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A Novel Synthetic Cannabinoid Derivative Inhibits Inflammatory Liver Damage via Negative Cytokine Regulation

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ABSTRACT

The therapeutic potential of cannabinoids has been described previously for several inflammatory diseases, but the molecular mechanisms underlying the anti-inflammatory properties of cannabinoids are not well understood. In this study, we investigated the mechanism of action of a novel synthetic cannabinoid, [(+)-(6aS,10aS)-6,6-Dimethyl-3-(1,1-dimethylheptyl)-1-hydroxy-9-(1H-imidazol-2-ylsulfanylmethyl]-6a,7,10,10a-tetrahydro-6H-dibenzo[*b*,*d*]pyran (PRS-211,092) that has no psychotropic effects but exhibits immunomodulatory properties. Treatment with PRS-211,092 significantly decreased Concanavalin A-induced liver injury in mice that was accompanied by: 1) promotion of early gene expression of interleukin (IL)-6 and IL-10 that play a protective role in this model; 2) induction of early gene expression of the suppressors of cytokine signaling (SOCS-1 and 3), followed by 3) inhibition of several pro-inflammatory mediators, including IL-2, monocyte chemoattractant protein-1 (MCP-1), IL-1β, interferon-γ, and tumor necrosis factor α. Based on these results, we propose a mechanism by which PRS-211,092 stimulates the expression of IL-6, IL-10 and the SOCS proteins that, in turn, negatively regulates the expression of pro-inflammatory cytokines. Negative regulation by PRS-211,092 was further demonstrated in cultured T cells, where it inhibited IL-2 production and nuclear factor of activated T cells activity. These findings suggest that this cannabinoid derivative is an immunomodulator that could be developed as a potential drug for hepatitis as well as for other short- or long-term inflammatory diseases.

The identification of Δ^9-tetrahydrocannabinol as the active component of marijuana (*Cannabis sativa*) prompted medicinal chemists to develop numerous cannabinoid analogs and opened a new era in research on the pharmaceutical applications of these compounds. Mechoulam (2000) discovered that the undesirable psychotropic effects of cannabinoids lie in a specific configuration of the molecule. This situation was best exemplified with the HU-210 and HU-211 enantiomeric pair of synthetic cannabinoids, HU-210 has the natural configuration and is 100 times more psychoactive than Δ^9-tetrahydrocannabinol, whereas HU-211 has the opposite configuration and is devoid of cannabimimetic side effects while retaining important therapeutic benefit (Mechoulam et al., 1990). Pharmos has synthesized chemical derivatives of HU-211 and has identified among them a novel compound, PRS-211,092, that has no psychotropic effects but exhibits superior anti-inflammatory properties. Cannabinoids are known as modulators of immune responses and inflammation, but many of the cellular components that mediate these actions have not yet been defined (Klein et al., 2001). Induction of liver injury in mice by the mitogenic plant lectin Concanavalin A (Con A) is a well established model for viral or autoimmune hepatitis (Koziel, 1999; McFarlane, 1999). Con A directly activates immune cells that secrete inflammatory mediators such as cytokines, chemokines, and acute phase response proteins, which determine the degree of liver damage (Tiegs et al., 1992). Among the pro-inflammatory cytokines, tumor necrosis factor α (TNFα) and interferon γ play a major role in Con A-induced liver cell damage (Mizuhara et al., 1994; Gantner et al., 1995; Kusters et al., 1996; Mizuhara et al., 1996). These cytokines are negatively regulated by interleukin (IL)-10, whereas IL-6 plays a bimodal role in liver inflammation (Louis et al., 1997; Tagawa et al., 2000; Kato et al., 2001). Recent studies have shown that IL-10 and IL-6 counteract inflammation by a mechanism that involves the induction of the suppressor of cytokines signaling (SOCS) family of proteins. Among these, SOCS1 and SOCS3 limit cellular cytokine responses via down-regulation of the Janus

I.L. and T.S. contributed equally to this work.

ABBREVIATIONS: HU-210, (−)-(6a*R*,10a*R*)-6,6-dimethyl-3-(1,1-dimethylheptyl)-1-hydroxy-6a,7,10,10a-tetrahydro-6H-dibenzo[*b*,*d*]pyran-9-methanol; HU-211, (+)-(6a*S*,10a*S*)-6,6-dimethyl-3-(1,1-dimethylheptyl)-1-hydroxy-6a,7,10,10a-tetrahydro-6H-dibenzo[*b*,*d*]pyran-9-methanol; PRS-211,092, [(+)-(6a*S*,10a*S*)-6,6-dimethyl-3-(1,1-dimethylheptyl)-1-hydroxy-9-(1H-imidazol-2-ylsulfanylmethyl)-6a,7,10,10a-tetrahydro-6H-dibenzo[*b*,*d*]pyran; Con A, Concanavalin A; TNF, tumor necrosis factor; IL, interleukin; SOCS, suppressor of cytokines signaling; FK 506, tacrolimus; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; A23187, 4-bromocalcimycin; NF-AT, nuclear factor of activated T cells; AP-1, activating protein-1; PCR, polymerase chain reaction.

tyrosine kinase/signal transducer and activator of transcription signaling pathway (Yasukawa et al., 2000). We used the Con A-induced liver injury mouse model to investigate whether the reduction of inflammation by PRS-211,092 in vivo is mediated through transcriptional regulation of cytokines and suppressors of cytokine signaling.

Materials and Methods

Animals. Female BALB/c mice (age, 6–8 weeks; weight, 18–22 g) were supplied by Harlan (Indianapolis, IN). All mice were maintained under controlled lighting (12 h on and 12 h off) and temperature (22°C) and allowed free access to standard laboratory chow and tap water. All procedures used in this study were done according to internal guidelines for usage of animals in medical research and Israeli law.

Drug Administration. All test compounds including PRS-211,092, vehicle [Cremophor/ethanol; 70:30 (w/w)], saline (B. Braun, Melsungen, Germany) and FK 506 (Prograf; Fujisawa Healthcare, Deerfield, IL) were injected at the indicated time points before or after Con A injection. Con A (Sigma, St. Louis, MO) and all other compounds were administered intravenously through the tail vein.

Alanine Aminotransferase and IL-10 Determination in the Plasma. Blood (500 μl) was collected into EDTA-containing tubes by retroorbital puncture 1 and 8 hours after injection of 15 mg/kg Con A for IL-10 and ALT, respectively. After brief centrifugation (3000 rpm for 2 min) plasma was recovered and stored at −80°C until use. The ALT test was carried out by automated procedure (AML Laboratory, Herzelia, Israel). Plasma IL-10 was measured by ELISA using a commercially available kit (Endogen, Woburn, MA) according to the manufacturer's instructions.

RNA and cDNA Preparation. Mice were killed by cervical dislocation at 15 min and 1, 4, and 8 h after Con A administration, and the liver and spleen were removed. Total RNA was prepared using SV total RNA kit (Promega, Madison, WI). cDNA was prepared from 2 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT) according to manufacturer instructions for first-strand cDNA synthesis.

TABLE 1
Primer sequences used

	Direction	Sequence
Mouse		
IL-2	Forward	5′-GAAACTCCCCAGGATGCTCAC
	Reverse	5′-GCCGCAGAGGTCCAAGTTC
IL-6	Forward	5′-AGAAGGAGTGGCTAAGGACCAA
	Reverse	5′-GGCATAACGCACTAGGTTTGC
MCP-1	Forward	5′-AACTGCATCTGCCCTAAGGTCT
	Reverse	5′-TGCTTGAGGTGGTTGTGGAA
IL-10	Forward	5′-GCCCTTTGCTATGGTGTCCTT
	Reverse	5′-TCCCTGGTTTCTCTTCCCAA
IL-1β	Forward	5′-ACACTCCTTAGTCCTCGGCCA
	Reverse	5′-CCATCAGAGGCAAGGAGGAA
SOCS-1	Forward	5′-GCATCCCTCTTAACCCGGTACT
	Reverse	5′-AATAAGGCGCCCCCACTTA
SOCS-3	Forward	5′-AGGCACTCCCCGGGAGTAC
	Reverse	5′-GGCCACGTTGGAGGAGAGA
TNFα	Forward	5′-AAGGACTCAAATGGGCTTTCC
	Reverse	5′-CCTCATTCTGAGACAGAGGCAAC
INFγ	Forward	5′-TGAAAATCCTGCAGAGCCAGAT
	Reverse	5′-TGATTCAATGACGCTTATGTTGTTG
Haptoglobin	Forward	5′-GCTGGGATCCTGAGCTTTGA
	Reverse	5′-TTGGCCATGGTTTCCTGAAC
SAA-3	Forward	5′-CAGAAGTTCACGGGACATGGA
	Reverse	5′-CCAGCAGGTCGGAAGTGGT
Cyclophilin	Forward	5′-TCGCCATTGCCAAGGAGTAG
	Reverse	5′-GGTCACCCCATCAGATGGAA
COX-1	Forward	5′-GACGTGGGCTTCAACCTTGT
	Reverse	5′-GGGTAATCTGGCACACGGAA
Human		
IL-2	Forward	5′-GGGACTTAATCAGCAATATCAACGT
	Reverse	5′-TTCTACAATGGTTGCTGTCTCATCT
Cyclophilin A	Forward	5′-GCATACGGGTCCTGGCATC
	Reverse	5′-TGCCATCCAACCACTCAGTCT

SAA-3, serum amyloid-3; COX, cyclooxygenase.

Quantitative Real-Time Polymerase Chain Reaction. Reaction mixture included 1 μl of cDNA, 300 nM concentrations of the appropriate forward and reverse primers (Sigma-Genosys Ltd., Cambridgeshire, UK), and 7.5 μl of the master mix buffer containing nucleotides, *Taq* polymerase, and SYBR green (SYBR Green master mix; Applied Biosystems, Warrington, UK), in a total volume of 15 μl. Gene amplification was carried out using the GeneAmp 5700 sequence detection system (Applied Biosystems). Amplification included one stage of 10 min at 95°C followed by 40 cycles of a two-step loop: 20 s at 95°C and 1 min at 60°C. Results are expressed as -fold increase of gene expression in samples from Con A-injected animals above those from saline-injected control animals. The results for gene expression in the liver were normalized to the Cyclophilin gene in samples from the liver and to the COX-1 gene in samples from the spleen, because the level of these genes was not affected by Con A or treatments. Primer sequences used are shown in Table 1.

Histology and Histochemistry. Formalin-fixed, paraffin-embedded liver tissue was sectioned at 5 μm in thickness, deparaffinized in xylene, and rehydrated through a series of decreasing concentrations of ethanol. For histological analysis, sections were stained with hematoxylin and eosin. Liver sections were then analyzed under a light microscope. Histopathological scoring was done at AML Laboratory (Herzelia, Israel). The samples were evaluated by three parameters using a scoring range from 0 (normal) to 4 (highest severity): A) portal tract: degree of vascular congestion, dilatation, teleangiectasia; B) centrilobular area: degree of vascular congestion and dilatation; and C) midzone liver parenchyma: degree of inflammatory cell infiltration, liver cell degeneration, and necrosis. The final average scores were calculated for each group and statistical analysis was performed.

Statistical Analysis. The results were analyzed using *t* test. All values for the different parameters measured in the study are expressed as an average of n individual mice ($n \geq 8$). $P \leq 0.05$ was considered significant.

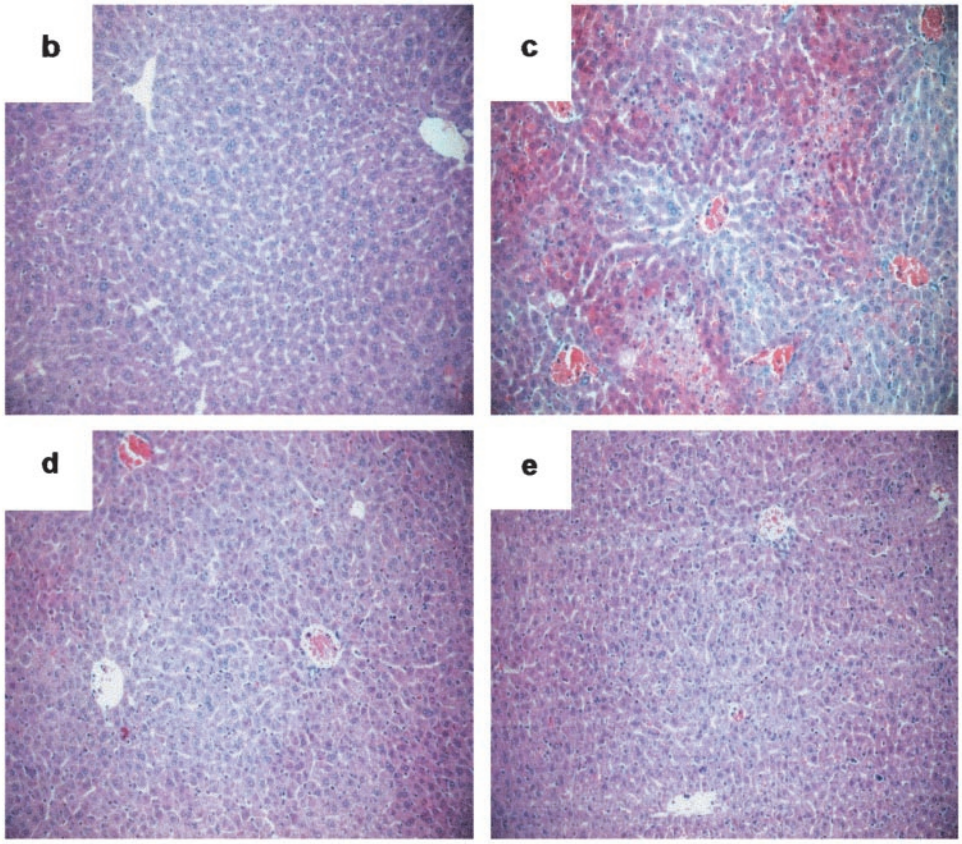
Determination of IL-2 RNA and Protein Levels in T Cells. Jurkat cells (human acute lymphoma T cell line; American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum. One day before activation, the cells were plated at 10^6 cells/well/ml on a 24-well plate. The cells were incubated for 1 h before stimulation with vehicle (0.1% DMSO), PRS-211,092, or cyclosporin A (Novartis, Basel, Switzerland). The cells were stimulated using 10 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma) and 1 μM of calcium ionophore A23187 (Sigma). For the measurement of IL-2 transcription, the cells were harvested 6 h after stimulation, washed twice with PBS, and used for RNA and cDNA preparation followed by real-time PCR analysis as described above. Primer sequences used are shown in Table 1.

A parallel experimental set was incubated for 24 h after activation, and the supernatant from each well was collected and analyzed for the presence of secreted IL-2 by ELISA using commercially available kit (Endogen) according to the manufacturer's instructions.

Reporter Gene Assay. Jurkat cells were stably transfected with a nuclear factor of activated T cells (NF-AT) or activating protein-1 (AP-1) luciferase reporter vector (BD Biosciences Clontech, Palo Alto, CA) together with a pcDNA3.1A vector (Invitrogen) bearing a neomycin resistance gene for selection. The stable cell lines were maintained in a complete RPMI 1640 medium as described above supplemented with 300 μg/ml G418 (Life Technologies). On the day of activation, the stable cells were plated at 1×10^6 cells/ml/well on a 24-well plate in fresh RPMI complete medium without G418.

a

Drug	Levels of ALT (IU/L) according to time of drug injection		
	30 min before Con A	30 min after Con A	1 Hr after Con A
Saline	1562 ± 411.6	1562 ± 411.6	1562 ± 411.6
Vehicle	1720 ± 428.8	1335 ± 387.3	1742 ± 420.4
PRS-211, 092	742 ± 145.0 (*)	664 ± 182.7 (*)	838 ± 204.6 (*)
FK 506	57 ± 16.6 (*)	53 ± 19.4 (*)	103 ± 5.9 (*)



f

Drug	Levels of gene expression			
	4 hours after treatment		8 hours after treatment	
	SAA3	haptoglobin	SAA3	haptoglobin
Vehicle	117.5± 19.4	1.9 ± 0.12	1157.4 ± 79.4	7.7 ± 1.6
PRS-211, 092	66.5± 16.2 (*)	0.9 ± 0.29 (*)	602.7 ± 293 (*)	3.0 ± 1.5 (*)
FK 506	6.2± 2.0 (*)	0.5 ± 0.19 (*)	45.9 ± 45.8 (*)	4.8 ± 2.1

Fig. 1. Treatment with PRS-211,092 reduces Con A-induced hepatitis. Female Balb/C mice (20 per group) were injected with 15 mg/kg Con A and treated with vehicle (Cremophor/ethanol), 5 mg/kg PRS-211,092, or 1 mg/kg FK 506. Control mice were injected with saline alone. The Con A-induced liver damage was evaluated at 8 h after Con A challenge. *, $p < 0.05$ compared with vehicle treated mice. a, the plasma levels of ALT were measured 8 hours after Con A challenge in mice that were treated with either saline, vehicle, PRS-211, 092, or FK506 at the indicated time points, before or after Con A. b to e, mice were treated first with compounds and 30 min later with Con A. Liver damage was evaluated by hematoxylin-eosin staining. All treatments were compared with Con A-untreated mice (b). Compared with vehicle (c), PRS-211,092 (d) reduced inflammation and liver damage. FK 506 (e) inhibited Con A-induced liver damage and the inflammation almost completely. f, PRS-211,092 reduced acute phase protein gene expression. Mice ($n = 8$) were treated with compounds and 30 min later with Con A. Serum amyloid A-3 and haptoglobin mRNA levels in the liver were measured by real-time PCR at the indicated time points after Con A.

PRS-211,092 was diluted in DMSO and added to the cell medium at 0.1 to 10 μM final concentrations 1 h before cell activation. The cells were activated with PMA and calcium ionophore as described above. After 6 h of incubation, the cells were washed once with ice-cold PBS and lysed for 15 min on ice with 50 μl of luciferase lysis buffer (Promega). Luciferase enzymatic activity in cell extracts was determined by the addition of 10 μl of cell extract and 90 μl of luciferase substrate (Promega) to a well of a flat black 96-well plate. Luminescence was immediately quantified using SPECTRAFluor Plus reader (Tecan, Durham, NC).

Results

Treatment with the synthetic cannabinoid PRS-211,092 attenuated Con A-induced liver damage as demonstrated by the following indications. Plasma levels of ALT, measured 8 h after Con A injection, were decreased by 51 to 57% in mice treated with 5 mg/kg PRS-211,092 compared with those in animals treated with vehicle alone (Fig. 1a). This effect of PRS-211,092 occurred whether the compound was administered 30 min before or up to 1 h after Con A challenge (Fig. 1a). Pretreatment (30 min) with 5 mg/kg PRS-211,092 was still effective when tested 13 h after Con A injection, reducing ALT levels by 69% compared with vehicle alone [434 $\pm$ 214 (n = 12) versus 1418 $\pm$ 390 IU/l (n = 13), respectively; $p <$ 0.05]. In addition, hematoxylin-eosin liver staining showed that PRS-211,092 reduced disruption of liver tissue compared with vehicle alone (Fig. 1, b-d). This reduction was confirmed by histopathology scoring for the degree of vascular congestion and dilatation as well as inflammatory cell infiltration and liver cell degeneration. Whereas average pathological score for the vehicle-treated group was 7.2 $\pm$ 1.3 (n = 5), the score for the PRS-211,092 treated group was 4.7 $\pm$ 0.3 (n = 5, p = 0.051). Acute phase proteins are secreted by hepatocytes during severe inflammation. Gene expression of the acute phase proteins serum amyloid A-3 and haptoglobin were elevated in liver 4 h after Con A injection and further increased at 8 h after Con A injection. Treatment with PRS-211,092 reduced the expression of both proteins by half (Fig. 1f). Taken together, treatment with PRS-211,092 prevented Con A-induced liver injury by approximately 50% as assessed by all parameters tested. T-cell activation by Con A is the initial event in the inflammatory process that leads to liver damage. The immunosuppressant FK 506 blocks Con A-induced T cell activation and liver damage (Mizuhara et al., 1994). Using FK 506 for comparison, we show that administration of 1 mg/kg FK 506 blocked ALT release (Fig. 1a), reduced liver tissue damage (Fig. 1e), and inhibited expression of acute phase proteins (Fig. 1f).

To investigate whether PRS-211,092 reduces liver damage through moderation of inflammatory cytokine secretion, we measured changes in mRNA levels of several key cytokines in the liver at four different time points after Con A challenge. An immediate early elevation of IL-1β, TNFα, and IFNγ was observed in liver 15 min after Con A injection. Strikingly, IL-6, which was not induced by Con A alone at this time point, was elevated 4.7-fold after pretreatment with PRS-211,092 (Fig. 2a). A time course experiment for 1, 4, and 8 h after Con A injection revealed that at the peak of cytokine expression, PRS-211,092 attenuated IL-2, IL-1β, monocyte chemoattractant protein-1 (MCP-1), TNFα, and IFNγ gene expression (Fig. 2, b-f). In contrast, the increase in IL-6 levels

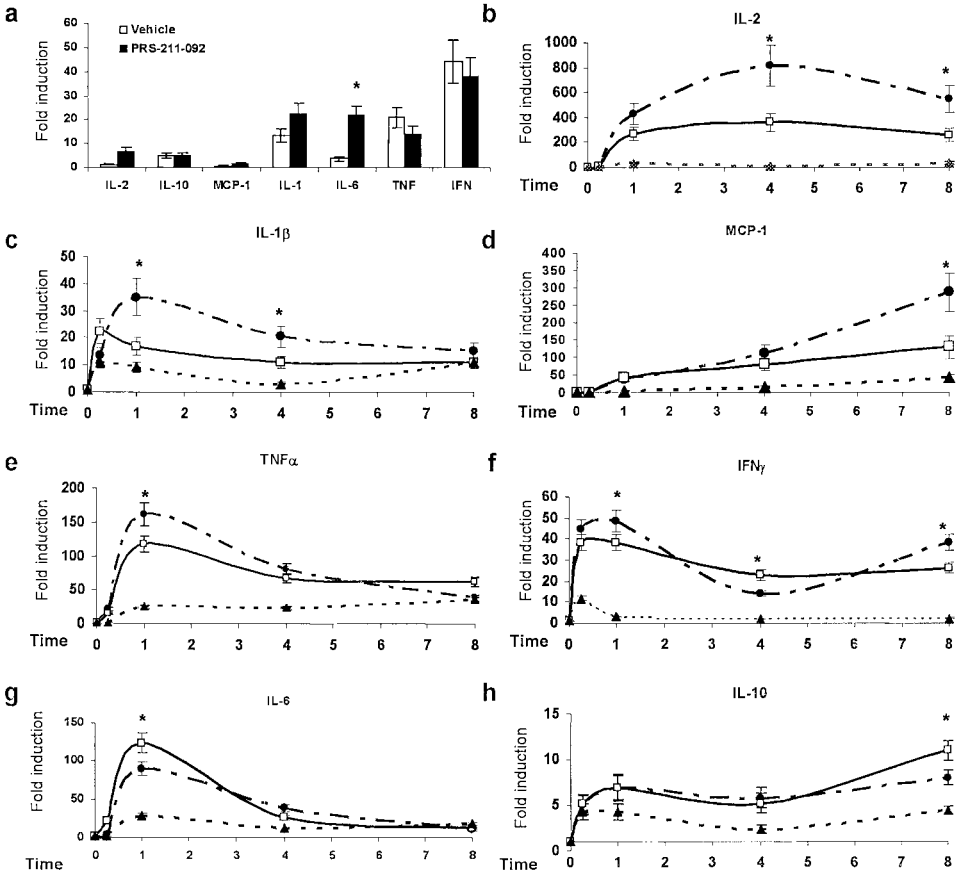


Fig. 2. PRS-211,092 modulates cytokine gene expression in the liver. a, mice (n = 8) were treated with vehicle or with PRS-211,092 30 min before Con A injection. Cytokine mRNA levels were measured in the liver 15 min after Con A challenge. Fold increases are expressed relative to that in saline-treated mice and are normalized to cyclophilin. *, $p < 0.05$ compared with vehicle treated mice. b to h, mice (n = 8) were treated with vehicle (●), PRS-211,092 (□) or FK 506 (▲) 30 min before Con A injection. Cytokine mRNA levels were measured in the liver at the indicated time points. PRS-211,092 reduced the expression peak of pro-inflammatory cytokines (b–f) and induced the expression of IL-6 (g) at the early phase and IL-10 at the late phase (h). Cytokine -fold induction is relative to saline-treated mice and normalized to cyclophilin. *, $p <$ 0.05 compared with vehicle treated mice.

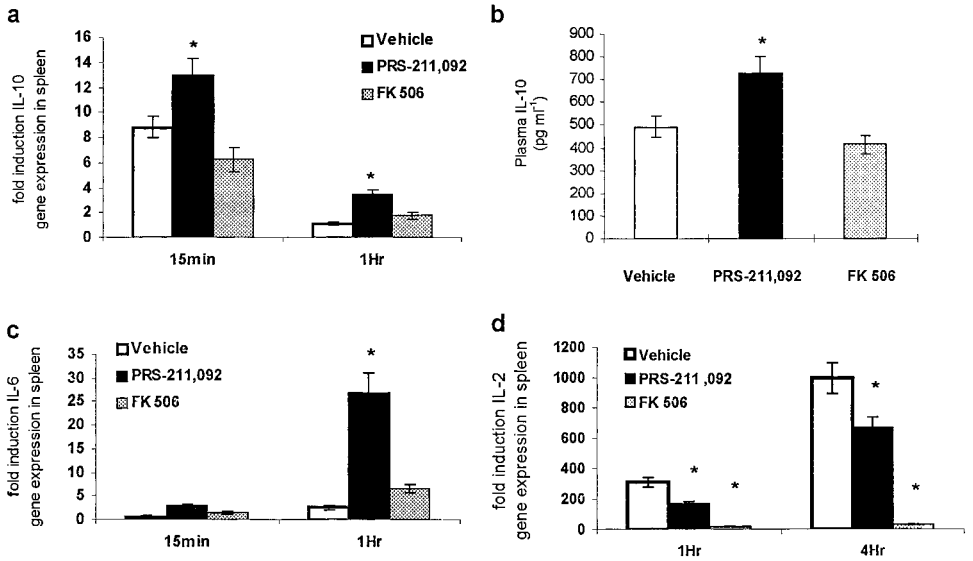


Fig. 3. PRS-211,092 elevates IL-10 and IL-6 expression, as well as IL-10 plasma level and reduces IL-2 gene expression in the spleen. Mice ($n = 8$) were treated with vehicle, PRS-211,092 or FK 506, 30 min before Con A injection. a, the expression of IL-10 was determined in the spleen by measuring mRNA levels at the time points indicated after Con A injection (b). Plasma IL-10 was measured 1 h after Con A injection. (c) IL-6 mRNA expression was determined in the spleen at the indicated time points after Con A injection. (d) IL-2 mRNA expression was measured in the spleen at the indicated time points after Con A injection. -Fold increases (a, c, d) in gene expression are relative to saline-treated mice and normalized to COX-1. *, $p < 0.05$ compared with vehicle-treated mice.

by PRS-211,092 observed 15 min after Con A injection (Fig. 2a) continued for 1 h and then decreased with time (Fig. 2g). IL-10 levels were induced by Con A in the liver and further augmented by PRS-211,092 at 8 h after Con A administration (Fig. 2h). FK 506 strongly inhibited or completely blocked expression of all cytokines assayed (Fig. 2b-h).

We also determined whether PRS-211,092 affects cytokine levels in spleen and plasma. An increase in IL-10 gene expression in the spleen occurred 15 min after Con A challenge, and this was augmented by PRS-211,092 (Fig. 3a). IL-10 induction in the spleen was followed by an increase in IL-10 plasma levels 1 h after Con A administration and further augmented by 150% with PRS-211,092 (Fig. 3b). In addition to IL-10, Con A slightly induced IL-6 mRNA in the spleen, but this induction was dramatically augmented by 10-fold after PRS-211,092 treatment 1 h after Con A injection (Fig. 3c). In contrast to PRS-211,092, FK 506 inhibited both IL-6 and IL-10 in the spleen of Con A-challenged mice (Fig. 3, a-d). As in the liver, the induction of IL-6 and IL-10 in spleen of PRS-211,092–treated mice was accompanied by the reduction of IL-2 mRNA levels (Fig. 3d). The anti-inflammatory action of IL-6 and IL-10 involves stimulation of SOCS-1 and -3 (Yasukawa et al., 2000). To investigate whether PRS-211,092 affects SOCS expression, SOCS-1 and 3 mRNA levels were determined in Con A-injected mice. Con A induced SOCS-1 and -3 gene expression in both spleen and liver (Fig. 4) and treatment with PRS-211,092 markedly augmented SOCS-1 stimulation by 282% in the liver and by 250% in the spleen 1 h after Con A injection (Fig. 4, a and b). Treatment with PRS-211,092 also augmented SOCS-3 expression in spleen (188%, 1 h after Con A) and liver (200%, 4 h after Con A) (Fig. 4, c and d). In contrast, FK 506 inhibited SOCS-1 and -3 in both liver and spleen (Fig. 4). Cytokine regulation by PRS-211,092 may be mediated by immune cells, such as T cells, which play a central role in Con A-induced liver damage (Sass et al., 2002). To test this, we used a human T cell line, Jurkat, stimulated with PMA and calcium ionophore. Similar to Con A, this induction leads to production and secretion of IL-2, a key event that is required for the resting T cells to become functional effector cells. Preincubation with increasing concentrations of PRS-211,092 led to a dose-dependent inhibition of IL-2 gene expression (Fig. 5a) and IL-2 cytokine secretion (Fig. 5b) at 0.1 to 10 μM range. In addition, we have tested the effect of PRS-211,092 on NF-AT, a major component of the IL-2 transcriptional machinery. Using a luciferase reporter gene assay, we could show that increasing doses of PRS-211,092 gradually reduced NF-AT activity in stimulated Jurkat cells (Fig. 5c). AP-1, another important regulator of the IL-2 promoter, was not affected by PRS-211,092 (Fig. 5d).

Discussion

Mice injected with Con A are used as an experimental model for hepatitis mediated by cellular immunity (Tiegs et al., 1992). We demonstrate here that PRS-211,092 is highly effective in attenuating Con A-induced liver damage (Fig. 1). The involvement of several cytokines in the onset of hepatitis has been reported (Sass et al., 2002), and we show that PRS–211,092 reduced the pro-inflammatory cytokines TNFα, IL-1β, IL-2, and interferon γ (Figs. 2 and 3). Suppression of these cytokines has been previously reported to inhibit Con A-induced liver injury (Mizuhara et al., 1994, 1996; Gantner et al., 1995; Kusters et al., 1996). In addition, PRS-211,092 also inhibited the expression of MCP-1 8 h after Con A injection (Fig. 2d), at a time when liver infiltration by activated lymphocytes occurs (Bradham et al., 1998). MCP-1 promotes the migration and activation of monocytes and plays a pivotal role in the development of long-term inflammation. Recently, it has been shown that propagermanium, an anti-inflammatory drug used clinically for the treatment of chronic hepatitis, prevented Con A-induced liver injury through interference with monocyte/macrophage recruitment mediated by MCP-1 (Yokochi et al., 2001). Several

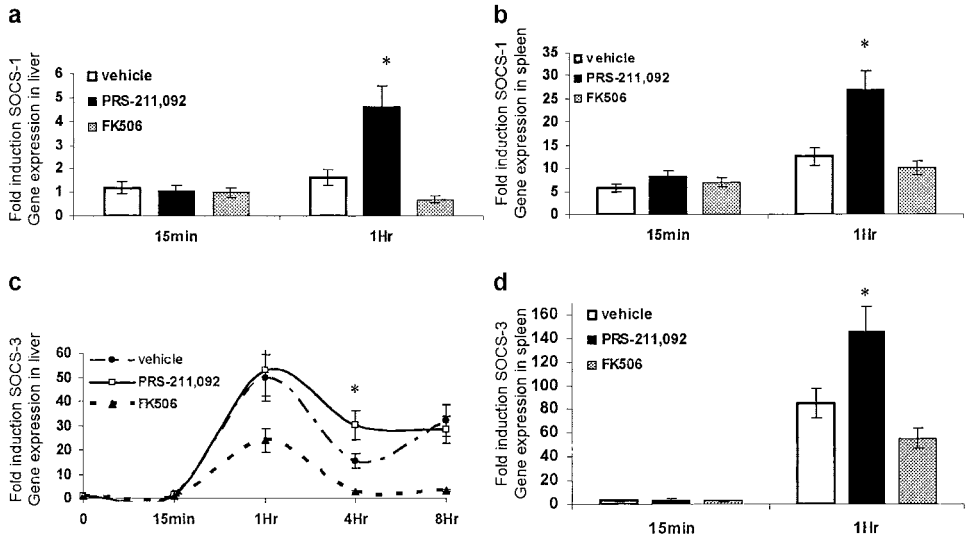


Fig. 4. PRS-211,092 elevates SOCS 1 and -3 gene expression in liver and spleen. a to d, mice ($n = 8$) were treated with vehicle, PRS-211,092, or FK 506, 30 min before Con A injection. mRNA expression levels were measured in liver (a and c) or spleen (b and d) at the time points indicated after Con A injection. Fold increases are relative to those of saline-treated mice and normalized to cyclophilin (liver) and COX-1 (spleen). *, $p < 0.05$ compared with vehicle-treated mice.

studies show that cytokines such as IL-6, IL-10, and TGF-β are also up-regulated during the onset of hepatitis and play a role in reducing liver inflammation (Mizuhara et al., 1994; Kato et al., 2001). In the spleen, PRS-211,092 augmented IL-10 gene expression by 150% at 15 min and 1 h after Con A challenge (Fig. 3a). In parallel, IL-10 protein concentration in plasma increased by 150% in PRS-211,092–treated mice 1 h after Con A challenge (Fig. 3b). In the liver, there was an elevation of IL-10 gene expression after Con A, which was further augmented (140%) by PRS-211,092 at 8 h after Con A challenge (Fig. 2h). We therefore assume that the augmentation of IL-10 by PRS-211,092 in the plasma originated from the spleen and probably plays an important role in reducing liver inflammation. This is consistent with previous reports showing that administration of exogenous IL-10 inhibited production of TNFα and IFNγ and reduced ALT plasma levels after Con A injection. In addition, ALT levels were increased both in IL-10 knockout mice as well as in mice receiving anti-IL-10 monoclonal antibody (Louis et al., 1997; Di Marco et al., 1999). Recombinant IL-10 treatment of patients with chronic hepatitis C decreased inflammation and scarring in the liver, with serum ALT activity returning to normal in most patients by the end of the study (Nelson et al., 2000). These findings support our hypothesis that the eleva-

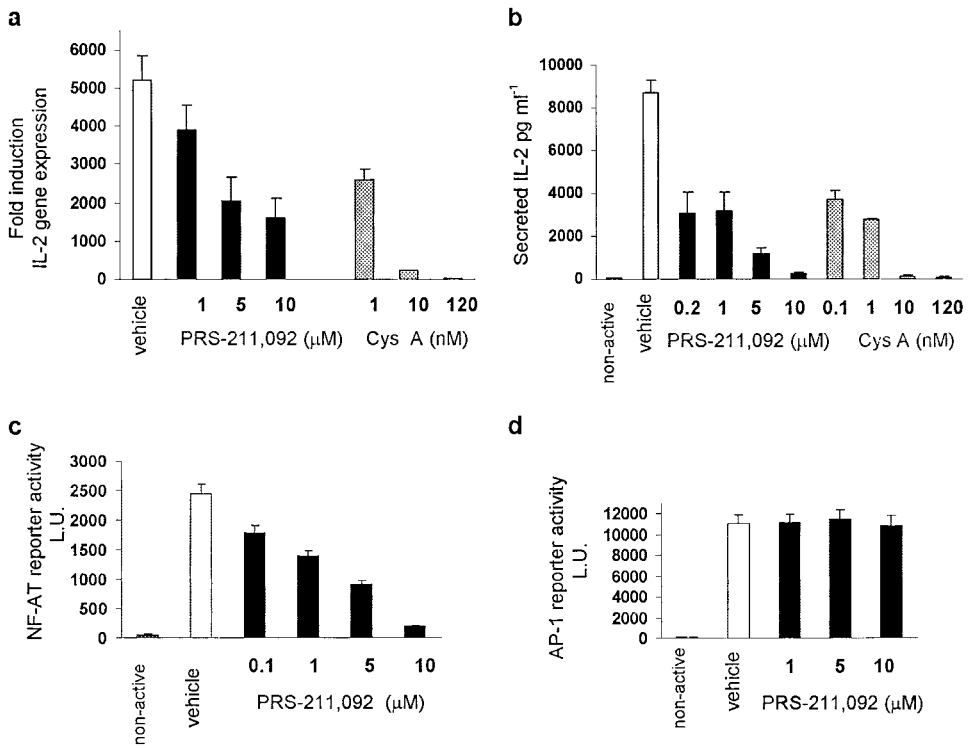


Fig. 5. PRS-211,092 down-regulates IL-2 transcription, IL-2 cytokine levels, and NF-AT activity in cultured T cells. a, Jurkat human T cells were incubated with vehicle (0.1% DMSO), PRS-211,092, or cyclosporin A (Cys A) as a positive control for 1 h followed by stimulation with PMA and calcium ionophore for additional 6 h. The cells were harvested and IL-2 gene expression was measured by real-time reverse transcription-PCR. -Fold inductions are expressed relative to that in nonactivated cells and are normalized to cyclophilin. b, Jurkat cells were treated and stimulated as in a for 24 h. IL-2 cytokine levels were measured in cell supernatants by ELISA. c and d, Jurkat cells, stably transfected with an NF-AT (c) or AP-1 (d) luciferase reporter gene, were treated as in a. 6 h after stimulation, the cells were harvested and luciferase activity was measured in cell extracts. L.U., luminescence units. All experiments were done in triplicate.

tion of IL-10 by PRS-211,092 contributes to the attenuation of liver damage. In addition to IL-10, Con A induced an increase in IL-6 expression, which was markedly augmented by PRS-211,092. In the liver, PRS-211,092 induced IL-6 levels by 470% and 140% 15 min and 1 h after Con A, respectively (Fig. 2, a and g). A strong augmentation of IL-6 (1030%) was also observed in the spleen of PRS-211,092–treated mice 1 h after Con A administration (Fig. 3c). However, at later time points, IL-6 levels in the liver decreased and were not changed by PRS-211,092 (Fig. 2g). A bimodal role has been suggested for IL-6 in the Con A–induced model of hepatitis as well as in other inflammatory conditions. Experiments using IL-6–neutralizing antibodies indicated that the presence of IL-6 in an early phase after Con A challenge is critical for evoking a strong hepatoprotective effect, whereas continuous high levels of the cytokine are toxic for the liver (Tagawa et al., 2000). Furthermore, Mizuhara et al. (1994) found that administration of recombinant IL-6 before Con A injection completely inhibited the release of transaminases. Therefore, we suggest that the transient early elevation of IL-6 by PRS-211,092 contributed to its protective effect on Con A-induced liver injury. In addition to elevation of IL-10 and IL-6, PRS-211,092 increased SOCS-1 and -3 gene expression in both liver and spleen after Con A challenge (Fig. 4). The SOCS proteins are activated by more than 40 cytokines and growth factors and negatively regulate the duration of cytokine-induced signaling by interfering with the Janus tyrosine kinase/signal transducer and activator of transcription pathway. Con A markedly induces SOCS-1 and -3 in mouse liver (Hong et al., 2002) and evidence from SOCS-1 gene knockout mice indicates that it is crucial for counteracting inflammation, especially in the liver (Starr et al., 1998; Marine et al., 1999). Recent data indicate that the induction of SOCS-1 and -3 correlates with IL-6 elevation (Starr et al., 1997). After partial hepatectomy, SOCS-3 transcripts and protein are induced during the priming phase of liver regeneration, and this induction is greatly diminished in IL-6–deficient mice. Moreover, IL-6 injection induces SOCS-3 in the IL-6 KO animals, showing that IL-6 is required for SOCS-3 induction (Campbell et al., 2001). It was also demonstrated that injection of IL-10 into mice induced mRNA expression of SOCS-3 in the liver (Shen et al., 2000) and that SOCS-3 is involved in IL-10-induced anti-inflammatory responses in macrophages (Berlato et al., 2002). Thus, we assume that the elevation we observed in SOCS-1 and 3 gene expression was as a consequence of IL-6 and IL-10 induction by PRS-211,092.

Cytokine regulation by PRS-211,092 in both spleen and liver may be a result of its effect on immune cells such as resident or infiltrating T cells, which are known to be critical for the development of Con A-induced hepatitis (Sass et al., 2002). Our results show that IL-2, a hallmark of T-cell activation, was inhibited by PRS-211,092 in the liver and spleen (Figs. 2b and 3d). We therefore investigated the effect of PRS-211,092 on IL-2 in a T cell line (Jurkat) stimulated in vitro with PMA and calcium ionophore. We found out that PRS-211,092 can inhibit IL-2 gene expression (Fig. 5a) and IL-2 secreted protein (Fig. 5b) in a similar dose-dependent manner. Interestingly, the same dose range of PRS-211,092 was required for these cells to selectively inhibit NF-AT but not AP-1 (Fig. 5, c and d), both essential transcription factors for IL-2 induction. We therefore suggest that NF-AT may be a component in a yet-to-be-explored signaling pathway that is targeted by PRS-211,092 and contributes to its effect on cytokine modulation.

In conclusion, we propose that PRS-211,092 reduces liver injury through early induction of IL-6 and IL-10 followed by an increase in SOCS proteins, which in turn down-regulate pro-inflammatory cytokine and chemokine signaling. This is the first time, to our knowledge, that evidence has been presented for the induction of SOCS-1 and 3 by any natural or synthetic cannabinoid. Because there are indications that Δ^9-tetrahydrocannabinol and endocannabinoids such as anandamide have immunomodulatory properties (Berdyshev, 2000), we suggest that the negative feedback control of cytokine signaling by the SOCS may be a generic feature of cannabinoids. In contrast to PRS-211,092, which partially inhibits pro-inflammatory cytokine expression, FK 506, the commonly used immunosuppressant, completely shuts down the immune response after Con A challenge (Figs. 2 and 4). Thus, unlike FK 506, PRS-211,092 down-modulates inflammation but maintains an active immune defense system.

In summary, the protective effect of PRS-211,092 on immune-mediated hepatitis through cytokine modulation suggests that this compound and its congeners may provide the basis for developing immunomodulatory drugs for treating hepatitis, as well as for other short- and long-term inflammatory diseases.

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