# Measurement of B lymphocyte signalling using PhosFlow

SYGNATURE DISCOVERY

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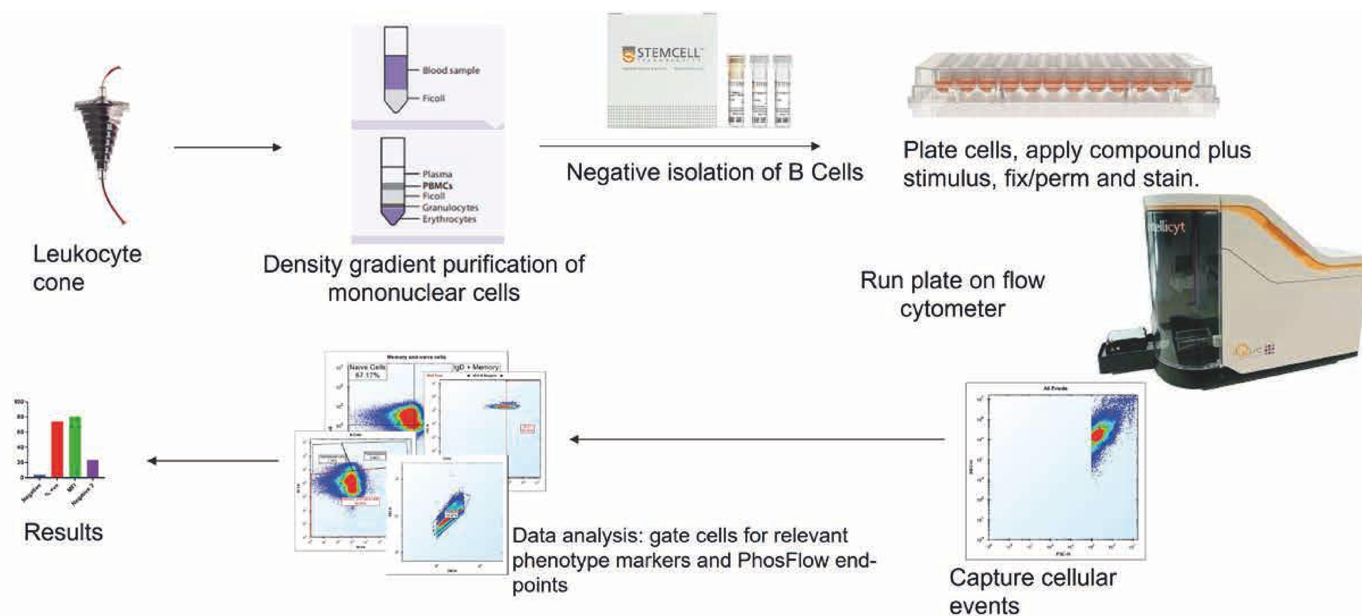
## Introduction

B lymphocytes (B cells) play a critical role in humoral immunity as part of adaptive immune system with roles in antigen presentation, cytokine secretion and antibody production (as differentiated plasmablasts). B cell receptors (BCR) are composed of an immunoglobulin plus CD79 (transduction moiety). BCR signals through a complex protein cascade that includes phosphorylation points; including Spleen Tyrosine Kinase (SYK), Bruton's Tyrosine Kinase (BTK) and Phospholipase C gamma 2 (PLCγ2). This signalling is crucial for B cell survival, proliferation and activity and represents a desirable target for B cell related malignancies, including chronic lymphocytic leukaemia, rheumatoid arthritis and systemic lupus erythematosus[1]. Indeed, zanubrutinib, a BTK inhibitor, has approval for treatment of B cell lymphomas, whilst the SYK inhibitor BAY-61-3606 is used to research BCR signalling[2]. Protein phosphorylation within the BCR signalling cascade can be studied using Flow Cytometry ("phosflow"). This permits relatively high throughput analysis of phosphorylation signalling cascades.

## Methods

Peripheral mononuclear blood cells (PBMCs) were enriched from leukocyte cones via density centrifugation.
B cells isolated from PBMCs by negative magnetic separation (StemCell Technologies).
Cells seeded into 96 well plates and compounds added using acoustic dispensing on an ECHO 650 (Beckman Coulter). Cells incubated for 1 h at 37 °C.
Anti-IgM added and B cells stimulated for 5 min.
Cells were fixed, permeabilised & labelled with cell surface plus phosphorylation antibodies.
Cells analysed by Flow Cytometry using an IntelliCyt® iQue Screener plus Forecyt® software (Sartorius).




## Anti-IgM time-course

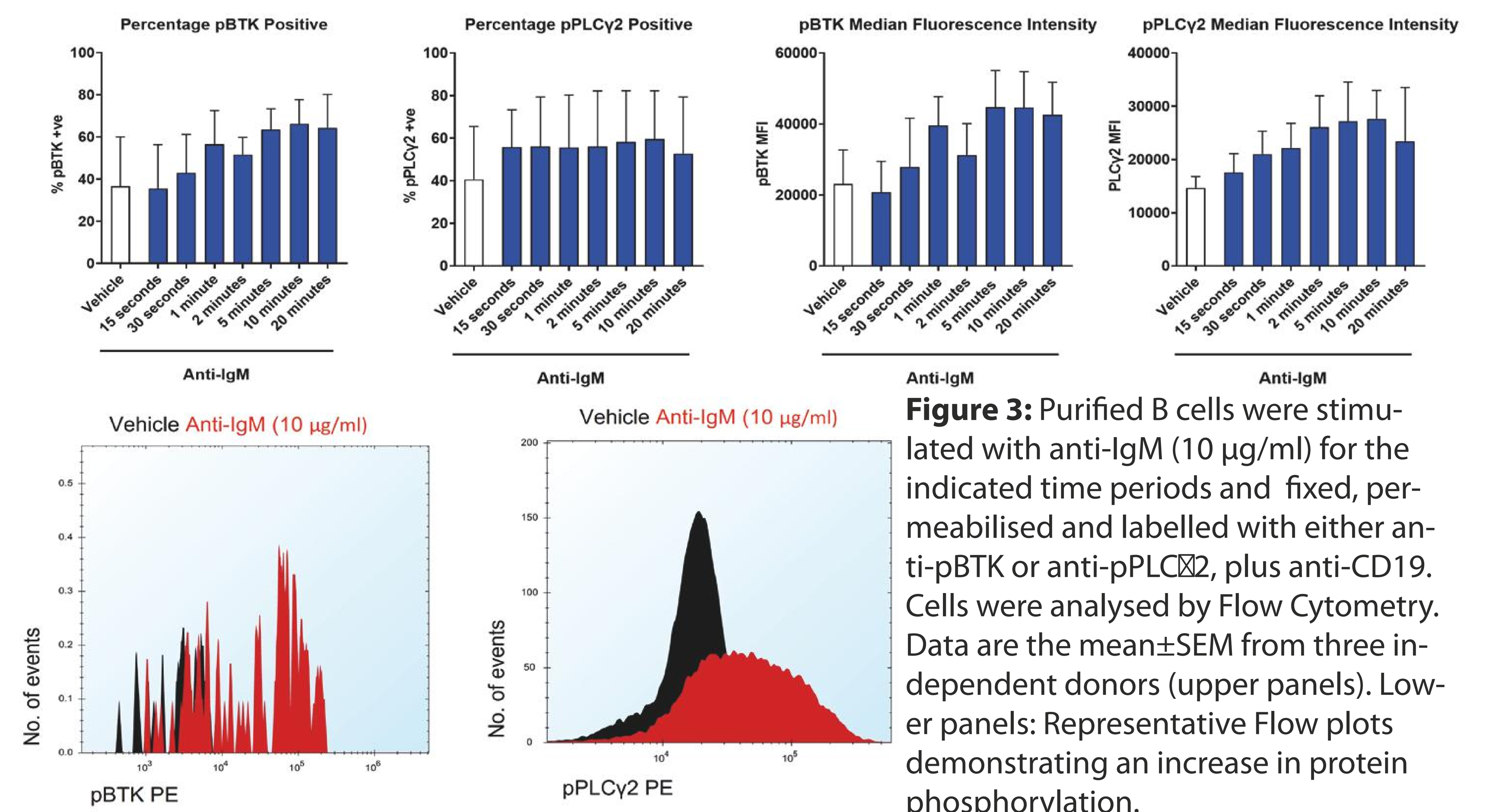



**Figure 3:** Purified B cells were stimulated with anti-IgM (10 μg/ml) for the indicated time periods and fixed, permeabilised and labelled with either anti-pBTK or anti-pPLCγ2, plus anti-CD19. Cells were analysed by Flow Cytometry. Data are the mean±SEM from three independent donors (upper panels). Lower panels: Representative Flow plots demonstrating an increase in protein phosphorylation.

## Anti-IgM concentration-response

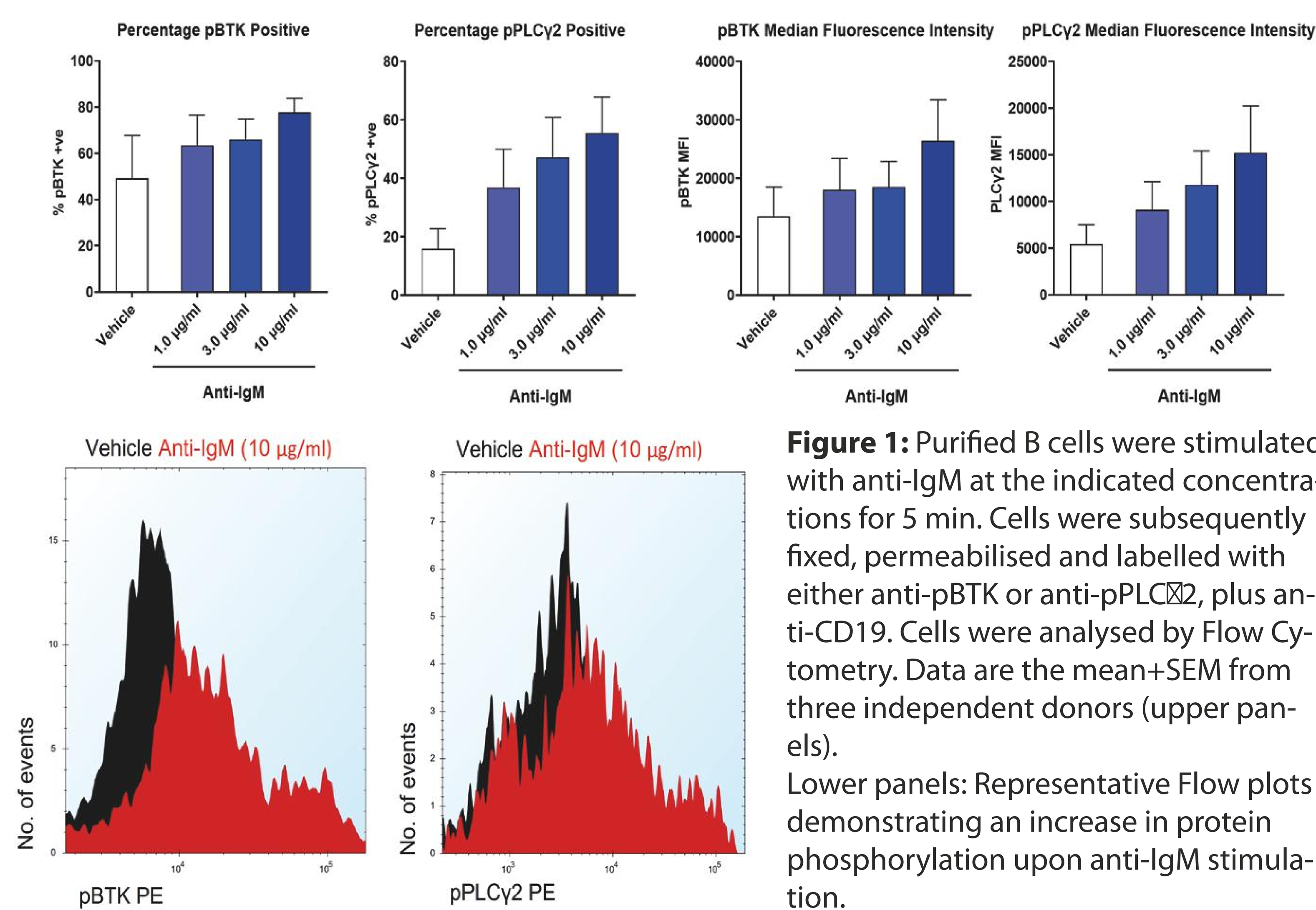



**Figure 1:** Purified B cells were stimulated with anti-IgM at the indicated concentrations for 5 min. Cells were subsequently fixed, permeabilised and labelled with either anti-pBTK or anti-pPLCγ2, plus anti-CD19. Cells were analysed by Flow Cytometry. Data are the mean+SEM from three independent donors (upper panels).
Lower panels: Representative Flow plots demonstrating an increase in protein phosphorylation upon anti-IgM stimulation.

## Effects of compound treatment

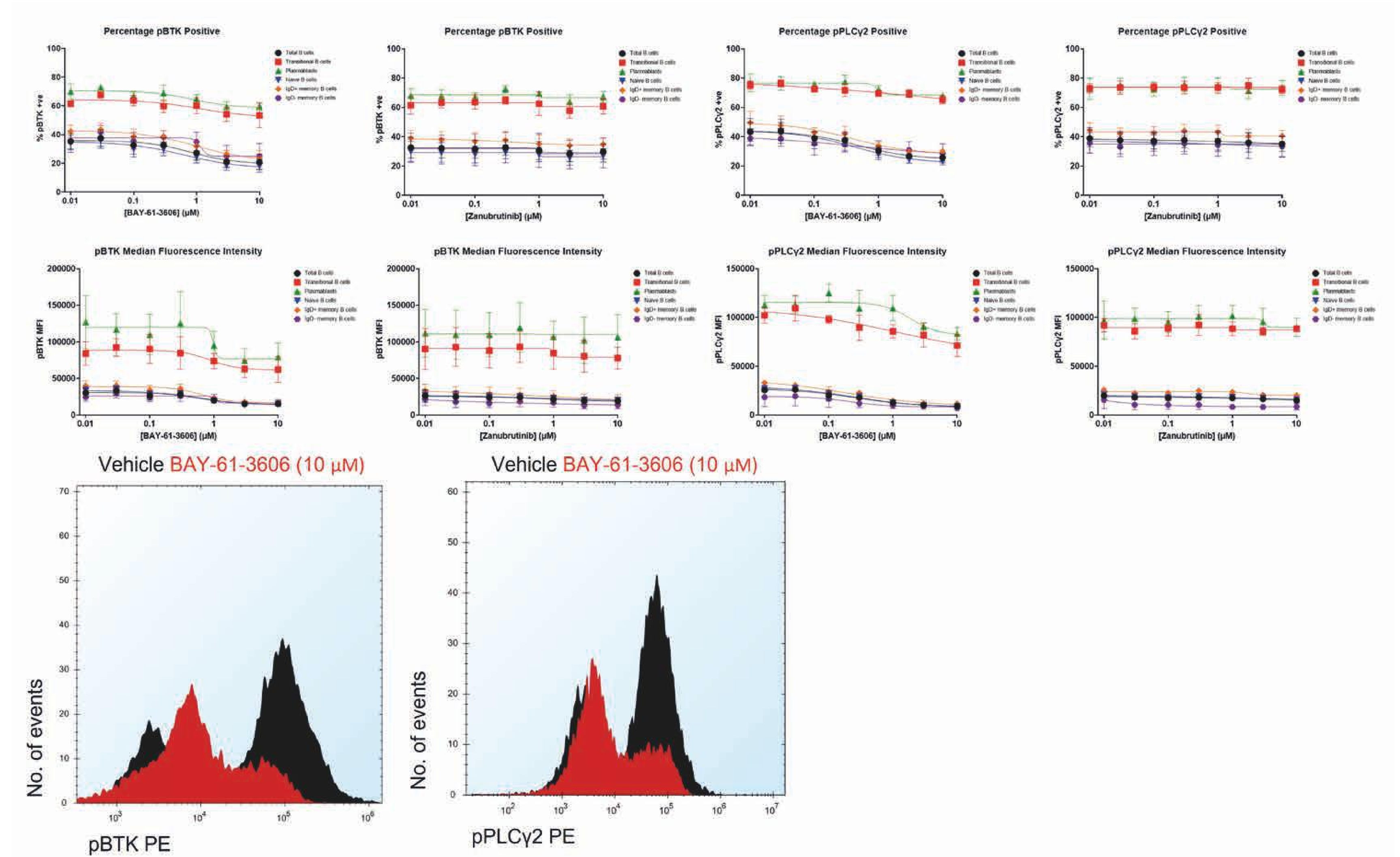



**Figure 4:** Purified B cells were incubated with compound at the indicated concentrations for 1 h. B cells were subsequently stimulated with anti-IgM (10 μg/ml) for 5 min and fixed, permeabilised and labelled with either anti-pBTK or anti-pPLCγ2, plus cell surface markers (anti-CD19, CD27, CD38 and IgD). Cells were analysed by Flow Cytometry. Data are the mean ± SEM from three independent donors (upper panels).
Middle panels: Representative Flow plots demonstrating a decrease in protein phosphorylation upon BAY-61-3606 (10 μM) treatment.

## B cell phenotyping and purity

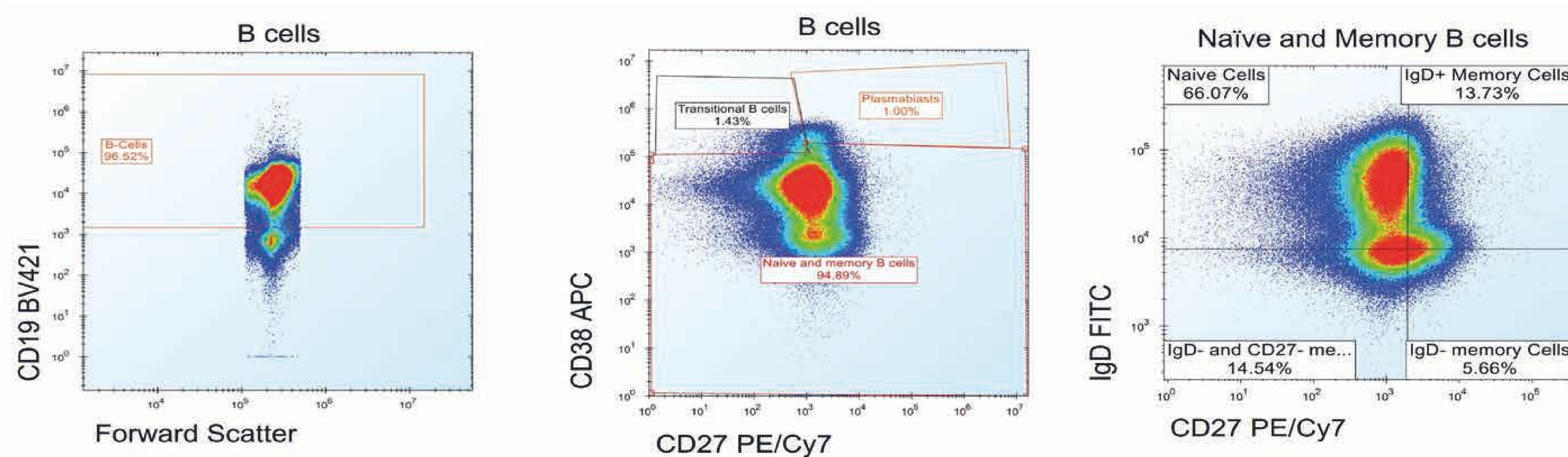



**Figure 2:** Purified B cells were incubated with compound for 1 h. B cells were subsequently stimulated with with anti-IgM for 5 min and fixed, permeabilised and labelled with either anti-pBTK or anti-pPLCγ2, plus cell surface markers (anti-CD19, CD27, CD38 and IgD). Cells were analysed by Flow Cytometry. **Left Panel:** Representative Flow plots highlighting the purity of B cells post-isolation. All donors used had greater than 95% purity. **Middle and right panel:** Representative Flow plots demonstrating detailed B cell phenotyping undertaken. Percentages of cell subsets reflective of literature values.

## Summary

B cell signalling can be studied in detail using Flow Cytometry. BAY-61-3606, but not zanubrutinib, decreases protein phosphorylation in a concentration dependent manner. Phosflow provides a powerful platform for the kinetic study of protein phosphorylation in immune cell subsets.

## References

1. Bag-Ozbek A, Hui-Yuen JS. Emerging B-Cell Therapies in Systemic Lupus Erythematosus. Ther Clin Risk Manag. 2021 Jan 14;17:39-54.
2. Moore DC, Thompson D. A Review of the Bruton Tyrosine Kinase Inhibitors in B-Cell Malignancies. J Adv Pract Oncol. 2021 May;12(4):439-447.

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