Inhibition of PI3K p110δ activity reduces IgE production in IL-4 and anti-CD40 stimulated human tonsil B cell cultures

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Abstract

Background: Phosphoinositide 3-kinase (PI3K) p110δ signalling negatively regulates the production of mouse IgE. However, there are disparities between the mouse and human IgE biology, and the role of PI3K p110δ in the production of human IgE is yet to be determined.

Objective: To investigate the effect of PI3K p110δ inhibition in the production of human IgE.

Methods: Human tonsil B cells were stimulated with IL-4 and anti-CD40 antibody to induce class switching to IgE and IgG1 in the presence or absence of IC87114, a small molecule inhibitor of PI3K p110δ. Using FACS, RT-PCR and ELISA we examined the effect of PI3K p110δ inhibition on IgE production and also determined the mechanisms involved.

Results: Unlike in mice, we observed that PI3K p110δ inhibition significantly reduces the number of IgE+ switched cells and the amounts of secreted IgE in IL4 and anti-CD40 cultures. However, the number of IgG1+ cells and secreted IgG1 were largely unaffected by PI3K p110δ inhibition. The expression levels of AID, ε and γ1 germinal transcripts or other factors involved in the regulation of CSR to IgE and IgG1 were also unaffected by IC87114. However, we found that IC87114 significantly decreases the proliferation of tonsil B cells stimulated with IL-4 and anti-CD40, specifically reducing the frequency of cells that had undergone 4 divisions or more.

Conclusion: Our data identifies proliferation as the key mechanism through which PI3K p110δ regulates CSR of human B cells to IgE and highlights PI3K p110δ as a pharmacological target for the treatment of allergic disease.

Keywords

IgE, allergy, class-switching, human B cells, Phosphoinositide 3-kinases p110δ, signal transduction

Abbreviations

AID	Activation-induced cytidine deaminase
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CSR	Class switch recombination
FACS	Fluorescence activated cell sorting
GLT	Germline transcription
NFκB	Nuclear factor-kappa B
PI3K	Phosphoinositide 3-kinase
PTEN.	Phosphatase and tensin homolog
STAT6	Signal transducer and activator of transcription-6

66 **Introduction**

67 Immunoglobulin (IgE) antibodies play a fundamental role in the pathogenies of
68 allergic disease. They bind to the high affinity IgE receptor (FcεRI) on mast cells
69 and basophils, which, when cross-linked by multivalent allergens, trigger cell
70 activation and the release of various mediators of immediate hypersensitivity
71 and allergic inflammation [1,2]. Since its discovery, over a half a century ago, we
72 have made significant progress not only in understanding the immunological
73 mechanisms associated with type 1 hypersensitivity reactions[1,2] but also in our
74 understanding of the mechanisms involved in the regulation of IgE production[3–
75 9].

76 The commitment of a B cell to undergo CSR to IgE is dependent on signals
77 downstream of the tumour necrosis factor receptor superfamily member CD40,
78 after its interaction with the CD40 ligand (CD40L) on the surface of T cells, and
79 IL4/L13 binding to their receptors on B cells[2,10]. These signals activate the
80 nuclear factor-κ B (NFκB) and signal transducer and activator of transcription
81 6 (STAT6) transcription factors that act synergistically to regulate CSR to IgE[10–
82 12]. Both NFkB and STAT6 bind to the Iε promoter and initiate the synthesis of ε
83 germline transcripts (εGLT) and make ε switch region available for genetic
84 recombination. Furthermore, NFκB and STAT6 can also activate the
85 expression of the enzyme activation-induced cytidine deaminase (AID)[13], an
86 important regulator of CSR and SMH, which produces DNA breaks in the switch
87 regions that precede the Cε exon[10].

88

89 Several molecular mechanisms have evolved to ensure rigorous regulation of
90 IgE production[10,14]. In mice, they also include signalling by PI3K p110δ[15–17], a

member of the class IA PI3K catalytic isoforms, which is activated downstream of various receptors on B cells[18]. Inhibition of the PI3K p110δ signalling in mouse B cells, both *in vitro* and *in vivo,* increases IgE production[15–17]. This effect of PI3K p110δ inhibition was found to be associated with increased εGLT and AID expression levels, suggesting that under normal conditions PI3K p110δ signalling supresses IgE production by repressing εGLT and AID expression. This was surprising since signalling by PI3K p110δ is also required for the production of type 2 cytokines such as IL4 and IL13[19,20]. It was later suggested that PI3K p110δ signalling limits IgE production in a B cell-intrinsic manner, a mechanism that involves the activation of Bcl6[16], a known negative regulator of CSR to IgE[21].

However, the B cell regulatory systems of IgE production in humans differ from those in mice[7], and the role of PI3K p110δ signalling in the production of human IgE is yet to be determined. Here, we isolated human tonsil B cells and stimulated CSR to IgE with IL4 and anti-CD40. To investigate the involvement of PI3K p110δ signalling in human IgE production, the B cell cultures were treated with IC87114, a small molecular inhibitor of p110δ also used in mouse B cell cultures[15,17]. The data show that IC87114 reduces the IgE production in tonsil B cell cultures stimulated with IL-4 and anti-CD40 antibody. Interestingly, this effect was shown to be specific for IgE, with the production of IgG1 being largely unaffected. This result is in striking contrast with the effect of PI3K p110δ inhibition in mouse B cells[19,20].

116 **Materials and methods**

117

118 **Ethics**

119 Following full informed written consent, tonsils were obtained from patients
120 undergoing routine tonsillectomies. The study was conducted at and in
121 accordance with the recommendations of King's College London and Guy's and
122 St Thomas's NHS Fundation Trust and the protocol was approved by the
123 London Bridge Research Ethics Committee (REC number 08/H0804/94).

124

125 **Cell culture**

126 Tonsils were obtained from patients undergoing routine tonsillectomies. The
127 protocol was approved by the London Bridge Research Ethics Committee (REC
128 number 08/H0804/94), and the study was conducted at and in accordance with
129 the recommendations of Guy's and St Thomas's NHS Fundation Trust and
130 King's College London. The untouched resting B cells were isolated from the
131 human tonsils by depletion of other cells using MojoSort Human B Cell (CD43-
132) Isolation Kit (BioLegend), whereas the naïve B cells were isolated using the
133 EasySep Human Naïve B cell isolation kit (Stem Cell Technologies). Purified B
134 cells were then cultured at 0.5 x10^6 cells/mL in RPMI 1640 (Lonza) containing
135 penicillin (100 IU/ml), streptomycin (100μg/ml) and glutamine (2mM, Invitrogen)
136 and 10% Foetal Calf Serum (Hyclone; Perbio Biosciences). To induce CSR to
137 IgE, cultures were stimulated with IL-4 (200 IU/ml; R&D Europe Systems Ltd)
138 and anti-CD40 antibody (0.5 μg/ml; G28.5; American Type Culture Collection).
139 To investigate the role of PI3K p110δ signalling in human IgE production, cell
140 cultures were also treated with different concentration of IC87114 (LKT

141 Laboratories Inc), a small molecule inhibitor of PI3K p110δ signalling used in
142 the mouse studies. Considering that IC87114 has an IC_{50} of 0.5μM, it was used
143 at concentrations below and above this IC_{50}; 0.125μM, 0.25μM, 0.5μM, 1μM,
144 and 2μM. The cells were then incubated for up to 10 days at 37°C with 5%
145 CO_2.
146
147 **Flow cytometry**
148 To determine the effect of the PI3K p110δ inhibition on STAT6 and NFκB
149 phosphorylation levels, cells were harvested 24 hours after culture with IL-4
150 and anti-CD40 and fixed with 2% paraformaldehyde (PFA), washed with PBS
151 and permeabilised with 1 ml of ice cold methanol for 20 mins on ice. The
152 permeabilised cells were then intracellularly stained for 1 hour at room
153 temperature (RT) with anti-STAT6^{Tyr641} Alexa Fluor 647 (A15137E;
154 Biolegend) and anti-NFκB p65^{Ser536} Alexa Fluor 488 (93H1; Cell Signalling
155 Technology). To determine the effect of the PI3K p110δ inhibition on CSR to
156 IgE, cells were harvested on day 10 of culture and stained with a live/dead
157 fixable stain dye (Life Technologies Ltd) followed by fixation with 2%
158 paraformaldehyde and permeabilization with PBS containing 0.5% Triton X-
159 100, and 0.5% saponin. The cells were then stained with anti-human IgE FITC
160 (IgE21; eBioscience) and anti-IgG1-PE human (IS11-3B2.2.3; Miltenyi Biotec)
161 for 30 minutes at room temperature in the dark. Following the staining cells
162 were washed and resuspended in FACS buffer and acquired with BD FACS
163 Canto.
164

165 **Proliferation**

166 Following the CFSE labelling, performed using CellTrace™ CFSE Cell
167 proliferation kit (C34554, Molecular Probes™ Invitrogen), the cells were
168 cultured as above, with IL-4 and anti-CD40 antibody, in the presence or
169 absence of 2μM IC87114. After 10 days of culture, cells were harvested and
170 acquired using FACSCanto. The data was analysed using FlowJo and the
171 proliferative capacity of cultured tonsil B cells was determined based on the
172 dilution of the CFSE labelling.

173

174 **ELISA**

175 Maxisorp plates (Nunc) were coated with polyclonal mouse anti-human IgE
176 (Dako Agilent Technologies) or mouse anti-human IgG1 (BD Pharmingen) in
177 pH 9.8 carbonate buffer overnight at 4°C. Unbound sites were blocked with
178 2.5% BSA (Sigma-Aldrich) for 2 hours at RT and then washed 4 times with
179 PBS+0.05% Tween-20. Supernatants from day 10 cultures were then added at
180 appropriate dilutions and plates incubated overnight at 4°C. Human serum IgE
181 (NIBSC) or IgG1 (Sigma-Aldrich) were used to construct standard curves.
182 Binding was detected by polyclonal goat anti-human IgE-HRP (Sigma-Aldrich)
183 or mouse anti-human IgG HRP (BD Pharmingen) in 1% PBS/BSA for 2 h at
184 37°C. The color reaction was developed using the 1- StepTM Ultra TMB-ELISA
185 substrate solution (Thermo Scientific) and plates were read at 450nm using the
186 Labtech LT 4500 microplate reader. IgE and IgG1 concentrations were then
187 calculated from the standard curve using Prism 9.2 software (GraphPad).

188

189

190 ***qRT-PCR***

191 Total RNA was isolated from cells using the Qiagen RNeasy Plus Mini Kit
192 (Qiagen) and the RNA concentrations were measured using the NanoDrop
193 2000 (Thermo Scientific). The isolated RNA was then reverse transcribed into
194 cDNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR
195 (Invitrogen). To evaluate the expression levels of certain genes we used
196 TaqMan MGB gene expression assays and TaqMan Universal PCR Master Mix
197 on Viia7 RT-PCR machine (Applied Biosystems). Gene expression was
198 standardised to an endogenous reference gene 18s rRNA (Hs99999901_s1,
199 Applied Biosystems). AID (Hs00757808_m1) and BCL6 (Hs00153368_m1)
200 gene specific qPCR assays (Applied Biosystems) were used with Taqman
201 MGB chemistry. εGLT and γ1GLT were analysed using previously published
202 Taqman primers and probes [22]. The sequences of forward and reverse primers
203 for εGLT (fw: 5′-CTGTCCAGGAACCCGACAGA-3′; rev: 5′-TGCA
204 GCAGCGGGTCAAG-3′) and γ1GLT (fw: 5'-CCAGGGCAGGGTCAGCA-3';
205 rev: 5'-GGTGCTCTTGGA GGAGGGT-3') (Sigma-Aldrich) and their
206 corresponding FAM-labelled MGB probes (εGLT: 5′-AGGCACCAAATG-3′) and
207 γ1GLT (γ1GLT: 5 ′-CTCAGCCAGGACCAAG-3′) (Applied Biosystems) were
208 designed in house. All gene specific assays were multiplexed with the 18s
209 endogenous control assay and run-in triplicate. SDS software was used to
210 quantify the target cDNA relative to 18s according to the $2^{(-\Delta ct)}$ method.

211

212

213 **STATISTICAL ANALYSIS**

214 Statistical analysis was performed using the One-Way ANOVA, with Tuky's
215 correction, or unless otherwise stated. A p value of < 0.05 was considered
216 significant (*$p < 0.05$,**$p < 0.01$,***$p < 0.001$).

217

218 **RESULTS**

219

220 **IC87114 inhibits CSR of human tonsil B cells to IgE *in vitro***

221 Studies in mice have shown that CSR to IgE can be enhanced both *in vivo* and
222 *in vitro* when the activity of PI3K p110δ is inhibited[15–17]. This effect of p110δ
223 inhibition is not exclusive to CSR to IgE, with CSR to IgG1 also being enhanced
224 in mouse B cells[15,17,23]. To investigate whether CSR to IgE and IgG1 in human
225 B cells is also affected by the inhibition of the p110δ signaling, as in the mouse
226 system, the isolated untouched tonsil ($CD43^-$) B cells were cultured with IL-4
227 and anti-CD40 and different concentrations of the IC87114. After 10 days of
228 culture, we observed an IC87114 dose dependent reduction in the frequency
229 of IgE^+ cells (Figure 1A, B). While the number of IgE^+ cells in cultures treated
230 with 0.125μM and 0.25μM of IC87114 were not significantly different to the IL-
231 4 and anti-CD40 cultures only, cultures treated with 0.5μM, 1μM and 2μM of
232 IC87114 had a significant reduction in the number of IgE^+ cells (Figure 1A, B).
233 When quantifying the amounts of secreted IgE antibody in culture supernatants
234 we observed that the effects of IC87114 on secreted IgE were much more
235 drastic (Figure 1D). Indeed, cultures treated with 1μM and 2μM of IC87114 had
236 almost 80% of their secreted IgE reduced compared to the IL-4 and anti-CD40
237 cultures only. Furthermore, we can also observe a significant reduction of the

238 secreted IgE in cultures treated with 0.25μM of IC87114. Surprisingly, IC87114
239 had a negligible effect on the number of IgG1$^+$ cells, and we could only observe
240 a significant IgG1$^+$ cell reduction in cultures treated with 2μM of IC87114
241 (Figure 1A, C). Similar effects of IC87114 on CSR were also observed in naïve
242 B cell cultures stimulated with IL-4 and anti-CD40 (Figure 1F, G). These
243 observations are in striking contrast to those reported by the mouse studies and
244 suggests that PI3K p110δ regulation of CSR to IgE and IgG1 differs between
245 the mouse and the human system.
246

247 **PI3K p110δ inhibition does not affect the class switch signals**
248 **downstream of the IL-4R and CD40**

249 To identify the mechanism by which PI3K p110δ regulates CSR to IgE and IgG1
250 in our cultured tonsil B cells, we first examined the expression levels of
251 phosphorylated and thus activated, STAT6 and NF-κB. As it can be seen in
252 Figure 2A, 24 hours post culture with IL-4 and anti-CD40, the phosphorylation
253 levels of STAT6 and NF-κB were unaffected by the inhibition of p110δ PI3K with
254 2μM IC87114. However, previous mouse studies have reported that IC87114
255 regulates CSR to IgE and IgG1 by upregulating the expression of AID, εGLT
256 and γ1GLT. Therefore, to confirm that the class switch signals downstream of
257 the IL-4R and CD40 in human B cells are unaffected by the p110δ PI3K
258 inhibition we also evaluated the expression levels of AID, εGLT and γ1GLT on
259 day 5 of the cell culture. The RT-PCR data showed that the treatment of the
260 cell culture with 2μM IC87114 has no significant effect in the expression levels
261 of AID, εGLT and γ1GLT (Figure 2B). This is consistent with the unchanged
262 STAT6 and NFκB phosphorylation levels and suggests that unlike in the mouse

263 system, the effect of PI3K p110δ inhibition on CSR of human B cells to IgE and
264 IgG1 occurs independently of class switch signals downstream of the IL-4R and
265 CD40.
266
267 **PI3K p110δ inhibition does not affect the expression levels of BCL6 in**
268 **human B cells.**
269 Previously, it was reported that PI3K p110δ regulates CSR of mouse B cells
270 to IgE in a B cell intrinsic manner by modulating BCL6 expression[16]. BCL6 is
271 a transcription factor that is important for GC reaction but also is known to
272 compete with STAT6 at the εGLT binding site leading to the repression of its
273 transcription and that of CSR to IgE[10,21]. Therefore, in mice the reduction of
274 the BCL6 by the inhibition of p110δ can lead to enhanced CSR to IgE[16].
275
276 To test if this is also true in the human B cells, we evaluated the expression
277 levels of BCL6 by RT-PCR. As can be seen from Figure 3, we did not observe
278 any changes in the BCL6 expression levels between cell cultures that were
279 untreated and those treated with 2μM IC87114.

280 Overall, this data, and that from above, suggests that there are fundamental
281 differences in the mechanisms through which PI3K p110δ signalling regulates
282 CSR to IgE in human and mouse B cells.
283
284
285

286 **Inhibition of the PI3K p110δ activity reduces the proliferative capacity of**
287 **human B cells stimulated to undergo CSR to IgE.**

288 Cell division plays an important role in CSR[24,25]. For CSR to IgE to happen B
289 cells are required to divide a minimum of 5 times, whereas CSR to IgG1
290 requires only 2 cell divisions[24,25]. Mouse studies investigating the role of p110δ
291 PI3K in IgE production did not report any significant effect of the p110δ PI3K
292 inhibition on the IL-4 and anti-CD40 induced B cell proliferation[15,17,23]. To
293 determine if the p110δ PI3K inhibition reduces the proliferative capacity of the
294 cultured tonsil B cells, thus, reducing the CSR of human B cells to IgE, we first
295 labelled the isolated tonsil B cells with CFSE and analyzed cell division after 10
296 days of culture. The representative FACS histograms and cumulative data
297 (Figure 4A, B) show that the number of proliferating B cells in cell cultures
298 treated with 2μM IC87114 was reduced by half. Considering that the minimum
299 number (5) of cell divisions required for CSR to IgE to occur, we also
300 investigated the number of cell divisions by measuring the halving of the CFSE
301 intensity. We found that the number of B cells undergoing 4 cell divisions, or
302 more is significantly reduced when cell cultures were treated with 2μM IC87114
303 (Figure 4C, D). In contrast, the number of B cells undergoing 2 and 3 divisions,
304 which are decisive for CSR to IgG1 (22) was slightly, but not significantly,
305 reduced.
306
307 The data suggests that the modulation of the B cell proliferation is the main
308 mechanism through which PI3K p110δ signalling regulates CSR to IgE in
309 human B cells stimulated with IL-4 and anti-CD40.

310 **Discussion**

311 Mast cells lacking the p110δ activity have a considerable reduction in IgE-FcεRI
312 mediated degranulation and release of proinflammatory mediators[26,27].
313 Inhibition of the p110δ signalling in mouse models of asthma is also associated
314 with reduced Th2 cytokines as well as reduced lung cellular influx, including
315 tissue esonophilia, airway mucus production and airway hyper-
316 responsiveness[28–30]. These studies confirm PI3K p110δ as a potential target for
317 the treatment of allergic disease. Surprisingly, however, other mouse studies
318 have reported that PI3K p110δ signaling negatively regulates CSR to IgE, thus
319 its inhibition enhances IgE production[15–17]. In this study, we investigated the
320 effect of PI3K p110δ inhibition in cultures of human tonsil B cells stimulated to
321 undergo CSR to IgE. We find that the inhibition of the PI3K p110δ signaling in
322 these human B cell cultures reduces IgE production. This contradiction
323 between the human and mouse responses to PI3K p110δ inhibition highlights
324 the differences in their B cell regulatory systems of IgE production.

325 PI3K p110δ is activated downstream of various receptors on B cells and plays
326 critical roles in the development and function of B cells[18,31–33]. Several studies
327 have also implicated PI3K signalling in the regulation of CSR[15,17,23,34]. The first
328 line of evidence came from mouse B cells lacking PTEN, a phosphatase that
329 negatively regulates PI3K activity[34]. These PTEN-deficient mouse B cells,
330 which have enhanced PI3K signalling, fail to undergo CSR both *in vivo* and *in*
331 *vitro*. IC87114 reverses the effect of PTEN-deficiency on CSR, suggesting that
332 CSR is predominantly regulated by the PI3K p110δ isoform. Furthermore,
333 inhibition of PI3K p110δ was able to also enhance CSR in wild type mouse B

334 cells, suggesting that under normal circumstances PI3K p110δ negatively
335 regulates CSR. In the case of mouse IgE and IgG1, PI3K p110δ negatively
336 regulates their IL4 and anti-CD40 induced CSR *in vitro* by downregulating the
337 levels of AID as well as that of εGLT and γ1GLT, respectively[15,17]. Interestingly,
338 our data from the human B cell cultures were inconsistent with these
339 observations made in the mouse systems. IC87114 treatment leads to a
340 significant reduction in the number of IgE^+ cells in the IL-4 and anti-CD40
341 stimulated tonsil B cell cultures. The effect of IC87114 on $IgG1^+$ cells was not
342 as marked, and a significant reduction was observed only with the highest
343 concentration of 2μM IC87114. It has also been reported that upon
344 immunisation with T dependent antigens, both total and antigen specific IgE
345 titters are dramatically reduced in mice treated with IC87114 and those with B
346 cell deficient PI3K p110δ ($CD19^{Cre}p110\delta^{flox/flox}$)[15,35]. In contrast, the titers of
347 IgG1 and IgM are unaffected, suggesting that the PI3K p110δ selectively
348 regulates IgE production *in vivo.* Further investigations indicated that PI3K
349 p110*δ* regulates CSR to IgE in a B cell-intrinsic manner by modulating the
350 expression levels of BCL6 [16]. However, we did not observe an effect of IC87114
351 on the class switch signals downstream of IL4R and CD40 and the levels of
352 BCL6 expression were unchanged, suggesting that the mechanisms through
353 which PI3K signalling regulates CSR to IgE differs between the human and the
354 mouse systems.
355
356 PI3K p110*δ* signalling plays a key role in B cell proliferation as it regulates the
357 expression levels of different components of the cell cycle machinery[26,32,33]. Our
358 findings show that regulation of the B cell proliferative capacity is the key

359 mechanism through which PI3K p110δ signalling regulates CSR to IgE in our
360 IL4 and anti-CD40 stimulated B cell cultures. Indeed, IC87114 significantly
361 reduces the frequency of cells that have undergone 4 or more cell divisions.
362 This contradicts the previous observations made in mouse, which showed that
363 the proliferation of mouse B cells, stimulated with IL-4 and anti-CD40, is
364 unaffected by the PI3K p110δ inhibition[3,15,17,23]. Furthermore, the effect of the
365 IC87114 on the number of B cells undergoing 4 or more cell divisions may
366 explain why we see a more pronounced effect on CSR to IgE, which requires a
367 minimum of 5 divisions, compared to CSR to IgG1, which only requires two cell
368 divisions.
369 An important feature of both mouse and human IgE+ B cells is their tendency
370 to differentiate into the PC lineage[6,8,9,36]. Recent studies in mice revealed that
371 this process is promoted by the mIgE signalling independently of antigen and
372 involves the PI3K signalling pathway[4,5,37]. The drastic effects of IC87114 on the
373 amounts of secreted IgE suggests that in addition to inhibiting the CSR to
374 human IgE, the loss of PI3K p110δ signalling might also inhibit the generation
375 of IgE producing PCs. Our future studies will aim to elucidate the role of PI3K
376 p110δ signalling in the differentiation of human IgE+ B cells.

377 Overall, our data have demonstrated that the regulation of IgE production by
378 PI3K p110δ differs between the mouse and the human systems. In human B
379 cell cultures, the PI3K p110δ regulates IgE production by modulating the
380 proliferative capacity of the IL-4 and anti-CD40 stimulated B cells. In addition,
381 these data highlights the therapeutic potential of small molecule inhibitors of
382 PI3K p110δ in the treatment of allergic disease.

383

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534 **Figures**

535 **Figure 1**. PI3K p110δ inhibition reduces class switching of human B cells to
536 IgE.

537 (A) IL4 and anti-CD40 stimulated cells were harvested from the day 10 cultures
538 containing different concentrations of IC87114 and intracellularly stained for IgE
539 and IgG1. The percentages of total IgE$^+$ cells (**B**) and IgG1$^+$ cells (**C**) relative to
540 the IL-4 and anti-CD40 only cultures. Secreted IgE (**D**) and IgG1(**E**) analyzed
541 by ELISA. Data represent the mean +/- standard error of mean (SEM) and
542 show the amounts of secreted IgE and IgG1 [ng/mL] made relative to the IL-4
543 and anti-CD40 only cultures. The percentages of total IgE$^+$ cells (**F**) and IgG1$^+$
544 cells (**G**) from naïve B cell cultures made relative to the IL-4 and anti-CD40 only
545 cultures.

546

547 **Figure 2.** IC87114 has no effect on the class switch signalling downstream of
548 IL-4R and CD40.

549 (**A**) After 24h of IL-4 and anti-CD40 stimulation, B cells were fixed,
550 permeabilized and subsequently stained with anti-STAT6 phospho (Tyr641)
551 and anti-NFκB p65 (Ser536). The data show the fold change in median
552 fluorescence intensity (MFI) relative to IL-4 and anti-CD40 only cultures. **(B)**
553 The expressions of AID, εGLT and γ1GLT were quantified with qRT-PCR from
554 RNA isolated from day 5 of the tonsillar B cell culture. Gene expression was
555 normalised with 18s rRNA.

556 **Figure 3**. PI3K p110δ inhibition does not affect the expression levels of BCL6
557 in IL-4 and anti-CD40 stimulated human B cells.

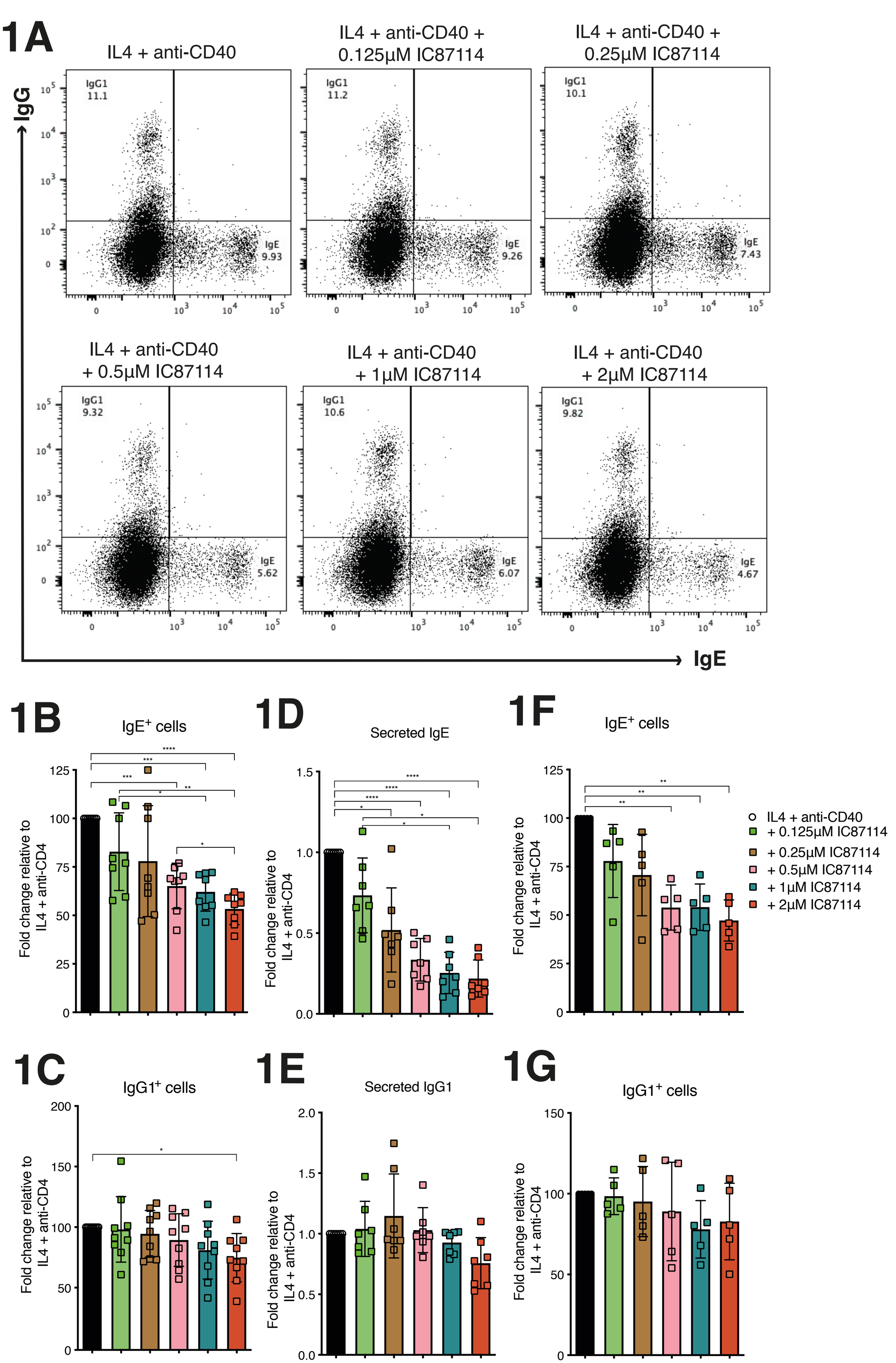
558 The RNA was isolated from cell pellets collected on day 5 of the B cell culture
559 and used to generate cDNA, which was used to quantify the expression levels
560 of BCL6 by RT-PCR. Values on the graphs are expressed in relation to
561 endogenous control, 18s x 10,000. Data represent means and SEM from 7
562 independent experiments. NOTE: I used RNA from 3 other experiments by Dr.
563 Ramadani that used the same culture conditions.

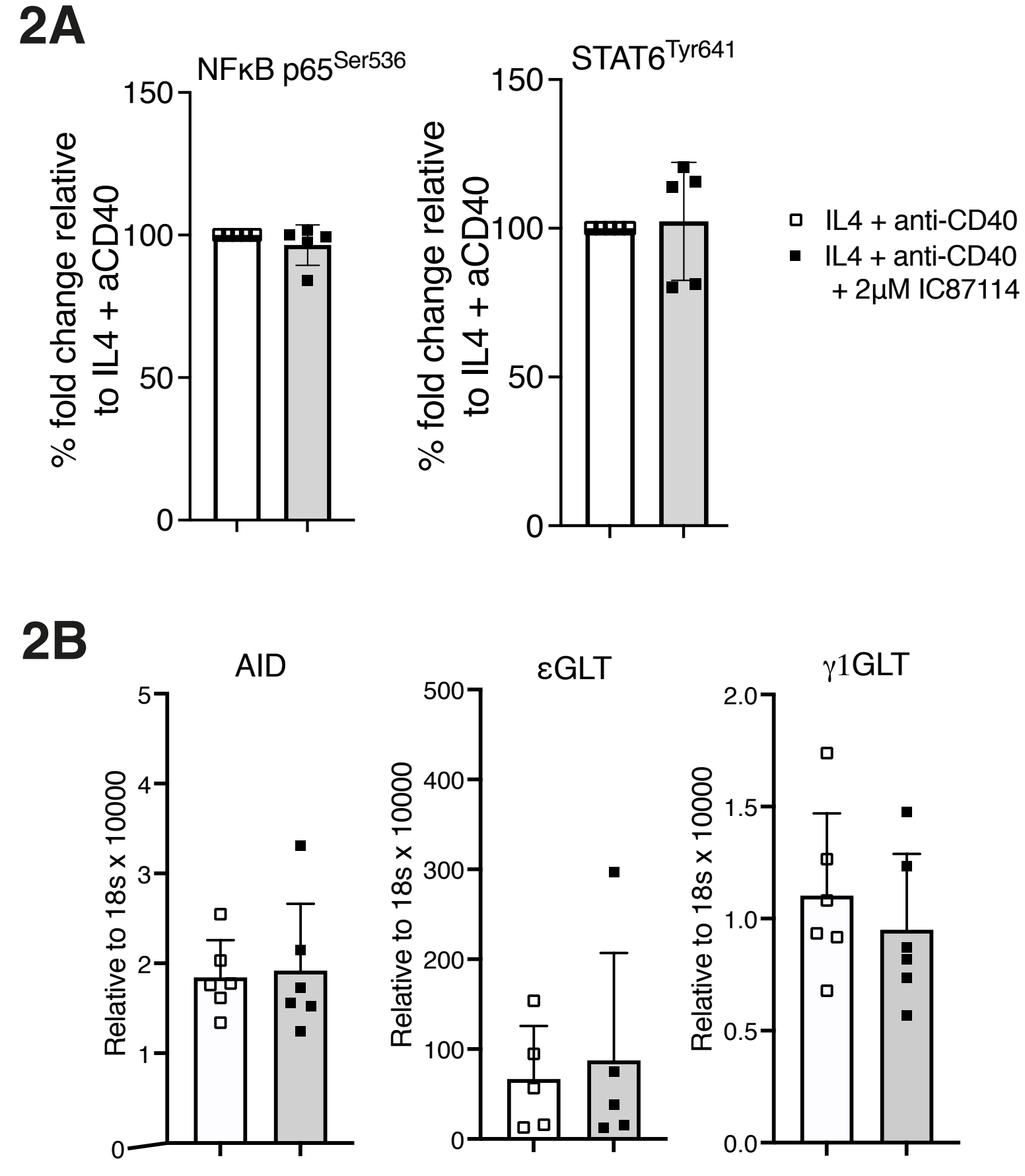
564

565 **Figure 4**. IC87114 inhibits the proliferative capacity of the IL-4 and anti-CD40
566 stimulated B cells.

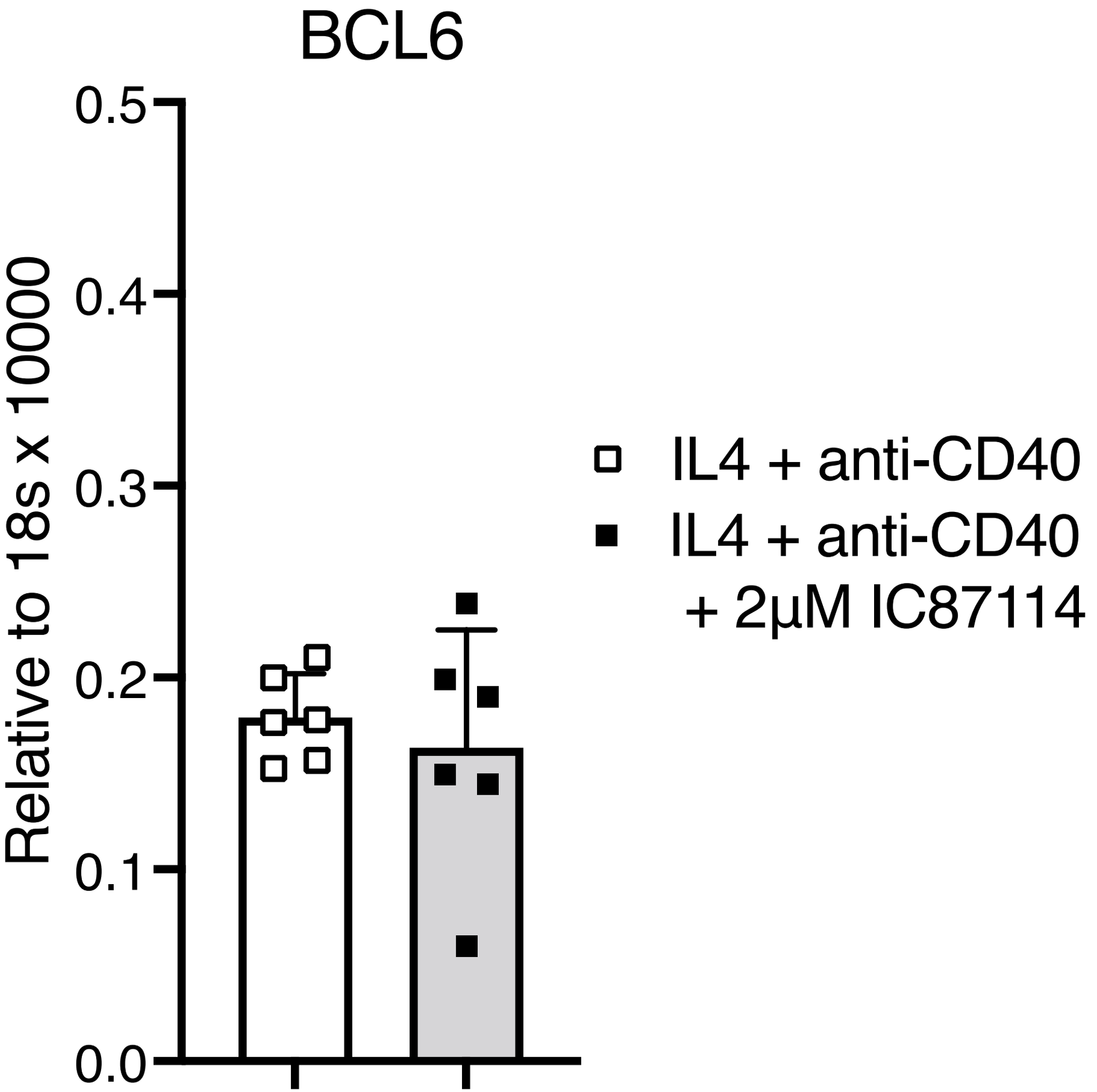
567 Isolated tonsil B cells were stained with CFSE and cultured for 10 days with IL-
568 4 and anti-CD40 alone or with 2μM IC87114. **(A)** The FACS histograms show
569 the percentage of proliferating B cells after 10 days of culture, whereas **(B)**
570 shows the fold change in proliferation relative to the IL-4 and anti-CD40 only
571 culture. The statistical comparisons were performed using the paired t-test. ****
572 $P < 0.0001$ (n= 5). (**C**) The FACS plots show the number of divisions and the
573 percentage of cells at each division that the IL-4 and anti-CD40 stimulated B
574 cells, with or without IC87114, were undergoing. Based on the CFSE dilution,
575 six numbers of cell divisions (D1-D6+) were identified and gated. (D) Graph
576 shows the frequency of B cells at divisions 1 to 6+ (D1 to D6+). The data
577 represent the mean and SEM

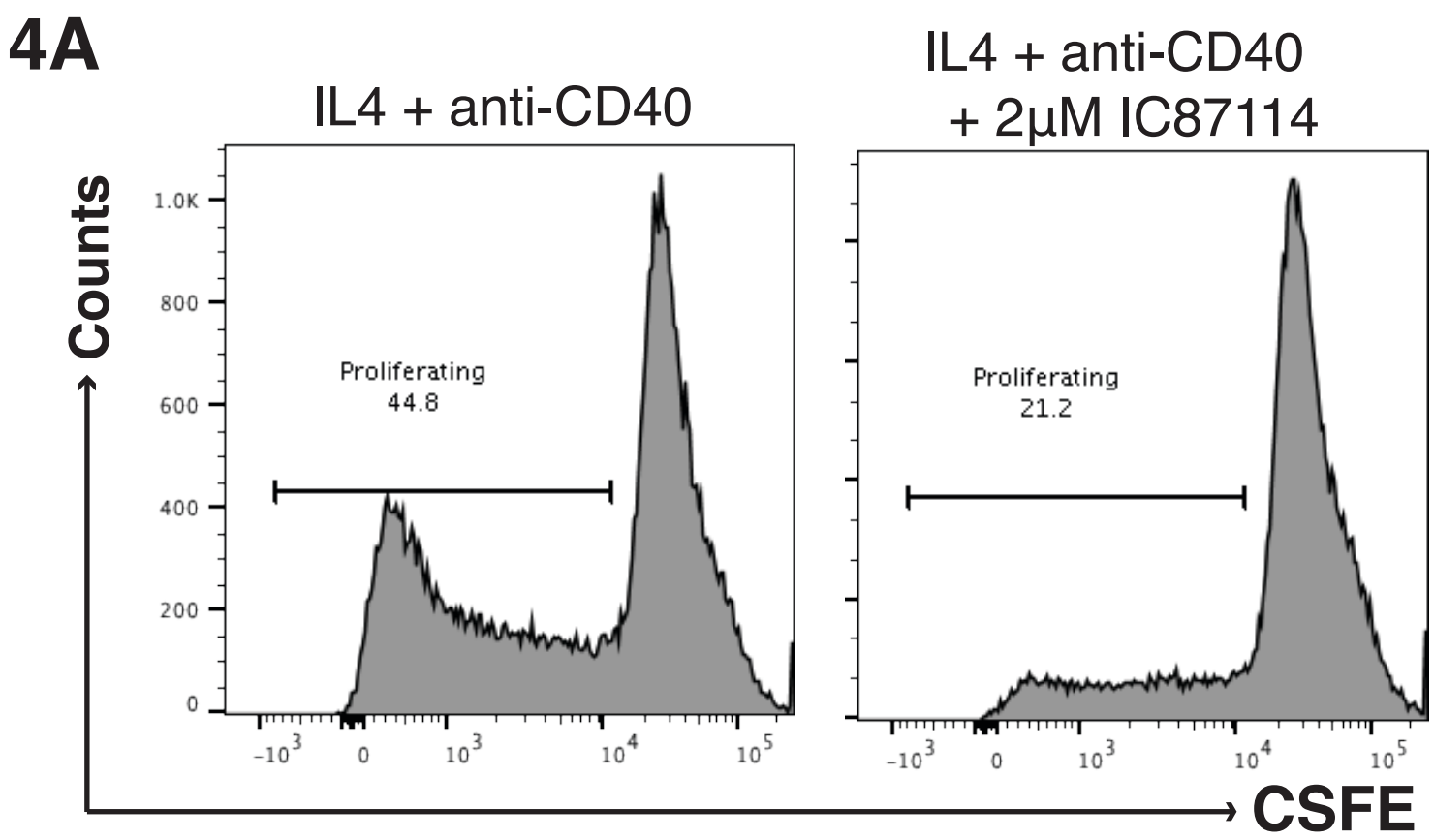
578

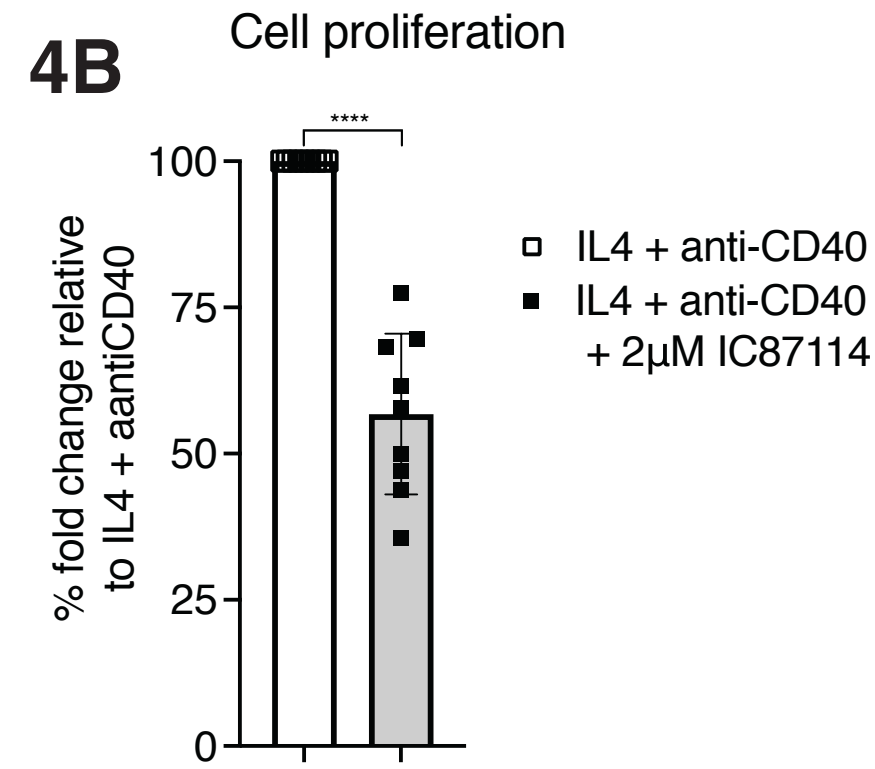

1A
IL4 + anti-CD40
IL4 + anti-CD40 + 0.125μM IC87114
IL4 + anti-CD40 + 0.25μM IC87114
IL4 + anti-CD40 + 0.5μM IC87114
IL4 + anti-CD40 + 1μM IC87114
IL4 + anti-CD40 + 2μM IC87114
IgG1 11.1
IgE 9.93
IgG1 11.2
IgE 9.26
IgG1 10.1
IgE 7.43
IgG1 9.32
IgE 5.62
IgG1 10.6
IgE 6.07
IgG1 9.82
IgE 4.67
IgG
IgE
1B
IgE+ cells
Fold change relative to IL4 + anti-CD4
1D
Secreted IgE
1F
IgE+ cells
IL4 + anti-CD40
+ 0.125μM IC87114
+ 0.25μM IC87114
+ 0.5μM IC87114
+ 1μM IC87114
+ 2μM IC87114
1C
IgG1+ cells
1E
Secreted IgG1
1G
IgG1+ cells

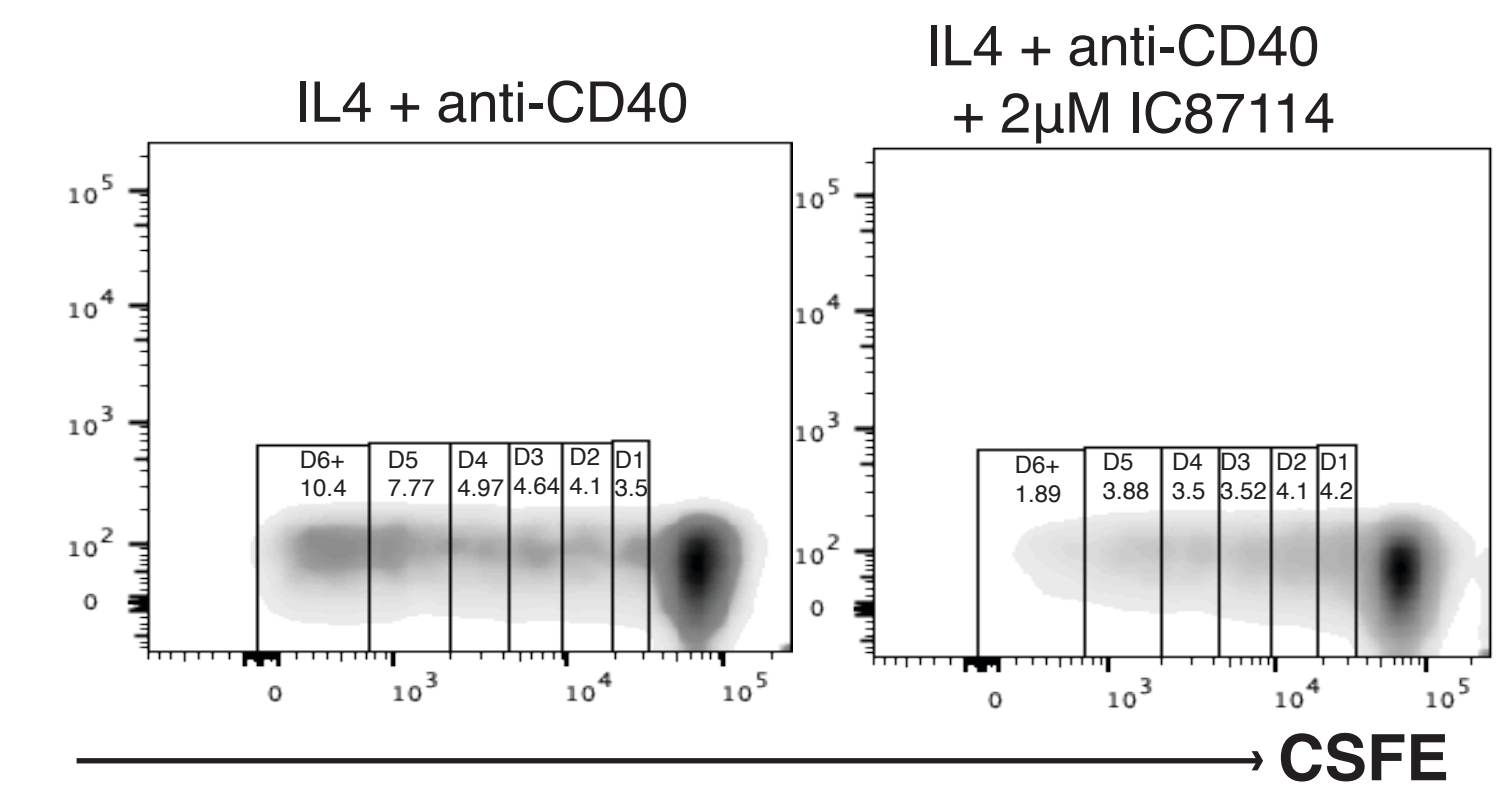

2A
NFκB p65Ser536
STAT6Tyr641
% fold change relative to IL4 + aCD40
150
100
50
0
IL4 + anti-CD40
IL4 + anti-CD40 + 2μM IC87114
2B
AID
εGLT
γ1GLT
Relative to 18s x 10000
5
4
3
2
1
0
500
400
300
200
100
2.0
1.5
1.0
0.5
0.0

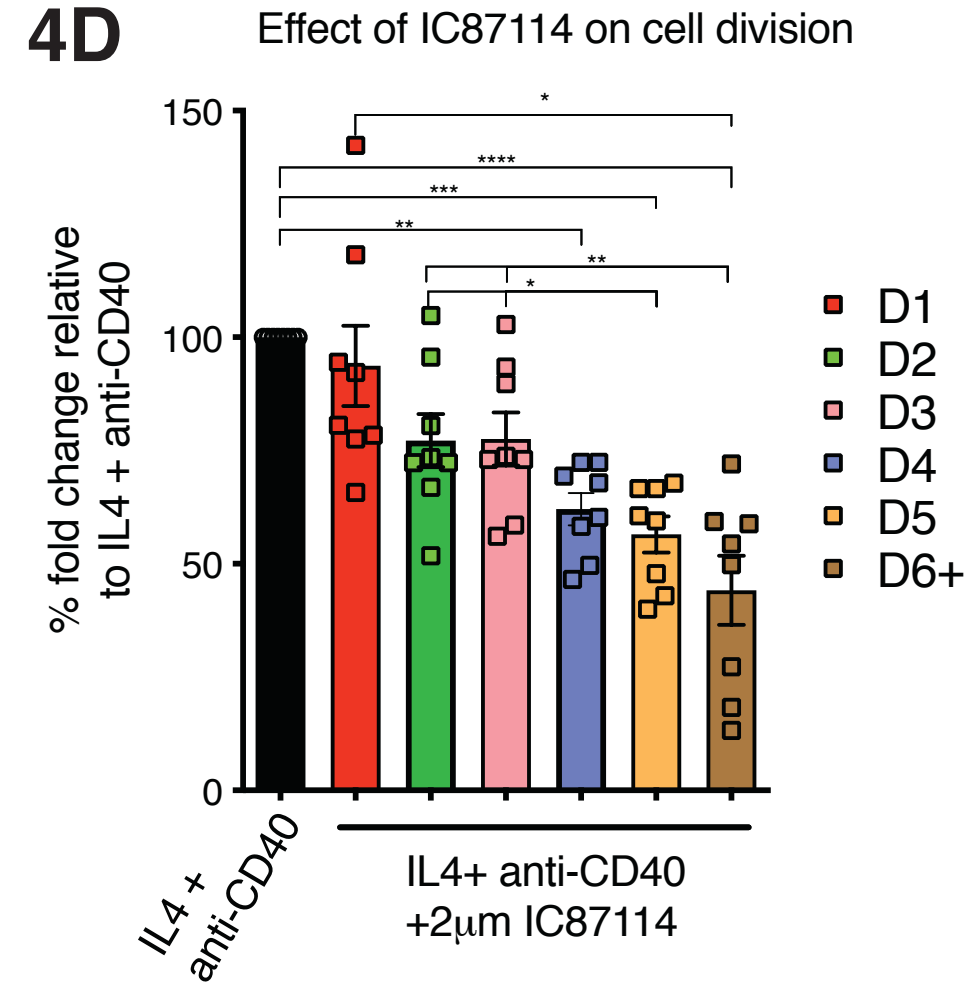
3


BCL6
Relative to 18s x 10000
0.5
0.4
0.3
0.2
0.1
0.0
IL4 + anti-CD40
IL4 + anti-CD40
+ 2μM IC87114


4A
IL4 + anti-CD40
IL4 + anti-CD40
+ 2µM IC87114
Counts
Proliferating
44.8
Proliferating
21.2
CSFE


4B
Cell proliferation
% fold change relative
to IL4 + aantiCD40
IL4 + anti-CD40
IL4 + anti-CD40
+ 2µM IC87114


4C
IL4 + anti-CD40
IL4 + anti-CD40
+ 2µM IC87114
D6+ 10.4
D5 7.77
D4 4.97
D3 4.64
D2 4.1
D1 3.5
D6+ 1.89
D5 3.88
D4 3.5
D3 3.52
D2 4.1
D1 4.2
CSFE


4D
Effect of IC87114 on cell division
% fold change relative
to IL4 + anti-CD40
D1
D2
D3
D4
D5
D6+
IL4 + anti-CD40
IL4+ anti-CD40
+2µm IC87114