Supporting Information for

Cannabinoid Receptor 2 (CB2) Signals via G-alpha-s and Induces IL-6 and IL-10 Cytokine Secretion in Human Primary Leukocytes

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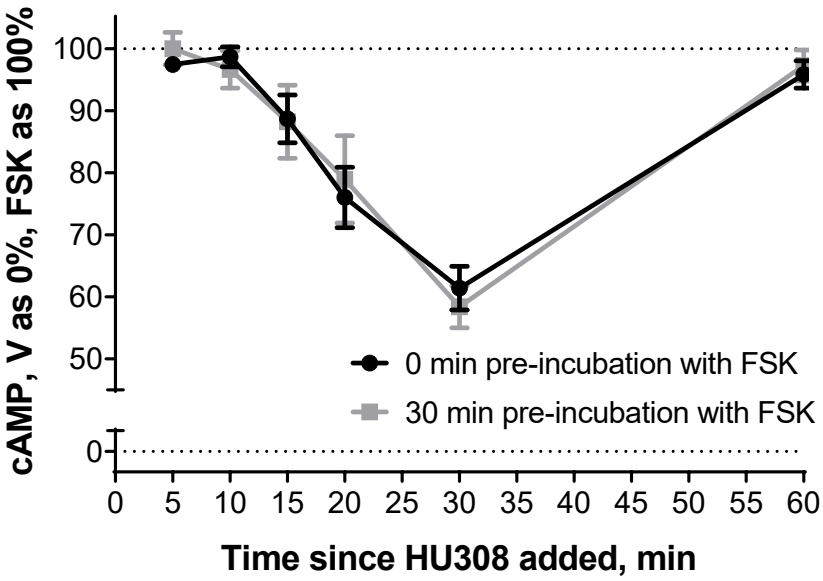
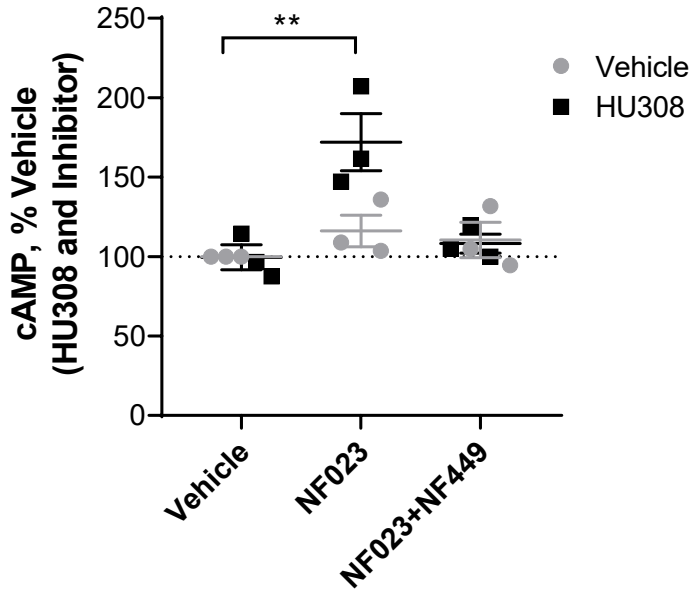
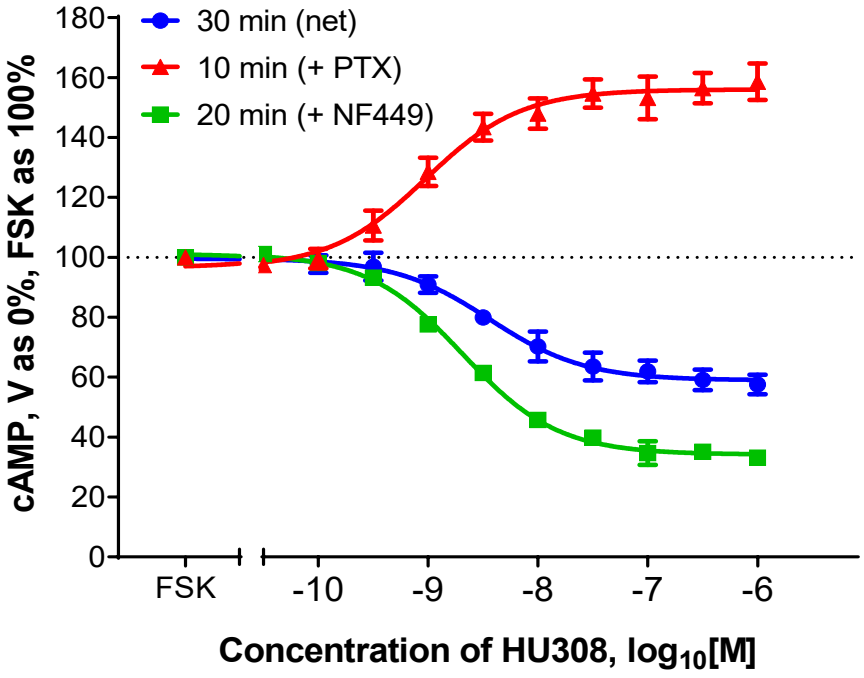
Figures S1 to S10



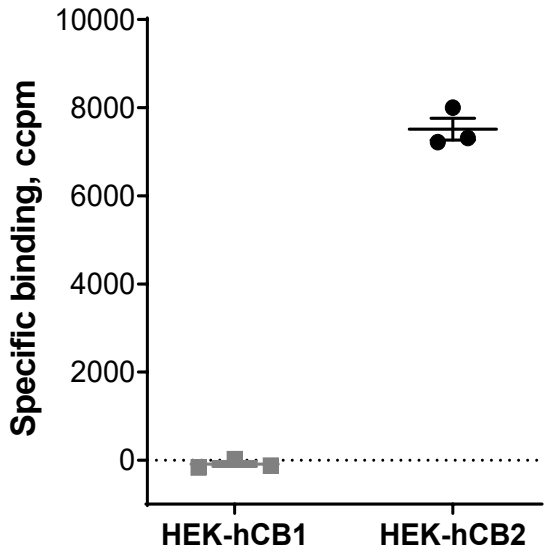
Supplementary Fig. 1. cAMP signaling in human primary PBMC pre-incubated with forskolin. Time-course of 1 μM HU308 with 10 μM forskolin (FSK) added at the same time (black) or after a 30-min pre-incubation with forskolin (grey). Data are normalized to vehicle control (0%) and forskolin alone (100%). Graph shows mean ± SEM for three independent experiments performed in technical triplicate, each on a separate subject (three subjects in total).



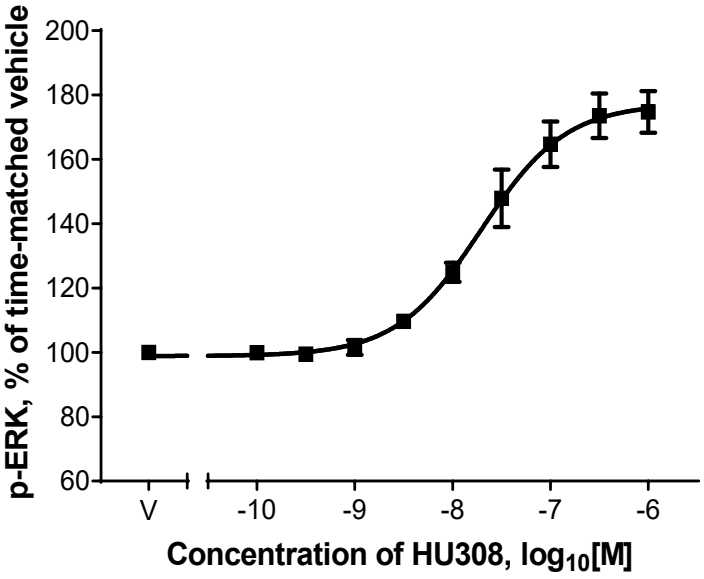
Supplementary Fig. 2. Stimulatory cAMP signaling in human primary PBMC without forskolin. PBMC were pre-treated for 30 min with a vehicle control, a $G\alpha_i$ inhibitor NF023 (10 μM), or a $G\alpha_s$ inhibitor NF449 (10 μM), and then HU308 (1 μM) was applied for 10 min (apparent signaling peak for stimulatory cAMP response). The responses are normalized to vehicle control (100%). All conditions were compared to the vehicle control. Only "NF023 + HU308" condition is significantly different from vehicle ($P < 0.01$, **), Tukey's multiple comparisons test. Graph shows independent experiment means (from technical triplicate) as well as overall mean ± SEM of these three independent experiments each performed with cells from a separate subject (three subjects in total). Note that NF023 reveals the stimulatory cAMP response, while NF449 blocks it.



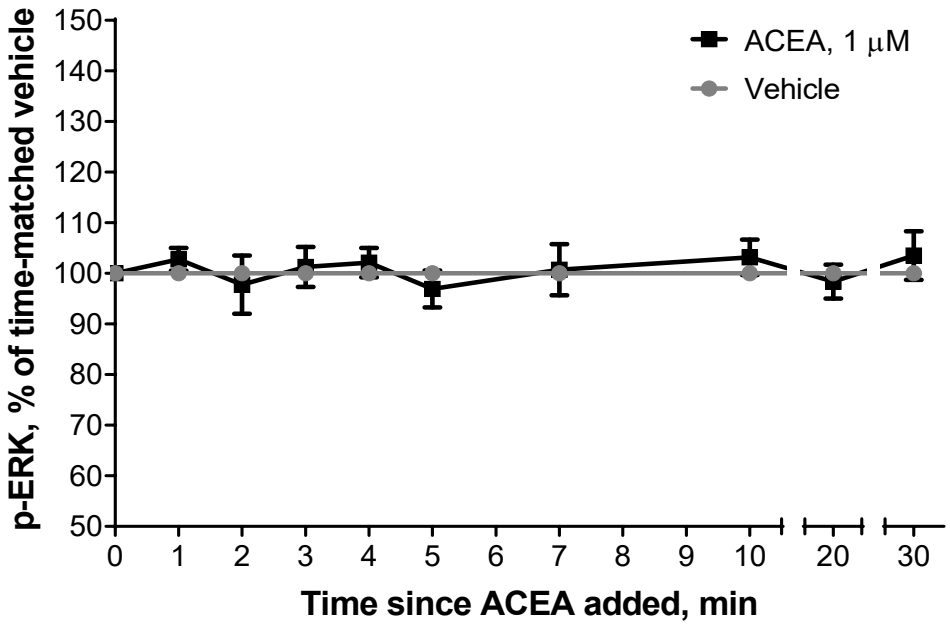
Supplementary Fig. 3. Concentration-dependent effects of HU308 on cAMP responses in human primary PBMC. Concentration-responses in the presence of 10 μM forskolin (FSK) and HU308 without inhibitors (●), or pre-treated with NF449 (■), or PTX (▲) and then stimulated with HU308 for 30, 10, or 20 min, respectively (at signaling peaks). Graph shows mean ± SEM for three experiments performed independently, each on a separate subject (three subjects in total).



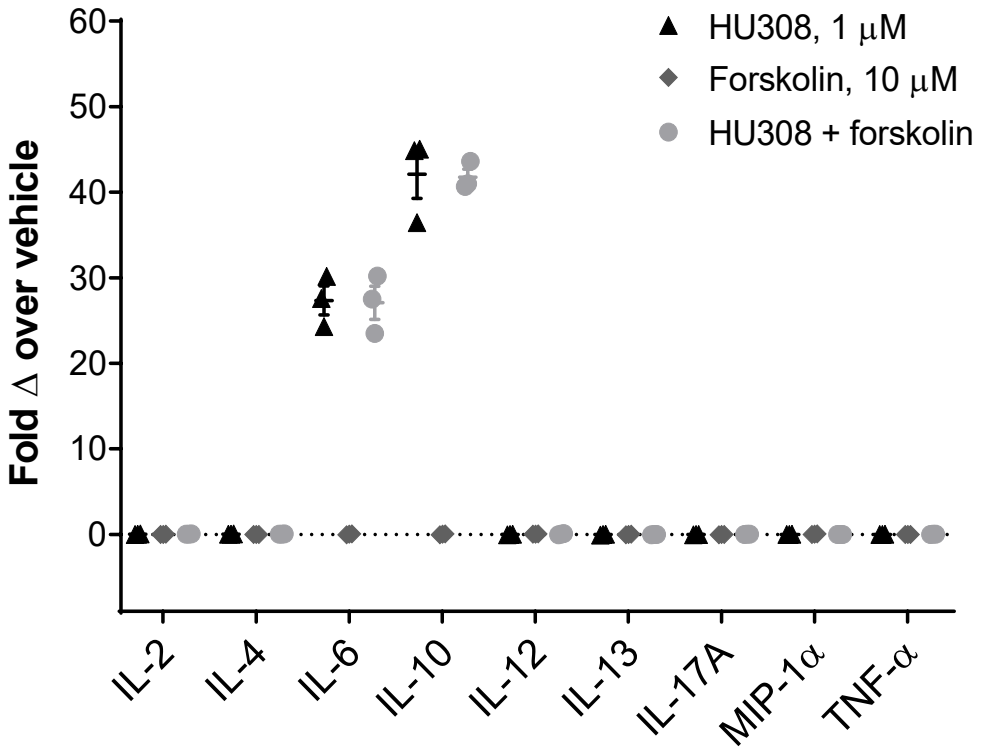
Supplementary Fig. 4. Selectivity of SR144528 for CB_2 over CB_1. HEK cells transfected with human CB_1 (HEK-hCB_1) or CB_2 (HEK-hCB_2) were incubated for 3 h at 15°C with [^{3}H]-CP55940 (5 nM) in the presence of a displacer SR144528 (1 μM, displaceable binding), or a vehicle control (total binding). The difference between total and displaceable binding was considered specific binding, plotted as ccpm – counts per minute corrected with a factory pre-set [^{3}H] detector normalization. HEK-hCB_1 is a positive control for CB_1, HEK-hCB_2 is a positive control for CB_2. Graph shows independent experiment means (from technical triplicate) as well as overall mean ± SEM of these three independent experiments each performed with cells from a separate subject (three subjects in total). Note absence of SR144528 displacement of [^{3}H]-CP55940 binding in CB_1-expressing cells.



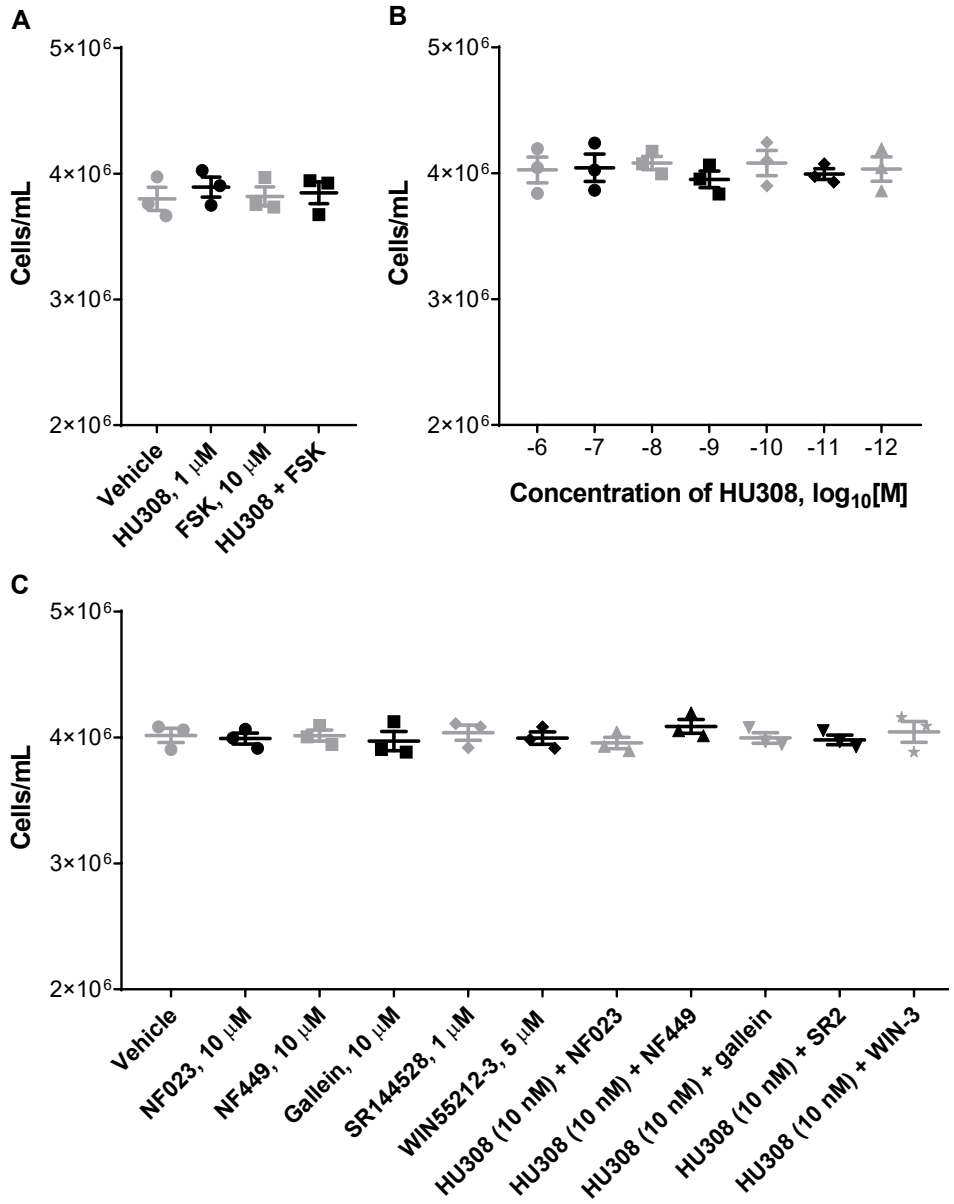
Supplementary Fig. 5. Concentration-dependent effect of HU308 on p-ERK response in human primary PBMC. HU308 concentration-response curve at 3 min, “V” indicates vehicle control. Graph shows mean ± SEM for three experiments performed independently, each with cells from a separate subject (three subjects in total).



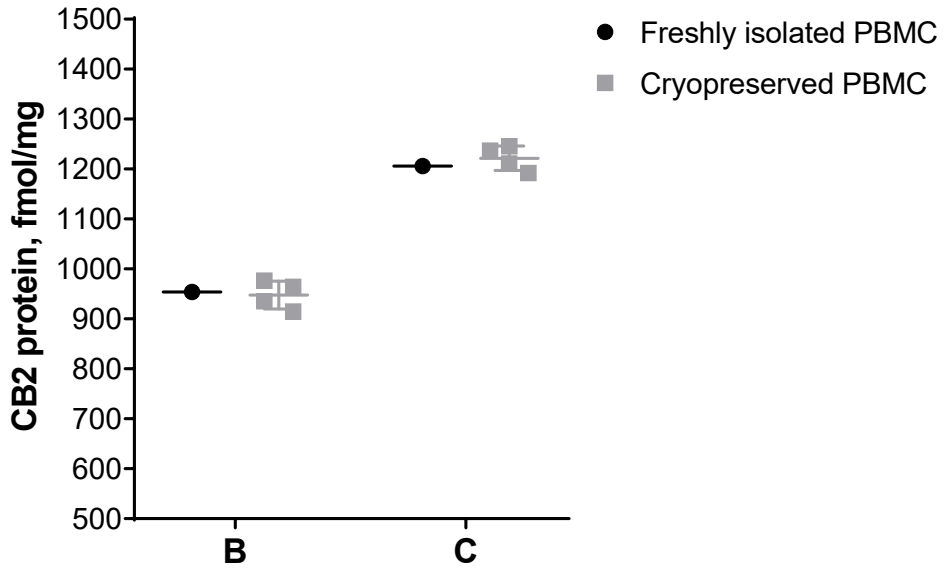
Supplementary Fig. 6. p-ERK1/2 time-course in human primary PBMC. Cells were treated with 1 μM ACEA (■) or a vehicle control (●). Responses are normalized to vehicle control at each time point. Graph shows mean ± SEM for three independent experiments performed in technical triplicate, each on a separate subject (three subjects in total). Note absence of ERK1/2 signaling in PBMC treated with a CB_1-selective agonist ACEA.



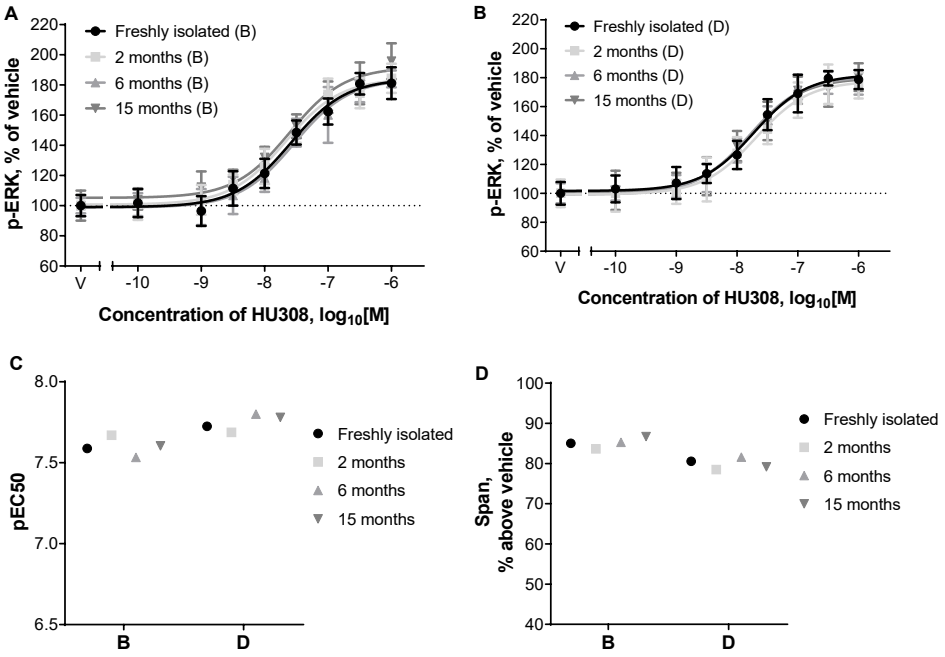
Supplementary Fig 7. Induction of cytokine secretion by PBMC. Cytokines in conditioned media from PBMC in response to HU308 (1 μM) in the presence or absence of forskolin (10 μM) after 12 h incubation. Graph shows independent experiment means (from technical triplicate) as well as overall mean ± SEM of these three independent experiments each performed with cells from a separate subject (three subjects in total).



Supplementary Fig. 8. Cell concentrations in PBMC samples used for cytokine secretion analysis. PBMC were seeded at 4.5 × 10^5 cells/well, and after incubations with the specified ligands or vehicle controls for 12 h (**A**) or 6 h (**B, C**) and supernatant collections, remaining cell pellets were re-suspended and counted in a hemocytometer. FSK – forskolin, SR2 – SR144528, WIN-3 – WIN55212-3. Note absence of cell concentration changes in all conditions. Graphs show independent experiment means (from technical triplicate) as well as overall mean ± SEM of these three independent experiments each performed with cells from a separate subject (three subjects in total).



Supplementary Fig. 9. CB_2 expression in freshly isolated and cryopreserved human primary PBMC. Freshly isolated and cryopreserved (then resuscitated after 4 days, 2, 6, and 14 months) PBMC from subjects B and C were incubated for 3 h at 15°C with [^{3}H]-CP55940 (5 nM) in the presence of a displacer HU308 (1 μM, displaceable binding), or a vehicle control (total binding). The difference between total and HU308-displaceable binding was considered specific binding, and utilized to calculate total binding sites (B_{max}) and then converted to fmol/mg of total protein as described in methods. Graph shows means from 4 independent experiments performed in technical triplicate, with overall mean ± SD, with samples from two donors (B, C). Note that there is no difference in CB_2 expression in freshly isolated and cryopreserved for up to 14 months PBMC.



Supplementary Fig. 10. p-ERK1/2 concentration-responses with freshly isolated and cryopreserved human primary PBMC. A and **B**: freshly isolated and cryopreserved (then resuscitated after 2, 6, and 15 months) PBMC from subjects B and D, respectively, were treated with a concentration series of HU308 or a vehicle control (indicated as "V"); data is normalized to vehicle controls (100%); concentration-response curves were obtained by fitting three-parameter nonlinear regression curves (Hill slope constrained to 1) using GraphPad Prism Software. **C**. Potencies of HU308 in freshly isolated and cryopreserved PBMC from subjects B and D calculated from the curves A and B, respectively. **D**. Efficacies of HU308 in freshly isolated and cryopreserved PBMC from subjects B and D calculated as spans of the curves A and B, respectively, above vehicle controls. Graphs A and B show mean ± SD of four independent experiments performed in technical triplicate, each with PBMC samples from the same isolation, subjects B and D, respectively; graphs C and D show calculated parameters from each concentration-response curve (shown in A and B). Note that there is no difference in concentration-responses with freshly isolated and cryopreserved for up to 15 months PBMC.