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2 **Intracellular BH3 profiling reveals shifts in anti-apoptotic dependency in B-cell**
3 **maturation and activation**

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13 **Short Title:** iBH3 profiling of normal and activated B-cell subsets

14

15 **Abstract**

16 Apoptosis is critical to B-cell maturation, but studies of apoptotic regulation in primary
17 human B cells is lacking. Previously, we found that infecting human B cells with Epstein-Barr
18 virus induces two different survival strategies (Price *et al.*, 2017). Here, we sought to better
19 understand the mechanisms of apoptotic regulation in normal and activated B cells. Using
20 intracellular BH3 profiling (iBH3), we defined the Bcl2-dependency of B-cell subsets from human
21 peripheral blood and tonsillar lymphoid tissue as well as mitogen-activated B cells. We found
22 that naïve and memory B cells were BCL-2 dependent, while germinal center B cells were MCL-
23 1 dependent and plasma cells were BCL-XL dependent. Proliferating B cells activated by CpG
24 or CD40L/IL-4 became more dependent upon MCL-1 and BCL-XL. As B-cell lymphomas often
25 rely on survival mechanisms derived from normal and activated B cells, these findings offer new
26 insight into potential therapeutic strategies for lymphomas.

27

28 **Introduction**

29 The maturation of B cells into antibody-secreting cells is critical to immunity and is
30 accomplished through either T-independent or T-dependent B-cell maturation. In T-independent
31 B-cell maturation, antigen binding to the B-cell receptor (BCR) stimulates extra-follicular naïve
32 and memory B cells to proliferate (Bortnick and Allman, 2013). B cells can also be activated via
33 pathogen-associated molecular patterns (PAMPs), like CpG DNA, binding to Toll-like receptors
34 (TLR) (Bernasconi et al., 2002; Krieg et al., 1995). Activated B cells can differentiate into short-
35 lived plasmablasts or migrate into secondary lymphoid tissue to undergo T-dependent
36 maturation. In the germinal center (GC) reaction, the affinity for antigen is further refined through
37 somatic hypermutation and selection by apoptosis (Victora and Nussenzweig, 2012). B cells
38 also compete for survival signals in the form of antigen binding to the BCR and CD40 receptor
39 binding by to cognate T follicular helper cells. Outcompeted B cells are eliminated by apoptosis
40 and surviving B cells exit the GC as long-lived memory B cells or plasma cells.

41 Ultimately, the humoral repertoire is shaped by apoptosis dependent on the multi-
42 domain Bcl-2 family proteins that select which B cells die and which continue to propagate
43 (Peperzak et al., 2012). Apoptosis is initiated when BAX and BAK dimerize and permeabilize
44 the mitochondrial outer membrane to release cytochrome C into the cytosol, which activates a
45 cascade of caspases that mediate DNA fragmentation, protein degradation, and cellular
46 blebbing (Fischer et al., 2003). To prevent aberrant activation, anti-apoptotic proteins bind and
47 sequester BAX and BAK as well as the pro-apoptotic BH3-only proteins (named as such
48 because they share homology in their BH3 domain). Dysregulation of apoptosis typically
49 accompanies the development of lymphomas or autoimmunity, and understanding the
50 mechanisms of apoptosis regulation is key to provide insight and develop new therapeutic
51 strategies for these diseases (Vo et al., 2012).

52 New breakthroughs in studying apoptosis regulation during B-cell maturation have been
53 made possible by the development of BH3 mimetics, a class of small molecular inhibitors that
54 mimic pro-apoptotic proteins and have shown great promise as cancer therapeutics (Oltersdorf
55 et al., 2005). In mice, naïve and memory B cells are sensitive to the BH3 mimetic ABT-737,
56 which inhibits BCL-2, BCL-XL, and BCL-W, but pre-existing GC and plasma cells are resistant
57 (Carrington et al., 2010), suggesting that apoptosis is regulated differently at various stages of
58 B-cell maturation. Recently, human tonsillar GC B cells were found to be sensitive to MCL-1
59 inhibition, which supports previous findings that MCL-1 is essential for GC formation in mice
60 (Peperzak et al., 2017; Vikstrom et al., 2010). Nonetheless, there remain challenges associated
61 with studying apoptosis in primary human B cells, which have short life-spans in culture,
62 spontaneously undergo apoptosis, and are difficult to transfect. In addition, how apoptotic
63 regulation changes specifically during B-cell activation poorly understood. As a result, we
64 utilized intracellular BH3 (iBH3) profiling to uncover the dominant anti-apoptotic mechanism at
65 the mitochondria of resting and activated primary human B cells. The principles behind iBH3
66 profiling are based upon the selective interactions between the pro- and anti-apoptotic proteins
67 of the Bcl2-family (Deng et al., 2007; Youle and Strasser, 2008). In short, the anti-apoptotic
68 proteins that are present at the mitochondria dictate which pro-apoptotic BH3-only peptides can
69 induce mitochondrial depolarization and cytochrome C release. In this way, we can identify the
70 anti-apoptotic dependency, or Bcl2-dependency, of the sample. iBH3 profiling can also assay the
71 Bcl2-dependency of multiple populations in heterogeneous samples, thereby eliminating the
72 need for time-consuming and expensive sorting experiments to study rare populations (Ryan et
73 al., 2016).

74 With the advent of iBH3 profiling and BH3 mimetics, we are equipped with tools that
75 allow us to study how apoptosis is regulated in human B-cell subsets and activated B
76 cells. Therefore, we have used iBH3 profiling to determine the BH3 profiles of normal, resting,

77 and activated B cells, and validated the hypotheses generated by these profiles by treating
78 normal and activated human B-cell subsets with BH3 mimetics.

79

80 **Results**

81 **Naïve and memory B cells circulating in the periphery depend upon BCL-2 for survival**

82 To ensure the reliability of iBH3 profiling, we sought to determine the mechanism of
83 apoptosis regulation in peripheral blood B cells from within the bulk PBMC population
84 (Carrington et al., 2010). After staining for cell-specific surface markers, cells were incubated
85 with recombinant BH3-only peptides and digitonin, which selectively permeabilizes the outer
86 cellular membrane and exposes intact mitochondria to the peptide treatment (**Figure 1A**).
87 Following peptide treatment, the cells were fixed, incubated with saponin to permeabilize
88 mitochondria, and stained with a fluorescently-labeled antibody to quantify the remaining levels
89 of intracellular cytochrome C. The extent of cytochrome C release was measured as the
90 dynamic range between total levels of cytochrome C, determined by treating with an inert
91 recombinant PUMA BH3-only peptide (PUMA2A) and alamethicin (Alm), which can induce
92 100% cytochrome C release.

93 Naïve (CD19⁺ IgD⁺) and memory (CD19⁺ IgD⁻) B cells were distinguished by IgD
94 expression (**Figure 1B**). iBH3 profiling revealed that the mitochondria in naïve and memory B
95 cells in the peripheral blood were highly sensitive to BIM, BAD, PUMA, and BMF, and relatively
96 resistant to NOXA and HRK (**Figure 1C**). This pattern of sensitivity suggested that naïve and
97 memory B cells were dependent upon BCL-2 for survival (**Figure 1D**).

98 To validate this hypothesis, we treated PBMCs with the following BH3 mimetics: ABT-
99 737 (BCL-2, BCL-XL, BCL-W inhibitor), ABT-199 (BCL-2 inhibitor), or WEHI-539 (BCL-XL
100 inhibitor) (**Figure 1E**). Naïve and memory B cells were resistant to WEHI-539 (IC₅₀ >1 μM) and

101 were sensitive to ABT-737 and ABT-199 ($IC_{50} < 1 \mu M$), thereby confirming our iBH3 profiling
102 hypotheses and previously published results of BCL-2 dependency (**Figure 1F**). ABT-199 was
103 used to distinguish between BCL-2 and BCL-W, which have identical binding partners (**Figure**
104 **1D**). Staurosporine (STS) is not a BH3 mimetic, but rather it is a pan-kinase inhibitor that
105 induces apoptosis and was used here to distinguish cells that can undergo apoptosis from those
106 that can't, such as cells that lack the effector molecules BAX and BAK (Wei et al., 2001).

107 **B-cell maturation is associated with changes in Bcl-2 family dependency**

108 To determine the changes in apoptotic regulation that take place during human B-cell
109 maturation, we queried the apoptotic regulation of CD19⁺ naïve (IgD⁺ CD38⁻), memory (IgD⁻
110 CD38⁻), germinal center (GC; IgD⁻ CD38^{mid}) B cells, and plasma cells (IgD⁻ CD38^{hi}) from tonsillar
111 tissue (**Figure 2A**). Like naïve and memory B cells in peripheral blood, tonsillar naïve and
112 memory B cells were highly sensitive to BIM, BAD, PUMA, and BMF and were predicted to be
113 BCL-2 dependent (**Figure 2B-C**). GC B cells, however, had a modestly lower response to BAD
114 and a heightened response to NOXA, suggesting that GC B cells were dependent upon MCL-1
115 for survival. Plasma cells were much less responsive to BH3 peptide treatments compared to
116 the other B-cell subsets. However, we hypothesized that within the reduced dynamic range of
117 plasma cell responses, lower NOXA sensitivity as compared to BAD and HRK suggested
118 dependence on BCL-XL for survival.

119 To validate the hypotheses generated from iBH3 profiling, we treated primary tonsillar B
120 cells with ABT-199, ABT-737, WEHI-539, and STS (**Figure 2D-E**). All cells were sensitive to
121 STS, indicating that all subsets were capable of intrinsic apoptosis. As predicted, naïve and
122 memory B cells were sensitive to BCL-2 inhibition by ABT-199 and ABT-737. Plasma cells were
123 uniquely sensitive to BCL-XL inhibition by WEHI-539, confirming their dependence on BCL-XL.
124 The difference in the sensitivity of plasma cells to ABT-737 and WEHI-539 may be due to a
125 difference in their binding affinities for BCL-XL (ABT-737 $IC_{50}=35nM$ vs WEHI-539 $IC_{50}=1.1nM$)

126 (Lessene et al., 2013; Oltersdorf et al., 2005). GC B cells were resistant to ABT-737, ABT-199,
127 and WEHI-539 since none of these drugs inhibited MCL-1. However, GC B cells were also
128 resistant to the MCL-1 specific BH3 mimetic, A-1210477 (**Figure 2 Figure Supplement 1**),
129 which is likely due to its weak cellular potency (i.e. sensitivity in the micromolar range) and
130 nonspecific toxicity (Kotschy et al., 2016; Levenson et al., 2015). In sum, these findings support
131 an existing model that B cells adjust their Bcl-2 family dependency throughout T-dependent B
132 cell maturation. Moreover, iBH3 profiling proved to be reliable in querying heterogeneous
133 populations simultaneously.

134 **CD40L/IL-4 stimulated B cells are sensitive to NOXA and HRK peptides, yet remain**
135 **sensitive to BCL-2 inhibition**

136 We next sought to model B-cell maturation with mitogen stimulation of peripheral blood
137 B cells. To track proliferation, PBMCs were stained with CellTrace Violet (CTV), which becomes
138 diluted upon cell division (**Figure 3A**). CTV was retained during permeabilization with digitonin,
139 thereby making it suitable in iBH3 profiling. We induced proliferation in peripheral blood B cells
140 *ex vivo* by treating with CD40L and IL-4 to mimic T-cell derived signals important in B-cell
141 survival in the GC. CD19+ B cells began proliferating 3 days after treatment with CD40L/IL-4, as
142 shown previously (Hawkins et al., 2007; Nikitin et al., 2014).

143 CD40L/IL-4 stimulated B cells were iBH3 profiled six days post-stimulation (**Figure 3B**).
144 Proliferating B cells displayed modestly increased sensitivity to NOXA and HRK peptide
145 treatment relative to nonproliferating B cells, suggesting an increasing dependence on MCL-1
146 and BCL-XL for survival. The robust increase in HRK sensitivity correlated with increased
147 susceptibility of proliferating B cells to the BCL-XL inhibitor, WEHI-539 (**Figure 3C-D**). However,
148 these cells remained sensitive to ABT-199 and ABT-737, suggesting that BCL-2 still plays an
149 important role in CD40L/IL-4 stimulated B-cell survival (**Figure 3C-D**). This also indicates that
150 NOXA sensitivity observed in proliferating B cells did not confer MCL-1 dependence.

151 **A CD38^{hi}, plasmablast-like subpopulation in CpG-stimulated B cells becomes sensitive to**
152 **NOXA and HRK in iBH3 profiling and becomes resistant to BCL-2 inhibition**

153 In B cells activated to proliferate *ex vivo* by CpG DNA, a PAMP that stimulates the TLR9
154 pathway, we observed similar changes in apoptotic regulation in proliferating B cells. However,
155 in CpG-stimulated cells, B cells that have undergone the most cellular divisions upregulate high
156 levels of CD38 surface expression, suggesting that they have differentiated into plasmablast-like
157 B cells (CTV^{lo} CD38^{hi}; **Figure 4A**) (Bernasconi et al., 2002; Huggins et al., 2007). This
158 population consisted predominantly of memory B cells and is noticeably absent in CD40L/IL-4
159 stimulated B cells because IL-4 downregulates CD38 expression through serine/threonine
160 kinases (Shubinsky and Schlesinger, 1996). In iBH3 profiling of CpG-stimulated B cells,
161 nonproliferating B cells were predicted to be BCL-2 dependent and as they proliferate they
162 become more responsive to NOXA and HRK peptides with CD38^{hi} B cells as the most
163 responsive to NOXA and HRK (**Figure 4B**).

164 Nonproliferating and CD38^{lo} proliferating B cells were within the same range of
165 sensitivity to ABT-199 and ABT-737 and were refractory to WEHI-539, indicating that as B cells
166 proliferated in response to CpG, BCL-2 remained important in regulating survival (**Figure 4C-D**).
167 CD38^{hi} B cells, in contrast, were less sensitive to ABT-199, ABT-737, and slightly more sensitive
168 to WEHI-539. Interestingly, it appeared that WEHI-539 increased the number of proliferating
169 CD38^{lo} memory B cells (**Figure 4C**), suggesting that in T-independent B-cell maturation, BCL-
170 XL may be involved in the differentiation of plasmablasts derived from memory B cells. This also
171 indicates that the perceived sensitivity of the CD38^{hi} cells to WEHI-539 may not be due to cell
172 death, but rather from a block in differentiation.

173 The overall increase in apoptosis resistance in the CD38^{hi} subset is not because of
174 impaired BAX and BAK activation, since CD38^{hi} cells were sensitive to STS, but rather it may be
175 due to an increasing dependence upon MCL-1 as indicated by increasing sensitivity to NOXA in

176 their iBH3 profile. In fact, of the proliferating subsets, only the response of the CD38^{hi} subset to
177 NOXA was significantly different from that of nonproliferating B cells (* p=0.0292). Thus, to
178 inhibit both BCL-XL and MCL-1, we tested the efficacy of combining WEHI-539 with A-1210477
179 in CpG-stimulated PBMCs (Chou, 2010). CpG-stimulated B cells were refractory to high
180 concentrations of A-1210477 (**Figure 4 Figure Supplement 1A-B**). However, a combination of
181 A-1210477 and sub-IC₅₀ levels of WEHI-539 resulted in synergism (Combination Index, CI<1) in
182 the CD38^{hi} subpopulation (**Figure 4 Figure Supplement 1C**). This effect was specific to MCL-1
183 as ABT-199 did not synergize with WEHI-539 (CI≈1) (**Figure 4 Figure Supplement 1D**).

184

185 **Discussion**

186 Proper regulation of apoptosis is critical for B-lymphocyte development and maturation.
187 Much of our mechanistic understanding of B-cell apoptosis is derived from murine studies due
188 to technical limitations in studying B-cell subsets in humans (Carrington et al., 2010; Peperzak
189 et al., 2013; Vikstrom et al., 2010). While two recent studies have characterized Bcl-2 family
190 dependency in human primary and secondary lymphoid tissue (Peperzak et al., 2017; Sarosiek
191 et al., 2017), in this study we use the newly developed iBH3 profiling technique to query the
192 mechanisms underlying apoptotic regulation both in heterogeneous lymphoid tissue as well as
193 upon activation of normal B-cell subsets *ex vivo* (Ryan and Letai, 2013; Ryan et al., 2016). Our
194 experimental approach couples proliferation tracking with cell surface markers to measurement
195 of intracellular cytochrome C release in response to BH3 peptides to define Bcl2 family member
196 dependency at the single cell level during B-cell activation. Validation of iBH3 profiling with BH3
197 mimetics defined transitions during B-cell maturation from BCL-2 dependency in naïve and
198 memory B cells, to MCL-1 in GC B cells, and to BCL-XL in plasma cells from secondary
199 lymphoid tissue. Then, upon activation by CpG or CD40L/IL-4, the Bcl2-dependency of
200 proliferating B cells shifts from BCL-2 to a combination of BCL-XL and MCL-1. This shift is

201 apparent by the increased resistance to BCL2-specific BH3 mimetics and is most pronounced in
202 the most differentiated subset of CpG-stimulated B cells.

203 Our findings corroborate several *in vivo* studies in mice that suggest that stage-specific
204 Bcl2-dependency occurs throughout B-cell maturation. Our work indicates that human naïve
205 and memory B cells, like those in mice, are sensitive to BCL-2 inhibition, whereas GC and
206 plasma cells are refractory (Carrington et al., 2010). In mouse GC B cells or those induced to
207 differentiate into plasma cells *ex vivo*, MCL-1 is important in promoting survival (Peperzak et al.,
208 2013; Vikstrom et al., 2010). Furthermore, murine plasma cells depend on BCL-XL protection
209 from apoptosis due to the unfolded protein response (Gaudette et al., 2014). These parallels in
210 mouse studies with our findings in human B cells suggest that intrinsic apoptotic regulation in B-
211 cell maturation is highly conserved and functionally critical to humoral immunity.

212 Proliferating B cells in the GC as well as *ex vivo* mitogen-stimulated B blasts display
213 evidence of oxidative and metabolic stress as well as DNA damage, which if left unresolved can
214 lead to apoptosis (Jellusova et al., 2017; Nikitin et al., 2014; Ranuncolo et al., 2007; Wheeler
215 and Defranco, 2012). Our findings indicate that GC cells display a heightened response to
216 NOXA in iBH3 profiling suggesting an increased dependence on MCL-1. In addition, our lab has
217 found that following EBV infection of primary human B cells, which is characterized by
218 hyperproliferation and activation of the DNA damage response (Nikitin et al., 2010), there is a
219 shift in Bcl2-dependency from BCL-2 to MCL-1 (Price et al., 2017). This suggests that upon
220 proliferation, MCL-1 expression is rapidly induced to respond to apoptosis-inducing intracellular
221 stress resulting from rapid expansion and differentiation.

222 Constitutive activation of gene expression and survival programs found in normal B-cell
223 maturation can promote the development of lymphomas. For example, in follicular lymphoma, a
224 t(14;18) chromosomal translocation induces BCL-2 over-expression in naïve B cells thereby
225 promoting survival in the B-cell follicle of a GC (Kridel et al., 2012; Tsujimoto et al., 1985).

226 Consistent with our findings and that of others linking GC survival to MCL-1 up-regulation
227 (Peperzak et al., 2017; Vikstrom et al., 2010), a subset of DLBCLs that arise from the GC often
228 display MCL-1 copy number gains (Wenzel et al., 2013). Indeed, c-Myc-driven Burkitt's
229 lymphomas are thought to have arisen from GC B cells, express high levels of MCL-1 and are
230 sensitive to MCL-1 inhibition (Dave et al., 2006; Kelly et al., 2014; Klein et al., 1995; Kotschy et
231 al., 2016). MCL-1 is also often over-expressed in plasma-cell derived multiple myelomas,
232 mimicking the requisite up-regulation of MCL-1 induced by stromal interactions in the bone
233 marrow (Gupta et al., 2017; Wulleme-Toumi et al., 2005). Interestingly, a subset of plasma-cell
234 derived tumors also display co-dependence of MCL-1 and BCL-XL (Morales et al., 2011),
235 similar to our findings in CpG-induced CD38^{hi} plasmablasts. Overall, these data strongly support
236 the importance of defining normal and activated human B-cell survival mechanisms towards
237 understanding the underlying basis of B-lymphoma cell survival.

238

239

240 **Materials and Methods**

241 **Cells and mitogens**

242 Peripheral B cells were obtained from normal human donors through the Gulf Coast
243 Regional Blood Center (Houston, TX). Buffy coats were layered over Ficoll Histopaque-1077
244 gradient (Sigma, H8889) and washed three times with FACS buffer (5% FBS in PBS) and
245 cultured in RPMI supplemented with 15% FBS (Corning), 2 mM L-Glutamine, 100 U/ml
246 penicillin, 100 µg/ml streptomycin (Invitrogen), and cyclosporin A. To track proliferation, PBMCs
247 were stained with CellTrace Violet (Invitrogen, C34557) for 20min in PBS at 37°C, washed in
248 FACS buffer, and then cultured with the appropriate mitogens.

249 Tonsillar B cell subsets were obtained from discarded, anonymized tonsillectomies from
250 the Duke Biospecimen Repository and Processing Core (Durham, NC). Tonsillectomies were
251 manually disaggregated, filtered through a cell strainer, and isolated by Ficoll Histopaque-1077
252 (Sigma, H8889). The harvested lymphocyte layer was washed three times with FACS buffer and
253 cultured in RPMI supplemented with 15% FBS (Corning), 2 mM L-Glutamine, 100 U/ml
254 penicillin, 100 µg/ml streptomycin (Invitrogen), and cyclosporin A.

255 TLR9 ligand CpG oligonucleotide (ODN 2006) was purchased from IDT and used at 2.5
256 µg/mL (Krieg et al., 1995). Human recombinant interleukin-4 (IL-4; Peprotech, AF200-04) was
257 used at 20ng/mL. HA-tagged CD40 ligand (R&D Systems, 6420-CL) was used at 5ng/mL with
258 an anti-HA cross-linking peptide (R&D Systems, MAB060; RRID:AB_10719128) at a
259 concentration of 0.2 µg/µL.

260 **Intracellular BH3 profiling and validation**

261 Intracellular BH3 (iBH3) profiling was performed with recombinant peptides synthesized
262 by New England Peptides (sequences listed in table). Stock peptides were resuspended at
263 10mM in DMSO and aliquoted and stored at -80°C. To prepare peptides for iBH3 profiling,

264 peptides were diluted to 200 μ M in 0.004% digitonin in DTEB buffer (135mM trehalose (Sigma,
265 T9449), 20 μ M EDTA (Sigma, E6758), 20 μ M EGTA (Sigma, E3889), 5mM succinic acid (Sigma,
266 S3674), 0.1% IgG-free BSA (VWR, 100182-742), 10mM HEPES (Sigma H4034), 50mM
267 potassium chloride (Sigma, P9541), adjusted to pH 7.5 with potassium hydroxide). Bim peptides
268 were used at 10 μ M, 1 μ M, and 0.1 μ M Alamethicin (Enzo, BML-A150-0005) was used at 25 μ M.

269 PBMCs and tonsillar cells were iBH3 profiled immediately after processing, while
270 mitogen-stimulated cells were profiled on day 6 post-stimulation. To prepare samples for iBH3
271 profiling, cells were stained with the Zombie Aqua viability dye (Biolegend, 423101) in serum-
272 free PBS for 15min at room temperature and then stained with FACS antibodies for surface
273 markers for 30min at 4°C. Because Zombie Aqua and CellTrace Violet fluoresce in the same
274 channel, mitogen-stimulated cells were centrifuged at low speed and washed to remove dead
275 cells (ie Trypan positive) before staining. After staining, cells were pelleted and resuspended at
276 4x10⁶ cells/mL in DTEB buffer. Equal volumes (50 μ L) of cells and peptides were incubated in
277 polypropylene tubes for 1hr at room temperature in the dark. To stop the reaction, 30 μ L of 4%
278 PFA in PBS was added and incubated at room temperature for 10min. To neutralize the fixation,
279 30 μ L of neutralization buffer (1.7M TRIS base, 1.25M glycine, pH 9.1) was added for 5min.
280 Intracellular levels of cytochrome C were probed by adding 20 μ L of staining buffer (1% saponin,
281 10% BSA, 20% FBS, 0.02% sodium azide in PBS) with 1 μ L antibody for human cytochrome C.
282 Samples were stained overnight and then transferred into polystyrene tubes for analysis the
283 next day.

Peptide	Amino acid sequence
Bad	Ac-LWAAQRYGRELRRMSDEFEGSFKGL-NH2
Bim	Ac-MRPEIWIAQELRRIGDEFNA-NH2
Bmf	Ac-HQAEVQIARKLQLIADQFHRY-NH2

Hrk	Ac-WSSAAQLTAARLKALGDELHQ-NH2
Noxa	Ac-AELPPEFAAQLRKIGDKVYC-NH2
Puma	Ac-EQWAREIGAQLRRMADDLNA-NH2
Puma2A	Ac-EQWAREIGAQARRMAADLNA-NH2

284 Ac – Acetyl, NH2 – Amide.

285 **Flow cytometry**

286 To track proliferation, cells were stained with CellTrace Violet (Invitrogen, C34557). Cells
287 were washed in FACS bufer (5% FBS in PBS), stained with fluorescently-conjugated antibodies
288 for 30min-1hr at 4°C in the dark, and then washed again before being analyzed on a BD FACS
289 Canto II. To normalize acquisition, Spherotech Accucount (ACBP-50-10) beads were included in
290 each tube.

Antibodies used for flow-cytometry:		
PE Mouse Anti-Human IgD	BD Pharmingen, 555779	(no RRID)
APC Mouse Anti-Human CD38	Biologend, 303510	RRID:AB_314362
PECy7 Mouse Anti-Human CD19	eBioscience, 25-0198-42	RRID:AB_10671548
FITC Mouse Anti-Human cytochrome C	Biologend, 612304	RRID:AB_2090159

291

292 **Dose-response curves and isobolograms**

293 Small molecule inhibitors used in this study were ABT-737 (Selleckchem, S1002), ABT-
294 199 (Adooq Biosciences, A12500), WEHI-539 (APExBIO, A3935), A1210477 (Selleckchem,
295 S7790), and staurosporine (Sigma, S5921-0.5MG). Dose-response curves were generated by
296 treating primary cells for 24 hours at 37°C. Mitogen-activated cells were treated on day 3 post-
297 stimulation for 3 days and assayed on day 6. Cell counts were performed by flow cytometry and

298 normalized to a DMSO (vehicle only) control. Curves were drawn in GraphPad Prism 7 to
299 calculate IC₅₀ values and 95% confidence intervals based on a Hill slope factor of 1.

300 Isobolograms were generated based on dose-response curves with individual drugs
301 (Drug 1, Drug 2) and combinations of drugs (Drug 1 + Drug 2) (Chou, 2010). Individual drug
302 dose-response curves were used to identify drug concentrations at IC₃₀, IC₅₀, and IC₇₀. Similarly,
303 at a constant concentration of Drug 1, the concentrations of Drug 2 were determined at the IC₃₀,
304 IC₅₀, and IC₇₀ points of the combination dose-response curve. To calculate the Combination
305 Index (CI), the following equation was used:

$$306 \quad CI_{50} = \frac{[\text{Drug 1}] (\text{IC}_{50}, \text{in combination})}{[\text{Drug 1}] (\text{IC}_{50}, \text{alone})} + \frac{[\text{Drug 2}] (\text{IC}_{50}, \text{in combination})}{[\text{Drug 2}] (\text{IC}_{50}, \text{alone})}$$

307 The CI was also calculated for CI₃₀ (with IC₃₀ values) and for CI₇₀ (with IC₇₀ values). We
308 classified the interaction between the combination of drugs based on the following CI values:

309 CI > 1, Antagonistic; CI = 1, Additive; CI < 1, Synergistic.

310 **Statistical analysis**

311 Student's 2-way ANOVA and two-tailed t-test were calculated using GraphPad Prism 5.0
312 software. *p < 0.05 was considered significant. IC₅₀ values were calculated by non-linear
313 regression using GraphPad and 95% confidence intervals were reported.

314

315 **Author Contributions**

316 J.D. and M.A.L designed research; J.D. performed research; J.D. and M.A.L. analyzed
317 data; J.D. and M.A.L. wrote the paper.

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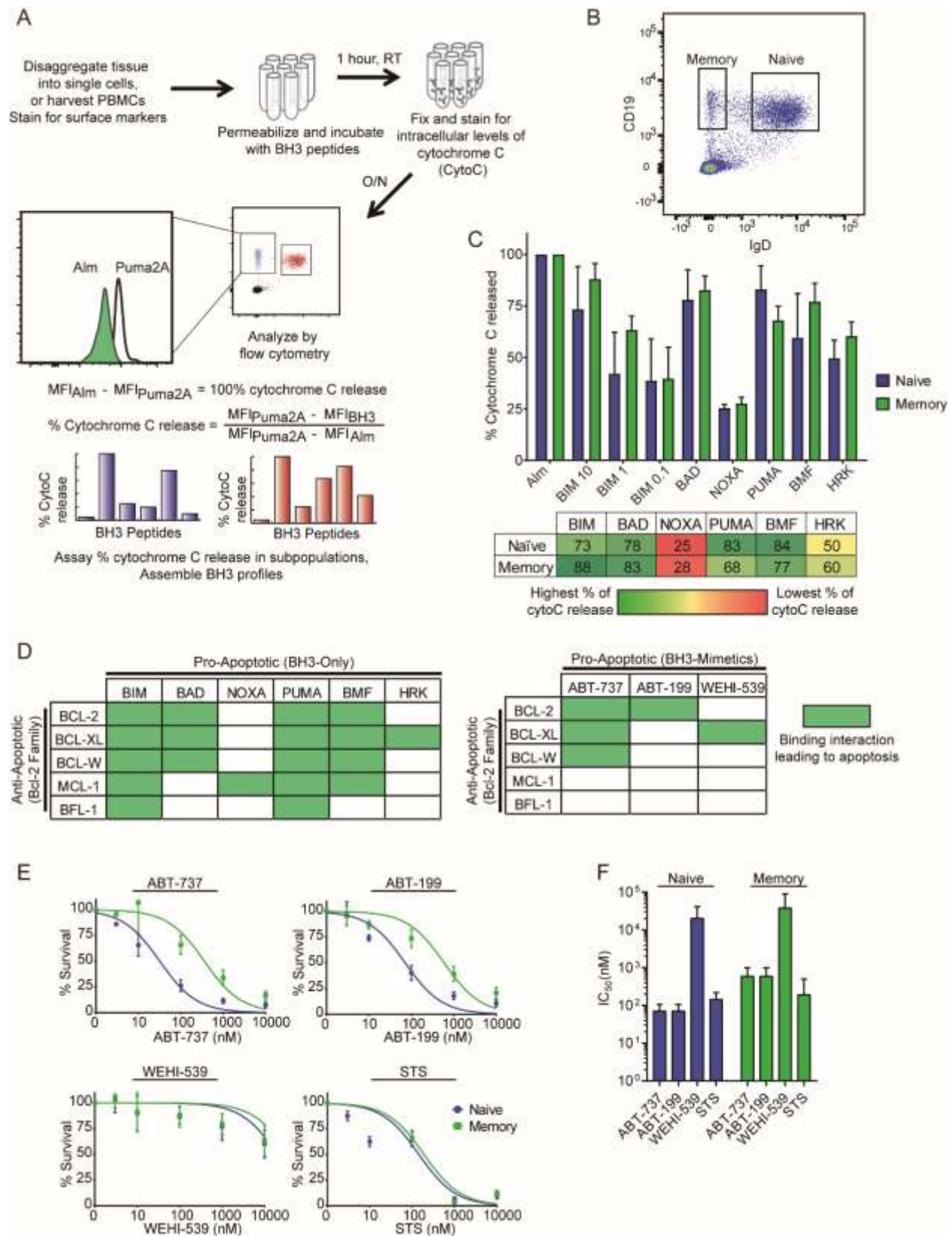
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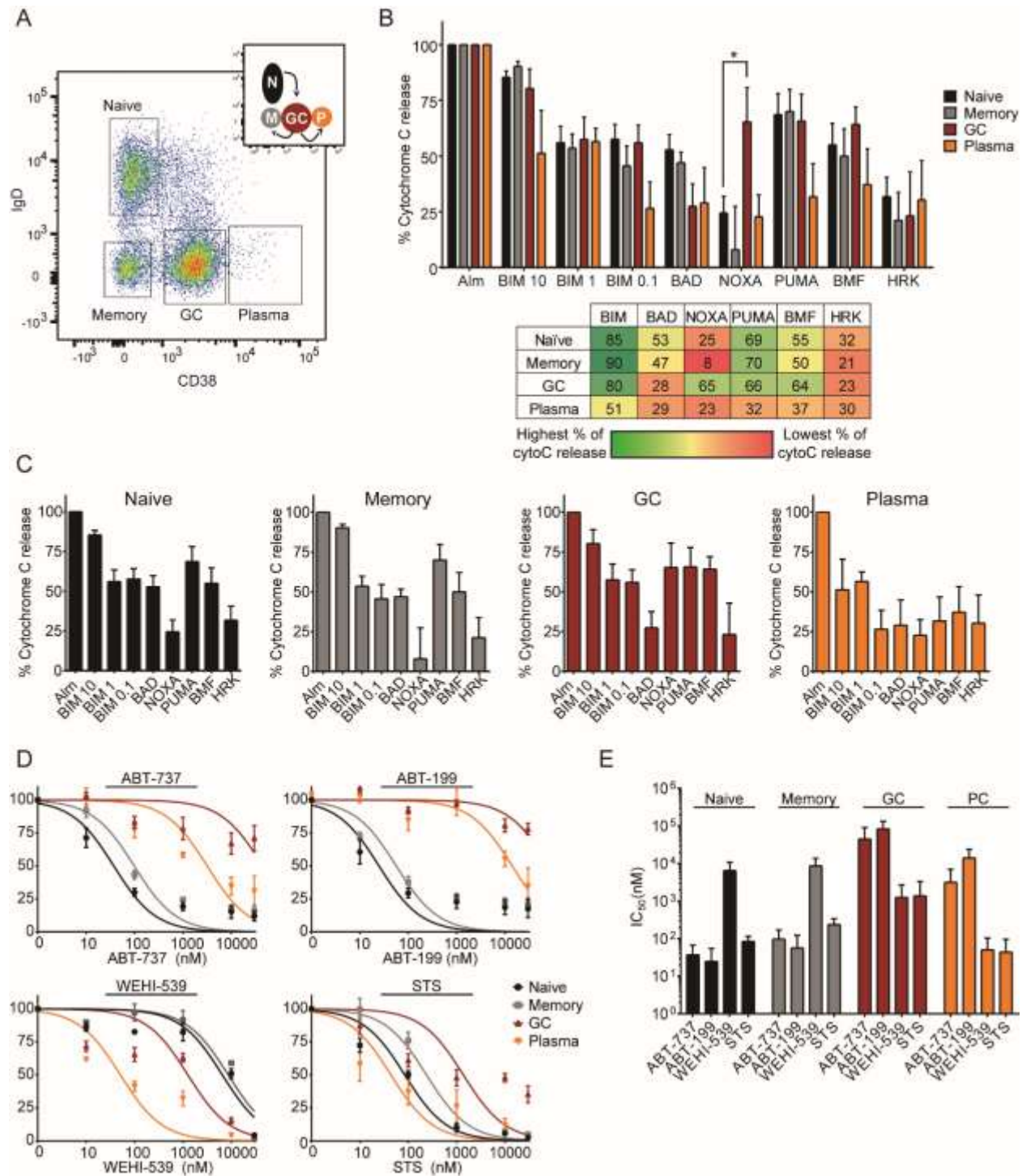
446 **Figure 1. The iBH3 profiles of normal, resting human B cells from the peripheral blood.**

447 A) Schematic of the intracellular BH3 (iBH3) process, including how percentage of cytochrome
448 C release is quantified. B) Representative flow cytometry plot of CD19⁺ naïve (IgD⁺) and
449 memory B cells (IgD⁻) from human peripheral blood mononuclear cells (PBMCs). C) iBH3
450 profiles of naïve and memory B cells from three human donors. Mean and SEM are plotted;
451 mean values are summarized in table below. Percentage of cytochrome C release is normalized
452 to alamethicin (Alm) positive control and Puma2A negative control. BIM 10 - 10 μ M BIM, BIM 1 -
453 1 μ M BIM, BIM 0.1 - 0.1 μ M BIM D) Left, schematic of selective interactions between pro- and
454 anti-apoptotic members of the Bcl-2 protein family and, right, BH3 mimetics and the anti-
455 apoptotic proteins that they inhibit. Green boxes indicate a binding interaction between the two
456 proteins that leads to cytochrome C release. E) Dose-response curves generated from treating
457 PBMCs for 24 hours with ABT-737 (BCL-2, BCL-XL, BCL-W inhibitor; 8 human donors), ABT-
458 199 (BCL-2 inhibitor; 8 human donors), WEHI-539 (BCL-XL inhibitor; 3 human donors) and
459 staurosporine (STS; Naïve, 9 human donors; Memory, 8 human donors), a broad kinase
460 inhibitor that can induce apoptosis. Percent survival is measured as a percentage of DMSO-
461 treated controls. F) Average IC₅₀ values with 95% confidence intervals for naïve and memory B
462 cells are plotted from (E).

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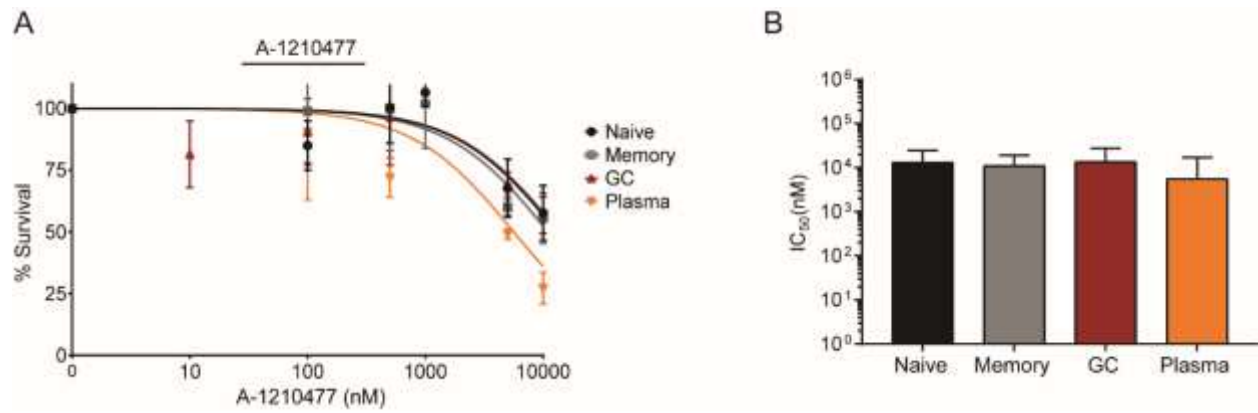
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467 **Figure 2. The iBH3 profiles of normal, resting human B cells from tonsillar lymphoid**
 468 **tissue.** A) Representative flow cytometry plot of CD19⁺ B cell subsets found in human tonsillar
 469 tissue: naïve (IgD⁺ CD38⁻), memory (IgD⁻ CD38⁻), germinal center (GC; IgD⁻ CD38^{mid}), and
 470 plasma cells (IgD⁻ CD38^{hi}). Inset, a color-coded schematic of the GC reaction. B) iBH3 profiles
 471 of tonsillar B cell subsets from 4-5 human donors. Mean and SEM are plotted; mean values are
 472 summarized in the table below. Percentage of cytochrome C release is normalized to
 473 alamethicin (Alm) positive control and Puma2A negative control. In samples treated with Noxa-

474 derived recombinant peptides, * $p=0.0467$, by unpaired two-tailed Student's t-test. C) Same as
475 in (B), but showing each subset's profile individually. D) Dose-response curves generated from
476 treating eight human donors for 24 hours with ABT-737 (BCL-2, BCL-XL, BCL-W inhibitor; 3
477 human donors), ABT-199 (BCL-2 inhibitor; 3 human donors), WEHI-539 (BCL-XL inhibitor; 3
478 human donors) and staurosporine (STS; 4-6 human donors), a broad kinase inhibitor that can
479 induce apoptosis. Percent survival is measured as a percentage of DMSO-treated controls. E)
480 Average IC_{50} values with 95% confidence intervals for each subset are plotted from (D). Plasma
481 cells, PC.

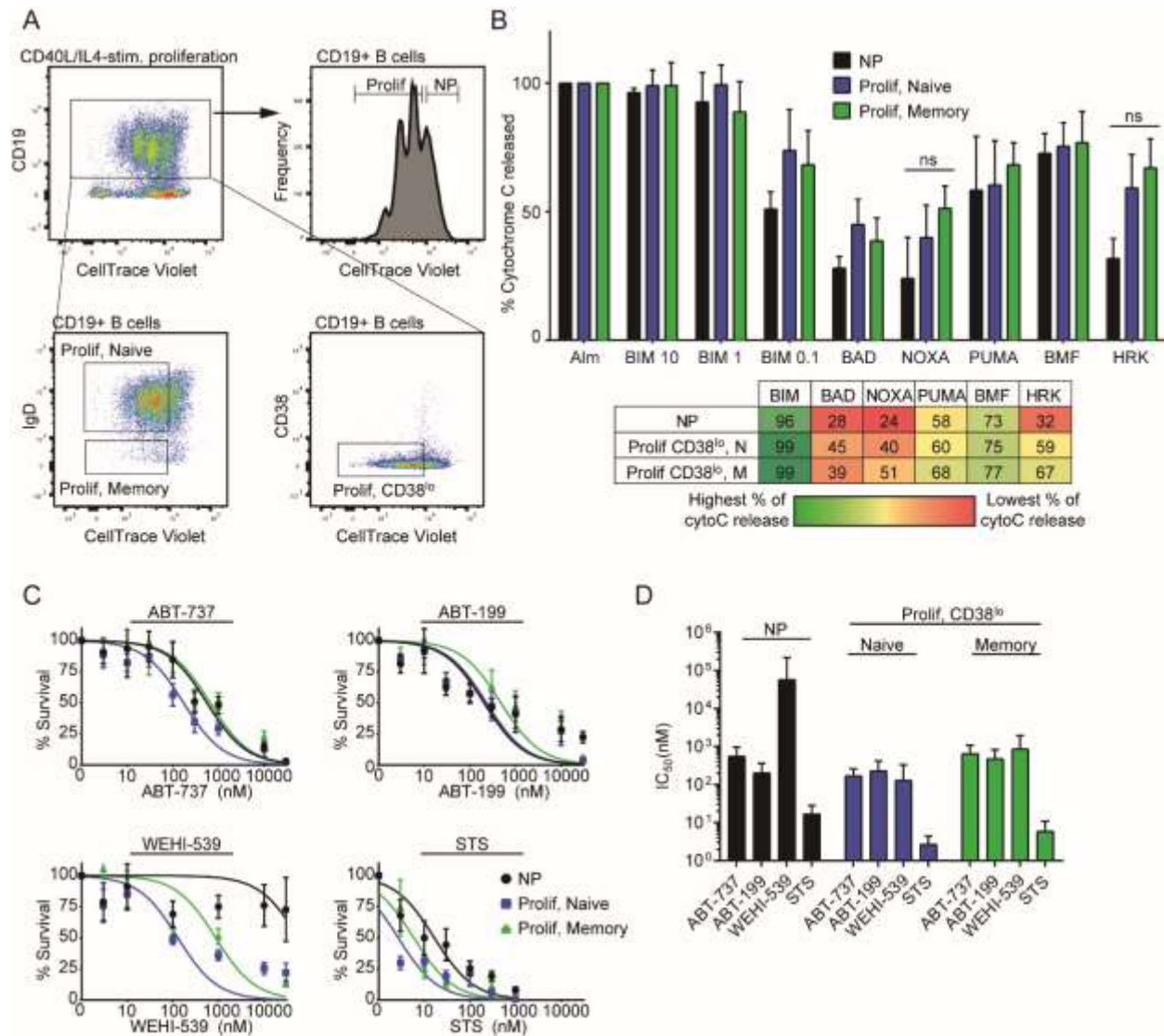
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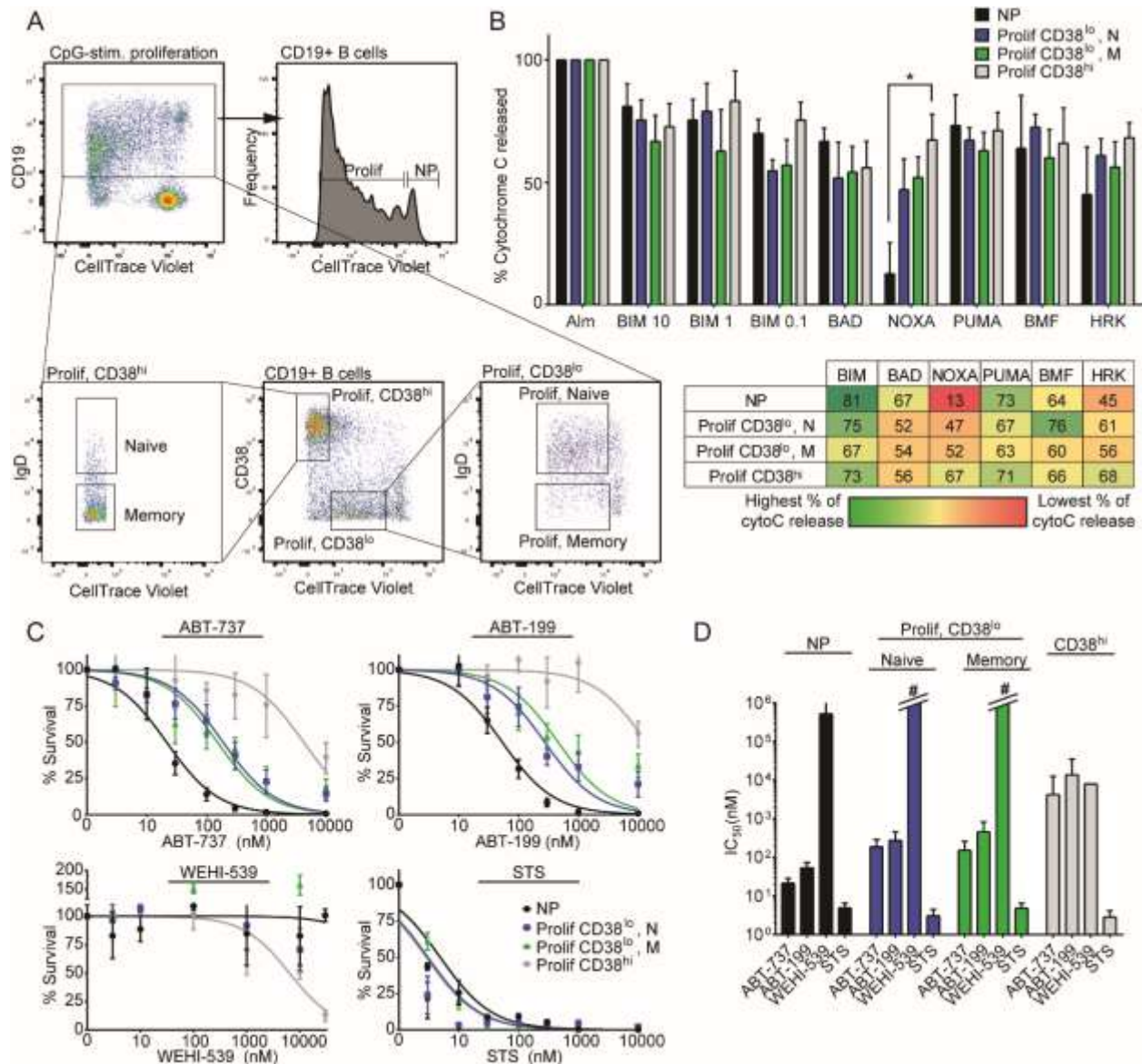
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485 **Figure 2 Figure Supplement 1. Tonsillar B cell subsets are largely refractory to the MCL-1**
486 **inhibitor A-1210477.** A) Dose-response curves generated from treating 5 human donors with
487 A-1210477 (MCL-1 specific BH3 mimetic) for 24 hours. B) Average IC₅₀ values with 95%
488 confidence intervals for each subset are plotted from (A).

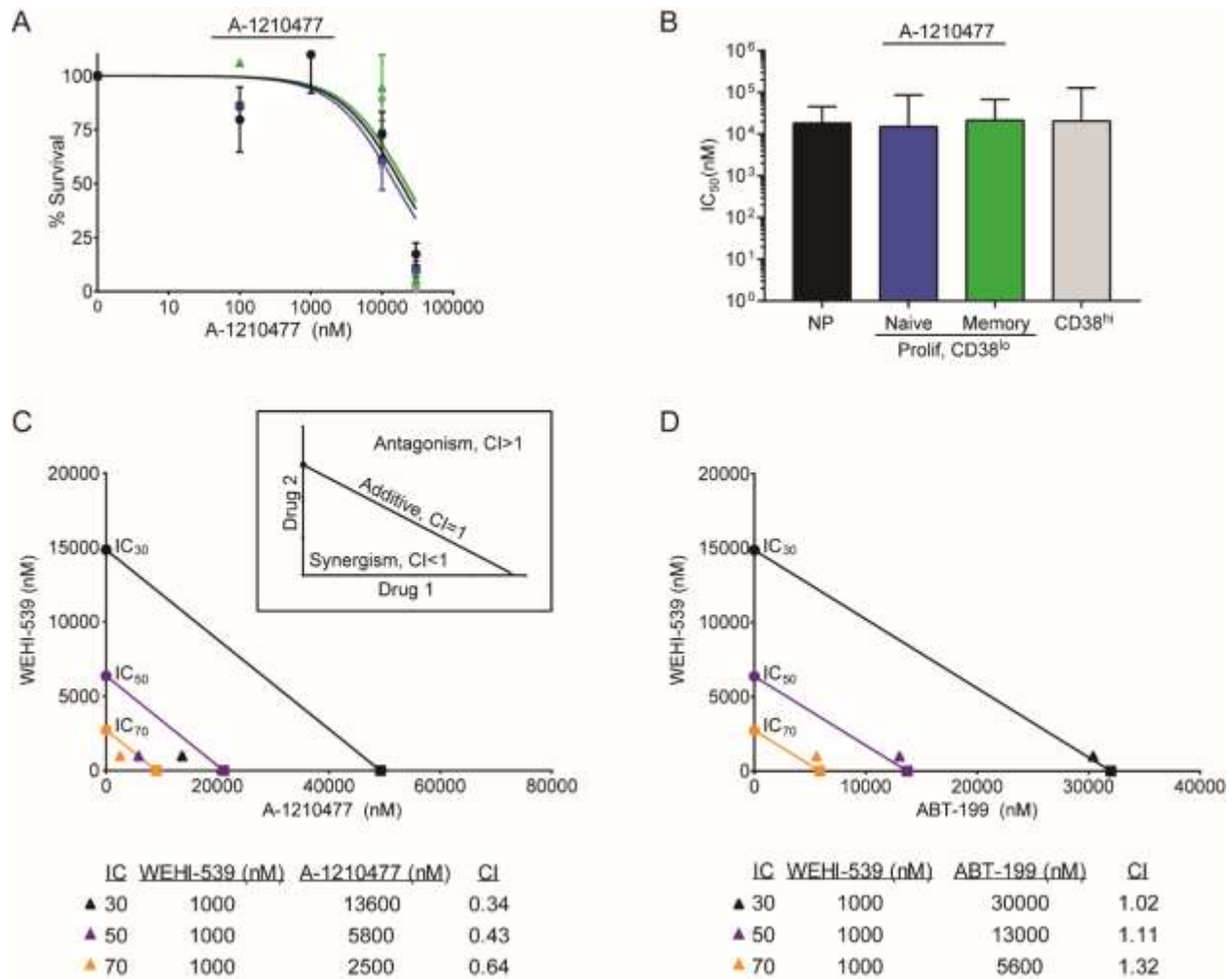


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490 **Figure 3. Changes in apoptotic regulation in activated, proliferating B cells stimulated by**
 491 **CD40L/IL-4.** A) Representative flow cytometry plot of CD19⁺ B cells that have been stimulated
 492 to proliferate by CD40L/IL-4, Day 7 after stimulation. Dilution of CellTrace Violet with each
 493 division distinguishes proliferating (Prolif; CTV^{lo}) cells from nonproliferating (NP; CTV^{hi}) cells.
 494 Cells that have proliferated the furthest also upregulate CD38 surface expression to some
 495 extent, but the size of this population has been inconsistent across donors. Both naïve and
 496 memory B cells proliferate in response to CD40L/IL-4 stimulation. B) iBH3 profiles of different
 497 subsets of CD40L/IL-4 stimulated B cells from 3-5 human donors. Mean and SEM are plotted;
 498 mean values are summarized in the table below. Percentage of cytochrome C release is
 499 normalized to alamethicin positive control and Puma2A negative control. ns, not significant by
 500 unpaired two-tailed Student's t-test. C) Dose-response curves generated from treating three
 501 human donors for 3 days with ABT-737 (6 human donors), ABT-199 (4-9 human donors),
 502 WEHI-539 (3 human donors), and STS (6 human donors). D) Average IC₅₀ values with 95%
 503 confidence intervals for each subset are plotted from (C).



504
 505 **Figure 4. Changes in apoptotic regulation in activated, proliferating B cells stimulated by**
 506 **CpG.** A) Representative flow cytometry plot of CD19⁺ B cells that have been stimulated to
 507 proliferate by CpG, Day 6 after stimulation. Dilution of CellTrace Violet with each division
 508 distinguishes proliferating (Prolif) cells from nonproliferating (NP) cells. Cells that have
 509 proliferated the most also upregulate high CD38 surface expression (CD38^{hi}). Both naïve and
 510 memory B cells proliferate in response to CpG stimulation. B) iBH3 profiles of different subsets
 511 of CpG-stimulated B cells from 4 human donors. Mean and SEM are plotted; mean values are
 512 summarized in the table below. Percentage of cytochrome C release is normalized to
 513 alamethicin (Alm) positive control and Puma2A negative control. In samples treated with Noxa-
 514 derived peptide, * p=0.0292, by unpaired two-tailed Student's t-test. C) Dose-response curves
 515 generated from CpG-stimulated PBMCs for 3 days with ABT-737 (5-7 human donors), ABT-199
 516 (5-7 human donors), WEHI-539 (3 human donors), and STS (6-7 human donors). D) Average
 517 IC₅₀ values with 95% confidence intervals for each subset are plotted from (C). #, for these
 518 subsets, there is no detectable sensitivity and therefore no calculable IC₅₀ value.



519

520 **Figure 4 Figure Supplement 1. Combined BCL-XL and MCL-1 inhibition is cytotoxic to the**
 521 **CD38^{hi} subset of CpG-stimulated B cells.** A) Dose-response curves generated from treating
 522 CpG-stimulated PBMCs for 3 days with A1210477 (3 human donors). B) Average IC₅₀ values
 523 with 95% confidence intervals for each subset are plotted from (A). C) Isobologram generated
 524 from treating CpG-stimulated PBMCs for 3 days with WEHI-539 (from Figure 4C), A1210477
 525 (from Figure 4 Figure Supplement 1A), and in combination. IC₃₀ (black), IC₅₀ (purple), and IC₇₀
 526 (orange) values are plotted for WEHI-539 alone (●), A1210477 alone (■), and A1210477 in
 527 combination with 1000nM WEHI-539 (▲). Combination index (CI) values are listed below. Inset,
 528 a schematic of the combination index (CI) values of synergistic, additive, and antagonistic
 529 interactions and where they fall on an isobologram. D) Isobologram generated from treating
 530 CpG-stimulated PBMCs for 3 days with WEHI-539 (from Figure 4C), ABT-199 (from Figure 4C)
 531 and in combination. IC₃₀ (black), IC₅₀ (purple), and IC₇₀ values are plotted for WEHI-539 alone
 532 (●), ABT-199 alone (■), and ABT-199 in combination with 1000nM WEHI-539 (▲). Combination
 533 index (CI) values are listed below.

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