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Investigating the kinetics of the B-cell receptor signaling network in a dynamic environment

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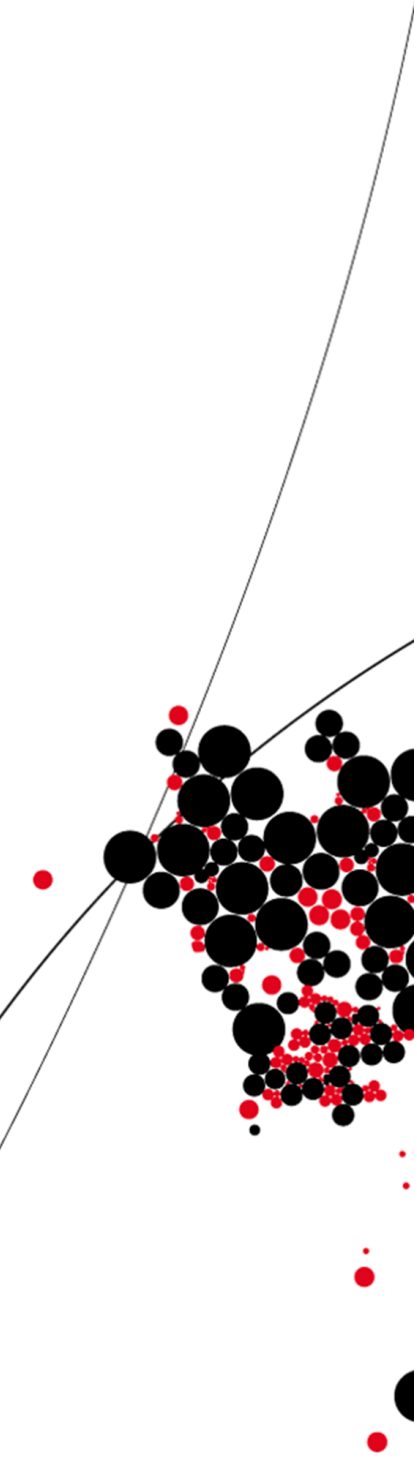

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Investigating the kinetics of the B-cell receptor signaling network in a dynamic environment

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Signal transduction is an important mechanism employed by cells to sense and respond to changes in their microenvironment. These changes are translated into a functional response by signaling networks consisting of receptors, kinases, phosphatases, transcription factors, and other molecules. Improving our understanding of these cellular signaling networks helps with identifying disease-associated signaling components and the development of new drug treatments. The BCR signaling network is an important network that is engaged in B-cell activation, development, proliferation, differentiation, and apoptosis. Malignant B-cells have elevated levels of ROS such as H_2O_2, due to their high metabolic activity. Upregulation of antioxidants in malignant B-cells brings their intrinsic ROS to a level that promotes chronic proliferation and differentiation, resulting in a poor prognosis. While the response of the BCR signaling network to H_2O_2 has been studied extensively, it has only been studied in response to step-wise increases of H_2O_2, which fails to replicate the dynamic *in vivo* environment. In this work, the differences in BCR signaling response of the DLBCL cell line HBL1 in response to static inputs and dynamic inputs of H_2O_2 have been compared. Our results show the contrast between activation patterns of the BCR signaling proteins CD79a, PLCγ2, and Syk in HBL1 cells stimulated with H_2O_2 step increases and H_2O_2 gradients. We demonstrate that, compared to step increases to the same concentration H_2O_2, gradients induce a signaling response that is stronger, weaker, or not not present at all, depending on the cell density, end concentration of H_2O_2, and flow rate.

INTRODUCTION

Within every cell, signal transduction pathways translate extracellular signals from the microenvironment into a specific response [1]. Most often these extracellular signals (e.g. hormones, growth and differentiation factors, interleukins) are bound by receptors present on the surface of a cell, which activates the downstream signaling network. A signaling network steers a cell toward functional responses such as proliferation, differentiation, and apoptosis. Tyrosine kinase and phosphatase enzymes play a fundamental role in a signaling network [1–3]. Kinases can transfer a phosphate group from ATP to the tyrosine residues of other signaling proteins, thereby activating them. Their counterpart, tyrosine phosphatase, acts as a signaling inhibitor by removing phosphate groups from phosphorylated proteins. Unraveling the crosstalk between kinases, phosphatases and other components within a signaling network is beneficial for the discovery of signaling components associated with disease, and for the development of new drug treatments (e.g. small molecules [4] or antibodies [5] that block or enhance signaling). In this work, the dynamics of the B-cell receptor (BCR) signaling network are investigated. The BCR is the main receptor on the surface of B cells, which is composed of the antigen-binding membrane immunoglobulin and a non-covalent associated Iga/Igb signaling subunit [6, 7]. The BCR plays a key role in B-cell activation, development, proliferation, differentiation, and apoptosis. The BCR signaling network (appendix fig. 7) can be inadvertently activated in several B-cell malignancies, which results in a less favorable prognosis [8–10]. One mechanism through which the BCR signaling network of malignant B cells can be activated is reactive oxygen species (ROS) induced inhibition of the phosphatases [11]. ROS are by-products of cellular respiration and metabolism, that have a variety of effects on the cell, depending on the source, location, concentration, as well as the exposure time [12]. Under normal circumstances, ROS are maintained at low concentrations and play a key role in cellular signaling, biosynthesis, and host defense [13–15]. At elevated concentrations, ROS will react with proteins, lipids, and nucleic acids, causing irreversible damage that leads to apoptosis. Increased ROS levels are a characteristic of malignant cells due to their oncogene activation, high metabolic activity, and ROS generated extrinsically in the tumor microenvironment (TME) [16, 17]. Malignant cells have an upregulation of antioxidants to counteract the oxidative stress and lower the intrinsic ROS to a concentration that is tumor-promoting. By lowering the concentration of intracellular ROS of malignant cells

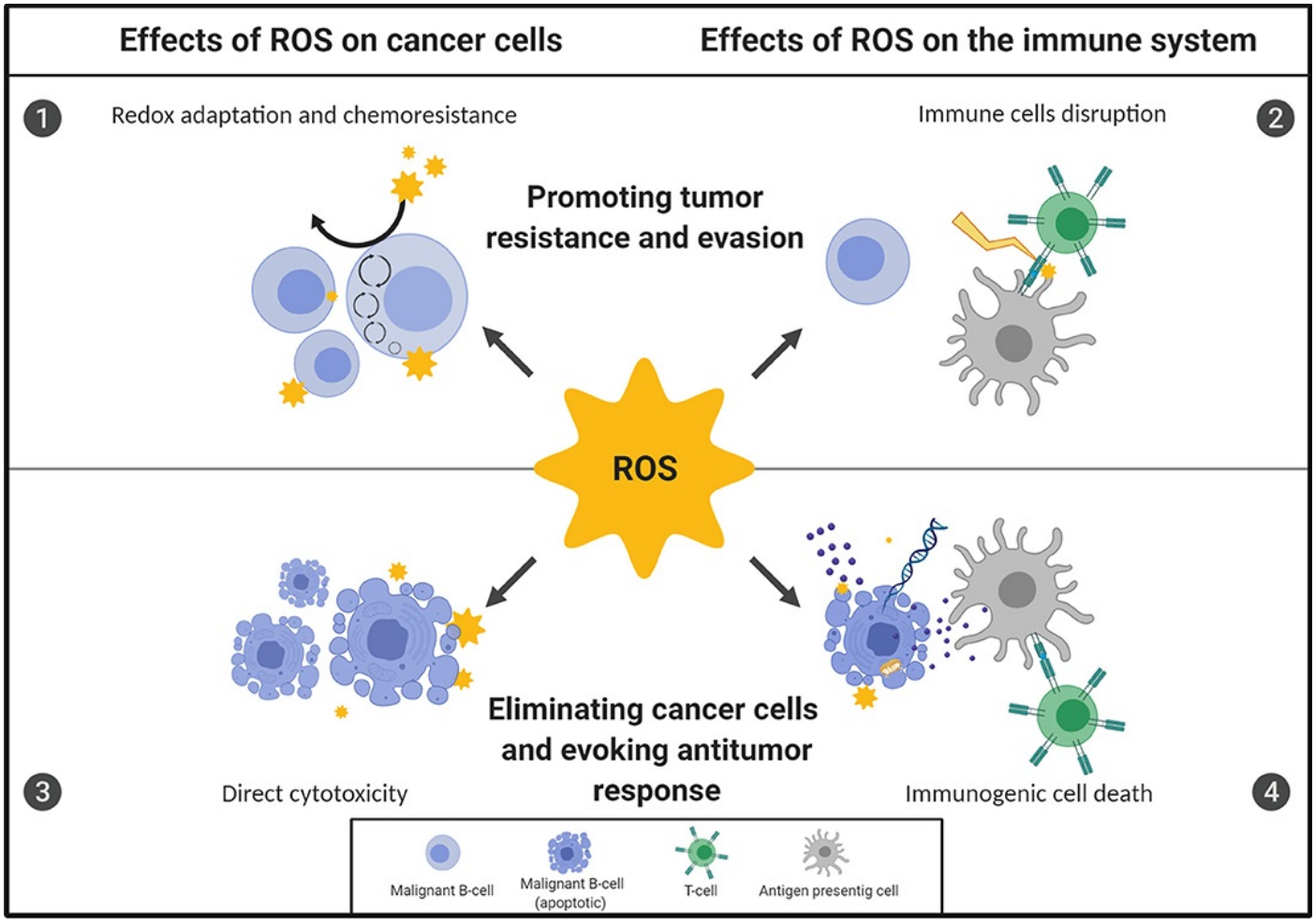

Fig. 1. The effects of ROS on malignant cells and the immune system, explained in a simple illustration [18]. Malignant cells have an upregulation of antioxidants that counteracts the effects of oxidative stress. This allows these cells to maintain the intrinsic ROS at a level that promotes proliferation and survival (**1**), and shields them from cytotoxic immune cells that are not able to function properly due to the elevated ROS levels in the microenvironment (**2**) [15, 19]. Oxidative-stress mediated cytotoxicity can be induced with therapies that either target specific antioxidant pathways within malignant cells, or inhibit the antioxidants in the TME (**3**). Through prooxidant treatments, the concentration of ROS within the microenvironment can be lowered, inducing activation of immune cells which can lead to an antitumor response (**4**).

through prooxidant treatments, or increasing it by inhibiting transcription factors of antioxidants, we can steer malignant cells to a cytostatic or cytotoxic state (Fig. 1).

While efforts are being made to decipher the dynamics between ROS and BCR signaling in B-cell lymphomas, most of these studies analyze the cellular response in a static environment, where ROS are added in a step increase manner [11, 20–23]. Within any organism, cells are exposed to a dynamic microenvironment where input signals such as internal and external stressors, hormones, and drugs (pharmacokinetics) continuously change over time. The dynamics of input signals are crucial for the cellular response [24–27]. So have recent studies for example discovered rate thresholds that regulate the stress response of yeast cells, temporal dynamics of stressors in the kidney that regulate specific signaling responses in kidney- and immune cells, and rate-sensitive signaling of hepatoma cells to gradients of insulin [27]. Results such as these demonstrate the importance of gaining a better understanding of the signaling response of cells within a dynamic environment. To study the kinetics between ROS and the BCR signaling network in a dynamic environment, a (micro)fluidic setup needs to be utilized to expose cells to ROS in temporal gradients. In recent years there has been an increase in research on signaling networks in dynamic environments [27]. Thiemicke *et al* developed an experimental methodology to precisely control the microenvironment of suspension cultures, by utilizing a syringe pump system (fig. 2). The setup was used to demonstrate that yeast cells have a different osmotic stress response when exposed to a linear gradient of NaCl, compared to a step increase of NaCl.

To summarize, characterizing an intracellular signaling network is useful for the discovery of signaling components that are involved in disease, as well as finding treatments to block or enhance the signaling of such components. Recent work has shown that the cell signaling response to static increases of stimulants, often performed *in vitro*, is different than in a dynamic environment such as the body, discovering rate thresholds and rate-sensitive signaling in a variety of cells. Thus it is important to analyze the signaling networks of cells using

dynamic stimulation profiles (e.g. oscillating gradients [28], linear gradients [25], and quadratic gradients [26]). In this report, a setup inspired by Thiemicke *et al* is used to study the response of human diffuse large B-cell lymphoma to dynamic inputs of hydrogen peroxide (H_2O_2), the main ROS produced in B cells [16].

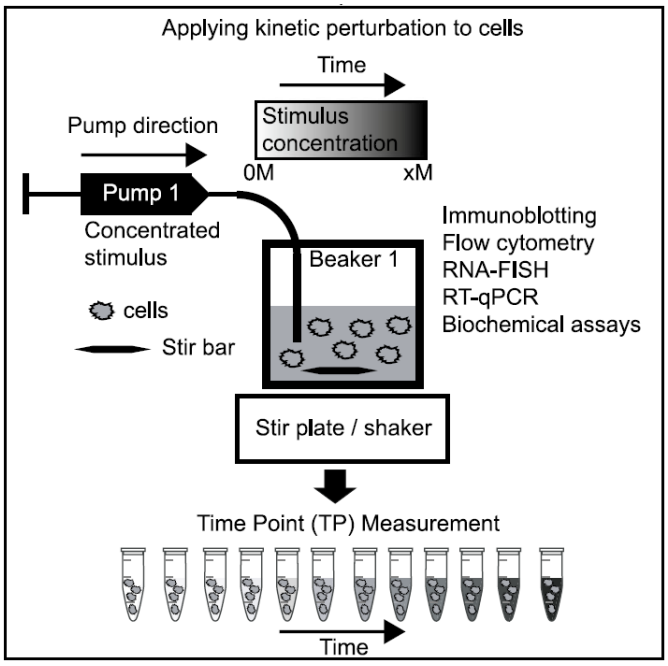

Fig. 2. The fluidic setup designed by Thiemicke *et al*, that enables stimulation of suspension cultures with dynamic input signals [25]. Stimulants can be added to the system but not removed, which makes the setup suitable for gradients that increase over time.

METHODS

Cell Culture

HBL1 cells were cultured in RPMI 1640 media (Gibco, Cat. no. 52400-025) containing 10% FBS (Gibco, Cat. No. A3160801), 100 U/mL Penicillin-Streptomycin (Gibco, Cat. No. 15140-122) at 37°C and 5% CO_2. The cells were passaged into new culture flasks (ThermoFisher, Cat. No. 156340) every 2-3 days, to a cell density of 2.5- to $5 \cdot 10^5$ cells/mL.

Cell stimulation setups

HBL1 cells were serum-starved with 2% RPMI medium (2% FBS and 100 U/mL Penicillin-Streptomycin) 90 minutes before stimulation. The cells were stimulated in either a 96-round bottom wells plate (Corning, Cat. No. 10693652) or a round bottom flask, depending on the experiment. Experiments with only step increase profiles were done in a 96-wells plate, and experiments including linear or quadratic gradients were done in a round bottom flask inside a laminar flowhood.

Step increase experiments

Step increase experiments in 96 wells-plates were carried out with a cell density of $2.2 \cdot 10^6$ cells/mL. Before starting the cell stimulation, 45 µL of a 2% RPMI/H_2O_2 mixture was added to the wells. To each well 180 µL of serum-starved cell suspension was added, starting at the longest time-point, working towards the shortest. In between time points, the plate was sealed and placed in an incubator. After adding cell suspension to the last well (t = 0), the cells were fixated immediately by adding 75 µL 4x paraformaldehyde (PFA) (Merck, Cat. No. 1040051000) to the wells using a multichannel pipette. After 15 min of fixation at RT, the wells plate was centrifuged at 800g for 5 min. Subsequently, the supernatant was removed and the cells were resuspended in 200 µL PBS (Gibco, Cat. No. 14190-094). The wells plate was stored at 4°C until analysis, for a maximum of 2 days.

Gradient experiments

H_2O_2 gradients experiments were done with a cell density of either 1.0- or $2.0 \cdot 10^6$ cells/mL. To expose the cells to linear and quadratic gradients of H_2O_2, a Nemesys pump system (Cetoni GmbH) was utilized. Syringes (B. Braun, Cat. No. 9161406V) were connected to the pump, and tubing was used to guide the H_2O_2 from the syringe to the round bottom flask containing the cells. To keep the cells at 37°C during the experiments, the round bottom flask was hung in a beaker filled with water that was placed on a hotplate stirrer connected to a temporal probe. At each time-point, a sample of $5 \cdot 10^5$ cells was taken and added to a FACS tube containing 2x PFA in a volume equal to the taken sample, to fixate the cells. After 15 min of incubation at RT, the tube was spun down for 5 min at 800g to remove the supernatant. The cells were and resuspended in 200 to 400 μL PBS and stored at 4°C until analysis.

Antibody staining and fluorescence-activated cell sorting

The activity of the signaling proteins of the HBL1 cells was measured using a flow cytometry protocol, utilizing fluorophore-conjugated antibodies (appendix table 1) targeting phosphorylated signaling proteins. All centrifugation steps were carried out at 800g for 5 minutes. Each sample obtained during cell stimulation was added to a 96-round bottom wells plate. The wells plate was spun down to remove the supernatant, and the cells were resuspended in 200 µL permeabilization buffer (100 mM Tris-HCl pH 7.5, 100 mM NaCl, and 0.1% Triton X-100 (ThermoFisher, Cat. No. 85112) in nuclease-free water). After 10 min of incubation at RT, the samples were centrifuged to remove the permeabilization buffer. The cells were washed once with a FACS buffer (0.1% BSA (Sigma, Cat. No. A45503-50G), 0.05% NaN_3 (Merck, Cat. No. RTC0000681L) and 0.5 mM EDTA (Lonza, Cat. No. 51201) in PBS). The cells were centrifuged, resuspended in 40 μL staining solution, and incubated for 1 hour at RT in the absence of light. After incubation, three washing steps with a FACS buffer were performed, once with 150 μL and twice with 200 μL. After the last washing step, the cells were resuspended in 150 μL FACS buffer and analyzed using flow cytometry. For each sample, between 5000 and 50.000 cells were analyzed. The phosphoresponse of the cells was determined by setting a fluorescence threshold on the 97.5th percentile of t = 0, for each stimulation condition and fluorophore individually.

RESULTS

BCR signaling of HBL1 cells following stimulation with a H_2O_2 step increase is dose-responsive and bimodal

The response of HBL1 cells to stimulation with H_2O_2 was established through a series of step increase experiments. In an initial experiment, HBL1 cells were stimulated for 10 min with H_2O_2 in concentrations from 50 µM to 1 mM. The activated forms of the signaling proteins CD79a, PLCγ2, and Syk were

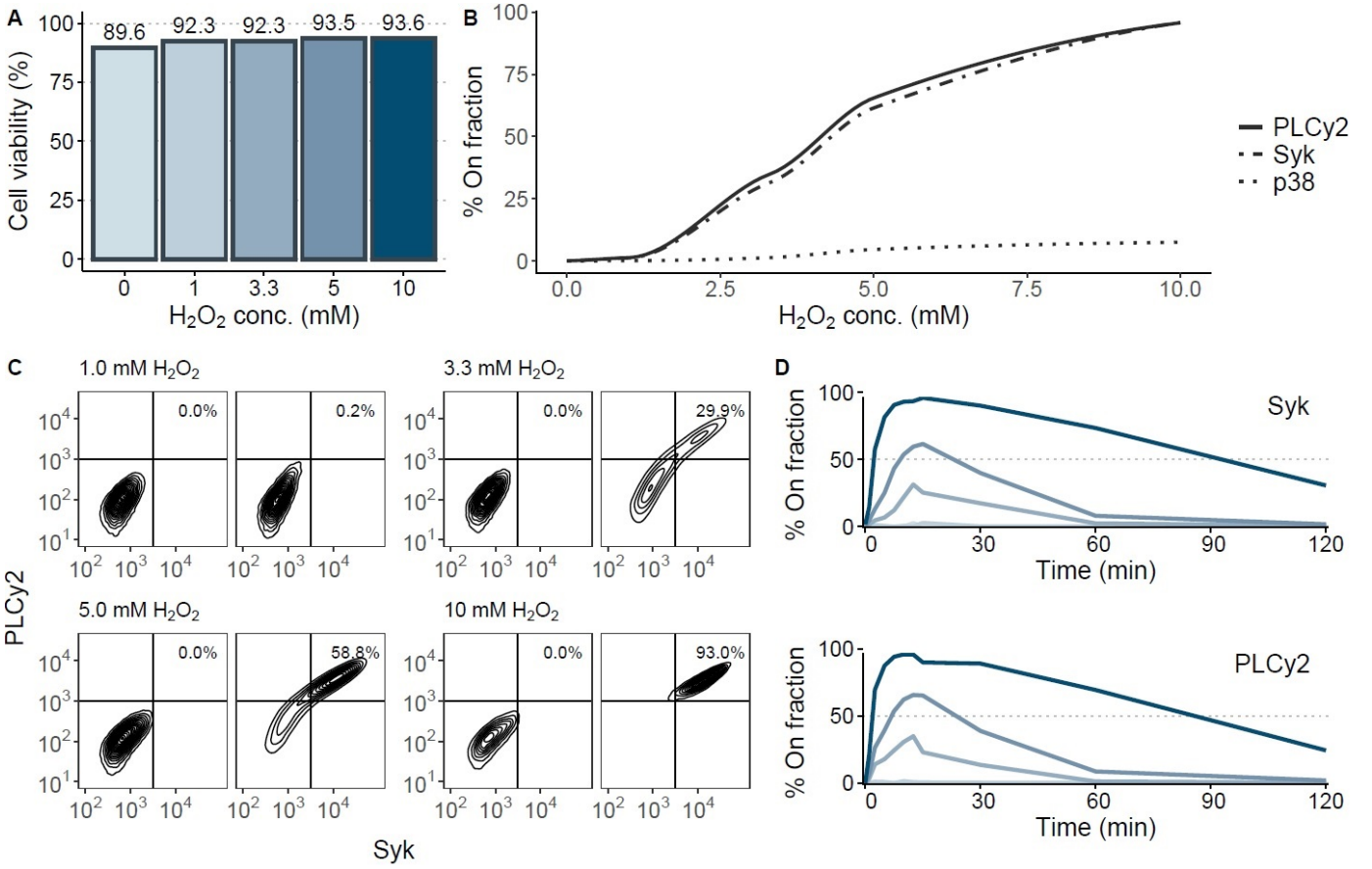

Fig. 3. A) Barplots demonstrating the viability of HBL1 cells after 120 min of incubation with 1 mM, 3.3 mM, 5 mM, and 10 mM H_2O_2. **B)** Dose-response curves of the PLCγ2, Syk, and p38 activation following a step increase stimulation with H_2O_2. **C)** Contour plots showing the bimodal PLCγ2 and Syk activation of a population of HBL1 cells in response to H_2O_2 step increases. **D)** H_2O_2-induced activation of PLCγ2 and Syk in a population of HBL1 cells over a duration of 120 min.

measured. Low concentrations of H_2O_2 induced little activation of CD79a, PLCγ2, and Syk within the HBL1 cells, however, a fold change of the median fluorescence intensity (MFI) that increased with the concentration was observed (appendix fig. 9). This experiment was followed by a step increase experiment with higher H_2O_2 concentrations, 1 mM, 3.3 mM, 5 mM, and 10 mM respectively. Activation of the kinases PLCγ2, Syk, and p38 was measured over a time period of 120 min. The viability of the cells was determined through a measurement of the activated states of apoptosis markers Caspase 3 and Poly(ADP-ribose) polymerase (PARP). Stimulation with H_2O_2 up to 10 mM does not affect the viability of HBL1 cells in a concentration of $2.2 \cdot 10^6$ cells/mL (fig. 3A). H_2O_2 induced a weak activation of the kinase p38 (<10%) at each H_2O_2 concentration. A dose-response activation of Syk and PLCγ2 was measured from H_2O_2 concentrations of 3.3 mM and up. The level of activation increased with the concentration (fig. 3B). When stimulated with 3.3 mM and 5 mM H_2O_2, the HBL1 cells formed bimodal populations (fig. 3C), with part of the population displaying more activated PLCγ2 and Syk, while the other part stayed at the base level. The HBL1 cells had a fast response to H_2O_2, with peak activation being reached at 15 min. This is followed by a slow decline over the course of 60 to 120 min (fig. 3D). A part of the HBL1 cells stimulated with 10 mM H_2O_2 remains active after 120 min, while the HBL1 stimulated with lower concentrations are back at their base level.

The BCR signaling response of HBL1 cells generally follows the input of H_2O_2

In order to assess the signaling response of HBL1 cells to dynamic inputs of H_2O_2, linear gradients to low, intermediate, and high H_2O_2 concentrations (0.125-, 0.25- and 0.5 pmol H_2O_2/cell respectively) were performed, and compared to their corresponding step increases. To analyze the BCR signaling activity in the cells, CD79a, PLCγ2, and Syk activation was measured using flow cytometry. For high H_2O_2 concentrations, a step increase was compared to linear gradients of 20-, 60-, and 90 min, as well as a quadratic gradient of 60 min. The step increases induced strong unimodal activation of the signaling proteins, while HBL1 cells stimulated with high concentration H_2O_2 gradients go through a bimodal phase during the gradient (Fig. 4A). We observed that for step increases and gradients to high concentrations of H_2O_2, the activation of the signaling proteins CD79a, PLCγ2, and Syk generally follows the input of H_2O_2 (Fig. 4B and D). However, the signaling response of HBL1 cells to 60- and 90-min H_2O_2 gradients were similar at each time point. When the final H_2O_2 concentration was reached, each stimulation profile induced approximately the same amount of signaling activation. When analyzing the signaling response of HBL1 cells to high H_2O_2 concentration gradients, we observed that 60 min gradients induce more signaling response than a 20 min gradient during the stimulation, at the intermediate

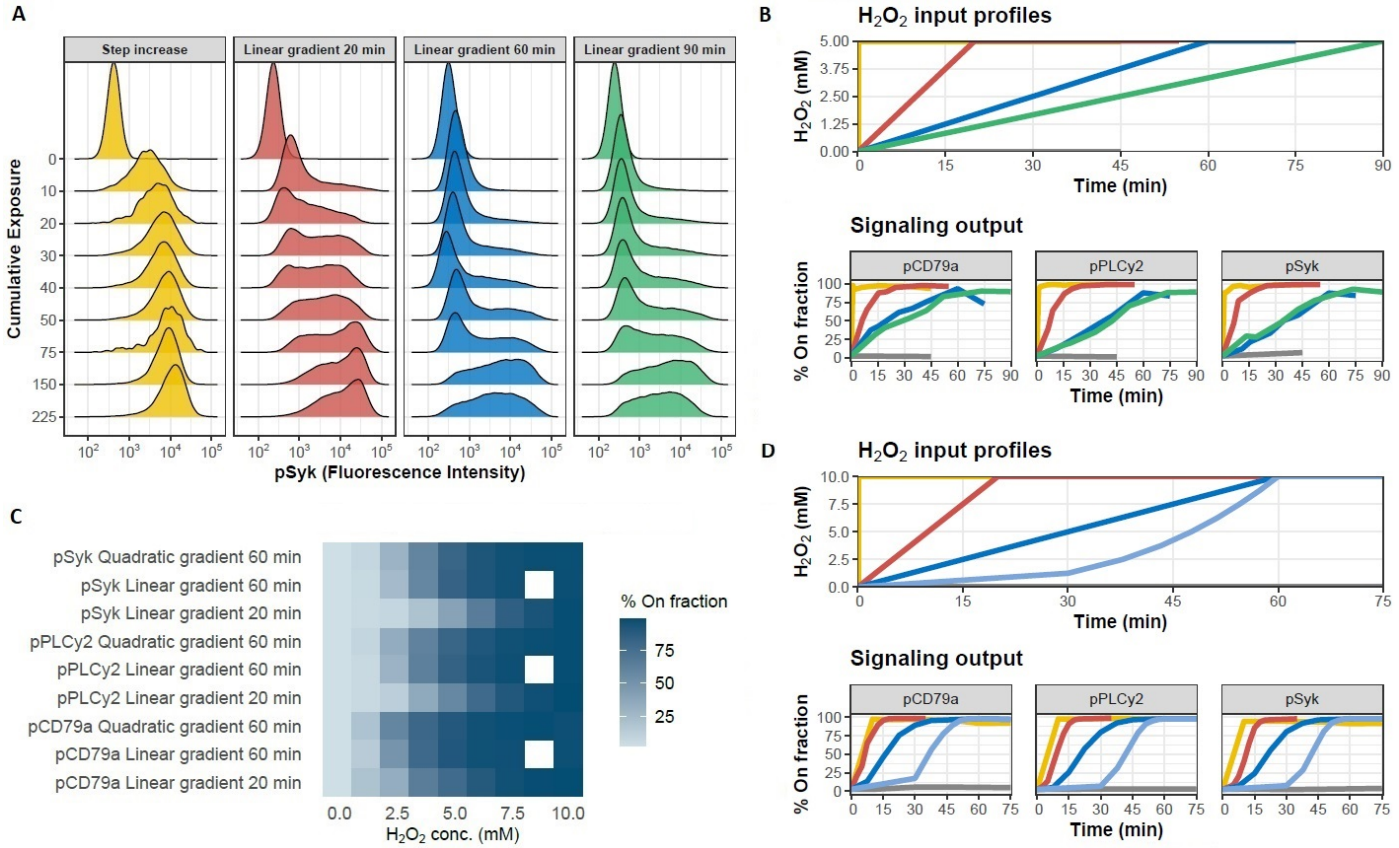

Fig. 4. A) Syk activation patterns of HBL1 cells stimulated with a high concentration of H_2O_2 in a step increase manner, and linear gradients of 20, 60, and 90 min. **B** and **D)** The signaling response of HBL1 cells during stimulation with a high concentration of H_2O_2 in different stimulation profiles. **C)** Heat map of CD79a, PLCγ2, and Syk activation in HBL1 cells during a 20 min linear, 60 min linear and 60 min quadratic gradients to a high H_2O_2 concentration.

concentrations of H_2O_2 (Fig. 4C). Interestingly, the signaling in response of HBL1 cells to a 60 min linear gradient and a 60 min quadratic gradient was approximately the same at each concentration of H_2O_2.

Low- and intermediate H_2O_2 concentrations gradients reveal a rate-dependency in the BCR signaling response of HBL1 cells

The signaling response of HBL1 cells to linear gradients of 20- and 60 min to an intermediate H_2O_2 concentration was compared with a corresponding step increase. The density- and line plots of the CD79a, PLCy2, and Syk activation are shown in figure 5. HBL1 cells stimulated with an intermediate concentration step increase formed a bimodal population between 10 and 30 min, reaching a max CD79a, PLCy2, and Syk activation of approximately 60%. HBL1 cells stimulated with linear gradients to an intermediate H_2O_2 concentration reached a bimodal state near the end of the gradient. The 20 min linear gradient induced an overall lower amount of signaling activation than the step increase. The most activation was observed in HBL1 cells stimulated with a 60 min linear gradient, with approximately 75% being of the cells reaching an activated state 15 min after the end of the gradient.

The signaling response of HBL1 cells to a low H_2O_2 concentration step increase was compared with the signaling response to a low H_2O_2 concentration 60 min linear gradient. The results of this experiment are shown in figure 6. A step increase induced a low signaling response, with CD79a reaching the highest activation, followed by Syk, and lastly PLCγ2. The density plots of this experiment (Fig. 6B) show activation of these signaling proteins in a small fraction of the cell population. Interestingly, adding the same amount of H_2O_2 in a 60 min linear gradient induced no activation of the signaling proteins at all, which implies there is a rate-threshold in the addition of H_2O_2. The max percent of Syk activation in response to gradients with different flow rates to a low concentration of H_2O_2 has been plotted in figure 6C, to demonstrate the relationship between the H_2O_2 flow rate and the signaling response.

Technical improvements in the cell stimulation and fluorescence-activated cell sorting protocol

In order to optimize the cell stimulation, fixation, and flow cytometry protocols, experiments were carried out to distinguish important variables within the methodology. One of these variables is the condition of the cell fixation, specifically the temperature during fixation and the fixation time. An experiment was carried out to determine if the fixation conditions could lead to variation between replicates. Four different fixation conditions were tested, to examine which condition could minimize the variation between replicates. A stimulation protocol with a 10 μg/mL anti-Ig step increase was performed on HBL1 cells. Fixation was done on ice or at RT, for either 15 or 60 min. For each fixation condition, three replicates were performed to get an idea of the variance between the

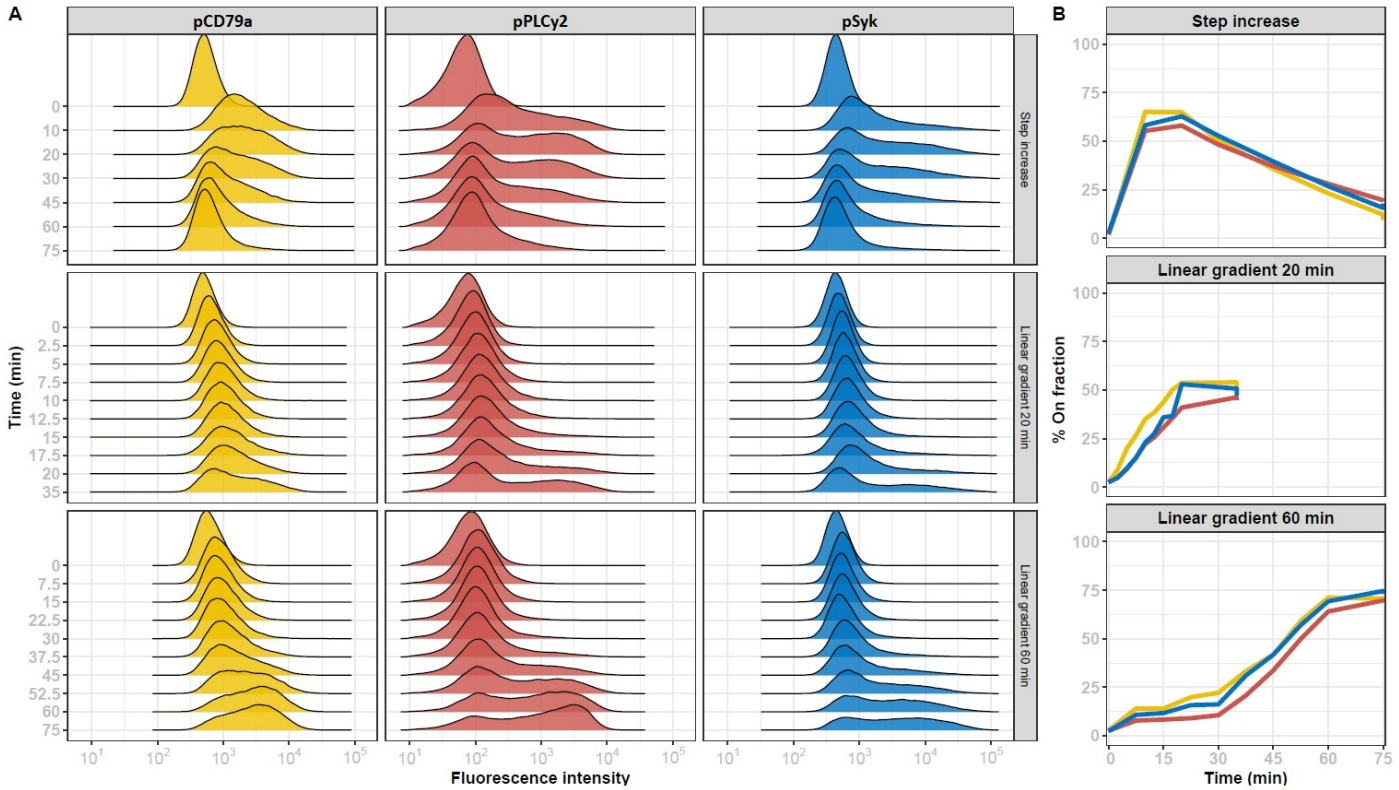

Fig. 5. Signaling response of HBL1 cells following stimulation with an intermediate H_2O_2 concentration in a step increase, 20 min linear gradient, and 60 min linear gradient. **A)** Density plots illustrating the CD79a, PLCγ2, and Syk activation of the HBL1 population. **B)** Line plots of the percentage of CD79a, PLCγ2, and Syk activation over time.

replicates. The standard deviations of the replicates are plotted in appendix figure 8. Another important variable to consider is the cell density that is used in an experiment. Within the cell stimulation experiments of this work, different densities were used based on the needs for the flow cytometry analysis. A control experiment was performed to analyze the effect of the concentration of HBL1 cells on the H_2O_2-induced activation of the signaling proteins. The experiment was done in a 96-wells plate, which was placed in an incubator set at 37°C and 5% CO_2 during the stimulation with H_2O_2. The cell densities were selected based on the densities used in earlier experiments. The results are shown in the appendix figure 12.

DISCUSSION

This study shows the BCR signaling response of the diffuse large B-cell lymphoma cell line HBL1 to stimulation with H_2O_2 in different concentrations, as well as the difference in the signaling response of HBL1 cells stimulated with static H_2O_2 inputs (step increases) and dynamic H_2O_2 inputs (gradients). The BCR signaling proteins CD79a, Syk, PLCγ2, p38, and apoptosis markers Caspase 3 and PARP were analyzed with flow cytometry. These signaling proteins were selected based on their location within the BCR signaling network. Due to an overlap in the emission wavelengths of the fluorophore-conjugated antibodies targeting these signaling proteins, only three BCR signaling proteins could be analyzed simultaneously. The first step of this study was to establish the response of the HBL1 cell line to static inputs H_2O_2, through a series of step increase experiments. BCR signaling of the HBL1 cells was analyzed in response to static input of very low H_2O_2 concentrations (50 µM - 1 mM), intermediate H_2O_2 concentrations (3.3 mM - 5 mM), and a high H_2O_2 concentration (10 mM). In the step increase experiments, we analyzed the activation of the BCR signaling proteins PLCγ2, Syk, and p38. Step increases with very low H_2O_2 concentrations induced little to no activation of the signaling proteins PLCγ2, Syk, and p38. However, within this range the measured MFI of the signaling proteins did increase with the H_2O_2 concentration, demonstrating a small amount of phosphatase inhibition within the cells. The results imply that the antioxidant capacity within the HBL1 cells is high enough to be able to decompose H_2O_2 concentrations below 1 mM and stop any significant inhibition of their phosphatases, which prevents additional activation of the BCR signaling network. At intermediate and high H_2O_2 concentrations, step increases induced activation of Syk and PLCγ2, which increased with the H_2O_2 concentration. The kinase p38, which is located all the way downstream in the BCR signaling network, had a lower response to H_2O_2. This lower response can be expected, since p38 is not directly inhibited by phosphatases (appendix fig. 7). We observed that step increases with intermediate concentrations of H_2O_2 results in the formation of bimodal populations, where one population of cells stays at the base level of signaling, while the other population reaches an activated signaling state. In our work, increasing the H_2O_2 of the step increase to a high concentration (10 mM) steered the HBL1 towards a strong, unimodal phosphoresponse. After 120 min of incubation with H_2O_2, the cell viability was still above 90% for each analyzed concentration. This implies that the bimodal response originates from a variability in the response threshold of the HBL1 cells,

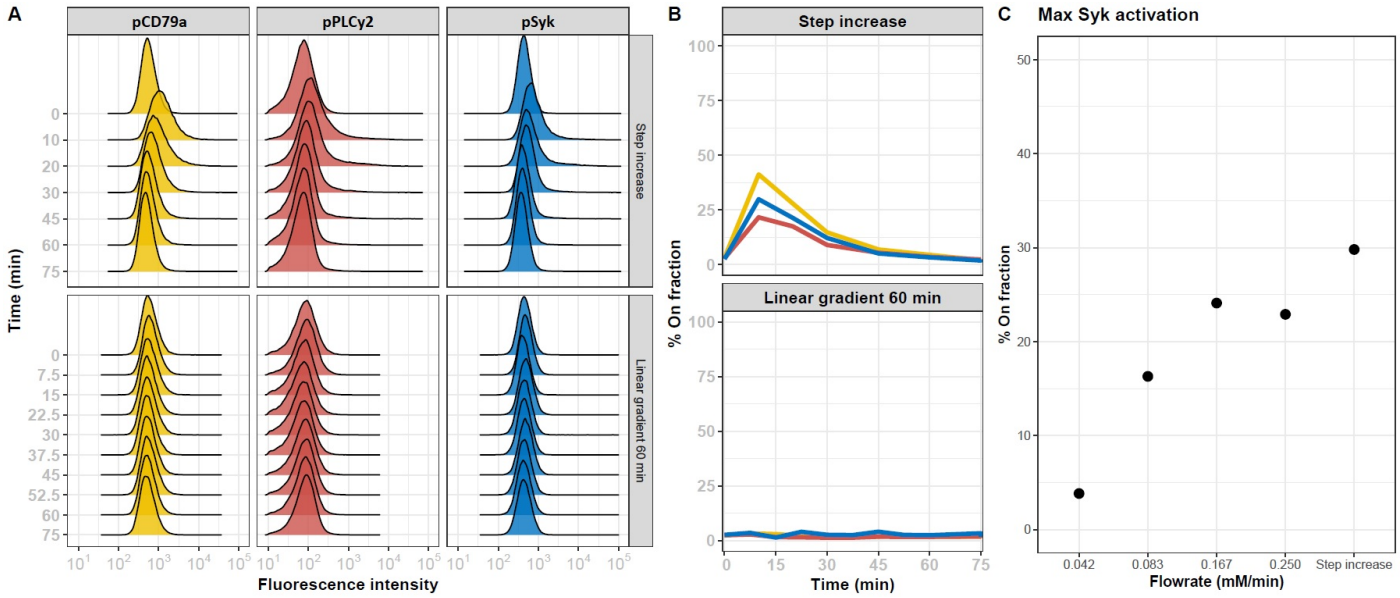

Fig. 6. Signaling response of HBL1 cells following stimulation with a low H_2O_2 concentration in a step increase and 60 min linear gradient. **A)** Density plots illustrating the CD79a, PLCγ2, and Syk activation of the HBL1 population. **B)** Line plots of the percentage of CD79a, PLCγ2, and Syk activation over time. **C)** The max percent Syk activation in HBL1 cells stimulated with different flow rates of H_2O_2 to low concentration of 0.125 pmol H_2O_2/cell.

rather than cell death. Heterogeneity in the phosphoresponse of CLL cells to intermediate concentrations of H_2O_2 has been reported in earlier work [11, 20, 23, 29], however this behavior was not yet observed in DLBCL cells. Ziegler *et al* disclosed that this bimodal response originates from the clustering of the B cell receptors and positive feedback through the kinases Syk and Lyn.

Although step increases with stimulants are a convenient way to study the cellular signaling response, it is important that the signaling response is also analyzed in a dynamic environment that more closely represents the *in vivo* microenvironment. In the microenvironment, it is unlikely that cells get exposed to an instantaneous increase of a stimulant. Studies have shown the importance of analyzing the cell signaling response to gradients of stimulants, stressors and nutrients, demonstrating that rate-thresholds in cell signaling have functional and phenotypic consequences in a variety of cells [27]. Malignant cells have an upregulation of antioxidants due to their increased metabolism [18]. We hypothesized that this upregulation of antioxidants could mean that when the flow rate of a H_2O_2 gradient is low enough, the HBL1 cells have enough time to metabolize the H_2O_2, and prevent activation of their signaling networks. To assess this hypothesis, we stimulated HBL1 cells with H_2O_2 in a variety of linear and quadratic gradients to low concentrations (0.125 pmol H_2O_2/cell), intermediate concentrations (0.25 pmol H_2O_2/cell) and high concentrations (0.5 pmol H_2O_2/cell), and compared the phosphoresponse to that of HBL1 cells stimulated with corresponding step increases. Our results show that HBL1 cells indeed have a different signaling response to H_2O_2 step increases and H_2O_2 gradients.

For high inputs of H_2O_2, we compared a step increase with linear gradients of 20 min, 60 min and 90 min, and a quadratic gradient of 60 min. In the density plots of these experiments, we notice differences in the activation patterns of the cell populations between the stimulation conditions. Step increases of high H_2O_2 concentrations induce a strong, unimodal signaling response, as we have shown in our step increase experiments as well. However, when stimulated with high concentration H_2O_2 gradients, the HBL1 cells go through a bimodal phase during the intermediate concentrations of the gradient. In one of these experiments, in which a cell density of $1 \cdot 10^6$ cells/mL was used, broad distributions of the PLCy2 and Syk signaling activity were formed in the cell populations stimulated with 60- and 90 min linear gradients. More interestingly, when we doubled the cell concentration, as well as the concentration of the gradients, we observed a more unimodal signaling response at the end of the gradient. It appears that the low-responding population of HBL1 cells needs a higher extracellular concentration of H_2O_2 to reach a similar level of activation as the high-responding population. More experiments with lower cell densities have to be performed to further explain this signaling behavior. For gradients to high H_2O_2 concentrations, we show that over time, the activation of the signaling proteins CD79a, PLCγ2, and Syk follows the input of the H_2O_2. When examining the activation of the signaling proteins at each concentration of H_2O_2 during the linear gradients, we observed that linear gradients with a lower flow rate induced more activation of the signaling proteins at the intermediate concentrations of H_2O_2. When the final concentration was reached, each H_2O_2 gradient induced approximately the same amount of activation as the H_2O_2 step increase. Moreover, although having very different input profiles, a 60 min linear gradient and 60 min quadratic gradient induced the same amount of signaling activation at each concentration of H_2O_2.

From the signaling response of our HBL1 cells stimulated with high concentration H_2O_2 gradients, we can observe that the slower gradients induce more signaling at the intermediate H_2O_2 concentrations during the gradient. The next step was to investigate the signaling response of HBL1 cells to gradients that

stop at these intermediate of H_2O_2. We compared the signaling response of HBL1 cells to intermediate H_2O_2 concentrations in a step increase, 20 min linear gradient, and 60 min linear gradient. As seen during our high concentration gradients, a 60 min linear gradient induced more signaling response than the 20 min linear gradient at intermediate concentrations of H_2O_2. During the first half of the 60 min linear gradient to an intermediate H_2O_2 concentration, the signaling response was still very low ($\approx$ 20%). During the second half of this gradient, the signaling response rose quickly, to an even higher value than the corresponding step increase. Interestingly, the linear gradient of 20 min induced less activation than the step increase. This implies that the BCR signaling of HBL1 cells in response to stimulation with H_2O_2 is rate-sensitive activation, with some flowrates inducing a higher signaling response, and others a lower response. To further investigate this rate-sensitive activation, we analyzed the signaling response to a low-concentration H_2O_2 step increase, with the signaling response to a low-concentration H_2O_2 60 min linear gradient. This gradient had the lowest flow rate that we had tested (0.042 mM/min). We observed a low amount of activation of the signaling proteins CD79a, PLCγ2, and Syk, following the low-concentration H_2O_2 step increase. The 60 min linear induced no activation at all, implying there is, in addition to a rate-sensitive behavior, a rate threshold as well. It could well be that adding H_2O_2 to the HBL1 cells with a low enough flow rate, gives these cells enough time to decompose the H_2O_2 and prevent activation of their signaling network. Data from multiple gradients with different flow rates, that surpassed this low H_2O_2 concentration (0.125 pmol H_2O_2/cell), was gathered. The max percent activation of the signaling proteins CD79a, PLCγ2, and Syk of each gradient at this low concentration was analyzed. In this analysis, we observed that for low concentrations of H_2O_2, activation of PLCγ2 and Syk decreased with the flow rate. Activation of CD79a was less dependent on flow rate.

CONCLUSIONS

This work showed the differences in the BCR signaling response of HBL1 cells stimulated with H_2O_2 step increases, linear gradients, and quadratic gradients. The BCR signaling response is dose-dependent and bimodal, with one part of the cell population not responding to low- and intermediate concentrations of H_2O_2. Stimulation with gradients to low- and intermediate concentrations of H_2O_2 revealed a rate threshold and rate sensitivity in the H_2O_2-induced BCR signaling of HBL1 cells.

FUTURE OUTLOOK

Our work demonstrate that BCR signaling of DLBCL cells in response to H_2O_2, is rate sensitive and has a rate-threshold. In future work, we can use our syringe pump system to precisely emulate the flow rate of H_2O_2 within the microenvironment of tumor cells. BCR stimulation could be performed with perfusion cell culture systems that contain a cell retention system. Such systems allow for oscillating gradients, to mimic the microenvironment even better. Gradients of H_2O_2 can eventually be combined with drugs such as small molecule inhibitors, to gain a better understanding of the response of DLBCL cells to these drugs in an environment that more closely resembles the microenvironment.

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REFERENCES

1. P van der Geer. Signal Transduction. pages 436–439. Academic Press, 2013.
2. Ayse Basak Engin and Atilla Engin. Protein kinase-mediated decisions between life and death, 2021.
3. Howard Schulman. Chapter 12 - Intracellular Signaling. pages 335–370. Academic Press, Burlington, 2004.
4. Frank McCormick. Small-molecule inhibitors of cell signaling. *Current Opinion in Biotechnology*, 11:593–597, 2000.
5. Ruei-Min Lu, Yu-Chyi Hwang, I-Ju Liu, Chi-Chiu Lee, Han-Zen Tsai, Hsin-Jung Li, and Han-Chung Wu. Development of therapeutic antibodies for the treatment of diseases. *Journal of Biomedical Science*, 27, 2020.
6. Libin Abraham, Jia Wang, Madison Bolger-Munro, and Michael Gold. Structure, Function, and Spatial Organization of the B Cell Receptor. *Encyclopedia of Immunobiology*, 2016.
7. Franklin R Toapanta, Paula J Bernal, and Marcelo B Sztein. Diverse phosphorylation patterns of B cell receptor-associated signaling in naïve and memory human B cells revealed by phosphoflow, a powerful technique to study signaling at the single cell level. *Frontiers in cellular and infection microbiology*, 2, 2012.
8. Carsten U Niemann and Adrian Wiestner. B-cell receptor signaling as a driver of lymphoma development and evolution. *Seminars in Cancer Biology*, 23:410–421, 2013.
9. Ondrej Havranek, Stefan Koehrer, Jingda Xu, Lisa Becker, Justin M Comer, Anusha R Karri, Zhiqiang Wang, Wencai Ma, Jason R Westin, Tomasz Zal, Jan A Burger, and R Eric Davis. B-Cell Receptor Signaling in Diffuse Large B-Cell Lymphoma: Tonic Alone in the Germinal Center B-Cell Subtype, Plus Self Antigen-Induced in the Activated B-Cell Subtype. *Blood*, 126, 2015.
10. Dimitar G Efremov, Sven Turkalj, and Luca Laurenti. Mechanisms of B Cell Receptor Activation and Responses to B Cell Receptor Inhibitors in B Cell Malignancies. *Cancers*, 12, 2020.
11. Chiara Cavallini, Roberto Chignola, Ilaria Dando, Omar Perbellini, Elda Mimiola, Ornella Lovato, Carlo Laudanna, Giovanni Pizzolo, Massimo Donadelli, and Maria Teresa Scupoli. *Blood*, pages 1942–1954.
12. Jennifer N Moloney and Thomas G Cotter. ROS signalling in the biology of cancer. *Seminars in cell & developmental biology*, 80:50–64, 2018.
13. K Brieger, S Schiavone, F J Jr Miller, and K-H Krause. Reactive oxygen species: from health to disease. *Swiss medical weekly*, 142, 2012.
14. Christine C Winterbourn. The biological chemistry of hydrogen peroxide. *Methods in enzymology*, 528:3–25, 2013.
15. Ling Wang, Zheng Kuang, Duo Zhang, Yifan Gao, Mingzhen Ying, and Tengjiao Wang. Reactive oxygen species in immune cells: A new antitumor target. *Biomedicine & Pharmacotherapy*, 133, 2021.
16. Yugang Wang, Yan Xia, and Zhimin Lu. Metabolic features of cancer cells., 2018.
17. Claudia Lennicke, Jette Rahn, Rudolf Lichtenfels, Ludger A Wessjohann, and Barbara Seliger. Hydrogen peroxide - production, fate and role in redox signaling of tumor cells. *Cell communication and signaling : CCS*, 13, 2015.
18. Krzysztof Domka, Agnieszka Goral, and Malgorzata Firczuk. cROSsing the Line: Between Beneficial and Harmful Effects of Reactive Oxygen Species in B-Cell Malignancies. *Frontiers in immunology*, 11, 2020.
19. Nada S Aboelella, Caitlin Brandle, Timothy Kim, Zhi-Chun Ding, and Gang Zhou. Oxidative Stress in the Tumor Microenvironment and Its Relevance to Cancer Immunotherapy. *Cancers*, 13, 2021.

20. M Lia Palomba, Kelly Piersanti, Carly G K Ziegler, Hugo Decker, Jesse W Cotari, Kurt Bantilan, Ivelise Rijo, Jeff R Gardner, Mark Heaney, Debra Bemis, Robert Balderas, Sami N Malek, Erlene Seymour, Andrew D Zelenetz, Marcel R M van den Brink, and Grégoire Altan-Bonnet. Multidimensional single-cell analysis of BCR signaling reveals proximal activation defect as a hallmark of chronic lymphocytic leukemia B cells. *PloS one*, 9, 2014.
21. Christine Wolf, Carsten Maus, Michael R O Persicke, Katharina Filarsky, Eugen Tausch, Christof Schneider, Hartmut Döhner, Stephan Stilgenbauer, Peter Lichter, Thomas Höfer, and Daniel Mertens. Modeling the B-cell receptor signaling on single cell level reveals a stable network circuit topology between nonmalignant B cells and chronic lymphocytic leukemia cells and between untreated cells and cells treated with kinase inhibitors. *International journal of cancer*, 151:783–796, 2022.
22. Kinetics of B cell receptor signaling in human B cell subsets mapped by phosphospecific flow cytometry. *Journal of immunology (Baltimore, Md. : 1950)*, 177:1581–1589, 2006.
23. Adam L Palazzo, Erik Evensen, Ying-Wen Huang, Alessandra Cesano, Garry P Nolan, and Wendy J Fantl. Association of reactive oxygen species-mediated signal transduction with in vitro apoptosis sensitivity in chronic lymphocytic leukemia B cells. *PloS one*, 6, 2011.
24. Benoit Sorre, Aryeh Warmflash, Ali H. Brivanlou, and Eric D. Siggia. Encoding of Temporal Signals by the TGF-β Pathway and Implications for Embryonic Patterning. *Developmental Cell*, 30:334–342, 2014.
25. Alexander Thiemicke, Hossein Jashnsaz, Guoliang Li, and Gregor Neuert. Generating kinetic environments to study dynamic cellular processes in single cells. *Scientific reports*, 9, 2019.
26. Amanda N Johnson, Guoliang Li, Hossein Jashnsaz, Alexander Thiemicke, Benjamin K Kesler, Dustin C Rogers, and Gregor Neuert. A rate threshold mechanism regulates MAPK stress signaling and survival. *Proceedings of the National Academy of Sciences of the United States of America*, 118, 2021.
27. Alexander Thiemicke and Gregor Neuert. Rate thresholds in cell signaling have functional and phenotypic consequences in non-linear time-dependent environments. *Frontiers in cell and developmental biology*, 11, 2023.
28. L J Bugaj, A J Sabnis, A Mitchell, J E Garbarino, J E Toettcher, T G Bivona, and W A Lim. Cancer mutations and targeted drugs can disrupt dynamic signal encoding by the Ras-Erk pathway. *Science (New York, N.Y.)*, 361, 2018.
29. Carly G K Ziegler, Joel Kim, Kelly Piersanti, Alon Oyler-Yaniv, Kimon V Argyropoulos, Marcel R M van den Brink, M Lia Palomba, Nihal Altan-Bonnet, and Grégoire Altan-Bonnet. Constitutive Activation of the B Cell Receptor Underlies Dysfunctional Signaling in Chronic Lymphocytic Leukemia. *Cell reports*, 28:923–937, 2019.
30. Cell Signaling Technology. B Cell Receptor Signaling, 2014.

APPENDIX

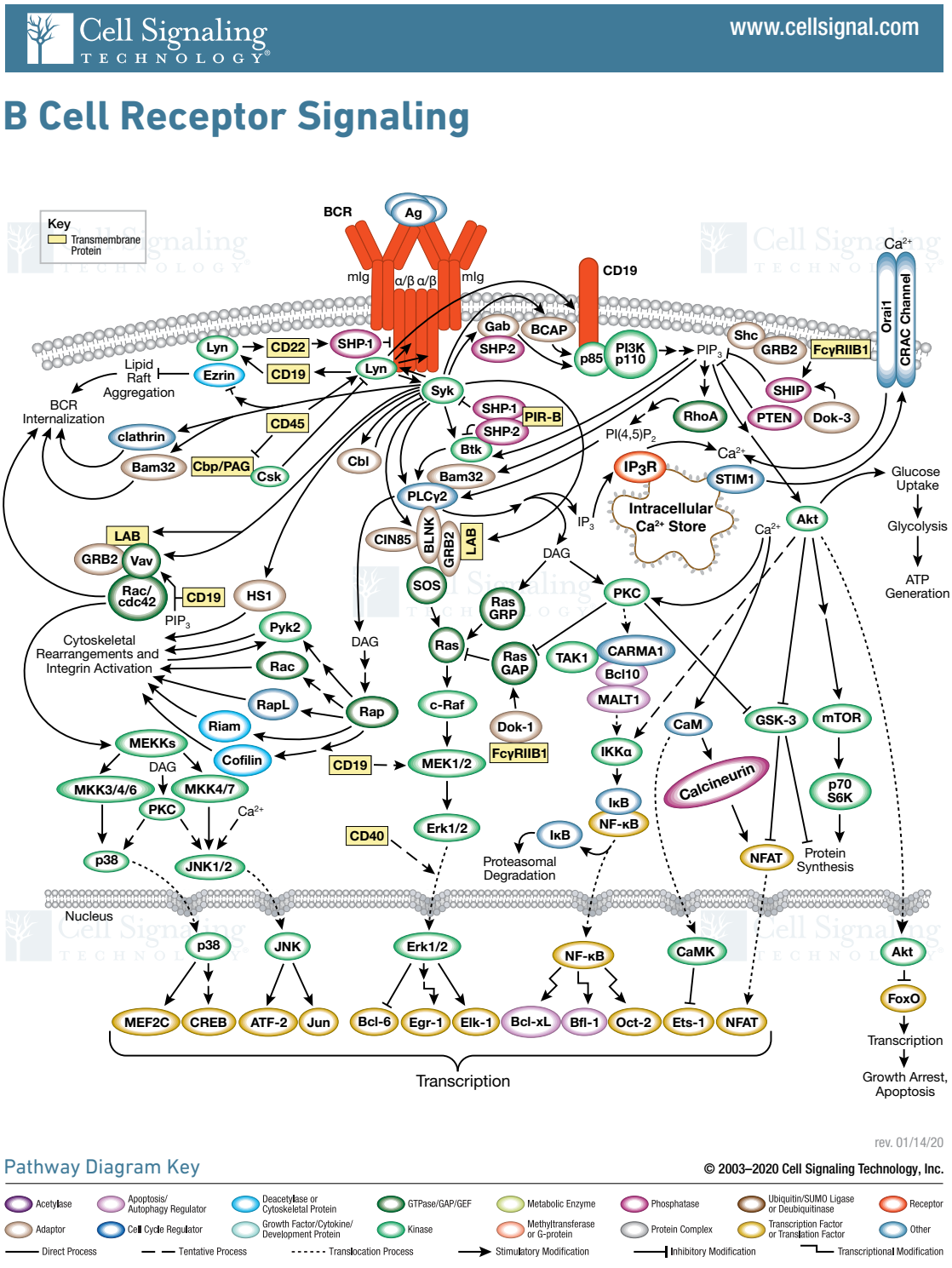

Fig. 7. The BCR signaling pathway. Revised by Cell Signaling Technology Inc. in 2014 [30].

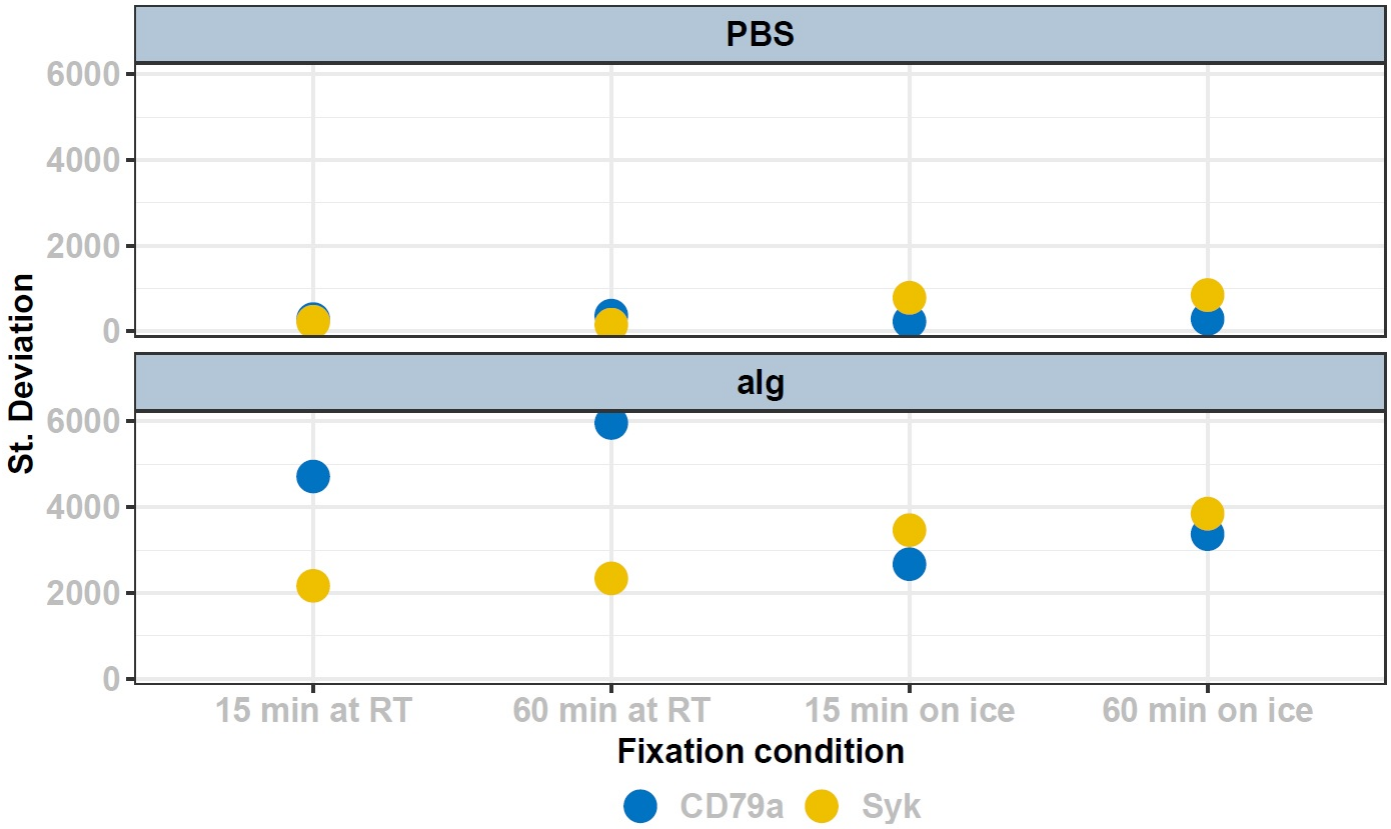

Fig. 8. The standard deviation between three replicates of stimulated (aIg) and unstimulated (PBS) HBL1 cells, fixated on ice or at RT, for 15 or 60 minutes.

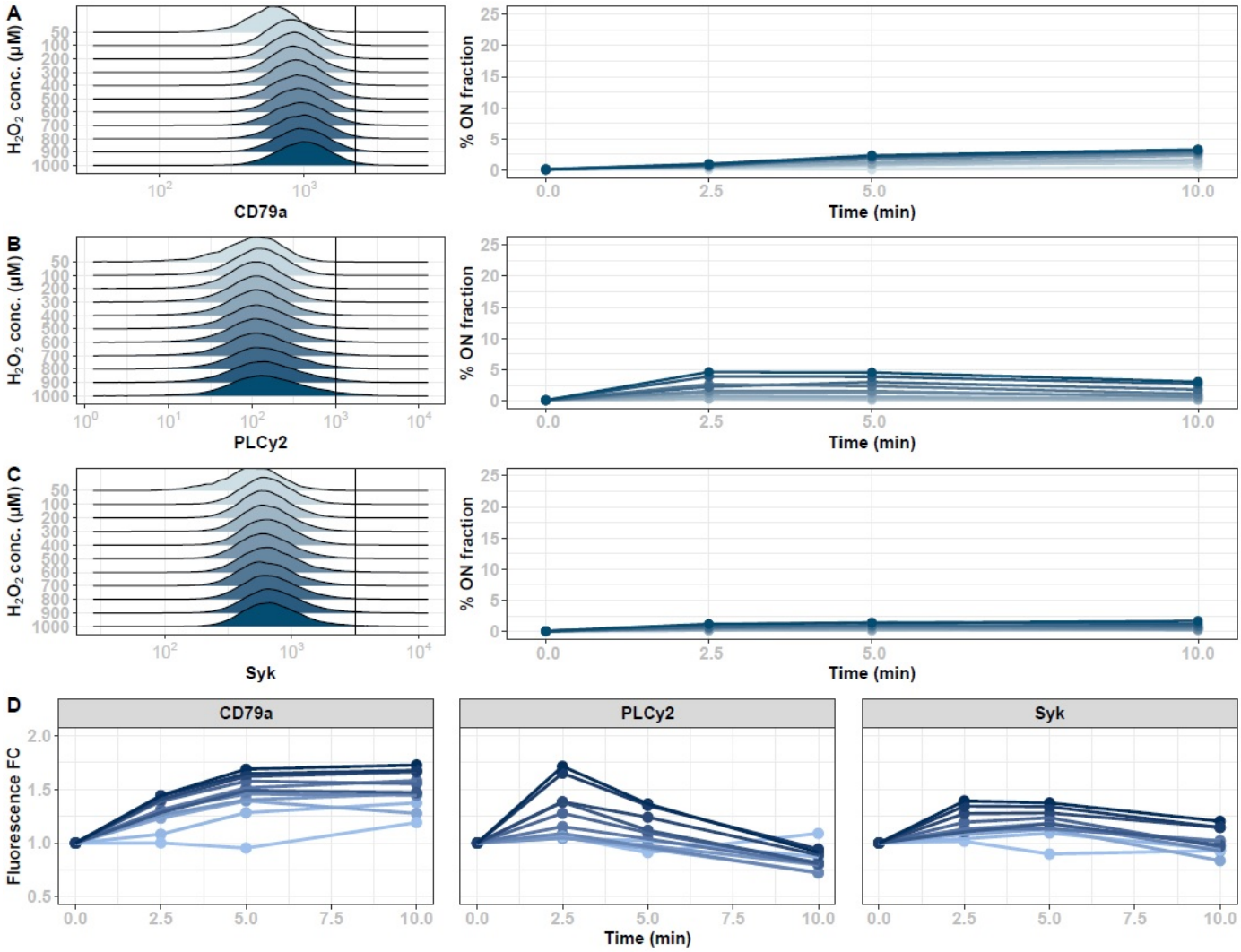

Fig. 9. The signaling response of HBL1 cells stimulated with step increases of H_2O_2 in concentrations ranging from 50 μM to 1000 μM.

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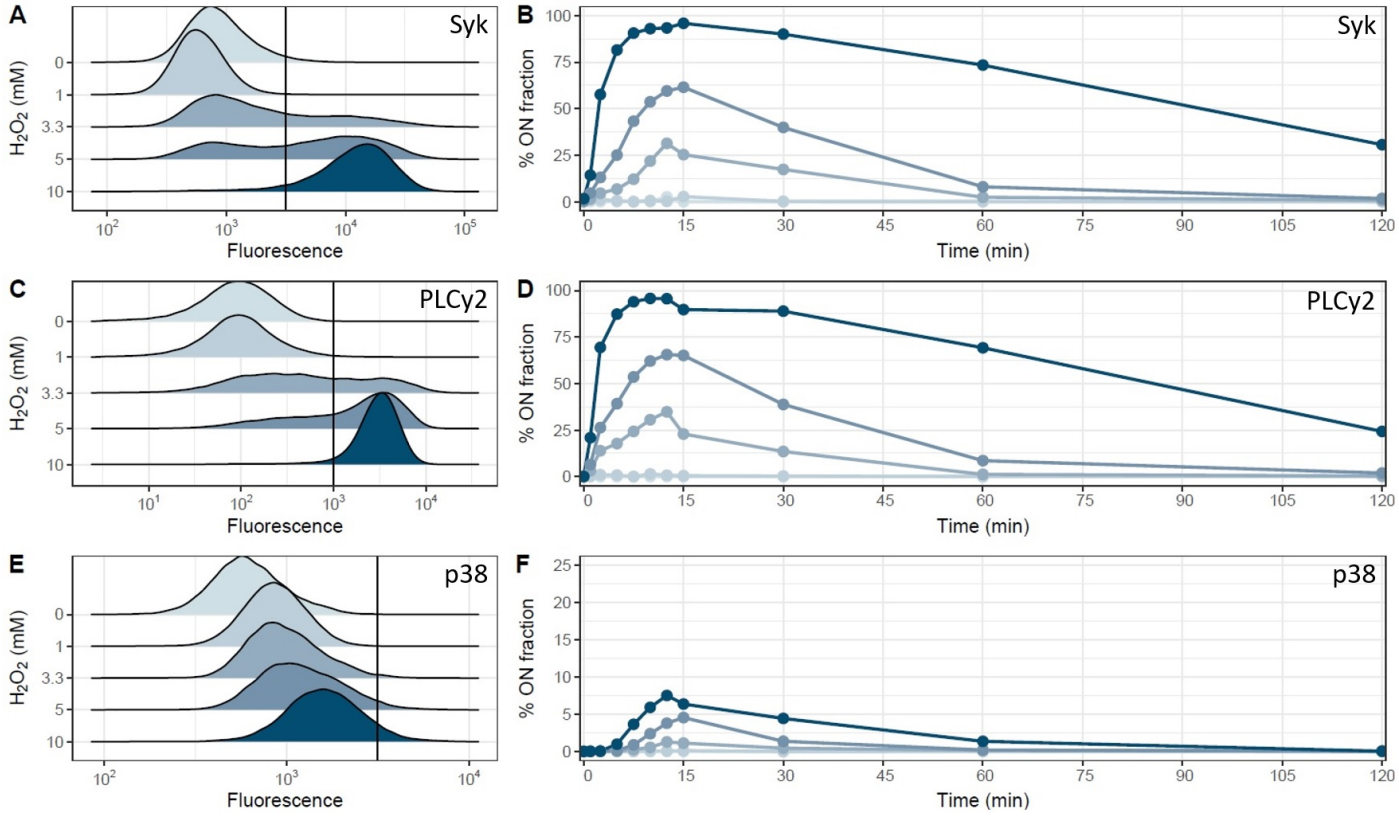

Fig. 10. Density plots and line plots of the activation of the signaling proteins Syk (**A, B**), PLCγ2 (**C, D**) and p38 (**E, F**). The density plots display the signaling proteins in HBL1 cells after 12.5 min of incubation with H_2O_2.

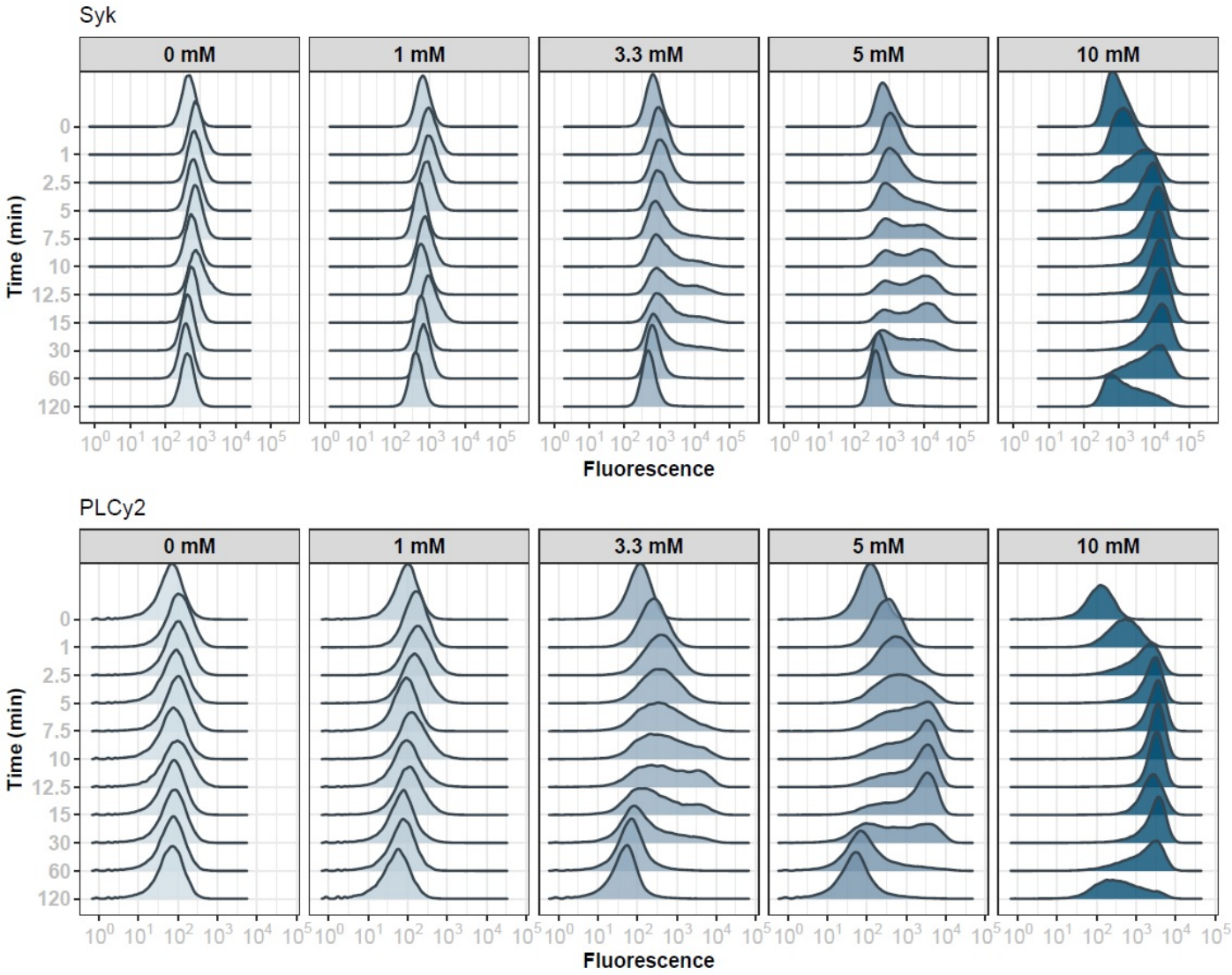

Fig. 11. Density plots demonstrating the activation of PLCγ2 and Syk within a population HBL1 cells, in response to step increases with H_2O_2 concentrations ranging from 1 to 10 mM.

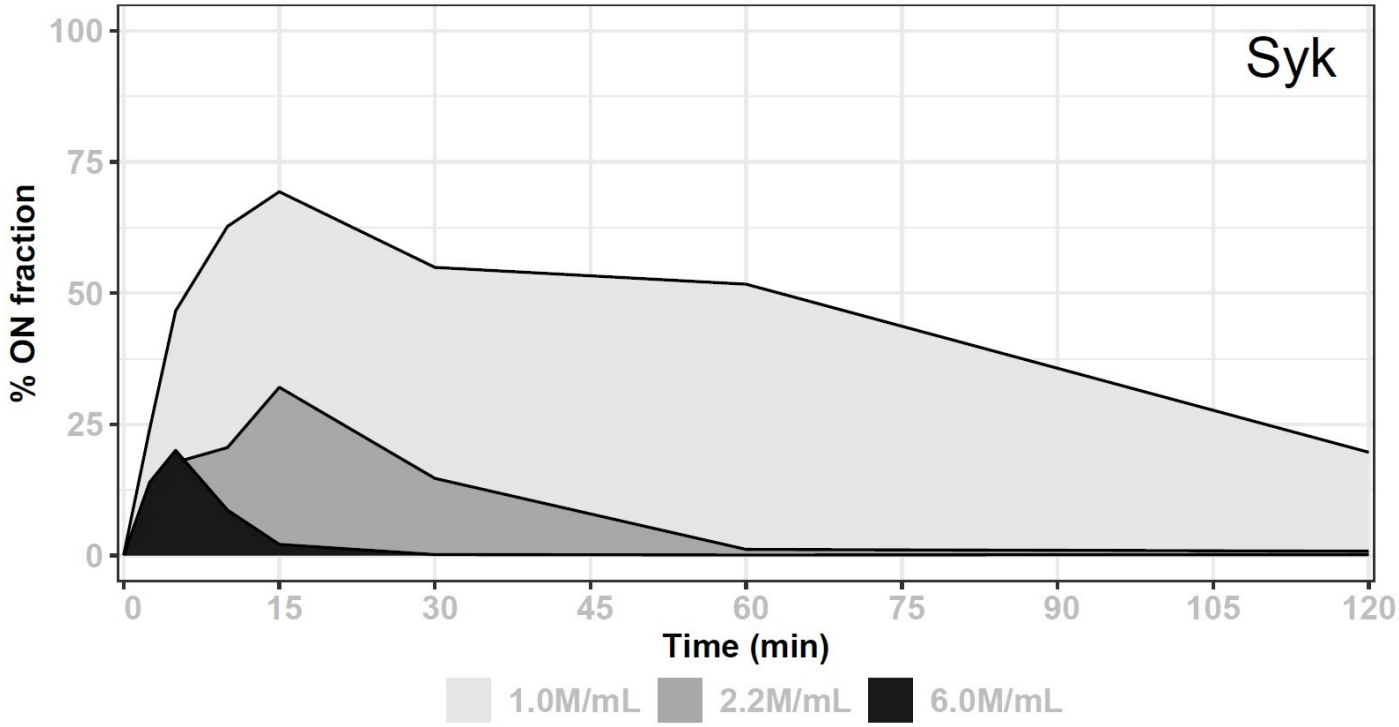

Fig. 12. The effect of cell concentration on the H_2O_2-induced activation of the signaling protein Syk. HBL1 cells in concentrations of 1.0-, 2.2-, and $6.0 \cdot 10^6$ cells/mL were stimulated with a 5 mM H_2O_2 step increase.

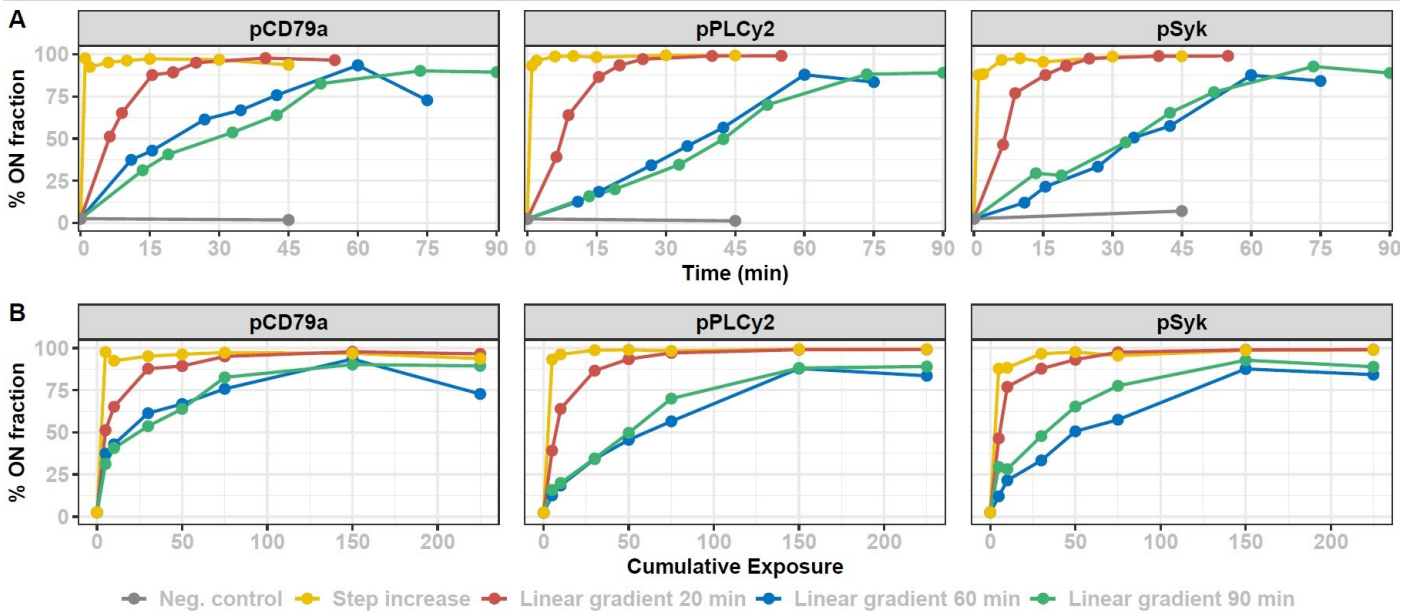

Fig. 13. Syk, PLCγ2 and CD79a activation in HBL1 cells exposed to 5 mM H_2O_2 in either a step increase, 20 min linear gradient, 60 min linear gradient or 90 min linear gradient. **A)** Protein activation plotted against time. **B)** Protein activation plotted against cumulative exposure.

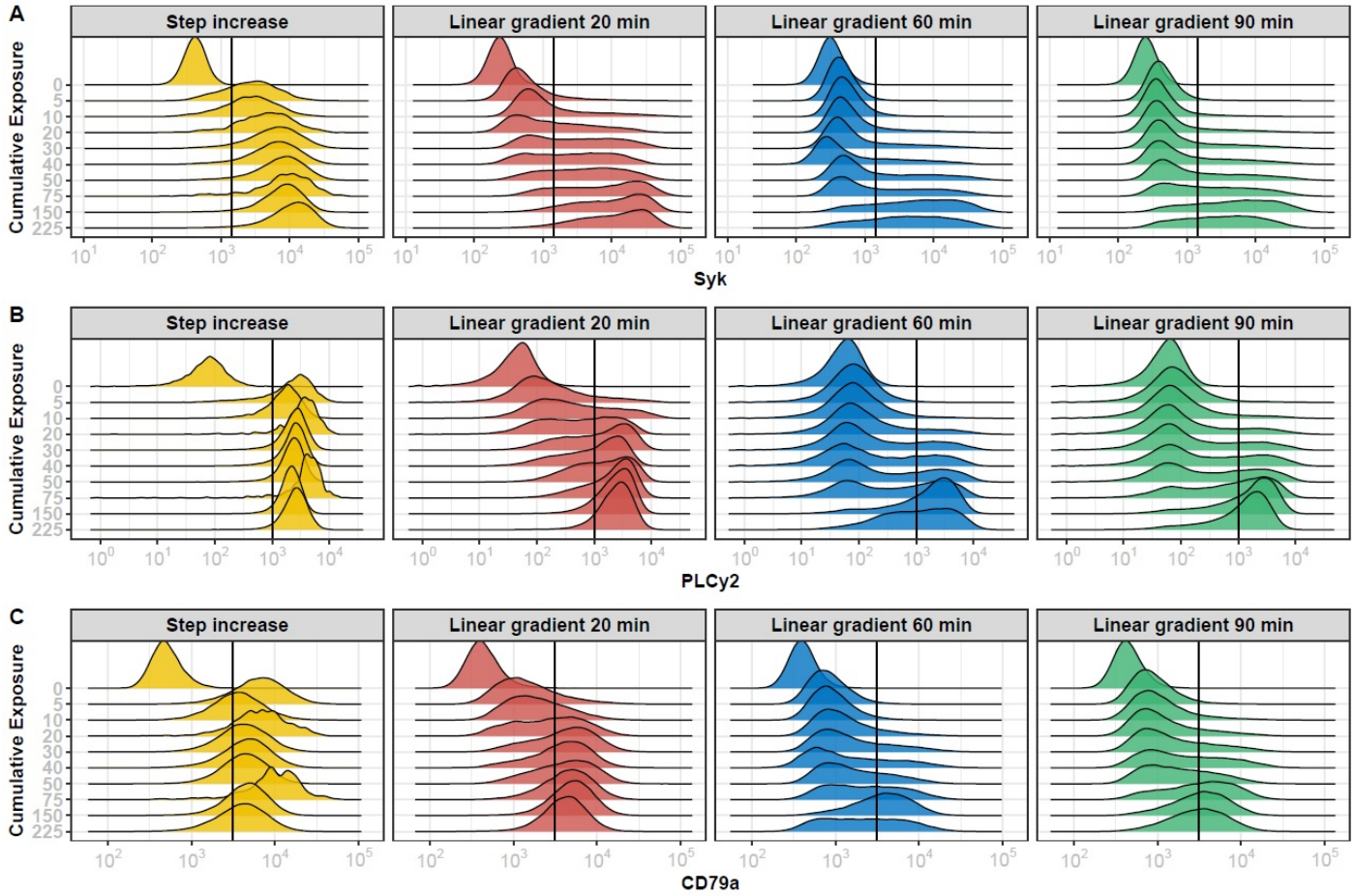

Fig. 14. Density plots corresponding to the results shown in figure 13, displaying the activation of Syk, PLCγ2 and CD79a in HBL1 cells after stimulation with 0.5 pmol H_2O_2/cell added in either a step increase or linear gradient.

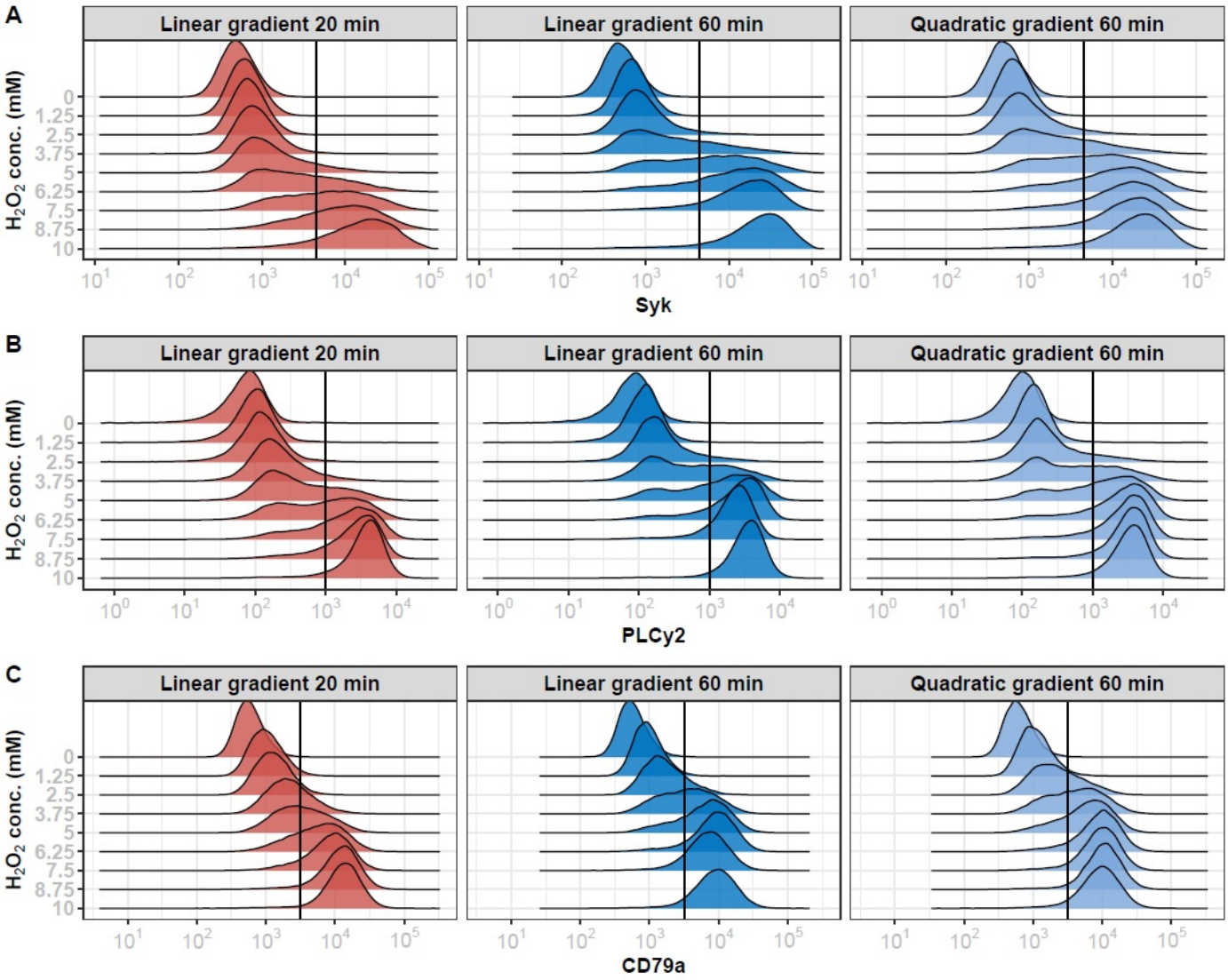

Fig. 15. Density plots corresponding to the results shown in figure **??**, displaying the activation of Syk, PLCγ2 and CD79a in HBL1 cells after stimulation with 0.5 pmol H_2O_2/cell added in either a step increase, linear gradient, or quadratic gradient.

Table 1. Fluorophores used to stain the activated signaling proteins

Target	Fluorophore	Product	Manufacturer	Cat.No.	Isotype	Excitation	Emission	Dilution
PARP	Brilliant Violet 421	BV421 Mouse Anti-Cleaved PARP (Asp 214)	BD Biosciences	564129	Mouse IgG1	405 nm	423 nm	1/500
Caspase 3	Violet Proliferation 450	V450 Rabbit Anti-Active Caspase-3	BD Biosciences	560627	Rabbit IgG	405 nm	450 nm	1/500
pCD79a	Alexa Fluor 488	Phospho-CD79A (Tyr182) (D1B9) Rabbit mAb (Alexa Fluor® 488 Conjugate)	Cell Signaling	52821	Rabbit IgG	490 nm	525 nm	1/250
pp38	Alexa Fluor 488	Phospho-p38 MAPK (Thr180/Tyr182) (3D7) Rabbit mAb (Alexa Fluor® 488 Conjugate)	Cell Signaling	41768	Rabbit IgG	490 nm	525 nm	Jan/50
pSyk	Phycoerythrin	Phospho-Syk (Tyr525/526) (C87C1) Rabbit mAb (PE Conjugate)	Cell Signaling	6485	Rabbit IgG	565 nm	573 nm	1/250
pPLCγ2	Alexa Fluor 647	Alexa Fluor® 647 Mouse anti-PLC-2 (pY759)	BD Biosciences	558498	Rabbit IgG	653 nm	669 nm	1/100