

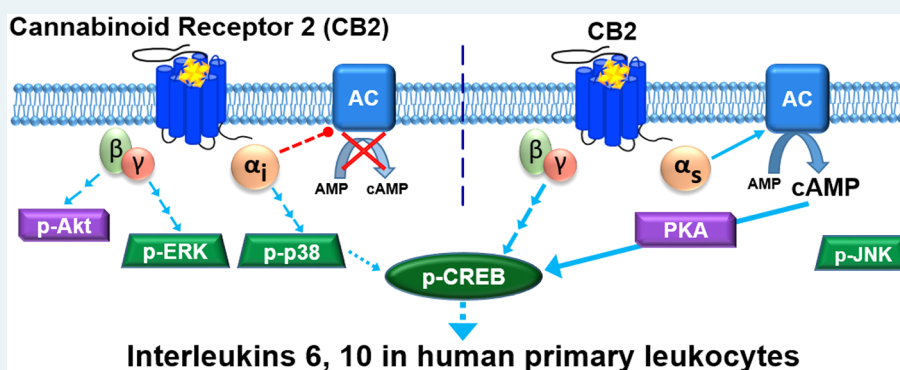
Cannabinoid Receptor 2 (CB₂) Signals via G-alpha-s and Induces IL-6 and IL-10 Cytokine Secretion in Human Primary Leukocytes

Yurii Saroz,^{†,‡} Dan T. Kho,^{‡,§} Michelle Glass,^{||} Euan Scott Graham,^{‡,§} and Natasha Lillia Grimsey^{*,†,‡,||}

[†]Department of Pharmacology and Clinical Pharmacology, School of Medical Sciences, Faculty of Medical and Health Sciences, [‡]Centre for Brain Research, Faculty of Medical and Health Sciences, and [§]Department of Molecular Medicine and Pathology, School of Medical Sciences, Faculty of Medical and Health Sciences, University of Auckland, 85 Park Road, Grafton, Auckland, 1023, New Zealand

^{||}Department of Pharmacology and Toxicology, School of Biomedical Sciences, Division of Health Sciences, University of Otago, Dunedin, 9016, New Zealand

Supporting Information



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 human primary immunocompetent cells. We applied an experimental paradigm which closely models the *in situ* state of human primary leukocytes (PBMC; peripheral blood mononuclear cells) to characterize activation of a number of signaling pathways in response to a CB₂-selective ligand (HU308). We observed a “lag” phase of unchanged cAMP concentration prior to development of classically expected G_{α_i}-mediated inhibition of cAMP synthesis. Application of G protein inhibitors revealed that this apparent lag was a result of counteraction of G_{α_i} effects by concurrent G_{α_s} activation. Monitoring downstream signaling events showed that activation of p38 was mediated by G_{α_i}, whereas ERK1/2 and Akt phosphorylation were mediated by G_{α_i}-coupled βγ. Activation of CREB integrated multiple components; G_{α_s} and βγ mediated ~85% of the response, while ~15% was attributed to G_{α_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.

KEYWORDS: cannabinoid receptor 2 (CB₂), G protein-coupled receptor (GPCR), signaling, leukocytes, interleukin 6, interleukin 10

Cannabinoid Receptor 2 (CB₂) is a class A G protein-coupled receptor (GPCR) which is expressed primarily in the immune system^{1,2} and is a promising therapeutic target for immune modulation in a wide range of disorders.³ CB₂ couples to G_{α_{i/o}} proteins inhibiting adenylyl cyclases,^{4,5} and limited evidence indicates coupling to G_{α_q}.⁶ To date, there is no evidence for CB₂ coupling to G_{α_s}; although increased cyclic adenosine monophosphate (cAMP) synthesis has been reported, this was coupled to G_{α_{i/o}}, likely via Gβγ.^{7,8} Downstream of G protein coupling, and perhaps involving modulation by β-arrestins,⁹ activation of extracellular signal-

regulated kinases 1 and 2 (ERK1/2) is a widely studied consequence of CB₂ activation reported in cell lines heterologously expressing this receptor¹⁰ and in rodent¹¹ and human immunocompetent cell lines,^{8,12} with limited published evidence for such signaling in human primary leukocytes.^{8,13} The paucity of studies on human primary leukocytes may in part be due to methodological difficulties such as low cell yields. Mitogenic or antigenic proliferative stimulations are

Received: July 17, 2019

Published: October 1, 2019

widely used approaches to overcome some of these practical limitations.^{13,14} However, these methods cause lymphocytes to dedifferentiate and turn into lymphoblasts—mitotically overactive cells—which do not closely model the *in situ* state of leukocytes.¹⁵

The limited literature regarding CB₂ signaling via other types of mitogen-activated protein kinases (MAPKs) indicates context-dependence of activation. CB₂ can induce p38 phosphorylation (p-p38)^{12,16,17} which has been linked to growth inhibition of cancer cells, while dendritic cells¹⁸ and LPS-stimulated monocytes¹² were nonresponsive. Akt kinase (protein kinase B), an important mediator of immunomodulation¹⁹ can be activated,²⁰ inhibited,²¹ or unaffected²² by CB₂ activation. A stress-activated protein kinase JNK (c-Jun NH₂-terminal kinase), involved in pro-inflammatory signaling,²³ has been shown to be activated^{24,25} and inhibited¹² via CB₂.

The ultimate outcomes of CB₂ activation in immune cells include inhibition of proliferation, induction of apoptosis, influences on cytokine/chemokine networks, and regulation of adhesion and migration.²⁶ In human immunocompetent cells CB₂ activation inhibits IL-2,²⁷ IL-17, INF- γ , TNF- α ²⁸ secretion, and stimulates IL-4²⁹ and TGF- β ³⁰ secretion. Murine *in vitro* and *in vivo* models indicate that cannabinoids inhibit IL-2,³¹ IL-12, and IFN- γ ,^{32,33} and induce IL-4 secretion.^{32,33} However, these studies stimulated leukocytes with mitogens or antibodies. Species differences in cannabinoid signaling notwithstanding,⁵ cytokine secretion is generally highly dependent on the cellular environment. Indeed, cannabinoid effects on the cytokine network are sensitive to the method of cell activation.³⁴

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 modeling normal human physiology. We endeavored to carry out a comprehensive study of CB₂ signaling in unstimulated human primary leukocytes (peripheral blood mononuclear cells, PBMC) under conditions closely preserving their *in vivo* state. We have observed an unprecedented CB₂-mediated signaling and functional profile, including coupling to G α _s.

RESULTS

CB₂, but Not CB₁, Is Expressed in Unstimulated Human Primary PBMC. We quantified expression of CB₁ and CB₂ protein by whole cell radioligand binding with high affinity CB₁/CB₂ ligand [³H]-CP55940 displaced by CB₁- and CB₂-selective ligands, ACEA³⁵ and HU308³⁶, respectively. Although CB₂ was expected to be the primary cannabinoid-responsive receptor in human PBMC, CB₁ transcripts have also been detected in some contexts.¹ Whereas CB₁ protein could not be detected in PBMC from any of three healthy human donors, CB₂ was readily detectable (Figure 1). Intrasubject variability in CB₂ expression was low (independent experiments are from three blood draws from the same donor taken over the course of up to 8 months), while intersubject expression was within the same magnitude, ranging from 704 to 1323 fmol/mg. PBMC samples from these same three donors were utilized in subsequent experiments.

CB₂ Activation Induces Simultaneous G α _i and G α _s Coupling, Producing Delayed cAMP Flux. CB₂ is widely recognized to inhibit cAMP production via G α _{i/o} inhibition of adenylyl cyclases. Depending on basal adenylyl cyclase activity

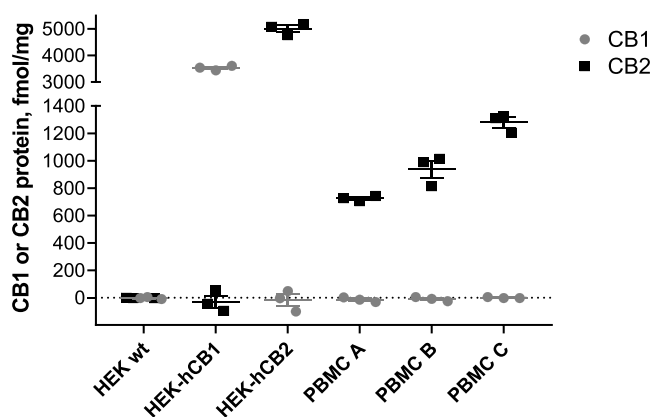


Figure 1. CB₁ and CB₂ protein quantification by whole cell radioligand binding. Cells were incubated with [³H]-CP55940 (5 nM) in the presence and absence of a CB₁-selective displacer ACEA (1 μ M), or a CB₂-selective displacer HU308 (1 μ M). Total binding sites (B_{max}) were calculated (see Methods) and 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. The graph shows independent experiment means (from technical triplicate) as well as overall mean \pm SEM of these three independent experiments each performed with cells from a separate subject (three subjects in total).

in the sample of interest, stimulation of adenylyl cyclase (e.g., with forskolin) is a typical prerequisite for measuring inhibition.^{4,10} We first studied the time-course of cAMP signaling in PBMC in response to CB₂-selective agonist HU308 (1 μ M), and observed inhibition of forskolin-stimulated cAMP synthesis in line with our general expectations (Figure 2A). However, there was a lag to the onset of measurable inhibition which lasted at least 10 min, with the 20–35 min time-points being significantly different from time-matched vehicle control ($p < 0.0001$ – 0.0005 ; two-way repeated measures [RM] ANOVA with Holm-Šidák posthoc test). This was followed by a steady reduction in cAMP concentration, reaching a maximum extent by 30 min ($60.3 \pm 2.8\%$ of forskolin-stimulated), with cAMP subsequently returning back to the forskolin-induced level by 60 min. Prior studies in cells overexpressing CB₂ describe an immediate onset and earlier peak cAMP inhibition.³⁷ Given that we cannot readily measure inhibition of cAMP production without forskolin being present, we hypothesized that low basal cAMP and/or slow 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, preincubation with forskolin prior to HU308 addition did not influence the signaling latency, time to peak response, nor efficacy, therefore ruling out this hypothesis (Supporting Information, Figure S1).

Preincubation with pertussis toxin (PTX), a G α _i³⁸ and G α _i-derived $\beta\gamma$ ³⁹ inhibitor, followed by its coapplication with HU308 revealed a wave of increased cAMP (Figure 2A). 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, with the 7–25 min time-points being significantly different from time-matched vehicle control ($p < 0.0001$ – 0.011 ; two-way RM ANOVA with Holm-Šidák posthoc test). Given that increased cAMP is associated with

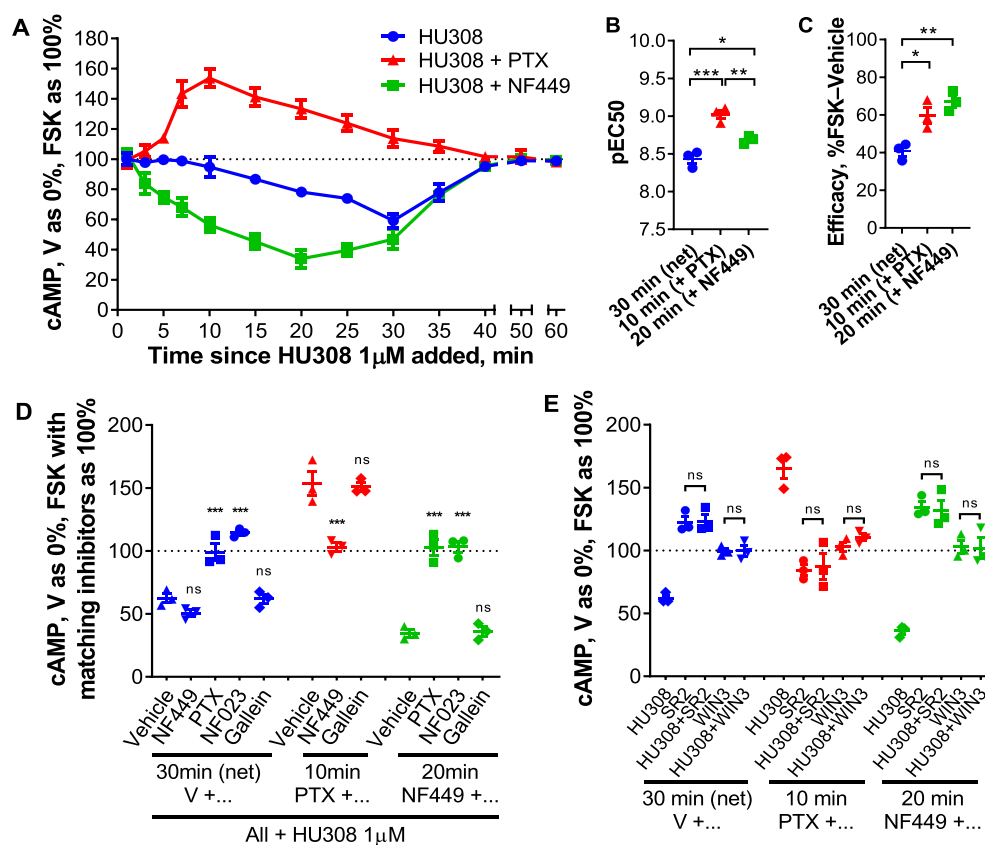


Figure 2. cAMP signaling in human primary PBMC. (A) PBMC were incubated with 10 μ M forskolin (FSK) and HU308 (1 μ M) without inhibitors (blue \bullet), or after pretreatment with NF449 (10 μ M for 30 min, green \blacksquare), or with PTX (100 ng/mL for 6 h, red \blacktriangle). The graph shows mean \pm SEM of three independent experiments performed in technical triplicate, each with cells from a separate subject (three subjects in total). (B,C) HU308 concentration–response curve parameters; potency (B) and efficacy (C; span of curves as in Figure S3). Statistical comparisons by one-way ANOVA with Tukey posthoc test. (D,E) Stimulations were with 10 μ M forskolin and 1 μ M HU308 or vehicle for 30 min for net cAMP flux, 10 min for the stimulatory pathway (revealed by PTX), or 20 min for the inhibitory pathway (revealed by NF449). (D) PBMC were additionally pretreated as indicated with PTX, NF449, NF023, or gallein. Data are normalized to vehicle control as 0% and forskolin controls with matching inhibitors as 100%. Statistical comparisons are to the vehicle plus HU308 control within each set, measured by one-way ANOVA with Tukey posthoc test. (E) PBMC were pretreated as indicated, then coincubated with forskolin (10 μ M), SR144528 (SR2, 1 μ M), or WIN55212-3 (WIN-3, 5 μ M), and HU308 (100 nM) or vehicle for 10, 20, or 30 min. Data are normalized to vehicle control as 0% and forskolin control as 100%. All SR2 and WIN-3 conditions were significantly different from “HU308” control for peak signaling within each set ($p < 0.0001$ – 0.004). Within each set, condition “SR2” was compared to “SR2 + HU308”, “WIN3” was compared to “WIN3 + HU308”, and had no significant difference. Statistical comparisons by one-way ANOVA with Tukey posthoc test. Graphs B–E show independent experiment means (from technical triplicate) as well as overall mean \pm SEM of these three independent experiments each performed with cells from a separate subject (three subjects in total).

G_{α_s} coupling, we tested the effect of G_{α_s} -blocker NF449⁴⁰ 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, with the 5–35 min time-points being significantly different from time-matched vehicle control ($p < 0.001$ – 0.035 ; two-way RM ANOVA with Holm–Šidák posthoc test). The time-courses for onset of reduction and return to forskolin-stimulated cAMP levels for this now apparently “pure” inhibitory signaling had similar timing.

These findings indicate that when no signaling inhibitors are present the opposing influences of G_{α_i} and G_{α_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_{α_i} versus G_{α_s} coupling to adenylyl cyclase(s) result in transient net inhibition of cAMP synthesis. Increased cAMP in response to HU308 was detectable even without forskolin stimulation, although inhibition of G_{α_i} was required to reveal this as was the case when forskolin was present (Figure S2).

cAMP fluxes for the three types of responses at their respective peak time-points (net cAMP, 30 min; cAMP accumulation, 10 min; cAMP inhibition, 20 min) were of nanomolar potency, typical for CB_2 -mediated signaling by HU308,^{5,41} with subtle differences in potencies between the three cAMP responses with rank order: stimulatory cAMP > “pure” inhibitory > net cAMP (Figure 2B, Figure S3). Not surprisingly from the opposing effects on cAMP concentration, the peak stimulatory and “pure” inhibitory cAMP responses revealed by the G_{α} inhibitors had greater efficacies than the maximal net cAMP response (Figure 2C, Figure S3). Between-subject variability was small.

We further probed the contribution of G proteins (Figure 2D) and investigated CB_2 -specificity (Figure 2E). As expected from the time-course data, G_{α_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_{α_i} inhibitor NF023⁴² 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

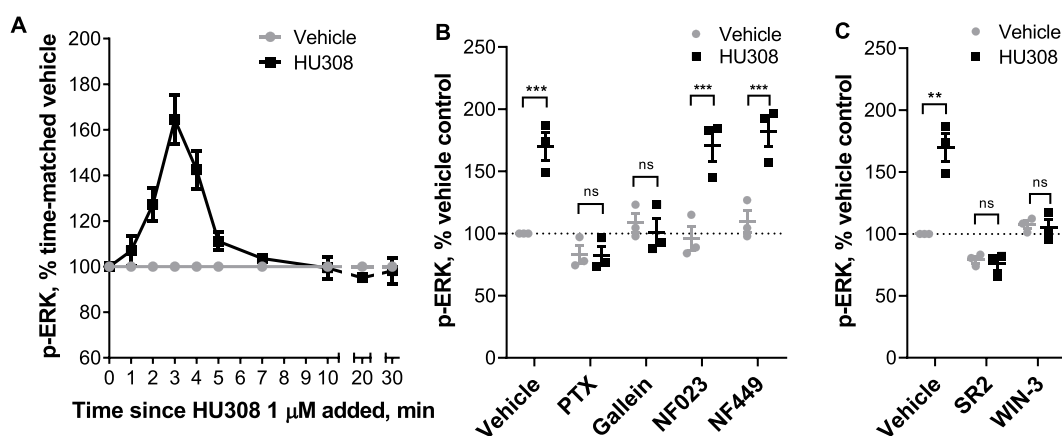


Figure 3. ERK1/2 signaling in human primary PBMC. **A.** Time-course with 1 μM HU308 (■) or a vehicle control (gray ●). The 2, 3, and 4 min time-points are significantly different from time matched vehicle controls ($p < 0.0001$; two-way RM ANOVA with Holm-Sidak posthoc test). The graph shows mean \pm SEM of three independent experiments performed in technical triplicate, each with cells from a separate subject (three subjects in total). **(B)** Incubation (3 min) with HU308 (1 μM) or vehicle control in the presence of G protein inhibitors PTX, gallein, NF023, NF449, or vehicle control. **(C)** Incubation (3 min) with HU308 (100 nM) or vehicle control in the presence of SR144528 (SR2, 1 μM), WIN55212-3 (WIN-3, 5 μM), or vehicle control. Data are normalized to vehicle control (100%). Graphs B and C show independent experiment means (from technical triplicate) as well as overall mean \pm SEM of these three independent experiments each performed with cells from a separate subject (three subjects in total). Statistical comparisons by two-way RM ANOVA with Holm-Sidak posthoc test.

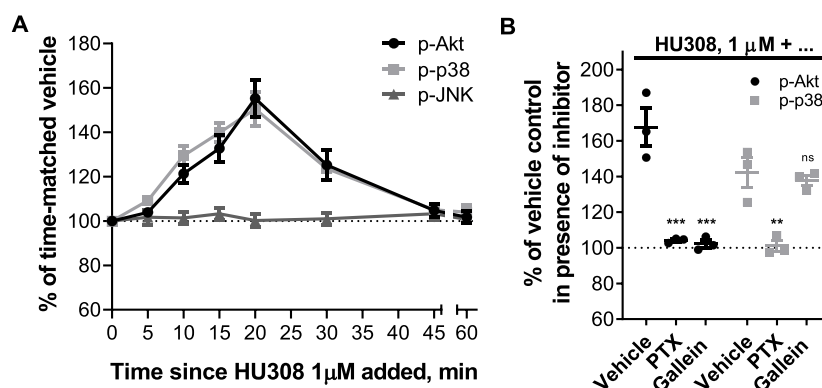


Figure 4. Phosphorylation of Akt, JNK, and p38 MAP kinases in human primary PBMC. **(A)** Time-courses with HU308 (1 μM) and vehicle control. For p-Akt and p-p38 time-points 10–30 min are significantly different from time-matched vehicle controls ($p < 0.0001$ – 0.0002 ; two-way RM ANOVA with Holm-Sidak posthoc test). For p-JNK, all points are indistinguishable from 0 min ($p = 0.988$; one-way ANOVA). The graph shows mean \pm SEM of three independent experiments performed in technical triplicate, each with cells from a separate subject (three subjects in total). **(B)** Responses to 20 min 1 μM HU308 after pretreatment with PTX, gallein, or vehicle control. Data are normalized and statistically compared to the corresponding vehicle plus HU308 control by two-way RM ANOVA with Holm-Sidak posthoc test. The graph shows independent experiment means (from technical triplicate) as well as overall mean \pm SEM of these three independent experiments each performed with cells from a separate subject (three subjects in total).

peak stimulation of cAMP synthesis (measured in the presence of PTX) was completely blocked by $G\alpha_s$ inhibitor NF449. A $G\beta\gamma$ inhibitor gallein⁴³ had no effect on cAMP signaling, indicating lack of $G\beta\gamma$ involvement (Figure 2D). As well as the potencies of HU308-induced cAMP flux being consistent with CB_2 -specific responses, inverse agonist SR144528⁴⁴ at 1 μM (a concentration at which it binds to CB_2 , but not to CB_1 , Figure S4) completely blocked cAMP signaling induced by HU308 (Figure 2E). Inverse agonism of SR144528 was readily measurable for the inhibitory and net cAMP pathways, in which it increased cAMP above forskolin-induced levels ($p < 0.0001$, $p = 0.0005$, respectively; two-way RM ANOVA with Holm-Sidak posthoc test). There was a trend toward inverse agonism in the stimulatory cAMP pathway, but this reduction in cAMP was not significantly different from forskolin alone ($p = 0.78$; two-way RM ANOVA with Holm-Sidak posthoc test). A CB_1/CB_2 neutral antagonist WIN55212-3^{45,46} (no

significant differences from forskolin controls, $p = 0.80$ – 0.98 ; two-way RM ANOVA with Holm-Sidak posthoc test) also completely abrogated all HU308-induced cAMP signaling (Figure 2E). Given the lack of CB_1 protein in PBMC (Figure 1), WIN55212-3 most likely acted via CB_2 , and taken together with the SR144528 data and response potencies provides strong evidence that the observed signaling is CB_2 -mediated.

CB_2 -Stimulated Phosphorylation of ERK1/2 Is Mediated by $G\alpha_i$ - but Not $G\alpha_s$ -Coupled $G\beta\gamma$. Phosphorylation of ERK1/2 (p-ERK) is a widely observed CB_2 response, but the potential pathways to stimulation are many and varied and p-ERK can act as an integrator of responses.⁴⁷ Time-courses with 1 μM HU308 revealed transient activation of p-ERK, with a peak at around 3 min (Figure 3A). HU308 activated ERK1/2 in a concentration-dependent manner (Figure S5), with approximately one log unit lower potency than observed for cAMP fluxes ($pEC_{50} 7.69 \pm 0.06$). Responses were very

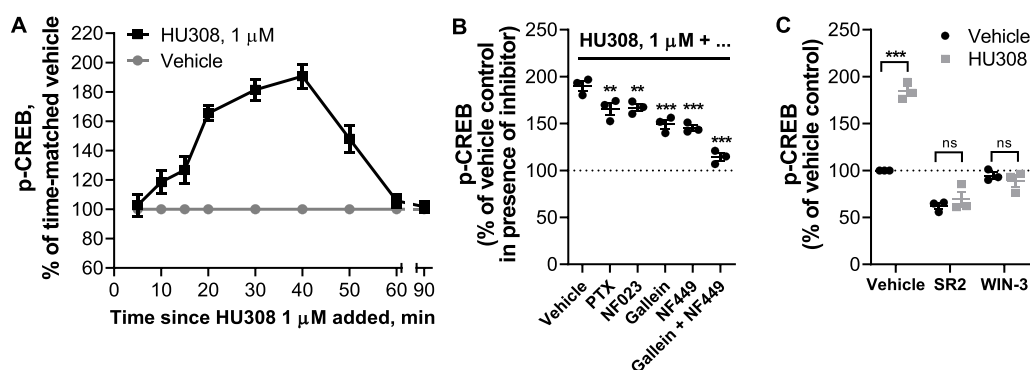


Figure 5. Phosphorylation of CREB in human primary PBMC. (A) Time-course with HU308 (1 μ M) and vehicle control. 10–50 min time-points are significantly different from time-matched vehicle controls ($p < 0.0001$ – 0.0022 ; two-way RM ANOVA with Holm-Sidak posthoc test). The graph shows mean \pm SEM of three independent experiments performed in technical triplicate, each with cells from a separate subject (three subjects in total). (B) p-CREB stimulated by HU308 (1 μ M) for 40 min in the presence of G protein inhibitors gallein, NF023, NF449, PTX, or vehicle control; data are normalized to responses in the absence of HU308 (100%); statistical comparisons are to the HU308 response without inhibitors (one-way RM ANOVA with Holm-Sidak posthoc test). (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 μ M), a CB₁/CB₂ neutral antagonist WIN55212-3 (WIN-3, 5 μ M), or vehicle control; data are normalized to vehicle control (100%). Two-way RM ANOVA with Holm-Sidak posthoc test. Graphs B and C show independent experiment means (from technical triplicate) as well as overall mean \pm SEM of these three independent experiments each performed with cells from a separate subject (three subjects in total).

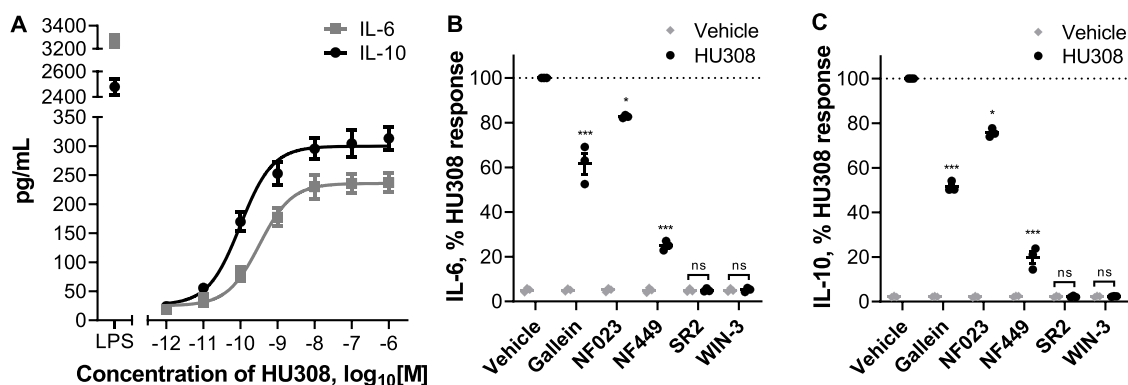


Figure 6. Induction of cytokine secretion by PBMC. (A) Induction of IL-6 and IL-10 secretion in response to 6 h incubation with HU308. Lipopolysaccharide (LPS, 10 ng/mL) is a positive control. The graph shows mean \pm SEM of three independent experiments performed in technical triplicate, each with cells from a separate subject (three subjects in total). (B) IL-6 and (C) IL-10 accumulation in the presence of G protein inhibitors or CB₂ antagonists: PBMC were pretreated with gallein, NF023, NF449, SR144528 (SR2, 1 μ M), or WIN55212-3 (WIN-3, 5 μ M) for 30 min followed by 6 h treatments with HU308 (10 nM) with the inhibitors/antagonists present for the entire duration of incubation; data are normalized to HU308 responses without inhibitors/antagonists. Effects of inhibitors on HU308 responses were statistically compared with the corresponding HU308 response without inhibitor via two-way RM ANOVA with Holm-Sidak posthoc test. In the presence of SR2 and WIN-3, there was no statistical difference between HU308 and the HU308 vehicle control. Graphs B and C show independent experiment means (from technical triplicate) as well as overall mean \pm SEM of these three independent experiments each performed with cells from a separate subject (three subjects in total).

consistent between the three donors. Neither $G\alpha_i$ inhibitor NF023 nor $G\alpha_s$ inhibitor NF449 had an effect on the observed ERK1/2 signaling (Figure 3B), whereas $G\alpha_{i/o}/\beta\gamma$ inhibitor PTX and $G\beta\gamma$ inhibitor gallein both completely blocked activation of p-ERK. This indicates that p-ERK1/2 induced by HU308 is mediated by $G\alpha_i$ -linked $\beta\gamma$ dimers. We further verified that the observed p-ERK1/2 response was CB₂-mediated by coapplying HU308 with SR144528 or WIN55212-3 (Figure 3C). Both antagonists completely blocked p-ERK, 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 μ M (Figure S6), a concentration which binds to CB₁ but not CB₂ (Figure 1) and can induce CB₁-mediated p-ERK.⁴⁸

CB₂ Induces Phosphorylation of p38 via $G\alpha_i$ and Akt via $G\beta\gamma$, but Does Not Activate JNK. Time-courses stimulating PBMC with 1 μ M HU308 revealed phosphorylation of Akt and p38 with signaling peaks at around 20 min, and with similar overall kinetics (Figure 4A). Conversely, there was no phosphorylation of JNK detected. Application of PTX completely abrogated Akt and p38 phosphorylation (Figure 4B), implicating the involvement of $G\alpha_i/\beta\gamma$ proteins. $G\beta\gamma$ inhibitor gallein completely blocked p-Akt, but did not influence p-p38 (Figure 4B). HU308 therefore induces phosphorylation of p38 downstream of $G\alpha_i$ subunits, while Akt is activated via $G\alpha_i$ -coupled $\beta\gamma$ heterodimers.

CB₂ Induces p-CREB Downstream of $G\alpha_s$ and $G\beta\gamma$. CREB phosphorylation is a classical downstream consequence of $G\alpha_s$ and cAMP-mediated activation of protein kinase A (PKA).⁴⁹ A time-course with PBMC stimulated by 1 μ M

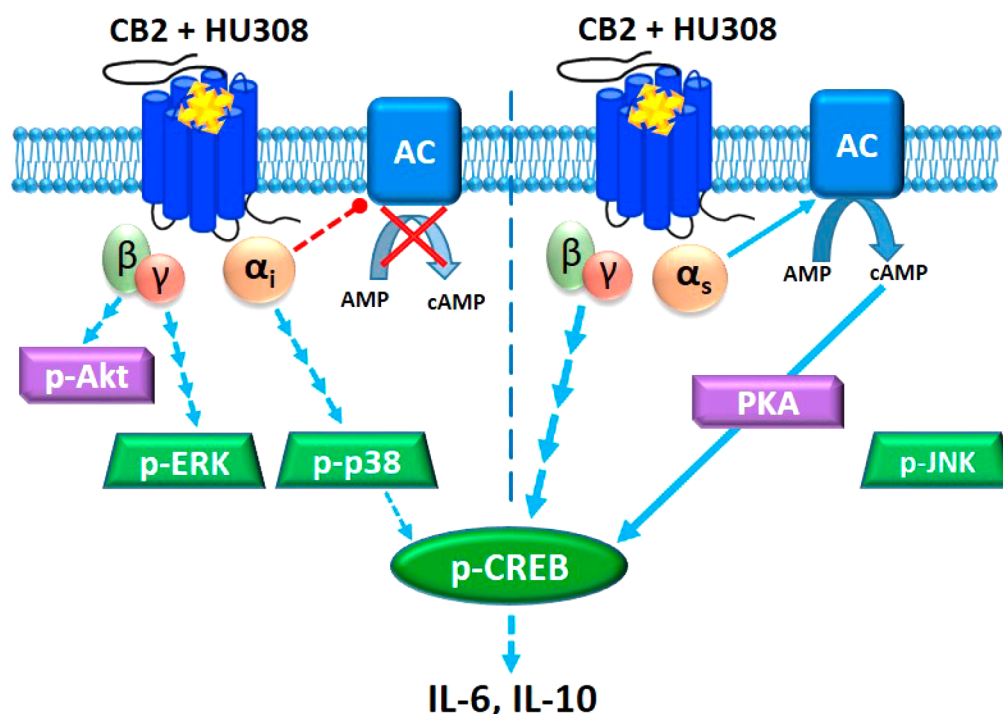


Figure 7. CB₂ signaling network in mixed human primary leukocytes. Binding of HU308 to CB₂ leads to activation of G α β γ and G α β γ heterotrimers. G α _i inhibits adenylyl cyclase(s) (AC) and activates a cascade leading to phosphorylation of p38 and CREB. β γ from G α β γ activates Akt and initiates a cascade leading to phosphorylation of ERK. G α _s stimulates AC which leads (likely via protein kinase A, PKA) to p-CREB. β γ from non-G α β γ (likely from G α β γ) also contributes to p-CREB activation. On the basis of inhibitor effects (Figure 5B), G α _s and non-G α β γ are the major mediators of p-CREB, whereas p-p38 is a minor p-CREB mediator. JNK phosphorylation was not observed. Given that G α _i-mediated inhibition of AC does not inhibit p-CREB activation it is likely that G α _i and G α _s modulations of AC are either segregated in separate compartments within one cell, or occur in different cells (represented by vertical dotted line). p-CREB likely mediates IL-6/IL-10 induction, which might be released from one cell or from different cell types.

HU308 (Figure 5A) revealed a wave of CREB phosphorylation which was maximal around 30–40 min and resolved by 60 min. We applied G protein inhibitors NF023, NF449, gallein, and PTX (Figure 5B), and found that both NF449 and gallein each partially inhibit CREB phosphorylation to a similar degree, but almost completely block the HU308 response when coapplied indicating that G α _s and β γ 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 α _i but not G α _i-coupled β γ in p-CREB activation. SR144528 and WIN55212-3 completely blocked the peak CREB phosphorylation (Figure 5C), indicating that this pathway activation occurs downstream of CB₂. SR144528 reduced p-CREB relative to the vehicle control indicating that this compound acted as an inverse agonist ($p = 0.013$; two-way RM ANOVA with Holm-Šidák posthoc test).

CB₂ Activation Induces Secretion of Cytokines IL-6 and IL-10. We stimulated PBMC with HU308 under the same conditions as used for the signaling assays and detected cytokines released into the assay media. After 12 h, interleukin 6 (IL-6) and 10 (IL-10) concentrations were significantly different from vehicle-treated cells (both $p < 0.0001$; two-way RM ANOVA with Holm-Šidák posthoc test), whereas the remaining seven cytokines in our panel (IL-2, IL-4, IL-12, IL-13, IL-17A, MIP-1 α , and TNF- α) were indistinguishable from vehicle-treated cells (all $p \geq 0.999$; Figure S7). HU308 was also coapplied with forskolin (10 μ M) to mimic conditions of our cAMP signaling assays; forskolin did not induce detectable cytokine secretion by itself ($p = 0.733$; two-way RM ANOVA;

Figure S7) and did not influence the HU308-mediated IL-6 and IL-10 levels ($p = 0.834$; two-way RM ANOVA; Figure S7). The responses were very similar between PBMC from three subjects and cell numbers were unaffected by the applied ligands (Figure S8). Subsequent interleukin experiments were carried out *without* forskolin present.

IL-6 and IL-10 accumulation were also detectable at an earlier time-point—after 6 h of HU308 stimulation (Figure 6A). This was concentration-dependent, with HU308 having greater potency for inducing IL-10 (pEC₅₀ 10.06 \pm 0.08 and 9.55 \pm 0.04; $p = 0.005$, unpaired t test). Application of G protein inhibitors revealed that HU308-induced IL-6 and IL-10 secretion are almost fully blocked by G α _s inhibitor NF449, partially blocked by β γ inhibitor gallein, and to a lesser extent sensitive to G α _i inhibitor NF023 (Figure 6B,C). Responses to HU308 (10 nM) were fully abrogated by SR144528 (1 μ M) and WIN55212-3 (5 μ M) down to levels below the assays' limits of quantitation. SR144528 and WIN55212-3 had no measurable effects on IL-6 or IL-10 production when applied alone.

DISCUSSION

To our knowledge this study represents the first investigation of CB₂ signaling and functional implications thereof in unstimulated human primary PBMC. Cells were kept in culture for as little time as practically possible (up to 6 h prior to stimuli of interest) and in 10% serum in order to mimic the state of leukocytes *in vivo*; a unique experimental paradigm in comparison with the vast majority of other cannabinoid studies

to date. 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 7.

We found that human primary PBMC respond in a concentration-dependent manner to CB₂-selective agonist HU308 with a 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. Application of G α inhibitors revealed 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. Response potencies were consistent with CB₂ specificity, and signaling was also sensitive to SR144528 and WIN55212-3, indicating that the observed signaling is CB₂-mediated. Although the idea that CB₂ may couple promiscuously to G proteins is not unprecedented,^{6,10} to our knowledge CB₂ coupling to G α_s has not been reported previously.

Börner and colleagues⁸ observed stimulatory effects of CB₁/CB₂ mixed agonists JWH015, THC, and methanandamide on cAMP concentrations in IL-4-stimulated human primary T lymphocytes after prolonged cannabinoid incubations; however, cAMP increases were sensitive to PTX and therefore concluded to be downstream of G α_i , likely via $\beta\gamma$.⁸ Promiscuous coupling of other receptors to G α_i and G α_s has been observed, including CB₁ in our own hands.⁵⁰ However, we did not observe G α_s coupling of human CB₂ in this same model. Studies in other models have also applied PTX and not revealed CB₂ signaling consistent with G α_s coupling.^{10,51,52} We therefore speculate that a critical component of the CB₂ immune cell signaling landscape has not been captured in previously studied models.

As we have studied mixed PBMC 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 α_i -predominant versus G α_s -predominant cell types. Leukocytes are known to express G α_i ,^{53,54} G α_o ,³⁹ and G $\alpha_{i/s}$ -sensitive adenylyl cyclases.⁵⁵ However, balance between G α and G $\beta\gamma$ subunit expression may determine overall cAMP responses.⁵⁶ As well, adenylyl cyclase isoforms can differ in their G α sensitivities, and expression levels can vary between immune cell types.⁵⁵ Furthermore, G protein coupling “switches” have been reported for receptors in heterodimers.^{57,58} The particular profile and stoichiometries of interaction partners can give rise to differential signaling “units” between cell types.⁵⁹ There are also various explanations for potential dual coupling within the same cell. Signaling subcellular compartmentalization and/or sequestration of signaling components is one example;⁶⁰ indeed, subcellular distribution of CB₂ can influence CB₂ signaling patterns,⁶ and CB₂ seems to have a particularly unique distribution in immune cells.^{61–63} Homologous signaling feedback upon CB₂ activation which results in rapid desensitization of G α_s coupling with little or slower influence on G α_i coupling such as via differential phosphorylation by G Protein-Coupled Receptor Kinases or PKA^{64–66} might assist in explaining the observed cAMP signaling profile. G $\beta\gamma$ exhaustion/sequestration may also hinder G α_s activation.^{67,68} It is also important to note and intriguing regarding the downstream functional implications, that subcellular compart-

mentalization of G proteins and/or adenylyl cyclase subtypes could give rise to localized cAMP fluxes, implying that simultaneous effects of both cAMP increases and decreases are possible even when the net cAMP concentration is unchanged.⁶⁹

The ERK1/2 signaling cascade regulates cell cycle progression,⁷⁰ immunological functions, including differentiation of T lymphocytes,¹⁴ polarization of CD4+ T lymphocytes,⁷¹ and regulation of cytokine network,⁷² and is dysregulated in a number of disease states.²³ We find that human primary PBMC respond to CB₂ activation by HU308 with rapid and transient stimulation of p-ERK. As this was completely sensitive to both G $\beta\gamma$ inhibitor gallein and G $\alpha_{i/o}$ / $\beta\gamma$ inhibitor PTX, we conclude that ERK1/2 phosphorylation induced by HU308 is mediated by $\beta\gamma$ dimers of G $\alpha\beta\gamma$ heterotrimeric proteins. Although in some systems gallein may inhibit GRK2 and possibly associated arrestin signaling,⁷³ 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.⁷⁴ Interestingly NF023, which we confirmed in cAMP assays inhibits G α_i , did not block the p-ERK response, indicating that this blocker specifically inhibits G α subunits without influencing G $\beta\gamma$ activity as also observed previously.⁷⁵ HU308 also induced phosphorylation of p38 and Akt. p-p38 was mediated by G $\alpha_{i/o}$ subunits of G proteins (possibly via Src kinase),⁷⁶ as it was completely blocked by G $\alpha_{i/o}$ / $\beta\gamma$ inhibitor PTX but not by G $\beta\gamma$ inhibitor gallein, whereas p-Akt was mediated by G $\alpha_{i/o}$ -coupled $\beta\gamma$ heterodimers (fully blocked by PTX and gallein).

In contrast with prior studies of human promyelocytic leukemia HL60 cells²⁵ and CB₂-overexpressing HEK cells²⁴ we did not observe JNK phosphorylation. 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.¹² Another explanation may be a different CB₂-mediated functional profile (i.e., biased agonism) between HU308 and the previously studied ligands.^{5,77} There may also be signal-modifying influences of serum as has been shown for CB₁.⁷⁸ The serum factors that circulating immune cells would be exposed to *in situ* are likely to influence signaling patterns.⁷⁹ We therefore 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. However, we must acknowledge that 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.⁸⁰ However, careful attention to vehicle controls, the potency of HU308 effects, only subtle inverse agonist effects, and lack of effects of a neutral antagonist when applied alone, indicate that any influence in this regard was minimal to nonexistent in our study.

cAMP/PKA, MAP kinases ERK and p38, and Akt/PKB all have the potential to induce phosphorylation of CREB.⁴⁹ Immunological functions of p-CREB include antiapoptotic effects on macrophages via p-38,⁸¹ proliferative effects on T lymphocytes via p-38⁸² and p-ERK,⁸³ and activation/proliferation of B lymphocytes via p-ERK.⁸⁴ CB₂-mediated p-CREB activation in primary PBMC is mediated primarily and to a similar degree by G α_s subunits and non-G α_i -derived $\beta\gamma$ dimers, and to a limited extent by G α_i subunits. Though G α_s -coupled $\beta\gamma$ is not widely appreciated to induce signaling

events, at least a few examples exist in the literature.^{85,86} p-CREB is unlikely to be downstream of pERK or p-Akt in our system given that these pathways were both associated with $\beta\gamma$ from $G\alpha\beta\gamma$ heterotrimers, however $G\alpha_i$ -coupled p-p38 may be involved to a minor degree. One of the primary p-CREB activation pathways is likely via $G\alpha_s$ leading to cAMP-activated PKA. This is interesting given that when measuring cAMP concentrations we did not detect any increase unless a $G\alpha_i$ blocker was present. This implies that the consequences of $G\alpha_i$ versus $G\alpha_s$ activation on cAMP concentration are distinct, supporting 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 study a physiologically relevant outcome, we detected a CB₂-mediated induction of interleukins 6 and 10 at 6 and 12 h. Both were sensitive to NF449 > gallein > NF023, indicating that $G\alpha_s$ is the main contributor to these responses. This pattern of influence of G protein inhibitors was similar to that for p-CREB, and given that p-CREB can induce transcription of IL-6 and IL-10 genes,⁸⁷ it is very feasible that CREB phosphorylation is an important mediator of HU308-induced IL-6 and IL-10. Although in some instances IL-6 may be secreted from a preformed pool,⁸⁸ the observed induction of IL-6 by HU308 at 6 and 12 h likely involves its *de novo* synthesis.⁸⁹ To our knowledge, PBMC do not have intracellular granules of IL-10, and so it is most likely being synthesized *de novo* and then secreted. Kinetics of mRNA induction for IL-6 and IL-10^{90,91} are consistent with a multihour lag in their production after CB₂ activation.

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-6/10 induction. p-p38 and p-Akt have also been linked to both IL-6^{92,93} and IL-10 production.^{94,95} Given the indicated involvement of CREB we were surprised to find that forskolin, a strong activator of signaling pathways downstream of adenylyl cyclases, did not influence cytokine secretion. We postulate that a costimulus enabled by CB₂ activation but not by forskolin might be required for IL-6/10 induction.⁴⁹ It is also plausible that the potential for subcellular compartmentalization and/or temporal integration of cAMP fluxes play critical roles. Application of specific signaling pathway inhibitors would be useful to more directly delineate the exact signaling pathways responsible.

The degree of cytokine induction we observed was small in comparison with the positive control utilized, LPS—a potent mediator of bacterial sepsis,⁹⁶ which models systemic inflammation *in vitro*. 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.

We did not observe modulation of cytokines IL-2, IL-4, IL-12, IL-13, IL-17A, MIP-1 α , 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.

We also noted that HU308 did not influence cell number of PBMC treated for 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.^{17,97,98} Furthermore, this response has been found to be cell type- and ligand-dependent,⁹⁹ and is not always mediated by cannabinoid receptors.¹⁰⁰

IL-10 is an anti-inflammatory cytokine expressed by many immunocompetent cell types which regulates T-lymphocytic responses via direct mechanisms¹⁰¹ and by inhibiting effector functions of antigen-presenting cells.^{102,103} IL-10 can also protect 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.¹⁰⁴ On the basis of these paradigms the combined release of IL-6 and IL-10 seems counterintuitive. However, IL-6 can context-dependently exert anti-inflammatory effects, and indeed simultaneous production of IL-6 and IL-10 from mixed human primary immune cells has been observed previously.¹⁰⁵

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, seems 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 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*.^{106,107} Low dose recombinant IL-6 can also increase plasma IL-10 and have other anti-inflammatory effects in humans.¹⁰⁸ However, given the acute time frame within which we detected both IL-6 and IL-10 (by 6 h), 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.¹⁰⁹ IL-6 and IL-10 have classically been thought to be produced by T helper 2 (Th2) T lymphocytes,^{110,111} while concurrent production has been observed in monocytes, tumor-associated macrophages, and dendritic cells.^{112–114} 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.

There is some precedent for cannabinoid mediation of both IL-6 and IL-10. Endocannabinoids upregulated IL-6 in LPS-stimulated U937 cells⁸⁰ and in whole blood cultures.¹¹⁵ Cannabinoid-mediated IL-10 induction has been shown *in vivo* in mice,¹¹⁶ in murine LPS-stimulated macrophages,¹¹ and in murine encephalitogenic T lymphocytes;¹¹⁷ 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.^{118,119} Increased serum IL-10 has been correlated with cannabis consumption,¹²⁰ while elevated IL-6 and other pro-inflammatory markers were found in individuals with cannabis dependency.¹²¹ In a trial of THC in multiple sclerosis, cannabinoid treatments were found not to influence serum concentrations of IL-10 or CRP (a surrogate measure of IL-6), nor other measured cytokines, though sample sizes were small and variability between subjects large.¹²² Further, THC is a partial agonist at CB₂ and so may have only subtle effects in comparison with full 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 serious adverse effects from illicit synthetic cannabinoids, some of which are potent CB₂ agonists.^{123,124} Although these adverse symptoms may be mediated by a noncannabinoid-receptor target,¹²⁵ we wonder whether a potent and efficacious influence on cytokine balance and/or different cytokine profile arising from biased agonism via CB₂ could contribute to deleterious effects.

The results of this study greatly expand our knowledge of CB₂ signaling and functional implications in human primary PBMC under conditions closely preserving their *in situ* state. The discovery of unprecedented CB₂ coupling to stimulatory (G_{α_s}) proteins, their simultaneous activation alongside inhibitory (G_{α_i}) proteins in a cAMP pathway, of G_{α_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 effects of CB₂ agonism that 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.

METHODS

Preparation of Human PBMC. Human primary PBMC were isolated from blood samples collected from three healthy volunteers (male and female, aged 22–26) after obtaining written informed consents in accordance with ethical approval from the University of Auckland Human Participants Ethics Committee (#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 K₂EDTA-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 min at 800g at room temperature. The collected PBMC were washed twice with RPMI by centrifugation for 10 min at 250g, resuspended in a culture medium—RPMI supplemented with 10% v/v fetal bovine serum (FBS; New Zealand origin, Moredun 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 (Figure S9) or HU308 signaling responses of PBMC (Figure S10).

Cell Lines and Cell Culture. Human embryonic kidney (HEK) cell lines stably transfected with human CB₁ (HEK-hCB1¹²⁶) or human CB₂ (HEK-hCB2⁵⁰), 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. Human primary PBMC or HEK cells were pelleted by centrifugation for 10 min at 250g or 5 min at 125g, respectively, and resuspended in binding medium (RPMI with 1 mg/mL BSA, 2 mM L-Glutamine, 25 mM HEPES). PBMC (3 × 10⁵ cells per assay point), HEK-hCB₁ or HEK-hCB₂ (0.4 × 10⁵ diluted in 1.6 × 10⁵ HEK wt to avoid ligand depletion), or HEK wt (2 × 10⁵) 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) [³H]-CP55940 (PerkinElmer), and an appropriate displacer to measure nonspecific displacement of the radioligand or a vehicle (DMSO) control to measure total binding of the radioligand. The displacer was either ACEA at 1 μM (arachidonyl-2-chloroethylamide, synthesized from arachidonic acid as described in ref 127), or 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 (PerkinElmer, MA, USA) pretreated with 0.1% polyethylenimine (PEI, Sigma-Aldrich). After three washes with ice-cold wash buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 1 mg/mL BSA) filter plates were dried overnight, then sealed, and 50 μL per well of Irgasafe Plus scintillation fluid (PerkinElmer) was dispensed. After 30 min, plates were counted in a Wallac MicroBeta TriLux liquid scintillation counter (PerkinElmer). 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} , the concentration of binding sites for the radioligand,¹²⁸ using the following formula based on eq 7.5.21 from ref 129:

$$B_{\max} = \frac{\text{specific binding}}{\text{fractional occupancy}} \\ = \frac{\text{specific binding}}{[\text{radioligand}]/(K_d + [\text{radioligand}])}$$

B_{\max} values were then converted to moles 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 [³H]-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. PBMC were resuspended in assay medium (RPMI with 2 mM L-glutamine, 25 mM HEPES, 10% v/v FBS) and seeded at 1 × 10⁵ 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% CO₂ 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. The assays were performed without any phosphodiesterase inhibitors to allow for assessment of signaling kinetics.

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 of 24 μL using 2× concentrated detection mix. The signal was detected on a CLARIOstar plate reader (BMG Labtech) using recommended TR-FRET settings. Assay sample cAMP concentrations were interpolated from standard curves. 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). PBMC were pelleted by centrifugation for 10 min at 250g, resuspended in assay medium, and seeded at 1 × 10⁵ cells in 10 μL per well into Falcon 384-well plates. Plates were incubated in a humidified atmosphere at 37 °C and 5% CO₂ 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 at 10 μL per well at requisite time points. At the end of incubation, plates were placed on ice, and cells were lysed by the 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. The signal was detected on a CLARIOstar plate reader with AlphaLISA-compatible filters. Counts were normalized to vehicle controls (100%) to allow compilation of data from independent experiments.

Akt, p38, JNK, CREB Phosphorylation Assays. 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. In experiments with PTX (Sigma-Aldrich), cells were pelleted, resuspended in the assay medium containing PTX 100 ng/mL or vehicle, and incubated for 5 h in a humidified atmosphere at 37 °C and 5% CO₂. After the incubation, the cells were counted and resuspended in the same assay media with PTX or vehicle and equilibrated for 1 h as described for the signaling assays (bringing the total PTX incubation to 6 h). Then ligands/vehicles were added for signaling experiments. In gallein (Santa Cruz Biotechnology) or suramin derivatives NF023 (Abcam) and NF449 (Abcam) experiments, the inhibitors or their vehicles were added to cells at 10 μM 30 min prior to adding ligands or vehicles. All ligand and vehicle solutions used in assays with inhibitors were prepared in assay media containing the inhibitors or vehicles, so that inhibitors or vehicle controls

at 1× concentration were present for the entire duration of stimulations.

Analysis of Cytokine Secretion. PBMC were seeded at 4.5 × 10⁵ cells per well in assay medium into 96 Well V-Bottom plates (Interlab) and incubated for 30 min. If applicable, cells were pretreated 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 (multiplex screen) or 6 h (quantitative IL-6/IL-10 analysis) incubation in a humidified atmosphere at 37 °C and 5% CO₂, plates were centrifuged for 10 min at 250g, and the supernatants were collected and frozen at −80 °C. The remaining cell pellets were resuspended, and aliquots (in technical duplicate) were diluted in Trypan blue for hemocytometer counting. Interleukins 2, 4, 6, 10, 12, 13, and 17A, macrophage inflammatory protein (MIP-1α), and tumor necrosis factor (TNF-α) were detected using Cytometric Bead Array (CBA) technology (BD Biosciences) and assayed on an Accuri C6 flow cytometer (BD Biosciences) as described previously.¹³⁰ 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 calculated. Concentrations of IL-6 and IL-10 were interpolated using FCAP Array software (v. 3.1, BD Biosciences) from standard curves. 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 interleukins 2, 4, 6, 10, 12, 13, 17A, MIP-1α, and TNF-α, respectively.

Data Analysis. All analysis was performed in GraphPad Prism Software (v. 8.0; GraphPad Software Inc.). Data were 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. Normalized data were utilized for statistical analysis of selected experiments (Figures 2D,E, 4B, and 5B, as described in figure legends). Otherwise, statistical analyses were performed on raw data. Repeated measures (RM) designs were utilized to reflect that each independent experiment was carried out on a separate subject. Normal distribution and equality of variance were verified with Shapiro-Wilk and Brown-Forsythe tests. If these tests did not pass, a decimal logarithm transformation was performed which enabled adherence to parametric test assumptions. If a statistically significant difference ($p < 0.05$) was detected in one- or two-way ANOVA, data were further analyzed using either Holm-Sidak (for comparisons to control) or Tukey (when comparisons between multiple conditions were of interest) posthoc tests with significance levels indicated graphically as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), nonsignificant (ns).

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acspstci.9b00049.

Preincubation with forskolin has no effect on net cAMP kinetics in human primary PBMC; stimulatory cAMP signaling is detectable without forskolin; effects of HU308 on cAMP and p-ERK responses in PBMC are concentration-dependent; SR144528 is selective for CB₂ over CB₁; PBMC do not respond to a CB₁-selective agonist ACEA in p-ERK assay; HU308 induces interleukins 6 and 10 but not IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17A, MIP-1 α or TNF- α in PBMC; HU308 has no effect on cell concentrations after 6 or 12 h of incubation; cryopreservation of PBMC has no effect on CB₂ expression, nor p-ERK response (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: n.grimsey@auckland.ac.nz. Tel.: +64 9 923 1886.

ORCID

Michelle Glass: 0000-0002-5997-6898

Natasha Lillia Grimsey: 0000-0003-3941-4537

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank John McGowan (University of Auckland, NZ) for synthesizing the ACEA. This research was supported by funding from the Auckland Medical Research Foundation and the Health Research Council NZ (to N.G.). Y.S. was supported by Dean's International Doctoral Scholarship, Faculty of Medical and Health Sciences, The University of Auckland. The Accuri C6 flow cytometer was purchased with funding from the NZ Lottery Health Board (to E.S.G.).

REFERENCES

- (1) Galiegue, S., Mary, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G., and Casellas, P. (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem.* 232, 54–61.
- (2) Gonsiorek, W., Hesk, D., Chen, S.-C., Kinsley, D., Fine, J. S., Jackson, J. V., Bober, L. A., Deno, G., Bian, H., Fossetta, J., Lunn, C. A., Kozlowski, J. A., Lavey, B., Piwinski, J., Narula, S. K., Lundell, D. J., and Hipkin, R. W. (2006) Characterization of peripheral human cannabinoid receptor (hCB2) expression and pharmacology using a novel radioligand, [³⁵S]Sch225336. *J. Biol. Chem.* 281, 28143–51.
- (3) Pacher, P., and Kunos, G. (2013) Modulating the endocannabinoid system in human health and disease—successes and failures. *FEBS J.* 280, 1918–43.
- (4) Bouaboula, M., Poinot-Chazel, C., Marchand, J., Canat, X., Bourrié, B., Rinaldi-Carmona, M., Calandra, B., Le Fur, G., and Casellas, P. (1996) Signaling pathway associated with stimulation of CB2 peripheral cannabinoid receptor. Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. *Eur. J. Biochem.* 237, 704–11.
- (5) Soethoudt, M., Grether, U., Fingerle, J., Grim, T. W., Fezza, F., de Petrocellis, L., Ullmer, C., Rothenhäusler, B., Perret, C., van Gils, N., Finlay, D., MacDonald, C., Chicca, A., Gens, M. D., Stuart, J., de Vries, H., Mastrangelo, N., Xia, L., Alachouzos, G., Baggelaar, M. P., Martella, A., Mock, E. D., Deng, H., Heitman, L. H., Connor, M., Di Marzo, V., Gertsch, J., Lichtman, A. H., Maccarrone, M., Pacher, P., Glass, M., and van der Stelt, M. (2017) Cannabinoid CB2 receptor ligand profiling reveals biased signalling and off-target activity. *Nat. Commun.* 8, 13958.
- (6) Brailoiu, G. C., Deliu, E., Marcu, J., Hoffman, N. E., Console-Bram, L., Zhao, P., Madesh, M., Abood, M. E., and Brailoiu, E. (2014) Differential activation of intracellular versus plasmalemmal CB2 Cannabinoid receptors. *Biochemistry* 53, 4990–4999.
- (7) Rhee, M. H., Bayewitch, M., Avidor-Reiss, T., Levy, R., and Vogel, Z. (1998) Cannabinoid receptor activation differentially regulates the various adenylyl cyclase isozymes. *J. Neurochem.* 71, 1525–1534.
- (8) Börner, C., Smida, M., Höllt, V., Schraven, B., and Kraus, J. (2009) Cannabinoid receptor type 1- and 2-mediated increase in cyclic AMP inhibits T cell receptor-triggered signaling. *J. Biol. Chem.* 284, 35450–35460.
- (9) Nogueras-Ortiz, C., Roman-Vendrell, C., Mateo-Semidey, G. E., Liao, Y.-H., Kendall, D. A., and Yudowski, G. A. (2017) Retromer stops beta-arrestin 1-mediated signaling from internalized cannabinoid 2 receptors. *Mol. Biol. Cell* 28, 3554–3561.
- (10) Shoemaker, J. L., Ruckle, M. B., Mayeux, P. R., and Prather, P. L. (2005) Agonist-directed trafficking of response by endocannabinoids acting at CB2 receptors. *J. Pharmacol. Exp. Ther.* 315, 828–38.
- (11) Correa, F., Mestre, L., Docagne, F., and Guaza, C. (2005) Activation of cannabinoid CB 2 receptor negatively regulates IL-12p40 production in murine macrophages: Role of IL-10 and ERK1/2 kinase signaling. *Br. J. Pharmacol.* 145, 441–448.
- (12) Gertsch, J., Leonti, M., Raduner, S., Racz, I., Chen, J.-Z., Xie, X.-Q., Altmann, K.-H., Karsak, M., and Zimmer, A. (2008) Beta-caryophyllene is a dietary cannabinoid. *Proc. Natl. Acad. Sci. U. S. A.* 105, 9099–9104.
- (13) Coopman, K., Smith, L. D., Wright, K. L., and Ward, S. G. (2007) Temporal variation in CB2R levels following T lymphocyte activation: evidence that cannabinoids modulate CXCL12-induced chemotaxis. *Int. Immunopharmacol.* 7, 360–71.
- (14) Yamashita, M., Kimura, M., Kubo, M., Shimizu, C., Tada, T., Perlmutter, R. M., and Nakayama, T. (1999) T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. *Proc. Natl. Acad. Sci. U. S. A.* 96, 1024–1029.
- (15) Nowell, P. C. (1960) Phytohemagglutinin: An Initiator of Mitosis in Cultures of Normal Human Leukocytes. *Cancer Res.* 20, 462–466.
- (16) Herrera, B., Carracedo, A., Diez-Zaera, M., Guzman, M., and Velasco, G. (2005) p38 MAPK is involved in CB2 receptor-induced apoptosis of human leukaemia cells. *FEBS Lett.* 579, 5084–5088.
- (17) Gustafsson, K., Christensson, B., Sander, B., and Flygare, J. (2006) Cannabinoid receptor-mediated apoptosis induced by R(+)-methanandamide and Win55,212–2 is associated with ceramide accumulation and p38 activation in mantle cell lymphoma. *Mol. Pharmacol.* 70, 1612–20.
- (18) Do, Y., McKallip, R. J., Nagarkatti, M., and Nagarkatti, P. S. (2004) Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NF-kappaB-dependent apoptosis: novel role for endogenous and exogenous cannabinoids in immunoregulation. *J. Immunol.* 173, 2373–2382. [pii].
- (19) Zhang, Y., Wang, X., Yang, H., Liu, H., Lu, Y., Han, L., and Liu, G. (2013) Kinase AKT controls innate immune cell development and function. *Immunology* 140, 143–152.
- (20) Gomez, O., Sanchez-Rodriguez, A., Le, M. Q. U., Sanchez-Caro, C., Molina-Holgado, F., and Molina-Holgado, E. (2011) Cannabinoid receptor agonists modulate oligodendrocyte differentiation by activating PI3K/Akt and the mammalian target of rapamycin (mTOR) pathways. *Br. J. Pharmacol.* 163, 1520–1532.
- (21) Utomo, W. K., Peppelenbosch, M. P., Braat, H., Parikh, K., de Vries, M., Fuhler, G. M., Comalada, M., Bruno, M. J., and van Goor, H. (2017) Modulation of Human Peripherial Blood Mononuclear Cell Signaling by Medicinal Cannabinoids. *Front. Mol. Neurosci.* 10, 1–11.
- (22) Gómez del Pulgar, T., Velasco, G., and Guzmán, M. (2000) The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. *Biochem. J.* 347, 369–73.
- (23) Kim, E. K., and Choi, E.-J. (2010) Pathological roles of MAPK signaling pathways in human diseases. *Biochim. Biophys. Acta, Mol. Basis Dis.* 1802, 396–405.

- (24) Li, A.-L., Lin, X., Dhopeswarkar, A. S., Thomaz, A. C., Carey, L. M., Liu, Y., Nikas, S. P., Makriyannis, A., Mackie, K., and Hohmann, A. G. (2019) Cannabinoid CB2 Agonist AM1710 Differentially Suppresses Distinct Pathological Pain States and Attenuates Morphine Tolerance and Withdrawal. *Mol. Pharmacol.* 95, 155–168.
- (25) Sugiura, T., Kishimoto, S., Oka, S., Gokoh, M., and Waku, K. (2004) Metabolism and physiological significance of anandamide and 2-arachidonoylglycerol, endogenous cannabinoid receptor ligands, in Arachidonate Remodeling and Inflammation (Fonteh, A. N., and Wykle, R. L., Eds.), pp 211–237. Birkhäuser Basel, Basel. DOI: [10.1007/978-3-0348-7848-7_11](https://doi.org/10.1007/978-3-0348-7848-7_11).
- (26) Rom, S., and Persidsky, Y. (2013) Cannabinoid receptor 2: potential role in immunomodulation and neuroinflammation. *J. Neuroimmune Pharmacol.* 8, 608–20.
- (27) Ihenetu, K., Molleman, A., Parsons, M., and Whelan, C. (2003) Pharmacological characterisation of cannabinoid receptors inhibiting interleukin 2 release from human peripheral blood mononuclear cells. *Eur. J. Pharmacol.* 464, 207–15.
- (28) Cencioni, M. T., Chiurchiù, V., Catanzaro, G., Borsellino, G., Bernardi, G., Battistini, L., and Maccarrone, M. (2010) Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB2 receptors. *PLoS One* 5, e8688.
- (29) Yuan, M., Kiertscher, S. M., Cheng, Q., Zoumalan, R., Tashkin, D. P., and Roth, M. D. (2002) Delta 9-Tetrahydrocannabinol regulates Th1/Th2 cytokine balance in activated human T cells. *J. Neuroimmunol.* 133, 124–31.
- (30) Gardner, B., Zu, L. X., Sharma, S., Liu, Q., Makriyannis, A., Tashkin, D. P., and Dubinett, S. M. (2002) Autocrine and paracrine regulation of lymphocyte CB2 receptor expression by TGF-beta. *Biochem. Biophys. Res. Commun.* 290, 91–6.
- (31) Massi, P., Sacerdote, P., Ponti, W., Fuzio, D., Manfredi, B., Viganò, D., Rubino, T., Bardotti, M., and Parolaro, D. (1998) Immune function alterations in mice tolerant to Δ9-tetrahydrocannabinol: Functional and biochemical parameters. *J. Neuroimmunol.* 92, 60–66.
- (32) Newton, C. A., Klein, T. W., and Friedman, H. (1994) Secondary immunity to *Legionella pneumophila* and Th1 activity are suppressed by delta-9-tetrahydrocannabinol injection. *Infect. Immun.* 62, 4015–4020.
- (33) Klein, T. W., Newton, C. A., Nakachi, N., and Friedman, H. (2000) 9-Tetrahydrocannabinol Treatment Suppresses Immunity and Early IFN-gamma, IL-12, and IL-12 Receptor 2 Responses to *Legionella pneumophila* Infection. *J. Immunol.* 164, 6461–6466.
- (34) Nakano, Y., Pross, S., and Friedman, H. (1993) Contrasting effect of delta-9-tetrahydrocannabinol on IL-2 activity in spleen and lymph node cells of mice of different ages. *Life Sci.* 52, 41–51.
- (35) Hillard, C. J., Manna, S., Greenberg, M. J., DiCamelli, R., Ross, R. A., Stevenson, L. A., Murphy, V., Pertwee, R. G., and Campbell, W. B. (1999) Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). *J. Pharmacol. Exp. Ther.* 289, 1427–33.
- (36) Hanus, L., Breuer, A., Tchilibon, S., Shiloah, S., Goldenberg, D., Horowitz, M., Pertwee, R. G., Ross, R. A., Mechoulam, R., and Fride, E. (1999) HU-308: a specific agonist for CB(2), a peripheral cannabinoid receptor. *Proc. Natl. Acad. Sci. U. S. A.* 96, 14228–33.
- (37) Oyagawa, C. R. M., de la Harpe, S. M., Saroz, Y., Glass, M., Vernall, A. J., and Grimsey, N. L. (2018) Cannabinoid Receptor 2 Signalling Bias Elicited by 2,4,6-Trisubstituted 1,3,5-Triazines. *Front. Pharmacol.* 9, 1202.
- (38) Locht, C., and Antoine, R. (1995) A proposed mechanism of ADP-ribosylation catalyzed by the pertussis toxin S1 subunit. *Biochimie* 77, 333–340.
- (39) Milligan, G., and Kostenis, E. (2006) Heterotrimeric G-proteins: A short history. *Br. J. Pharmacol.* 147 (147), S46.
- (40) Hohenegger, M., Waldhoer, M., Beindl, W., Bing, B., Kreimeyer, A., Nickel, P., Nanoff, C., Freissmuth, A. M., and Gilman, A. G. (1998) Gs alpha-selective G protein antagonists. *Proc. Natl. Acad. Sci. U. S. A.* 95, 346–351.
- (41) Dhopeswarkar, A., and Mackie, K. (2016) Functional selectivity of CB2 cannabinoid receptor ligands at a canonical and non-canonical pathway. *J. Pharmacol. Exp. Ther.* 358, 342–351.
- (42) Freissmuth, M., Boehm, S., Beindl, W., Nickel, P., Ijzerman, A. P., Hohenegger, M., and Nanoff, C. (1996) Suramin analogues as subtype-selective G protein inhibitors. *Mol. Pharmacol.* 49, 602–11.
- (43) Lehmann, D. M., Seneviratne, A. M. P. B., and Smrcka, A. V. (2008) Small Molecule Disruption of G Protein Subunit Signaling Inhibits Neutrophil Chemotaxis and Inflammation. *Mol. Pharmacol.* 73, 410–418.
- (44) Rinaldi-Carmona, M., Barth, F., Millan, J., Derocq, J. M., Casellas, P., Congy, C., Oustric, D., Sarran, M., Bouaboula, M., Calandra, B., Portier, M., Shire, D., Breliere, J. C., and Le Fur, G. L. (1998) SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J. Pharmacol. Exp. Ther.* 284, 644–650.
- (45) Savinainen, J. R., Kokkola, T., Salo, O. M. H., Poso, A., Järvinen, T., and Laitinen, J. T. (2005) Identification of WIN55212–3 as a competitive neutral antagonist of the human cannabinoid CB 2 receptor. *Br. J. Pharmacol.* 145, 636–645.
- (46) Sugiura, T., Kondo, S., Miyashita, T., Kodaka, T., Suhara, Y., Takayama, H., and Waku, K. (2000) Evidence That 2-Arachidonoylglycerol but Not N -Palmitoylethanolamine or Anandamide Is the Physiological Ligand for the Cannabinoid CB2 Receptor. *J. Biol. Chem.* 275, 605–12.
- (47) Arkun, Y., Yasemi, M., and Thattai, M. (2018) Dynamics and control of the ERK signaling pathway: Sensitivity, bistability, and oscillations. *PLoS One* 13, e0195513 DOI: [10.1371/journal.pone.0195513](https://doi.org/10.1371/journal.pone.0195513).
- (48) Compagnucci, C., Di Siena, S., Bustamante, M. B., Di Giacomo, D., Di Tommaso, M., Maccarrone, M., Grimaldi, P., Sette, C., and Androutsellis-Theotokis, A. (2013) Type-1 (CB1) Cannabinoid Receptor Promotes Neuronal Differentiation and Maturation of Neural Stem Cells. *PLoS One* 8, e54271 DOI: [10.1371/journal.pone.0054271](https://doi.org/10.1371/journal.pone.0054271).
- (49) Johannessen, M., Delghandi, M. P., and Moens, U. (2004) What turns CREB on? *Cell. Signalling* 16, 1211–1227.
- (50) Finlay, D. B., Cawston, E. E., Grimsey, N. L., Hunter, M. R., Korde, A., Vemuri, V. K., Makriyannis, A., and Glass, M. (2017) Gas signalling of the CB1 receptor and the influence of receptor number. *Br. J. Pharmacol.* 174, 2545–2562.
- (51) Felder, C. C., Joyce, K. E., Briley, E. M., Mansouri, J., Mackie, K., Blond, O., Lai, Y., Ma, A. L., and Mitchell, R. L. (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol. Pharmacol.* 48, 443–50.
- (52) Slipetz, D. M., O'Neill, G. P., Favreau, L., Dufresne, C., Gallant, M., Gareau, Y., Guay, D., Labelle, M., and Metters, K. M. (1995) Activation of the human peripheral cannabinoid receptor results in inhibition of adenylyl cyclase. *Mol. Pharmacol.* 48, 352–361.
- (53) Scherzer, J. A., Lin, Y., McLeish, K. R., and Klein, J. B. (1997) TNF translationally modulates the expression of G1 protein alpha(i2) subunits in human polymorphonuclear leukocytes. *J. Immunol* 158, 913–918.
- (54) Kuwano, Y., Adler, M., Zhang, H., Groisman, A., and Ley, K. (2016) Gai2 and Gai3 Differentially Regulate Arrest from Flow and Chemotaxis in Mouse Neutrophils. *J. Immunol.* 196, 3828–3833.
- (55) Duan, B., Davis, R., Sadat, E. L., Collins, J., Sternweis, P. C., Yuan, D., and Jiang, L. I. (2010) Distinct Roles of Adenylyl Cyclase VII in Regulating the Immune Responses in Mice. *J. Immunol.* 185, 335–344.
- (56) Leander, R., and Friedman, A. (2014) Modulation of the cAMP Response by G alpha i and G beta gamma: A Computational Study of G Protein Signaling in Immune Cells. *Bull. Math. Biol.* 76, 1352–1375.
- (57) Glass, M., and Felder, C. C. (1997) Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. *J. Neurosci.* 17, 5327–33.

- (58) Navarro, G., Reyes-Resina, I., Rivas-Santisteban, R., Sánchez de Medina, V., Morales, P., Casano, S., Ferreiro-Vera, C., Lillo, A., Aguinaga, D., Jagerovic, N., Nadal, X., and Franco, R. (2018) Cannabidiol skews biased agonism at cannabinoid CB1 and CB2 receptors with smaller effect in CB1-CB2 heteroreceptor complexes. *Biochem. Pharmacol.* 157, 148–158.
- (59) Ferré, S. (2015) The GPCR heterotetramer: challenging classical pharmacology. *Trends Pharmacol. Sci.* 36, 145–52.
- (60) Ellisdon, A. M., and Halls, M. L. (2016) Compartmentalization of GPCR signalling controls unique cellular responses. *Biochem. Soc. Trans.* 44, S62–7.
- (61) Kleyer, J., Nicolussi, S., Taylor, P., Simonelli, D., Furger, E., Anderle, P., and Gertsch, J. (2012) Cannabinoid receptor trafficking in peripheral cells is dynamically regulated by a binary biochemical switch. *Biochem. Pharmacol.* 83, 1393–1412.
- (62) Castaneda, J. T., Harui, A., Kiertscher, S. M., Roth, J. D., and Roth, M. D. (2013) Differential Expression of Intracellular and Extracellular CB 2 Cannabinoid Receptor Protein by Human Peripheral Blood Leukocytes. *J. Neuroimmune. Pharmacol.* 8, 323–332.
- (63) Castaneda, J. T., Harui, A., and Roth, M. D. (2017) Regulation of Cell Surface CB2 Receptor during Human B Cell Activation and Differentiation. *J. Neuroimmune Pharmacol.* 12, 544–554.
- (64) Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997) Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* 390, 88–91.
- (65) Tesmer, V. M., Kawano, T., Shankaranarayanan, A., Kozasa, T., and Tesmer, J. J. G. (2005) Snapshot of activated G proteins at the membrane: the Gα_q-GRK2-Gβγ complex. *Science* 310, 1686–90.
- (66) Zhu, W., Petrashevskaya, N., Ren, S., Zhao, A., Chakir, K., Gao, E., Chuprun, J. K., Wang, Y., Talan, M., Dorn, G. W., Lakatta, E. G., Koch, W. J., Feldman, A. M., and Xiao, R.-P. (2012) Gi-biased β₂AR signaling links GRK2 upregulation to heart failure. *Circ. Res.* 110, 265–74.
- (67) Olanas, M. C., Ingianni, A., and Onali, P. (1998) Role of G protein βγ subunits in muscarinic receptor-induced stimulation and inhibition of adenylyl cyclase activity in rat olfactory bulb. *J. Neurochem.* 70, 2620–7.
- (68) Uezono, Y., Kaibara, M., Murasaki, O., and Taniyama, K. (2004) Involvement of G protein βγ-subunits in diverse signaling induced by G(i/o)-coupled receptors: study using the *Xenopus* oocyte expression system. *Am. J. Physiol. Cell Physiol.* 287, C885–94.
- (69) Conti, M., Mika, D., and Richter, W. (2014) Cyclic AMP compartments and signaling specificity: role of cyclic nucleotide phosphodiesterases. *J. Gen. Physiol.* 143, 29–38.
- (70) Roskoski, R. (2012) ERK1/2 MAP kinases: Structure, function, and regulation. *Pharmacol. Res.* 66, 105–143.
- (71) Agrawal, A., Dillon, S., Denning, T. L., and Pulendran, B. (2006) ERK1–/– Mice Exhibit Th1 Cell Polarization and Increased Susceptibility to Experimental Autoimmune Encephalomyelitis. *J. Immunol.* 176, 5788–5796. [pii].
- (72) Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J. H., Patriotis, C., Jenkins, N. A., Copeland, N. G., Kollias, G., and Tschlis, P. N. (2000) TNF-α induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 103, 1071–1083.
- (73) Kamal, F. A., Travers, J. G., Schafer, A. E., Ma, Q., Devarajan, P., and Blaxall, B. C. (2017) G Protein–Coupled Receptor–G–Protein βγ – Subunit Signaling Mediates Renal Dysfunction and Fibrosis in Heart Failure. *J. Am. Soc. Nephrol.* 28, 197–208.
- (74) van Der Lee, M. M. C., Bras, M., van Koppen, C. J., and Zaman, G. J. R. (2008) beta-Arrestin recruitment assay for the identification of agonists of the sphingosine 1-phosphate receptor EDG1. *J. Biomol. Screening* 13, 986–98.
- (75) Saitoh, F., Wakatsuki, S., Tokunaga, S., Fujieda, H., and Araki, T. (2016) Glutamate signals through mGluR2 to control Schwann cell differentiation and proliferation. *Sci. Rep.* 6, 1–14.
- (76) Simo-Cheyrou, E. R., Vardatsikos, G., and Srivastava, A. K. (2016) Src tyrosine kinase mediates endothelin-1-induced early growth response protein-1 expression via MAP kinase-dependent pathways in vascular smooth muscle cells. *Int. J. Mol. Med.* 38, 1879–1886.
- (77) Dhopeswarkar, A., and Mackie, K. (2014) CB2 Cannabinoid receptors as a therapeutic target—what does the future hold? *Mol. Pharmacol.* 86, 430–7.
- (78) Graham, E. S., Ball, N., Scotter, E. L., Narayan, P., Dragunow, M., and Glass, M. (2006) Induction of Krox-24 by Endogenous Cannabinoid Type 1 Receptors in Neuro2A Cells Is Mediated by the MEK-ERK MAPK Pathway and Is Suppressed by the Phosphatidylinositol 3-Kinase Pathway. *J. Biol. Chem.* 281, 29085–29095.
- (79) Gurevich, V. V., and Gurevich, E. V. (2018) Arrestins and G proteins in cellular signaling: The coin has two sides. *Sci. Signaling* 11, eaav1646.
- (80) Marazzi, J., Kleyer, J., Paredes, J. M. V., and Gertsch, J. (2011) Endocannabinoid content in fetal bovine sera — Unexpected effects on mononuclear cells and osteoclastogenesis. *J. Immunol. Methods* 373, 219–228.
- (81) Park, J. M., Greten, F. R., Wong, A., Westrick, R. J., Arthur, J. S. C., Otsu, K., Hoffmann, A., Montminy, M., and Karin, M. (2005) Signaling Pathways and Genes that Inhibit Pathogen-Induced Macrophage Apoptosis— CREB and NF-κB as Key Regulators. *Immunity* 23, 319–329.
- (82) Yu, C.-T., Shih, H. -m., and Lai, M.-Z. (2001) Multiple Signals Required for Cyclic AMP-Responsive Element Binding Protein (CREB) Binding Protein Interaction Induced by CD3/CD28 Costimulation. *J. Immunol.* 166, 284–292.
- (83) Grady, G. C., Mason, S. M., Stephen, J., Zuniga-Pflucker, J. C., and Michie, A. M. (2004) Cyclic Adenosine 5′-Monophosphate Response Element Binding Protein Plays a Central Role in Mediating Proliferation and Differentiation Downstream of the Pre-TCR Complex in Developing Thymocytes. *J. Immunol.* 173, 1802–1810.
- (84) Yasuda, T., Sanjo, H., Pagès, G., Kawano, Y., Karasuyama, H., Pouyssegur, J., Ogata, M., and Kurosaki, T. (2008) Erk Kinases Link Pre-B Cell Receptor Signaling to Transcriptional Events Required for Early B Cell Expansion. *Immunity* 28, 499–508.
- (85) Bomsel, M., and Mostov, K. E. (1993) Possible role of both the alpha and beta gamma subunits of the heterotrimeric G protein, Gs, in transcytosis of the polymeric immunoglobulin receptor. *J. Biol. Chem.* 268, 25824–35.
- (86) McIntire, W. E., MacCleery, G., and Garrison, J. C. (2001) The G protein beta subunit is a determinant in the coupling of Gs to the beta 1-adrenergic and A2a adenosine receptors. *J. Biol. Chem.* 276, 15801–9.
- (87) Wen, A. Y., Sakamoto, K. M., and Miller, L. S. (2010) The Role of the Transcription Factor CREB in Immune Function. *J. Immunol.* 185, 6413–6419.
- (88) Baló-Banga, J. M., Schweitzer, K., Lakatos, S., and Sipka, S. (2015) A novel rapid (20-minute) IL-6 release assay using blood mononuclear cells of patients with various clinical forms of drug induced skin injuries. *World Allergy Organ. J.* 8, 1.
- (89) McHugh, S. M., Wilson, A. B., Deighton, J., Lachmann, P. J., and Ewan, P. W. (1994) The profiles of interleukin (IL)-2, IL-6, and interferon-gamma production by peripheral blood mononuclear cells from house-dust-mite-allergic patients: a role for IL-6 in allergic disease. *Allergy* 49, 751–9.
- (90) Fan, J., Nishanian, P., Breen, E. C., McDonald, M., and Fahey, J. L. (1998) Cytokine gene expression in normal human lymphocytes in response to stimulation. *Clin. Diagn. Lab. Immunol.* 5, 335–40.
- (91) Breen, E. C., McDonald, M., Fan, J., Boscardin, J., and Fahey, J. L. (2000) Cytokine gene expression occurs more rapidly in stimulated peripheral blood mononuclear cells from human immunodeficiency virus-infected persons. *Clin. Diagn. Lab. Immunol.* 7, 769–73.
- (92) Wysk, M., Yang, D. D., Lu, H. T., Flavell, R. A., and Davis, R. J. (1999) Requirement of mitogen-activated protein kinase kinase 3 (MKK3) for tumor necrosis factor-induced cytokine expression. *Proc. Natl. Acad. Sci. U. S. A.* 96, 3763–8.

- (93) Mortaz, E., Lazar, Z., Koenderman, L., Kraneveld, A. D., Nijkamp, F. P., and Folkerts, G. (2009) Cigarette smoke attenuates the production of cytokines by human plasmacytoid dendritic cells and enhances the release of IL-8 in response to TLR-9 stimulation. *Respir. Res.* 10, 47.
- (94) Horie, K., Ohashi, M., Satoh, Y., and Sairenji, T. (2007) The role of p38 mitogen-activated protein kinase in regulating interleukin-10 gene expression in Burkitt's lymphoma cell lines. *Microbiol. Immunol.* 51, 149–161.
- (95) Pengal, R. A., Ganesan, L. P., Wei, G., Fang, H., Ostrowski, M. C., and Tridandapani, S. (2006) Lipopolysaccharide-induced production of interleukin-10 is promoted by the serine/threonine kinase Akt. *Mol. Immunol.* 43, 1557–1564.
- (96) Morrison, D. C., and Ulevitch, R. J. (1978) The effects of bacterial endotoxins on host mediation systems. A review. *Am. J. Pathol.* 93, 526–618.
- (97) Galve-Roperh, I., Sánchez, C., Cortés, M. L., Gómez del Pulgar, T., Izquierdo, M., and Guzmán, M. (2000) Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat. Med.* 6, 313–9.
- (98) Sánchez, C., de Ceballos, M. L., Gomez del Pulgar, T., Rueda, D., Corbacho, C., Velasco, G., Galve-Roperh, I., Huffman, J. W., Ramón y Cajal, S., and Guzmán, M. (2001) Inhibition of glioma growth in vivo by selective activation of the CB(2) cannabinoid receptor. *Cancer Res.* 61, 5784–9.
- (99) Cridge, B. J., and Rosengren, R. J. (2013) Critical appraisal of the potential use of cannabinoids in cancer management. *Cancer Manage. Res.* 5, 301–13.
- (100) Maccarrone, M., Lorenzon, T., Bari, M., Melino, G., and Finazzi-Agro, A. (2000) Anandamide induces apoptosis in human cells via vanilloid receptors. Evidence for a protective role of cannabinoid receptors. *J. Biol. Chem.* 275, 31938–31945.
- (101) Joss, A., Akdis, M., Faith, A., Blaser, K., and Akdis, C. A. (2000) IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway. *Eur. J. Immunol.* 30, 1683–90.
- (102) Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M., and O'Garra, A. (1991) IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* 147, 3815–22.
- (103) Corinti, S., Albanesi, C., la Sala, A., Pastore, S., and Girolomoni, G. (2001) Regulatory activity of autocrine IL-10 on dendritic cell functions. *J. Immunol.* 166, 4312–8.
- (104) Schett, G. (2018) Physiological effects of modulating the interleukin-6 axis. *Rheumatology* 57, ii43–ii50.
- (105) Janský, L., Reymanová, P., and Kopecký, J. (2003) Dynamics of cytokine production in human peripheral blood mononuclear cells stimulated by LPS or infected by *Borrelia*. *Physiol. Res.* 52, 593–8.
- (106) McGeachy, M. J., Bak-Jensen, K. S., Chen, Y., Tato, C. M., Blumenschein, W., McClanahan, T., and Cua, D. J. (2007) TGF- β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell-mediated pathology. *Nat. Immunol.* 8, 1390–1397.
- (107) Jin, J.-O., Han, X., and Yu, Q. (2013) Interleukin-6 induces the generation of IL-10-producing Tr1 cells and suppresses autoimmune tissue inflammation. *J. Autoimmun.* 40, 28–44.
- (108) Steensberg, A., Fischer, C. P., Keller, C., Møller, K., and Pedersen, B. K. (2003) IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *Am. J. Physiol. - Endocrinol. Metab.* 285, E433–E437.
- (109) Lee, S., Lee, T. A., Lee, E., Kang, S., Park, A., Kim, S. W., Park, H. J., Yoon, J. H., Ha, S. J., Park, T., Lee, J. S., Cheon, J. H., and Park, B. (2015) Identification of a subnuclear body involved in sequence-specific cytokine RNA processing. *Nat. Commun.* 6, 1–14.
- (110) Fiorentino, D. F., Bond, M. W., and Mosmann, T. R. (1989) Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170, 2081–95.
- (111) Mosmann, T. R., and Sad, S. (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today* 17, 138–46.
- (112) Schuerwegh, A. J., De Clerck, L. S., Bridts, C. H., and Stevens, W. J. (2003) Comparison of intracellular cytokine production with extracellular cytokine levels using two flow cytometric techniques. *Cytometry 55B*, 52–58.
- (113) Jang, S., Uematsu, S., Akira, S., and Salgame, P. (2004) IL-6 and IL-10 induction from dendritic cells in response to *Mycobacterium tuberculosis* is predominantly dependent on TLR2-mediated recognition. *J. Immunol.* 173, 3392–7.
- (114) Qu, Q.-X., Xie, F., Huang, Q., and Zhang, X.-G. (2018) Membranous and Cytoplasmic Expression of PD-L1 in Ovarian Cancer Cells. *Cell. Physiol. Biochem.* 43, 1893–1906.
- (115) Raduner, S., Majewska, A., Chen, J.-Z., Xie, X.-Q., Hamon, J., Faller, B., Altmann, K.-H., and Gertsch, J. (2006) Alkylamides from Echinacea are a new class of cannabinomimetics. Cannabinoid type 2 receptor-dependent and -independent immunomodulatory effects. *J. Biol. Chem.* 281, 14192–206.
- (116) Zhu, L. X., Sharma, S., Stolina, M., Gardner, B., Roth, M. D., Tashkin, D. P., and Dubinett, S. M. (2000) Delta-9-Tetrahydrocannabinol Inhibits Antitumor Immunity by a CB2 Receptor-Mediated, Cytokine-Dependent Pathway. *J. Immunol.* 165, 373–380.
- (117) Kozela, E., Juknat, A., Kaushansky, N., Rimmerman, N., Ben-Nun, A., and Vogel, Z. (2013) Cannabinoids Decrease the Th17 Inflammatory Autoimmune Phenotype. *J. Neuroimmune Pharmacol.* 8, 1265–1276.
- (118) Massi, P., Vaccani, A., and Parolaro, D. (2006) Cannabinoids, immune system and cytokine network. *Curr. Pharm. Des.* 12, 3135–46.
- (119) Eisenstein, T. K., and Meissler, J. J. (2015) Effects of Cannabinoids on T-cell Function and Resistance to Infection. *J. Neuroimmune Pharmacol.* 10, 204–16.
- (120) Pacifici, R., Zuccaro, P., Pichini, S., Roset, P. N., Poudevida, S., Farré, M., Segura, J., and De la Torre, R. (2003) Modulation of the immune system in cannabis users. *J. Am. Med. Assoc.* 289, 1929–31.
- (121) Bayazit, H., Selek, S., Karababa, I. F., Cicek, E., and Aksoy, N. (2017) Evaluation of oxidant/antioxidant status and cytokine levels in patients with cannabis use disorder. *Clin. Psychopharmacol. Neurosci.* 15, 237–242.
- (122) Katona, S., Kaminski, E., Sanders, H., and Zajicek, J. (2005) Cannabinoid influence on cytokine profile in multiple sclerosis. *Clin. Exp. Immunol.* 140, 580–5.
- (123) Cannaeert, A., Storme, J., Franz, F., Auwärter, V., and Stove, C. P. (2016) Detection and Activity Profiling of Synthetic Cannabinoids and Their Metabolites with a Newly Developed Bioassay. *Anal. Chem.* 88, 11476–11485.
- (124) Doi, T., Tagami, T., Takeda, A., Asada, A., and Sawabe, Y. (2018) Evaluation of carboxamide-type synthetic cannabinoids as CB1/CB2 receptor agonists: difference between the enantiomers. *Forensic Toxicol.* 36, 51–60.
- (125) Riley, S. B., Sochat, M., Moser, K., Lynch, K. L., Tochtrop, R., Isbell, T. S., and Scalzo, A. (2019) Case of brodifacoum-contaminated synthetic cannabinoid. *Clin. Toxicol.* 57, 143–144.
- (126) Cawston, E. E., Redmond, W. J., Breen, C. M., Grimsey, N. L., Connor, M., and Glass, M. (2013) Real-time characterization of cannabinoid receptor 1 (CB1) allosteric modulators reveals novel mechanism of action. *Br. J. Pharmacol.* 170, 893–907.
- (127) Lin, S., Khanolkar, A. D., Fan, P., Goutopoulos, A., Qin, C., Papahadjis, D., and Makriyannis, A. (1998) Novel analogues of arachidonylethanolamide (anandamide): Affinities for the CB1 and CB2 cannabinoid receptors and metabolic stability. *J. Med. Chem.* 41, 5353–5361.
- (128) Hulme, E. C., and Trevethick, M. A. (2010) Ligand binding assays at equilibrium: Validation and interpretation. *Br. J. Pharmacol.* 161, 1219–1237.
- (129) Motulsky, H. J., and Neubig, R. R. (2010) Analyzing binding data. *Curr. Protoc. Neurosci.* 52, 7.5.1.
- (130) O'Carroll, S. J., Kho, D. T., Wiltshire, R., Nelson, V., Rotimi, O., Johnson, R., Angel, C. E., and Graham, E. S. (2015) Pro-inflammatory TNF α and IL-1 β differentially regulate the inflamma-

tory phenotype of brain microvascular endothelial cells. *J. Neuroinflammation* 12, 1–18.