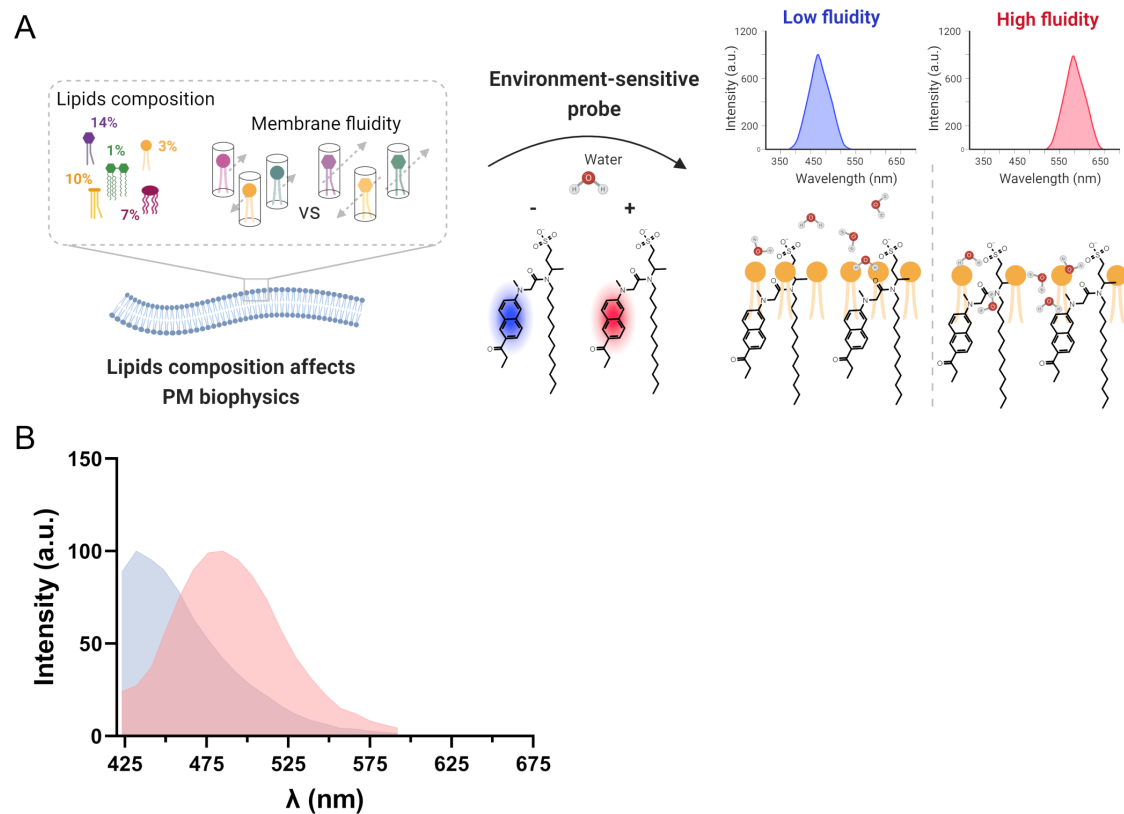


Plasma membrane order maps functional diversity in immune cells

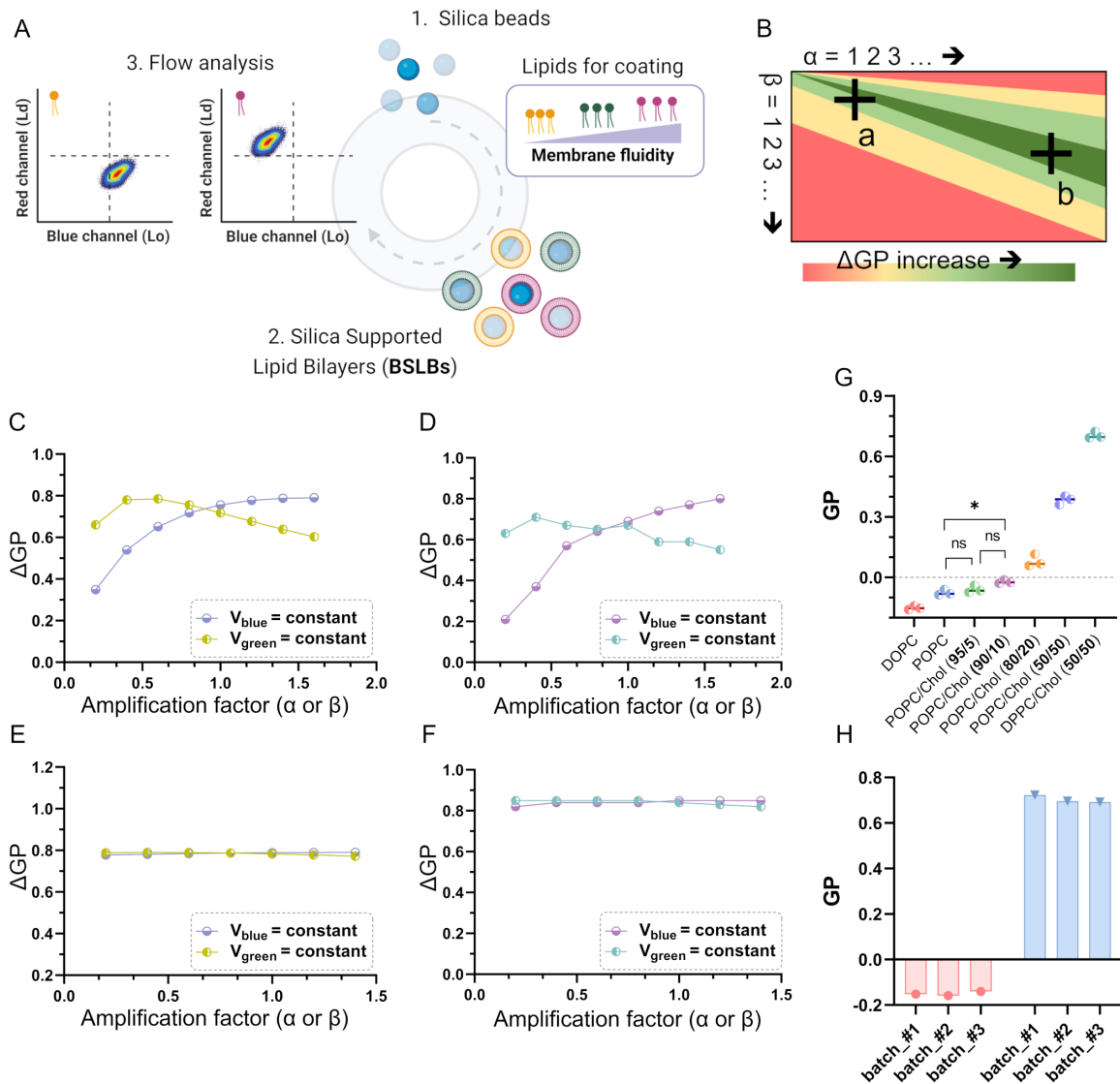
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Supplementary Figure 1. Pro12A's emission shift reflects local PM Order. **A** Schematic of the mechanism for environment sensing for Pro12A dye. **B** Emission spectra of Pro12A staining silica beads coated with either low (blue curve, lipids composition = DPPC:Cholesterol 50:50%) or high (red curve, lipids composition DOPC 100%) order membranes.



Supplementary Figure 2. Optimized flow parameters allow to resolve small differences in PM Order of BSLBs. **A** Schematic of the synthesis of Silica Supported Lipid Bilayers (BSLBs) used for optimization of the flow setup. **B** Theoretical dependence of ΔGP on the amplification factors of the green (α) and blue (β) channels, calculated from the emission spectra of Pro12A in either low (DPPC:Cholesterol 1:1) or high (DOPC) order membranes. The black crosses indicate regions of constant high ΔGP at either low (a) or high (b) absolute intensities. **C-F** Theoretical (**C**, **E**) and experimental (**D**, **F**) ΔGP values as a function of the amplification factor within the region a (**C**, **D**) or b (**E**, **F**). **G** GP values from BSLBs with different membrane order (order increases from left to right). Each dot refers to the median GP value from one replicate. **H** GP values calculated from different batches of BSLBs coated with either low (DPPC:Cholesterol 1:1, blue bars) or high (DOPC, red bars) order membranes. Batches were prepared over ~270 days, demonstrating the reproducibility of BSLBs and usability as calibration beads.

Supplementary Note 1

The first step in our optimization process for PM order measurement was to assess both resolution and dynamic range of our flow cytometry setup by analysing model membranes with known membrane order. To do so, we prepared silica bead supported lipid bilayers (BSLBs) using pre-defined mixtures of lipids with different saturation degrees. BSLBs were stained with Pro12A and analysed in flow cytometry. Specifically, we collected the intensities in the blue (430-470 nm) and green (500-550 nm) regions of the emission spectrum and calculated the generalized polarization (GP) (*i.e.*, an experimental readout for membrane order) for individual beads. GP values were normalized to the most disordered membrane (*i.e.*, DOPC-coated beads) and transformed into ΔGP values. Individual ΔGP values were defined according to the formula $\Delta GP = \text{abs} | GP_{i\text{-th}} - \text{median} GP_{\text{DOPC}} |$ (Eq. 1), where $GP_{i\text{-th}}$ and $\text{median} GP_{\text{DOPC}}$ represent the membrane order of the *i-th* bead and the median GP of DOPC-coated beads, respectively (according to simplified version of Eq. 2).

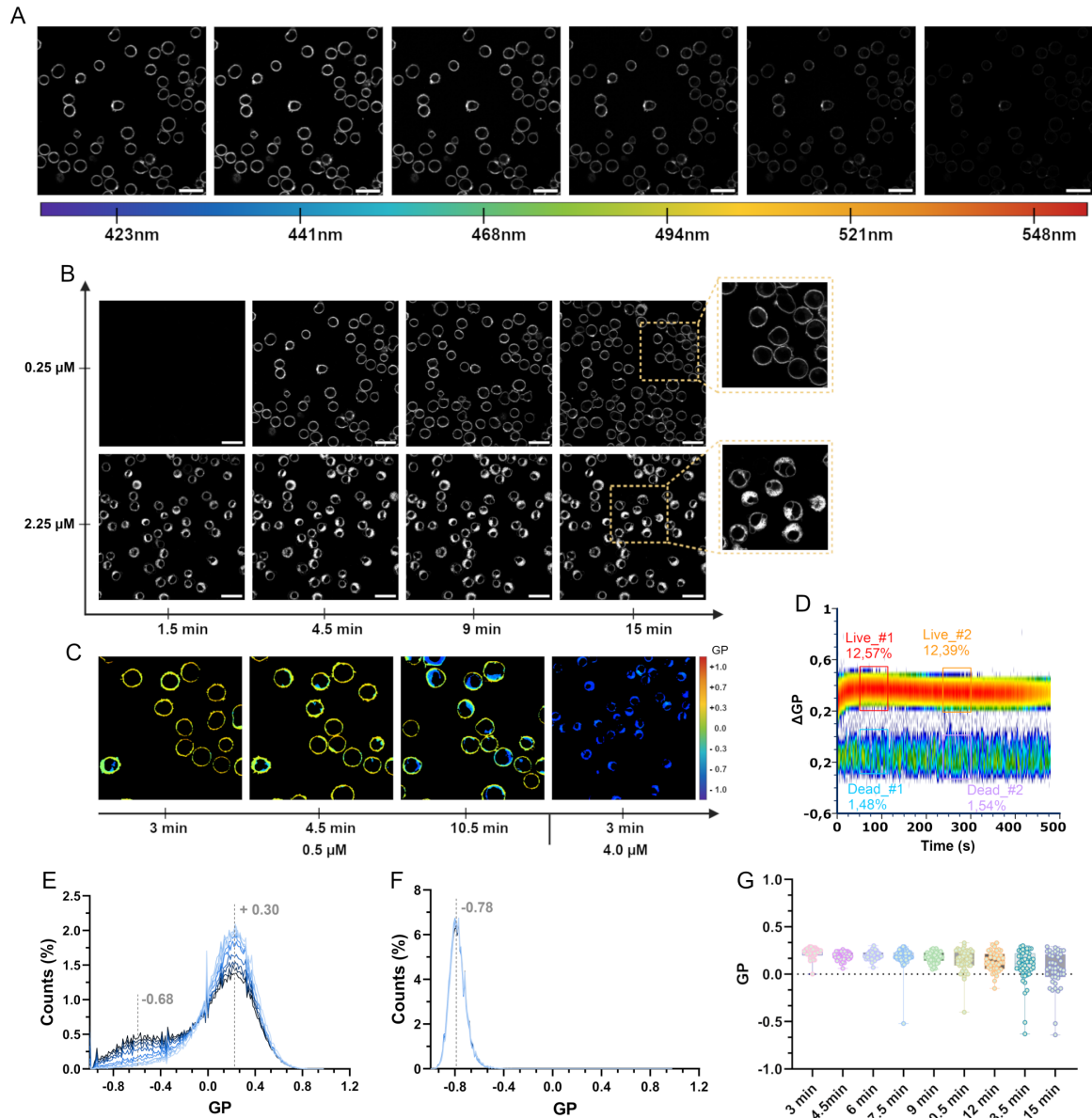
When utilizing independent photomultiplier tubes (PMTs) detectors to measure intensities in the two channels, GP values can be potentially affected by different photons' amplification occurring at the two detectors. Therefore, we should consider two additional terms in the canonical equation for GP (see Eq. 1).

$$GP = \frac{(\alpha I_b - \beta I_g)}{(\alpha I_b + \beta I_g)}$$

Eq. 2

The α and β values represent the photons amplification factors in the blue and green channels, whereas I_b and I_g are the respective channel intensities. Using Eq. 2, we generated a theoretical 2D-map of ΔGP values (*i.e.*, $GP_{\text{DPPCChol}} - GP_{\text{DOPC}}$, Fig. 2) as a function of pair-wise combination of α/β factors, starting from the emission spectrum of Pro12A in DOPC- and DPPC:Cholesterol 1:1-coated beads. Interestingly, the region of high ΔGPs (green band in Fig. 2B) widens with an increase in the amplification factors magnitude. Figures 2C-F show the theoretical and experimental ΔGP values obtained by systematically varying one amplification factor (*i.e.*, PMT voltage) at a time within the regions identified by the two black crosses in Fig. 2C. From our volttration experiments, we selected that pair of PMT voltages in the blue and green channels which yielded the highest and most stable ΔGP value.

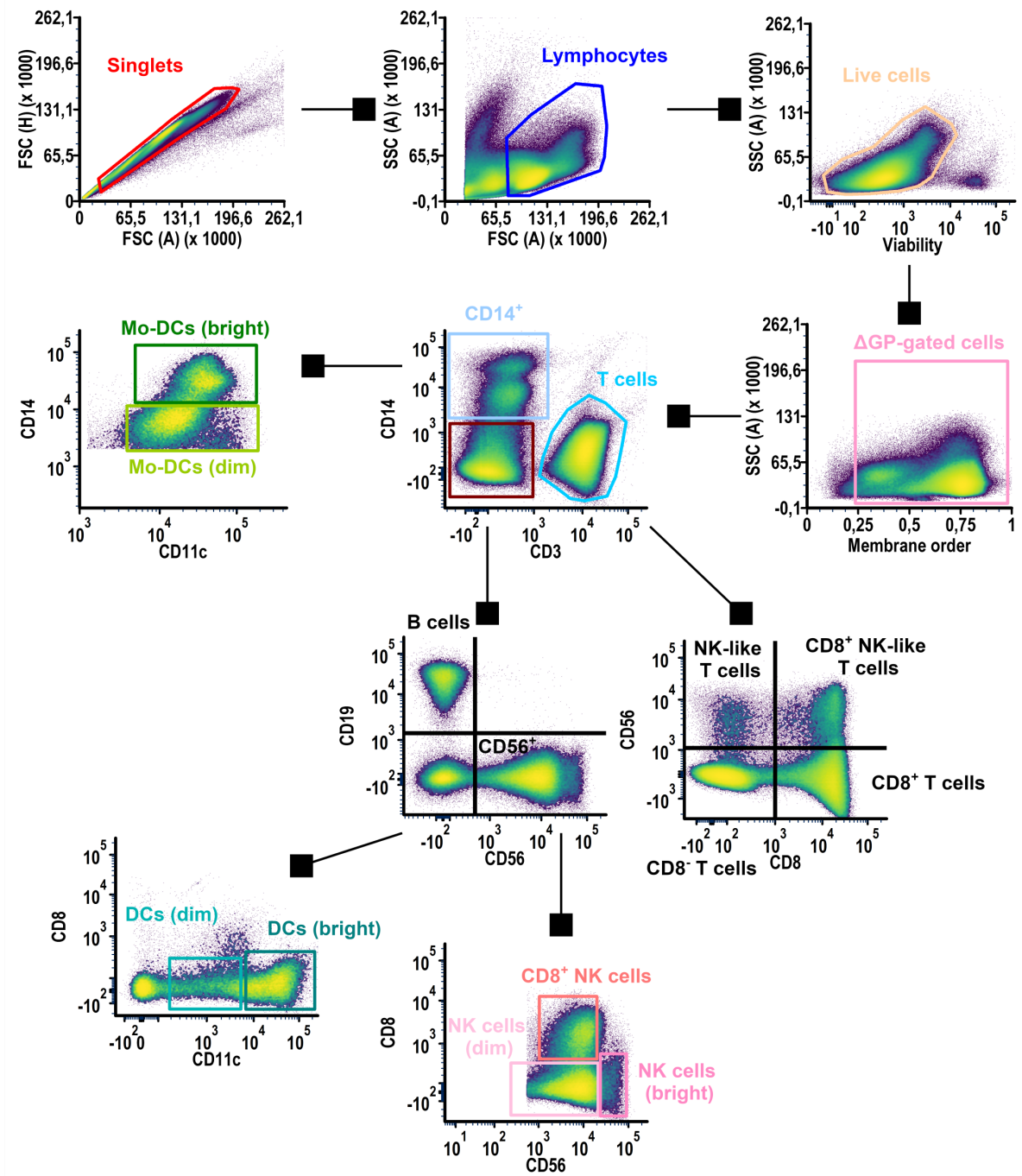
As shown in Fig. 2G, using optimal PMT voltages ensured a wide dynamic range, enabling to detect differences in membrane with small changes in lipid order. Furthermore, given the stability and reproducibility of GP values from BSLBs over many days (Fig. 2H), we used 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC)-coated beads at the beginning of each experiment on cells, to calibrate our flow setup and normalize inter-day ΔGP measurements.



Supplementary Figure 3. Pro12A concentration is critical to allow selective staining of plasma membrane by Pro12A. **A** Example of a λ -stack obtained with our spectral confocal microscope. Each image corresponds to a 9 nm-wide emission window. **B** Images showing the internalization of Pro12A in CEM cells as a function of time and at two different dye concentrations (i.e., 0.25 μ M and 2.25 μ M). **C** GP-colour coded images showing the shift in the emission of Pro12A (i.e., GP value) between the plasma membrane and the intracellular environment, as a function of time and at two different dye concentrations. **D** Flow trajectory reporting Δ GP values over time in CEM cells. **E-F** Distributions of pixel-wise GP values as a function of time after staining (from light to dark blue curves) with either low (**E**) or high (**F**) concentration of Pro12A. The time lag between two consecutive curves is 3 minutes. The observed decrease in GP values (i.e., increase of peak at -0.68 over time) is due to slow flipping of Pro12A to the inner leaflet of PM and, to a less extent, internalization of dye. At high probe concentration Pro12A becomes cytotoxic, and GP values decrease even further due to extensive internalization of dye and consequently staining of intracellular membranes in dead cells. **G** Single-cell GP median values as a function of time after staining, suggesting heterogeneity of PM permeability in CEM cells.

Supplementary Note 2

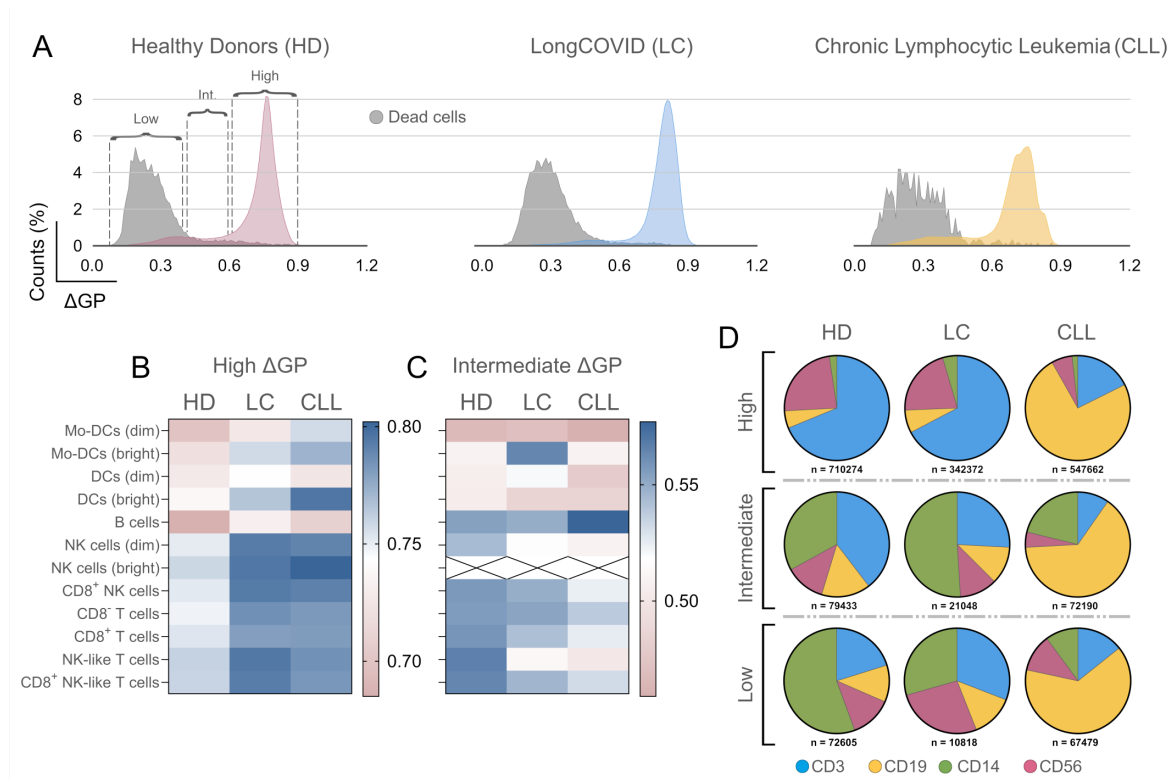
Next in the optimization protocol for the cytometry platform, we tested the compatibility of our pipeline with cell analysis. Pro12A is selective for the outer leaflet of PM but high concentrations might promote flipping to the inner leaflet and internalization¹. To this end, we first screened different concentrations of Pro12A and staining times *via* spectral confocal microscopy on CEM cell lines (as model system for live cells), to identify optimal conditions for preventing internalization and cytotoxicity. By a proper titration of the dye, we were able to selectively stain the plasma membrane and prevent internalization up to ~20 minutes after staining (typical measurements of PM order in flow cytometry require ~5-6 min), thus demonstrating the greater performance of Pro12A compared to other commercially available probes (Fig. 3A-C)¹. Lack of Pro12A internalization was also verified in our flow pipeline by analysing variations in ΔGP values over time (Fig. 3D). Indeed, following an initial stabilization time (due to the kinetic of Pro12A intercalation into the membranes), ΔGPs remained constant throughout the measurement. Furthermore, the negligible toxicity of our probe was confirmed by the unaltered ratio between the percentage of live and dead cells over time. Stability of ΔGP values over time was confirmed under confocal microscopy analysis by monitoring both distributions and cell-specific (CEM cell lines) medians of ΔGP values (Fig. 3E-G).



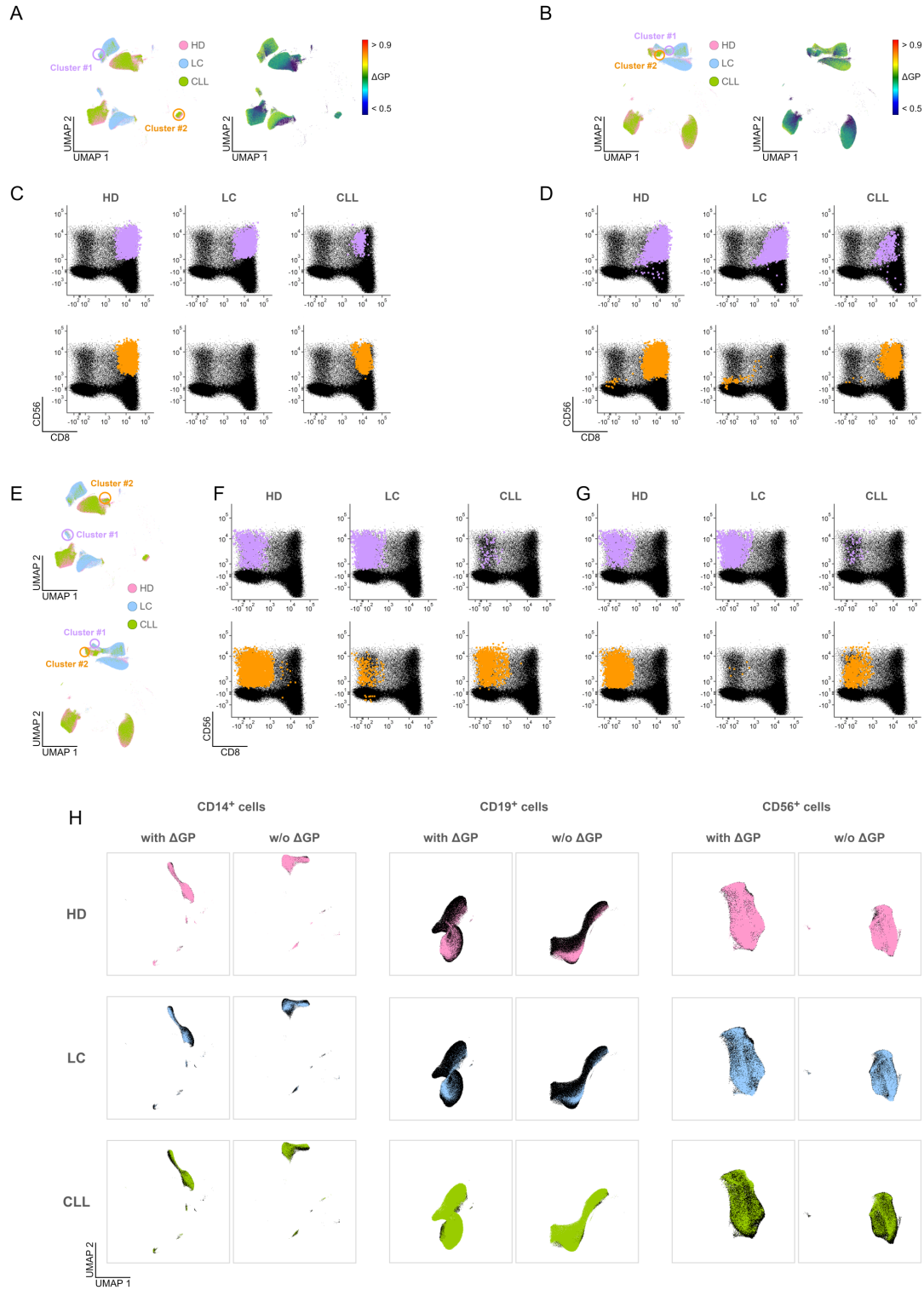
Supplementary Figure 4. Co-staining of Pro12A and cell lineage markers to profile cell type-specific PM Order. Gating strategy followed to identify the 12 subpopulations of immune cells from healthy and diseased patients. The example in the Figure 4 refers to pulled PBMCs isolated from healthy donors.

Supplementary Note 3

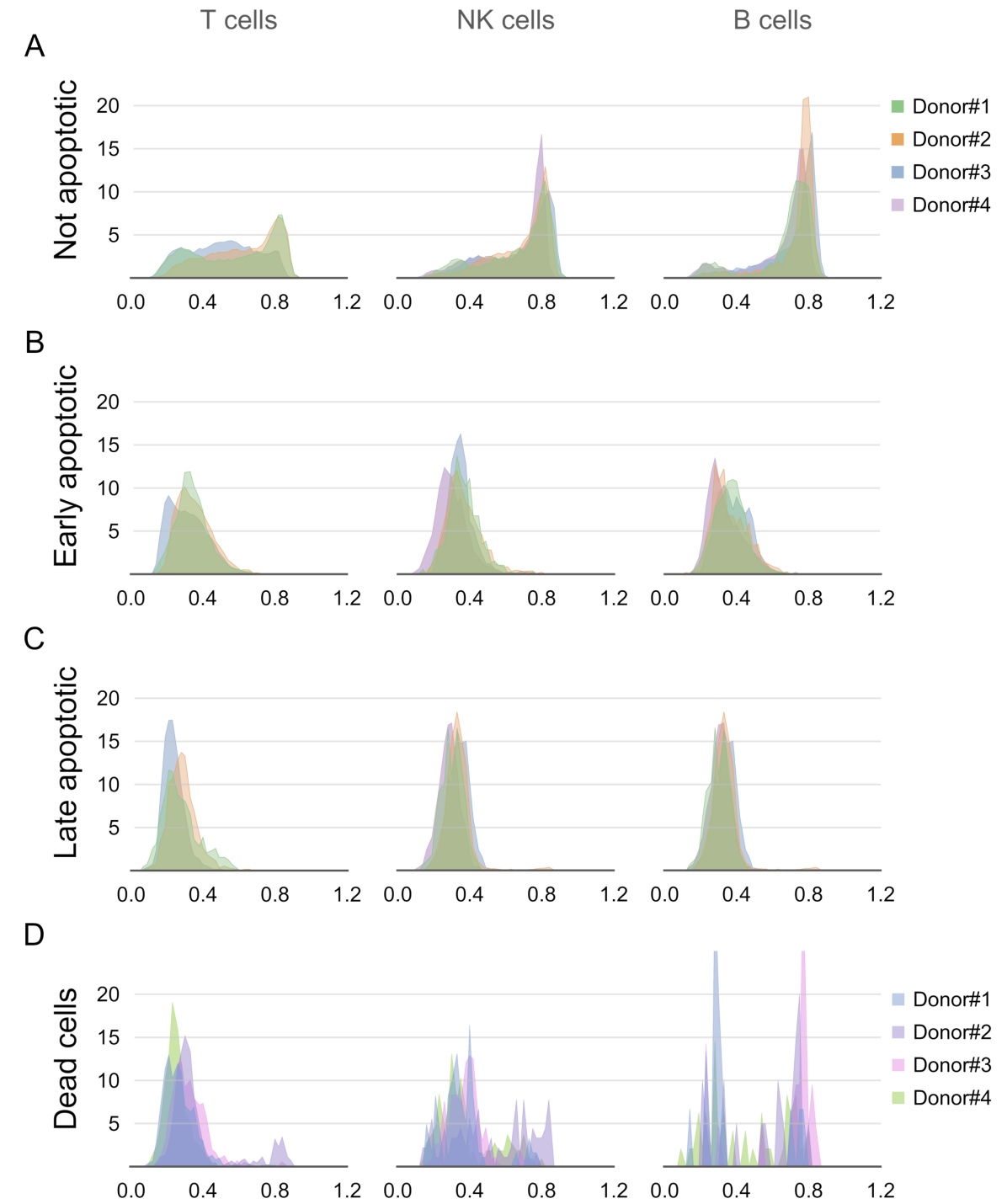
Flow cytometry data were first gated on singlets, non-debris, and live (viability dye–negative) cells. Subsequent gating identified each population as follows: Monocytes-derived Dendritic cells dim for CD11c (Mo-DCs (dim)): CD3⁻CD14⁺CD11c⁺; Monocytes-derived Dendritic cells bright for CD11c (Mo-DCs (bright)): CD3⁻CD14⁺⁺CD11c⁺; Dendritic cells dim for CD11c (DCs (dim)): CD3⁻CD14⁻CD56⁻CD19⁻CD11c⁺; Dendritic cells bright for CD11c (DCs (bright)): CD3⁻CD14⁻CD56⁻CD19⁻CD11c⁺⁺; B cells: CD3⁻CD14⁻CD56⁻CD19⁺; NK cells dim for CD56 (NK cells (dim)): CD3⁻CD14⁻CD19⁻CD56⁺CD8⁻; NK cells bright for CD56 (NK cells (bright)): CD3⁻CD14⁻CD19⁻CD56⁺⁺CD8⁻; CD8⁺ NK cells: CD3⁻CD14⁻CD19⁻CD56⁺CD8⁺; CD8⁻ T cells: CD3⁺CD14⁻CD56⁻CD8⁻; Cytotoxic T cells (CD8⁺ T cells): CD3⁺CD14⁻CD56⁻CD8⁺; NK-like T cells: CD3⁺CD14⁻CD56⁺CD8⁻; and CD8⁺ NK-like T cells: CD3⁺CD14⁻CD56⁺CD8⁺.



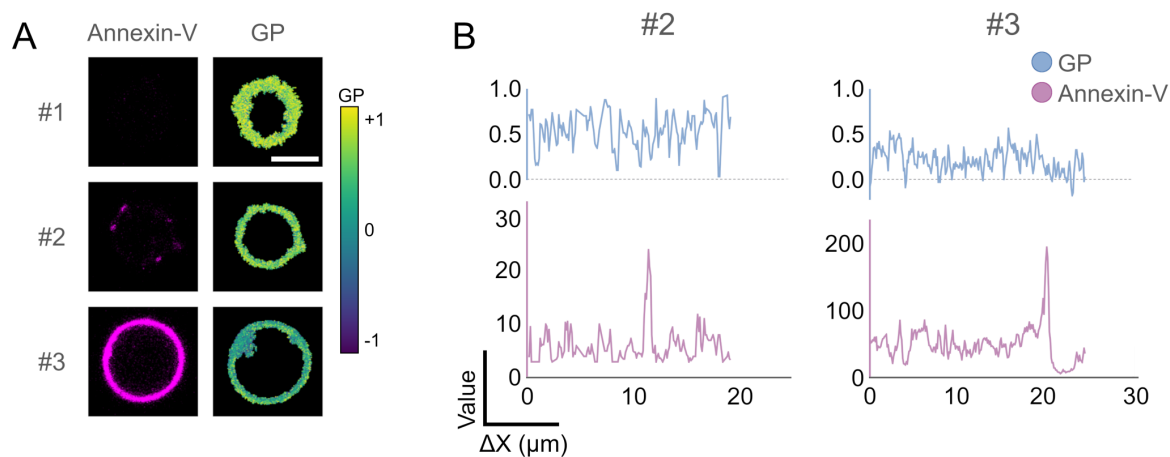
Supplementary Figure 5. PM Order heterogeneity across cell type and donors. **A** PM order (ΔGP) distribution from pulled PBMCs from healthy and diseased donors. The grey distribution refers to the corresponding dead-gated cells. **B-C** Heatmaps showing variations in median ΔGP values for each immune cell subpopulation within the high ΔGP -gated and intermediate ΔGP -gated cells. **D** Pie charts showing the relative abundances of main immune cell types in each sample and within high, intermediate and low ΔGP -gated cells.



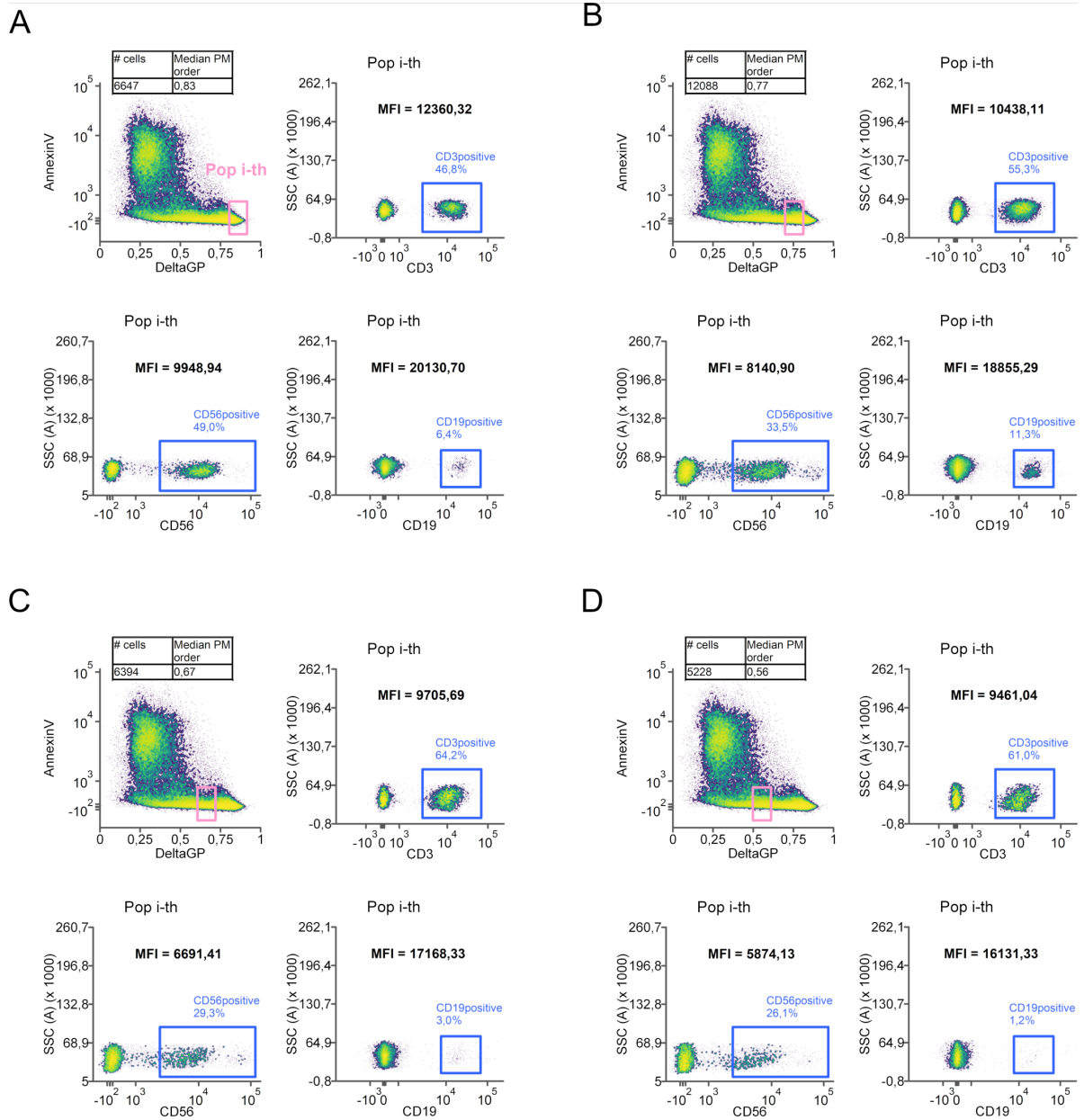
Supplementary Figure 6. PM Order enhances phenotype clustering in UMAP analysis. **A-B** UMAP projections of merged HD, LC, and CLL samples with **(A)** or without **(B)** ΔGP included in dimensionality reduction. Left panels show sample clustering; right panels show ΔGP values. Cluster #1 and Cluster #2 correspond to the same gated $CD8^+$ NK-like T-cell population. **C-D** $CD56$ versus $CD8$ expression plots for cells in Cluster #1 (purple) and Cluster #2 (orange), with **(C)** or without **(D)** ΔGP in the UMAP analysis. **E** UMAP projections of merged samples with (top) or without (bottom) ΔGP , showing sample-specific clustering of T-cell subpopulations; clusters correspond to the same gated NK-like T-cell population. **F-G** $CD56$ versus $CD8$ expression plots for Cluster #1 and Cluster #2 cells, with **(F)** or without **(G)** ΔGP . **H** UMAP projections of different immune cell types generated with or without ΔGP . Black dots indicate all cells; colored dots represent individual samples.



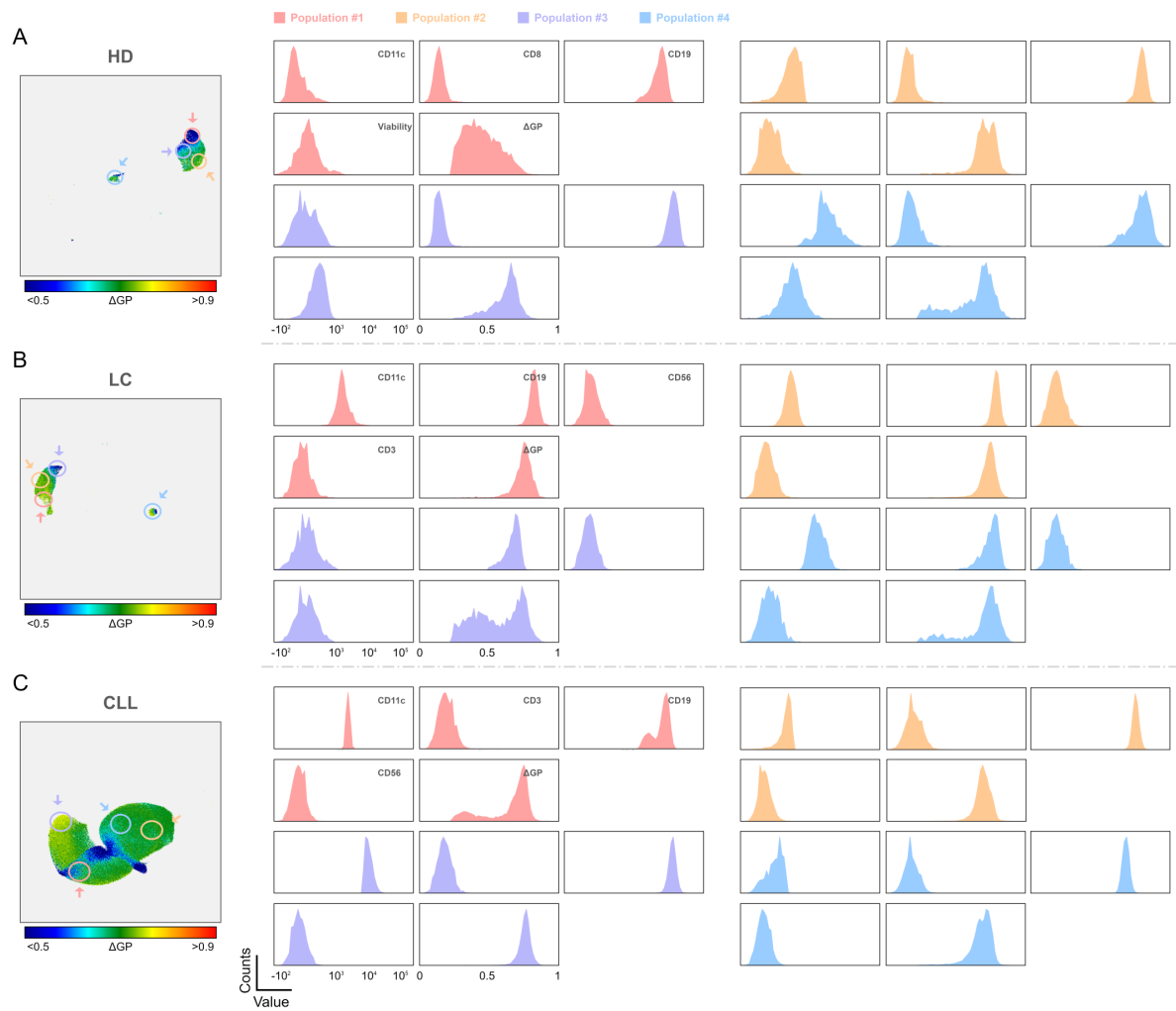
Supplementary Figure 7. PM Order is highly heterogeneous in non-apoptotic cells. Apoptosis analysis on pulled PBMCs from four healthy donors. Plots show the overlap of PM order (ΔGP) distributions from individual donors for not apoptotic- (**A**), early apoptotic- (**B**), late apoptotic- (**C**) and dead-gated (**D**). Cells were stained with Annexin V–Alexa Fluor 647 (AnV647) and FVS780 for viability and defined as follows: non-apoptotic cells (AnV647⁻ FVS780⁻), early apoptotic cells (AnV647⁺ FVS780⁻), late apoptotic cells (AnV647⁺ FVS780⁺), and dead cells (AnV647⁻ FVS780⁺).



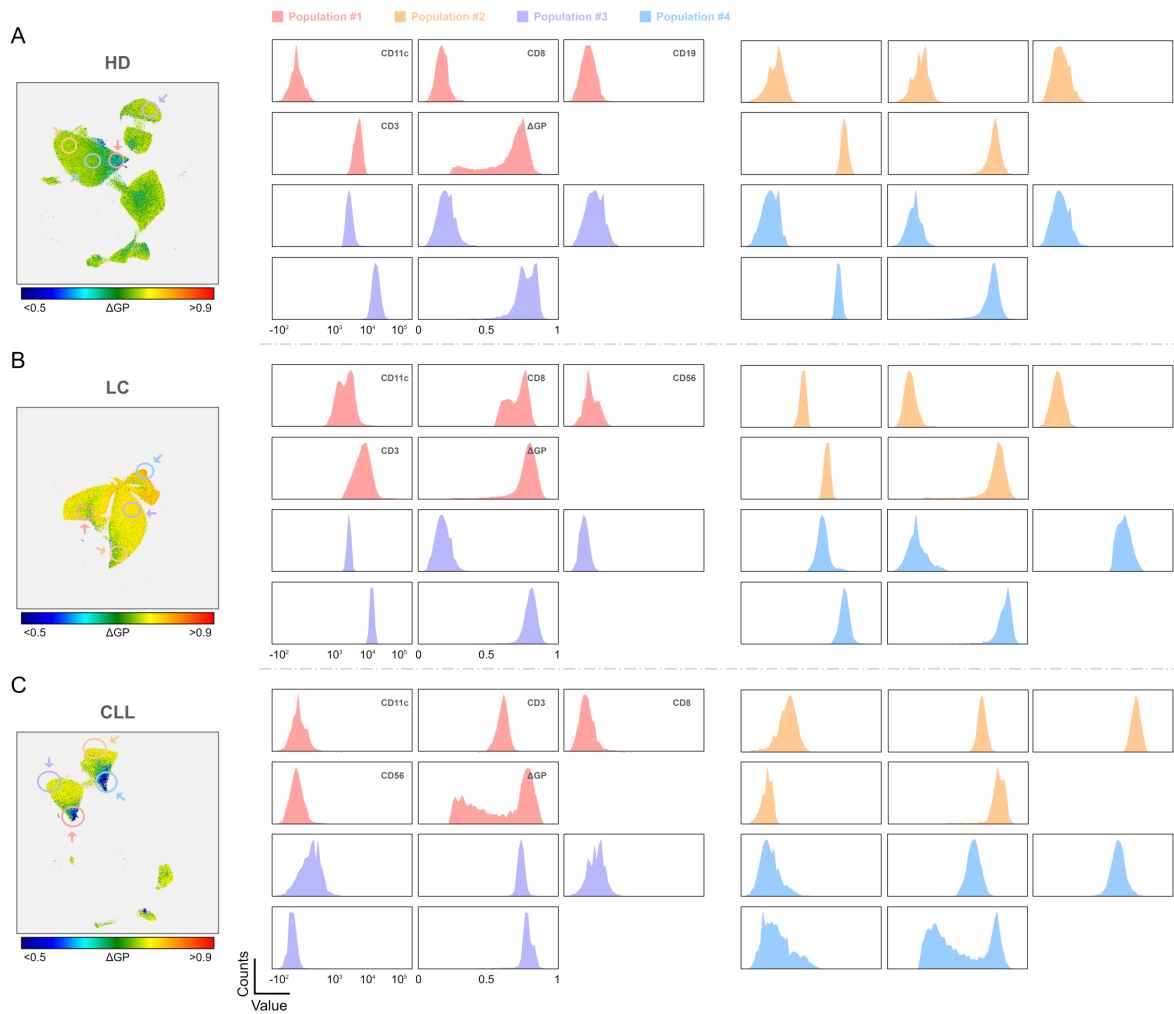
Supplementary Figure 8. PM Order is orthogonal to Annexin-V staining for apoptosis. **A** Microscopy images acquired with a spectral confocal microscope, showing three different cells (#1, #2 and #3) from PBMCs isolated from healthy donors, after being stained with both Pro12A and Annexin-V-AF647. Magenta and pseudo-coloured images refer to Annexin-V and calculated GP values, respectively. The scale bar indicates 5 μm distance and the white arrows indicate Annexin-V puncta. **B** Trajectories of GP value (blue line) and Annexin-V intensity (violet line) profiled along the membrane of cells #2 and #3.



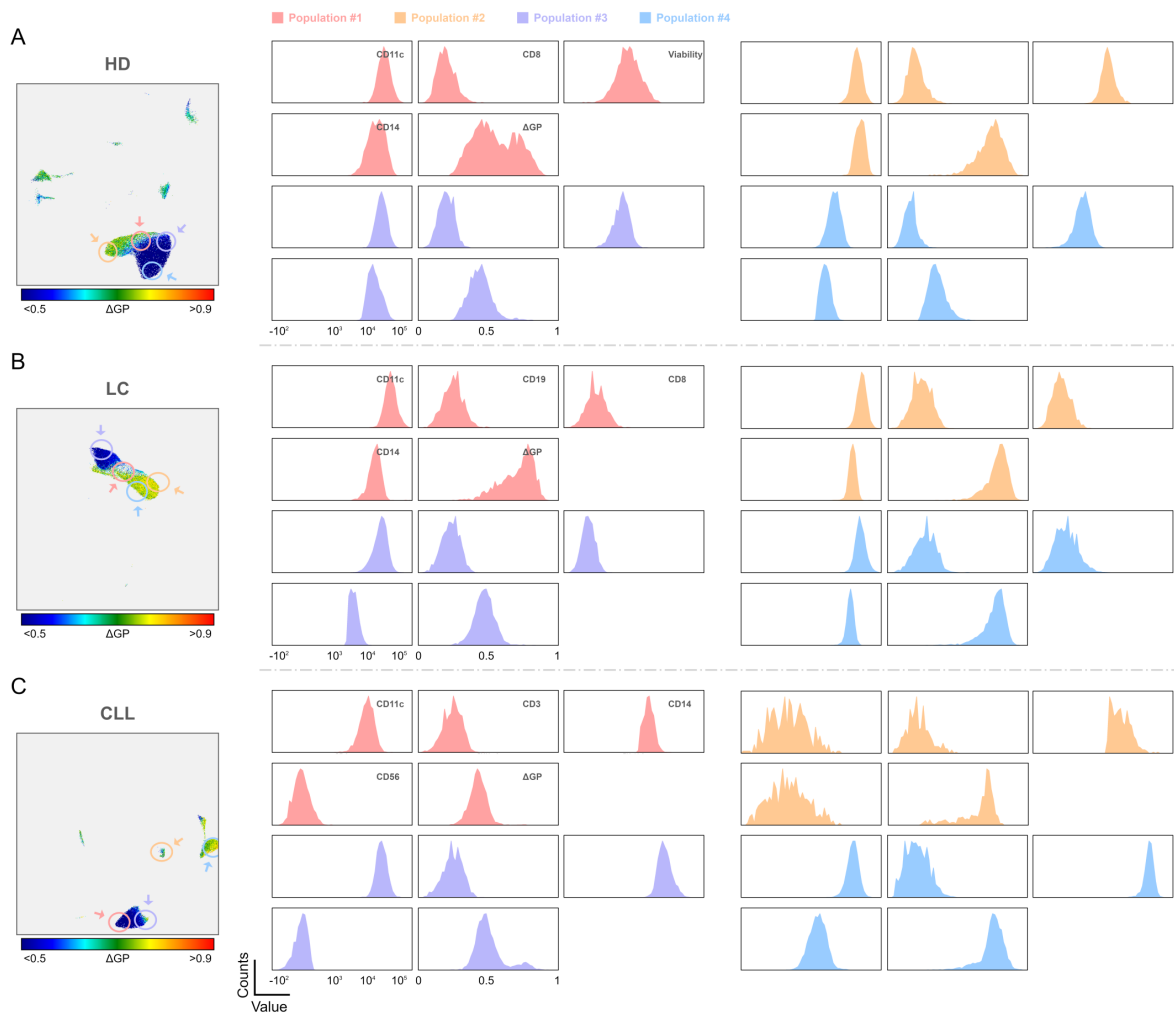
Supplementary Figure 9. Lower PM Order correlates with a slight decrease in surface markers for cell lineage. Expression level of main surface biomarkers for cell lineage in apoptosis studies. The expression of CD3, CD56 and CD19 was quantified from a subset of cells having median ΔGP equal to 0.83 (A), 0.77 (B), 0.67 (C) and 0.56 (D).



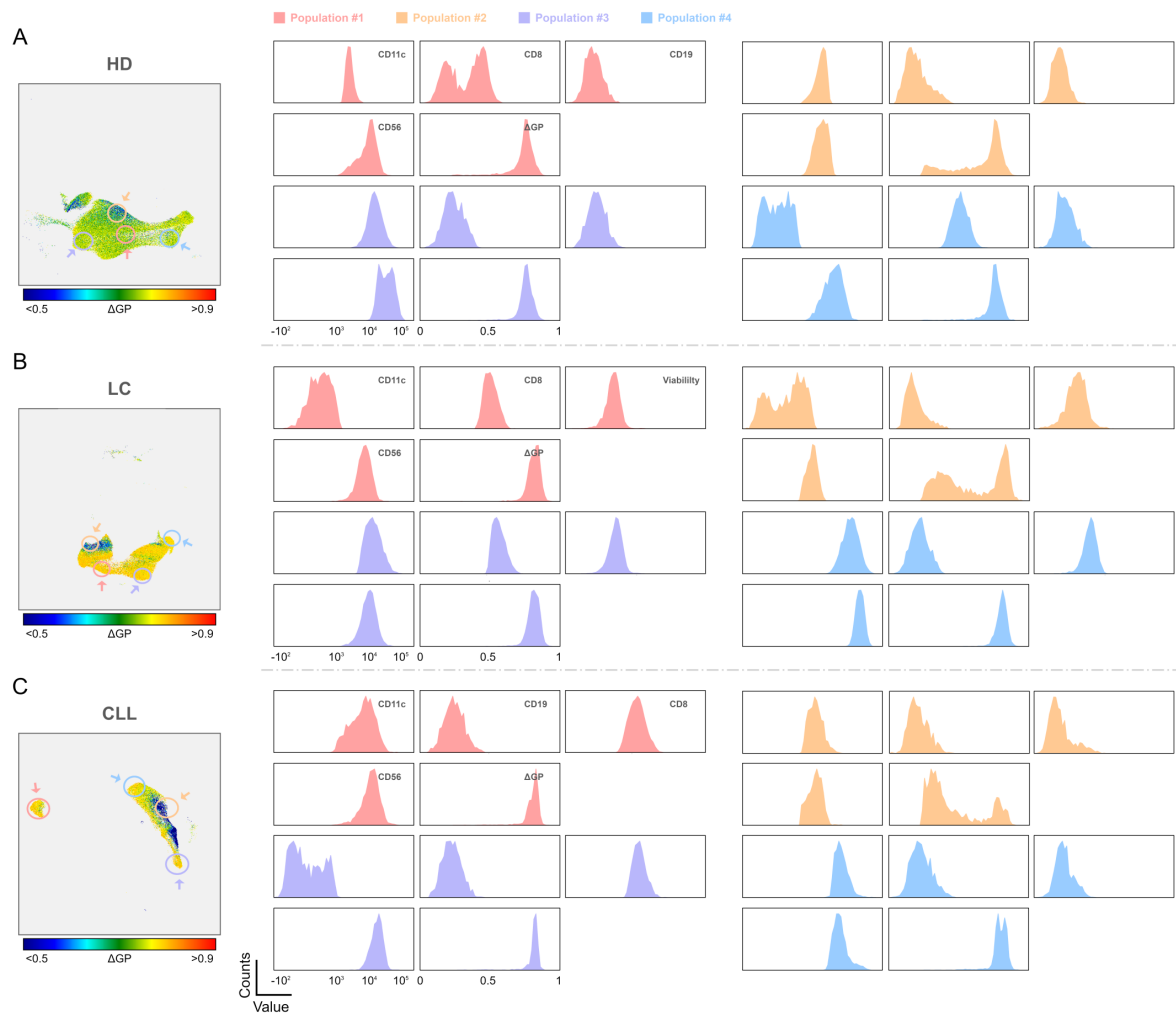
Supplementary Figure 10. PM Order is orthogonal to cell surface markers in B cells. **A** UMAP projection (left box) and histograms showing the intensity distribution of different biomarkers and the distribution of ΔGP values (right panels). Plots refer to B cells ($CD19^+$) from healthy donors (**A**), Long COVID (**B**) and Chronic lymphocytic leukaemia (**C**) patients. Each coloured circle and corresponding arrow identify a different population within the B cells subset. The biomarker intensity histograms are rearranged in the same way across panels within the same sample.



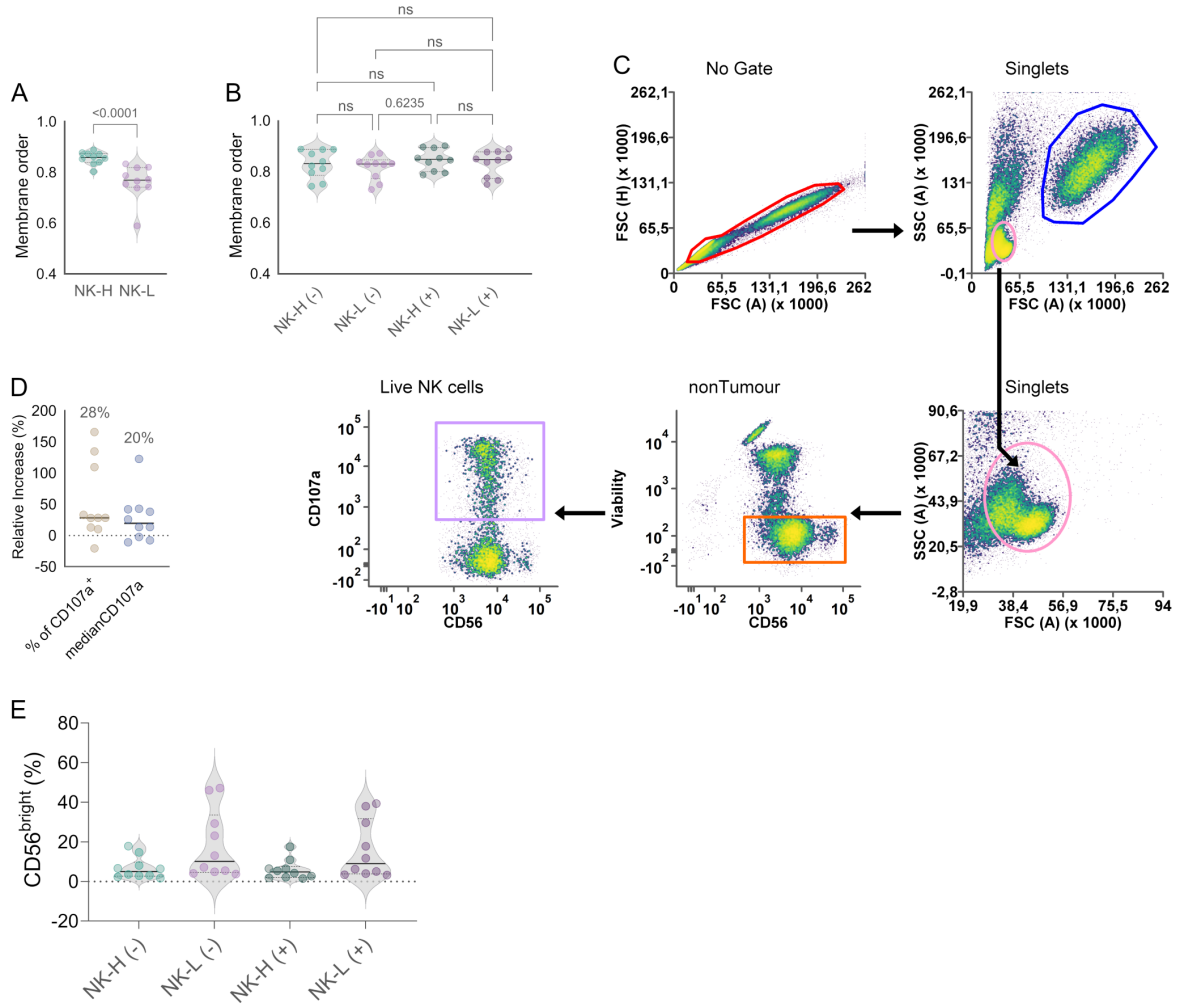
Supplementary Figure 11. PM Order is orthogonal to cell surface markers in T cells. **A** UMAP projection (left box) and histograms showing the intensity distribution of different biomarkers and the distribution of ΔGP values (right panels). Plots refer to T cells ($CD3^+$) from healthy donors (**A**), Long COVID (**B**) and Chronic lymphocytic leukaemia (**C**) patients. Each coloured circle and corresponding arrow identify a different population within the T cells subset. The biomarker intensity histograms are rearranged in the same way across panels within the same sample.



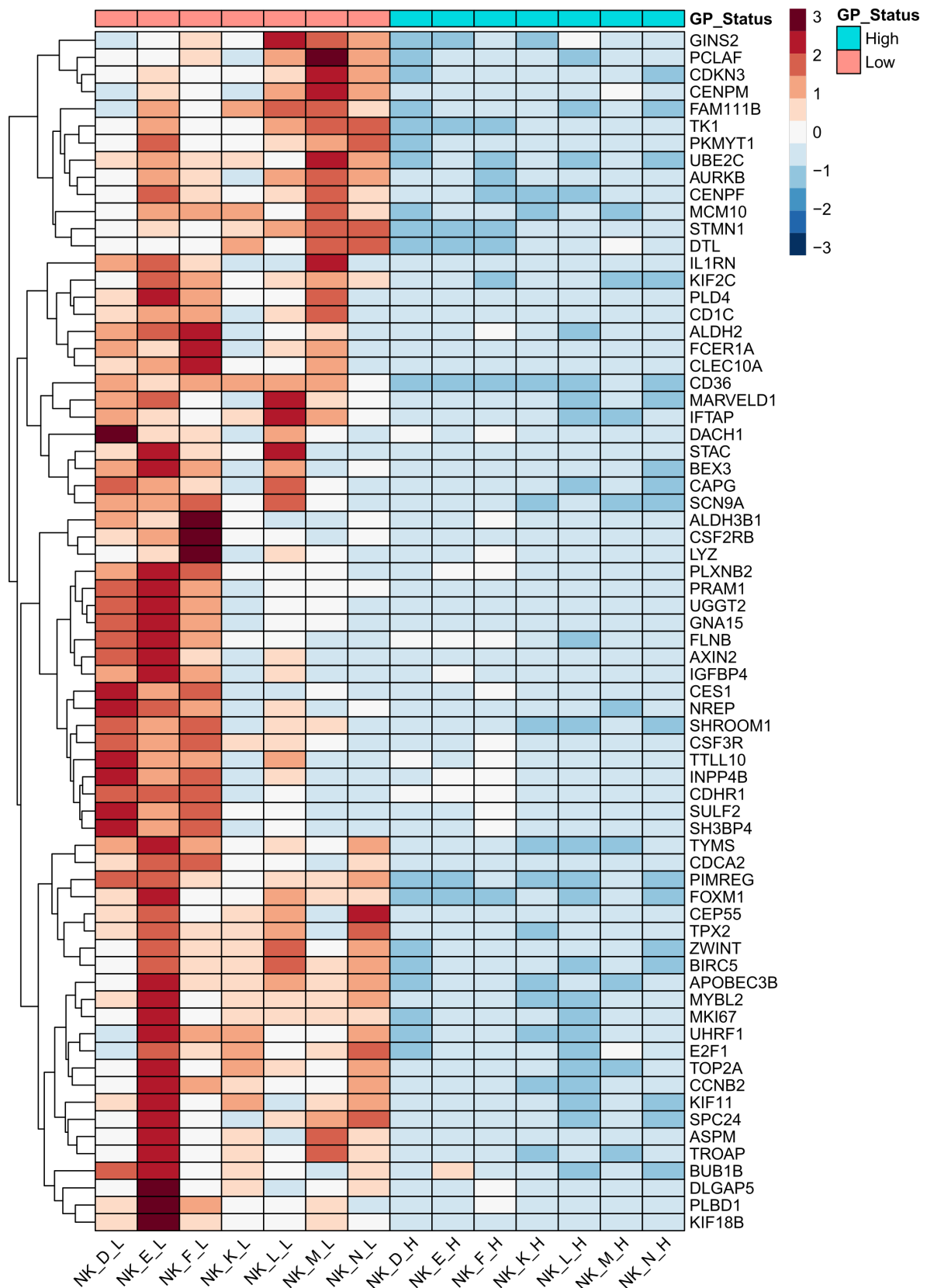
Supplementary Figure 12. PM Order is orthogonal to cell surface markers in Monocytes-derived Dendritic cells. **A** UMAP projection (left box) and histograms showing the intensity distribution of different biomarkers and the distribution of ΔGP values (right panels). Plots refer to Mo-DCs cells ($CD14^+CD11c^+$) from healthy donors (**A**), Long COVID (**B**) and Chronic lymphocytic leukaemia (**C**) patients. Each coloured circle and corresponding arrow identify a different population within the Mo-DCs cells subset. The biomarker intensity histograms are rearranged in the same way across panels within the same sample.



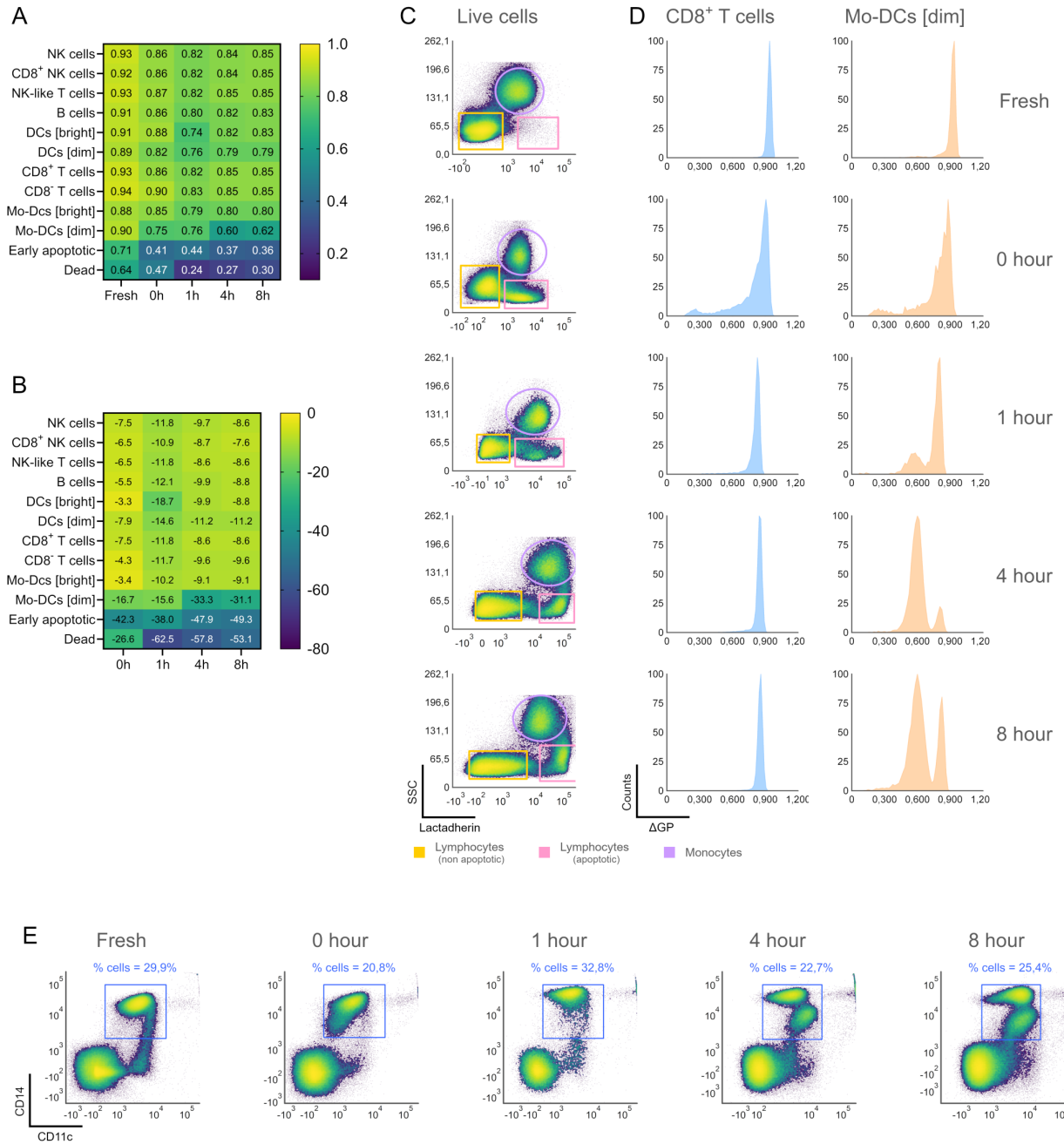
Supplementary Figure 13. PM Order is orthogonal to cell surface markers in NK cells. **A** UMAP projection (left box) and histograms showing the intensity distribution of different biomarkers and the distribution of ΔGP values (right panels). Plots refer to NK cells (CD56⁺) from healthy donors (**A**), long COVID (**B**) and Chronic lymphocytic leukaemia (**C**) patients. Each coloured circle and corresponding arrow identify a different population within the NK cells subset. The biomarker intensity histograms are rearranged in the same way across panels within the same sample.



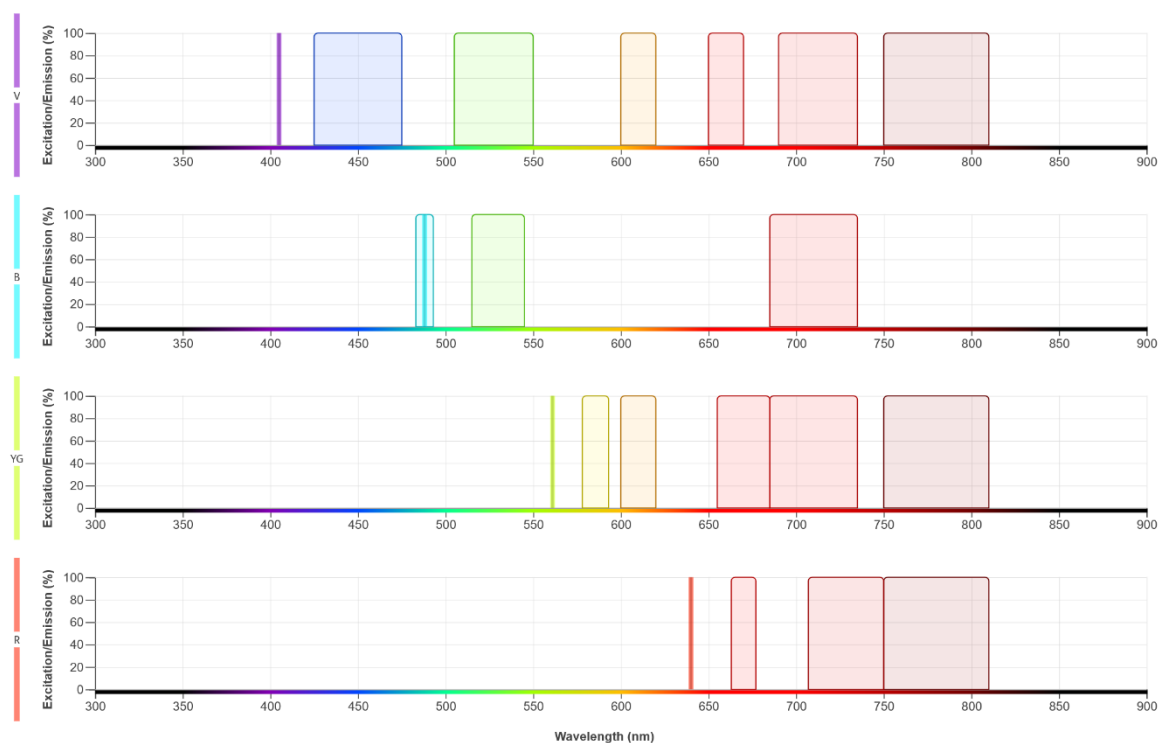
Supplementary Figure 14. PM Order in biophysically sorted NK cells. **A** Median PM order values of NK cells from different healthy donors right after sorting. **B** Median membrane order values of sorted NK cells after 24 hours of recovery in cell medium, with (+) or w/o (-) incubation with K562 tumour cells. **C** Gating strategy to estimate number of live NK cells and degranulation percentage. **D** Relative percentage increase of CD107a expression level (intensity) and positive (CD107a⁺) cells. **E** Violin plots showing the percentage of CD56^{bright} cells within NK-H and NK-L subsets before (-) and after (+) exposure to tumour cells. In A and B, the small number represent the adjusted *p*-values calculated via the Kruskal-Wallis statistical test and the Dunn's test for multiple comparison. The threshold for statistical significance was set at $p = 0.05$.



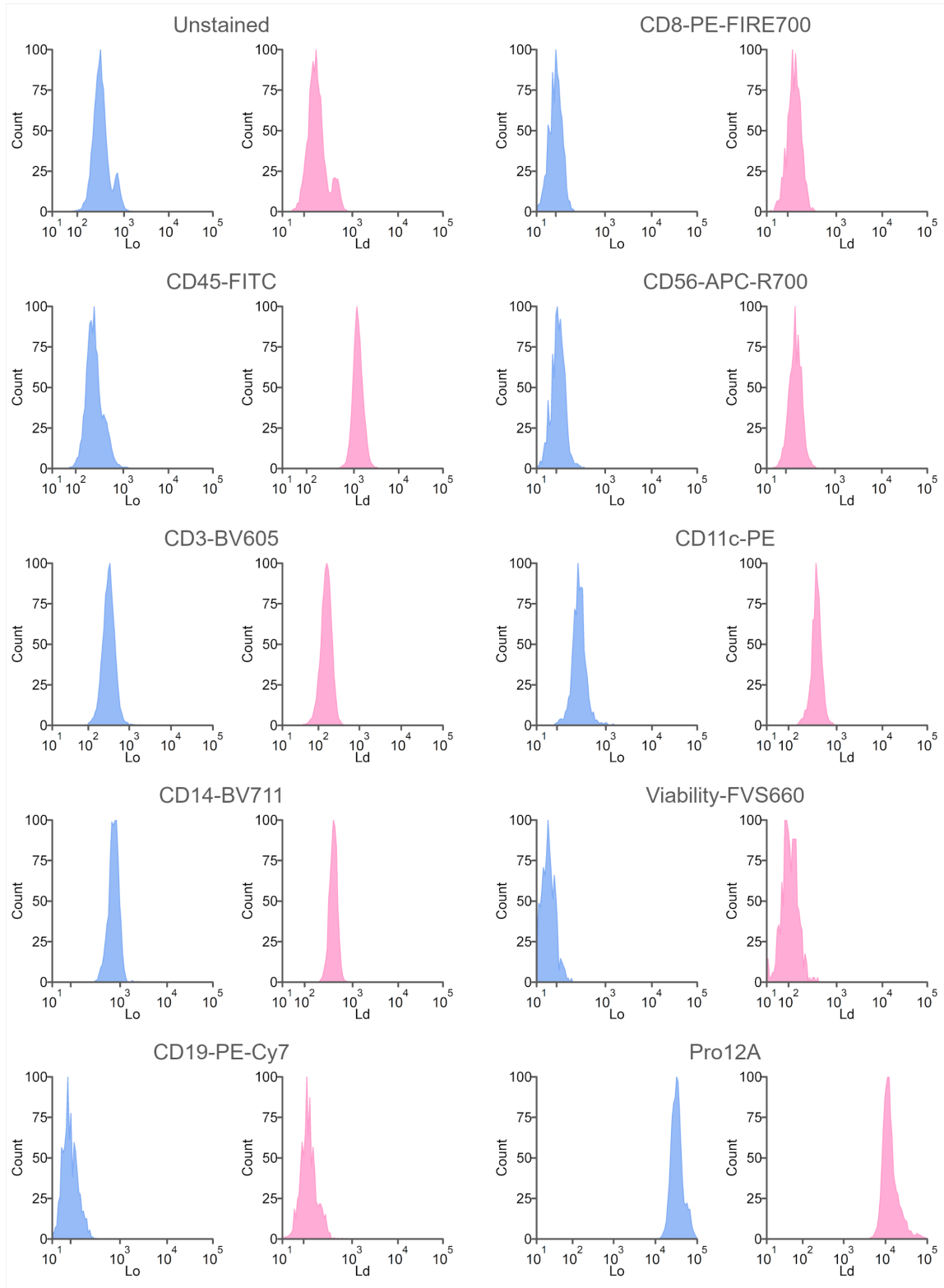
Supplementary Figure 15. NK cells sorted according to their PM order show different transcriptomic profiles. Heatmap showing the top 70 significant genes which were differentially expressed between NK-H and NK-L subsets. Each column within the NK-H and NK-L clusters refer to a different donor.



Supplementary Figure 18. Effects of freezing-thawing steps on PM Order. **A** Heatmap showing the median ΔGP values from PBMCs right after isolation (fresh) or after different post-thawing recovery times. **B** Relative decrease percentage of ΔGP values compared to freshly (no freezing) analysed PBMCs. **C-D** SSC versus Lactadherin plots (**C**) and ΔGP distributions (**D**) from freshly isolated PBMCs and cells after different post-thawing recovery times. **E** Percentage of CD14⁺ cells from freshly isolated PBMCs or from frozen samples after different times in culture for post-thawing recovery.



Supplementary Figure 19. Optical configuration used for the flow cytometer. Configuration of our flow cytometer Fortessa BD LSRFortessa 16-colour, showing the lasers for excitation and the optical filters for wavelengths selection in the different channels.



Supplementary Figure 20. The flow cytometry antibodies panel shows no overlap with Pro12A emission. Spillover (i.e., intensity distribution) from antibody-conjugated fluorophores used to stain PBMCs from healthy donors and patients into the channels occupied by Pro12A signal. The *Lo* (ordered lipid phase) and *Ld* (disordered lipid phase) channels were used to measure membrane order.

Supplementary Note 4

Pro12A is generally measured using the BV421 and BV510 channels. Therefore, depending on the flow cytometer configuration, some commercial fluorochromes might not be suitable for coupling with Pro12A given their spectral spillover. In our flow cytometry setup (Supplementary Fig. 19), the following dyes are not compatible with PM order measurement via Pro12A: BUV395, BUV496, BV421, BV480, BV510, AlexaFluor405. The fluorochromes BUV563 and BV570 might require additional optimization and spillover assessment given the spectral proximity with Pro12A detection channels. On the other hand, the following fluorochromes can be safely coupled with Pro12A for PM order measurement: BUV615, BUV661, BUV737, BUV805, BV605, BV650, BV711, BV786. In addition, Pro12A can be coupled with all fluorochromes from the PE and PerCP family as well as the other AlexaFluor derivatives and the red-excitable dyes.

Supplementary Table 2

Panel	H	df	N	P value	ε²
panel C	9.326	2	33	0.0094	0.24
panel F, Mo-DCs (bright)	8.818	2	32	0.0122	0.24
panel F, Mo-DCs (dim)	9.825	2	33	0.0074	0.26
panel F, DCs (bright)	8.294	2	33	0.0158	0.21
panel F, DCs (dim)	1.236	2	33	0.5392	0.00
panel F, B cells	3.803	2	33	0.1493	0.06
panel F, NK cells (dim)	7.507	2	33	0.0234	0.18
panel G, CD8⁻ T cells	6.608	2	33	0.0367	0.15
panel G, CD8⁺ T cells	5.693	2	33	0.0580	0.12
panel G, NK-like T cells	8.629	2	33	0.0134	0.22
panel G, CD8⁺ NK-like T cells	7.628	2	33	0.0221	0.19
hpanel G, NK cells (bright)	10.720	2	32	0.0047	0.30
panel G, CD8⁺ NK cells	7.999	2	33	0.0183	0.20

H, Kruskal-Wallis test statistic; **df**, degrees of freedom; **N**, total number of observations included in the analysis; **P value**, exact two-sided P value from the Kruskal-Wallis test; **ε²**, epsilon-squared effect size.