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BCR affinity differentially regulates colonization of the subepithelial dome and infiltration into germinal centers within Peyer's patches

Document Version:

Accepted author manuscript (peer-reviewed)

Citation for published version:

Biram, A, Stromberg, A, Winter, E, Stoler-Barak, L, Salomon, R, Addadi, Y, Dahan, R, Yaari, G, Bemark, M & Shulman, Z 2019, 'BCR affinity differentially regulates colonization of the subepithelial dome and infiltration into germinal centers within Peyer's patches', *Nature Immunology*, vol. 20, no. 4, pp. 482-492. <https://doi.org/10.1038/s41590-019-0325-1>

Total number of authors:

10

Digital Object Identifier (DOI):

[10.1038/s41590-019-0325-1](https://doi.org/10.1038/s41590-019-0325-1)

Published In:

Nature Immunology

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1 **BCR affinity differentially regulates colonization of the subepithelial**
2 **dome and infiltration into germinal centers within Peyer's patches**

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15

16 **Abstract**

17 Gut-derived antigens trigger immunoglobulin A (IgA) immune responses that are
18 initiated by cognate B cells in the Peyer's patch (PP). These cells colonize the
19 subepithelial domes (SEDs) of the PP, and subsequently infiltrate into pre-existing
20 germinal centers (GCs). Here, we defined the pre-GC events and the microanatomical site
21 at which affinity-based B cell selection occurred in PPs. Using whole-organ imaging, we
22 showed that the affinity of the B cell antigen receptor (BCR) regulated infiltration of
23 antigen-specific B cells into GCs, but not clonal competition in the SED. Follicular
24 helper-like T cells resided in the SED and promoted its B cell colonization, independently
25 of the magnitude of BCR affinity. Imaging and immunoglobulin sequencing indicated
26 that selective clonal-expansion ensued during infiltration into GCs. Thus, in PPs, in
27 contrast to draining lymph nodes and spleen, T cells predominantly promoted expansion
28 of B cells without clonal selection during pre-GC events. These findings have major
29 implications for the design of oral vaccines.

30

31 The initiation of an effective antibody immune response and establishment of enduring
32 protection from invading pathogens depends on formation of long-lived plasma and
33 memory B cells¹⁻³. Generation of these cells takes place primarily in germinal centers
34 (GCs) where B cells undergo diversification by somatic hypermutation (SHM) and
35 affinity-based clonal selection⁴⁻⁷. These events lead to an increase in immunoglobulin
36 affinity over time, a process known as antibody affinity maturation^{2,4,8}. In draining lymph
37 nodes (LNs) and in the spleen, antigen-specific B cells compete for GC entry at the
38 boundary between the T cell zone and the B cell rich follicles based on the affinity of the
39 B cell antigen receptor (BCR)⁹⁻¹¹. Selection of B cells for GC seeding or for
40 differentiation into early plasmablasts is regulated by T follicular helper cells (T_{FH} cells),
41 which physically interact with B cells and discern high- versus low-affinity clones based
42 on the capacity of their BCR to take up and present antigen on their surface^{9,12}. However,
43 it remained unknown whether these findings also apply to mucosal-associated lymphoid
44 organs.

45 The major immunoglobulin isotype produced in intestinal tissues, IgA, is secreted by
46 plasma cells that originate primarily from Peyer's patches (PPs), lymphoid organs that
47 are located along the small intestine¹³⁻¹⁵. As opposed to LNs, which drain lymphatics and
48 typically lack B cell activity in the absence of stimulation, PPs lack afferent lymphatic
49 vessels and host chronically-induced and T cell-dependent GCs that are formed in
50 response to commensal bacteria and food-derived immunogens^{14,16}. Antigens are
51 predominantly transported from the gut lumen through the follicle-associated epithelium
52 that covers the PP by specialized epithelial cells, known as microfold (M) cells¹⁷. These
53 cells transcytose luminal antigens into the subepithelial domes (SEDs) in PPs, where B

54 cells and antigen presenting cells reside^{16,18}. CCR6 is a G-protein coupled receptor that
55 mediates chemotactic migration of immune cells towards its ligand, CCL20^{19,20}. In PPs,
56 CCR6 plays a critical role in B cell entry into the SED, where the necessary signals for
57 class switch recombination to the IgA isotype are provided^{20,21}. Subsequently, antigen-
58 specific B cells do not migrate towards the T cell zone¹¹ but rather infiltrate preformed
59 GCs that support immune responses to different antigens^{20,22}. Whether affinity-based
60 selection takes place during the pre-GC events in the SED and the role of T cells in this
61 process was not examined.

62 In the current study, we used whole-organ imaging by light sheet fluorescence
63 microscopy (LSFM) for detection of transgenic B cells bearing high or low affinity BCRs
64 in PPs. This technique allowed us to efficiently visualize multiple niches within intact
65 PPs and examine the impact of BCR affinity and T cell help on B cell colonization of the
66 SED and GC compartments. We found that effective B cell expansion in the SED
67 predominantly depended on local delivery of T_{FH} cell-derived signals to antigen-specific
68 B cells, independently of clonal selection.

69

70 **Results**

71 **Whole-organ imaging captures all the SEDs and GCs in intact PPs**

72 B cells interact with antigen, express activation-induced cytidine deaminase (AID) and
73 class switch to IgA within the SED compartment^{20,23}. To visualize all B cell
74 compartments in intact PPs, we used LSFM in AID fate-reporter mice
75 *Aicda*^{Cre/+}*Rosa26*^{Stop-tdTomato/+}, in which B cells express tdTomato fluorescent protein
76 during and following AID expression²⁴. LSFM imaging of intact PPs revealed GC

77 structures that were coupled to clusters of tdTomato⁺ B cells within the SED facing the
78 lumen of the gut (**Fig. 1a, Supplementary Video 1**). Expression of CCR6 on multiple
79 cell types, is essential for proper formation and organization of the SED immune
80 niche^{20,21,23}. Accordingly, PPs derived from *Ccr6*^{-/-}*Aicda*^{Cre/+}*Rosa26*^{Stop-tdTomato/+} hosted
81 significantly fewer and smaller GCs compared to wild-type mice and no tdTomato⁺ B
82 cells were detected in proximity to the lumen (**Fig. 1a-c**), indicating that whole-organ
83 imaging detected B cells in the SEDs.

84 To study the immune response of antigen-specific B cells in PPs, splenic B cells were
85 isolated from mice that express DsRed or GFP ubiquitously and carry the *Igh*^{B1-8hi} heavy
86 chain allele that generate a BCR specific for 4-hydroxy-3-nitrophenylacetyl (NP) when
87 coupled with Igλ light chain²⁵. B1-8^{hi} DsRed⁺ Igλ⁺ B cells derived from these mice were
88 transferred into wild-type mice prior to oral immunization. To effectively deliver a
89 cognate oral immunogen into PPs through M cells, we used NP coupled to cholera toxin
90 (NP-CT) as an antigen²². This type of oral immunization did not induce damage to the
91 mucosal surfaces (**Supplementary Fig. 1**). To visualize B cell immune responses in
92 defined GC and SED niches, we imaged transferred B1-8^{hi} DsRed⁺ B cells in mice that
93 carry an *Aicda*-transgene fused to *GFP* (AID-GFP)²⁴. One day following cell transfer,
94 AID-GFP host mice were orally immunized with NP-CT. Nine days after antigen
95 administration, we detected B1-8^{hi} DsRed⁺ B cells among host AID-GFP⁺ B cells in the
96 GCs and SEDs of the PP (**Fig. 1d, Supplementary Video 2**), while almost no B1-8^{hi}
97 DsRed⁺ B cells were detected in the PPs of mice that did not receive NP-CT (**Fig. 1d**).
98 Imaging of B1-8^{hi} *Ccr6*^{GFP/+} DsRed⁺ B cells in wild-type mice immunized with NP-CT
99 confirmed that B cells expressed CCR6 in the SED, but not in the GC (**Fig. 1e**)^{20,21}. As

100 expected, transferred B1-8^{hi} DsRed⁺ B cells were found next to CD11c⁺ cells (DCs and B
101 cells) and CX3CR1⁺ macrophages in the SED (**Fig. 1f,g, Supplementary Video 3 and**
102 **4**)^{16,26}. These results indicate that LSFM can be used for large-scale analysis of B cell
103 immune responses in multiple compartments within the PP.

104

105 **BCR affinity regulates B cell GC entry but not SED colonization**

106 To assess whether an affinity-based B cell selection takes place in the SED, we examined
107 the capacity of B1-8^{hi} GFP⁺ Igλ⁺ and B1-8^{lo} DsRed⁺ Igλ⁺ B cells which express BCRs
108 with a 40-fold difference in affinity for NP, to colonize different PP compartments²⁵. To
109 minimize competition of transferred cells with endogenous B cells, we used MD4 host
110 mice, in which nearly all B cells (99%) are specific for hen egg lysozyme (HEL)^{9,27,28}.
111 MD4 mice had pre-existing GCs in their PPs that contained HEL-specific B cells without
112 prior exposure to antigen (HEL) (**Supplementary Fig. 2a**)²⁷. Transferred B1-8^{hi} GFP⁺ B
113 cells had entered and expanded in the SED 4 days after NP-CT administration, and were
114 detected in the GCs by day 9 (**Fig. 2a-c, Supplementary Fig. 2b and Video 5**). B1-8^{lo}
115 DsRed⁺ B cells expanded in the SED four days after NP-CT delivery, but were not
116 detected in the GCs at day 9 and instead accumulated in the SED (**Fig. 2a-c,**
117 **Supplementary Video 6**). These observations indicate that the magnitude of the BCR
118 affinity regulated the infiltration of B cells into pre-existing GCs; however, B cells with
119 low affinity BCRs were able to colonize the SEDs.

120 To determine whether affinity-based competition took place in the SED, we co-
121 transferred a mixture of B1-8^{hi} GFP⁺ and B1-8^{lo} DsRed⁺ B cells at a 1:1 ratio into MD4
122 hosts, followed by NP-CT delivery. After nine days, LSFM imaging indicated that B1-8^{hi}

123 GFP⁺ and B1-8^{lo} DsRed⁺ B cells colonized the SED compartment to a similar extent,
124 while the GC was composed entirely of B1-8^{hi} GFP⁺ B cells (**Fig. 2b,d**). Accordingly,
125 flow cytometric analysis revealed that very few transferred B1-8^{lo} DsRed⁺ B cells (less
126 than 9 %) expressed GC markers (FAS⁺CD38⁻) in MD4 host mice in response to NP-CT
127 at day 9 post-antigen delivery, whether or not B1-8^{hi} GFP⁺ B cells were present (**Fig. 2e**
128 **and Supplementary Fig. 2c**). Thus, B cell affinity-based competition did not take place
129 during SED colonization; however, GC infiltration and/or early GC competition
130 depended on BCR affinity.

131 Next, we investigated whether BCR affinity controlled B cell proliferative capacity in the
132 SED. Intravenous infusion of the nucleoside analog 5-ethynyl-2'-deoxyuridine (EdU)
133 into wild-type mice followed by flow cytometry analysis revealed that CD38⁺CCR6⁺IgA⁺
134 B cells in the SED were highly proliferative compared to CD38⁻CCR6⁻FAS⁺IgA⁺ GC B
135 cells (**Fig. 2f and Supplementary Fig. 2d**). We also measured EdU incorporation by
136 transferred B1-8^{hi} GFP⁺ and B1-8^{lo} DsRed⁺ B cells in wild-type mice that received NP-
137 CT orally. Flow cytometric analysis did not reveal a significant difference in EdU uptake
138 between the two cell types (**Fig. 2g**), indicating that BCR affinity did not dictate the
139 magnitude of B cell proliferation in the SED.

140 Next, we examined whether BCR affinity plays a role in plasma cell formation within
141 PPs. Two-photon laser scanning microscopy (TPLSM) imaging of PPs explanted from
142 reporter mice, which carry a *YFP* transgene under the control of the *Prdm1* promoter
143 (Blimp-1-YFP) indicated the presence of scattered YFP⁺ plasma cells within the SED and
144 in the interfollicular regions of the PP (**Fig. 2h and Supplementary Fig. 3a**). To
145 examine whether B cells have the capacity to differentiate into plasmablasts early during

146 the response, B1-8^{hi} Blimp-1-YFP DsRed⁺ B cells were transferred into wild-type mice,
147 and PPs were examined five days after NP-CT administration. TPLSM analysis of intact
148 PPs revealed individual B1-8^{hi} DsRed⁺ B cells that expressed Blimp-1-YFP in the SED
149 compartment, as well as in the interfollicular regions (**Fig. 2h, and Supplementary Fig.**
150 **3b-c and Video 7**). Flow cytometric analysis showed that on average 4.25 % of the
151 transferred B1-8^{hi} Blimp-1-YFP DsRed⁺ B cells were Blimp-1-YFP⁺ five days after NP-
152 CT administration (**Supplementary Fig. 3d**). Flow cytometry quantification of the
153 frequency of CD138⁺ plasma cells among transferred B1-8^{hi} GFP⁺ or B1-8^{lo} DsRed⁺ B
154 cells showed no significant difference between the two types of transferred B cells at day
155 5 after NP-CT delivery (**Fig. 2i**), indicating BCR affinity did not influence plasma cell
156 differentiation. These results indicate that in the SED, B cells rapidly proliferated and had
157 the capacity to differentiate into early antibody forming cells; however, this process was
158 not affected by the magnitude of their BCR affinity.

159

160 **T_{FH} cells promote effective colonization of the SED and GC entry**

161 In LNs, after engagement with antigen, B cells interact with cognate T cells and receive
162 help signals that promote clonal selection^{9,11}. To investigate the role of T cells in the
163 SED, B1-8^{hi} GFP⁺ B cells were transferred into wild-type mice and allowed to colonize
164 this compartment, followed by an intravenous dose of depleting antibody against CD4
165 five days after NP-CT delivery. LSFM analysis nine days after immunization revealed
166 that B1-8^{hi} GFP⁺ B cells did not infiltrate the GCs and the size of the SED compartment
167 was reduced by 1.77-fold compared to untreated wild-type mice (**Fig. 3a-c**). To
168 specifically examine the role of T cell help in this process, we used *Sh2d1a*^{-/-} mice, which

169 lack expression of the SLAM-associated protein (SAP) as hosts. In these mice CD4⁺ T
170 cells are unable to interact with antigen-specific B cells and deliver them with help
171 signals and as a result, the T cell-dependent antibody immune response is severely
172 impaired^{29,30}. LSFM imaging indicated that B1-8^{hi} GFP⁺ B cells transferred into *Sh2d1a*^{-/-}
173 mice colonized the SED 2.85-fold less efficiently than in wild-type mice, and no GCs
174 were formed (**Fig. 3a-c**). These results indicate that optimal SED colonization of B cells
175 was dependent on T cell help, while GC infiltration was strictly T cell dependent.

176 To examine whether B cells physically interacted with helper T cells in the SED, we
177 performed whole-mount staining of CD4⁺ cells within intact PPs dissected from
178 *Aicda*^{Cre/+} Rosa26^{Stop-tdTomato/+} mice. Using TPLSM, CD4⁺ T cells were detected in close
179 contact with polyclonal tdTomato⁺ B cells, which typically respond to food and bacterial
180 antigens²⁰ (**Fig. 3d**). Similar results were obtained by visualization of transferred B1-8^{hi}
181 tdTomato⁺ B cells (derived from *Igh*^{B1-8hi} Rosa26^{tdTomato/+} mice) in wild-type hosts four
182 days after NP-CT administration (**Fig. 3d**). These observations suggest that CD4⁺ T cells
183 regulated B cell functions in the SED by forming physical contacts with B cells.

184 To characterize the CD4⁺ T cells in the SED, we labeled immune cells *in situ* within the
185 SED and examined their identity by flow cytometric analysis^{31,32}. For this, we generated
186 mixed bone marrow chimeric mice in which 10% of the hematopoietic cells were derived
187 from AID-GFP mice (used as a landmark for the SED) and 90% of the hematopoietic
188 cells were derived from mice that express photoactivatable GFP (PA-GFP) ubiquitously
189 (**Fig. 3e,f**). In response to two-photon irradiation, PA-GFP shifts its excitation peak from
190 415nm (inactive-PA-GFP), to 495nm (active-PA-GFP); however, irradiated cells also
191 retain the inactive-PA-GFP form³¹. Flow cytometric analysis of inactive-PA-GFP⁺ active-

192 PA-GFP⁺ cells derived from the SED revealed that on average, 23.6 % of the local
193 resident cells were CD4⁺ T cells, and on average 97.1 % of the CD4⁺ T cells were
194 CD44⁺CD62L⁻ activated (CD44⁺CD62L⁻) T cells (**Fig. 3g-j**). Within the CD44⁺CD62L⁻
195 CD4⁺ T cell fraction, 27.5 % on average expressed the T_{FH} cell markers CXCR5 and PD-
196 1, whereas naïve CD4⁺ T cells did not express these receptors (**Fig. 3i,j**). Similar analysis
197 of GC T cells indicated that on average 93.5 % of the CD4⁺ T cells in the GCs were
198 CD44⁺CD62L⁻ activated T cells and on average 47.6 % of the photoactivated CD4⁺ T
199 cells were PD-1⁺CXCR5⁺ T_{FH} cells (**Supplementary Fig. 4a-d**). The CD38⁺FAS⁺ GC B
200 cells/CD44⁺CD62L⁻PD-1⁺CXCR5⁺ T_{FH} cells ratio among the photoactivated cells in the
201 GC was similar to the ratio observed in the SED (**Supplementary Fig. 4e**), suggesting
202 similar availability of T_{FH} cells for a given number of B cells in the two niches. Together,
203 these results indicate that T_{FH}-like cells resided in the SED compartment and supported B
204 cell functions.

205

206 **B cell competition for T cell help ensues during entry into GCs**

207 In draining LNs, high and low affinity clonal variants are subjected to selective forces
208 imposed by the limiting number of T_{FH} cells that ensue prior to GC seeding^{33,34}. To
209 examine where competition for T cell help occurs in PPs, we compared SED colonization
210 and GC infiltration by B cells that express the same BCR, but elicit differential T cell
211 help signals. Expression of ICAMs on B cells is not required for GC formation; however,
212 they serve as amplifiers of T cell help signals and promote clonal selection for GC
213 seeding¹². Mice that express GFP ubiquitously and carry *Igh*^{B1-8hi/+} were crossed to *Icam1*^{-/-}
214 and *Icam2*^{-/-} mice (herein, *Icam1/2*^{-/-}). LSFM indicated that transferred B1-8^{hi} *Icam1/2*^{-/-}

215 GFP⁺ B cells colonized the SED and GCs in wild-type mice to a similar extent as B1-8^{hi}
216 *Icam1/2*^{+/+} DsRed⁺ B cells at day 9 after NP-CT immunization (**Fig. 4a,b**). Furthermore,
217 no significant difference in the SED colonization capacity was detected between B1-8^{hi}
218 *Icam1/2*^{-/-} GFP⁺ B cells and B1-8^{hi} *Icam1/2*^{+/+} DsRed⁺ B cells when co-transferred (1:1
219 ratio) into wild-type mice and analyzed nine days after NP-CT delivery (**Fig. 4c,d**). In
220 contrast, the GC compartment was mainly composed of B1-8^{hi} *Icam1/2*^{+/+} DsRed⁺ B
221 cells, 20.94-fold more than B1-8^{hi} + *Icam1/2*^{-/-} GFP B cells (**Fig. 4c,d**). LSFM imaging
222 revealed that transferred B1-8^{hi} *Icam1/2*^{-/-} GFP⁺ B cells were found in close proximity to
223 the GC structure, suggesting that these cells were outcompeted after departure from the
224 SED, during or after entry into the GC. Thus, T cell help is a limiting factor for B cell
225 infiltration into GCs, but not for initial colonization of the SED niche.

226

227 **T cell help is insufficient to promote clonal selection in SEDs**

228 Orally delivered antigens are subjected to dilution and proteolysis along the intestinal
229 tract. To examine if variable amounts of antigen throughout the small intestine affect
230 SED and GC colonization, we used flow cytometry to quantify the frequency of B1-8^{hi}
231 GFP⁺ GC B cells in single PPs in mice, nine days after NP-CT administration. B1-8^{hi}
232 GFP⁺ B cells efficiently infiltrated pre-existing GCs in the duodenum PP (36 % of total
233 GC cells), whereas this process was progressively less efficient towards the ileum PP (6.4
234 % of total GC cells) (**Fig. 5a**). However, the frequency of antigen-specific
235 CD38⁺CCR6⁺IgA⁺ SED B cells in individual PPs dissected from different parts of the
236 small intestine was roughly comparable (except the most distal PP) (**Fig. 5a**). The
237 differences in GC and SED colonization were not a result of differential homing of naïve

238 B cells to these PPs (**Supplementary Fig. 5a,b**). Similar results were obtained in wild-
239 type mice treated with antibiotics (**Supplementary Fig. 5c,d**), indicating that the
240 presence of commensal bacteria did not affect the magnitude of the GC response. As
241 such, the amount of antigen delivered into the PP imposes a major limitation on antigen-
242 specific B cell infiltration into the GCs, whereas colonization of the SED is less affected.

243 Antigen availability greatly influences the ability of cognate B cells to take up
244 antigen and promote effective interactions with T cells⁹. DEC205 (encoded by *Ly75*) is
245 expressed on activated B cells and mediates antigen endocytosis followed by peptide
246 loading on MHCII molecules³¹. To determine whether antigen presentation by B cells
247 was a limiting factor in promoting competition in the SED, we artificially loaded antigen
248 on MHCII by using a chimeric DEC205 antibody fused to a dominant peptide derived
249 from CT β subunit (CTB)³⁵. A mixture of B1-8^{hi} *Ly75*^{-/-} CD45.1⁺ and B1-8^{hi} *Ly75*^{+/+}
250 tdTomato⁺ B cells (~30:70) was transferred into wild-type mice followed by NP-CT
251 immunization. Five days later, we intravenously injected DEC-CTB antibody or control
252 DEC-OVA antibody or PBS into host mice and analyzed the GC and SED compartments
253 after additional 4 days (**Supplementary Fig. 5e**). Injection of DEC-CTB antibody
254 increased the proportion of IgA⁺CCR6⁺ B cells in the SED compartment by 1.55-fold
255 compared to control treatments (**Fig. 5b,c**) suggesting that T cell help promotes B cell
256 expansion in the SED. Next, to investigate whether selective clonal expansion takes
257 place, we normalized the frequency of the transferred B cells to their initial frequency in
258 the transferred mixture. B1-8^{hi} *Ly75*^{+/+} tdTomato⁺ B cells were enriched 1.65-fold in the
259 SED compared to B1-8^{hi} *Ly75*^{-/-} CD45.1⁺ B cells in mice treated with DEC-CTB
260 antibody (**Fig. 5b,d**). In the GCs of DEC-CTB treated mice, the frequency of B1-8^{hi}

261 *Ly75^{+/+}* tdTomato⁺ B cells was increased by 2.58-fold compared to B1-8^{hi} *Ly75^{-/-}* CD45.1⁺
262 B cells (**Fig. 5e,f**). These data demonstrate that high antigen presentation by B cells
263 provided a selective advantage in the SED and promoted effective affinity-based
264 infiltration and/or competition in the GC compartment.

265

266 **The BCR actively signals in the SED independent of its affinity**

267 During B cell immune responses, the BCR triggers a series of phosphorylation events
268 essential for B cell activation³⁶. *Nurr77* expression (encoded by *Nr4a1*) is induced upon
269 BCR triggering³⁷. To understand whether BCR signaling in the SED played a role in
270 BCR affinity-dependent GC infiltration, we examined PP-derived B cells from reporter
271 mice that express GFP under *Nr4a1* transgene promoter (herein, *Nurr77*-GFP) by flow
272 cytometry. CD38⁺CCR6⁺IgA⁺ B cells in the SED had a 5-fold higher expression of
273 *Nurr77*-GFP compared to CD38⁻FAS⁺IgA⁺ GC B cells (**Fig. 6a**). Flow cytometry analysis
274 of NP-specific B cells by staining PP cells of NP-CT immunized *Nurr77*-GFP mice with
275 NP-PE indicated higher *Nurr77*-GFP expression in CD38⁺CCR6⁺IgA⁺ SED B cells
276 compared to CD38⁻FAS⁺IgA⁺ GC B cells (**Fig. 6b and Supplementary Fig. 5f and**
277 **6a,b**). Furthermore, intracellular staining indicated that the B cells in the SED had higher
278 expression of pErk and pSyk compared to GC and naïve B cells (**Fig. 6c,d**), suggesting
279 that the BCRs on SED B cells were actively engaged in signaling.

280 Next, to investigate whether the magnitude of the BCR affinity regulated the
281 phosphorylation events within SED B cells, we transferred either B1-8^{hi} GFP⁺ or B1-8^{lo}
282 DsRed⁺ B cells into wild-type host mice prior to NP-CT immunization. Five days later, at
283 the time of SED colonization, intracellular staining showed no significant difference in

284 pErk or pSyk between B1-8^{hi} GFP⁺ or B1-8^{lo} DsRed⁺ B cells (**Fig. 6e,f**). Thus, BCR
285 affinity did not play a role in the magnitude of downstream activation events in the SED.

286

287 **Selective clonal expansion occurs after initial SED colonization**

288 To investigate whether our observations apply to polyclonal B cells that respond to gut-
289 derived antigens, we examined B cell clonal variations and inter-clonal relationships in
290 *Aicda*^{Cre/+}*Rosa26*^{Stop-tdTomato/+} mice. Individual tdTomato⁺CCR6⁺FAS⁺GL-7⁺IgA⁺ SED B
291 cells and tdTomato⁺CCR6⁻FAS⁺GL-7⁺IgA⁺ GC B cells were sorted from a single PP and
292 subjected to *Igh* mRNA sequencing and CDR3-based clustering (**Supplementary Fig.**
293 **6c**). We found limited V segment usage in the GC B cells, and nearly all the cells were
294 clonally expanded (90 % on average) (**Fig. 7a,b**). Conversely, V segment usage was
295 highly diverse in SED B cells and fewer clones were detected more than once (38 % on
296 average) (**Fig. 7b,c**). These results suggest that clonal selection for extensive expansion
297 and GC seeding took place after initial SED colonization.

298 Class-switched B cells were shown to depart the SED and infiltrate GCs^{20,24}.
299 Accordingly, we observed that an average of 7 % of IgA⁺ clones in the SED were also
300 represented in the GC compartment; however, half of the highly-expanded clones (>5
301 clonal members) were present in both compartments (**Fig. 7b,c**). Comparison of SHM
302 indicated that GC B cells had more mutations in the *Igh* genes than SED B cells (**Fig.**
303 **7d**). Clones detected in both compartments had a similar number of SHM in the *Igh*
304 transcript, while non-shared clones derived from the SED compartment carried 2.4-fold
305 fewer mutations compared to the shared clones (**Fig. 7e**). These results suggest that
306 highly mutated clones in the SED originated from the GC.

307 To gain additional insight into the clonal relationships in PPs, we constructed
308 evolutionary phylogenetic trees of the B cell clones found in both compartments. We
309 found several clonal families in which clonal members in the SED compartment had
310 generated highly diversified descendants in the GCs (**Fig. 7f and Supplementary Fig.**
311 **7**). In addition, we found highly-diversified clones within the GC compartment that had
312 generated clonal members in the SED compartment (**Fig. 7f**). These observations indicate
313 that diversified IgA⁺ GC B cells re-entered the SED compartment as mutated GC-derived
314 memory B cells.

315

316 **Discussion**

317 In the PPs, activated B cells occupy two major niches, the SED, located under the
318 follicle-associated epithelium, and the GC structures, positioned distal to the gut lumen
319 within the B cell follicles¹⁶. Here, we identified the site at which affinity-based B cell
320 selection was initiated in PPs and define the role of BCR affinity in regulating B cell
321 responses. We found that several processes that regulate pre-GC events in the SED
322 differed from those that occur in the draining LN and spleen: in the PP, antigen-specific
323 B cells were not subjected to affinity-based competition in the SED prior to GC entry; T
324 cells predominantly promoted B cell expansion without clonal selection at the pre-GC
325 stage; B cell affinity-based competition ensued during or immediately after GC
326 infiltration; and GC-derived memory cells and plasmablasts were found in the SED.

327 The nature of the intestinal environment, as well as the requirement for active transport of
328 luminal antigens into the PPs, greatly affect the amount of antigen available for triggering
329 a cognate B cell immune response¹⁵. In germ free mice the IgA response is triggered only

330 when a large dose of commensal bacteria is administered³⁸, indicating that a large amount
331 of antigen is required for delivery of antigens into the PPs and/or initiation of the GC
332 response. In support of these observations, we demonstrated that both BCR affinity and
333 antigen availability promoted effective infiltration into the GC structures. In sharp
334 contrast, the magnitude of B cell activation dictated by these two factors had only a small
335 impact on initial BCR signaling, as well as proliferation in the SED, once a minimal
336 threshold was reached. This suggests that in PPs the threshold for GC entry is higher than
337 the threshold that triggers BCR signaling and B cell proliferation in the SED. The GCs
338 contain follicular dendritic cells (FDC) that reside in the light zone and capture antigens
339 on their surface and present it to B cells³⁹. These cells have the capacity to augment
340 effective BCR triggering by increasing antigen density on their surface using receptors
341 that capture antibody-antigen complexes³⁹. Generation of plasmablasts early during the
342 response in the SED and interfollicular regions of the PPs, could potentially provide
343 antibodies that complex with cognate antigens on FDCs and support effective clonal
344 competition in the GCs over an extended period of time, even when antigen levels are
345 extremely low.

346 T_{FH} cells play an important role in regulating the B cell response in the SED without
347 promoting affinity-based clonal selection, most likely through CD40 ligation on B cells²⁰.
348 The inability of T cells to promote clonal selection in the PP SED indicates that TCR
349 binding of peptide-loaded MHCII (pMHCII) on B cells are not sufficient to support this
350 process. Artificial increase of antigen presentation on B cells endowed them with a
351 selective advantage, suggesting that in response to vaccination, the density of pMHCII
352 was too low to promote clonal selection. We suggest that there are two different T cell

353 help thresholds in PPs: one for maintaining B cells in the SED, and one for promoting
354 clonal selection, which depends on higher surface presentation of pMHCII. Clonal
355 selection in the SED might take place when cognate antigen levels in the gut are
356 extremely high, such as during breach of the gut epithelium and invasion of commensal
357 or pathogenic bacteria into the host tissues.

358 Consistent with T_{FH} cell functions, we found that SAP-dependent T cell help promoted B
359 cell SED colonization. Nonetheless, although T cells and CD40 signaling play an
360 important role in entry into the SED²⁰, antigen-specific B cells could access this
361 compartment, independently of effective T cell help. BCR engagement is sufficient for
362 CCR6 expression^{20,40,41}, suggesting that B cells that encounter antigen can gain access to
363 the SED niche independently of T cell functions. Indeed, we found that B cells in the
364 SED were constantly engaged in signaling, independently of the BCR affinity. This
365 indicates that signals derived from T_{FH}-like cells synergize with BCR signals to promote
366 entry and/or non-selective expansion of antigen-specific B cells in the SED.

367 The major purpose of the GC is to produce long-lived memory and plasma cells that
368 produce protective high-affinity antibodies¹⁴. We identified highly-mutated CCR6⁺IgA⁺
369 B cells that typically reside in the SED and provided evidence for the presence of GC-
370 derived memory B cells at this site; whether these cells directly migrate from the GC to
371 the SED or first enter the circulation is yet to be determined^{42,43}.

372 Collectively, we found that the SED niche was important for the initial expansion of B
373 cells bearing high- and low-affinity BCRs, followed by competitive GC infiltration into
374 preformed GC structures. These findings have far ranging implications in oral vaccine

375 design for the generation of memory GC-derived IgA⁺ B cells with high-affinity BCRs
376 specific for intestinal pathogens.

377 **Accession code:** PRJEB30525.

378 **Acknowledgments:** Z.S. is supported by the European Research Council (ERC) grant
379 No. 677713, Human Frontiers of Science Program (CDA-00023/2016), Israel Science
380 Foundation (ISF) grant no. 1090/18, Azrieli Foundation, Rising Tide Foundation, and the
381 Morris Kahn Institute for Human Immunology. Z.S. is a member in the European
382 Molecular Biology Organization (EMBO) Young Investigator Program and is supported
383 by grants from The Benozio Endowment Fund for the Advancement of Science, The Sir
384 Charles Clore Research Prize, Comisaroff Family Trust, Irma & Jacques Ber-Lehmsdorf
385 Foundation, Gerald O. Mann Charitable Foundation and David M. Polen Charitable
386 Trust. Imaging was made possible thanks to the “The de Picciotto-Lesser Cell
387 Observatory in memory of Wolf and Ruth Lesser”.

388

389 **Author contributions:** A.B. designed and conducted the experiments, performed data
390 analysis and wrote the manuscript; A.S. prepared the in-house antigen used in this study;
391 E.W. analyzed single-cell immunoglobulin sequencing data; L.S.B. and Y.A. assisted
392 with light sheet imaging; R.S. and R.D. produced antibodies used in this study. G.Y.
393 supervised immunoglobulin-sequencing analysis; M.B. advised and helped in the design
394 of some of the experiments; and Z.S. designed experiments, supervised the study and
395 wrote the manuscript.

396 **Competing interests:** The authors declare no competing interests.

397

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- 500

501 **Figure Legends**

502

503 **Figure 1. Visualization of SED niches and GC compartments in intact Peyer's**

504 **patches using light sheet fluorescence microscopy. a,** Schematic representations of PP

505 positions (upper panels). Middle and lower panels, show GCs and SEDs in a PP of

506 *Aicda*^{Cre/+}*Rosa26*^{Stop-tdTomato/+} *Ccr6*^{+/+} or *Ccr6*^{-/-} mice. Arrows indicate SED structures.

507 Scale bars, 300 μ m and 200 μ m respectively. **b,c,** Quantification of the number (b), and

508 volume (c) of GCs and SEDs of *Ccr6*^{+/+} and *Ccr6*^{-/-} mice. Each dot represents an

509 individual PP in (b) or individual GC or SED in (c). Imaging analyses were performed in

510 two independent experiments with a total of four mice per group (n=4), including 11

511 *Ccr6*^{+/+} and 17 *Ccr6*^{-/-} PPs, line represents mean. *** P<0.0001, two tailed Student's t

512 test. **d,** B1-8^{hi} DsRed⁺ B cells (red) in a PP of an AID-GFP (green) mouse without antigen

513 administration, or nine days after NP-CT administration. Scale bar, 300 μ m. **e,** B1-8^{hi}

514 *Ccr6*^{GFP/+} DsRed⁺ B cells transferred into a WT host, nine days after oral antigen

515 administration. Scale bar, 300 μ m. **f,g,** B1-8^{hi} DsRed⁺ B cells (red) in a PP of CD11c-YFP

516 or Cx3cr1^{GFP/+} reporter mice, nine days after antigen administration. The SED area is

517 marked with a dashed line. Scale bar, 300 μ m. In d-g, representative images of two

518 independent experiments with three mice in each experiment (n=6) are shown.

519 **Figure 2. BCR affinity controls GC infiltration but not proliferation nor early**

520 **plasmablast formation in the SED. a,b,** Images showing transferred B1-8^{hi} GFP⁺, B1-

521 8^{lo} DsRed⁺ B cells or a mixture in MD4 hosts, 4 (a), or 9 days (b) after NP-CT

522 immunization. Scale bar, 300 μ m. **c,** Quantification of SED and GC volumes. Each dot

523 represents one compartment. Analyses were performed in two independent experiments

524 with a total of four mice per group (n=4), including 17 B1-8^{hi} GFP⁺ and 26 B1-8^{lo}

525 DsRed⁺ GCs and coupled SEDs at day 4 and 19 and 36 at day 9, respectively. Line
526 represents mean. **d**, Quantification of SED and GC volumes in MD4 mice that received a
527 1:1 mixture of B1-8^{hi} GFP⁺ and B1-8^{lo} DsRed⁺ B cells. Data are pooled from two
528 independent experiments using a total of 12 compartments from three mice (n=3). Line
529 represents mean. **e**, Flow cytometry analysis of transferred cells as in b-d. Gating is
530 depicted in Supplementary Fig. 2d. Data are pooled from two independent experiments
531 with a total of five or six mice per group with (n=5) and without competition (n=6),
532 respectively, line represents mean. **f,g**, Proliferation analysis of endogenous naïve, GC
533 and SED B cells, 2.5 hours following EdU administration (f) or in transferred B1-8^{hi}
534 GFP⁺ and B1-8^{lo} DsRed⁺ B cells, nine days after NP-CT immunization (g). Gating
535 strategy and additional details are described in Supplementary Fig. 2e. Data are pooled
536 from two independent experiments with six mice per group (n=12) (f) or with three mice
537 per group (n=6) (g), line represents mean. **h**, Blimp-1-YFP cells in the SED (left) or
538 transferred B1-8^{hi} Blimp-1-YFP DsRed⁺ cells in the SED, five days after immunization
539 (right). Arrowheads indicate Blimp-1-expressing cells. Scale bar, 100 μ m. Images
540 represent two independent experiments with two mice in each experiment. **i**, Analysis of
541 CD138⁺ cells gated on B1-8^{hi} tdTomato⁺ or B1-8^{lo} DsRed⁺ B cells, five days after
542 immunization. Line represents mean. ** P<0.01, *** P<0.0001, one-way ANOVA with
543 Bonferroni posttest in (c) and (f), and two-tailed Student's t test in (d,e,g,i). ns, not
544 significant.

545 **Figure 3. Effective SED colonization by antigen-specific B cells depends on T_{FH}-like**
546 **cells. a**, Images of transferred B1-8^{hi} GFP⁺ B cells in WT, *Sh2d1a*^{-/-}, and α CD4-treated
547 mice (days 5 and 7), nine days after NP-CT immunization. Scale bar, 300 μ m.

548 Representative images from three experiments showed similar results. **b,c**, Volume
549 quantification of SEDs (b), and GCs (c). Data are pooled from two independent
550 experiments with three mice in each experiment (n=6) with 35 (WT), 28 (α CD4) and 19
551 (*Sh2d1a*^{-/-}) GCs and coupled SEDs, line represents mean. *** P<0.0001, one-way
552 ANOVA with Bonferroni posttest. **d**, Upper panels: SED of *Aicda*^{Cre/+}*Rosa26*^{Stop-tdTomato/+}
553 mouse stained for CD4. Lower panels: B1-8^{hi} tdTomato⁺ B cells in the SED, four days
554 after NP-CT immunization. Scale bar, 100 μ m and 30 μ m in zoom in images. Two
555 independent experiments with two mice in each experiment (n=4) showed similar results.
556 **e**, SED of AID-GFP:PA-GFP (10:90) chimeric mouse prior to and after photoactivation.
557 Scale bar, 100 μ m. **f**, Flow cytometry analyses of inactive- and active-PA-GFP cells. **g**,
558 CD4⁺ cell frequency among the active-PA-GFP cells. **h**, Merged plot of
559 CD4⁺CD44⁺CD62L⁻ cells among total CD4⁺ cells (black) and active-PA-GFP⁺CD4⁺ cells
560 (green). **i**, Plots showing PD-1^{hi}CXCR5^{hi} cells in the active-PA-
561 GFP⁺CD4⁺CD44⁺CD62L⁻ compartment. Lack of markers on naïve cells is shown as
562 control. **j**, T cell frequencies are summarized in the graph as mean; each dot represents a
563 mouse, bar represents mean. In f-j, data are pooled from three independent experiments
564 using a total of four mice (n=4).

565 **Figure 4. Competition for T cell help takes place during infiltration into pre-existing**
566 **GC sites.** **a**, Representative images of B1-8^{hi} *Icam1/2*^{+/+} DsRed⁺ or B1-8^{hi} *Icam1/2*^{-/-}
567 GFP⁺ B cells transferred separately into WT hosts, nine days following oral antigen
568 administration. **b**, Volumetric quantification of B cells in the SEDs and GCs of WT mice
569 PPs under noncompetitive conditions. Data are pooled from two independent experiments
570 with three mice in each experiment (n=6) and two PPs from each mouse, corresponding

571 to 36 GC and coupled SED structures. Line represents mean. Scale bar 300 μm . **c**,
572 Representative images of B1-8^{hi} *Icam1/2*^{+/+} DsRed⁺ or B1-8^{hi} *Icam1/2*^{-/-} GFP⁺ B cells co-
573 transferred at a 1:1 ratio into WT hosts, nine days following oral NP-CT immunization. A
574 single PP (upper panels) and a single GC (lower panels) are shown. **d**, Volumetric
575 quantification of B cells in SEDs and GCs of WT PPs transferred with cells as in (c).
576 Data are pooled from two independent experiments with a total of seven mice (n=7), two
577 PPs from each mouse and 56 GC and coupled SED structures, line represents mean. Scale
578 bar, 300 μm . *** P<0.0001, two-tailed Student's t test. ns, not significant.

579 **Figure 5. Antigen presentation by B cells in the SED is insufficient for promoting**
580 **competition for T cell help. a**, Frequency of B1-8^{hi} GFP⁺ B cells in GCs (FAS⁺CD38⁻)
581 or SEDs (IgA⁺CD38⁺CCR6⁺) of individual PPs. Data are pooled from two independent
582 experiments with single PPs taken from two mice in each experiment (n=4). Each dot
583 represents mean of cell frequency; error bars indicate s.e.m. **b**, Flow cytometry plots of
584 transferred B1-8^{hi} *Ly75*^{+/+} tdTomato⁺ and B1-8^{hi} *Ly75*^{-/-} CD45.1⁺ B cells in the SED of
585 immunized mice following intravenous injection of 50 μg DEC205-CTB or DEC205-
586 OVA antibodies or PBS as control, as described in Supplementary Fig. 5a. **c**, Frequencies
587 of SED B cells following treatment with DEC205-CTB Ab or control, line represents
588 mean. Each dot represents a single mouse. **d**, Flow cytometric analysis of transferred B1-
589 8^{hi} *Ly75*^{+/+} tdTomato⁺ and B1-8^{hi} *Ly75*^{-/-} CD45.1⁺ B cells as in b, normalized to the initial
590 percentage of the transferred cells. Line represents mean. **e,f**, Flow cytometry plots (e)
591 and quantification of transferred cells within the GC (f) following immunization and Ab
592 treatment, as in (b). Each B cell subset in the GC was normalized to the percentage of the
593 transferred cells. Data shown in c-f are pooled from two independent experiments with

594 three mice per experiment (n=6). Each dot represents an individual mouse, line represents
595 mean. * P<0.05, *** P<0.0001, two-tailed Student's t test in (c) and one-way ANOVA
596 with Bonferroni posttest in (d) and (f). ns, not significant.

597

598 **Figure 6. Active BCR signaling in the SED is independent of its affinity. a,**
599 Representative histogram showing Nurr77 expression in SED (IgA⁺CD38⁺CCR6⁺) and
600 GC (FAS⁺CD38⁻) B cells of Nurr77-GFP reporter mice. Geometric mean fluorescence
601 intensity (gMFI) is summarized in the graph. Data are pooled from two independent
602 experiments with three mice in each experiment (n=6), each dot represents a mouse; line
603 represents mean. **b,** Nurr77 expression as in (a) within NP specific B cells detected using
604 NP-PE (Supplementary Fig. 5f), 9 days after NP-CT administration. Data are pooled
605 from two independent experiments with three mice in each experiment (n=6), each dot
606 represents a mouse; line represents mean. *** P<0.0001, two-tailed Student's t test. **c,d,**
607 Representative histograms showing pErk (c) or pSyk (d) in naïve, SED and GC B cells in
608 WT mice. gMFI values are summarized in the graph. Data are pooled from two
609 independent experiments with four mice in each experiment (n=8), each dot represents a
610 mouse; line represents mean. ** P<0.01, *** P<0.0001, one-way ANOVA with
611 Bonferroni posttest. ns, not significant. **e,f,** Representative histograms showing pErk (e)
612 or pSyk (f) expression in B1-8^{hi} GFP⁺ or B1-8^{lo} DsRed⁺ B cells, 5 days after NP-CT
613 administration. Naïve cell population expression is shown as negative control. gMFI
614 values are summarized in the graph. Data are pooled from two independent experiments
615 with three mice in each experiment (n=6), line represents mean. ns, not significant; two-
616 tailed Student's t test.

617

618 **Figure 7. Selective clonal expansion and diversification takes place in the GCs, but**

619 **not in the SED compartment. a,** The number of V regions detected in IgA sequences

620 of GC or SED-derived B cells recovered from a single PP explanted from

621 *Aicda*^{Cre/+}*Rosa26*^{Stop-tdTomato/+} mice. Data are pooled from two independent experiments

622 with one mouse per experiment (n=2), bar represents mean. **b,** Clonal distribution based

623 on CDR3 sequences as in (a). Colored fractions represent expanded CDR3 sequences

624 (>2), white fraction represents single clones. Each graph represents one mouse (n=2). The

625 number of sequenced cells is indicated in the center circle. **c,** The number of clonal

626 members detected in the GC and SED, each bar represents a clone. **d,** Number of

627 mutations per B cell in the GC and SED. Each dot represents a cell and 183 SED and 174

628 GC cells are shown; line represents mean. *** P<0.0001, two-tailed Student's t test. **e,**

629 The mutation ratio of SED and GC clones that were either unique or shared between the

630 niches, each dot represents a mouse (n=2), bar represents mean. **f,** Lineage-tree analysis

631 of clonally related sequences. The number of mutations between neighboring nodes is

632 indicated and includes synonymous, non-synonymous, and reverse mutations to the

633 germline sequence. GL, germline. UCA, unique common ancestor, inferred from the

634 sequence analysis. Additional lineage-trees are available in Supplementary Fig. 7. Data are

635 pooled from two independent experiments with one mouse per experiment.

636

637

638

639 **Methods**

640 **Mice**

641 Transgenic knock-in mice carrying the *Igh*^{B1-8hi} or *Igh*^{B1-8lo} alleles²⁵ or *Ly75*^{-/-} mice were a
642 gift of M. Nussenzweig (The Rockefeller University). AID-GFP mice were generated by
643 R. Casellas (NIAMS, NIH) and provided by M. Nussenzweig. GFP-, DsRed-, PA-GFP-
644 expressing mice and MD4, *Aicda*^{Cre}, *Rosa26*^{Stop-tdTomato}, *Sh2d1a*^{-/- 30}, Blimp-1-YFP, Nurr-
645 77-GFP and *Ccr6*^{GFP/+} mice were purchased from the Jackson Laboratories. GFP- and
646 DsRed-expressing mice were bred to mice that carry either the *Igh*^{B1-8hi} or *Igh*^{B1-8lo}
647 alleles²⁵. tdTomato⁺ mice were generated by crossing *Rosa26*^{Stop-tdTomato} mice to a Cre
648 expressing strain. *Icam1/2*^{-/-} mice were provided by B. Engelhardt (University of Bern)
649 and bred with *Igh*^{B1-8hi} GFP-expressing mice. *Ly75*^{-/-} mice were crossed with mice
650 carrying the *Igh*^{B1-8hi} and the congenic marker CD45.1 to produce *Igh*^{B1-8hi} *Ly75*^{-/-}
651 CD45.1-expressing mice. CD11c-YFP and *Cx3cr1*^{GFP/+} mice were provided by S. Jung
652 (Weizmann Institute). Wild-type mice (C57BL/6) were purchased from Harlan. All
653 experiments with mice were approved by the Weizmann Institute Animal Care and Use
654 Committee (IACUC).

655 **Adoptive cell transfer**

656 B cells were purified by forcing spleen tissue through mesh into PBS containing 2% fetal
657 calf serum and 1 mM EDTA. Resting B cells were purified using negative selection anti-
658 CD43 magnetic beads (Miltenyi Biotec). For Igλ B cell purification, cells were incubated
659 with anti- Igκ-PE antibody for 30 min at 4°C. Cells were washed, and Igκ B cells were
660 depleted using anti-PE magnetic beads (Miltenyi Biotec.). Igλ enrichment was verified by
661 flow cytometry prior to cell transfer. Isolated cells (100K, Igλ) were injected

662 intravenously into host mice one day before oral antigen administration. In competition
663 experiments, the ratio of adoptively transferred cells (B1-8^{hi} GFP⁺ and B1-8^{lo} DsRed⁺ B
664 cells or B1-8^{hi} *Icam1/2*^{+/+} DsRed⁺ and B1-8^{hi} *Icam1/2*^{-/-} GFP⁺ B cells) was 1:1 (1x10⁵ of
665 each cell type). In experiments involving transfer of B1-8^{hi} *Ly75*^{-/-} CD45.1⁺ and B1-8^{hi}
666 *Ly75*^{+/+} tdTomato⁺ B cells, the ratio was either 3:7 or 1:1 and confirmed by flow
667 cytometer analysis of the cell mixture prior to injection of the cells into the mice. Cell
668 frequencies (% of cells in the GC and SED compartments) were normalized to the initial
669 cell transfer ratio.

670 **Chimeric mice**

671 For generation of chimeric mice, hosts were irradiated with 950 rad followed by injection
672 of fresh BM cells. For generating chimeric mice for photoactivation experiments
673 CD45.2⁺ hosts were reconstituted with a mixture of BM cells containing 90% BM cells
674 derived from PA-GFP-expressing mice, and 10% BM cells derived from AID-GFP
675 reporter mice. Chimeric mice experiments were performed 8 weeks following bone
676 marrow transplantation.

677 **Oral antigen administration and treatments**

678 NP was conjugated to CT as previously described²². Mice received a single
679 administration of 500µl PBS containing 3% NaHCO₃ and 10µg NP-CT by gavage. For
680 CD4⁺ T cell depletion experiments, 200 µg of rat anti-mouse CD4 mAb (clone GK1.5,
681 rIgG2b, BioXcell) was injected intravenously on days 5 and 7 following oral delivery of
682 NP-CT. For antibiotic treatment, mice were given a combination of ampicillin (1g/l),
683 vancomycin (0.5 g/l), neomycin sulfate (1 g/l), imipenem (250mg/l), and metronidazole

684 (1 g/l) (Sigma Aldrich) in their drinking water for two weeks⁴⁴. Mice were 5 weeks of
685 age at the beginning of antibiotic administration.

686 **Anti-DEC205 treatment**

687 For DEC-CTB cloning, the sequence of CTB peptide 81-100 was inserted into the
688 DEC205 vector³⁵. DEC-CTB and DEC-OVA antibodies were produced as chimeric
689 antibodies in 293T cell as previously described⁴⁵. Mice received 50µg of antibodies
690 intravenously 5 days after oral antigen administration.

691 **In vivo EdU proliferation assay**

692 For proliferation measurements, mice were injected intravenously with 2mg of the
693 nucleoside analog 5-ethynyl-2'-deoxyuridine (EdU) (Molecular Probes) in PBS. After 2.5
694 hours, mice were dissected and PP cells were then stained for surface antigens as
695 described, followed by EdU detection using Click-iT EdU Alexa Fluor 647 Flow
696 Cytometry Assay Kit (Molecular Probes) according to manufacturer's protocol.

697 **Flow cytometry**

698 Small intestines were excised and washed with ice cold PBS to remove fecal content.
699 Peyer's patches were harvested and forced through a mesh into PBS containing 2% fetal
700 calf serum and 1 mM EDTA. For blockade of Fc receptors, single cell suspensions were
701 incubated with 2 µg/ml anti-16/32 (clone 93) for 5 min. Cells were washed and incubated
702 with fluorescently labeled antibodies (Table S1) for 30 min. GC cells were gated as
703 live/single, B220⁺CD38⁻FAS⁺ or B220⁺GL-7⁺FAS⁺. SED B cells were gated as
704 B220⁺CD38^{hi}GL-7⁻FAS⁺CCR6⁺. Transferred cells within GCs were detected by staining
705 cell suspensions with GC markers, as indicated, along with the endogenous fluorescent
706 markers used for B1-8^{hi} and B1-8^{lo} B cell detection (GFP⁺, tdTomato and DsRed⁺,

707 respectively). In photoactivation experiments, T_{FH} cells were gated as
708 $CD4^+CD44^+CD62L^-PD-1^+CXCR5^+$ out of the photoactivated cells or out of total
709 inactive PA-GFP cells. PA-GFP fluorescence was measured using the V500 (inactive
710 PA-GFP) and FITC (active PA-GFP) channels. In BCR signaling experiments, B1-8
711 DsRed⁺ B cells were transferred into CD45.1 hosts and gated as $B220^+DsRed^+CD45.2^+$.
712 Intracellular staining for pErk and pSyk was performed using Foxp3 / Transcription
713 Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions.
714 Stained cell suspensions were analyzed using a CytoFlex flow cytometer (Beckman
715 Coulter).

716 **Tissue processing and image acquisition by LSFM**

717 Peyer's patches were excised and fixed in 4% PFA for 2 hours, followed by immersion in
718 FocusClear (CelExplorer Labs) for 1-4 days. Samples were imaged using a light sheet Z1
719 microscope (Zeiss Ltd.) equipped with two sCMOS PCO- Edge cameras, 10X
720 illumination objectives (LSFM clearing 10X/0.2), Clr Plan- Neofluar 20X/1,0
721 Corr $nd=1.45$, and detection objective designed for cleared samples in water-based
722 solution with a final refractive index (RI) of 1.45. Samples were loaded into a glass
723 capillary in a 1.5% low melting agarose solution (ROTH). Imaging was performed using
724 dual side illumination, with zoom 0.36 and multi view mode for tiling of multiple fields
725 of view, with overlap of 10%. GFP Excitation 488, Emission / detection - BP 505-545;
726 YFP Excitation 514, Emission / detection - BP 525-565; RFP Excitation 561, Emission /
727 detection - BP 575-615.

728 **Gut permeability assay**

729 Gut permeability assay was performed by evaluation of FITC dextran (Sigma Aldrich) in
730 the blood as previously described⁴⁶. WT mice received NP-CT by gavage or 3% NaHCO₃
731 as control. Gut permeability was assessed 24 hours after oral NP-CT administration. In
732 addition, sera of mice that did not receive NP-CT and treated with FITC dextran were
733 used as a negative control. For histological H&E analysis, mice received NP-CT orally
734 and tissue dissection was performed 1, 5 or 9 days after NP-CT administration.

735 **Whole mount staining**

736 PPs of *Aicda*^{Cre/+}Rosa26^{Stop-tdTomato} or WT cells transferred with B1-8^{hi} tdTomato⁺ B cells
737 were harvested as described, and stained with CD4 antibody conjugated to Alexa fluor
738 488 (1:100) (GK1.5, Biolegend) as previously described⁴⁷. Images were acquired by
739 TPLSM.

740 **Photoactivation and image acquisition by TPLSM**

741 Zeiss LSM 880 upright microscope fitted with Coherent Chameleon Vision laser was
742 used for photoactivation and CD4 visualization imaging experiments. Images were
743 acquired with a femtosecond-pulsed two-photon laser tuned to 940 nm. The microscope
744 was fitted with a filter cube containing 565 LPXR to split the emission to a PMT detector
745 (with a 579-631 nm filter for tdTomato fluorescence) and to an additional 505 LPXR
746 mirror to further split the emission to 2 GaAsp detectors (with a 500-550nm filter for
747 GFP fluorescence). PPs of chimeric mice were dissected, and photoactivation of the
748 region of interest was provided at 830 nm as previously described^{31,48}. Tile images were
749 acquired as a 100 μm Z-stacks with 5 μm steps between each Z-plane. The zoom was set
750 to 0.7, and pictures were acquired at 512 x 512 x-y resolution.

751 **Image analysis**

752 LSFM image dual side fusion was performed using ZEN software. The independent tiles
753 were stitched into a single image stack visualized in a volumetric mode using Arivis
754 Vision4D software. Quantification of B cells in GCs and SEDs size was performed using
755 Imaris software (Bitplane). Each compartment was segmented using the Imaris 3D
756 surface object. Samples with clear separation between SED and GCs were segmented
757 together. Under some conditions, in which many individual cells were located between
758 the two compartments, segmentation was done separately. TPLSM images were
759 processed by Imaris software (Bitplane).

760 **Single cell *Igh* sequencing**

761 Single PP was harvested from *Aicda*^{Cre/+}Rosa26^{Stop-tdTomato} and processed for flow
762 cytometry analysis. Cell suspensions were stained for dump (CD4, CD8, GR-1, F4/80)
763 and gated as dump⁻tdTomato⁺B220⁺GL-7⁺FAS⁺CCR6⁻IgA⁺ or tdTomato⁺B220⁺GL-7⁻
764 FAS⁺CCR6⁺IgA⁺ representing GC or SED cells, respectively. Cell sorting was performed
765 using a FACS Aria cell sorter (BD Bioscience). For total VDJ sequencing of Igα heavy
766 chains, GC and SED derived B cells were sorted into 96 well plates containing lysis
767 buffer (PBS containing 3 U/μl RNAsin, 10 mM dithiothreitol –DTT). cDNA was purified
768 using random primers (NEB) as previously described⁴⁹. Igα heavy chain sequence was
769 amplified twice using primers for the Igα constant region (5'-
770 ATCAGGCAGCCGATTATCAC-3' for the first reaction and 5'-
771 GAGGTGCAGCTGCAGGAGTCTGG-3' for the second reaction)⁴² together with a mix
772 of primers for the variable region (Table S2)⁵⁰. The PCR products were sequenced and
773 analyzed for CDR3 using web-based IgBlast and IMGT tools. Sequence alignment was

774 performed using SnapGene software (GSL Biotech). For schematic representation of the
775 results, sequences derived from single cells were clustered according to their CDR3
776 region and presented as percent of the total sequenced cells. Primer-derived mutations
777 were excluded from the analysis.

778 ***Igh* lineage analysis**

779 Ig Fasta sequences were aligned against the IMGT mouse heavy chain gene database
780 (Sep. 2017) using NCBI IgBlast (version 1.7.0)⁵¹. Post processing of IgBlast output, and
781 clonal clustering were performed using Change-O v0.3.7
782 (<http://changeo.readthedocs.io/>)⁵², Alakazam v0.2.8 (<http://alakazam.readthedocs.io/>),
783 SHazaM v0.1.8 (<http://shazam.readthedocs.io/>), and custom scripts within the R statistical
784 computing environment, as follows. V(D)J sequences were assigned to clonal groups by
785 partitioning sequences based on sequence similarity of IGHV gene annotations, IGHI
786 gene annotations, and junction region lengths. Within these groups, sequences differing
787 from one another by a hamming distance of 0.2 within the junction region were defined
788 as clones by single-linkage clustering. Distances were measured and normalized by the
789 length of the junction region. The clonal distance threshold was determined by manual
790 inspection to identify the local minima between the two modes of the within-sample
791 bimodal distance-to nearest histogram. Full-length germline sequences were
792 reconstructed for each clonal cluster with D segment and N/P regions masked (replaced
793 with Ns), with any ambiguous gene assignments within clonal groups resolved by the
794 majority rule. Lineage trees were constructed for each clone having at least two unique
795 sequences using PHYLIP (v3.695)⁵³ and Alakazam.

796 **Statistics and Reproducibility**

797 Statistical significance was determined with Graphpad Prism Version 5.0 using the tests
798 indicated in each figure. All null hypothesis testing was performed with a 95%
799 confidence interval; df, degrees of freedom. In Fig. 1 two-tailed Student's t test was
800 performed with $t = 5.3$, $df = 26$ in Fig. 1b, $t = 5.6$, $df = 63$ (SED) and $t = 4.0$, $df = 63$
801 (GC) in Fig. 1c. For Fig. 2 differences in SED and GC volumes were calculated using
802 one-way ANOVA with Bonferroni posttest, $df = 3$, $F = 8.5$ in (c). For Fig 2d, two-tailed
803 Student t test, $t = 1.9$, $df = 20$ (SED) and $t = 6.7$, $df = 20$ (GC). For Fig. 2e $t = 7.4$, $df = 10$
804 (no competition) and $t = 5.7$, $df = 8$ (competition), two-tailed Student's t test. For Fig. 2f,
805 $df = 2$, $F = 294.3$. For Fig. 2g, $t = 0.8$, $df = 10$, two-tailed Student's t test. For Fig. 2h. $t =$
806 0.9 , $df = 10$, two-tailed Student's t test. For Fig. 3b,c, $df = 2$, $F = 16.3$ (SED) and $df = 2$, F
807 $= 54.0$, one-way ANOVA with Bonferroni posttest. For Fig. 4, $t = 1.7$, $df = 68$ in a (SED,
808 no competition), $t = 0.3$, $df = 67$ (GC, no competition), $t = 1.6$, $df = 110$ (SED,
809 competition), $t = 11.54$, $df = 110$ (GC, competition), two-tailed Student's t test. For Fig. 5,
810 $t = 2.3$, $df = 10$ in (c), two-tailed Student's t test; $df = 3$, $F = 10.9$ in (d) and $df = 3$, $F =$
811 42.3 in (f), one-way ANOVA with Bonferroni posttest. For Fig.6, $t = 24.5$, $df = 10$ in (a),
812 $t = 11.6$, $df = 10$ in (b), two-tailed Student's t test; $df = 2$, $F = 24.6$ in (c), $df = 2$, $F = 14.4$
813 in (d), one-way ANOVA with Bonferroni posttest; $t = 0.5$, $df = 10$ in (e) and (f), two-
814 tailed Student's t test. In Fig. 7d, $t = 9.0$, $df = 342$, two-tailed Student's t test.

815 **Data availability:** All BCR sequencing data generated in this manuscript have been
816 deposited in the European nucleotide archive (ENA) under accession number
817 PRJEB30525. Custom scripts used for data analysis are available upon request. All other
818 data are available in the main text or the supplementary materials.

819 **Ethical compliance:** The experiments performed in this study were approved by the
820 Weizmann Institute Animal Care and Use Committee (IACUC) and followed all relevant
821 ethical regulations.

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824 **Methods References**

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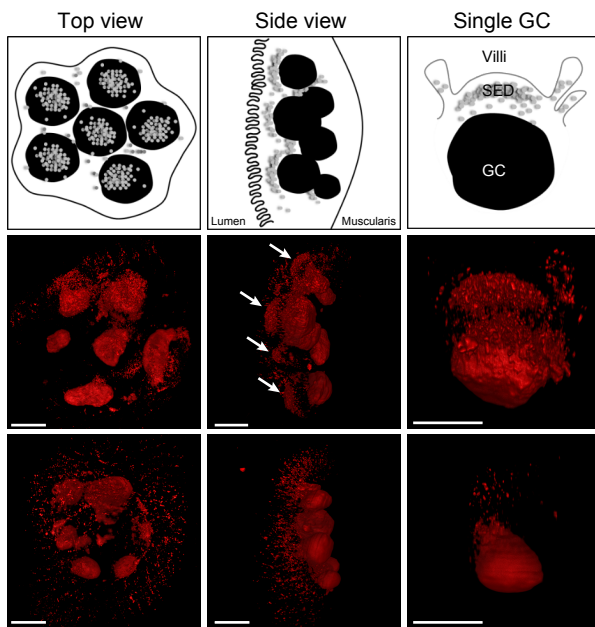
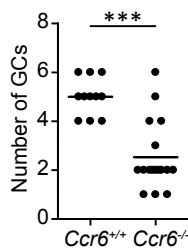
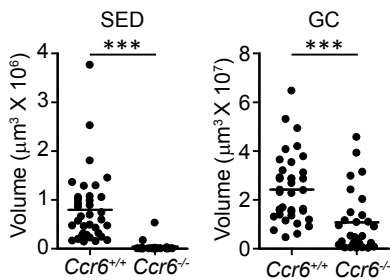
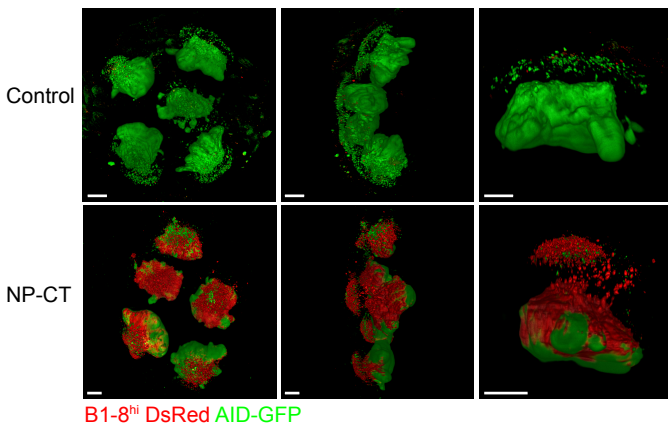
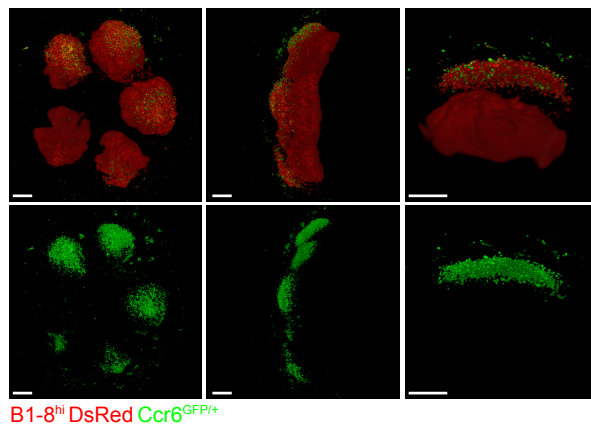
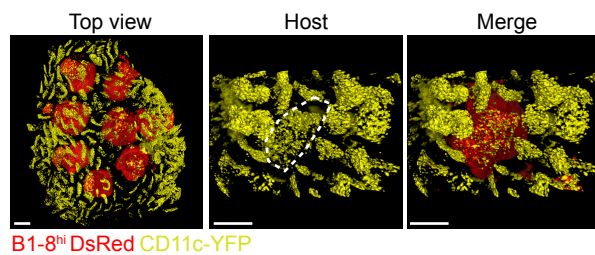
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