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Autocrine and Paracrine Regulation of Lymphocyte CB2 Receptor Expression by TGF-β

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The marijuana-derived cannabinoid Δ^9-tetrahydrocannabinol (THC) has been shown to be immunosuppressive. We report that THC induces the immunosuppressive cytokine TGF-β by human peripheral blood lymphocytes (PBL). The ability of THC to stimulate TGF-β production was blocked by the CB2 receptor specific antagonist SR144528 but not by the CB1 specific antagonist AM251. Furthermore, our data suggest that TGF-β actively regulates lymphocyte CB2 receptor expression in an autocrine and paracrine manner. Whereas the addition of recombinant TGF-β to PBL cultures downregulated CB2 receptor expression, anti-TGF-β antibody treatment increased CB2 receptor expression. We conclude that one mechanism by which THC contributes to immune suppression is by stimulating an enhanced production of lymphocyte TGF-β.

Key Words: **T lymphocytes; G proteins; cytokines.**

Marijuana smoke delivers milligram quantities of cannabinoids, including the psychoactive cannabinoid THC to the lung (1). In addition to its psychoactive effects, THC is an immune modulator. Both *in vivo* and *in vitro* studies have shown that THC has immunosuppressive effects on macrophages (2–5), natural killer cells and T lymphocytes (6, 7). Two specific cannabinoid receptors have been identified; the CB1 receptor is primarily expressed in the nervous system while the CB2 receptor is primarily expressed in the immune system (8, 9). The distribution of these receptors suggests that it may be possible to separate the immunomodulatory effects of THC from its psychoactive effects. In support of this, immunomodulation by cannabinoids was found to be absent in mice deficient in CB2 receptor expression (10). Moreover, in a murine model of lung cancer we have previously shown that THC-related impairment in anti-tumor immunity is predominantly CB2 receptor dependent. In the present study, we utilize isolated human peripheral blood lymphocytes to investigate cannabinoid-dependent signaling in the induction of the immunosuppressive cytokine TGF-β. We found that THC stimulates human lymphocyte TGF-β production via a CB2 receptor-dependent signaling pathway. In addition, we demonstrate that the THC-mediated production of TGF-β down regulates CB2 receptor expression. This study identifies TGF-β in the autocrine and paracrine regulation of lymphocyte CB2 receptor expression.

MATERIALS AND METHODS

Reagents. Δ^9-Tetrahydrocannabinol (THC) was obtained as a solution of 50 mg/ml in absolute ethanol from the National Institute on Drug Abuse (NIDA) of the National Institutes of Health. Methanandamide was purchased from Biomole (Plymouth Meeting, PA). Recombinant TGF-β, as well as the antibodies utilized for a TGF-β ELISA, were purchased from R & D Systems (Minneapolis, MN) and Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). Antiserum to human CB2 receptor was purchased from (Alexis Biochemicals, San Diego, CA). Cyclic AMP enzyme immunoassay kit was purchased from Cayman Chemical (Ann Arbor, MI). SR144528 was generously provided by Murielle Rinaldi-Carmona, Sanofi Recherche Montpellier France. Forskolin, 3-isobutyl-1-methylxanthin (IBMX), and pertussis toxin were purchased from Sigma (St. Louis, MO).

PBL preparation and culture. Human peripheral blood lymphocytes (PBL) were isolated from random donor leuko-packs (American Red Cross) by Ficoll density centrifugation, followed by monocyte depletion via adherence as previously described (11). For TGF-β

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Abbreviations used: THC, Δ^9-tetrahydrocannabinol; OPD, *o*-phenylenediamine dihydrochloride.

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induction assays PBL (10^6 cells/ml) were stimulated by culture in 25-cm^2 flasks or 24-well plates that had been precoated with anti-CD3 antibody (0.05 μg/ml) in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% human serum (Gemini Bioproducts, Calabasas, CA) unless otherwise noted. To determine TGF-β production, PBL were cultured in various concentrations of THC (0.05, 0.50, and 5.0 μg/ml) or diluent control in the presence or absence of forskolin (50 μM), IBMX (100 μM), pertussis toxin (10 ng/ml), SR144528, or AM251. Following an 18-h culture period, the supernatants were collected for the assays described below.

TGF-β ELISA. TGF-β concentrations were determined from PBL culture supernatants by specific ELISA as described previously (12). A 50-μl of sample or TGF-β standard was added to a 96-well ELISA plate (Corning, Newark, CA) that had been coated with 4 μg/ml mouse anti-TGF-β at 4°C overnight. The supernatants (500 μl) were first acidified for 30 min at room temperature with 5.0 N HCl (5 μl) and then neutralized with 2.5 M NaOH, 0.5 M Hepes (10 μl). Following incubation for 1 h, the plate was washed with PBS/0.05% Tween 20. The plate was then incubated with 4 μg/ml chicken anti-TGF-β1 for 30 min. Following washing, peroxidase labeled goat anti-chicken IgG was added and incubated for 30 min. After washing, 100 μl *o*-phenylenediamine dihydrochloride 0.08% (OPD) substrate was added, color development was stopped after 15 min by addition of 100 μl of 2 N H_2SO_4, and the subsequent change in color was read with a Molecular Devices spectrophotometer (Sunnyvale, CA). The sensitivity limit of the TGF-β ELISA was 15 pg/ml.

Total RNA preparation and Northern blot analysis. To determine if the increased levels of TGF-β seen in response to THC were accompanied by increased expression of TGF-β mRNA, RNA isolation and Northern blot analysis were performed as previously described (13). For Northern blot analysis, 10 μg of total RNA that had been isolated from lymphocytes cultured with or without 5 μg/ml THC was subjected to denaturing 1% formaldehyde–agarose gel electrophoresis. Following staining the gel with ethidium bromide, RNA was transferred to a nylon membrane and immobilized on the membrane by a UV cross-linker (Stratagene, La Jolla, CA). The membrane was hybridized with a [^{32}P]dCTP-labeled plasmid TGF-β or β-actin cDNA probes. After washing, the membrane was exposed to Kodak XAR-5 film at −70°C. The relative intensity of TGF-β mRNA versus β-actin mRNA was determined with a Model 1650 transmittance/reflectance scanning densitometer (Bio-Rad Laboratories, Hercules, CA) and quantified using an Ambis image analysis system.

cAMP enzyme immunoassay (EIA). cAMP levels in THC-treated PBL were determined by a cAMP EIA kit obtained from Cayman Chemical Co. (Ann Arbor, MI) and performed according to the manufacturer's instructions. The results are expressed as percentages of cAMP levels in PBL normalized to diluent control.

Western blotting. Non-stimulated and anti-CD3 stimulated PBL treated with different concentrations of TGF-β or 5 μg/ml anti-TGF-β were harvested and lysed in ice-cold buffer containing 1% NP-40, 10 mM Tris (pH 7.8), 150 mM NaCl, 10 mM NaF, 2 mM PMSF, 1 mM sodium orthovanadate, 10 mg/ml leupeptin and 2 u/ml aprotinin. Lysates were cleared by centrifugation at 12,000 rpm for 10 min. Total protein was assayed using a protein assay kit (Bio-Rad). Samples containing 30 μg of total protein were separated utilizing a Mini-PROTEAN II and Ready Gel Precaste Gels (Bio-Rad). The proteins were transferred to PVDF membrane using Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). ECL detection of CB2 receptor protein levels was performed with rabbit antiserum to human CB2 receptor according to the manufacturer's instructions (ECL systems; Amersham, Arlington Heights, IL). A protein band with a relative molecular mass of 40 kDa was detected which corresponds to the relative molecular mass of the hCB2 receptor (14). As an internal control, β-actin protein levels were detected with goat anti-human β-actin and rabbit anti-goat (Santa Cruz, CA). The relative intensity of the CB2 receptor protein band versus β-actin was quantified by densitometry as described for Northern blot analysis.

RESULTS AND DISCUSSION

Cannabinoids Induce Lymphocyte TGF-β Production

THC is an immunosuppressive agent that has been shown to enhance tumor growth in immune competent murine models (15) and to mediate decreased host responsiveness in infectious disease models (16). One possible mechanism for THC-induced immunosuppression is the induction of immunosuppressive cytokines. In murine models, THC treatment led to an increase in the production of TGF-β in both tumor homogenates and splenocyte cultures (15). TGF-β antagonizes both CTL generation (17) and macrophage activities (18, 19). The presence of TGF-β at the tumor site may be one mechanism by which tumors evade an effective immune response and has been shown to be one mechanism of tumor-induced tolerance (20–22). In the immune therapy of established tumors the production of TGF-β was found to be higher in non-responsive tumors (23). Further, the blockade of TGF-β signaling in T cells resulted in immune-mediated eradication of implanted tumors (24). Thus, TGF-β potently suppresses cell-mediated immune responses (25).

To determine the capacity of THC to induce human lymphocyte TGF-β production, anti-CD3 stimulated PBL were cultured for 18 h in the presence of THC, and the concentrations of TGF-β in PBL culture supernatants were determined (Fig. 1A). There was a dose responsive increase in lymphocyte TGF-β production in response to THC. In PBL cultures treated with 0.05 μg/ml THC, TGF-β production increased by more than two fold, as the THC concentration was increased to 5.0 μg/ml TGF-β production increased fivefold over diluent treated controls. Endocannabinoids may provide a distinct pathway for immune regulation *in vivo.* To determine if endocannabinoids mediate PBL TGF-β production, PBL were treated with methanandamide, a metabolically stable analog of the endocannabinoid anandamide. Methanandamide treatment (5.0 μg/ml) resulted in a fivefold increase in TGF-β production (Fig. 1A).

THC Upregulates TGF-β mRNA Expression

To determine if the increased level of TGF-β seen in response to THC was accompanied by an increased expression of TGF-β mRNA, Northern blot analysis was preformed. PBL were cultured with or without THC (5 μg/ml). After a 16-h incubation, total RNA was extracted for Northern blot analysis (Fig. 1B). In PBL that were stimulated with anti-CD3 alone, TGF-β mRNA expression increased 20% over unstimulated controls. However, in PBL that were stimulated with anti-CD3 and treated with THC, TGF-β mRNA expression increased by more than 200% compared to unstimulated controls. Thus the observed increase of TGF-β concentration was accompanied by an increase

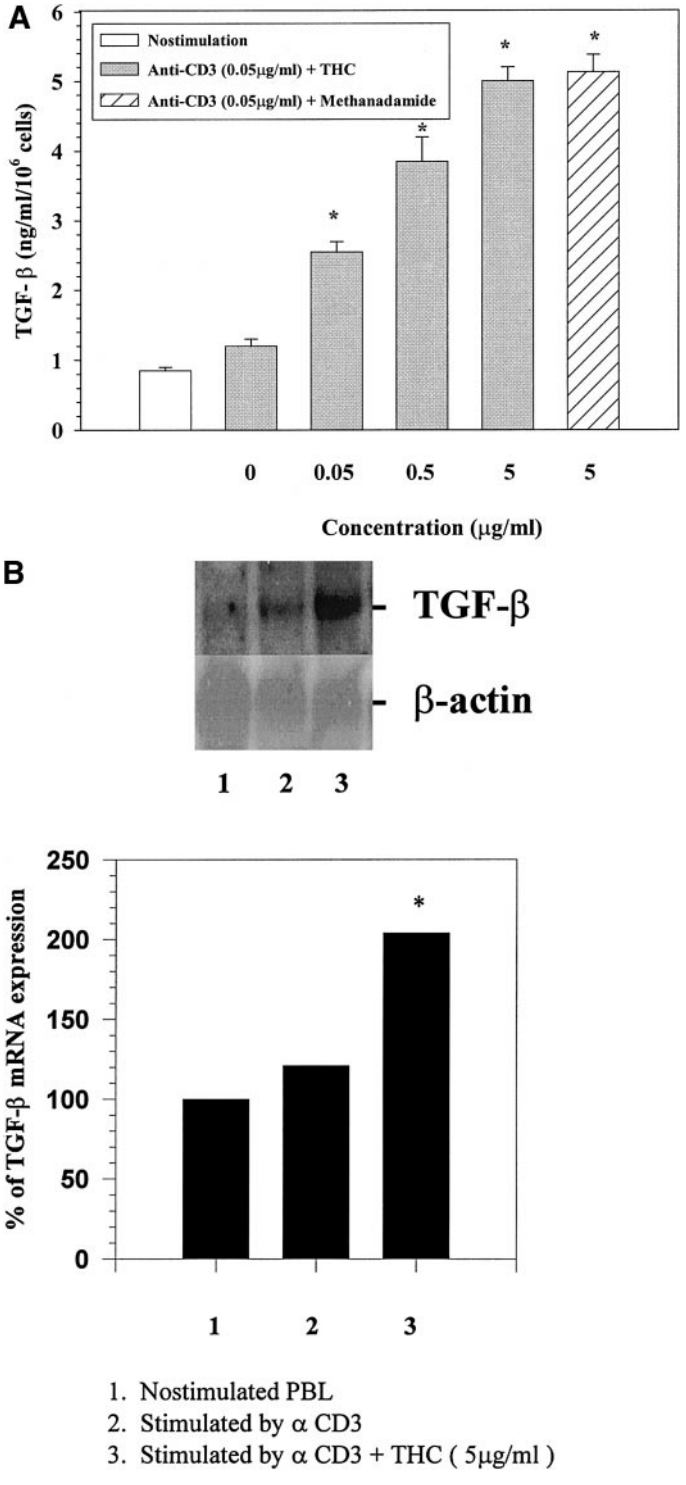


FIG. 1. Cannabinoids induce lymphocyte production of TGF-β. (A) THC or methanandamide induces lymphocyte production of TGF-β. Unstimulated or anti-CD3 stimulated PBL were cultured in the presence of THC (0.05–5.0 μg/ml) or methanandamide 5.0 μg/ml for 18 h and the production of TGF-β was determined by ELISA. THC increased PBL TGF-β production in a dose-responsive manner $^*P < 0.05$ compared to anti-CD3 stimulation alone ($n = 5$). Methanandamide stimulated PBL TGF-β production. $^*P < 0.05$ compared to diluent control ($n = 5$). (B) THC induces TGF-β mRNA expression. Unstimulated PBL or anti-CD3 stimulated PBL were cultured with or without 5 μg/ml THC. Following a 16-h incubation, total RNA from each condition was extracted for Northern analysis. The blots were performed using 10 μg of total RNA per lane. Filters were sequentially hybridized to the [^{32} P]cDNA probes TGF-β and β-actin (top). The relative expression of RNA products was determined by utilizing an Ambis Image Analysis System with the ratio of TGF-β to β-actin signals as arbitrary units (bottom). The blot analysis indicates that THC up-regulates TGF-β mRNA expression. $^*P < 0.05$ compared to anti-CD3 stimulation alone ($n = 2$).

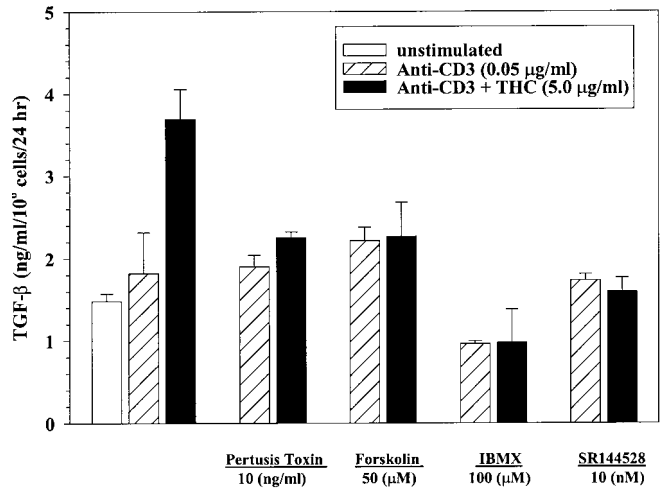


FIG. 2. THC-mediated PBL TGF-β production is CB2 receptor dependent. PBL were stimulated with anti-CD3 and treated with or without THC (5 μg/ml) in the presence or absence of 10 ng/ml pertussis toxin (incubated at 37°C for 24 h in advance), forskolin (50 μM), methylxanthine (IBMX, 100 μM), or a CB2 specific antagonist, SR144528 (10 nM). TGF-β concentrations were determined by ELISA. All three treatments abrogated THC-mediated induction of TGF-β production compared to THC alone ($n = 3$).

in TGF-β mRNA expression. These results suggest that tumor-associated lymphocytes may be one source of the THC-induced increase of TGF-β seen in murine tumor models. These findings, together with those of previous studies, suggest that cannabinoid-induced lymphocyte TGF-β production may be important in limiting an effective cell-mediated immune response.

THC-Mediated PBL TGF-β Production Is CB2 Receptor Dependent

The identification of specific receptors with a high affinity for cannabinoids has helped to elucidate potential pathways for immune modulation (26, 27). The cannabinoid receptors have been shown to signal through G-proteins and to inhibit adenyl cyclase activation (28, 29). Consistent with a G-protein coupled pathway, THC-mediated TGF-β production was pertussis toxin sensitive (Fig. 2). PBL were incubated with THC (5.0 μg/ml) with or without pertussis toxin (10 ng/ml). In pertussis toxin treated cells, the THC-mediated induction of TGF-β production was signifi-

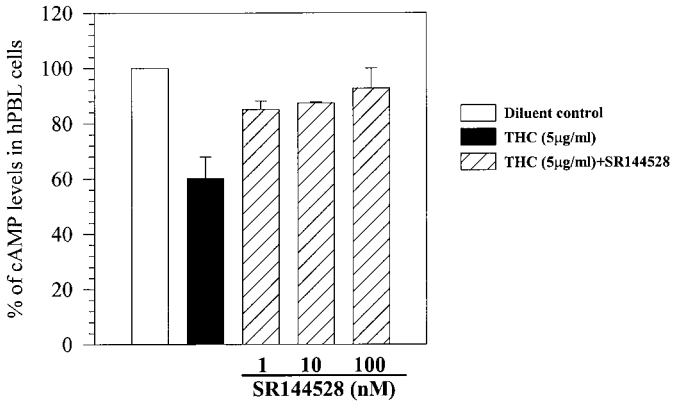


FIG. 3. A specific CB2 antagonist reverses THC mediated decrease in cAMP. PBL were anti-CD3-stimulated and incubated with THC (5 μg/ml) alone or in combination with 1 nM of the CB2 specific antagonist SR144528. cAMP levels were determined by enzyme immunoassay (EIA). The CB2 receptor antagonist reversed the THC-mediated decrease in cAMP concentration ($n = 2$).

cantly inhibited. To determine the relationship between THC mediated TGF-β production and adenyl cyclase activity, PBL were cultured with or without THC (5 μg/ml) and treated with an adenyl cyclase activator, forskolin, or a phosphodiesterase inhibitor, methylxanthine. Consistent with pertussis toxin sensitivity both forskolin and methylxanthine abrogated THC-mediated lymphocyte production of TGF-β (Fig. 2). Because the CB2 receptor is the dominant cannabinoid receptor expressed by immune cells (8, 29, 30), we

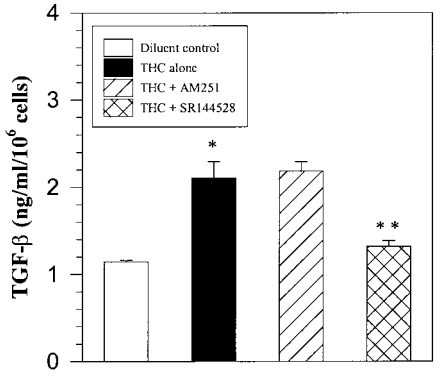
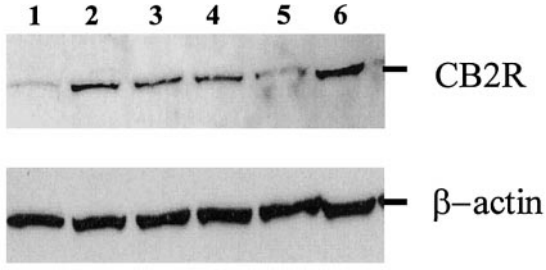


FIG. 4. THC induction of PBL TGF-β production is CB2 but not CB1 receptor dependent. Anti-CD3-stimulated PBL were cultured with THC at a concentration of 160 nM (0.05 μg/ml), THC + SR144528 (a specific CB2 receptor antagonist) at a concentration of 2.4 nM, THC + AM251 (a specific CB1 receptor antagonist) at a concentration of 30 nM for 18 h, and the TGF-β concentration was determined by ELISA. TGF-β production by THC-treated PBL are significantly higher than those in PBL treated with diluent control ($^*P < 0.05$). TGF-β production from cells treated with both THC and SR144528 were significantly different compared to cells treated with THC alone ($^{**}P < 0.05$). No significant difference is found between THC alone and THC + AM251. SR144528 or AM251 alone do not affect TGF-β production compared to diluent control (data not shown) ($n = 3$).



1.No stimulation
2.Stimulated by αCD3
3.Stimulated by αCD3 + 1.25 μg/ml TGF-β
4.Stimulated by αCD3 + 2.5 μg/ml TGF-β
5.Stimulated by αCD3 + 5 μg/ml TGF-β
6.Stimulated by αCD3 + 5 μg/ml TGF-β + 5μg/ml αTGFβ

FIG. 5. Autocrine regulation of lymphocyte CB2 receptor by TGF-β. PBL were stimulated by anti-CD3 and incubated with TGF-β from 0 to 5 ng/ml for Western blot analysis of CB2 receptor expression. To determine if endogenous TGF-β regulates lymphocyte CB2 receptor expression, anti-CD3-stimulated PBL were incubated with anti-TGF-β mAb (5 μg/ml). The expression of the CB2 receptor decreased with increasing concentrations of recombinant TGF-β. The concentrations of TGF-β found to decrease CB2 expression are within the range of concentrations induced by THC in PBL cultures. Anti-TGF-β mAb increased lymphocyte CB2 receptor expression. Isotype control antibody did not alter the anti-CD3-stimulated PBL CB2 receptor expression (data not shown) ($n = 2$).

hypothesized that THC-mediated TGF-β production is signaled through the CB2 receptor. To test this hypothesis, we incubated THC treated PBL with or without the CB2 receptor specific antagonist, SR144528 (1 nM) and measured TGF-β production. We found that CB2 receptor antagonism blocked THC-mediated induction of lymphocyte TGF-β production (Fig. 2). Thus, forskolin stimulation of adenyl cyclase, methylxanthine inhibition of phosphodiesterase, and SR144528 antagonism of the CB2 receptor all abrogated THC-mediated lymphocyte production of TGF-β, implicating CB2 receptor cAMP-dependent signaling. As a further confirmation that the THC-mediated increase in TGF-β concentration involves cAMP-dependent signaling, cAMP levels in THC-treated PBL were determined. Following THC treatment, the cAMP concentration in PBL was only 60% that of PBL treated with diluent control. When THC-treated PBL were incubated with the CB2 receptor-specific antagonist, SR144528, the THC-mediated decrease in cAMP concentration was reversed (Fig. 3).

THC Induction of PBL TGF-β Production Is CB2 Receptor but Not CB1 Receptor Dependent

While the CB2 receptor is the predominant cannabinoid receptor present on cells of the immune system,

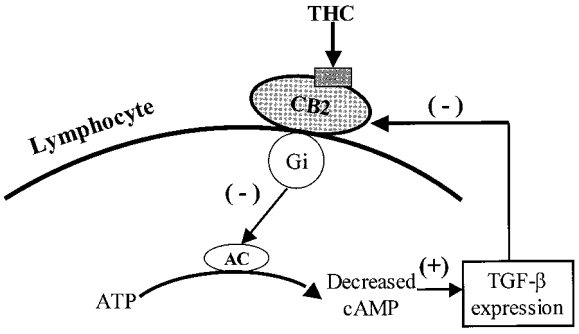


FIG. 6. TGF-β mediates autocrine regulation of lymphocyte CB2 receptor expression. THC stimulation of the lymphocyte CB2 receptor leads to a decrease in adenyl cyclase activity resulting in decreased cAMP concentration, which induces TGF-β production. The increased TGF-β results in decreased CB2 receptor expression, thus forming an autocrine regulatory loop.

the CB1 receptor is also expressed on immune cells (9). To determine if the CB1 receptor is involved in THC-mediated TGF-β production we employed the CB1 receptor specific antagonist, AM251, to block CB1 receptor signaling. The TGF-β concentration in PBL supernatants cultured with THC alone, or in the presence of either a CB1 or a CB2 receptor specific antagonist was determined. The CB2 receptor antagonist significantly reduced THC-induced PBL TGF-β production. In contrast, the CB1 receptor antagonist did not inhibit THC mediated PBL TGF-β production (Fig. 4). SR144528 or AM251 treatment alone did not affect PBL TGF-β production compared to the diluent control (data not shown). The CB2 receptor dependent induction of TGF-β is consistent with the observation that cannabinoid immunomodulation is absent in CB2 receptor knockout mice (10) and is consistent with our hypothesis that THC-mediated immune suppression is CB2 receptor dependent.

Autocrine and Paracrine Regulation of Lymphocyte CB2 Receptor Expression by TGF-β

Because stimulation of the CB2 receptor led to an increase in lymphocyte TGF-β production, we hypothesized that the increased TGF-β concentration could down regulate CB2 receptor expression and thus form a negative autocrine regulatory loop. To determine if this regulatory mechanism is operative in lymphocytes, we evaluated CB2 receptor expression on PBL cultured in the presence of exogenous recombinant TGF-β. We found that CB2 receptor expression decreased with increasing concentrations of recombinant TGF-β (Fig. 5). Further, the concentrations of recombinant TGF-β found to decrease CB2 receptor expression were within the range of concentrations induced by either THC or methanandamide in PBL cultures (Fig. 1A). To determine if endogenous TGF-β regulates CB2 receptor expression, anti-TGF-β antibody was incubated with anti-CD3 stimulated PBL. Anti-TGF-β antibody treatment resulted in an increase of CB2 receptor expression while control antibody did not alter CB2 receptor expression. Thus, the CB2 receptor-mediated increase in TGF-β production leads to a downregulation of CB2 receptor expression. These results suggest that the concentration of endogenous TGF-β may be sufficient to maintain autocrine regulatory control of CB2 receptor expression (Fig. 6). This is the first report of CB2 receptor signaling regulating TGF-β production inhuman lymphocytes. These findings suggest that exogenous and endogenous cannabinoid receptor ligands can play a role in the regulation of host immunity.

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