



Research article

Cannabidiol modulates classical and non-classical hla expression in human choriocarcinoma cell line[☆]

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ABSTRACT

Cannabidiol (CBD) modulates diverse signaling pathways with potential relevance to tumor immune escape; however, its impact on the regulation of classical and non-classical HLA class I molecules remains incompletely understood. Here, we examined the effects of CBD on HLA expression in JEG-3 choriocarcinoma cells, focusing on cannabinoid-related receptors and intracellular Ca²⁺ signaling. CBD increased the expression of classical HLA class I genes, most notably HLA-C, while reducing HLA-G levels, a non-classical HLA class I molecule associated with local immunosuppressive functions. Receptor profiling revealed constitutive expression of CB1 and CB2, whereas GPR55 and PPAR γ expression became detectable only after CBD exposure. Receptor inhibition assays showed that HLA-G downregulation was selectively attenuated by CB1 blockade, with no meaningful contribution from CB2 or GPR55. In contrast, CBD-induced HLA-C upregulation was significantly attenuated by GPR55 and CB2 inhibition, while remaining unaffected by CB1 blockade, suggesting distinct receptor-associated pathways for classical and non-classical HLA regulation. Calcium chelation using BAPTA further demonstrated that HLA-G modulation was highly sensitive to intracellular Ca²⁺ reduction, whereas classical HLA expression required higher BAPTA concentrations to be affected. Altogether, these findings support the possibility that CBD may promote coordinated immunomodulatory effects associated with differential regulation of classical and non-classical HLA molecules through receptor-associated and calcium-sensitive signaling pathways.

1. Introduction

The endocannabinoid system (ECS) plays a fundamental role in regulating multiple physiological processes, including metabolism, cell

survival, and immune responses [1–3]. It comprises endogenous ligands such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), their canonical receptors CB1 and CB2, and associated metabolic enzymes [4–7]. Dysregulation of the ECS has been implicated in various

Abbreviations: 2-AG 2, Arachidonoylglycerol; 5-HT1A, 5-Hydroxytryptamine; AEA, Anandamide; Ca²⁺, Calcium ion; CB1, Cannabinoid Receptor type 1; CB2, Cannabinoid Receptor type 2; CBD, Cannabidiol; CBRs, Cannabinoids Receptors; ECS, Endocannabinoid System; FAAH, Fatty Acid Amide Hydrolase; GABA, Gamma-Aminobutyric Acid; GPCR, G Protein Coupled Receptors; HLA, Human Leukocyte Antigen; HLAa, Classical Human Leukocyte Antigen class I; HLA Ib, Non classical Human Leukocyte Antigen class I; JEG-3, Human choriocarcinoma cell line; MHCIa, Classical Major Histocompatibility Complex class I; MHC Ib, Non classical Major Histocompatibility Complex class I; MTT, Methyl Thiazolyl Tetrazolium; NK, Natural Killer; PCR, Polymerase Chain Reaction; PD-1, Programmed Death-1; PDAC, Pancreatic Ductal Adenocarcinoma; PD-L1, Programmed Death-Ligand 1; PPAR, Peroxisome Proliferator-Activated Receptor; RCC7, Renal Cell Carcinoma cell line; RT-PCR, Reverse Transcription Polymerase Chain Reaction; THC, Δ 9-Tetrahydrocannabinol; TRPV, Transient Receptor Potential Vanilloid.

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pathological conditions, ranging from neurodegenerative and metabolic disorders to cancer. In addition, phytocannabinoids (the plant-derived cannabinoids from *Cannabis sativa*) exert their effects through interactions with CB1 and CB2 receptors, as well as multiple non-canonical targets. Among them, the two most abundant and therapeutically characterized compounds are Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD). CBD has very low affinity for CB1/CB2, although it displays pleiotropic activity by engaging alternative targets, including G protein-coupled receptors (GPR55, GPR18), TRP channels (TRPV1, TRPV4), serotonin 5-HT_{1A}, GABA receptors, and PPAR γ [8,9].

Cannabinoids have long been used for palliative purposes in oncology, particularly for their analgesic, antiemetic, and orexigenic effects [10–12]. However, increasing evidence indicates that they also exert direct antitumor effects in both *in vitro* and *in vivo* models. THC and CBD have been reported to inhibit proliferation, migration, and angiogenesis, as well as to induce apoptosis in several tumor types, including glioblastoma, breast, prostate, lung, and pancreatic cancers [13–17]. Nevertheless, the underlying mechanisms remain incompletely understood due to their pleiotropic signaling pathways and concentration-dependent effects. Furthermore, CBD offers notable therapeutic advantages. While the clinical use of THC is restricted by its psychotropic activity, CBD has no psychoactive effects and displays a more favorable safety profile, thereby supporting its potential as an immunomodulatory and therapeutically relevant compound in cancer biology. It should not be overlooked that the combined use of CBD and THC remains a relevant therapeutic possibility, as CBD can modulate THC-mediated signaling through its allosteric effects on CB1 and CB2 [18,19], allowing their contributions to be adjusted and potentially optimized for clinical applications.

Human Leukocyte Antigen (HLA) class I molecules, the human equivalent of MHC-I, are central mediators of immune recognition and play a pivotal role in shaping the balance between immune activation and tolerance. Classical HLA class I molecules (HLA-Ia: HLA-A, -B, -C), broadly expressed on all nucleated cells, are essential for presenting endogenous antigens to cytotoxic T lymphocytes, enabling recognition and elimination of infected or transformed cells. In contrast, non-classical subtypes (HLA-Ib: HLA-E, -F, -G, -H), display a more restricted tissue distribution and function. Among them, HLA-E and HLA-G are characterized by tolerogenic functions frequently exploited by viruses and tumors to evade the host immune responses [20]. HLA-G is predominantly expressed at the maternal–fetal interface and contribute to immune tolerance by interacting with inhibitory receptors on NK cells, T and B lymphocytes, and antigen-presenting cells [21,22]. However, its aberrant expression in tumors has been strongly associated with immune evasion and cancer progression [22–24].

Alterations in HLA expression are increasingly recognized as key determinants of tumor responsiveness to immunotherapies, particularly immune checkpoint inhibitors. Modulation of HLA molecules by tumor cells not only impairs antigen presentation but also promotes a tolerogenic microenvironment, thereby limiting the efficacy of cytotoxic T-cell responses. Recent evidence also suggests that calcium homeostasis may participate in the regulation of HLA expression in tumor cells. In the renal carcinoma cell line named RCC7, stable HLA-G expression was shown to induce an aberrant upregulation of calcium transporters [25]. Moreover, studies in pancreatic ductal adenocarcinoma (PDAC) indicate that dysregulated calcium influx promotes autophagy-mediated lysosomal degradation of MHC class I molecules [26,27]. Altogether, these observations raise the possibility that calcium-dependent pathways may influence classical and non-classical HLA molecules, although this remains largely unexplored. A better understanding of HLA regulation, including its pharmacological modulation, is therefore essential for developing strategies that enhance antitumor immunity and improve the effectiveness of current immunotherapeutic approaches.

Cannabinoids, through their diverse signaling pathways, have been shown to modulate several of the key molecules involved in tumor immunosurveillance. For instance, there is evidence that THC may

impair the efficacy of PD-L1/PD-1 immune checkpoints blockade by acting on CB2 receptors expressed on tumor-specific T cells [28]. In contrast, CBD has been associated with immunomodulatory effects that may favor antitumor immune responses, including the upregulation of classical HLA class I molecules [29] and modulation of PD-L1/PD-1-related pathways [30,31]. These findings suggest that distinct cannabinoids differentially regulate mechanisms associated with antigen presentation and immune responses, providing a strong rationale for investigating their role as potential immunomodulatory agents in cancer therapy.

Despite growing interest in the immunomodulatory properties of CBD, its potential to regulate classical and non-classical HLA expressions in tumor cells remains poorly understood. To address this gap, we used the JEG-3 human choriocarcinoma cell line, which constitutively expresses HLA-G in addition to the classical HLA class I molecules. This dual expression property provides a useful model to investigate cannabinoid–immune interactions in a single cellular context. In this framework, we examined both the modulation of HLA, classical and non-classical subtypes (focusing particularly on HLA-G) and the involvement of cannabinoid receptors and associated signaling pathways, aiming to elucidate the mechanisms by which CBD may influence tumor–immune dynamics.

2. Materials and methods

2.1. Drugs and chemicals

Cannabidiol (CBD, purity by HPLC: 99.8%), specific inhibitors of Cannabinoids Receptors (CBRs): LY320135 (CB1- selective antagonist), AM630 (CB2- selective antagonist) and CID16020046 (GPR55-specific inhibitor) and BAPTA-AM (cytoplasmic calcium chelator), were used.

The CBD was purchased from Cerilliant® (Texas, USA). It was initially dissolved in dimethyl sulfoxide (DMSO) to a concentration of 250 mM and stored at -20°C . CBD was further diluted with tissue culture medium for *in vitro* studies, keeping the DMSO concentration below 0.2%. LY320135 (CB1- selective antagonist) and AM630 (CB2-selective antagonist) and CID16020046 (GPR55- selective antagonist) were purchased from Tocris Bioscience (Bristol, UK). BAPTA-AM (cytoplasmic calcium ion chelator) was kindly provided by Dra. Eugenia Alzugaray (Universidad Nacional de La Plata, Argentina).

2.2. Cell culture

Human choriocarcinoma cell line (JEG-3) was used. It was generously provided by Instituto de Físicoquímica Biológica y Química, Universidad de Bioquímica y Farmacia (UBA-CONICET), Buenos Aires.

This cell line was cultured *in vitro* in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Gibco™) according to the manufacturer's protocols and 1% penicillin/streptomycin (Gibco™) in a 5% CO₂, humidified atmosphere at 37°C until a confluence state of 75%. Cells were regularly dissociated using Trypsine-EDTA 0.25% for further RNA extraction, cDNA synthesis, polymerase chain reaction (PCR) and reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis.

2.3. CBD treatments with specific receptor inhibitors

Cells were seeded in 6-multi-well plates at a density of 5×10^5 cells/well in complete culture medium and allowed to adhere for 24 h. Following adhesion, five independent assays were performed. Based on previously established viability assays in JEG-3 cells [32], 1 μM CBD was selected as the working non-cytotoxic concentration for all subsequent experiments.

Monomeric CBR inhibition assay. Cells were allocated into four experimental groups: (i) negative control (DMEM + 10% FBS + 0.4% DMSO), (ii) positive control (DMEM + 10% FBS + 0.4% DMSO + 1 μM

CBD), (iii) inhibitor treatment (DMEM + 10% FBS + 0.4% DMSO + CBR inhibitors), and (iv) inhibitor + CBD treatment (DMEM + 10% FBS + 0.4% DMSO + CBR inhibitors + 1 μ M CBD). LY320135, AM630, and CID16020046 were used at a final concentration of 5 μ M. Treatments were performed for 24 h, with medium replacement after 12 h.

Dimeric CBR inhibition assay. Cells were distributed into the same four groups as described above. Inhibitor treatments consisted of combinations targeting CB1/CB2, CB1/GPR55, or CB2/GPR55, each applied at a final concentration of 5 μ M. Treatments were carried out for 24 h, with medium replaced after 12 h of incubation.

After each experiment was completed, samples were collected in TRIzol for RNA extraction.

2.4. Cytoplasmic calcium chelation assay

To assess the potential involvement of intracellular calcium in the signaling pathway underlying CBD-mediated modulation of HLA expression, a cytoplasmic calcium chelation assay was performed. Cells were seeded in 6-multi-well plates at a density of 5×10^5 cells/well in complete culture medium and allowed to adhere for 24 h. Following adhesion, three independent assays were performed. The samples were assigned to four experimental conditions: (i) negative control (DMEM supplemented with 10% FBS and 0.4% DMSO); (ii) positive control (DMEM + 10% FBS + 0.4% DMSO + 1 μ M CBD); (iii) BAPTA-only treatments, comprising escalating BAPTA concentrations (0.1, 0.5, and 1 μ M); and (iv) BAPTA + CBD treatments, in which cells were pre-incubated for 1 h with BAPTA (0.1, 0.5, or 1 μ M) followed by the addition of 1 μ M CBD. All experimental conditions were maintained for 24 h, with medium replacement after 12 h.

2.5. RNA Extraction, cDNA Synthesis, and RT-PCR/ RT-qPCR analysis

For the analysis Cannabinoids Receptors (CBRs), HLA Ia and HLA-G expression in tumor cells cultured under different experimental conditions, reverse transcription-polymerase chain reaction (RT-PCR) was performed using specific primers. RNA extraction from JEG-3 cells, was performed with TRIzol Reagent (Invitrogen). For cDNA synthesis, 500–1000 ng of the total RNA was *retro*-transcribed with MMLV reverse transcriptase (Promega), according to manufacturer's instructions.

For the qualitative analysis of gene expression, PCR was performed using the DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Waltham, MA, USA, cat. no. K1081). Amplicons were subsequently separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

For the quantitative analysis of gene expression, RT-qPCR was performed on cDNA samples. The samples were diluted fivefold, and it was performed with StepOne Plus Real Time PCR System (Applied Biosystems). The FastStart Universal SYBR Green Master Mix (Roche) was used for all reactions. Primers efficiency and initial molecule (N_0) values were determined by LinReg software 3.0, and gene expression was normalized to RPL7 housekeeping gene, for each condition. All the oligonucleotide sequences are listed in Table 1.

2.6. Statistics

Significant differences were determined using Graph Pad Prism 8 (USA). Statistical significance was calculated using t-tests and ANOVA. Tukey-Kramer post hoc analyses were conducted when appropriate. The significance was set at $p < 0.05$. Data were expressed as mean $\pm$ standard error of mean (SEM).

3. RESULTS

3.1. Expression profile of cannabinoid-related receptors in JEG-3 cells

To investigate potential signaling pathways regulating HLA

Table 1

Oligonucleotide sequences of *hla classic*, *hla-g*, *CBRs*, *ppar γ* and *trpv1* gene and *rpl7* reference gene.

Gene	primers	5' – Oligo Seq – 3'
<i>hla-a</i>	Fw	CCTGCCCTTCCCTTGTGAC
	Rv	AAATTTTAGGAATCTTCTCC
<i>hla-b</i>	Fw	TCCTAGCAGTTGTGGTCATC
	Rv	AGGGTACACAGTGGACACAA
<i>hla-c</i>	Fw	GGCTGTCTGTTGTCTCTAG
	Rv	AGGGGTACAGGTGGACACGG
<i>hla-g</i>	Fw	AATGGCGAGGATGGCAAG
	Rv	TGACGAAGCGAAGAAGC
<i>cb1</i>	Fw	TGTGCAGATGAAGGCTCAGG
	Rv	GAGCATTGGTACTGCTCTGGT
<i>cb2</i>	Fw	ACACCTATGGGCATGTTCTC
	Rv	CAGCACTGGGAACCAACAGA
<i>gpr55</i>	Fw	AACCTTCATCGGCTCCTCTG
	Rv	AAGCACACGAAGTCTCTGG
<i>pparγ</i>	Fw	AGTGGGGATGTCTCATAATG
	Rv	CAAGGAGCGGAGGTCCGCA
<i>trpv1</i>	Fw	GGACCAACATGCTCTACTAC
	Rv	ACTCCACAGGCAGTGAGTTA
<i>rpl7</i>	Fw	GGAAGAGGAGACACGGAACA
	Rv	CCTTTGTTCAGCCACATTGG

expression in the JEG-3 choriocarcinoma cell line, we first examined the expression profile of cannabinoid-related receptors. PCR analysis showed that JEG-3 cells constitutively expressed CB1 and CB2 (Fig. 1A). In contrast, TRPV1 expression was not detected under basal conditions, whereas GPR55 showed only a faint amplification signal in untreated cells (Fig. 1A). PPAR γ expression was also undetectable under basal conditions (Fig. 1B). Primer specificity was confirmed using fibroblasts, which showed clear amplification of TRPV1 and GPR55.

We next assessed whether exposure to 1 μ M CBD modified receptor expression. Under these conditions, CB1 and CB2 expression patterns remained comparable to untreated cells, whereas GPR55 amplification became markedly more evident following CBD exposure (Fig. 1A). In addition, PPAR γ expression became detectable after CBD treatment (Fig. 1B). These observations guided the design of the subsequent cannabinoid receptor inhibition assays.

3.2. CBD upregulates the expression of classical HLA class I molecules in JEG-3 cells

RT-PCR analysis of HLA-A, HLA-B, and HLA-C in JEG-3 cells revealed that treatment with 1 μ M CBD increased the intensity of all classical HLA class I amplification bands, with the most evident changes observed for HLA-A and HLA-C (Fig. 2A). Consistently, RT-qPCR analysis demonstrated a significant upregulation ($p < 0.001$) of all classical HLA loci following CBD exposure, although the magnitude of induction differed among isotypes. Relative to untreated controls, CBD treatment induced approximately 2.4-fold, 1.5-fold, and 3.4-fold increases in HLA-A (2.61 ± 0.08 vs. 1.08 ± 0.09), HLA-B (1.67 ± 0.08 vs. 0.99 ± 0.09), and HLA-C (3.47 ± 0.08 vs. 1.04 ± 0.07) expression, respectively (Fig. 2B).

3.3. Cbd-induced HLA-C upregulation involves GPR55 and CB2, but not CB1 signaling

To characterize the cannabinoid receptors (CBRs) involved in CBD-mediated regulation of classical HLA molecules, JEG-3 cells were exposed to selective CBR inhibitors in the presence of 1 μ M CBD, focusing on HLA-C—the locus exhibiting the highest CBD-induced upregulation. Pharmacological inhibition of CB1 did not attenuate the CBD-induced increase in HLA-C expression, and band intensity remained comparable to that observed with CBD alone. In contrast, inhibition of either CB2 or GPR55 markedly reduced HLA-C expression toward basal levels (Fig. 3C).

RT-qPCR analysis allowed quantitative evaluation of the expression

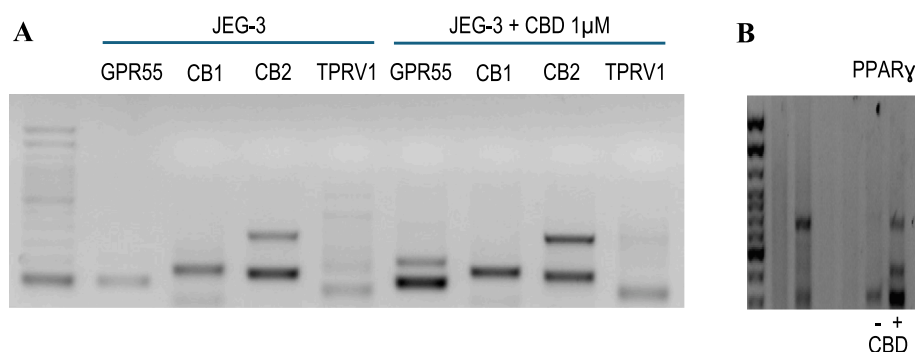


Fig. 1. PCR analysis of cannabinoid-related receptor expression in JEG-3 cells. (A) Agarose gel showing RT-PCR amplification of GPR55, CB1, CB2, and TRPV1 transcripts. Lane 1: 100 bp ladder; lanes 2–5: untreated control cells; lanes 6–9: cells treated with 1 μM CBD. Constitutive expression of CB1 and CB2 were detected under both conditions, whereas GPR55 amplification became more evident following CBD treatment. TRPV1 amplification was not detected in JEG-3 cells under the conditions analyzed. Additional faint bands observed for CB2 and GPR55 may correspond to nonspecific amplification products frequently observed in endpoint RT-PCR assays. (B) RT-PCR amplification of PPAR γ transcripts in untreated and CBD-treated JEG-3 cells. PPAR γ expression became detectable following treatment with 1 μM CBD. Faint bands shown on the left correspond to additional biological replicates exhibiting the same relative expression pattern. Five independent biological samples were analyzed for all experimental conditions (n = 5).

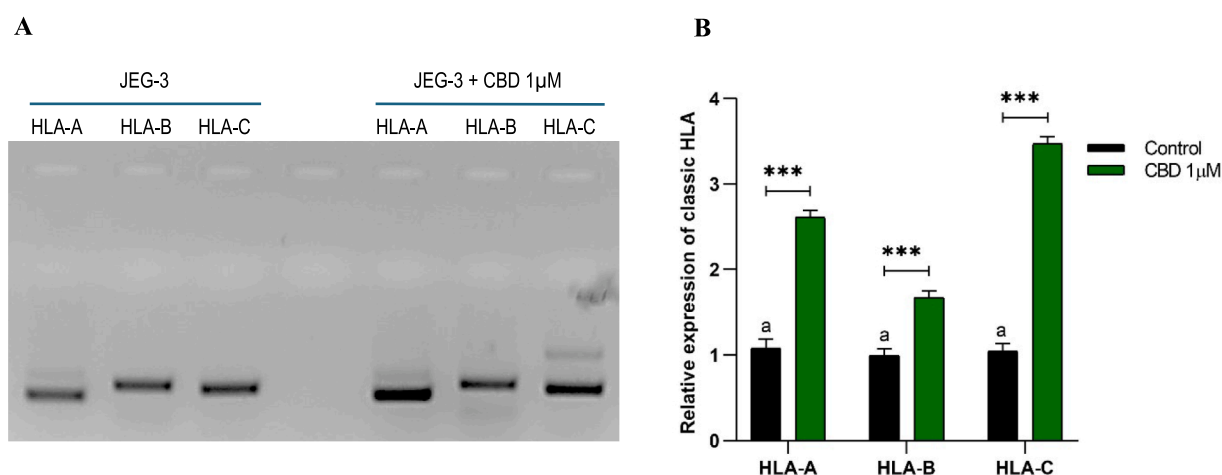


Fig. 2. Classical HLA class I expression in CBD-treated JEG-3 cells. (A) Agarose gel showing RT-PCR amplification of HLA-A, HLA-B, and HLA-C transcripts in untreated control cells (left) and cells treated with 1 μM CBD (right). Three independent biological samples were analyzed for each experimental condition. (B) Relative expression levels of classical HLA class I genes in control (black bars) and CBD-treated (green bars) cells. Bars represent mean $\pm$ SEM from three independent experiments (n = 3). ***p < 0.001. Groups sharing the letter “a” were not significantly different.

changes observed in the endpoint RT-PCR assays: HLA-C expression remained significantly elevated ($p < 0.001$) in cells treated with CBD alone (2.9 ± 0.1) or CBD plus CB1 inhibitor (2.7 ± 0.3), whereas co-treatment with CB2 (1.5 ± 0.3) or GPR55 (1.3 ± 0.2) inhibitors restored expression to levels not significantly different ($p > 0.05$) from untreated control cells (1.1 ± 0.1) (Fig. 3C).

The potential contribution of CBR dimers to CBD-induced HLA-C upregulation was further assessed by RT-qPCR. A significant increase ($p < 0.001$) in HLA-C expression was observed in cells treated with CBD alone (3.0 ± 0.1) or co-treated with CBD plus CB1/CB2 inhibitors (3.0 ± 0.2). In contrast, co-treatment with CBD and CB1/GPR55 (1.2 ± 0.1) or CB2/GPR55 (1.4 ± 0.1) inhibitors restored expression to values not significantly different ($p > 0.05$) from baseline levels (1.4 ± 0.1) (Fig. 3D).

3.4. Classical HLA (HLA-C) expression involves calcium signaling

Finally, the potential contribution of intracellular calcium signaling to HLA-C regulation was examined. RT-PCR analysis showed that HLA-C band intensity remained elevated in cells co-treated with CBD and low BAPTA concentrations, with only a modest reduction observed at the

highest BAPTA dose (Fig. 3A).

Consistently, RT-qPCR analysis demonstrated that co-treatment with CBD and low BAPTA concentrations (0.1 μM: 3.8 ± 0.6 ; 0.5 μM: 3.4 ± 0.5) did not significantly differ ($p > 0.05$) from CBD treatment alone (4.9 ± 0.8). In contrast, co-treatment with 1 μM BAPTA significantly reduced ($p < 0.05$) the CBD-induced HLA-C upregulation (2.3 ± 0.3), restoring expression levels close to baseline values.

Treatment with BAPTA alone at all tested concentrations (0.1 μM: 2.4 ± 0.2 ; 0.5 μM: 2.3 ± 0.4 ; 1.0 μM: 2.4 ± 0.1) did not significantly modify HLA-C expression relative to basal levels (1.4 ± 0.2). Nevertheless, HLA-C expression remained significantly elevated ($p < 0.001$) in CBD-treated cells and in those exposed to low BAPTA concentrations compared with untreated controls (Fig. 3B).

3.5. CB1 receptor inhibition partially restores HLA-G expression

We recently reported that treatment with 1 μM CBD strongly suppresses HLA-G expression in JEG-3 cells [32]. This inhibitory effect clearly contrasts with the CBD-induced upregulation observed for the classical HLA class I molecules described above.

RT-PCR analysis of JEG-3 cells co-treated with 1 μM CBD and 5 μM

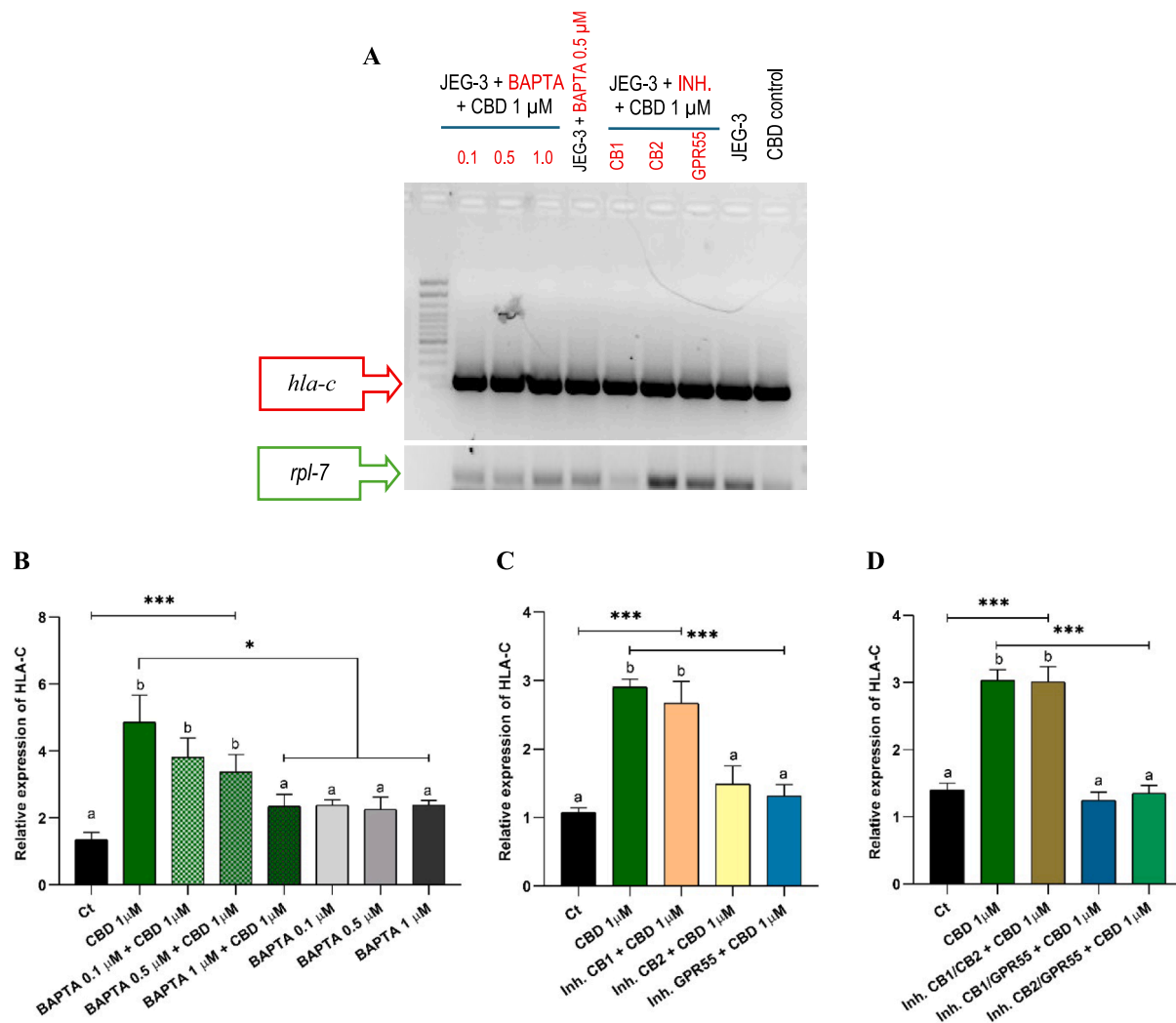


Fig. 3. Effects of calcium chelation and cannabinoid receptor inhibition on HLA-C expression in JEG-3 cells. (A) Agarose gel showing RT-PCR amplification of HLA-C and RPL7 transcripts under different experimental conditions. Lanes 1–4 correspond to cells co-treated with 1 μ M CBD and increasing concentrations of BAPTA (0.1, 0.5, and 1 μ M), together with cells treated with BAPTA alone (0.5 μ M). Lanes 5–7 correspond to cells co-treated with 1 μ M CBD and selective inhibitors of CB1, CB2, or GPR55 (5 μ M). Lane 8 represents basal HLA-C expression in untreated cells, and lane 9 corresponds to cells treated with 1 μ M CBD alone. Three independent biological samples were analyzed for each condition. (B) Relative HLA-C expression in cells treated with CBD (1 μ M), CBD combined with increasing concentrations of BAPTA (0.1, 0.5, and 1 μ M), or BAPTA alone ($n = 3$). (C) Relative HLA-C expression in untreated control cells, CBD-treated cells (1 μ M), and cells co-treated with CBD (1 μ M) and selective inhibitors of CB1, CB2, or GPR55 (5 μ M). (D) Relative HLA-C expression in untreated control cells, CBD-treated cells (1 μ M), and cells co-treated with CBD (1 μ M) and combinations of receptor inhibitors (CB1/CB2, CB1/GPR55, and CB2/GPR55; 5 μ M each). In panels C and D, bars represent mean $\pm$ SEM from five independent experiments ($n = 5$). * $p < 0.05$ and *** $p < 0.001$. Different letters indicate statistically significant differences between groups.

cannabinoid receptor inhibitors showed that inhibition of CB2 or GPR55 did not modify the suppressive effect of CBD, whereas CB1 inhibition partially restored HLA-G expression (Fig. 4A). These findings were corroborated by RT-qPCR analysis (Fig. 4C). No significant differences ($p > 0.05$) were observed between CBD-treated cells alone (0.11 ± 0.03) and co-treatment with CBD plus CB2 (0.08 ± 0.04) or GPR55 (0.10 ± 0.02) inhibitors. In contrast, co-treatment with a CB1 inhibitor significantly increased HLA-G expression (0.42 ± 0.03 ; $p < 0.001$) relative to CBD alone, restoring expression to approximately 40% of basal levels (1.06 ± 0.14).

Given the potential for functional interactions among cannabinoid-related receptors, we next evaluated the effects of combined receptor inhibition in CBD-treated JEG-3 cells. Simultaneous inhibition of CB1/CB2 or CB1/GPR55 significantly increased HLA-G expression relative to CBD treatment alone ($p < 0.001$), whereas co-inhibition of CB2/GPR55 did not significantly alter expression (Fig. 4B). Consistently, RT-qPCR analysis (Fig. 4D) showed that co-treatment with CBD and inhibitors

of CB1/CB2 (0.21 ± 0.04) or CB1/GPR55 (0.27 ± 0.04) significantly increased HLA-G expression compared with CBD alone (0.11 ± 0.01 ; $p < 0.001$), while co-treatment with CB2/GPR55 inhibitors (0.09 ± 0.01) produced no significant changes ($p > 0.05$).

Fig. 4E summarizes the comparative effects of CB1 inhibition alone or in combination with CB2 or GPR55 blockade. Although all CB1-containing inhibition conditions significantly increased HLA-G expression relative to CBD treatment alone, the magnitude of restoration remained partial compared with untreated control cells.

3.6. HLA-G downregulation is strongly associated with intracellular calcium signaling

In JEG-3 cells co-treated with CBD and the intracellular calcium chelator BAPTA, RT-PCR analysis showed a progressive restoration of HLA-G band intensity as BAPTA concentrations increased. In contrast, treatment with BAPTA alone produced no detectable changes in HLA-G

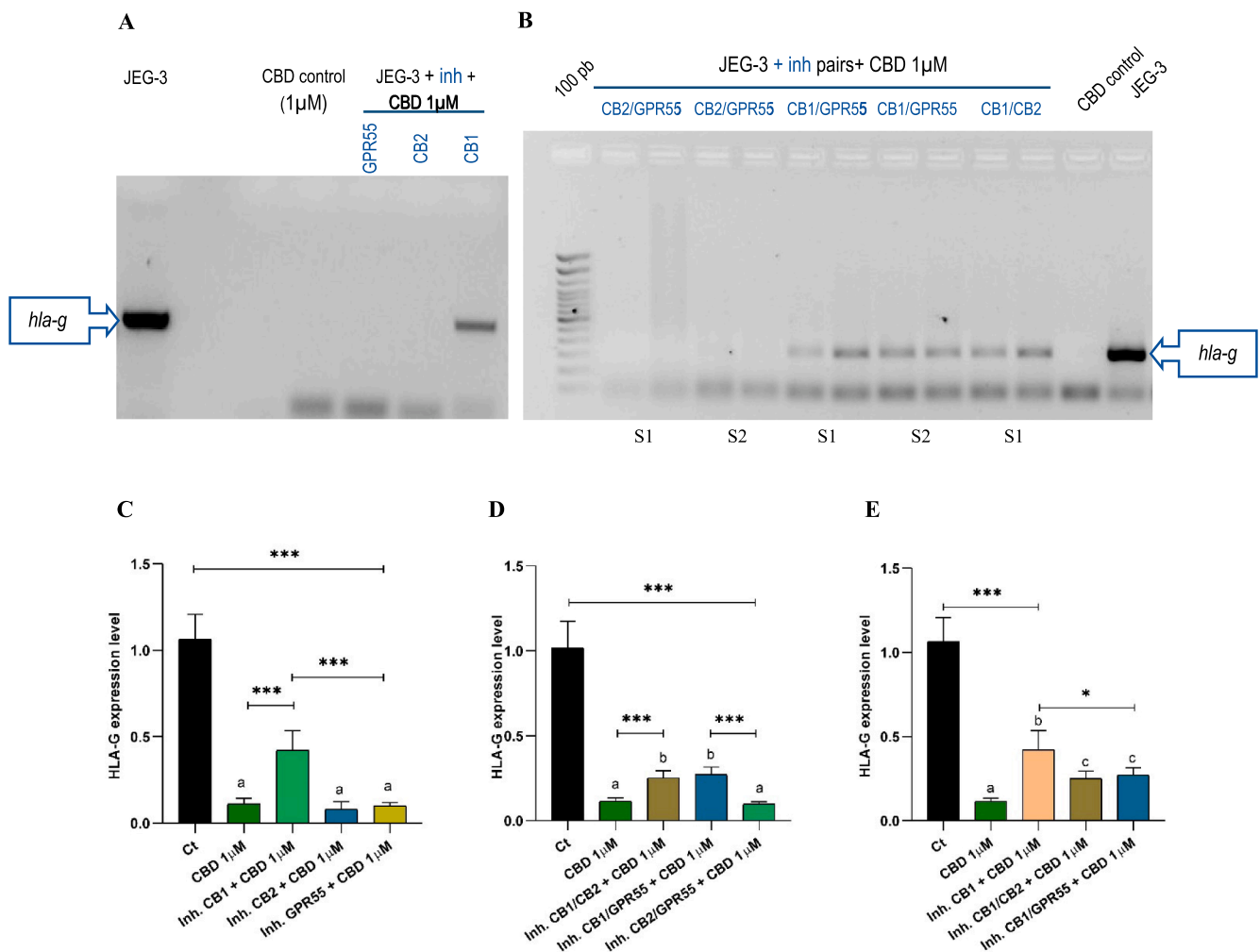


Fig. 4. Effects of single or combined cannabinoid receptor inhibition on HLA-G expression in JEG-3 cells. (A) Agarose gel showing RT-PCR amplification of HLA-G transcripts following single-receptor inhibition assays. Lane 1: untreated control cells; lane 2: cells treated with 1 μ M CBD; lanes 3–5: cells co-treated with 1 μ M CBD and selective inhibitors of CB1, CB2, or GPR55 (5 μ M), respectively. (B) Agarose gel showing RT-PCR amplification of HLA-G transcripts following combined receptor inhibition assays. Lane 1: 100 bp ladder; lanes 2–5: cells co-treated with 1 μ M CBD and CB2/GPR55 inhibitors (samples S1 and S2); lanes 6–9: cells co-treated with CBD and CB1/GPR55 inhibitors (samples S1 and S2); lanes 10–11: cells co-treated with CBD and CB1/CB2 inhibitors (sample S1); lane 12: CBD-treated control (C +); lane 13: untreated control (C -). (C) Relative HLA-G expression in untreated control cells, CBD-treated cells (1 μ M), and cells co-treated with CBD and single receptor inhibitors (CB1, CB2, or GPR55; 5 μ M). (D) Relative HLA-G expression in untreated control cells, CBD-treated cells (1 μ M), and cells co-treated with CBD and combinations of receptor inhibitors (CB1/CB2, CB1/GPR55, or CB2/GPR55; 5 μ M each). (E) Comparative analysis of HLA-G expression in untreated control cells, CBD-treated cells, cells co-treated with CBD and the CB1 inhibitor alone, and cells co-treated with CBD and inhibitor combinations involving CB1 (CB1/CB2 and CB1/GPR55). Bars represent mean $\pm$ SEM from five independent experiments (n = 5). *p < 0.05 and ***p < 0.001. Different letters indicate statistically significant differences between groups.

expression (Fig. 5A).

RT-qPCR analysis revealed a dose-dependent expression profile consistent with the endpoint RT-PCR results. Co-treatment with 1 μ M CBD and increasing concentrations of BAPTA resulted in a dose-dependent restoration of HLA-G expression ($p < 0.001$), reaching values of 0.34 ± 0.04 at 0.1 μ M, 0.46 ± 0.03 at 0.5 μ M, and 0.85 ± 0.03 at 1 μ M BAPTA, approaching basal expression levels. In contrast, treatment with BAPTA alone at the same concentrations (0.1 μ M: 0.90 ± 0.04 ; 0.5 μ M: 0.85 ± 0.03 ; 1.0 μ M: 0.83 ± 0.10) did not significantly differ ($p > 0.05$) from basal HLA-G expression levels (1.11 ± 0.20) (Fig. 5B).

4. Discussion

Tumor immune evasion represents a major challenge for cancer therapy. A comprehensive understanding of the tumor microenvironment and the adaptive mechanisms that enable cancer cells to avoid

immune recognition is essential for the design of new and more effective therapeutic strategies. One of the major pathways underlying this process involves dysregulation of HLA expression, which is responsible for antigen presentation in all nucleated cells and represents a key determinant of the immune system's ability to detect—or overlook—tumor cells. Classical HLA class I molecules (HLA-A, -B, -C) are required for cytotoxic T cell-mediated immune surveillance, whereas non-classical molecules such as HLA-G contribute to immune tolerance and promote tumor escape [33]. Different studies have described that dysregulation of MHC-I expression is associated with tumor progression [34,35]. Mutations or reduced expression levels of HLA molecules correlate with the development of laryngeal carcinoma and the metastatic spread of melanoma [36,37], underscoring the central role of HLA profiles in shaping antitumor immunity.

Although the antitumor properties of cannabinoids have been widely explored, their effects on immune regulation remain incompletely characterized and sometimes contradictory, depending on receptor

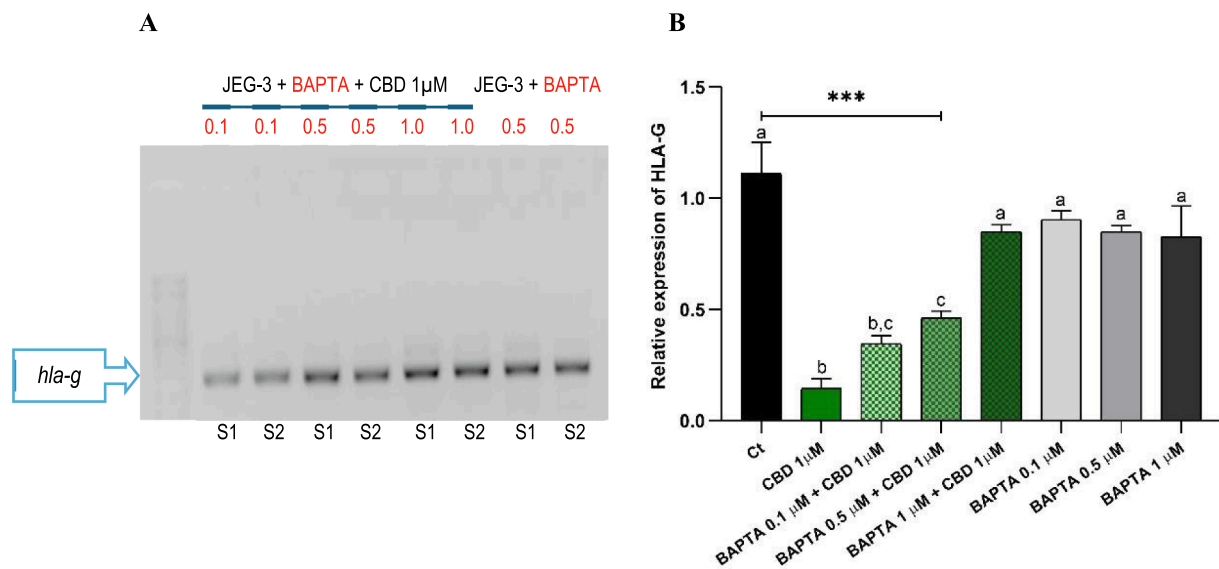


Fig. 5. Effects of calcium chelation on CBD-mediated HLA-G modulation in JEG-3 cells. (A) Agarose gel showing RT-PCR amplification of HLA-G transcripts following calcium chelation assays. Lane 1: 100 bp ladder; lanes 2–4: JEG-3 cells co-treated with 1 μM CBD and increasing concentrations of BAPTA (0.1, 0.5, and 1 μM); lane 5: cells treated with 0.5 μM BAPTA alone. Lanes 6–9 correspond to amplification of the reference gene RPL7 under the same experimental conditions. (B) Relative HLA-G expression levels in untreated control cells (Ct), cells co-treated with 1 μM CBD and increasing concentrations of BAPTA (0.1, 0.5, and 1 μM), and cells treated with 0.5 μM BAPTA alone. Bars represent mean ± SEM from three independent experiments (n = 3). ***p < 0.001. Different letters indicate statistically significant differences between groups.

subtype, tumor context, and ligand specificity [38]. Within this framework, cannabidiol (CBD) has gained considerable attention because of its ability to modulate both tumor cell behavior and immune-regulatory pathways [28–31], with the advantage that—unlike THC—it does not exert psychotropic effects. CBD has been shown to enhance immune-mediated tumor control by upregulating classical HLA molecules in metastatic cancer [29] and reducing PD-1 expression in colon carcinoma [31]. Our previous study provided the first evidence that CBD can downregulate the non-classical HLA-G molecule in JEG-3 choriocarcinoma cells [32], revealing a previously unrecognized connection between cannabinoid signaling and a key immune-evasive pathway. In the present work, we expand these findings by incorporating transcriptional analyses of classical HLA class I genes together with receptor-inhibition assays and calcium-modulation experiments, allowing us to explore the signaling pathways potentially involved in the coordinated regulation of classical and non-classical HLA molecules by CBD.

When analyzing the cannabinoid-related receptor profile in JEG-3 cells, several relevant observations emerged. CB1 and CB2 were constitutively expressed under basal conditions and remained detectable after CBD exposure, whereas GPR55 and PPARγ expression became markedly more evident following stimulation with 1 μM CBD. Although CBD has been reported to interact with CB receptors predominantly at higher micromolar concentrations in heterologous systems [10], our experimental model consistently showed significant biological responses at 1 μM, including modulation of HLA expression and reproducible pharmacological inhibition patterns. These findings suggest that, in JEG-3 cells, low-dose CBD is sufficient to engage signaling networks associated with cannabinoid-responsive pathways, likely reflecting the context-dependent pharmacology of CBD in native cellular systems. At the same time, the induction of GPR55 and PPARγ supports the notion that CBD-mediated signaling in trophoblastic tumor cells extends beyond canonical cannabinoid receptors and may involve multiple interconnected regulatory pathways [9,39–41].

Our receptor inhibition assays revealed a clear divergence between the mechanisms associated with HLA-G downregulation and those linked to the induction of classical HLA molecules. CBD-mediated suppression of HLA-G was significantly attenuated by CB1 blockade,

whereas independent inhibition of CB2 or GPR55 produced little or no effect. These findings support a predominant involvement of CB1-associated signaling in the regulation of HLA-G expression in JEG-3 cells. Nevertheless, considering the weak direct agonistic activity of CBD on CB1 [18,19], it is unlikely that this response results exclusively from classical orthosteric receptor activation. CBD is known to inhibit FAAH activity and thereby increase endogenous anandamide availability, which could indirectly potentiate CB1 signaling [42,43]. Additional indirect or context-dependent mechanisms cannot be excluded and are likely to contribute to the overall regulatory response observed in this model.

In contrast, the receptor profile associated with classical HLA upregulation was markedly different. HLA-C—selected as the representative classical HLA because it exhibited the strongest induction—was highly sensitive to GPR55 and CB2 inhibition, whereas CB1 blockade alone failed to significantly modify the CBD response. Moreover, combined inhibition experiments suggested that these receptors do not operate through a simple linear signaling cascade. GPR55 inhibition consistently abolished the CBD-induced increase in HLA-C expression, either alone or in combination with CB1 or CB2 antagonists, while CB2 blockade also attenuated the response under selected conditions. Together, these findings suggest the participation of a coordinated signaling network involving GPR55 and CB2 rather than an isolated receptor-specific mechanism.

Although the precise molecular interactions underlying this response remain unresolved, several studies have demonstrated that cannabinoid-related GPCRs can undergo functional cross-talk and heteromerization depending on receptor density, ligand availability, and cellular context [44,45]. Within this framework, it is plausible that receptor interactions involving GPR55 and CB2 may alter downstream signaling dynamics in JEG-3 cells [42,46], potentially favoring calcium-associated pathways under CBD exposure. However, we consider this interpretation to be mechanistic speculation supported indirectly by the pharmacological profile observed in our experiments rather than definitive evidence of receptor heteromerization.

The marked sensitivity of both HLA-G and HLA-C modulation to intracellular calcium chelation further supports the participation of

calcium-dependent signaling pathways in the immunomodulatory effects of CBD. Interestingly, the magnitude and sensitivity of this response differed substantially between classical and non-classical HLA molecules. HLA-G expression was strongly affected even at the lowest BAPTA concentration tested, whereas significant attenuation of HLA-C upregulation required the highest calcium-chelating condition. These observations suggest that intracellular Ca^{2+} may participate differently in each regulatory pathway, potentially reflecting distinct downstream signaling architectures.

Although CB1 and CB2 are classically associated with Gi/o-mediated inhibition of adenylate cyclase rather than calcium mobilization, cannabinoid signaling is increasingly recognized as highly context-dependent and capable of engaging non-canonical pathways through receptor cross-talk, PLC/IP3-associated signaling, and indirect modulation of intracellular calcium homeostasis [8,39,41]. In this regard, the involvement of GPR55—which is strongly linked to calcium mobilization—together with the pronounced effects observed after BAPTA treatment, provided a strong rationale for exploring calcium signaling in this model. Importantly, the existence of calcium-sensitive adenylate cyclase isoforms [47,48] raises the possibility that intracellular Ca^{2+} fluctuations may indirectly modulate cAMP-dependent pathways associated with CB1 signaling, potentially providing a mechanistic link between calcium dynamics, cAMP-associated signaling, and HLA-G regulation.

The coordinated modulation of classical HLA genes observed after CBD treatment raises the possibility that broader upstream transcriptional regulatory programs may be involved rather than isolated locus-specific mechanisms. In trophoblastic cells, HLA-C expression is known to be controlled by specialized regulatory networks distinct from canonical MHC-I pathways, including the recently described ELF3–NLRP2/NLRP7 axis [49]. Trophoblastic cells are classically characterized by restricted expression of certain classical HLA-I molecules, particularly HLA-A and HLA-B; however, basal expression of all three classical HLA loci was consistently detected in our JEG-3 cultures. Importantly, CBD treatment promoted coordinated upregulation of HLA-A, HLA-B, and HLA-C, supporting the possibility that the observed response may involve shared transcriptional regulatory mechanisms rather than isolated locus-specific modulation. While the molecular intermediates linking calcium-sensitive signaling pathways to these transcriptional programs remain unclear, our findings are consistent with the possibility that CBD-induced signaling convergence may influence broader regulatory networks governing HLA expression in JEG-3 cells. Our findings also reinforce the concept that CBD signaling in tumor cells is unlikely to depend on a single receptor or linear intracellular pathway. The increased expression of PPAR γ after CBD treatment [9], together with the known involvement of transient receptor potential (TRP) channels [41] and additional cannabinoid-responsive pathways described in other tumor models [13–17,39], suggests that multiple signaling systems may converge to shape the final immunomodulatory phenotype induced by CBD. In this context, the CB/GPR55 axis identified in our experiments should be interpreted as a relevant component of a broader and potentially highly integrated signaling network.

From an immunological perspective, the simultaneous downregulation of HLA-G and upregulation of classical HLA molecules may have important implications for tumor immune recognition. Although HLA-C can interact with inhibitory KIR receptors expressed by NK cells [35], it also plays a critical role in antigen presentation to cytotoxic T lymphocytes [50]. Therefore, the overall immunological impact of CBD treatment would likely depend on the balance between inhibitory NK signaling and enhanced adaptive immune recognition. In parallel, the reduction of HLA-G expression may contribute to weakening local immune tolerance mechanisms frequently exploited by trophoblastic and other tumor cells [23]. Together, these coordinated effects suggest that CBD could favor a shift toward a more immunologically visible tumor phenotype. In addition, although HLA-E was not directly evaluated in the present study, its constitutive expression in JEG-3 cells and its

dependence on leader-sequence peptides derived from classical HLA class I molecules raise the possibility that CBD-induced modulation of HLA-A, –B, and –C expression could indirectly influence HLA-E stabilization and associated immunoregulatory signaling.

We would like to highlight some limitations of the present study that we believe are worth addressing in future research. First, HLA modulation was evaluated at the transcriptional level, and additional protein-expression analyses will be necessary to confirm the functional impact of these findings. Likewise, although our pharmacological approach allowed us to identify receptor-associated signaling patterns, the use of additional agonists, broader inhibitor panels, and molecular interference strategies will be required to precisely define the signaling hierarchy involved. Finally, other potentially relevant mediators and immunoregulatory components—including PPAR γ , TRP channels, additional cannabinoid-responsive receptors such as GPR18, and the possible involvement of HLA-E-associated signaling pathways—remain to be explored in trophoblastic tumor cells.

Importantly, the trophoblastic origin of JEG-3 cells should also be considered when interpreting these findings. Unlike many somatic tumor models, trophoblastic cells exhibit highly specialized immunoregulatory programs associated with maternal–fetal tolerance [20,23], including distinct mechanisms controlling HLA-C expression and the constitutive expression of non-classical HLA molecules such as HLA-G and HLA-E [49,51]. Therefore, the downstream regulatory pathways engaged by CBD in this cellular context may differ from those operating in other tumor types. In this regard, the coordinated modulation of classical and non-classical HLA molecules observed here likely reflects a context-dependent immunoregulatory response shaped by the unique transcriptional landscape of trophoblastic cells [50]. Further studies in additional tumor models will be necessary to determine whether similar CBD-mediated immunomodulatory patterns are conserved in non-trophoblastic malignancies.

Overall, our findings position CBD as a multifaceted immunomodulatory compound capable of simultaneously reducing tumor immune escape through CB1-associated suppression of HLA-G while enhancing the expression of classical HLA molecules through pathways involving GPR55, CB2, and calcium-sensitive signaling mechanisms. Beyond its previously described antiproliferative and antitumoral properties, these observations broaden the potential relevance of CBD in tumor immunobiology and support further investigation into its possible integration with immunotherapeutic strategies.

CRediT authorship contribution statement

Kevin I. Martínez: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **María B. Palma:** Writing – review & editing, Methodology, Formal analysis. **Fernando J. Sepúlveda:** Writing – review & editing, Methodology. **Edgardo D. Carosella:** Writing – review & editing, Supervision. **Marcela N. García:** . **Fernando L. Riccillo:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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