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

Yurii Saroz[1,2], Dan T. Kho[2,3], Michelle Glass[4], E. Scott Graham[2,3], Natasha Lillia Grimsey[1,2,*]

[1] Department of Pharmacology and Clinical Pharmacology, School of Medical Sciences, Faculty of Medical and Health Sciences, University of Auckland, 85 Park Road, Grafton, Auckland 1023, New Zealand

[2] Centre for Brain Research, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand

[3] Department of Molecular Medicine and Pathology, School of Medical Sciences, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand

[4] Department of Pharmacology and Toxicology, School of Biomedical Sciences, Division of Health Sciences, University of Otago, Dunedin, New Zealand

* **Corresponding author**: n.grimsey@auckland.ac.nz, +64 9 923 1886.

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Author ORCIDs:

YS - https://orcid.org/0000-0003-4699-3127

DTK - https://orcid.org/0000-0001-7193-2954

MG - https://orcid.org/0000-0002-5997-6898

ESG – https://orcid.org/0000-0002-2925-9126

NLG - https://orcid.org/0000-0003-3941-4537

Significance statement

Cannabinoid receptor 2 (CB2) is a G protein-coupled receptor which plays a complex role in immunomodulation and is a promising target in a range of disorders with immune system involvement. However, to date the majority of the studies in this field have been performed on cell lines, rodent models, or stimulated primary cells. Here we provide a detailed account of CB2-mediated signaling in primary human immune cells under conditions which closely mimic their *in situ* state. We reveal a complex signaling system involving an unprecedented CB2 signaling pathway and leading to immunomodulatory functional outcomes. This work provides not only a critical foundation impacting CB2-targeted drug discovery, but reveals important wider considerations for GPCR signaling studies and model validity.

Abstract

Cannabinoid receptor 2 (CB2) is a promising therapeutic target for immunological modulation. There is, however, a deficit of knowledge regarding CB2 signaling and function in primary human immunocompetent cells. We applied an experimental paradigm which closely models the *in situ* state of primary human leukocytes (PBMC; peripheral blood mononuclear cells) to characterize activation of a number of signaling pathways in response to a CB2-selective ligand (HU308). Application of G protein inhibitors revealed that an apparent "lag" phase of unchanged cAMP concentration prior to development of classically-expected $G\alpha_i$-mediated inhibition of cAMP synthesis was a result of counteraction by concurrent $G\alpha_s$ activation, a phenotype not previously observed for CB2. Monitoring downstream signaling events, p38 was mediated by $G\alpha_i$ whereas ERK1/2 and Akt phosphorylation were mediated by $G\alpha_i$-coupled βγ. Activation of CREB integrated multiple components; $G\alpha_s$ and βγ mediated ~85% of the response, while ~15% was attributed to $G\alpha_i$. Responses to HU308 had an important functional outcome – secretion of interleukins 6 (IL-6) and 10 (IL-10). IL-2, IL-4, IL-12, IL-13, IL-17A, MIP-1α, and TNF-α were unaffected. IL-6/IL-10 induction had a similar G protein coupling profile to CREB activation. All response potencies were consistent with that expected for HU308 acting via CB2. Additionally, signaling and functional effects were completely blocked by a CB2-selective inverse agonist, giving additional evidence for CB2 involvement. This work expands the current paradigm regarding cannabinoid immunomodulation and reinforces the potential utility of CB2 ligands as immunomodulatory therapeutics.

Introduction

The immunomodulatory properties of cannabinoids, compounds derived from *Cannabis sativa* and their synthetic analogues, are promising therapeutic leads in a wide range of disorders with immune system involvement, including leukemia (1, 2), multiple sclerosis (3–5), rheumatoid arthritis (6, 7), atherosclerosis (8, 9), and stroke (10, 11). Cannabinoids exert their effects mainly via two cannabinoid receptors, CB1 and CB2, which are class A (rhodopsin-like) G protein-coupled receptors (GPCRs) (12). Unlike CB1, which is abundant in the central nervous system, CB2 is expressed primarily in the immune system (e.g 13, 14) and is a promising therapeutic target for immune modulation without inducing adverse psychotropic side effects. CB2 is widely accepted to couple to $G\alpha_{i/o}$ proteins which mediate inhibition of adenylyl cyclases (e.g. 15–19), and limited evidence indicates coupling to $G\alpha_q$ calcium signaling (20–22). To date, there is no direct evidence for CB2 coupling to $G\alpha_s$; although stimulation of cyclic adenosine monophosphate (cAMP) synthesis has been reported in specific contexts, this was suggested to be coupled to $G\alpha_{i/o}$, likely via $G\beta\gamma$ (23, 24). Downstream of G protein coupling, and perhaps involving modulation by β-arrestins (25), activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) is a widely studied signaling consequence of CB2 activation reported in model cell lines heterologously expressing this receptor (e.g. 17–19) and in rodent (e.g. 26, 27) and human immunocompetent cell lines (e.g. 21, 24, 28), with limited published evidence for such signaling in primary human leukocytes (29, 30). The paucity of studies on primary human leukocytes may be explained by methodological difficulties of working with blood-derived primary human cells (e.g. low yields or purity of isolated leukocytic populations). Mitogenic or antigenic proliferative stimulation of leukocytes are widely used approaches to overcome some of these practical limitations (e.g. 29, 30). However, these methods alter cellular phenotypes causing lymphocytes to de-differentiate and turn into lymphoblasts – mitotically overactive cells – which do not closely model *in situ* state of leukocytes (31).

CB2 signaling via other types of mitogen-activated protein kinases (MAPKs) has been less widely studied, and the limited literature indicates context-dependence of activation. CB2 can mediate induction of p38 phosphorylation (p-p38; (32–34), which has been linked to growth inhibition of cancer cells (33, 34), yet in LPS-stimulated monocytes p-p38 is decreased by a CB2-selective agonist (21), and B lymphocytes (34) and dendritic cells (35) are non-responsive to some cannabinoids in the p38 pathway. Akt kinase (protein kinase B), an important mediator of immunomodulation (reviewed in 36, 37) can be activated (38), inhibited (39), or unaffected (40) by CB2 activation. A stress-activated protein kinase JNK

(c-Jun NH2-terminal kinase), involved in pro-inflammatory signaling (reviewed in 41), has been shown to be activated (32) and inhibited (21) via CB2.

The ultimate outcomes of CB2 activation in immune cells include the inhibition of proliferation and induction of apoptosis, influences on cytokine/chemokine networks, and regulation of adhesion and migration (reviewed in 42). CB2 ligands influence cytokine networks in human immunocompetent cells, inhibiting IL-2 (e.g. 43), IL-17, INF-γ, TNF-α (e.g. 44) secretion, and stimulating IL-4 (45) and TGF-β (46) secretion. Murine cell models indicate that cannabinoids inhibit IL-2 (47), IL-12 and IFN-γ (48, 49), and induce IL-4 secretion (48, 49), with similar effects *in vitro* and *in vivo*. However, to our knowledge all studies of cannabinoid influence on cytokine networks to date have utilized human or murine leukocytes stimulated with mitogens (e.g. phytohemagglutinin, Concanavalin A) or anti-CD3/anti-CD28 antibodies. Species differences in cannabinoid signaling notwithstanding (19, 50, 51), cytokine secretion may well be highly dependent on experimental conditions and cellular environment. Indeed, cannabinoid-induced effects on cytokine network have been shown to be sensitive to the method of cell activation (52). This is not surprising given the considerable degrees of crosstalk in signaling pathways in general, particularly MAPK (reviewed in 53).

Therefore, close to the entirety of our current understanding of CB2 signaling and influence on the cytokine secretome has been obtained from models which exhibit considerable limitations in modelling normal human physiology (e.g. 54–56). We endeavored to carry out the first comprehensive study of CB2 signaling in unstimulated primary human leukocytes (peripheral blood mononuclear cells, PBMC) under conditions closely preserving their *in situ* state. We have observed an unprecedented CB2-mediated signaling and functional profile, including novel coupling to $G\alpha_s$.

Results

CB2, but not CB1, is expressed in unstimulated primary human PBMC

Prior to initiating signaling studies, we quantified expression of CB1 and CB2 protein by whole cell radioligand binding with high affinity CB1/CB2 ligand [^{3}H]-CP55940 displaced by a CB1- and a CB2- selective ligand, ACEA (57) and HU308 (58) respectively. Although CB2 was expected to be the primary cannabinoid-responsive receptor in human PBMC, CB1 transcripts have also been detected in some contexts (e.g. 13, 59).

Whereas CB1 protein could not be detected in PBMC from any of three healthy human donors, CB2 was readily detectable in all (Fig. 1). Intra-subject variability in CB2 expression was low (independent experiments are from three separate blood draws taken over the course of up to 8 months from the same donor), while inter-subject expression was within the same magnitude, ranging from 704 to 1323 fmol/mg. PBMC samples from these same three donors were utilized in subsequent signaling and functional experiments.

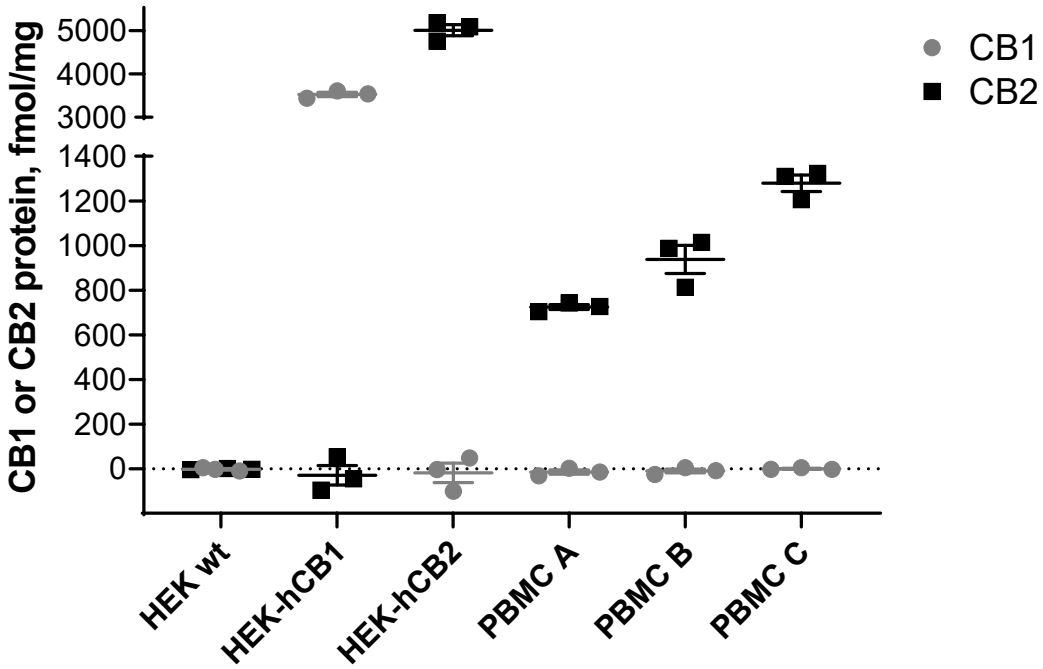

Fig. 1. CB1 and CB2 protein quantification by whole cell radioligand binding assay. Cells were incubated with [^{3}H]-CP55940 (5 nM) in the presence and absence of a CB1-selective displacer ACEA (1 μM), or a CB2-selective displacer HU308 (1 μM). Total binding sites (B_{max}) were calculated (see methods) and then converted to fmol/mg of total protein. HEK wt is a negative control (not expressing CB1 or CB2), HEK-hCB1 is a positive control for CB1, HEK-hCB2 is a positive control for CB2. PBMC A, B, and C are from subjects A, B, and C, respectively. Graph shows means from 3 independent experiments, with overall mean ± SEM.

CB2 activation induces simultaneous $G\alpha_i$ and $G\alpha_s$ coupling, producing delayed cAMP flux

Inhibition of cAMP is the primary recognized signaling pathway to which CB2 couples (e.g. 15–18, 60). This cAMP flux is mediated via $G\alpha_{i/o}$ inhibition of adenylyl cyclases, but can also be modulated by $G_{\beta\gamma}$. Depending on basal adenylyl cyclase activity in the sample of interest, stimulation of adenylyl cyclase is a frequent prerequisite for measuring inhibition, for example with canonical stimulant forskolin (e.g. 15–18, 60).

We first studied the time-course of cAMP signaling in PBMC in response to a CB2-selective agonist HU308 (1 μM), and observed inhibition of forskolin-stimulated cAMP synthesis in line with our general expectations (Fig. 2A). However, there was a lag to the onset of measurable inhibition which lasted 10 min. This was followed by a steady reduction in cAMP concentration, reaching a maximum extent by 30 min (to 60.3 ± 2.8% of forskolin-stimulated), with cAMP subsequently returning back to the forskolin-induced level by 60 min. Previous studies in cells overexpressing CB2 (e.g. 61, 62) describe an immediate onset and an earlier peak cAMP inhibition – as would be expected assuming cAMP flux is directly downstream of receptor activation. Given that we cannot readily measure inhibition of cAMP production without forskolin being present to stimulate adenylyl cyclase, we hypothesized that a low basal cAMP concentration and/or slower response to forskolin in the primary leukocytes might mask the ability to detect inhibition at early time-points and hence produce the observed delay. However, pre-incubation with forskolin prior to HU308 addition did not influence the signaling latency, time to peak response, nor efficacy, therefore ruling out this hypothesis (Fig. 2B).

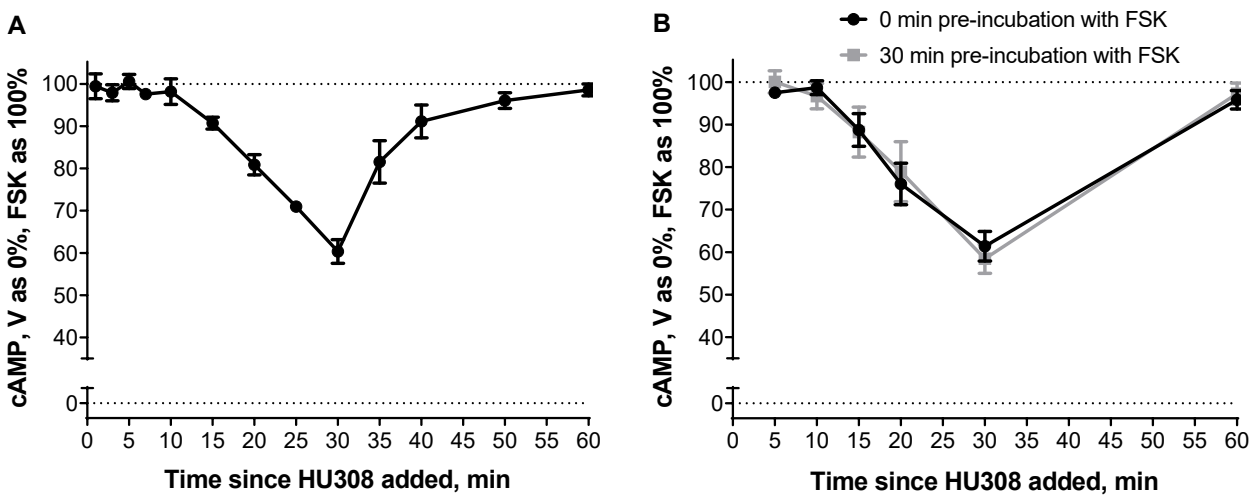

Fig. 2. cAMP signaling in primary human PBMC. A. Time-course of 1 μM HU308 with 10 μM forskolin (FSK) added simultaneously. **B.** Time-course of 1 μM HU308 with 10 μM

forskolin 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%). Graphs show mean ± SEM for three independent experiments performed in technical triplicate, each on a separate subject (three subjects in total).

Application of pertussis toxin (PTX), a $G\alpha_i$ (63–65) and $G\alpha_i$-derived βγ (66–68) inhibitor at the same time as HU308 stimulation, revealed a wave of increased cAMP in PBMC (Fig. 3A). This induction of cAMP accumulation had an earlier onset than the net cAMP inhibition (without PTX), with peak activation at around 10 min, and a slower return to forskolin-stimulated levels than the net cAMP flux.

Given that increased cAMP following GPCR activation is typically a result of $G\alpha_s$ coupling, we tested the effect of $G\alpha_s$-blocker NF449 (69–73) on HU308-stimulated cAMP flux. In comparison with net cAMP flux, not only was the peak of inhibition shifted to an earlier time-point (20 min), but the onset of cAMP inhibition had no apparent delay. The time-courses for onset of reduction and return to forskolin-stimulated cAMP levels for this now apparently "pure" inhibitory signaling were roughly symmetrical.

Taken together, these findings indicate that when no signaling inhibitors are present the opposing influences of $G\alpha_i$ and $G\alpha_s$ coupling produce a net apparent "lag" to the onset cAMP flux. At later time-points, the different time-courses and relative efficacies of $G\alpha_i$ versus $G\alpha_s$ coupling to adenylyl cyclase(s) results in transient net inhibition of cAMP synthesis, prior to resolution back to forskolin-stimulated levels. Consistent with the ability of CB2 to increase cAMP via activation of adenylyl cyclase, increased cAMP in response to HU308 was detectable even without forskolin stimulation, although inhibition of $G\alpha_i$ was required to reveal this as was the case when forskolin was present (Supplementary Fig. 1).

cAMP fluxes at their respective peaks (30 min for net cAMP, 10 min for cAMP accumulation, 20 min for cAMP inhibition) were concentration-dependent (Fig. 3B) and were of nanomolar potency, typical for CB2-mediated signaling by HU308 (19, 61). Potencies of the cAMP responses were subtly different between peaks, with pEC50 spanning 9.02 ± 0.06 to 8.44 ± 0.06, with the following rank order: stimulatory cAMP > "pure" inhibitory > net cAMP responses (Fig. 3C). Not surprisingly from the observed profiles and opposing effects on cAMP concentration, the peak stimulatory and "pure" inhibitory cAMP responses revealed by the Gα inhibitors had greater efficacies than the maximal net cAMP response (Fig. 3D). Between-subject variability was small; note that the data in Fig. 3 shows the mean and error from experiments performed on three different human donors.

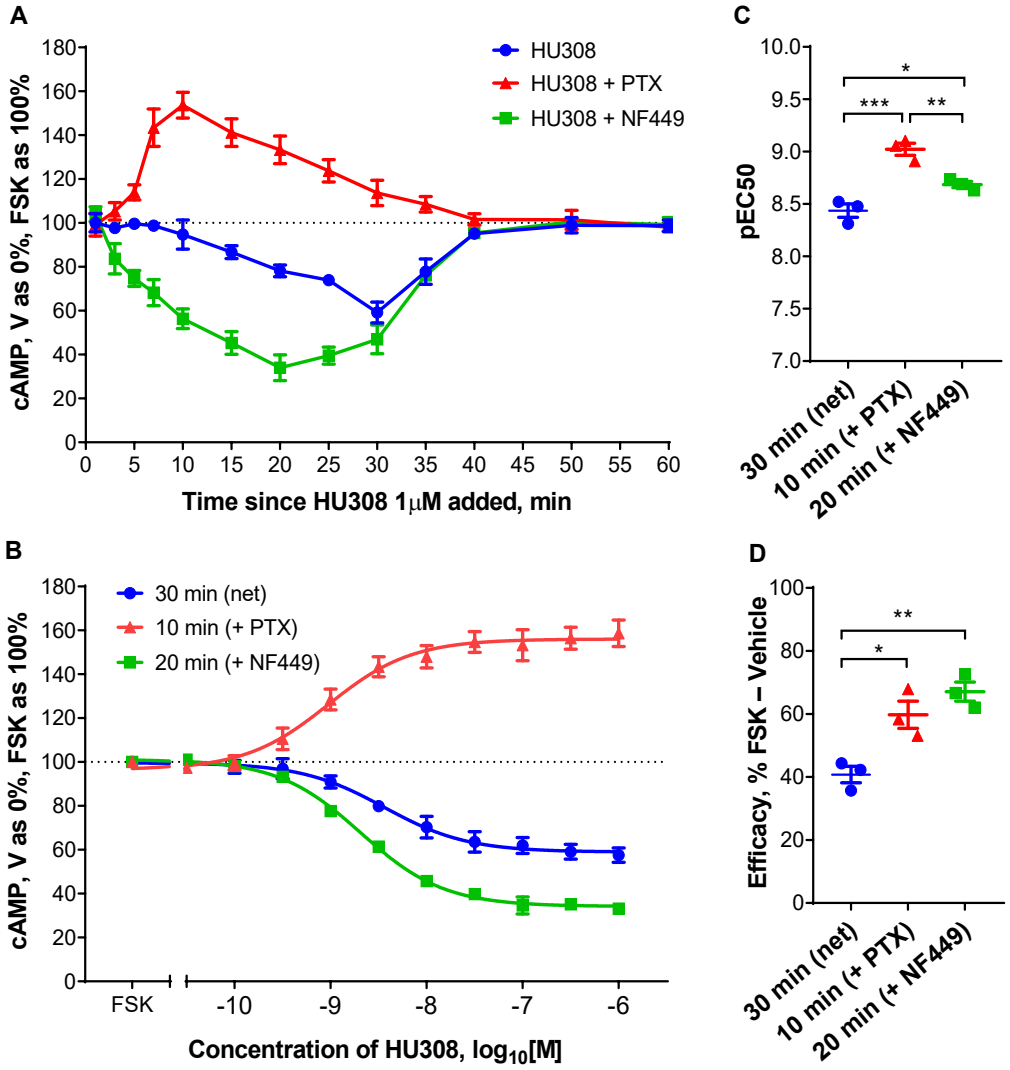

Fig. 3. Effects of PTX and NF449 on signaling kinetics and potency of cAMP responses in PBMC. A. PBMC were incubated with 10 μM forskolin (FSK) and HU308 (1 μM) for specified time periods without inhibitors (●), or after pre-treatment with NF449 (10 μM for 30 min, ■), or with PTX (100 ng/mL for 6 h, ▲). **B.** Concentration-responses with 10 μM forskolin (FSK) and HU308 without inhibitors (●), or pre-treated with NF449 (■), or PTX (▲) and then stimulated with varying concentrations of HU308 for 30, 10, or 20 min, respectively (at signaling peaks). **C, D.** HU308 concentration-response curve parameters; potency (**C**) and efficacy (**D**; span of curves normalized as in **B**). All graphs show mean ± SEM for three experiments performed independently, each on a separate subject (three subjects in total).

Focusing again on the peak response time points, we further probed the contribution of G proteins in the discovered cAMP signaling (Fig. 4A) and verified CB2-specificity (Fig. 4B). As expected from the prior time-course data, $G\alpha_s$ inhibitor NF449 had a minimal effect on the net cAMP response peak (30 min), while $G\alpha_{i/o}/\beta\gamma$ inhibitor PTX and $G\alpha_i$ inhibitor NF023 (72, 74, 75) blocked the HU308-induced inhibition of cAMP synthesis. The “pure” inhibitory response (in the presence of NF449) was also completely blocked by the $G\alpha_{i/o}$ inhibitors. In contrast, the peak stimulation of cAMP synthesis (measured in the presence of PTX) was completely blocked by $G\alpha_s$ inhibitor NF449. A Gβγ inhibitor gallein (72, 73, 75–78) had no effect on cAMP signaling, indicating lack of Gβγ involvement (Fig. 4A).

As well as the potencies of HU308-induced cAMP flux being consistent with CB2-specific responses, we also sought to verify CB2 specificity via competition with other CB2-selective ligands. Inverse agonist SR144528 (79) at 1 μM (a concentration at which it binds to CB2, but not to CB1, Supplementary Fig. 2) acted as an inverse agonist and completely blocked the net, stimulatory, and inhibitory cAMP signaling induced by HU308 (Fig. 4B), bringing all cAMP responses to the level of SR144528-induced response. A CB1/CB2 neutral antagonist WIN55212-3 (80, 81) also completely abrogated all HU308-induced cAMP signaling (Fig. 4B). Given the lack of CB1 protein in PBMC (Fig. 1), WIN55212-3 most likely acted via CB2 receptors, and taken together with the SR144528 effects and response potencies provides strong evidence that the observed signaling is CB2-mediated.

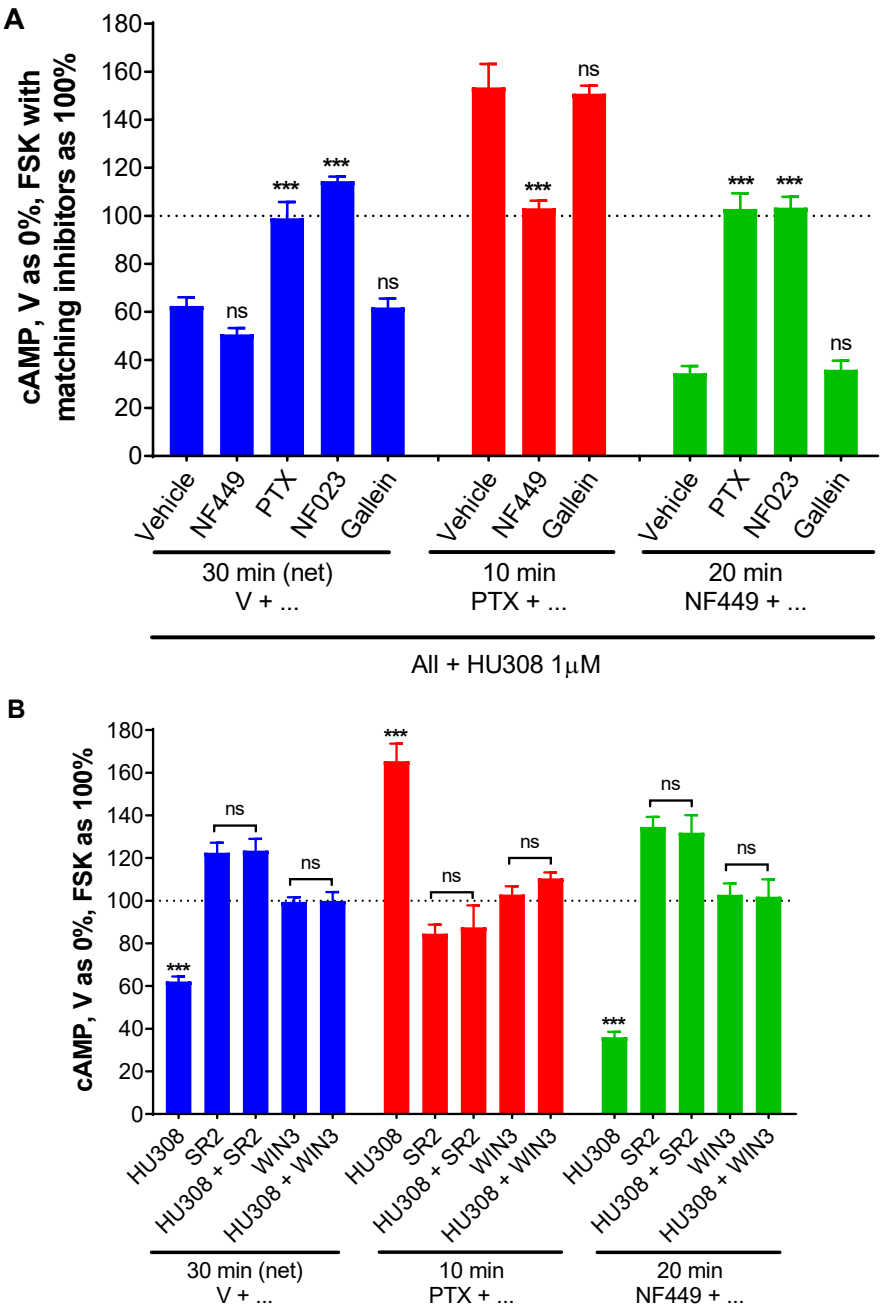

Fig. 4. Effects of G protein inhibitors and CB2 antagonists at cAMP signaling peaks in primary human PBMC. Stimulations were with 10 μM forskolin and 1 μM HU308 or vehicle for 30 min for net cAMP flux, 10 min for the stimulatory pathway (revealed by 6 h pre-treatment with PTX, 100 ng/mL), or 20 min for the inhibitory pathway (revealed by 30 min pre-treatment with NF449, 10 μM). **A.** PBMC were pre-treated as indicated with PTX, NF449, NF023 (30 min at 10 μM) or gallein (30 min at 10 μM). Data is normalized to vehicle control as 0% and forskolin controls with matching inhibitors as 100%. Statistical comparisons are to the vehicle control within each set (10, 20, 30 min). **B.** PBMC were pre-treated as indicated, then co-incubated with forskolin (10 μM), a CB2-selective inverse agonist/antagonist SR144528 (SR2, 1 μM) or a CB1/CB2 neutral antagonist WIN55212-3 (WIN-3, 5 μM), and HU308 (100 nM) or vehicle for 10, 20, or 30 min. Data is normalized to

a vehicle control as 0% and a forskolin control as 100%. All SR2 and WIN-3 conditions were significantly different from “HU308” control for peak signaling within each set (10, 20, 30 min) (*P < 0.001*). Within each set, condition “SR2” was compared to “SR2 + HU308”, “WIN3” was compared to “WIN3 + HU308”, and had no significant difference. Graphs show mean ± SEM for three experiments performed independently, each on a separate subject (three subjects in total).

CB2-stimulated phosphorylation of ERK1/2 is mediated by $G\alpha_i$- but not $G\alpha_s$-coupled Gβγ

We next sought to investigate downstream signaling consequences of CB2 activation in primary human PBMC. Phosphorylation of ERK1/2 (p-ERK) is a widely observed CB2 response, but the potential pathways to stimulation are many and varied, and as a result p-ERK can act as an integrator of responses (reviewed in 82). Time-courses with 1 μM HU308 revealed transient activation of p-ERK, with a peak at around 3 min (Fig. 5A). HU308 activated ERK1/2 in a concentration-dependent manner (Fig. 5B), with approximately 1 log unit lower potency than that observed for cAMP fluxes (pEC50 7.69 ± 0.06). Responses were very consistent between the three donors.

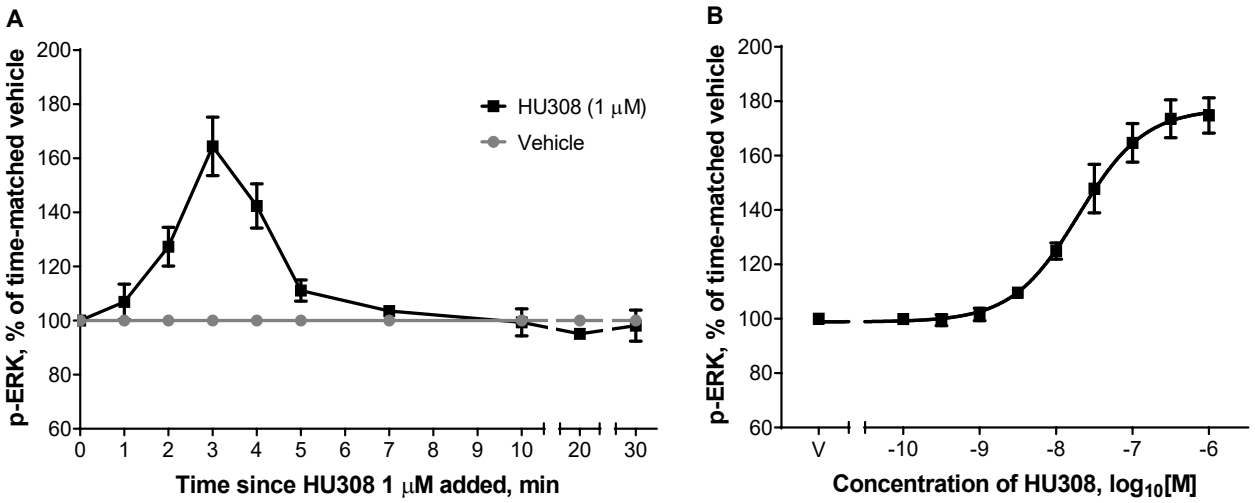

Fig. 5. ERK1/2 signaling in primary human PBMC. A. Time-course with 1 μM HU308 (■) or a vehicle control (●). **B.** HU308 concentration-response curve at 3 min, “V” indicates vehicle control. Graphs show mean ± SEM for three experiments performed independently, each with cells from a separate subject (three subjects in total).

To test the pathway via which CB2 induces p-ERK in primary PBMC, we again used $G\alpha_i$ inhibitor NF023 and $G\alpha_s$ inhibitor NF449, neither of which had an effect on the observed ERK1/2 signaling (Fig. 6A), whereas $G\alpha_{i/o}/\beta\gamma$ inhibitor PTX and $G\beta\gamma$ inhibitor gallein both completely blocked activation of p-ERK. Taken together, these findings indicate that ERK1/2 phosphorylation induced by HU308 is mediated by βγ dimers of $G\alpha_i\beta\gamma$ heterotrimeric proteins.

Analogous to our cAMP experiments, we further verified that the observed p-ERK1/2 response was CB2-mediated by co-applying HU308 with SR144528 or WIN55212-3 (Fig. 6B). Both antagonists completely blocked the peak signaling at 3 min (SR144528 producing inverse agonism), supporting the inference that HU308 binding to CB2 receptors is the only initiator of the measured p-ERK signal. Furthermore, we observed no p-ERK response for CB1-selective agonist ACEA at 1 μM (Supplementary Fig. 3), a concentration which we verified binds to human CB1 but not CB2 (Fig. 1) and can induce CB1-mediated p-ERK (e.g. 83).

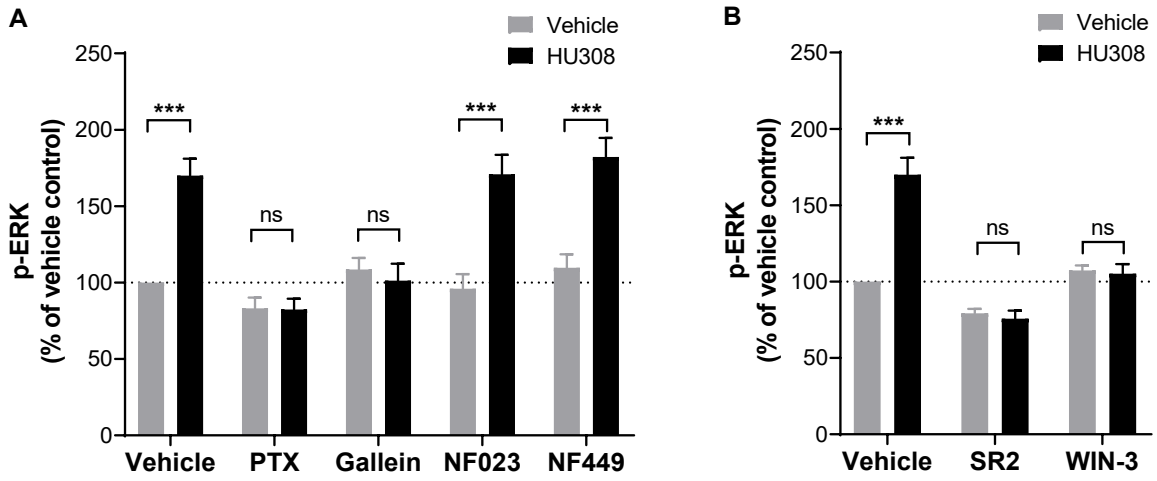

Fig. 6. Effects of CB2 pathway inhibitors and antagonists on p-ERK signaling in primary human PBMC at 3 min. A. p-ERK stimulated by HU308 (1 μM) or vehicle control in the presence of G protein inhibitors PTX (100 ng/mL, 6 h pre-treatment), gallein, NF023, NF449 (10 μM, 30 min pre-treatments) or vehicle control. **B.** p-ERK stimulated by a CB2-selective agonist HU308 (100 nM) or vehicle control in the presence of a CB2-selective inverse agonist SR144528 (SR2, 1 μM), a CB1/CB2 neutral antagonist WIN55212-3 (WIN-3, 5 μM), or vehicle control. Data is normalized to vehicle control (100%). Graphs show mean ± SEM for three experiments performed independently, each on a separate subject (three subjects in total).

CB2 induces phosphorylation of p38 via $G\alpha_i$ and Akt via Gβγ, but does not activate JNK

CB2 has previously, though inconsistently, been reported to stimulate phosphorylation of p38 (32–34), Akt (38), and JNK (32). Time-courses with 1 µM HU308 (Fig. 7A) revealed phosphorylation of Akt and p38 with signaling peaks at around 20 min, and with overall kinetics similar between the two protein kinases. Conversely, there was no phosphorylation of JNK in PBMC in response to HU308. Application of PTX completely abrogated HU308-induced Akt and p38 phosphorylation (Fig. 7B), implicating the involvement of $G\alpha_i$/βγ proteins. To further distinguish between $G\alpha_i$ and βγ signaling, we applied a Gβγ inhibitor gallein, and found that at the signaling peak (20 min) phosphorylation of Akt, but not of p-38, was completely blocked by gallein (Fig. 7C). Taken together, these finding show that HU308 induces phosphorylation of p38 downstream of $G\alpha_i$ subunits, while Akt is activated via $G\alpha_i$ –coupled βγ heterodimers.

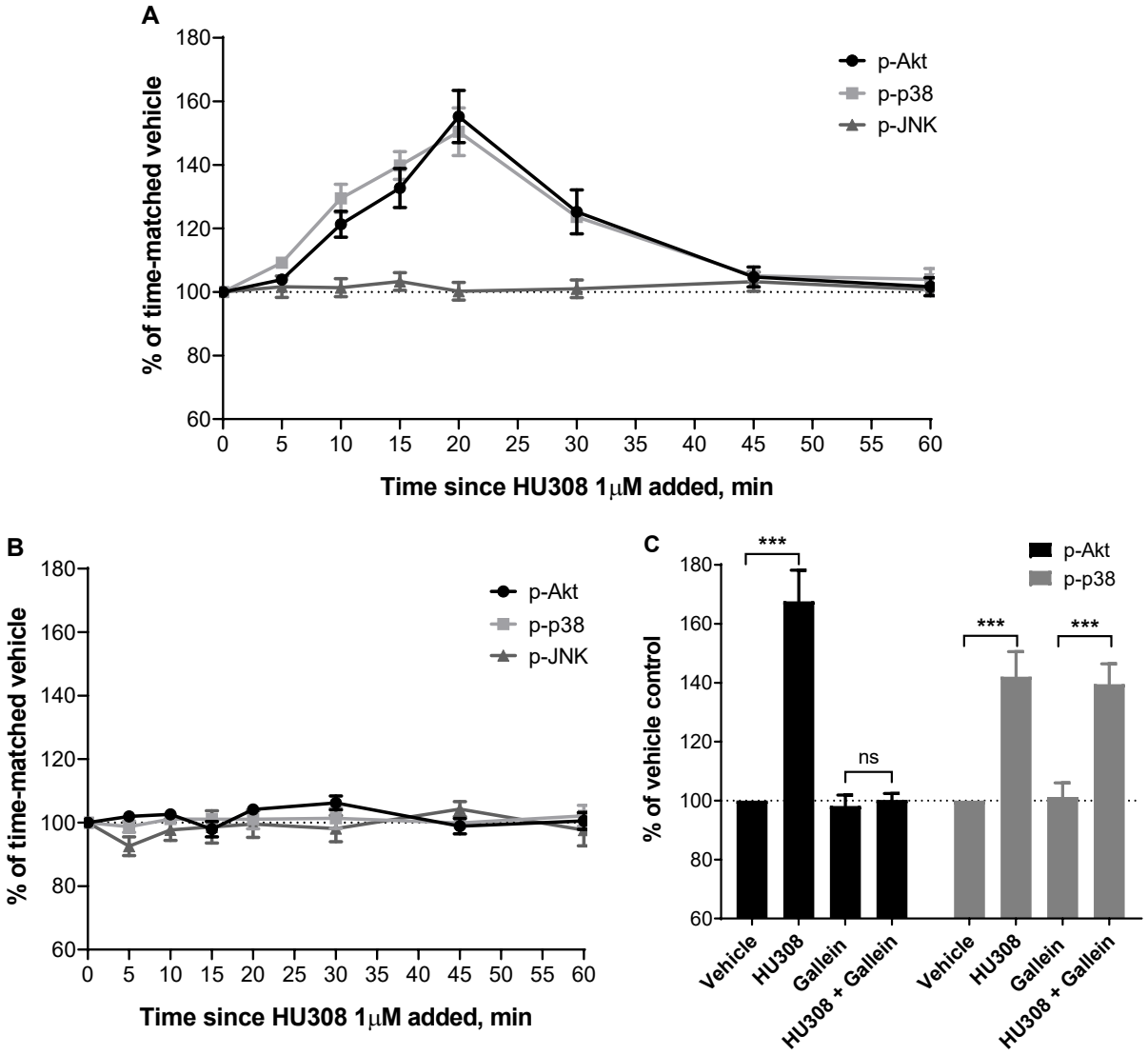

Fig. 7. Phosphorylation of Akt, JNK, and p38 MAP kinases in primary human PBMC. Time-courses with HU308 (1 μM) and vehicle control without PTX (**A**) or after 6 h of treatment with 100 ng/mL PTX (**B**). **C.** Responses to 1 μM HU308 after a 30-min pre-treatment with 10 μM gallein or vehicle control at 20 min (signaling peaks for p-Akt and p-p38). Data is normalized to vehicle control. Graphs show mean ± SEM for three experiments performed independently, each on a separate subject (three subjects in total).

CB2 induces phosphorylation of CREB downstream of $G\alpha_s$ and $G\beta\gamma$

Although to our knowledge not previously reported in the context of CB2, CREB phosphorylation is a classical downstream consequence of $G\alpha_s$ and cAMP-mediated activation of protein kinase A (PKA; reviewed in 84). A time-course with PBMC stimulated by 1 μM HU308 (Fig. 8A) revealed a wave of CREB phosphorylation which was maximal at around 30-40 min and resolved to basal level by 60 min. To investigate involvement of G

proteins in p-CREB signaling, we applied G protein inhibitors NF023, NF449, gallein and PTX (Fig. 8B), and found that both NF449 and gallein each partially inhibit CREB phosphorylation to a similar degree, but almost completely block the HU308 response when co-applied indicating that Gαs and βγ heterodimers are similarly important in CREB phosphorylation in this context. p-CREB was reduced by PTX and NF023 to a lesser extent, with equivalent efficacy to each other, implying a minor involvement of $G\alpha_i$ but not $G\alpha_i$-coupled βγ in p-CREB activation. SR144528 and WIN55212-3 completely blocked the peak CREB phosphorylation (Fig. 8C), indicating that this pathway activation occurs downstream of CB2. SR144528 reduced p-CREB relative to the vehicle control indicating that this compound again acted as an inverse agonist.

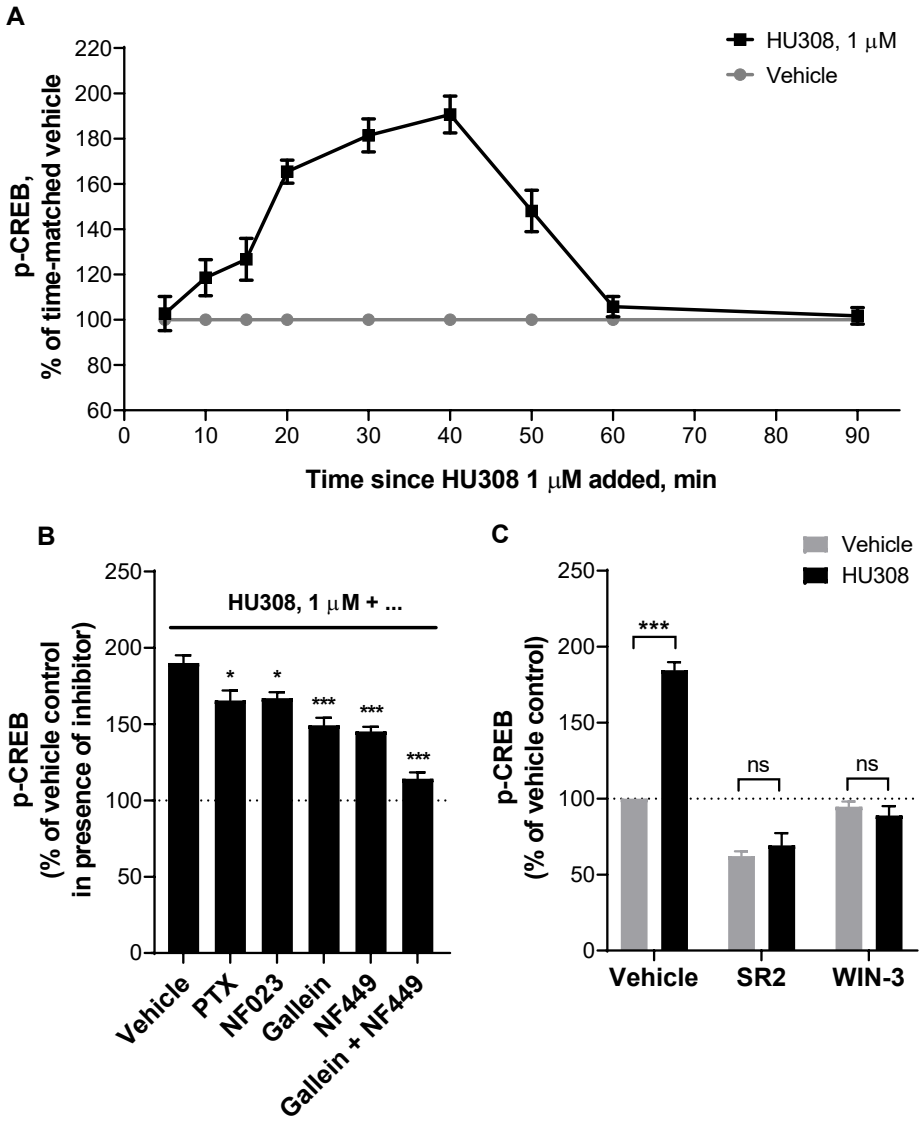

Fig. 8. Phosphorylation of CREB in primary human PBMC. A. Time-course with HU308 (1 μM) and vehicle control. **B.** p-CREB stimulated by HU308 (1 μM) for 40 min in the presence of G protein inhibitors gallein, NF023, NF449 (10 μM, 30 min pre-treatments), PTX (100 ng/mL, 6h) or vehicle control; data is normalized to responses in the absence of HU308

(100%); statistical comparisons are to the HU308 response without inhibitors. **C.** p-CREB stimulated by a CB2-selective agonist HU308 (100 nM) or vehicle control at 40 min in the presence of a CB2-selective inverse agonist/antagonist SR144528 (SR2, 1 μM), a CB1/CB2 neutral antagonist WIN55212-3 (WIN-3, 5 μM), or vehicle control; data is normalized to vehicle control (100%). Graphs show mean ± SEM for three experiments performed independently, each on a separate subject (three subjects in total).

CB2 activation induces secretion of cytokines IL-6 and IL-10

Modulation of the cytokine network is one of the mechanisms of cannabinoid-mediated immunomodulation (reviewed in 85). In order to study functional consequences of CB2 activation in primary human PBMC, we stimulated PBMC with HU308 under the same conditions as used for the signaling assays and detected cytokines released into the assay media. After a 12-hour incubation, interleukins 6 (IL-6) and 10 (IL-10) were detected at concentrations that were significantly different from vehicle-treated cells ($p < 0.01$), whereas the remaining 7 cytokines in our panel (IL-2, IL-4, IL-12, IL-13, IL-17A, MIP-1α, and TNF-α) were indistinguishable from vehicle-treated cells (Fig. 9A). Forskolin (10 μM) was included in the assay to mimic conditions of our cAMP signaling assays; this had no effect by itself and did not influence the HU308-mediated IL-6 and IL-10 levels. The responses were very similar between PBMC from 3 subjects and cell numbers were unaffected by the applied ligands (Supplementary Fig. 4).

IL-6 and IL-10 accumulation were also detectable at an earlier time-point – after 6 h of HU308 stimulation (Fig. 9B). This was concentration-dependent, with HU308 having greater potency for inducing IL-10 compared to IL-6 (pEC50 10.06 ± 0.08 and 9.55 ± 0.04; $p = 0.0052$, unpaired t test).

Application of G protein inhibitors revealed that HU308-induced secreted levels of both IL-6 and IL-10 are almost fully blocked by $G\alpha_s$ inhibitor NF449, partially blocked by βγ inhibitor gallein, and to a lesser extent sensitive to $G\alpha_i$ inhibitor NF023 (Fig. 9C).

Responses to HU308 (10 nM) were fully abrogated by SR144528 (1 μM) and WIN55212-3 (5 μM) down to levels below the assays' limits of quantitation (≤ 1.6 and ≤ 0.13 pg/mL for IL6 and IL10, respectively). SR144528 and WIN55212-3 had no measurable effects on IL-6 or IL-10 production when applied alone.

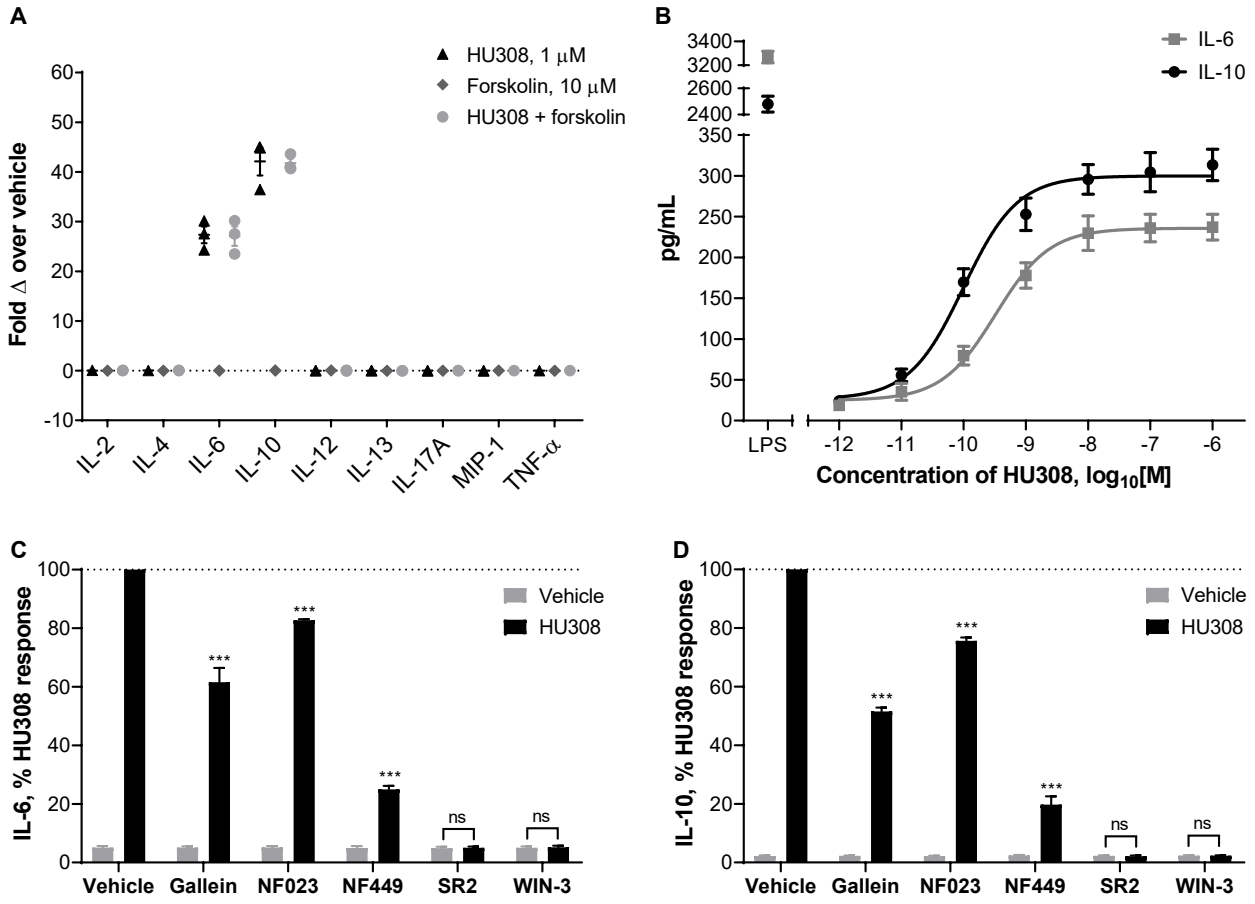

Fig. 9. Induction of cytokine secretion by PBMC. A. Multiplex screen of cytokine accumulation in conditioned media from PBMC in response to HU308 (1 μM) in the presence or absence of forskolin (10 μM) after 12 h of incubation; abbreviations: IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17A – human interleukins 2, 4, 6, 10, 12, 13, 17a, respectively; MIP-1α –human macrophage inflammatory protein 1α, TNF-α – human tumor necrosis factor α; means of three independent experiments (each performed on leukocytes from a separate subject, three subjects in total) and overall mean ± SEM. **B.** Induction of IL-6 and IL-10 secretion in response to a concentration series of HU308 after 6 h of incubation, with lipopolysaccharide (LPS, 10 ng/mL) as a positive control. **C.** IL-6 and **D.** IL-10 accumulation in the presence of G protein inhibitors or CB2 antagonists: PBMC were pre-treated with gallein, NF023, NF449 (all at 10 μM), SR144528 (SR2, 1 μM), or WIN55212-3 (WIN-3, 5 μM) for 30 min followed by 6-hour treatments with HU308 (10 nM) with the inhibitors/antagonists present for the entire duration of incubation; data normalized to HU308 responses without inhibitors/antagonists. Effects of inhibitors on HU308 responses were statistically compared with the corresponding HU308 response without inhibitor. In the presence of SR2 and WIN-3, there was no statistical difference between HU308 and the HU308 vehicle control. Graphs **B-D** show mean ± SEM of three independent experiments, each performed on leukocytes from a separate subject, three subjects in total.

Discussion

Despite CB2 being widely regarded as an important immune system modulator and promising therapeutic target, to our knowledge this study represents the first investigation of CB2 signaling and functional implications thereof in unstimulated primary human PBMC. Assays were performed on cells from three healthy donors with as limited time in culture as practically possible (up to 6 hours prior to stimuli of interest) and in the presence of 10% serum in order to closely mimic the state of leukocytes *in situ*; a unique experimental paradigm in comparison with the vast majority of other cannabinoid studies to date. We feel it is important to study CB2 in PBMC without stimulation by exogenous mitogens or antigens, as such proliferation-inducing stimuli cause complex changes in signaling and function (31) and alter CB1 and CB2 expression (86–89). Obtaining an accurate understanding of CB2 function in normal cells is critical to meaningful interpretation of CB2 function in abnormal conditions and in assessing therapeutic potential. The signaling profile we observed is summarized in Figure 10.

CB2-mediated inhibition of cAMP in response to cannabinoid treatment has been widely studied in cell models (e.g. 15–19). As a second messenger, cAMP elicits broad immunomodulatory effects, regulating both innate and adaptive immunological functions (reviewed in 90). Moreover, signaling pathways downstream of cAMP are associated with a number of disease states, ranging from cardiovascular disorders (e.g. 91, 92) to cancer (e.g. 93, 94). We found that primary human PBMC respond in a concentration-dependent manner to a CB2-selective agonist HU308 with net inhibition of forskolin-induced cAMP synthesis, however a surprising feature was a latency of approximately 10 min prior to any measurable change in cAMP concentration. We subsequently determined via use of Gα inhibitors that CB2 simultaneously activates stimulatory and inhibitory cAMP pathways, explaining the apparent delay in net cAMP inhibition which arises from these oppositely-directed responses balancing each other at early time-points. Subsequent to the peak of the $G\alpha_s$ component, the $G\alpha_i$-mediated inhibition of adenylyl cyclase becomes prominent and thereby produces the delayed net inhibition of cAMP accumulation. Both the apparent $G\alpha_s$- and “pure” $G\alpha_i$-mediated components, as well as the net cAMP flux, were all induced concentration-dependently with potencies consistent with CB2 specificity. cAMP signaling was also sensitive to a CB2 inverse agonist/antagonist SR144528 and a CB1/CB2 neutral antagonist WIN55212-3. For the latter, given that we could not detect CB1 expression nor functional effects, we take inhibition by WIN55212-3 to be supportive of a CB2-dependent mechanism. Taken together, this evidence is convincing that the observed signaling is CB2-mediated.

Although the idea that CB2 may couple promiscuously to G proteins is not unprecedented (e.g. 20–22), to our knowledge CB2 coupling to $G\alpha_s$ has not been reported previously. Börner and colleagues (24) observed stimulatory effects of CB1/CB2 mixed agonists JWH015, THC and methanandamide on cAMP concentrations in IL-4-stimulated primary human T lymphocytes after prolonged cannabinoid incubations, however cAMP increases were sensitive to PTX and therefore concluded to be downstream of $G\alpha_i$, likely via βγ (24). Promiscuous coupling of other receptors to $G\alpha_i$ and $G\alpha_s$ has been observed, including CB1 in our own hands (95). However, we did not observe $G\alpha_s$ coupling of human CB2 in this same model system. Studies of CB2 cAMP signaling in other model systems have also applied pertussis toxin and not revealed any signaling which would be consistent with $G\alpha_s$ coupling (e.g. 15, 16, 18). We therefore speculate that a critical component of the CB2 immune cell signaling landscape has not been captured in previously studied models.

Given that we have studied mixed PBMC and not attempted to define which cell type(s) produce CB2-mediated signaling, we cannot provide direct evidence as to whether both cAMP inhibition and stimulation have been induced in the same cell or if our findings arise as a result of assaying a mixture of $G\alpha_i$-predominant versus $G\alpha_s$-predominant cell types. Leukocytes are known to express $G\alpha_i$ (e.g. 96, 97) and ubiquitous $G\alpha_s$ (reviewed in 98), as well as $G\alpha_{i/s}$-sensitive adenylyl cyclase isoforms (e.g. 99). However, balance between Gα and Gβγ subunit expression has been suggested to be important in determining overall cAMP responses (100). As well, adenylate cyclase isoforms can differ in their Gα sensitivities and expression levels can vary between immune cell types (99). Furthermore, G protein coupling “switches” have been reported for receptors in heterodimers (e.g. 101, 102). The particular profile and stoichiometries of potential interaction partners in any given cell type could well give rise to differential signaling “units” between cell types (reviewed in 103). There are also various explanations which could give rise to dual coupling within the same cell. Signalsome subcellular compartmentalization and/or sequestration of signaling components is one example (reviewed in 104); indeed subcellular distribution of CB2 can influence CB2 signaling patterns (22), and CB2 seems to have a particularly unique distribution in immune cells (105–107). Homologous signaling feedback upon CB2 activation which results in rapid desensitization of $G\alpha_s$ coupling with little or slower influence on $G\alpha_i$ coupling (such as via differential phosphorylation by G Protein-Coupled Receptor Kinases, [108, 109] or PKA [110]) might assist in explaining the observed cAMP signaling profile. Gβγ exhaustion/sequestration has also been suggested to hinder $G\alpha_s$ activation (111, 112). It is also important to note, and intriguing regarding the downstream functional implications, that subcellular compartmentalization of G proteins and/or adenylyl cyclase subtypes could give

rise to localized cAMP fluxes (reviewed in 113, 114). This implies that simultaneous functional effects of both cAMP increases and decreases are possible even when the net cAMP concentration in a cell is unchanged.

Pursuing further downstream CB2-mediated signaling, we found that primary human PBMC respond to CB2 activation by HU308 with rapid and transient stimulation of p-ERK. The ERK 1/2 signaling cascade regulates cell cycle progression (reviewed in 90), immunological functions, including differentiation of T lymphocytes (29), polarization of CD4+ T lymphocytes (116), and regulation of cytokine network (e.g. 94, 95), and is dysregulated in a number of disease states (reviewed in 39, 91, 92). ERK1/2 is canonically thought to couple via $G\alpha_i$-coupled βγ proteins (68, 75, 121, 122), and when induced by cannabinoids is sensitive to a $G\alpha_{i/o}$/βγ inhibitor PTX (e.g. 17, 19). There is also evidence for arrestin-mediated p-ERK (25). Although in some systems gallein may inhibit GRK2 and possibly associated arrestin signaling (123), we do not know of any studies suggesting that PTX blocks arrestin-initiated signaling other than one report of a small potency shift for arrestin recruitment (124). Therefore, as the p-ERK signaling in PBMC was completely sensitive to both Gβγ inhibitor gallein and $G\alpha_{i/o}$/βγ inhibitor PTX, we conclude that ERK1/2 phosphorylation induced by HU308 is mediated by βγ dimers of $G\alpha_i\beta\gamma$ heterotrimeric proteins. Interestingly NF023, which we confirmed in cAMP assays inhibits $G\alpha_i$, did not block the p-ERK response, indicating that this blocker specifically inhibits Gα subunits without influencing Gβγ activity (as has also been observed previously; 75).

In addition to ERK1/2, HU308 induced phosphorylation of p38 and Akt. p-p38 signaling was mediated by $G\alpha_{i/o}$ subunits of G proteins (possibly via Src kinase 125–127), as it was completely blocked by $G\alpha_{i/o}$/βγ inhibitor PTX but not by Gβγ inhibitor gallein, whereas p-Akt was mediated by $G\alpha_{i/o}$–coupled βγ heterodimers (fully blocked by PTX and gallein). We did not observe JNK phosphorylation in contrast with a prior study in human promyelocytic leukemia HL60 cells (32). However, we note that activation of this pathway may be cell-context dependent given that in LPS-stimulated human monocytes JNK was inhibited by a CB2 ligand (21). Another possible explanation may be due to a different CB2-mediated functional profile (i.e. biased agonism) between HU308 and the previously studied ligands (19, 128). There may also be signal-modifying influences of serum as has been shown for CB1 (129). We must acknowledge that while we feel that inclusion of 10% fetal bovine serum is an important methodological improvement upon earlier studies carried out under low serum or serum-free conditions due to the strong potential for the serum factors that circulating immune cells would be exposed to *in situ* to influence signaling patterns (130),

this is of course not a perfect model of human serum from the matched donor. Some of the resistance to inclusion of serum in cannabinoid signaling studies is the potential for presence of endocannabinoids and precursor molecules which would complicate the interpretation of reductionist experiments (e.g. 131), however careful attention to vehicle controls, the potency of exogenously-applied HU308 effects, only subtle inverse agonist effects, and lack of effects of a neutral antagonist when applied alone, indicate that any potential influence in this regard was minimal to non-existent in our study.

Given the prevailing view that CB2 activation is typically immunosuppressive, it is perhaps surprising that p-p38 and p-Akt were induced given their known pro-inflammatory roles. Although p-p38 has pro-apoptotic effects on human T-lymphoblastic leukemic (33) and mantle cell lymphoma (34) cell lines, this pathway is widely regarded as having pro-inflammatory functions, e.g. stimulation of TNF-α (132) and IL-12 in macrophages (133), CD4+ T lymphocytes activation and differentiation into Type 1 helper cells (134), as well as induction of IL-6 (135, 136). Akt kinase plays an important role in innate immunity, contributing to macrophage activation and, again, synthesis of IL-6 (reviewed in 37).

cAMP/PKA, MAP kinases ERK and p38, and Akt/PKB all have the potential to induce phosphorylation of CREB as an integrative downstream effect of multiple signaling pathways (reviewed in 84). Immunological functions of p-CREB include anti-apoptotic effects on macrophages via p-38 (137), proliferative effects on T lymphocytes via p-38 (138) and p-ERK (139), and activation/proliferation of B lymphocytes via p-ERK (140). These three leukocytic subpopulations represent the majority of cell types that comprise the PBMC studied in this paper. CB2-mediated p-CREB activation in primary PBMC is mediated primarily and to a similar degree by $G\alpha_s$ subunits and non-$G\alpha_i$-derived βγ dimers, and to a limited extent by $G\alpha_i$ subunits. p-CREB is unlikely to be downstream of pERK or p-Akt in our system given that these pathways were both associated with βγ from $G\alpha_i\beta\gamma$ heterotrimers, however $G\alpha_i$-coupled p-p38 may be involved to a minor degree. One of the primary activation pathways for CB2-mediated p-CREB in PBMC is likely via $G\alpha_s$ activation of adenylate cyclase leading to cAMP-activated PKA. This is quite interesting given that when measuring cAMP concentrations directly we did not detect any increase in cAMP concentration in the overall cell population unless a $G\alpha_i$ blocker was present. This observation seems to imply that the consequences of $G\alpha_i$ versus $G\alpha_s$ activation on cAMP concentration are distinct, giving some evidence toward our theories that either CB2 is signaling differently between cell types or that subcellular compartmentalization can give rise to differential local cAMP concentrations with independent consequences for signaling.

Proceeding to measurement of a physiologically relevant immunological outcome, we detected a CB2-mediated induction of interleukins 6 and 10 at 6 and 12 h, which were both sensitive to NF449 > gallein > NF023, indicating that $G\alpha_s$ is the main contributor to IL-6 and IL-10 induction in PBMC. This pattern of influence of G protein inhibitors was generally similar to that observed for p-CREB, and given that p-CREB can induce transcription of IL-6 and IL-10 genes (reviewed in 141), it is very feasible that CREB phosphorylation is an important mediator of the effects of HU308 on IL-6 and IL-10 production. Given that p-p38 was blocked by PTX and not gallein, this is a candidate for the NF023-sensitive ($G\alpha_i$-requiring) component of IL-6 induction. p-p38 (142, 143) and p-Akt (144, 145) have also been linked to IL-10 production. Taken together, the simplest correlations between our signaling and cytokine induction data are a primary role of $G\alpha_s$ coupling, likely involving p-CREB, and more subtle role of $G\beta\gamma$ and $G\alpha_i$ signaling, perhaps via p-Akt (and/or p-ERK) and p-p38 respectively. Given the indicated involvement of CREB we were surprised to find that forskolin, a strong activator of signaling pathways downstream of adenylate cyclases, did not influence IL-6 or IL-10 at all. We postulate that a co-stimulus enabled by CB2 activation but not by forskolin might be required for IL-6/10 induction (147, reviewed in 84). It is also plausible that the aforementioned potential for subcellular compartmentalization of cAMP responses and/or temporal integration of cAMP fluxes play critical roles. Clearly, application of specific signaling pathway inhibitors would be required to more directly delineate the exact signaling pathways responsible for the IL-6/IL-10 production observed.

We did not observe induction of cytokines IL-2, IL-4, IL-12, IL-13, IL-17A, MIP-1α, or TNF-α, likely because we avoided activation of PBMC with mitogenic/antigenic proliferative or cytokine-inductive stimuli to preserve their *in vivo* phenotype and hence could not detect any potential inhibitory effects of HU308. Application of physiologically relevant stimuli under conditions of normal and dysregulated immunoreactivity represents an important direction for future research.

We also noted that HU308 did not influence cell number of treated PBMC at 6 and 12 h, which is *prima facie* unexpected given prior reports of cannabinoid-induced cell death. However, these prior studies were carried out under completely different experimental paradigms (e.g. 34, 148, 149), have been found to be cell type- and ligand-dependent (reviewed in 150), and are not always mediated by cannabinoid receptors (e.g. 151).

IL-10 is an anti-inflammatory cytokine expressed by many immunocompetent cell types which regulates T-lymphocytic responses via direct mechanisms, e.g. inhibiting CD4+ T cell proliferation (152), and by inhibiting effector functions of antigen-presenting cells, e.g.

activated macrophages and dendritic cells (153, 154). IL-10 can also exert protective functions from excessive immunoreactivity by inhibiting overproduction of cytokines, *including* IL-6 which is widely described as pro-inflammatory. Typical IL-6 effects include induction of acute phase proteins, monocyte recruitment and macrophage infiltration, activation of plasma cells, and CD4+ lymphocyte differentiation (reviewed in 155). Based on these paradigms the combined release of IL-6 and IL-10 seems counter-intuitive. However, IL-6 can context-dependently exert anti-inflammatory effects, and indeed simultaneous production of IL-6 and IL-10 from mixed primary human immune cells has been observed previously (156).

The HU308-induced production of both IL-6 and IL-10 within the same time-frame, and with strikingly similar potencies and patterns of influence by inhibitors, seem to imply that CB2 either stimulates release of one cytokine which induces the second, or that the two cytokines are induced via the same signaling pathways. Considerable literature supports the first possibility; for example, IL-6 in concert with other specific cytokines can stimulate differentiation of naïve CD4+ T cells into IL-10-producing regulatory type 1 helper T (Tr1) or IL-17-producing T helper (T_H-17) cells which have anti-inflammatory effects *in vivo* (157, 158). Low dose recombinant IL-6 can also increase plasma IL-10 and have other anti-inflammatory effects in humans (159). However, given the acute time-frame within which we detected both IL-6 and IL-10 (by 6h), and slightly greater potency of the IL-10 response, it seems unlikely that in our system CB2 activation first induces IL-6 which subsequently leads to IL-10 secretion from a different cell type.

It is possible that IL-6 and IL-10 were released independently from different cells, however, the near-identical profile of sensitivity to G protein inhibitors leads us to suspect that the most plausible scenario is that IL-6 and IL-10 are generated from the same cell type(s) in our system. Interestingly, these cytokines have coordinated mRNA regulation via the same nuclear site (160). IL-6 and IL-10 have classically been thought to be produced by T helper 2 (Th2) T lymphocytes (161, reviewed in 162), while concurrent production has been observed in monocytes, tumor-associated macrophages and dendritic cells (163–165). In future work it will be important to clarify the influence of CB2 activation on different immune subtypes and this knowledge will also assist in designing follow-on experiments to investigate other CB2-mediated immune cell functions.

We also note that the degree of cytokine induction we observed was small in comparison with the positive control utilized, LPS – a potent mediator of bacterial sepsis (reviewed in 166), which models systemic inflammation under experimental conditions, but is also

physiologically relevant for modeling vascular (167) and liver disorders (168). Given that HU308 was ~10 times less efficacious than LPS, it is unlikely that CB2 activation would result in dramatic systemic inflammatory reactions *in vivo*, but might rather elicit immunomodulatory effects with a potential for both pro- and anti-inflammatory functions – likely depending on the state of the immune system.

There is some precedent for cannabinoid mediation of both IL-6 and IL-10, though it has not before been studied in the specific context we present here. Endocannabinoids 2-AG and anandamide have upregulated IL-6 in LPS-stimulated U937 cells (131) and in whole blood cultures (169), respectively, with relatively high IL-6 levels compared to physiological concentrations (170). Cannabinoid-mediated IL-10 induction has been shown *in vivo* in mice (171), in murine LPS-stimulated macrophages (27), and in murine encephalitogenic T lymphocytes (172); the latter study also shows inhibition of IL-6 by cannabinoids, likely indicating species and/or cell type specificity. To our knowledge, CB2-mediated IL-10 production by human PBMC has never been shown before.

Historically, there is a considerable body of data regarding the general effects of cannabis and THC on human immune function, though somewhat surprisingly, little has been reported on cytokine profiles (reviewed in 173, 174). Increased serum IL-10 has been correlated with cannabis consumption (IL-6 was not measured, 175), while elevated IL-6 and other pro-inflammatory markers were found in individuals with cannabis dependency (IL-10 was not measured, 176). In a trial of THC and cannabis oil extract in multiple sclerosis patients, cannabinoid treatments were found not to influence serum concentrations of IL-10 or CRP (a surrogate measure of IL-6), nor other measured cytokines (comparing before versus 13 weeks after treatment), though the authors themselves point out the small sample sizes and large variability between subjects (177). Further, it is important to keep in mind that THC is at most a partial agonist at CB2 and so may have only subtle effects in comparison with full CB2 agonists. Only a few clinical trials of CB2-selective agonists have been undertaken and to our knowledge none have reported on cytokine profiles. We have also noted the serious adverse effects arising from administration of illicit synthetic cannabinoids, some of which have been found to be highly potent CB2 agonists (178, 179). Although it is speculated that these adverse symptoms are mediated by a non-cannabinoid-receptor target (e.g. 180), based on the results presented here we wonder whether a potent and efficacious influence on cytokine balance and/or different cytokine profile arising from biased agonism via CB2 could give rise to some of the deleterious effects.

The results of this study greatly expand our knowledge of CB2 signaling and functional implications in primary human PBMC under conditions closely preserving their *in situ* state. The discovery of unprecedented CB2 coupling to stimulatory ($G\alpha_s$) proteins, their simultaneous activation alongside inhibitory ($G\alpha_i$) proteins in a cAMP pathway, of $G\alpha_s$-mediated effects on CREB phosphorylation, and of IL-6 and IL-10 induction, offer new perspectives on CB2 signaling and function which may be useful for drug discovery and investigations of CB2 signaling phenomena including functional selectivity. The novel effects of CB2 agonism we have observed in this study raise a number of questions and potential challenges, but reinforce the potential utility of CB2 ligands as immunomodulatory therapeutics.

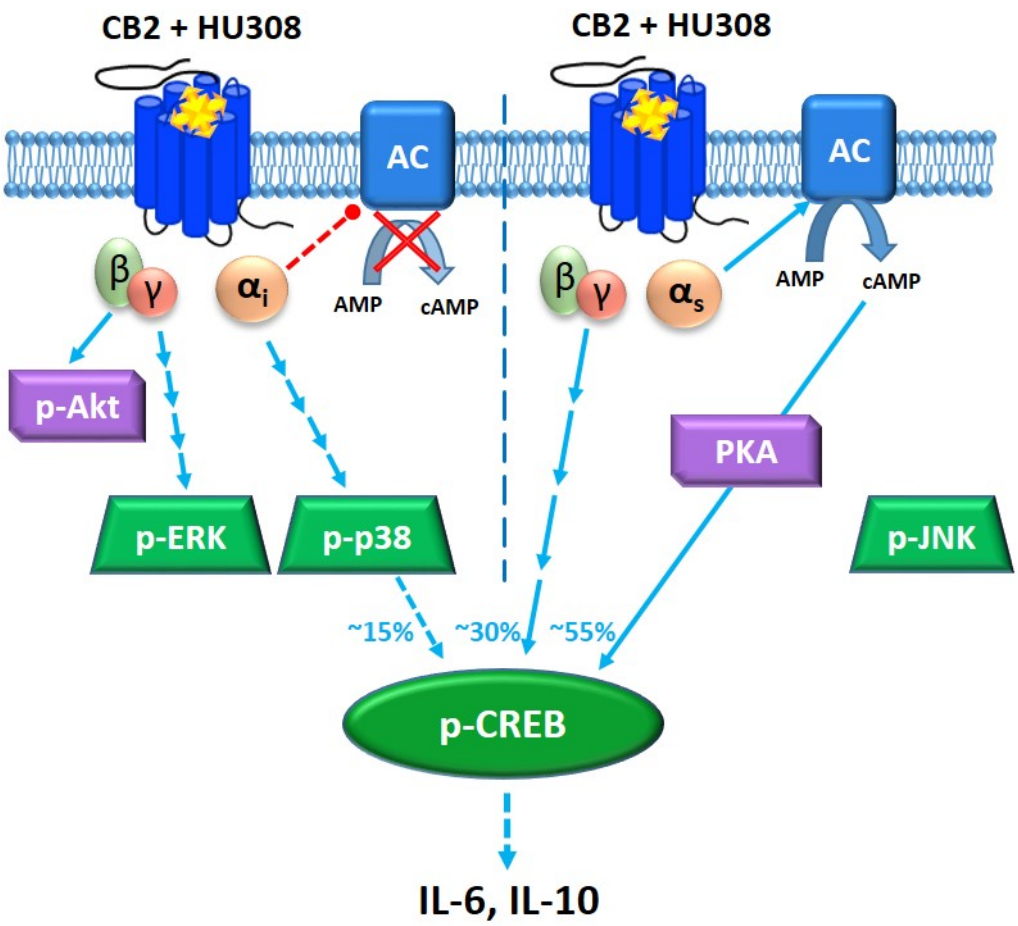

Fig. 10. CB2 signaling network in mixed primary human leukocytes. Binding of HU308 to CB2 leads to activation of $G\alpha_i\beta\gamma$ and $G\alpha_s\beta\gamma$ heterotrimers. $G\alpha_i$, inhibits adenylate cyclase(s) (AC) and activates a cascade leading to phosphorylation of p38 and CREB. βγ from $G\alpha_i\beta\gamma$ activates Akt and initiates a cascade leading to phosphorylation of ERK. $G\alpha_s$ stimulates AC which leads (likely via protein kinase A, PKA) to p-CREB. βγ from non-$G\alpha_i\beta\gamma$ (likely from $G\alpha_s\beta\gamma$) also contributes to p-CREB activation. JNK phosphorylation was not observed. Given that $G\alpha_i$-mediated inhibition of AC does not inhibit p-CREB activation it is likely that $G\alpha_i$ and $G\alpha_s$ modulation of AC are either segregated in separate compartments within one cell, or occur in different cells (represented by dotted line). p-CREB likely mediates IL-6/IL-10 induction, which might be released from one cell or from different cell types.

Methods

Preparation of Human PBMC

Primary human PBMC were isolated from blood samples collected from 3 healthy volunteers (male and female, aged 22-26) after obtaining written informed consents and in accordance with ethical approval from the University of Auckland Human Participants Ethics Committee (reference # 014035).

Whole venous blood samples, 50 mL per draw, 3-6 draws per donor (with each set of experiments performed on one draw from each donor) were collected into K_2EDTA-coated BD Vacutainer® Blood Collection Tubes (Becton, Dickinson and Company), transferred to polypropylene tubes (Corning), and diluted 1.25 volumes of blood with 1 volume RPMI 1640 medium (HyClone) supplemented with 2 mM L-Glutamine (Thermo Fisher Scientific).

PBMC were isolated by gradient centrifugation in Histopaque®-1077 (Sigma-Aldrich) in Greiner Leucosep® 50 mL tubes (Greiner Bio-One) for 15 minutes at 800 × g at room temperature. The collected PBMC were washed twice with RPMI by centrifugation for 10 minutes at 250 × g, re-suspended in a culture medium – RPMI supplemented with 10% v/v fetal bovine serum (FBS; New Zealand-origin, Moregate Biotech) and 2 mM L-Glutamine. Following isolation, PBMC were either used in assays immediately, or cryopreserved in 50% RPMI with 40% FBS and 10% DMSO and stored at -80°C for up to 12 months until later use. Cryopreservation did not affect CB2 expression (Supplementary Fig. 5) or HU308 signaling responses of PBMC (Supplementary Fig. 6).

Cell Lines and Cell Culture

Human embryonic kidney (HEK) cell lines stably transfected with human CB1 (HEK-hCB1, 181) or human CB2 (HEK-hCB2, 95), or an untransfected wild type HEK Flp-in cell line (HEK wt, Invitrogen, R75007) were cultured in high-glucose DMEM (HyClone) with 10% v/v FBS, in the presence of Zeocin (InvivoGen) at 250 µg/mL, Hygromycin B (Thermo Fisher Scientific) at 50 µg/mL, or Zeocin at 100 µg/mL, respectively.

Whole Cell Radioligand Binding

We utilized a whole cell competition radioligand binding method to quantitate CB1 and CB2 protein. Primary human PBMC or HEK cells were pelleted by centrifugation for 10 min at 250 × *g* or 5 min at 125 × *g* respectively, and re-suspended in binding medium (RPMI with 1 mg/mL BSA, 2 mM L-Glutamine, 25 mM HEPES). PBMC (3×10^5 cells per assay point), HEK-hCB1 or HEK-hCB2 (0.4×10^5 diluted in 1.6×10^5 HEK wt to avoid ligand depletion), or HEK wt (2×10^5) cells were dispensed in Deep Well 96-well Plates (Gene Era Biotech)

with reaction mix already present to produce a final 200 µL reaction volume. The reaction mix included 5 nM (final concentration) tritiated CB1/CB2 mixed agonist [^{3}H]-CP55940 (PerkinElmer) in binding medium, and an appropriate displacer to measure non-specific displacement of the radioligand, or a vehicle (DMSO) control to measure total binding of the radioligand. The displacer was either CB1-selective agonist ACEA at 1 µM (arachidonyl-2-chloroethylamide, synthesized from arachidonic acid as described in (182), or CB2-selective agonist HU308 at 1 µM (Tocris Bioscience).

Plates were incubated on the surface of a 15°C thermostatic bath for 3 h to reach steady-state, then placed on ice. Reactions were then rapidly filtered under vacuum through GF/C glass fiber filter-bottom 96-well microplates (Perkin Elmer, MA, USA) pre-treated for 1 h at room temperature with 0.1% polyethyleneimine (PEI, Sigma-Aldrich). After three washes with an ice-cold wash buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 1 mg/mL BSA) filter plates were dried overnight at room temperature, then sealed, and 50 µL per well Irgasafe Plus scintillation fluid (Perkin Elmer) was dispensed. After 30 minutes, plates were counted in a Wallac MicroBeta® TriLux Liquid Scintillation Counter (Perkin Elmer), 2 min per well, as ccpm – counts per minute corrected with a factory pre-set ^{3}[H] detector normalization. Each independent experiment was verified to have undergone < 10% ligand depletion. CB1 and CB2 protein quantification was based on the maximum binding parameter B_{max}, which corresponds to the concentration of binding sites for the radioligand (183), using the following formula (based on equation 7.5.21 from 184):

$$Bmax = \frac{Specific\ binding}{Fractional\ occupancy} = \frac{Specific\ binding}{[Radioligand] \big/ (Kd + [Radioligand])}$$

B_{max} were then converted to mol using the radioligand specific activity (as stated by the manufacturer), and then converted to units of fmol/mg of total protein added to an assay point (as measured by Bio-Rad DC Protein Assay Kit). Equilibrium dissociation constants (K_d) for [^{3}H]-CP55940 were measured by carrying out homologous competition binding under the same experimental conditions. pK_d values were: 7.94 ± 0.04 at CB1, 7.80 ± 0.05 at CB2 (*mean ± SEM, n=3*).

cAMP Assay

Primary human PBMC were re-suspended in assay medium (RPMI with 2 mM L-Glutamine, 25 mM HEPES, 10% v/v FBS) and seeded at 1 × 10^5 cells per well in 10 µL into Falcon 384-well plates (Corning). All ligand and vehicle solutions were prepared at 2× concentrations in the assay medium, and dispensed 10 µL per well in 384-well plates at requisite time points.

After equilibration in a humidified atmosphere at 37°C and 5% CO_2 for 1 h, cells were incubated with 10 µM forskolin (canonical adenylyl cyclase activator, Tocris Bioscience) with or without stimulations of interest and/or vehicle (DMSO) controls. At the end of incubation, plates were rapidly cooled by placing on ice, and cells were lysed by adding 10 µL per well of 3× concentrated ice-cold lysis buffer (formulation as per LANCE cAMP kit described below). The plates were placed on shakers at 500 RPM for 10 min at 4°C, and frozen at -80°C.

cAMP detection was performed in 1/2-area white 96-well plates (PerkinElmer), using the LANCE cAMP Kit (PerkinElmer) with a high sensitivity method with 12 µL per point lysate and total detection volume 24 µL using 2× concentrated detection mix. Signal was detected on a CLARIOstar plate reader (BMG Labtech) using recommended TR-FRET settings (excitation 320 nm, emission 665 nm). Concentration of cAMP in assay samples was interpolated from 8-point standard curves (0, 100 pM – 1 µM). Data were normalized to vehicle (set as 0%) and forskolin (set as 100%) controls to allow compilation of data from independent experiments.

ERK1/2 Phosphorylation Assay (p-ERK)

Primary human PBMC were pelleted by centrifugation for 10 min at 250 × *g*, re-suspended in assay medium, and seeded at 1 × 10^5 cells in 10 µL per well into Falcon 384-well plates. Plates were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 55 min, and then transferred to the surface of a water bath at 37°C and incubated for 5 min prior to adding ligands or vehicle controls. Treatments were prepared at 2× concentrations in assay medium, and dispensed 10 µL per well at requisite time points. At the end of incubation, plates were placed on ice, and cells were lysed by addition of 10 µL per well of 3× concentrated ice-cold lysis buffer (formulation as per AlphaLISA kit described below). The plates were placed on shakers (500 RPM, 10 min at 4°C) and frozen at -80°C. p-ERK detection was performed using the AlphaLISA SureFire Ultra p-ERK 1/2 (Thr202/Tyr204) Assay Kit (PerkinElmer) per the manufacturer's specifications in 1/2-area white 96-well plates. Signal was detected on a CLARIOstar plate reader with AlphaLISA-compatible filters (excitation 680 nm, emission 615 nm). Counts were normalized to vehicle controls (100%) to allow compilation of data from independent experiments.

Akt, p38, JNK, CREB Phosphorylation Assays (p-Akt, p-p38,p-JNK, and p-CREB)

PBMC were treated and lysed under the same conditions as described for the p-ERK assay. Analytes were detected using AlphaLISA SureFire Ultra p-Akt 1/2/3 (Ser473), p-JNK 1/2/3

(Thr183/Tyr185), p-p38 MAPK (Thr180/Tyr182), and p-CREB (Ser133) Assay Kits as described above.

Application of G Protein Inhibitors

Primary human PBMC were treated with G protein inhibitors equivalently between all signaling assays. In experiments with PTX (Sigma-Aldrich), cells were pelleted as described above, re-suspended in the assay medium containing PTX at 100 ng/mL or 1:2000 dilution of its vehicle (50% glycerol, 50 nM Tris-HCl pH 7.5, 10 mM glycine, 500 mM NaCl), and incubated for 5 h in a humidified atmosphere at 37°C and 5% CO_2. After the incubation, the cells were counted and re-suspended in the same assay media with PTX or its vehicle to concentrations specified for the signaling assays, equilibrated for 1 h as described for the signaling assays (bringing the total PTX treatment time to 6 h), and used the aforementioned signaling experiments.

In experiments involving treatments with gallein (Santa Cruz Biotechnology) or suramin derivatives NF023 (Abcam) and NF449 (Abcam), the inhibitors or their vehicles were added to cells to final concentration 10 μM 30 min prior to adding ligands or vehicles.

All ligand and vehicle solutions used in signaling assays with pathway inhibitors were prepared in assay media containing the inhibitors or vehicles, so that appropriate pathway inhibitors or vehicle controls at 1× concentration were present in cell suspensions for the entire duration of corresponding experiments.

Analysis of Cytokine Secretion

Primary human PBMC were seeded at 4.5×10^5 cells in 50 or 75 μL per well in assay medium into 96 Well V-Bottom plates (Interlab), incubated for 30 min, if applicable pre-treated for 30 min with 1 μM SR144528 (a kind gift from Roche Pharmaceuticals, Basel, Switzerland), 5 μM WIN55212-3 (Tocris), 10 μM G protein inhibitors (NF023, NF449, gallein), or vehicle control (DMSO), and subsequently treated with HU308 or vehicle, with or without forskolin and G protein inhibitors or antagonists if applicable, in 150 μL final volume. After 12 h (for multiplex screen) or 6 h (for quantitative analysis of IL-6 and IL-10 secretion) incubation in a humidified atmosphere at 37°C and 5% CO_2, plates were centrifuged for 10 min at 250 × *g*, and supernatants were collected and frozen at -80°C. Each remaining cell pellet was re-suspended and aliquots (in technical duplicate) diluted in Trypan blue solution for hemocytometer counting. Human interleukins 2, 4, 6, 10, 12, 13, 17A, macrophage inflammatory protein (MIP-1α) and tumor necrosis factor (TNF-α) were detected using the Cytometric Bead Array (CBA) technology (BD Biosciences) and assayed

on an Accuri C6 flow cytometer (BD Biosciences) as described previously (185). For the multiplex screen, two concentrations of standard in the lower and middle range of standard curves (78 and 625 pg/mL) were utilized as internal controls and the mean fluorescent intensity (MFI) of each cytokine was plotted in GraphPad Prism and compared to vehicle controls. Concentrations of IL-6 and Il-10 were interpolated using FCAP Array software (v. 3.1, BD Biosciences) from 10-point standard curves (0, 19.5 – 5000 pg/mL). Manufacturer stated limits of detection (LOD) were 11.2, 1.4, 1.6, 0.13, 7.9, 0.6, 0.3, 0.2, and 1.2 pg/mL for human interleukins 2, 4, 6, 10, 12, 13, 17A, MIP-1α, and TNF-α, respectively.

Data Analysis

All data analysis was performed using GraphPad Prism Software (v. 8.0; GraphPad Software Inc.). Data is presented as mean ± standard error of the mean (SEM) from three independent experiments performed in triplicate, each with samples from a separate subject (three subjects in total). Concentration-response curves were obtained by fitting three-parameter nonlinear regression curves (Hill slope constrained to 1). All datasets passed the Shapiro-Wilk test for normality and Brown-Forsythe test for equal variance ($p > 0.05$). If a statistically significant difference ($p < 0.05$) was detected in one-way ANOVA the data was further analyzed using Tukey's multiple comparisons post-hoc test with significance levels indicated graphically as: *$p < 0.05$* (*), *$p < 0.01$* (**), *$p < 0.001$* (***), *non-significant* (ns).

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