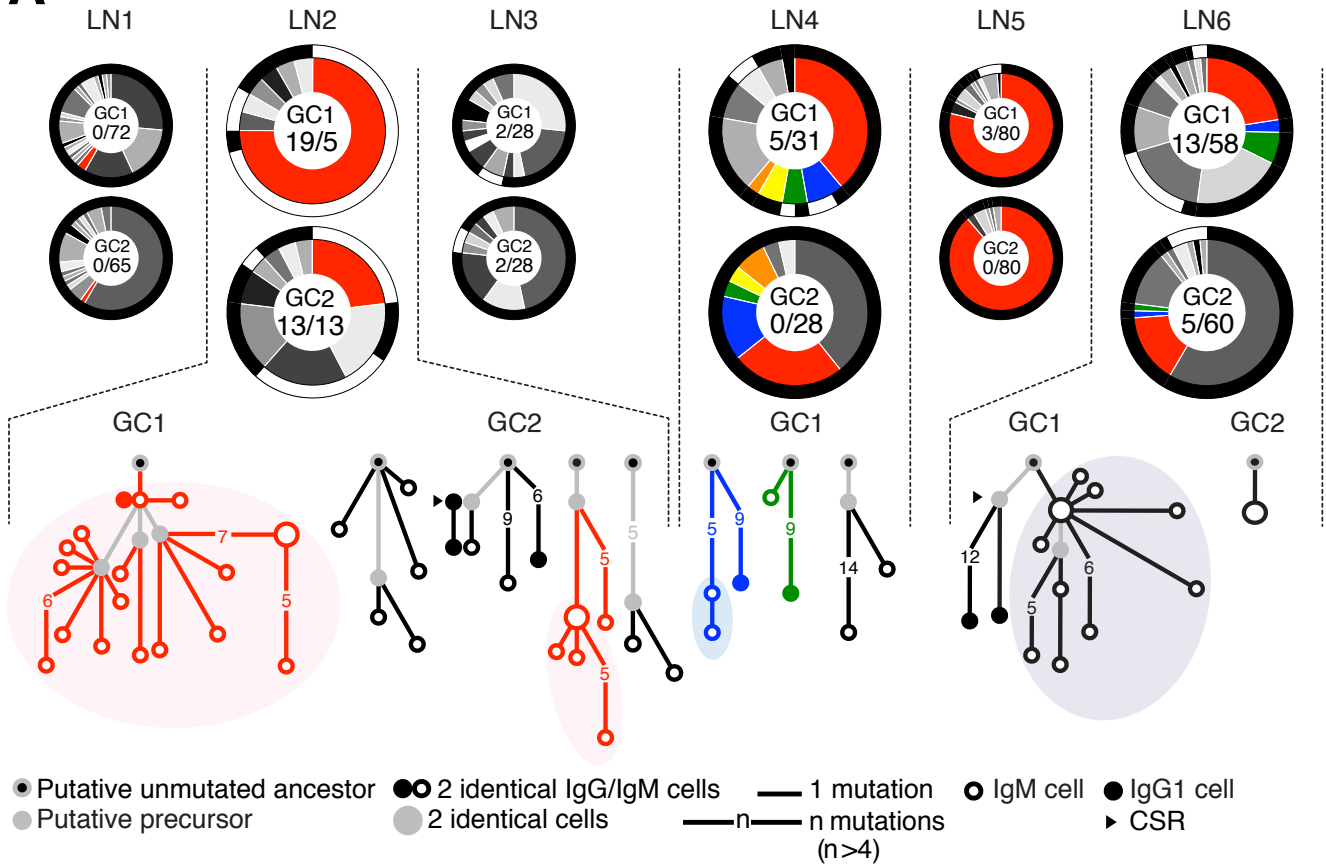
**A)** Gating strategy used to identify donor-derived B1-8<sup>hi</sup> tdT<sup>+</sup> B cells after adoptive transfer into congenic recipient mice immunized with NP-CGG (see Figure 4 for details).

**B)** Dot plot showing raw C<sub>T</sub> values for  $\gamma$ 1-switch circle transcript ( $\gamma$ 1-SWCT) and the reference gene *Actb*. Purified naïve B cells from C57BL/6 wild type mice were stimulated *in vitro* for 24h, 48h and 72h with IL-4 and LPS. Samples were pre-amplified for  $\gamma$ 1-SWCT for 22 cycles, and then analyzed by qPCR.

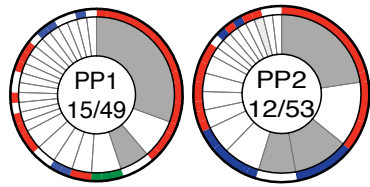
**C)** Analysis of  $\gamma$ 1-SWCT expression as shown in **(B)**. DNA fragments amplified by qPCR were resolved by electrophoresis in a 1.5% agarose gel. NTC = no template control.

# Figure S5

**A**



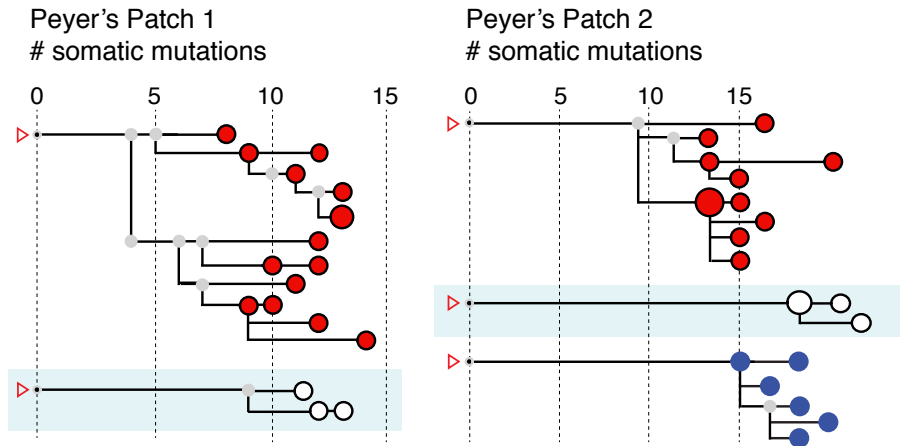
**B**



Legend:

- Unmutated ancestor
- Putative precursor
- IgM ● IgG2 ● IgA ● IgG1
- ▷ Clone without CSR
- 2,3 or 4 identical cells

**C**



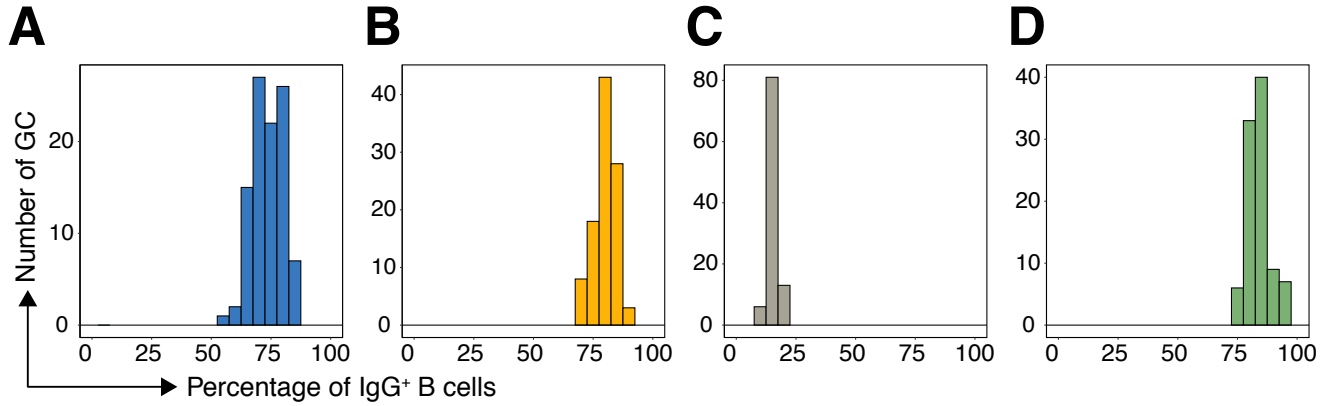
**Figure S5. Clonal analysis of photoconverted GC B cells in lymph nodes and Peyer's Patches. Related to Figure 5.**

**A)** Charts showing clonal diversity of GCs isolated from popliteal lymph nodes (pLNs) after photoactivation in mice immunized 15 days before with CGG-Alum. Two individual GCs per pLN were photoactivated and separately flow cytometry-sorted, as described ([Tas et al., 2016](#)). Pie charts show clonal distribution of sequenced Igh genes in each GC. In the inner ring, each slice represents one distinct clone represented in greyscale, colored slices indicate clones that were found in both GCs (top and bottom pie charts) from the same lymph node. In the outer ring the isotype IgG (black) or IgM (white) of each clone is indicated. Numbers in the center of each chart are the total number of IgM-/IgG cells sequenced. Clonal trees represent the phylogeny of IgM heavy-chain variable-segment ( $V_H$ ) sequences within each clone containing more than 2 cells per clone from pLNs that have more than 5 IgM cells (symbols according to the legend in the top panel). Pairs are from 5 different mice in 3 independent experiments.

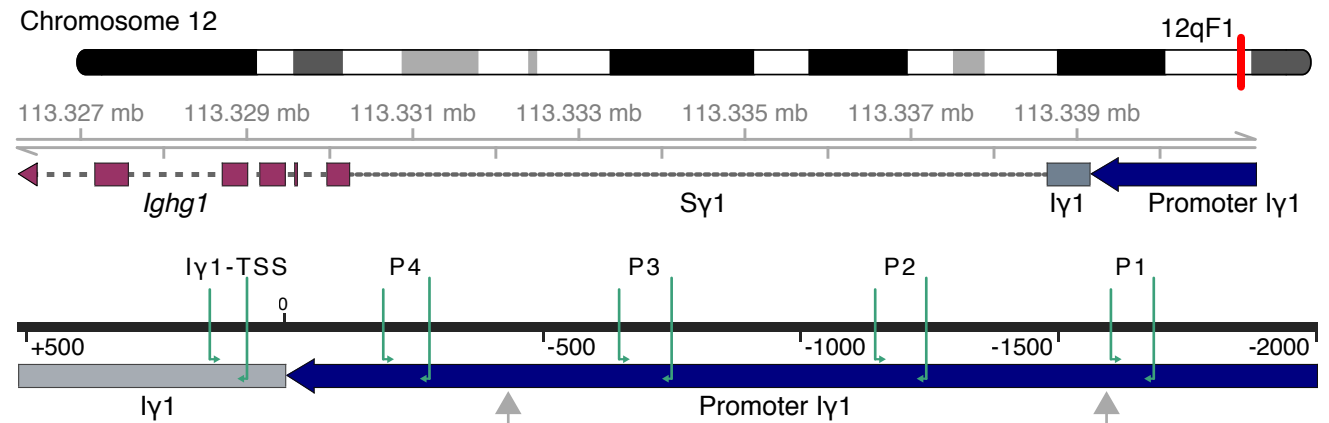
**B-C)** GCs were visualized in the Peyer's patches of unimmunized AID-Cre-Confetti mice. **(B)** Pies of isotype distribution across clones were assembled for single GCs from two separate mice. Inner segments denote clones, outer colored ring denotes isotype.  $n/n$  = number of clones/ total number of sequenced cells. **(C)** Clonal trees representing the phylogeny of  $V_H$ -segment sequences were constructed from the most heavily expanded clones containing more than 3 members per clone (grey segments in pies). Symbols correspond to the legend in the lower left panel.



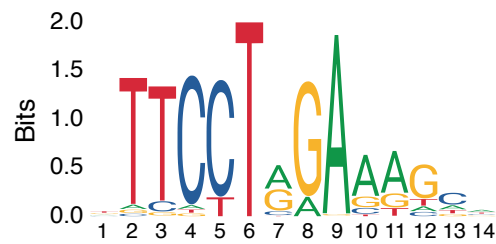
# Figure S6



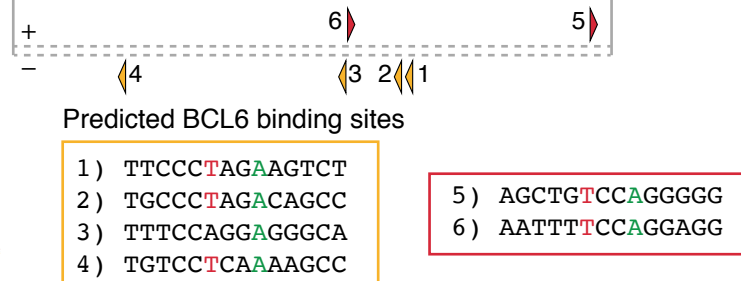
## E



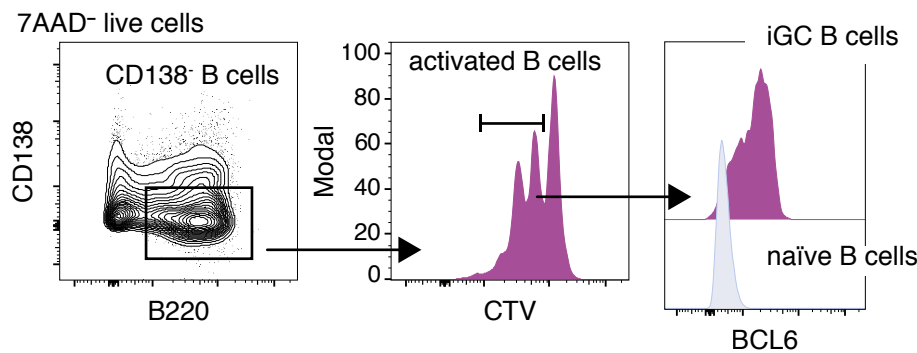
## F



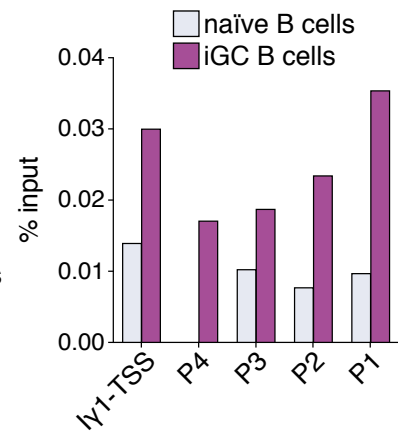
## G



## H



## I



**Figure S6. Mathematical modelling and ChIP-qPCR analysis of BCL6 in primary B cells.**  
**Related to Figure 5 and Figure 6.**

**A-B)** Same simulation as in Figure 5F with ongoing influx of IgM<sup>+</sup> B cells into the GC. The influx rate decays from 2 cells per hour to zero following a sigmoidal function with half value at day 10 post-GC onset and a width of 10 days.

**C-D)** Same analysis as in **(A-B)** but, instead of modulating the B cell influx duration, with IgM<sup>+</sup> B cells collecting twice as much antigen per interaction with FDC, which induces more signaling in the subsequent selection steps. In **(A-C)**, the sensitivity of GC reactions to ongoing influx and increased antigen uptake, respectively, is shown with unchanged ( $p=0.03$  per division) switching probability. In **(B-D)** the switching probability was adapted to  $p=0.05$  and  $p=0.18$  per division, respectively, in order to make the simulation consistent with the measured mean IgG dominance. Each graph represents 100 simulation replicates.

**E-I)** ChIP-qPCR analysis of BCL6 in primary B cells using the Nojima culture system. Splenic naïve B cells were magnetically purified and co-cultured for 72 h with Nojima cells supplemented with IL-4. These *in vitro* derived GC (iGC) B cells were flow cytometry purified to assess binding of BCL6 to the  $\gamma 1$ -GLT promoter by ChIP-qPCR.

**E)** Diagram depicting the chromosome location of the  $\gamma 1$ -GLT promoter (blue arrow) 2000 bp upstream of the Iy1 coding sequence (grey segment) in the mouse genome. Green arrows indicate the binding location of the primers used for ChIP-qPCR as shown in **(I)**.

**F)** Consensus binding motif for BCL6 in murine B cells.

**G)** Predicted BCL6-binding sites in the  $\gamma 1$ -GLT promoter scanned with the JASPAR 2018 database. Yellow and red triangles indicate the location of the predicted sites in the  $\gamma 1$ -GLT promoter.

**H)** Flow cytometric analysis of iGC B cells showing induction of BCL6 after 72h of culture with Nojima cells and IL-4.

**I)** Bar plot showing enrichment of BCL6 binding across the  $\gamma 1$ -GLT promoter in iGC B cells and naïve B cells by ChIP-qPCR. Data was normalized using the percent input method. Data is representative of three independent experiments.

**Table S1. Sequences of primers and probes used to detect GLT and *Actb* expression by qPCR. Related to Figure 1, 3 and 4.**

<b>Target</b>	<b>Primer Sequence (5' - 3')</b>	<b>Probe Sequence (5' - 3')</b>
<i>γ1-GLT</i>	F: CGAGAAGCCTGAGGAATGTGT R: GGAGTTAGTTTGGGCAGCAGAT	FAM-TGGTTCTCTCAACCTGTAGTCCATGCCA
<i>γ2b-GLT</i>	F: CGCACACCTACAGACAACCAG R: GTCACAGAGGAACCAGTTGTATC	FAM-CCAGGGGGCCAGTGGATAGACTGAT
<i>γ2c-GLT</i>	F: GGACCACTAAAGCTGCTGACACAT R: AACCCCTGACCAGGCATCCT	FAM-AGCCCCATCGGTCTATCCACTGGC
<i>γ3-GLT</i>	F: GACCAAATTCGCTGAGTCATCA R: ACCGAGGATCCAGATGTGTCA	FAM-CTGTCTATCCCTTGGTCCCTGGCTGC
<i>μ-GLT</i>	F: TCTGGACCTCTCCGAAACCA R: ATGGCCACCAGATTCTTATCAGA	FAM-ATGTCTTCCCCCTCGTCTCCTGCG
<i>Actb</i>	F: CGTGAAAAGATGACCCAGATCA R: TGGTACGACCAGAGGCATACAG	HEX-TCAACACCCCAGCCATGTACGTAGCC

**Table S2. Sequences of primers used in CHIP-qPCR studies. Related to STAR Methods.**

<b>Target</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>
I $\gamma$ 1-P1	5'-GCTCCACCTACCTTGTCTTTAT-3'	5'-GAGATGGGTTTCAGAGTGTCATAG-3'
I $\gamma$ 1-P2	5'-CACTCTCACTCCAGGGTATAGA-3'	5'-TGAGACCCAGAACACAGAATTAG-3'
I $\gamma$ 1-P3	5'-CTCCACAACCTGTACCTAAAT-3'	5'-GGACATGGAAGTAGAGGATCAAA-3'
I $\gamma$ 1-P4	5'-GTCAGGAAAGAGTGGGCATAA-3'	5'-CTGGCTGTACTCCTGTTTCTC-3'
I $\gamma$ 1-TSS	5'-GGGCAGGACCAAACAGGAA-3'	5'-TTTCCCTGCTGACCCCACTC-3'