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

Anna Cutrina-Pons[1*], Aloka De Sa[1*], David J Fear[2], Hannah J Gould[1] and Faruk Ramadani[1]

Author Affiliations: [1] Randall Centre of Cell & Molecular Biophysics, and [2] Division of Asthma, Allergy and Lung Biology, King's College London, United Kingdom. *The first two authors contributed equally to this article, and both should be considered first author.

Correspondence: Faruk Ramadani, Randall Centre of Cell and Molecular Biophysics, King's College London, London, SE1 1UL, UK; e-mail: faruk.ramadani@kcl.ac.uk or Phone: 44 (0)20 7848 6442; Fax: +44 (0)20 7848 6435.

Abstract

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. To investigate the effect of PI3K p110δ inhibition in the production of human IgE we isolated human B cells from tonsil tissue and stimulated them 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. 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. 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

Introduction

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

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

Several molecular mechanisms have evolved to ensure rigorous regulation of 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 $CD43^-$ 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, B cell cultures were treated with IC87114, a small molecular inhibitor of p110δ,–which was used to determine the role of PI3K p110δ signalling in IgE production by mouse B cells(15,17), and nemiralisib, a new generation of PI3K p110δ inhibitors (22). The data show that PI3K p110δ inhibition reduces 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(15–17,23).

Materials and methods

Ethics

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

Cell culture

The untouched resting B cells were isolated from the human tonsils by depletion of other cells using MojoSort Human B Cell (CD43-) Isolation Kit (BioLegend), whereas the naïve B cells were isolated using the EasySep Human Naïve B cell isolation kit (Stem Cell Technologies). The purity of the B cells used in these experiments was > 95%. Purified B cells were then cultured at 0.5 x10^6 cells/mL in RPMI 1640 (Lonza) containing penicillin (100 IU/ml), streptomycin (100µg/ml) and glutamine (2mM, Invitrogen) and 10% Foetal Calf Serum (Hyclone; Perbio Biosciences). To induce CSR to IgE, cultures were stimulated with IL-4 (200 IU/ml; R&D Europe Systems Ltd) and anti-CD40 antibody (0.5 µg/ml; G28.5; American Type Culture Collection). To investigate the role of PI3K p110δ signalling in human IgE production, cell cultures were also treated with different concentration of IC87114 (LKT Laboratories Inc), a small molecule inhibitor of PI3K p110δ signalling used in

the mouse studies. Considering that IC87114 has an IC_{50} of 0.5µM, it was used at concentrations below and above this IC_{50}; 0.125µM, 0.25µM, 0.5µM, 1µM, and 2µM. PI3K p110δ was also inhibited using nemiralisb (GSK2269557), a highly selective inhibitor of p110δ (pKi=9.9) (MedChemExpress LLC). The cells were then incubated for up to 10 days at 37°C with 5% CO_2.

Flow cytometry

To determine the effect of the PI3K p110δ inhibition on STAT6 and NFκB phosphorylation levels, cells were harvested 24 hours after culture with IL-4 and anti-CD40 and fixed with 2% paraformaldehyde (PFA), washed with PBS and permeabilised with 1 ml of ice cold methanol for 20 mins on ice. The permeabilised cells were then intracellularly stained for 1 hour at room temperature (RT) with anti-STAT6^{Tyr641} Alexa Fluor 647 (A15137E; Biolegend) and anti-NFκB p65^{Ser536} Alexa Fluor 488 (93H1; Cell Signalling Technology). To determine the effect of the PI3K p110δ inhibition on CSR to IgE, cells were harvested on day 10 of culture and stained with a live/dead fixable stain dye (Life Technologies Ltd) followed by fixation with 2% paraformaldehyde and permeabilization with PBS containing 0.5% Triton X-100, and 0.5% saponin. The cells were then stained with anti-human IgE FITC (IgE21; eBioscience) and anti-IgG1-PE human (IS11-3B2.2.3; Miltenyi Biotec) for 30 minutes at room temperature in the dark. Following the staining cells were washed and resuspended in FACS buffer and acquired with BD FACS Canto.

Proliferation

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

ELISA

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

qRT-PCR

Total RNA was isolated from cells using the Qiagen RNeasy Plus Mini Kit (Qiagen) and the RNA concentrations were measured using the NanoDrop 2000 (Thermo Scientific). The isolated RNA was then reverse transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). To evaluate the expression levels of certain genes we used TaqMan MGB gene expression assays and TaqMan Universal PCR Master Mix on Viia7 RT-PCR machine (Applied Biosystems). Gene expression was standardised to an endogenous reference gene 18s rRNA (Hs99999901_s1, Applied Biosystems). AID (Hs00757808_m1) and BCL6 (Hs00153368_m1) gene specific qPCR assays (Applied Biosystems) were used with Taqman MGB chemistry. εGLT and γ1GLT were analysed using previously published Taqman primers and probes (24). The sequences of forward and reverse primers for εGLT (fw: 5′-CTGTCCAGGAACCCGACAGA-3′; rev: 5′-TGCA GCAGCGGGTCAAG-3′) and γ1GLT (fw: 5’-CCAGGGCAGGGTCAGCA-3’; rev: 5’-GGTGCTCTTGGAGGAGGGT-3’) (Sigma-Aldrich) and their corresponding FAM-labelled MGB probes (εGLT: 5′-AGGCACCAAATG-3′ and γ1GLT: 5 ′-CTCAGCCAGGACCAAG-3′) (Applied Biosystems) were designed in house. All gene specific assays were multiplexed with the 18s endogenous control assay and run-in triplicate. SDS software was used to quantify the target cDNA relative to 18s according to the $2^{(-\Delta ct)}$ method.

STATISTICAL ANALYSIS

Statistical analysis was performed using the One-Way ANOVA, with Tuky's correction, or unless otherwise stated. A p value of < 0.05 was considered significant (*$p < 0.05$,**$p < 0.01$,***$p < 0.001$). Data shown represent mean +/- standard deviation (SD) or unless otherwise stated.

RESULTS

PI3K p110δ inhibition reduces CSR of human tonsil B cells to IgE *in vitro*

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

significant reduction of the secreted IgE in cultures treated with 0.25μM of IC87114. Surprisingly, IC87114 had a negligible effect on the number of $IgG1^+$ cells, and we could only observe a significant $IgG1^+$ cell reduction in cultures treated with 2μM of IC87114 (Figure 1A, C). Similar effects of IC87114 on CSR were also observed in naïve B cell cultures stimulated with IL-4 and anti-CD40 (Figure 1F, G). The effects of IC87114 on IgE production were also confirmed using nemiralisb, one of the most selective PI3K p110δ currently in development (22). As can be seen in Figure 2, the percentages of IgE^+ cells and the amounts of secreted IgE in cultures containing 1nM and 10nM nemiralisib are selectively reduced. These observations are in striking contrast to those reported by the mouse studies and suggests that PI3K p110δ regulation of CSR to IgE and IgG1 differs between the mouse and the human system.

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

To identify the mechanism by which PI3K p110δ regulates CSR to IgE and IgG1 in our cultured tonsil B cells, we first examined the expression levels of phosphorylated and thus activated, STAT6 and NFκB. As it can be seen in Figure 2A, 24 hours post culture with IL-4 and anti-CD40, the phosphorylation levels of STAT6 and NFκB were unaffected by the inhibition of p110δ PI3K with 2μM IC87114. However, previous mouse studies have reported that IC87114 regulates CSR to IgE and IgG1 by upregulating the expression of AID, εGLT and γ1GLT(15,17). Therefore, to confirm that the class switch signals downstream of the IL-4R and CD40 in human B cells are unaffected

by the p110δ PI3K inhibition we also evaluated the expression levels of AID, εGLT and γ1GLT on day 5 of the cell culture. The RT-PCR data showed that the treatment of the cell culture with 2μM IC87114 has no significant effect in the expression levels of AID, εGLT and γ1GLT (Figure 2B). This is consistent with the unchanged STAT6 and NFκB phosphorylation levels and suggests that unlike in the mouse system, the effect of PI3K p110δ inhibition on CSR of human B cells to IgE and IgG1 occurs independently of class switch signals downstream of the IL-4R and CD40**.**

PI3K p110δ inhibition does not affect the expression levels of BCL6 in human B cells.

Previously, it was reported that PI3K p110δ regulates CSR of mouse B cells to IgE in a B cell intrinsic manner by modulating BCL6 expression(16). BCL6 is a transcription factor that is important for GC reaction but also is known to compete with STAT6 at the εGLT binding site leading to the repression of its transcription and that of CSR to IgE(10,21). Therefore, in mice the reduction of the BCL6 by the inhibition of p110δ can lead to enhanced CSR to IgE(16).

To test if this is also true in the human B cells, we evaluated the expression levels of BCL6 by RT-PCR. As can be seen from Figure 3, we did not observe any changes in the BCL6 expression levels between cell cultures that were untreated and those treated with 2μM IC87114.

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

Inhibition of the PI3K p110*δ* activity reduces the proliferative capacity of human B cells stimulated to undergo CSR to IgE.

Cell division plays an important role in CSR(25,26). For CSR to IgE to happen B cells are required to divide a minimum of 5 times, whereas CSR to IgG1 requires only 2 cell divisions(25,26). Mouse studies investigating the role of p110δ PI3K in IgE production did not report any significant effect of the p110δ PI3K inhibition on the IL-4 and anti-CD40 induced B cell proliferation(15,17,23). To determine if the p110δ PI3K inhibition reduces the proliferative capacity of the cultured tonsil B cells, thus, reducing the CSR of human B cells to IgE, we first labelled the isolated tonsil B cells with CFSE and analyzed cell division after 10 days of culture. The representative FACS histograms and cumulative data (Figure 4A, B) show that the number of proliferating B cells in cell cultures treated with 2µM IC87114 was reduced by half. Considering that the minimum number (5) of cell divisions required for CSR to IgE to occur, we also investigated the number of cell divisions by measuring the halving of the CFSE intensity. We found that the number of B cells undergoing 4 cell divisions, or more is significantly reduced when cell cultures were treated with 2µM IC87114 (Figure 4C, D). In contrast, the number of B cells undergoing 2 and 3 divisions, which are decisive for CSR to IgG1 (22) was slightly, but not significantly, reduced.

The data suggests that the modulation of the B cell proliferation is the main mechanism through which PI3K p110δ signalling regulates CSR to IgE in human B cells stimulated with IL-4 and anti-CD40.

Discussion

Mast cells lacking the p110δ activity have a considerable reduction in IgE-FcεRI mediated degranulation and release of proinflammatory mediators(27,28). Inhibition of the p110δ signalling in mouse models of asthma is also associated with reduced Th2 cytokines as well as reduced lung cellular influx, including tissue esonophilia, airway mucus production and airway hyper-responsiveness(29–31). These studies confirm PI3K p110δ as a potential target for the treatment of allergic disease. Surprisingly, however, other mouse studies have reported that PI3K p110δ signaling negatively regulates CSR to IgE, thus its inhibition enhances IgE production(15–17). In this study, we investigated the effect of PI3K p110δ inhibition in cultures of human tonsil B cells stimulated to undergo CSR to IgE. We find that the inhibition of the PI3K p110δ signaling in these human B cell cultures reduces IgE production. This contradiction between the human and mouse responses to PI3K p110δ inhibition highlights the differences in their B cell regulatory systems of IgE production.

PI3K p110δ is activated downstream of various receptors on B cells and plays critical roles in the development and function of B cells(18,32–34). Several studies have also implicated PI3K signalling in the regulation of CSR(15,17,23,35). The first line of evidence came from mouse B cells lacking

329 PTEN, a phosphatase that negatively regulates PI3K activity(35). These
330 PTEN-deficient mouse B cells, which have enhanced PI3K signalling, fail to
331 undergo CSR both *in vivo* and *in vitro*. IC87114 reverses the effect of PTEN-
332 deficiency on CSR, suggesting that CSR is predominantly regulated by the
333 PI3K p110δ isoform. Furthermore, inhibition of PI3K p110δ was able to also
334 enhance CSR in wild type mouse B cells, suggesting that under normal
335 circumstances PI3K p110δ negatively regulates CSR. In the case of mouse
336 IgE and IgG1, PI3K p110δ negatively regulates their IL4 and anti-CD40
337 induced CSR *in vitro* by downregulating the levels of AID as well as that of
338 εGLT and γ1GLT, respectively(15,17). Interestingly, our data from the human
339 B cell cultures were inconsistent with these observations made in the mouse
340 systems. IC87114 treatment leads to a significant reduction in the number of
341 IgE^{+} cells in the IL-4 and anti-CD40 stimulated tonsil B cell cultures. The effect
342 of IC87114 on $IgG1^{+}$ cells was not as marked, and a significant reduction was
343 observed only with the highest concentration of 2μM IC87114. It has also
344 been reported that upon immunisation with T dependent antigens, both total
345 and antigen specific IgE titters are dramatically reduced in mice treated with
346 IC87114 and those with B cell deficient PI3K p110δ
347 ($CD19^{Cre}p110\delta^{flox/flox}$)(15,36). In contrast, the titers of IgG1 and IgM are
348 unaffected, suggesting that the PI3K p110δ selectively regulates IgE
349 production *in vivo.* Further investigations indicated that PI3K p110*δ* regulates
350 CSR to IgE in a B cell-intrinsic manner by modulating the expression levels of
351 BCL6 (16). However, we did not observe an effect of IC87114 on the class
352 switch signals downstream of IL4R and CD40 and the levels of BCL6
353 expression were unchanged, suggesting that the mechanisms through which

PI3K signalling regulates CSR to IgE differs between the human and the mouse systems.

PI3K p110δ signalling plays a key role in B cell proliferation as it regulates the expression levels of different components of the cell cycle machinery(27,33,34). Our findings show that regulation of the B cell proliferative capacity is the key mechanism through which PI3K p110δ signalling regulates CSR to IgE in our IL4 and anti-CD40 stimulated B cell cultures. Indeed, IC87114 significantly reduces the frequency of cells that have undergone 4 or more cell divisions. This contradicts the previous observations made in mouse, which showed that the proliferation of mouse B cells, stimulated with IL-4 and anti-CD40, is unaffected by the PI3K p110δ inhibition(3,15,17,23). Furthermore, the effect of the IC87114 on the number of B cells undergoing 4 or more cell divisions may explain why we see a more pronounced effect on CSR to IgE, which requires a minimum of 5 divisions, compared to CSR to IgG1, which only requires two cell divisions.

An important feature of both mouse and human IgE+ B cells is their tendency to differentiate into the PC lineage(6,8,9,37). Recent studies in mice revealed that this process is promoted by the mIgE signalling independently of antigen and involves the PI3K signalling pathway(4,5,38). The drastic effects of IC87114 on the amounts of secreted IgE suggests that in addition to inhibiting the CSR to human IgE, the loss of PI3K p110δ signalling might also inhibit the generation of IgE producing PCs. Our future studies will aim to elucidate the role of PI3K p110δ signalling in the differentiation of human IgE+ B cells.

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

Acknowledgments

We are grateful to the patients and the ENT surgical team at the Guy's & St Thomas' NHS Foundation Trust for their help and support in the collection of tonsils used in this research.

Funding

This study was supported by Asthma UK career development award (AUK-CDA-2019-412) to F.R. The work of H.G. was supported by the Medical Research Council (MR/M022943/1).

Author Contributions

A.C-P., A.d.S, and D.J.F performed experiments and analysed data, and critically reviewed the manuscript. H.J.G. provided guidance, interpreted data and critically reviewed the manuscript. F.R. designed and performed experiments, analysed data and wrote the paper. All authors reviewed the final manuscript.

Conflict-of-interest

The authors declare that they have no conflicts of interests.

References

1. Gould HJ, Sutton BJ. IgE in allergy and asthma today. *Nat Rev Immunol* (2008) 8:205–217. doi: 10.1038/nri2273
2. Dullaers M, de Bruyne R, Ramadani F, Gould HJ, Gevaert P, Lambrecht BN. The who, where, and when of IgE in allergic airway disease. *Journal of Allergy and Clinical Immunology* (2012) 129:635–645. doi: 10.1016/j.jaci.2011.10.029
3. Newman R, Tolar P. Chronic calcium signaling in IgE+ B cells limits plasma cell differentiation and survival. *Immunity* (2021) 54:2756-2771.e10. doi: 10.1016/j.immuni.2021.11.006
4. Yang Z, Robinson MJ, Chen X, Smith GA, Taunton J, Liu W, Allen CDC. Regulation of B cell fate by chronic activity of the IgE B cell receptor. *Elife* (2016) 5: doi: 10.7554/eLife.21238.001
5. Haniuda K, Fukao S, Kodama T, Hasegawa H, Kitamura D. Autonomous membrane IgE signaling prevents IgE-memory formation. *Nat Immunol* (2016) 17:1109–1117. doi: 10.1038/ni.3508
6. He JS, Meyer-Hermann M, Xiangying D, Zuan LY, Jones LA, Ramakrishna L, Vries VCD, Dolpady J, Aina H, Joseph S, et al. The distinctive germinal center phase of IgE+ B lymphocytes limits their contribution to the classical memory response. *Journal of Experimental Medicine* (2013) 210:2755–2771. doi: 10.1084/jem.20131539
7. Gould HJ, Ramadani F. IgE responses in mouse and man and the persistence of IgE memory. *Trends Immunol* (2015) 36:40–48. doi: 10.1016/j.it.2014.11.002
8. Ramadani F, Bowen H, Upton N, Hobson PS, Chan YC, Chen JB, Chang TW, McDonnell JM, Sutton BJ, Fear DJ, et al. Ontogeny of human IgE-expressing B cells and plasma cells. *Allergy* (2017) 72:66–76. doi: 10.1111/ALL.12911
9. Yang Z, Sullivan BM, Allen CDC. Fluorescent In Vivo Detection Reveals that IgE+ B Cells Are Restrained by an Intrinsic Cell Fate Predisposition. *Immunity* (2012) 36:857–872. doi: 10.1016/j.immuni.2012.02.009
10. Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. *Nat Rev Immunol* (2003) 3:721–732. doi: 10.1038/nri1181
11. Delphin S, Stavnezer J. Characterization of an Interleukln 4 (IL-4) Responsive Region in the Immunoglobulln Heavy Chain Germ!ine E Promoter: Regulation

by NF-IL-4, a C/EBP Family Member and NF-KB/p50. http://rupress.org/jem/article-pdf/181/1/181/1106000/181.pdf

12. Takeda H, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura SI, Nakanishi K, Yoshida N, Kishimoto T, Akira S. Essential role of Stat6 in IL-4 signalling. *Nature 1996 380:6575* (1996) 380:627–630. doi: 10.1038/380627a0

13. Dedeoglu F, Horwitz B, Chaudhuri J, Alt FW, Geha RS. Induction of activation-induced cytidine deaminase gene expression by IL-4 and CD40 ligation is dependent on STAT6 and NFκB. *Int Immunol* (2004) 16:395–404. doi: 10.1093/intimm/dxh042

14. Hellman L. Regulation of IgE homeostasis, and the identification of potential targets for therapeutic intervention. *Biomedicine and Pharmacotherapy* (2007) 61:34–49. doi: 10.1016/j.biopha.2006.10.001

15. Zhang T ting, Okkenhaug K, Nashed BF, Puri KD, Knight ZA, Shokat KM, Vanhaesebroeck B, Marshall AJ. Genetic or pharmaceutical blockade of p110δ phosphoinositide 3-kinase enhances IgE production. *Journal of Allergy and Clinical Immunology* (2008) 122: doi: 10.1016/j.jaci.2008.08.008

16. Zhang T, Makondo KJ, Marshall AJ. p110δ Phosphoinositide 3-Kinase Represses IgE Switch by Potentiating BCL6 Expression. *The Journal of Immunology* (2012) 188:3700–3708. doi: 10.4049/jimmunol.1103302

17. Doi T, Obayashi K, Kadowaki T, Fujii H, Koyasu S. PI3K is a negative regulator of IgE production. *Int Immunol* (2008) 20:499–508. doi: 10.1093/intimm/dxn009

18. Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nat Rev Immunol* (2003) 3:317–330. doi: 10.1038/nri1056

19. Soond DR, Bjørgo E, Moltu K, Dale VQ, Patton DT, Torgersen KM, Galleway F, Twomey B, Clark J, Gaston JSH, et al. PI3K p110delta regulates T-cell cytokine production during primary and secondary immune responses in mice and humans. *Blood* (2010) 115:2203–2213. doi: 10.1182/BLOOD-2009-07-232330

20. Nashed BF, Zhang T, Al-Alwan M, Srinivasan G, Halayko AJ, Okkenhaug K, Vanhaesebroeck B, HayGlass KT, Marshal AJ. Role of the phosphoinositide 3-kinase p110delta in generation of type 2 cytokine responses and allergic airway inflammation. *Eur J Immunol* (2007) 37:416–424. doi: 10.1002/EJI.200636401

21. Harris MB, Chang C-C, Berton MT, Danial NN, Zhang J, Kuehner D, Ye BH, Kvatyuk M, Pandolfi PP, Cattoretti G, et al. Transcriptional Repression of Stat6-Dependent Interleukin-4-Induced Genes by BCL-6: Specific Regulation of Iε Transcription and Immunoglobulin E Switching. (1999). 7264–7275 p.

22. Vanhaesebroeck B, Perry MWD, Brown JR, André F, Okkenhaug K. PI3K inhibitors are finally coming of age. *Nat Rev Drug Discov* (2021) 20:741–769. doi: 10.1038/s41573-021-00209-1

23. Chiu H, Mallya S, Nguyen P, Mai A, Jackson L v., Winkler DG, DiNitto JP, Brophy EE, McGovern K, Kutok JL, et al. The Selective phosphoinoside-3-kinase p110δ inhibitor IPI-3063 potently suppresses b cell survival, proliferation, and differentiation. *Front Immunol* (2017) 8: doi: 10.3389/fimmu.2017.00747

24. Ramadani F, Upton N, Hobson P, Chan YC, Mzinza D, Bowen H, Kerridge C, Sutton BJ, Fear DJ, Gould HJ. Intrinsic properties of germinal center-derived B cells promote their enhanced class switching to IgE. *Allergy: European Journal of Allergy and Clinical Immunology* (2015) 70:1269–1277. doi: 10.1111/all.12679

25. McCall MN, Hodgkin PD. Switch recombination and germ-line transcription are division-regulated events in B lymphocytes. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* (1999) 1447:43–50. doi: 10.1016/S0167-4781(99)00131-1

26. Hasbold J, Lyons AB, Kehry MR, Hodgkin PD. Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4. *Eur J Immunol* (1998) 28:1040–1051. doi: 10.1002/(SICI)1521-4141(199803)28:03<1040::AID-IMMU1040>3.0.CO;2-9

27. Ali K, Bilancio A, Thomas M, Pearce W, Gilfillan AM, Tkaczyk C, Kuehn N, Gray A, Giddings J, Peskett E, et al. Essential role for the p110delta phosphoinositide 3-kinase in the allergic response. *Nature* (2004) 431:1007–1011. doi: 10.1038/NATURE02991

28. Ali K, Camps M, Pearce WP, Ji H, Rückle T, Kuehn N, Pasquali C, Chabert C, Rommel C, Vanhaesebroeck B. Isoform-specific functions of phosphoinositide 3-kinases: p110 delta but not p110 gamma promotes optimal allergic responses in vivo. *J Immunol* (2008) 180:2538–2544. doi: 10.4049/JIMMUNOL.180.4.2538

29. Kim JS, Jeong JS, Kwon SH, Kim SR, Lee YC. Roles of PI3K pan-inhibitors and PI3K-δ inhibitors in allergic lung inflammation: a systematic review and meta-analysis. *Sci Rep* (2020) 10: doi: 10.1038/s41598-020-64594-0

30. Park SJ, Min KH, Lee YC. Phosphoinositide 3-kinase δ inhibitor as a novel therapeutic agent in asthma. *Respirology* (2008) 13:764–771. doi: 10.1111/j.1440-1843.2008.01369.x

31. Lee KS, Lee HK, Hayflick JS, Lee YC, Puri KD. Inhibition of phosphoinositide 3□kinase δ attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *The FASEB Journal* (2006) 20:455–465. doi: 10.1096/fj.05-5045com

32. Turner M, Hodson DJ. The role of PI3K signalling in the b cell response to antigen. *Advances in Experimental Medicine and Biology*. (2009). p. 43–53 doi: 10.1007/978-0-387-79311-5_5

33. Clayton E, Bardi G, Bell SE, Chantry D, Peter Downes C, Gray A, Humphries LA, Rawlings D, Reynolds H, Vigorito E, et al. A crucial role for the p110δ subunit of phosphatidylinositol 3-kinase in B cell development and activation. *Journal of Experimental Medicine* (2002) 196:753–763. doi: 10.1084/jem.20020805

34. Okkenhaug K, Bilancio A, Farjot G, Priddle H, Sancho S, Peskett E, Pearce W, Meek SE, Salpekar A, Waterfield MD, et al. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science* (2002) 297:1031–1034. doi: 10.1126/SCIENCE.1073560

35. Omori SA, Cato MH, Anzelon-Mills A, Puri KD, Shapiro-Shelef M, Calame K, Rickert RC. Regulation of Class-Switch Recombination and Plasma Cell Differentiation by Phosphatidylinositol 3-Kinase Signaling. *Immunity* (2006) 25:545–557. doi: 10.1016/j.immuni.2006.08.015

36. Rolf J, Bell SE, Kovesdi D, Janas ML, Soond DR, Webb LMC, Santinelli S, Saunders T, Hebeis B, Killeen N, et al. Phosphoinositide 3-Kinase Activity in T Cells Regulates the Magnitude of the Germinal Center Reaction. *The Journal of Immunology* (2010) 185:4042–4052. doi: 10.4049/JIMMUNOL.1001730

37. Ramadani F, Bowen H, Gould HJ, Fear DJ. Transcriptional analysis of the human IgE-expressing plasma cell differentiation pathway. *Front Immunol* (2019) 10:402. doi: 10.3389/FIMMU.2019.00402/BIBTEX

38. Newman R, Tolar P. Chronic calcium signaling in IgE+ B cells limits plasma cell differentiation and survival. *Immunity* (2021) 54:2756-2771.e10. doi: 10.1016/j.immuni.2021.11.006

Figure legends

Figure 1. IC87114 reduces class switching of human B cells to IgE.

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

Figure 2. Inhibition of PI3K p110δ with nemiralisib selectively reduces class switching to IgE.

Purified CD43- tonsil B cell cultures were stimulated with IL-4 and anti-CD40 alone or in the presence of 1nM and 10nM nemiralisib. (**A**) On day 10 of the culture, cells were harvested and intracellularly stained for IgE and IgG1. The percentages of total IgE^+ cells (**B**) and $IgG1^+$ cells (**C**) from several experiments were made relative to the IL-4 and anti-CD40 only cultures. Secreted IgE (**D**) and IgG1(**E**) analyzed by ELISA show the amounts of secreted IgE and IgG1 [ng/mL] made relative to the IL-4 and anti-CD40 only cultures.

Figure 3. IC87114 has no effect on the class switch signalling downstream of IL-4R and CD40.

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

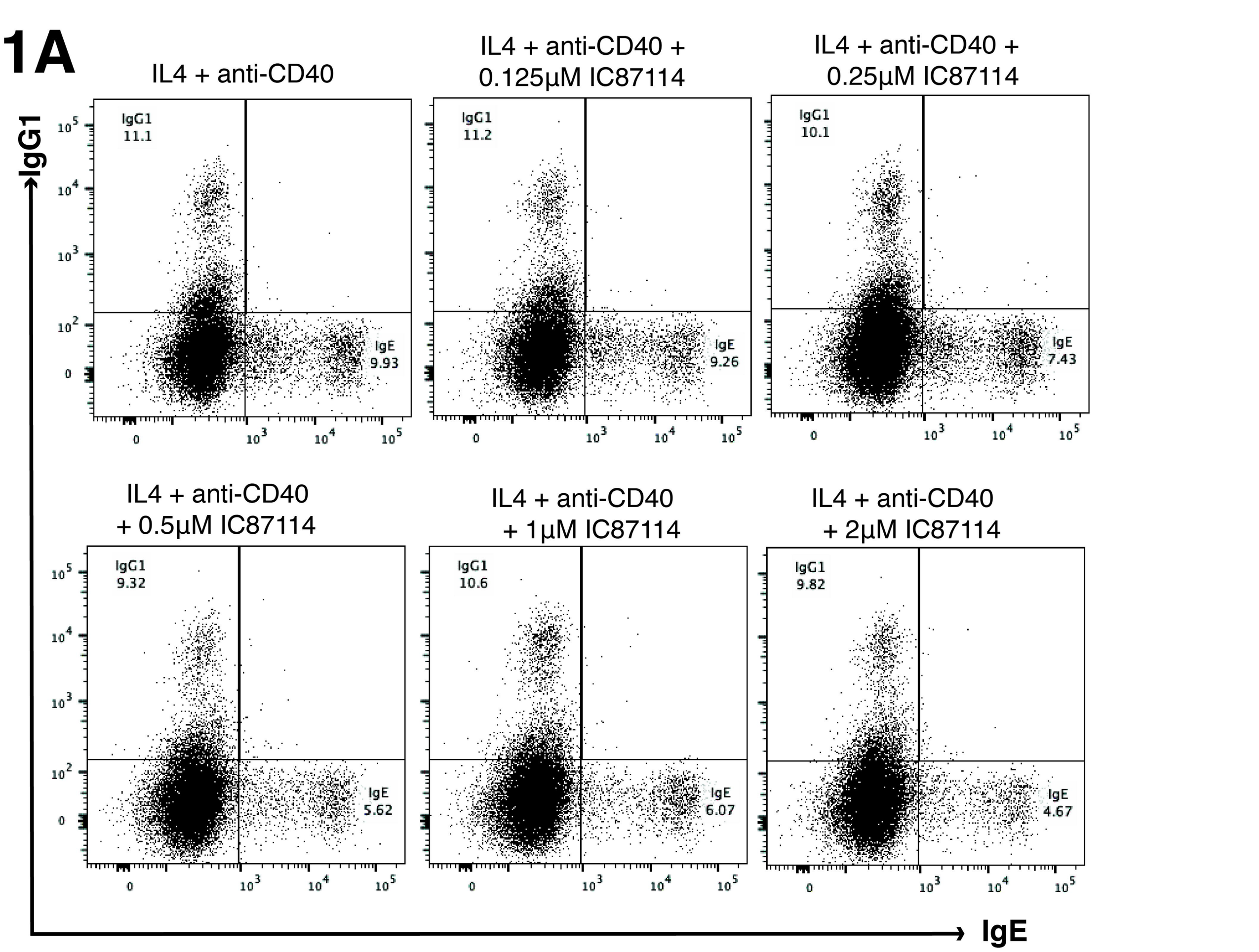
Figure 4. PI3K p110δ inhibition does not affect the expression levels of BCL6 in IL-4 and anti-CD40 stimulated human B cells.

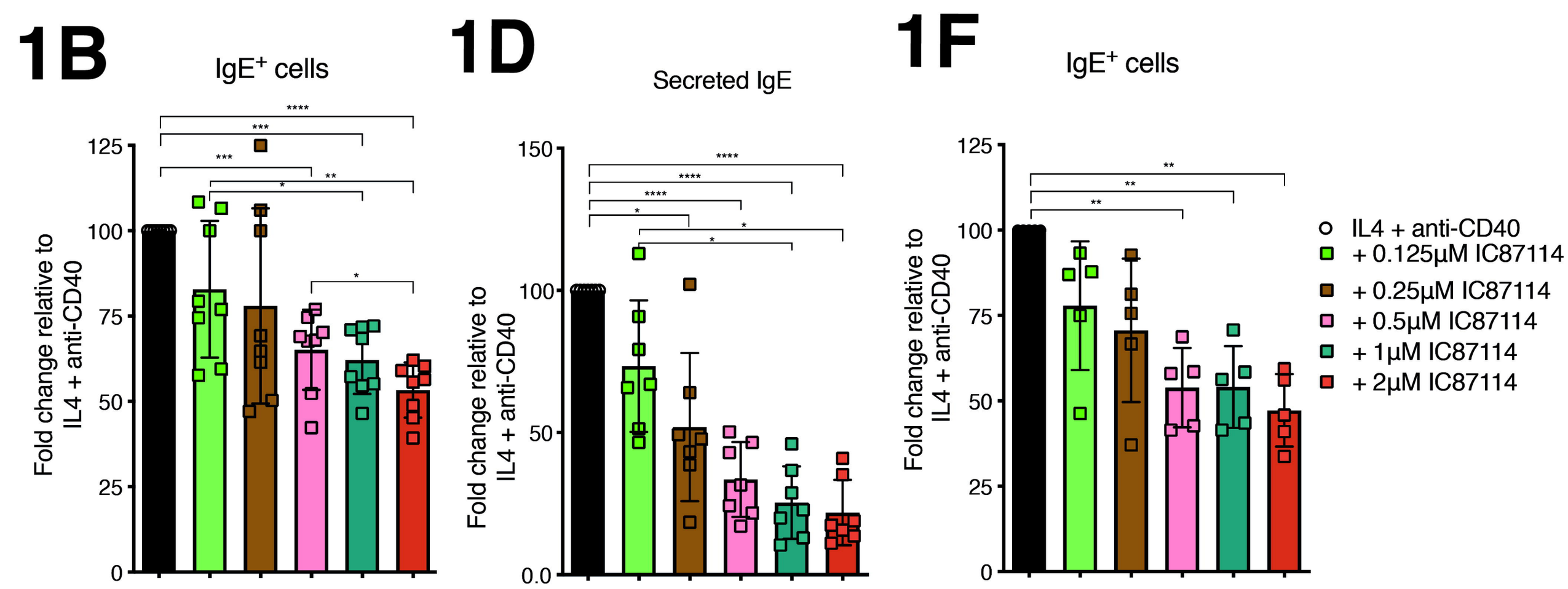
The expressions of BCL6 was quantified with qRT-PCR from RNA isolated from day 5 of the tonsillar B cell culture. Gene expression was normalised with 18s rRNA.

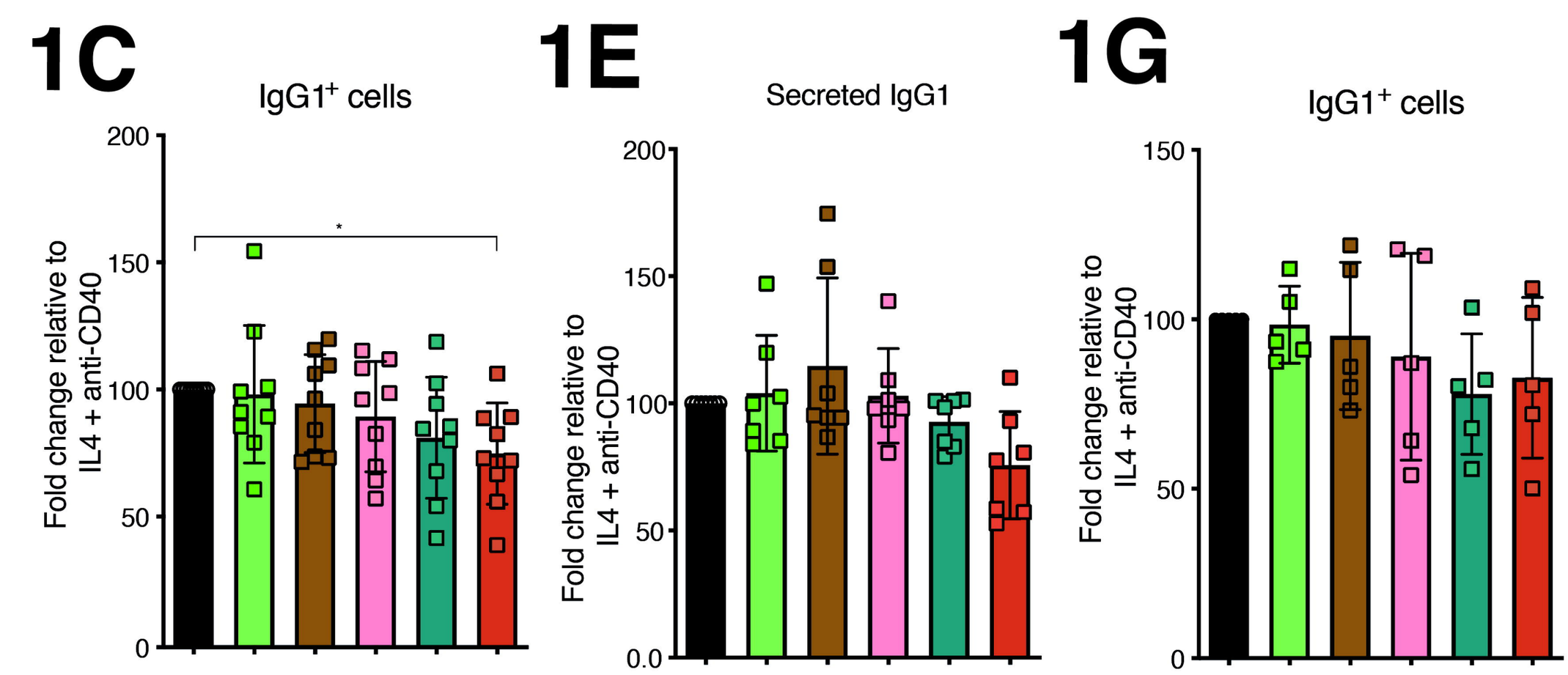
Figure 5. IC87114 inhibits the proliferative capacity of the IL-4 and anti-CD40 stimulated B cells.

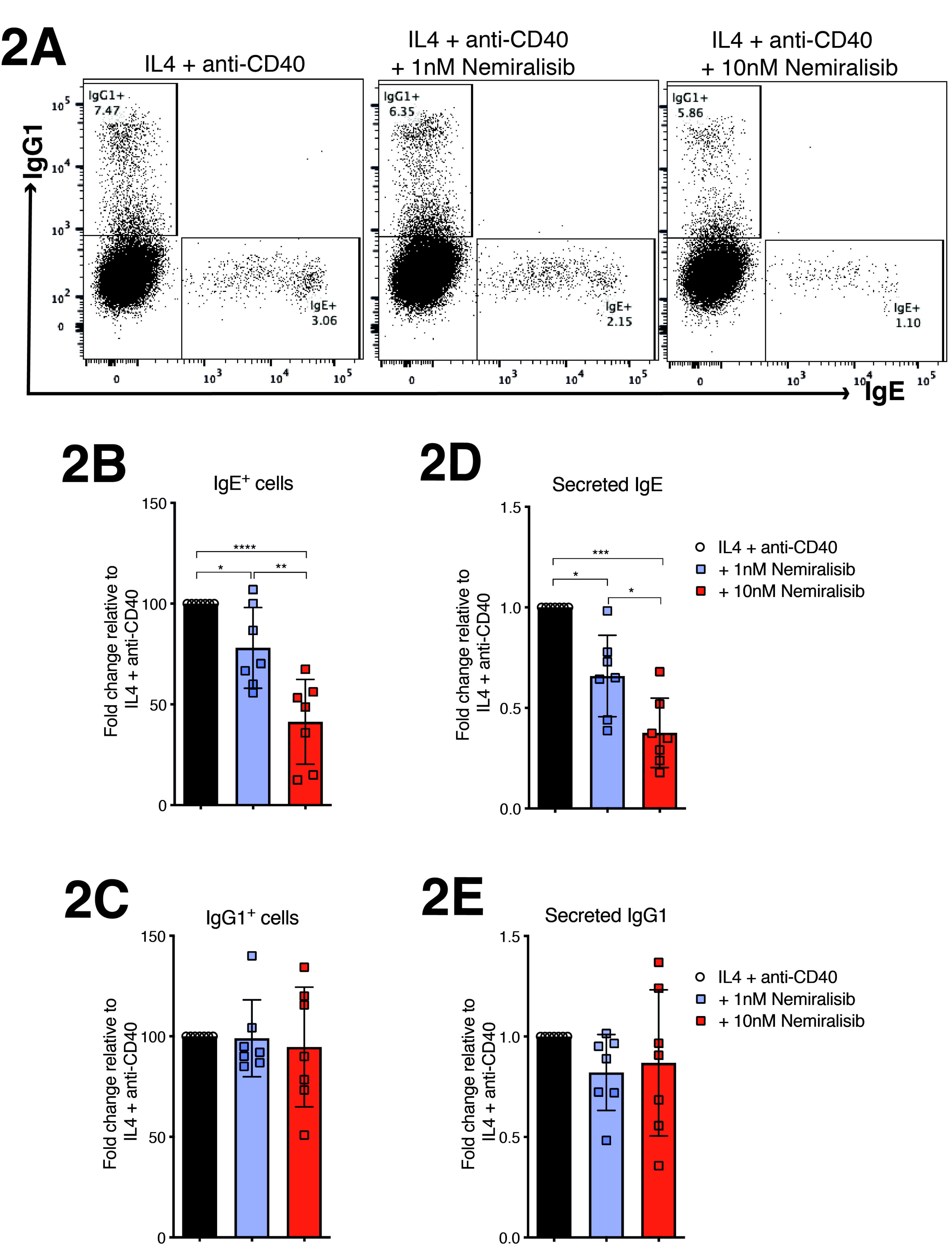
Isolated tonsil B cells were stained with CFSE and cultured for 10 days with IL-4 and anti-CD40 alone or with 2μM IC87114. **(A)** The FACS histograms show the percentage of proliferating B cells after 10 days of culture, whereas **(B)** shows the fold change in proliferation relative to the IL-4 and anti-CD40 only culture. The statistical comparisons were performed using the paired t-test. **** $P < 0.0001$. (**C**) The FACS plots show the number of divisions and the percentage of cells at each division that the IL-4 and anti-CD40 stimulated

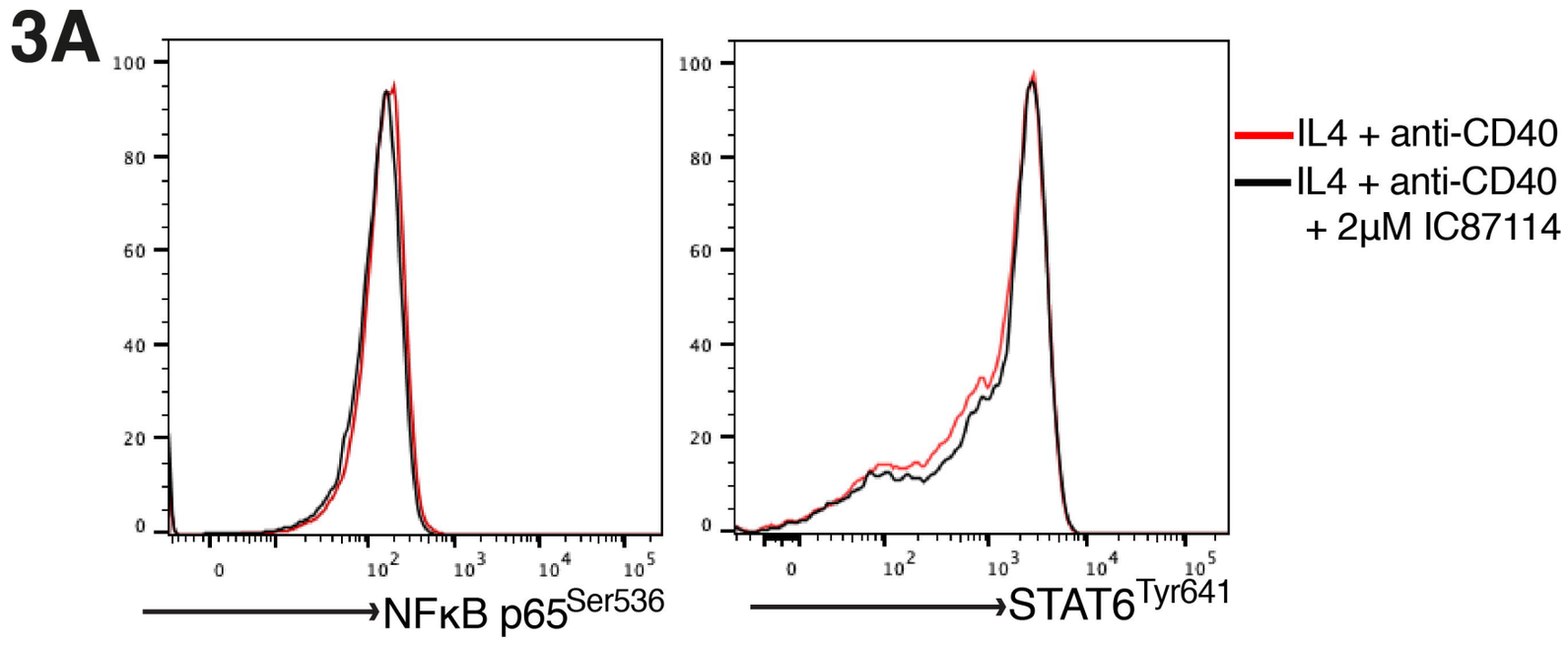
B cells, with or without IC87114, were undergoing. Based on the CFSE dilution, six numbers of cell divisions (D1-D6+) were identified and gated. (D) Graph shows the frequency of B cells at divisions 1 to 6+ (D1 to D6+).

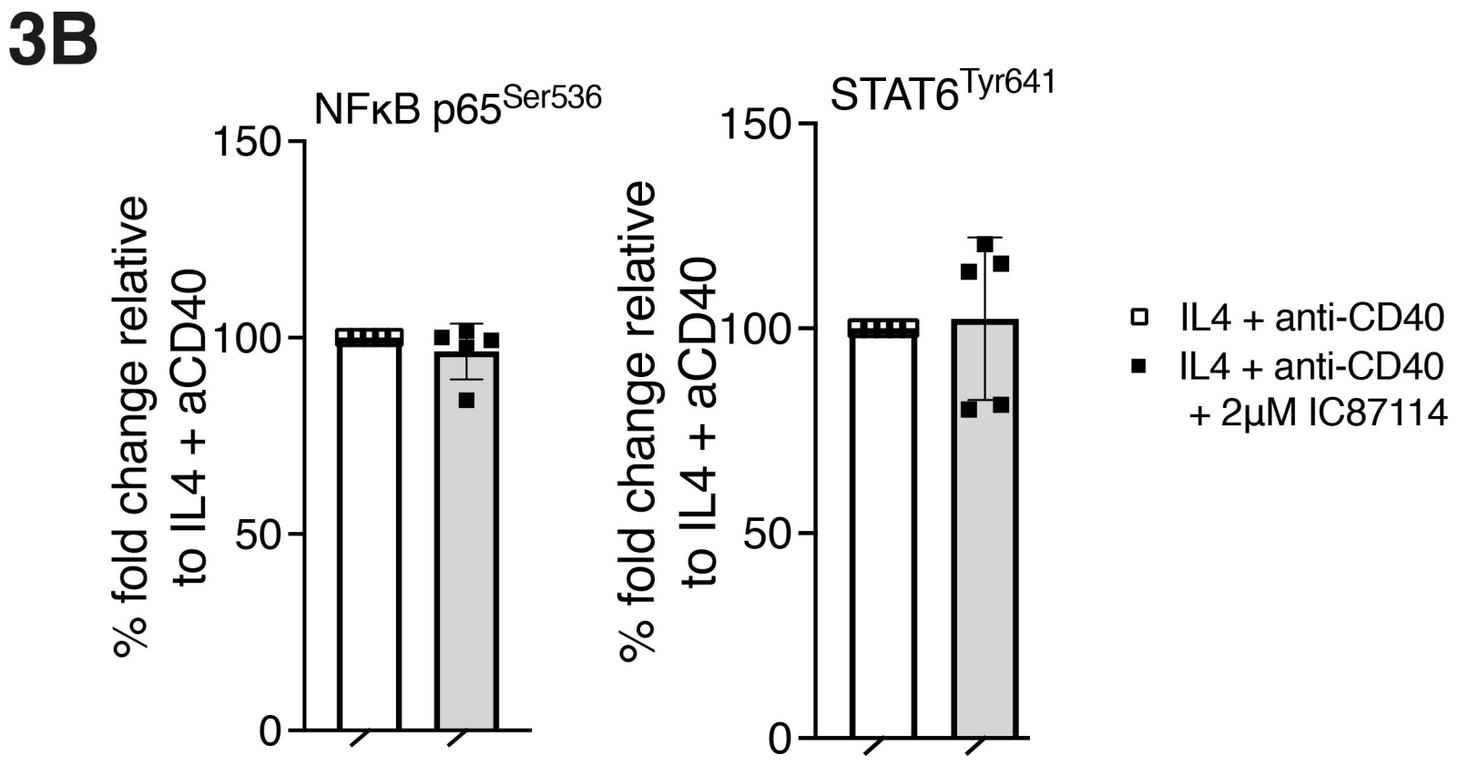

1A
IL4 + anti-CD40
IL4 + anti-CD40 + 0.125μM IC87114
IL4 + anti-CD40 + 0.25μM IC87114
IL4 + anti-CD40 + 0.5μM IC87114
IL4 + anti-CD40 + 1μM IC87114
IL4 + anti-CD40 + 2μM IC87114
IgG1
IgE
IgG1 11.1
IgE 9.93
IgG1 11.2
IgE 9.26
IgG1 10.1
IgE 7.43
IgG1 9.32
IgE 5.62
IgG1 10.6
IgE 6.07
IgG1 9.82
IgE 4.67

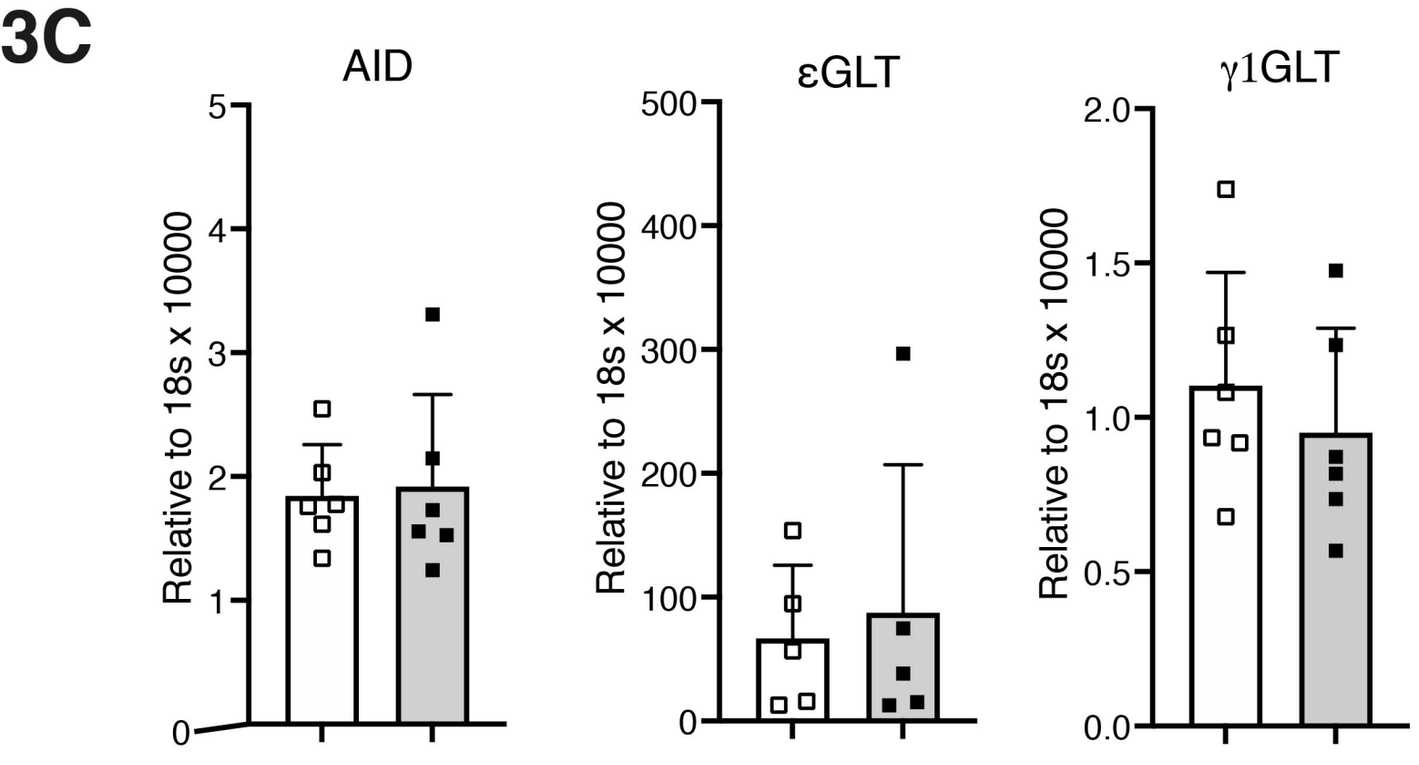

1B
IgE+ cells
1D
Secreted IgE
1F
IgE+ cells
Fold change relative to IL4 + anti-CD40
IL4 + anti-CD40
+ 0.125μM IC87114
+ 0.25μM IC87114
+ 0.5μM IC87114
+ 1μM IC87114
+ 2μM IC87114


1C
IgG1+ cells
1E
Secreted IgG1
1G
IgG1+ cells
Fold change relative to IL4 + anti-CD40

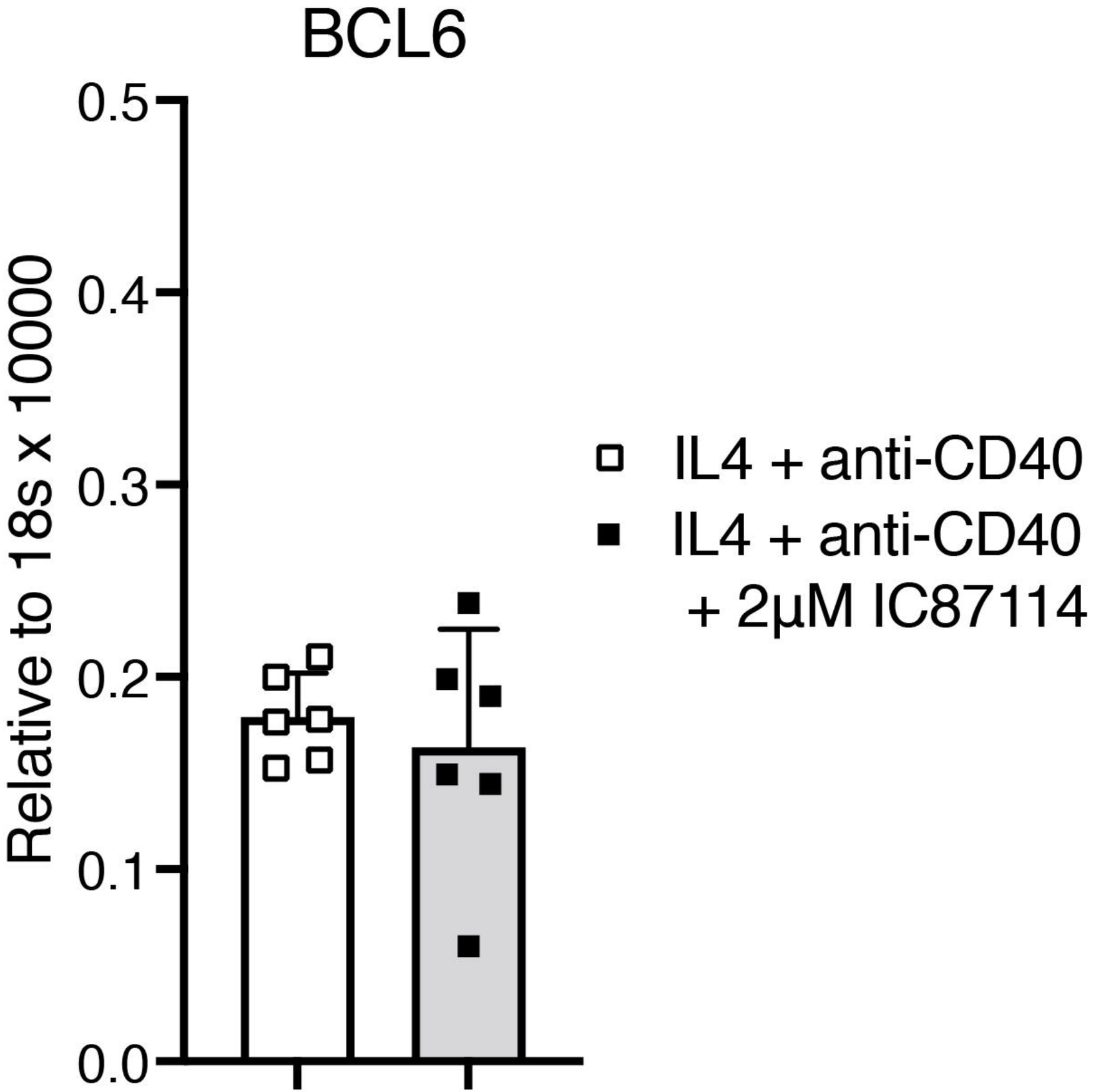

2A
IL4 + anti-CD40
IL4 + anti-CD40
+ 1nM Nemiralisib
IL4 + anti-CD40
+ 10nM Nemiralisib
IgG1
IgE
IgG1+
7.47
IgE+
3.06
IgG1+
6.35
IgE+
2.15
IgG1+
5.86
IgE+
1.10
2B
IgE+ cells
Fold change relative to
IL4 + anti-CD40
2D
Secreted IgE
IL4 + anti-CD40
+ 1nM Nemiralisib
+ 10nM Nemiralisib
2C
IgG1+ cells
2E
Secreted IgG1

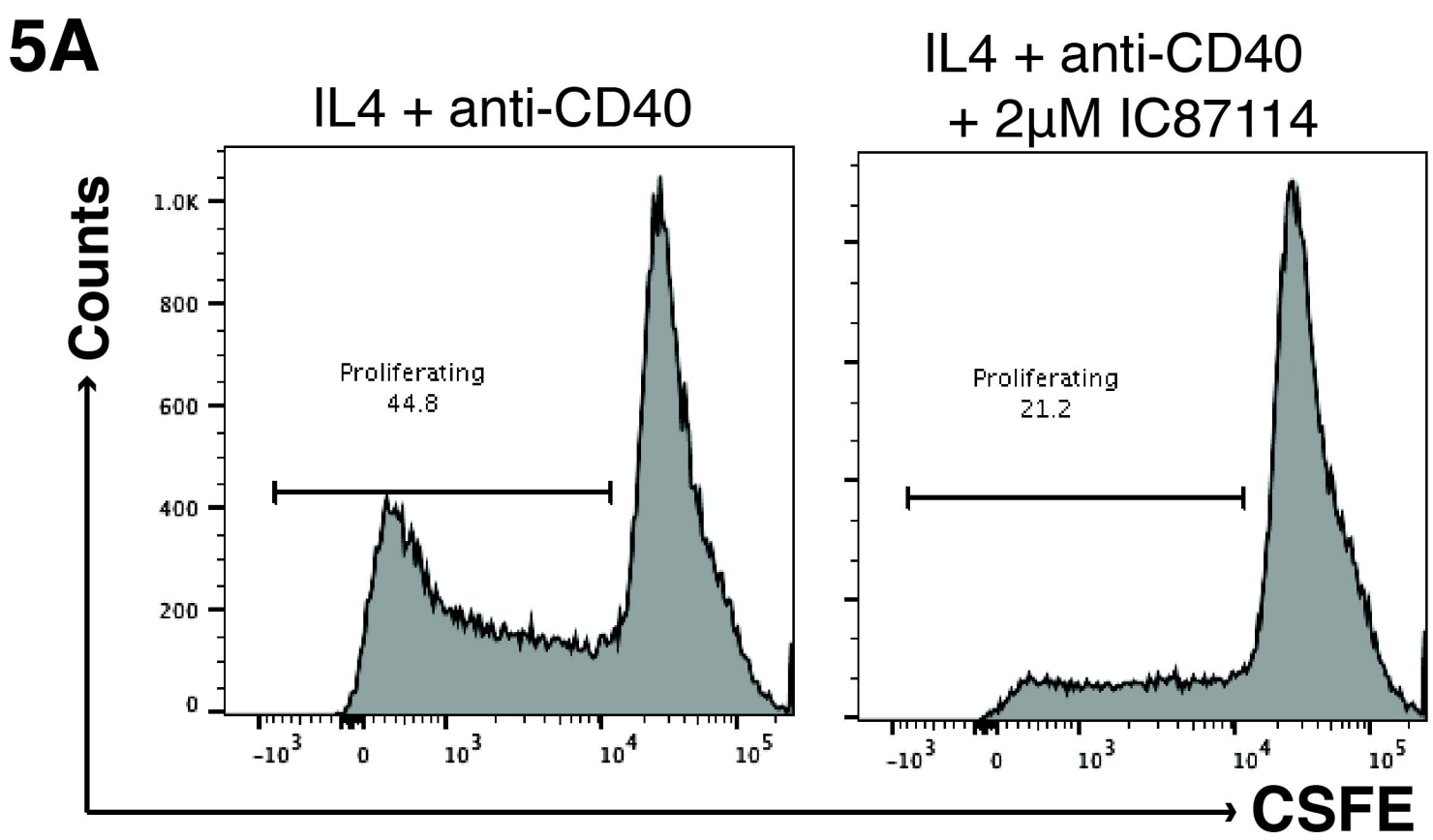

3A
IL4 + anti-CD40
IL4 + anti-CD40
+ 2μM IC87114
NFκB p65Ser536
STAT6Tyr641

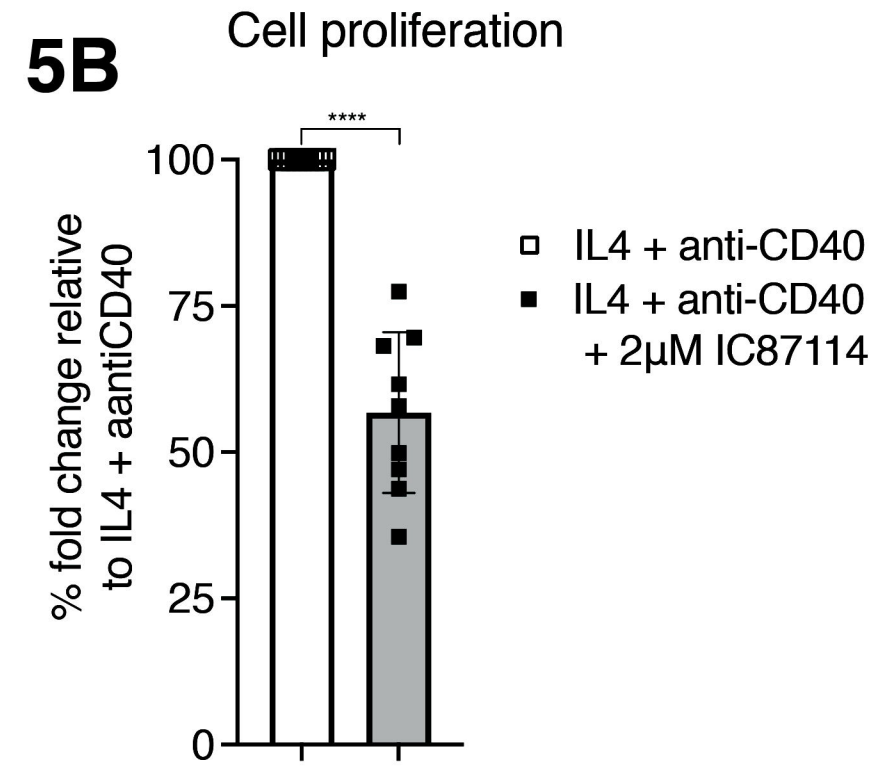

3B
NFκB p65Ser536
STAT6Tyr641
% fold change relative
to IL4 + aCD40
IL4 + anti-CD40
IL4 + anti-CD40
+ 2μM IC87114

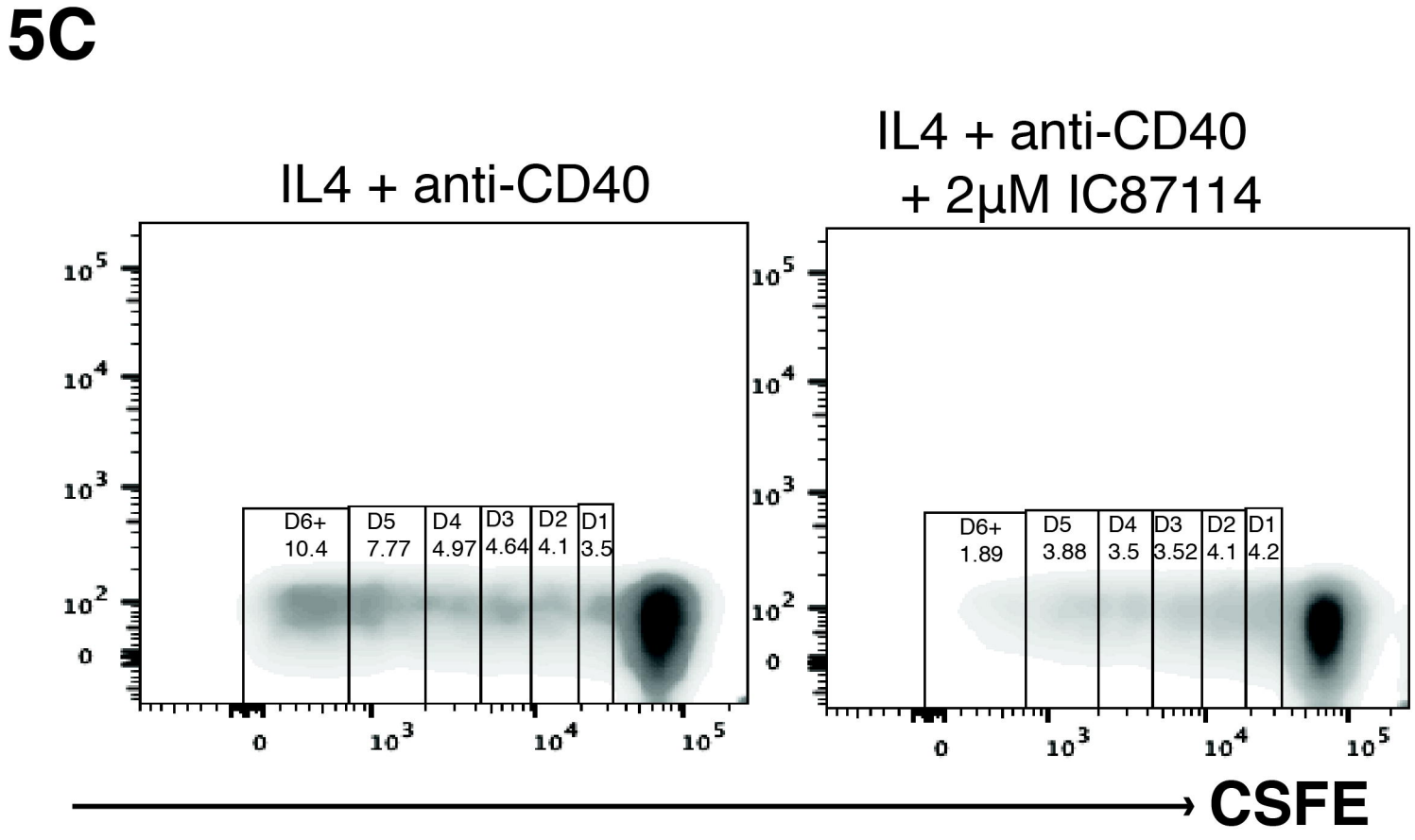

3C
AID
εGLT
γ1GLT
Relative to 18s x 10000

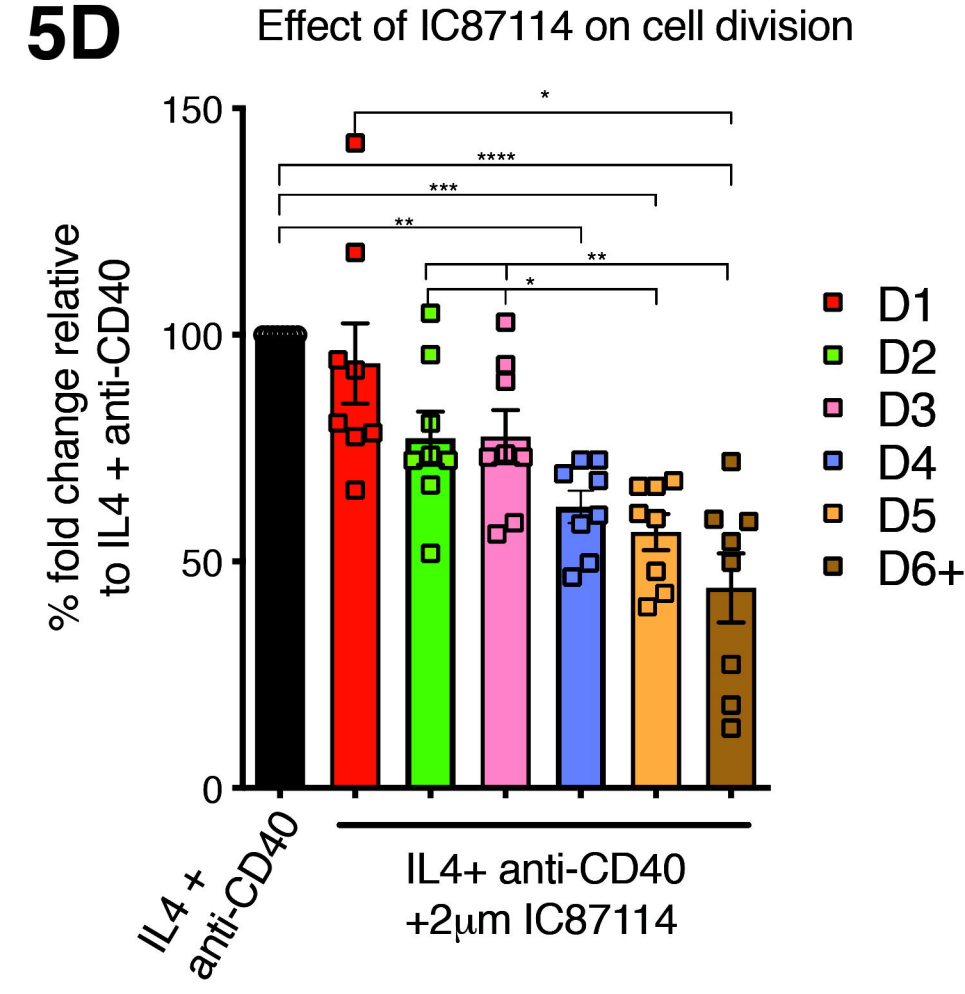
4


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


5A
IL4 + anti-CD40
IL4 + anti-CD40
+ 2µM IC87114
Counts
Proliferating
44.8
Proliferating
21.2
CSFE


5B
Cell proliferation
% fold change relative
to IL4 + aantiCD40
****
IL4 + anti-CD40
IL4 + anti-CD40
+ 2µM IC87114


5C
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


5D
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