A beneficial aspect of a CB1 cannabinoid receptor antagonist: SR141716A is a potent inhibitor of macrophage infection by the intracellular pathogen *Brucella suis*

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Abstract: The psychoactive component of marijuana, Δ^9-tetrahydrocannabinol (THC) suppresses different functions of immunocytes, including the antimicrobicidal activity of macrophages. The triggering of cannabinoid receptors of CB1 and CB2 subtypes present on leukocytes may account for these effects. We investigated the influence of specific CB1 or CB2 receptor antagonists (SR141716A and SR144528, respectively) and nonselective CB1/CB2 cannabinoid receptor agonists (CP55,940 or WIN 55212-2) on macrophage infection by *Brucella suis*, an intracellular gram-negative bacteria. None of the compounds tested affected bacterial phagocytosis. By contrast, the intracellular multiplication of *Brucella* was dose-dependently inhibited in cells treated with 10–500 nM SR141716A and 1 μM SR141716A-induced cells exerted a potent microbicidal effect against the bacteria. SR144528, CP55,940, or WIN 55212-2 did not affect (or slightly potentiated) the growth of phagocytized bacteria. However, CP55,940 or WIN 55212-2 reversed the SR141716A-mediated effect, which strongly suggested an involvement of macrophage CB1 receptors in the phenomenon. SR141716A was able to pre-activate macrophages and to trigger an activation signal that inhibited *Brucella* development. The participation of endogenous cannabinoid ligand(s) in *Brucella* infection was discussed. Finally, our data show that SR141716A up-regulates the antimicrobial properties of macrophages *in vitro* and might be a pharmaceutical compound useful for counteracting the development of intramacrophagic gram-negative bacteria. *J. Leukoc. Biol.* 67: 335–344; 2000.

Key Words: *gram-negative bacteria · marijuana · CP55,940 · WIN 55212-2*

INTRODUCTION

In addition to their psychotropic effects, cannabinoid ligands (CB) have been widely described as modulating immune responses [reviewed in ref. 1]. Indeed, the immunosuppressive effects of Δ^9-tetrahydrocannabinol (THC) have been well documented both *in vivo* [2–5] and *in vitro* [5, 6]. For instance, THC inhibits mitogen-induced proliferation of T lymphocytes or B lymphocytes [7], cytotoxic T cell activity [5, 8], antibody synthesis [9], interferon-γ (IFN-γ) production [10], and Th1 activity in *Legionella pneumophila*-infected mice [2]. THC also suppresses macrophage activities and inhibits tumor necrosis factor α (TNF-α) production [11, 12] and the antimicrobicidal response of these cells against *Legionella pneumophila* [13]. By contrast, THC and other CB were sometimes reported to enhance immune cell responses: they potentiate interleukin-1 (IL-1) bioactivity released by mouse resident macrophages or human macrophagic cell lines [14] and, at low serum concentration, CB display growth-enhancing activity on human tonsillar B cells [15]. Studies on CB receptors expressed on immune cells suggested that THC effects could be mediated through these receptors because THC is an agonist of these receptors [16–20].

Two distinct receptors (namely, CB1 and CB2) that interact with THC and several CB have been identified. They both couple to guanine nucleotide-binding proteins [reviewed in ref. 21]. The CB1 receptor is referred to as the brain cannabinoid receptor because of its predominant expression in the central nervous system. However, Bouaboula et al. [19] have demonstrated that these receptors are also expressed in several leukocyte populations, including monocytic cells. The CB2 receptor is referred to as the peripheral cannabinoid receptor because its gene is expressed primarily in peripheral tissues. CB2 receptors were identified on mouse and rat spleen cells [17], and it was suggested that these sites are also located on myeloid and/or B cells [18, 20]. There is some evidence that suggests CB2 receptors may be involved in CB-triggered modulation of lymphocyte [15, 22–23] or natural killer (NK) cell functions [23]. On the contrary, no CB1 receptor-mediated immune functions have been reported to date. Nevertheless, it was recently postulated that in rat, macrophage CB1 receptors might contribute to hemorrhagic hypotension [24], with anandamide produced by macrophages [24, 25] being a mediator of this effect.

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Received April 13, 1999; revised September 22, 1999; accepted October 29, 1999.

Macrophages are considered to be a target of infection by several facultative intracellular bacteria such as *Legionella pneumophila*, *Salmonella spp.*, *Mycobacterium spp.*, and *Brucella spp.* On the other hand, the macrophage itself is the host first line of defense and its response either leads a full immune response directed against the pathogen or permits the pathogen to multiply and spread. The development of the parasite greatly depends on the state of activation of the macrophage during its contact with the bacteria and the phagocytotic process. Consequently, triggering CB receptors expressed on monocytic cells [19] might interfere with phagocyte activity favoring either the development or the elimination of intracellular bacteria. This possibility could explain why *in vitro* treatment of murine macrophage cultures by THC, which is a nonselective agonist of CB1 and CB2 receptors, suppressed the growth restriction of *Legionella pneumophila*. Moreover, if as mentioned previously [24, 25], CB receptor endogenous agonists are produced by leukocytes, they might inhibit macrophage activation and help intracellular bacteria to circumvent the host defense. We thus investigated the influence of CB ligands on models of human and murine macrophage infection by the facultative intracellular gram-negative bacteria *Brucella suis* [28–30]. Nonselective CB1/CB2 cannabinoid receptor agonists (CP55,940 or WIN 55212-2 [1]) and specific antagonists of CB1 (SR141716A [26]) and CB2 (SR144528 [27]) receptors were assayed. We found that the recently described antagonist of CB1 receptor, SR141716A, was a potent inhibitor of *B. suis* intracellular multiplication in human and murine macrophages. By contrast, SR144528, CP55,940, or WIN 552212 did not change, or slightly potentiated, the growth of phagocytized bacteria. The SR141716A action was associated with macrophage CB1 receptors. It was postulated that SR141716A and other putative CB1 receptor antagonists might form an interesting family of pharmacological compounds for inducing macrophage activation and could be highly potent in counteracting intracellular bacterial infections.

MATERIALS AND METHODS

Reagents

CP55,940 [(-)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl) cyclohexan-1-ol hydrochloride] was generously provided by Pfizer; WIN55212-2 [(R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphtyl) methoanone] was purchased from RBI (Natick, MA). The CB1 receptor antagonist SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide] and the CB2 receptor antagonist SR144528 [N-(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] hepta-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide)] were synthesized at Sanofi Recherche [26–27]. Drugs were dissolved in dimethyl sulfoxide at 10^{-3} M.

Bacterial strains and media

B. suis 503 was a human isolate [28]. Some experiments were performed with *GFP-B. suis*: *B. suis* producing the green fluorescent protein [31]. Bacteria were grown at 37°C to a stationary phase in tryptic soy (TS) broth medium (GIBCO-BRL, Life Technologies, Cergy-Pontoise). In some experiments, bacteria were opsonized at 37°C for 45 min in 500 µL phosphate-buffered saline (PBS) containing 1/2000 heat-inactivated anti-*Brucella* antiserum [30].

Cell culture

THP-1 and J774A.1 cells were from the American Type Culture Collection (Rockville, MD). Cells were maintained at 37°C in 5% CO_2, RPMI-1640 medium supplemented with 5 mM glutamine (GIBCO-BRL) and 10% (v/v) heat-inactivated fetal calf serum (FCS; Sigma Chimie, Saint-Quentin, France). They were checked for the absence of mycoplasma by 4,6-diamino-2-phenylindole fluorescence.

Human monocytes

Human monocytes were prepared from blood of healthy donors as described previously [28].

THP-1-derived monocytes

THP-1 were differentiated into monocyte-like cells with 10^{-7} M 1,25-dihydroxyvitamin D_3 (VD3) (Hoffman-Laroche, Basle, Switzerland) [29]. They were then cultured overnight in serum-deprived conditions [serum-derived medium (SDM): RPMI medium supplemented with only 0.5% serum], before infection. Similarly, the night before infection, J774 A.1 cells were cultured overnight in SDM.

Analysis of CB1 and CB2 receptor mRNAs by reverse transcriptase-polymerase chain reaction (RT-PCR)

The expression of mRNAs for β_2-microglobulin, CB1, and CB2 receptors was examined by semi-quantitative RT-PCR. The mRNA purification from THP-1 or J774A.1 cells, followed by conversion to first-strand cDNA, were performed with the PolyATractR Serie 9600™ mRNA Isolation System with cDNA Synthesis (Promega, Charbonnières, France). DNA amplifications and PCR product analyses were carried out exactly as described [20].

The oligonucleotides were purchased from Genset (Paris, France). The primers used for the amplification mouse genes were: β_2-microglobulin primers, sense 5′-TGACCGGCTTGTATGCTATC-3′; antisense 5′-CAGTGTGAGCCAGGATATAG-3′. CB1 primers: sense 5′-TTGATGAAACCTACCTGATG-3′; antisense 5′-CACCTTGGCGATCTTAAC-3′. CB2 primers: sense 5′-GCCTGCAACTTTGTCATC-3′; antisense 5′ -GGCTTTCCAGAGGACATAC-3′. The expected amplicon sizes were 222 bp for β_2-microglobulin, 565 bp for CB1 receptor, and 368 bp for CB2 receptor. The sequence primers for the corresponding human genes were described previously [20].

cAMP assays

VD3-THP-1 cells were cultured overnight in 24-well plates (10^6 cells in 1 mL/well) in RPMI-1640, washed, and cultured in the same medium. They were then either pretreated or not with cannabinoid receptor ligands for 30 min before a 30-min stimulation with 1.5 µM forskolin (Sigma Aldricht, Saint Quentin, France) in the presence of 0.1 mM Ro-20-1724 (Tebu, Le Perray-en Yvelines, France [33]). Cells were lysed and their intracellular cAMP measured with a Biotrak cAMP EIA system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Values are means of triplicate determinations ± SEM.

Infection assay

Infection of human monocytes, THP-1-derived monocytes, or mouse J774A.1 cells with *B. suis* was performed, as described [28–30], in 24-well plates (Falcon, Becton Dickinson, Meylan, France). Briefly, bacteria were centrifuged and resuspended in RPMI-1640. Cells ($6–8 \times 10^5$) cultured overnight in 1 mL SDM were washed and incubated for 30 min at 37°C with 150 µL of bacterial RPMI suspension, corresponding to a bacteria-to-cell ratio of 20 for monocytes or THP-1-derived monocytes [29] and 50 for J774A.1 cells [30]. They were then extensively washed with PBS to remove non-phagocytized bacteria. Infected cells were reincubated for a further 60 min with 1 mL of SDM supplemented with 50 µg/mL of gentamicin sulfate to kill any remaining extracellular bacteria, and bacterial phagocytosis was measured (time 0 of culture). The gentamicin concentration was sufficient to kill bacteria within 60 min and did not impair the intracellular multiplication of *Brucella*. At various post-infection times, culture supernatant was removed and the number of intracellular viable bacteria evaluated by colony-forming unit (CFU) determination from replicate plates and serial dilutions of cell homogenates as previously described [28].

The results are expressed in CFU/well or multiplication index [MI = (CFU/well) at one time point/(CFU/well) at *time 0* of culture].

Statistical comparisons of intracellular bacteria development

To evaluate the effect of the different CB ligands on the *Brucella* multiplication, infection and post-infection cultures were performed in SDM supplemented with CB ligands. Bacteria MI were measured after 48 h post-infection and compared with bacteria MI in the control (parallel infection in the absence of compound). Data were analyzed by paired Student's *t* test. When indicated, cells were pretreated with the compound before infection.

Bactericidal and bacteriostatic effects induced by SR141716A

The SR141716A-induced bactericidal and bacteriostatic effects were defined (1) by the lowest concentration of drug that activated macrophages to eliminate phagocytized bacteria over 48 h and decrease 100-fold the counts of viable *B. suis* in macrophages and, (2) by the lowest concentration of drug in the presence of which no change in counts of viable *B. suis* occurred. (At concentrations indicated in the text, some decrease in the number of CFU/mL may have occurred, usually no more than 10%).

Fluorescence microscopy assessment of infection

J774 A.1 cells (10^5 cells/well in 400 µL MDS) were cultured overnight in eight-chamber culture slides (Lab-Tek, Nunc, Naperville, IL) with the different compounds and infected for 30 min at 37°C with 100 µL of opsonized or nonopsonized GFP-*B. suis* suspension (bacteria-to-cell ratio = 50) and treated as above. Infected cells were reincubated in 400 µL SDM, supplemented with 50 µg/mL of gentamicin together with the studied compound. They were then analyzed at different time periods. Infection was assayed by consecutive visualization of the *GFP-B. suis*-infected cells by phase-contrast microscopy and ultraviolet fluorescent microscopy (Leica DM IRB).

Toxicity of CP55,940, WIN55212-2, SR144528, and SR141716A

Cell toxicity of CB ligands was evaluated by measuring lactate dehydrogenase (LDH) activity in 100 µL infected culture supernatants with the CytoTox96 Promega kit (Promega, Charbonnières, France): phagocyte cultures treated for 48 h with 2 µM CP55,940, 5 µM WIN55212-2, 10 µM SR144528, 10 µM SR141716A exhibited no significant differences in cell viability between control and drug-supplemented cultures, i.e., 95–90% of cells remaining viable in the different conditions. In parallel, in *B. suis* cultures performed in TS medium (or in SDM) in the presence of different CB ligands at concentrations up to 10 µM, bacteria number measurements (CFU/mL of culture) at different times up to 24 h did not reveal any significant effect of drugs on bacterial development.

Analysis of macrophage membrane antigens

VD3-differentiated THP-1 cells treated with SR141716A (or not), were infected (or not) with *B. suis*. Forty-eight hours later, adherent cells were harvested with cell dissociation solution (Sigma Chimie). They were incubated with the monoclonal antibodies anti-CD14, anti-CD16, or anti-ICAM 1 (Immunotech, Marseille, France), then with a fluorescein-labeled anti-mouse IgG $F(ab')_2$ fragment (Immunotech), as previously described [32], and analyzed by cytofluorimetry.

Reactive oxygen species (ROS) measurement

Production of ROS ($O_2^{-}\cdot$ + $^{\cdot}NO$) was analyzed as previously described (34): (1) by monitoring the luminol-dependent chemiluminescence (LCDL) produced over 1 h by 5×10^5 SR141716A-pretreated VD3-THP-1 cells activated by 50 µL *B. suis* (or opsonized *B. suis;* MOI = 100). Experiments were performed in 1 mL phenol red-free RPMI medium containing 10^{-4} M luminol supplemented (or not) with 1 µM SR141716A in an automatic luminescence analyzer (Lumicon, Hamilton, Switzerland). Measurements were performed in the presence and absence of 2.5 mM N^G-monomethyl-L-arginine (L-NMMA) to differentiate the LCDL resulting from the different types of ROS ($O_2^{-}\cdot$ or $^{\cdot}NO$) produced during bacteria phagocytosis [34]. (2) By measuring nitrite and nitrite-derived nitrate concentrations with Griess reagent [30], in supernatants of 10^6 VD3-THP-1 (or SR141716A-treated VD3-THP-1 cells), infected in the conditions shown in Figure 1 (or not) with *B. suis* (or opsonized *B. suis*) and cultured for 24 and 48 h. In these experiments, Dulbecco's modified Eagle's medium (DMEM; with low NO_2 content) substituted for RPMI.

RESULTS

CB receptor expression on VD3-differentiated THP-1 cells

VD3-differentiated THP-1 cells phagocytose bacteria and constitute a convenient model to assess human macrophage infection by gram-negative bacteria [29]. We first confirmed the presence of CB1 and CB2 receptor transcripts in THP-1 cells by semi-quantitative RT-PCR, CB1 receptor mRNAs being expressed at a higher level than CB2 receptor mRNAs, as previously reported [19]. We then noticed that the cell differentiation by VD3 did not significantly modify transcript levels for both receptor types (data not shown). These observations, which suggested a possible expression of CB receptors on VD3-THP-1 cells, provided a molecular basis for evaluation of effects of cannabinoid ligands on phagocyte functions.

The effect of CP 55,940 on forskolin-stimulated cAMP synthesis was measured to indirectly analyze whether VD3-THP-1 cells really expressed functional cannabinoid receptors (**Table 1**). In serum-free medium, forskolin alone markedly activated adenylate cyclase, inducing a 10- to 15-fold elevation in intracellular cAMP compared with forskolin-unstimulated cells. Pretreatment of VD3-THP-1 cells with CP 55,940 decreased the forskolin-induced cAMP accumulation in a dose-related manner, thus verifying the expression of functional cannabinoid receptors on the cells [35]. Furthermore, as reported [33, 35, 36], the CB1 receptor antagonist SR141716A or the CB2 receptor antagonist SR144528 were able to reduce the CP55,940-induced inhibition of adenylate cyclase, respectively, with SR141716A exerting a more potent effect than SR144528. This regulation of cAMP level was consistent with an involvement of CB1 and CB2 receptors [33, 35–37], and

TABLE 1. Effect of SR141716A or SR144528 and CP55,940 on Forskolin-Stimulated cAMP in VD3-THP-1 Cells

Concentration of CP55.940	Intracellular AMPc (% of control)		
	Control	+SR141716A	+SR144528
1 nM	37.0 ± 13.8	71.0 ± 15.9	43.0 ± 12.3
10 nM	14.8 ± 2.5	51 ± 4.0	31.2 ± 7.8
100 nM	5.0 ± 3.5	33 ± 1.2	24.0 ± 4.7

VD3-THP-1 cells (10^6 cells/well) were cultured for 30 min in serum-free medium in the presence or absence of 250 nM SR141716A or SR144528, then with different concentrations of CP55,940 for an additional 30 min before a 30-min stimulation with 1.5 mM forskolin in the presence 0.1 mM Ro-20-1724. The reaction was stopped by addition of 50 mM Tris-HCl, 4 mM EDTA. cAMP levels were determined as in Materials and Methods and expressed as percentage of cAMP level in forskolin-stimulated cells. Results are means of triplicate determinations (±SD). The level of cAMP was 14 ± 2 pmol/well in forskolin-treated cells (control cells) and 0.9 ± 0.2 pmol/well in resting untreated cells.

agreed with the presence of CB1 receptor protein on THP-1 cells [38].

Effects of CB1 and CB2 receptor agonists or antagonists on *B. suis* multiplication within VD3-differentiated THP-1 cells

In accordance with our previous results [29], measurement of CFU over 48 h of infection showed that phagocytized *B. suis* survived, and after a limited decrease in their intracellular number, efficiently replicated in VD3-differentiated THP-1 cells, the number of intracellular bacteria increasing 500-fold in infected cultures supplemented with gentamicin (**Fig. 1**).

To assess the effect of different CB ligands on *B. suis* intramacrophagic multiplication, overnight SDM cultured cells were pretreated for 30 min with 1 μM CP55,940, WIN55212-2, SR141716A, and SR144528. They were then infected with *B. suis* and cultured with the same concentration of product. Figure 1 shows that cell treatment by CP55,940, WIN55212-2, and SR144528 did not significantly modify the phagocytosis index of the bacteria nor their intracellular multiplication. By contrast, in the presence of the CB1 receptor antagonist SR141716A, the macrophage controlled *Brucella* multiplication. SR141716A inhibited the intracellular bacterial development and in some experiments slightly lowered the number of live bacteria within the cells (Fig. 1A). Because the number of phagocytized bacteria in controls may vary two- to fourfold, depending on the cell and bacteria preparations, the preceding experiments were repeated 10 times. Figure 1B confirms that 48 h after infection, intracellular *Brucella* multiplication was not significantly different in CP55,940-, WIN55212-2-, or SR144528-treated cells, or in drug-free macrophage cells, whereas macrophage treatment by SR141716A totally prevented *Brucella* growth ($P < 0.005$) without affecting *B. suis* phagocytosis (Fig. 1A). The inhibitory effect induced by SR141716A did not result from cell toxicity, which could expose intracellular bacteria to gentamicin killing: infected VD3-THP-1 cells treated for 48 h with 10 μM SR141716A exhibited no significant differences in cell viability between control and drug-supplemented cultures, 90% of cells remaining viable in both conditions (data not shown).

Moreover, doubling the CP55,940 and WIN55212-2 concentrations or increasing the SR144528 concentrations up to 10 μM did not inhibit *B. suis* multiplication within macrophages (data not shown).

Effects of CB1 and CB2 receptor agonists or antagonists on *B. suis* multiplication within human monocytes and J774A.1 cells

CB1 and CB2 receptor mRNAs are expressed in human monocytes [20] or in J774A.1 murine macrophage cells (data not shown). In these cells, contrary to THP-1 cells [19], CB1 receptor mRNAs are expressed at a lower level than CB2 receptor mRNAs [20, and not shown]. The experiments performed on VD3 THP-1 cells were repeated with these mononuclear phagocytes, which can be infected by *B. suis* in a very similar manner as VD3-differentiated THP-1 cells [28, 30]. **Figure 2** shows that the treatment of human monocytes or J774.A1 cells by 1 μM SR141716A induced an inhibition of intracellular bacteria multiplication. On the other hand, CP55,940, WIN55212-2, and SR144528 had no effect (data not shown). These data thus confirmed those observed on VD3-THP-1 cells and showed that SR141716A controlled *B. suis* infection in different types of monocytic phagocytes.

Microbicidal activity of VD3-THP-1 cells exposed to SR141716A before and/or during *B. suis* infection

To specify how SR141716A exerted its effect on *Brucella* infection, VD3-THP-1 cells were treated with this compound

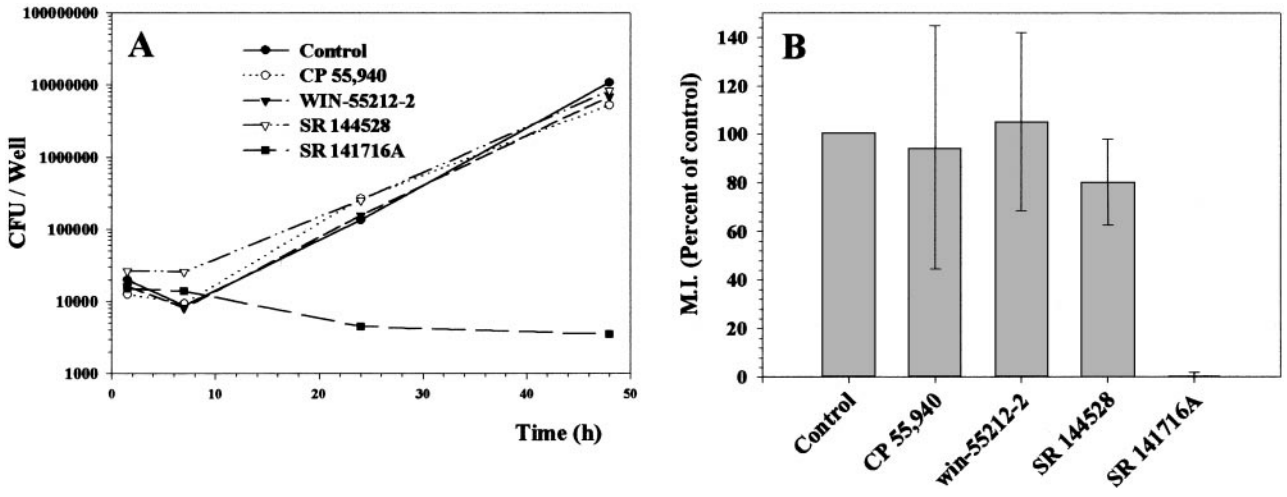

Fig. 1. Effect of cannabinoid ligands on VD3-treated THP-1 cell infection by *B. suis*. (A) Differentiated THP-1 cells were preincubated for 30 min without, or with different cannabinoid ligands, namely CP55,940, WIN55212-2, SR141716A, or SR144528, at 1 μM. Cells were then infected with *B. suis* and cultured for 48 h in the presence of the drug assessed. Intracellular cellular bacterial development was followed by CFU/well determination at different times. (B) Intracellular cellular bacterial development in the different cannabinoid-treated cells was measured at 48 h in 10 different experiments. For each drug, the MI were calculated at 48 h, as indicated in Materials and Methods, and compared with the MI of the control by a paired Student's *t* test. The (bacteria MI at 48 h in cannabinoid-treated cells)/(bacteria MI at 48 h in untreated cells) ratio was calculated. The histogram represents the mean ratio and the error bar the confidence interval of the mean.

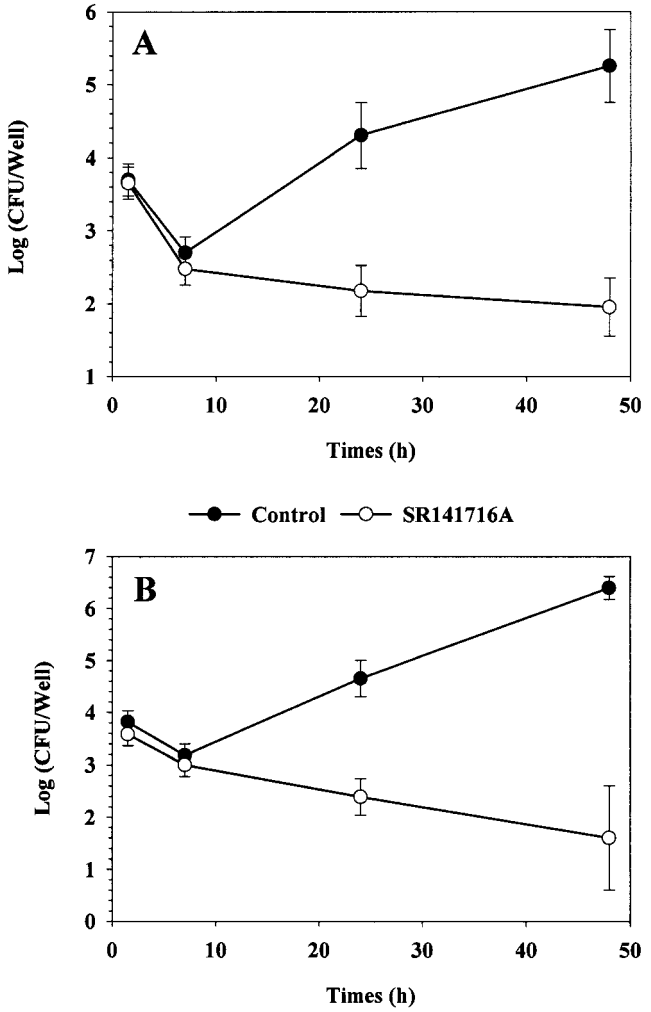

Fig. 2. SR141716A inhibits the infection of monocytes and J774A1 cells by *B. suis*. Human monocytes (A) or J774A1 cells (B) were pretreated or not for 24 h with 1 μM SR141716A concentrations, infected with *B. suis*, and cultured for a further 48 h in the absence or presence of 1 μM SR141716A. Intracellular cellular bacterial development was followed by CFU/well determination at different times as indicated in Materials and Methods. Data presented are means ± SEM ($n = 3$).

for different periods of time before and/or during infection. For each condition, the phagocytosis potential of the cells as well as bacterial intracellular growth were determined. **Figure 3** shows that: (1) cells pre-incubated for 24 h with 1 μM SR141716A phagocytized bacteria in a similar manner as 30-min-pretreated cells. However, in SR141716A-supplemented medium, 24-h-pretreated cells elicited a higher capacity to inhibit *B. suis* proliferation than 30-min-pretreated cells. Increasing the preincubation time with SR141716A from 24 to 48 h did not affect the capacity of the cells to inhibit bacteria growth (Fig. 3A). (2) The SR141716A-induced inhibition of *B. suis* development was not observed when SR141716A was washed (if necessary in case of preincubation) and omitted from the culture medium during the whole infection process, regardless of the duration of cell pretreatment (24 h, 30 min, or no pretreatment; Fig. 3A). (3) SR141716A was of reduced effectiveness when it was added to infected cells, i.e., after bacteria phagocytosis. A slower development of the bacteria was still observed, but the SR141716A-mediated inhibition, optimal when the drug was added at the onset of infection, decreased (Fig. 3B, SR141716A addition 6 h post-infection). When SR141716A was added after the beginning of the exponential phase of *B. suis* growth, it did not induce any inhibitory effect (Fig. 3B, SR141716A addition 10 h post-infection).

Pre-incubation time of macrophages with CP55,940, WIN55212-2, or SR144528 up to 48 h did not affect *B. suis* phagocytosis and development. By contrast, in some experiments, treatment with CP55,940 during infection slightly potentiated the bacterial growth (less than twofold) within the cells (data not shown).

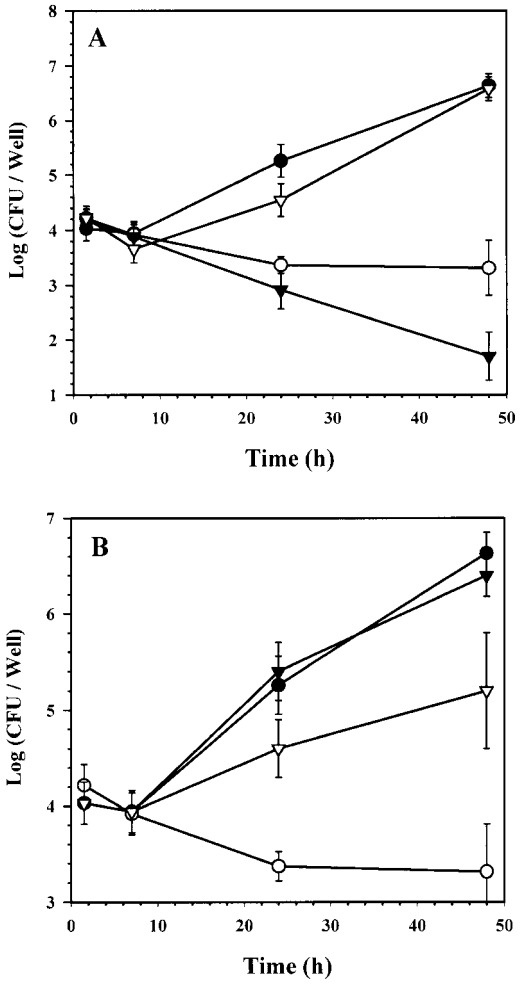

Fig. 3. *B. suis* growth in VD3-THP-1 cells exposed to SR141716A before and/or during *B. suis* infection. (A) Differentiated THP-1 cells were pretreated for 30 min (open circles) or 24 h (triangles) with 1 μM SR141716A, or not (filled circles). They were then either infected and cultured in the presence of 1 μM SR141716A (open circles, filled triangles) or infected and cultured in the absence of drug (filled circles, open triangles). (B) Cells were treated or not (filled circles) with 1 μM SR141716A 30 min before infection(open circles), or 6 h (open triangles) or 10 h (filled triangles) after the onset of infection and cultured in the presence (open circles, filled and open triangles) or absence (filled circles) of 1 μM SR141716A during the continuation of the experiment. The intracellular development of the bacteria was followed by determining CFU/well at different times post-infection. Data presented are means ± SEM (error bars) of four different experiments ($n = 4$).

Efficiency of SR141716A for inducing phagocyte microbicidal activity

VD3-differentiated THP-1 cells were pretreated for 24 h with different SR141716A concentrations, infected with *B. suis*, and cultured for a further 48 h in the presence of SR141716A at the same concentration as during pretreatment. Bacteria MI were then measured. Results are shown in **Figure 4**. (1) The drug concentration that induced a 100-fold decrease in the intracellular bacterial population (i.e., a bactericidal effect) was close to 1 μM, (2) the lowest concentration of SR141716A that triggered bacteriostatic properties in infected phagocytes (no change in counts of phagocytized *B. suis*) ranged near 500 nM; (3) the CB1 receptor antagonist promoted a 10-fold inhibition of *B. suis* development at 100 nM and exerted a significant effect (50% inhibition of bacterial growth) at 10 nM.

A similar dose-related effect of SR141716A was observed when J774A.1 cells or human monocytes were infected instead of VD3-THP-1 cells (data not shown).

Involvement of CB1 receptors in the anti-*Brucella* effect of SR141716A

In brain, SR141716A specifically antagonizes effects mediated by CB1 receptors. Because cannabinoid receptors were possibly expressed on monocytic phagocytes and were functional, the involvement of such receptors in the SR141716A-mediated inhibition of *B. suis* infection was analyzed. The CB ligands CP55,940 or WIN 55212-2 did not interfere with *B. suis* proliferating capacity within differentiated THP-1 cells or slightly increased it (Fig. 1). When added simultaneously with SR141716A in differentiated THP-1 cells, these compounds inverted the SR141716A-induced bactericidal effect against intramacrophagic *B. suis* (**Fig. 5**). CP55,940 and WIN 55212-2 also prevented the restriction of *B. suis* growth mediated by the

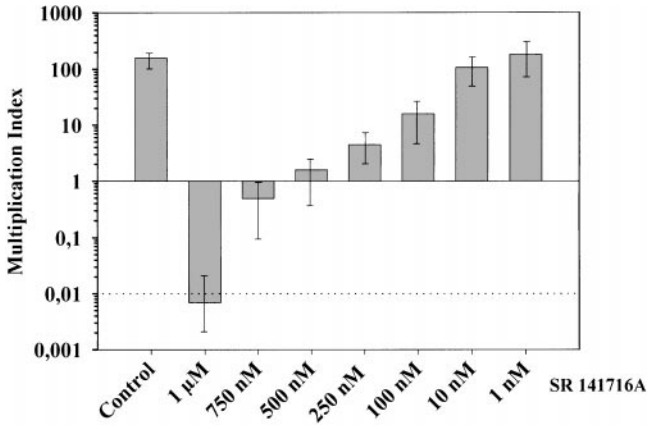

Fig. 4. *B. suis* infection of monocytic phagocytes is inhibited by SR141716A in a dose-dependent manner. VD3-differentiated THP-1 cells were pretreated for 24 h with different SR141716A concentrations, infected with *B. suis*, and cultured for a further 48 h in the presence of SR141716A at the concentration used during pretreatment. The multiplication index (MI) of the intracellular bacteria were then calculated as indicated in Materials and Methods. The continuous line corresponds to MI = 1 (bacteriostatic effect). Dotted line indicates MI = 0.01, (bactericidal effect). Each measurement of the number of viable bacteria represented the average of counts obtained in triplicate. Data presented are means ± SEM ($n = 5$).

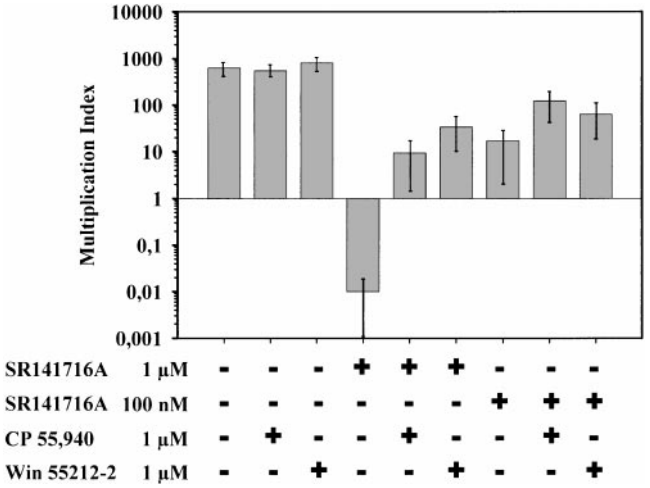

Fig. 5. CB1 agonists reversed the SR141716A-induced inhibition of *B. suis* infection. Differentiated THP-1 cells were pretreated with different combinations of CB ligands (for each condition. The concentration of the different assayed compounds is reported under corresponding bars). Cells were then infected with *B. suis* and cultured for a further 48 h in conditions similar to those of the pretreatment. The intracellular *Brucella* MI were then calculated for each experimental condition. Data presented are means ± SEM ($n = 4$).

CB1 receptor antagonist (Fig. 5). On the contrary, the CB2 antagonist SR144528, inefficient by itself, was unable to counteract the SR141716A-triggered microbicidal activity of VD3-THP-1 macrophage-like cells (data not shown).

Opsonization of the bacteria with specific antibodies increased the number of live phagocytized bacteria [30] and the use of the *GFP-B. suis* allowed direct evaluation of intramacrophagic bacterial development by fluorescence microscopy. Parallel observations of *GFP-B. suis*-infected J774A.1 cells by phase-contrast and fluorescence microscopy revealed a high number of bacteria within J774A.1 cells, 50–60% of cells being infected 48 h after the onset of infection (**Fig. 6A**). By contrast, in accordance with the above-reported results, in SR141716A-treated J774A.1 cell cultures, only a small percentage of cells (5–8%) were infected and bacterial development was dramatically reduced (Fig. 6B). Moreover, in the same experiment, CP55,940 treatment, which significantly supported the replication of *B. suis* (Fig. 6C), noticeably suppressed the growth-restricting potential of SR141116A in J774A.1 cells (Fig. 6D).

SR141716A potentiated *B. suis*-induced macrophage activation but not ROS production

CD14 is a lipopolysaccharide (LPS) receptor expressed on differentiated macrophages, and ICAM-1 is an adhesion molecule that is strongly induced on activated leukocytes. **Figure 7** shows that *B. suis*-infected THP-1 macrophages overexpressed these antigens, confirming their activation occurring during the *Brucella* infection process [29, 30]. Cell treatment by SR141716A affected neither CD14 nor ICAM-1 expression on differentiated THP-1 cells. By contrast, the CB1 receptor ligand was shown to enhance *B. suis*-induced CD14 expression, whereas it did not significantly change the *B. suis*-induced expression of ICAM-1 molecules. In parallel, the FCγRIII of immunoglobulins (CD16), which was expressed neither on

THP-1 cells nor on *B. suis*-infected cells was induced in SR141716A-treated cells infected by the bacteria.

The antimicrobial activity of macrophages can be regulated by formation of ROS during and after phagocytosis. On the other hand, THC was shown to attenuate nitric oxide synthase expression in LPS-induced macrophage RAW 264.7 cells [35]. Because *Brucella* is a poor inducer of ROS [39], we tested whether, on *Brucella* infection, SR141716-treated VD3-THP-1 cells could produce more superoxide anion and/or nitric oxide than untreated cells. Our experiments did not exhibit any increased production of ROS in *Brucella*-infected cells on SR141716A treatment (data not shown). In parallel, the weak production of NO (4 ± 2 µM) induced by *Brucella* in 48 h was not significantly modified in SR141716A-treated cells. These findings showed that the SR141716A effect was not mediated by an overproduction of ROS.

DISCUSSION

Most studies that have examined the effects of cannabinoids on immune functions to date generally demonstrate some degree of immunosuppression after immunocyte exposure to CB ligands [1]. Concerning bacterial infection, it was shown that THC-treated mice fail to develop any protective immunity against the intracellular bacteria *L. pneumophila* [2, 13]. This could be explained by a direct effect of the CB1 and/or CB2 receptor agonist THC on macrophages, with the THC treatment of macrophages in *in vitro* cultures preventing cells from exerting antibacterial activity [13]. Therefore, the involvement of CB receptors in the phenomenon is postulated. However, the nature of the receptor(s) involved remains unclear because THC does not discriminate between CB1 and CB2 receptors [21, 40].

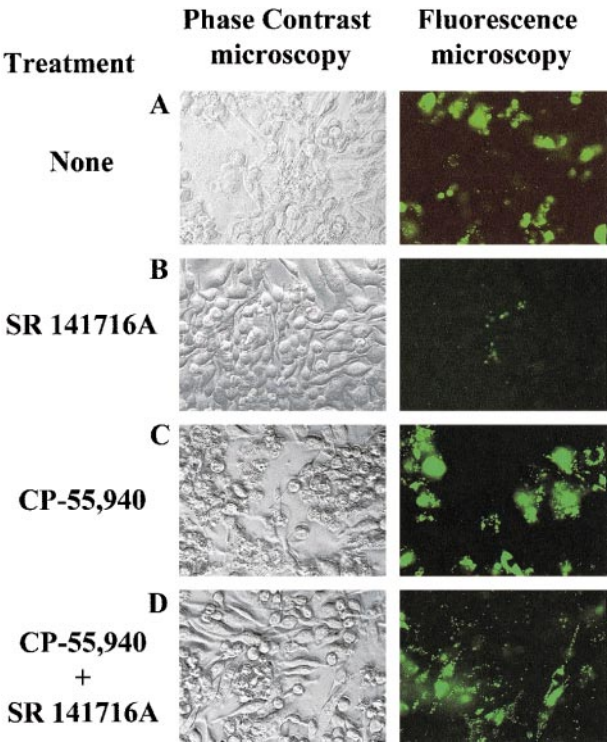

Fig. 6. Microscopic visualization of the inhibition of intracellular *B. suis* development in SR141716A-treated J774A1 cells. J774A1 cells were pretreated for 24 h with the cannabinoid ligands shown. Drugs were used at 1 µM. Cells were then infected with opsonized *GFP-B. suis* and cultured in conditions similar to those of pretreatment. Forty-eight hours after the onset of infection, intracellular bacterial development was characterized by concomitant visualization of the *GFP-B. suis*-infected cells by phase-contrast microscopy and ultraviolet fluorescent microscopy. The results shown are representative of three different experiments.

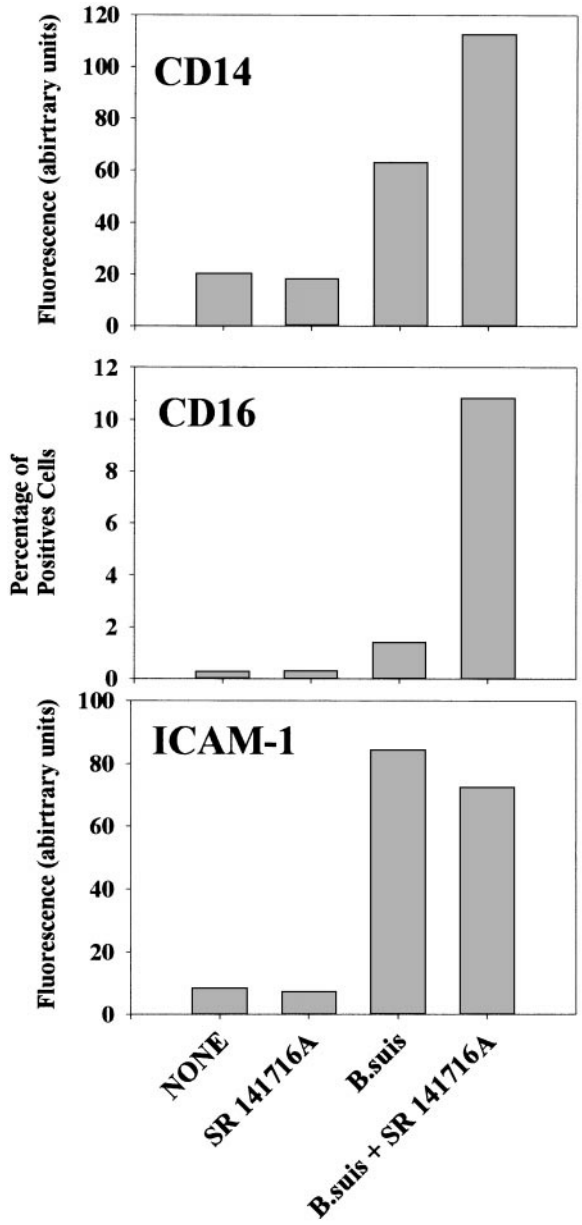

Fig. 7. CD14, CD16, and ICAM-1 expression in differentiated THP-1 cells treated with SR141716A and infected with *B. suis*. VD3-differentiated THP-1 cells treated (or not) with 1 µM SR141716A for 24 h were infected (or not) with *B. suis* (the different conditions are indicated on the *x*-axis). They were then cultured in conditions similar to those of the pretreatment. Forty-eight hours later, the adherent cells were harvested, incubated with the monoclonal antibodies anti-CD14, anti-CD16, or anti-ICAM 1, and treated with a fluorescein-labeled anti-mouse IgG $F(ab')_2$ fragment. They were then analyzed by cytofluorimetry. Because most of the infected cells were CD14 and ICAM-1 positive, the modulation of these antigens was analyzed by determination of the mean fluorescence intensity of cytofluorographs. CD16 expression was analyzed by evaluation of the percentage of CD16-positive cells.

Here, we report an unexpected effect of the CB1 receptor antagonist SR141716A: the growth of the intracellular bacteria *B. suis* was markedly inhibited in SR141716A-treated macrophage-like cells. In fact, SR141716A increases the microbicidal activity of phagocytes against intracellular *B. suis*: (1) in the absence of cells, SR141716A did not directly affect *B. suis* proliferation, (2) the bacteria phagocytosis was similar in control and SR141716A-treated cells, (3) the SR141716A-induced inhibition of *B. suis* growth depended on cell treatment conditions, and (4) 1 μM SR141716A-treated phagocytes eliminated the phagocytized *B. suis*.

The specificity of SR141716A was shown by comparing its effect with that induced by other CB synthetic ligands, including the CB2 antagonist SR144528. None inhibited the intramacrophagic *B. suis* growth. On the contrary, in line with previous observations reporting that macrophages treated by high concentrations (>5 μM) of THC were more permissive to *L. pneumophila* infection than untreated ones [13], 1 μM CP55,940 sometimes slightly favored intracellular bacteria proliferation. These different results observed in VD3-THP-1 cells and confirmed in J774A.1 cells and human monocytes were in favor of a specific action of SR141716A on human and mouse mononuclear phagocytes.

CB1 and CB2 receptors are most likely expressed in VD3-THP-1 cells, human monocytes, and J774A.1 cells, as suggested by the expression of both receptor type transcripts in these cells, the effect of CP55,940 on forskolin-activated adenylate cyclase in VD3-THP-1 cells, and the reversal of the effect of CP55,940 by specific CB1 or CB2 receptor antagonists [33, 35–36]. Based on previous observations [19, 41], this expression seems to be a general feature of monocytic phagocytes. In our experiments showing efficient inhibition of *B. suis* development, the SR141716A-mediated effect began at nanomolar concentrations and was maximum at 1 μM. This dose-related enhancement of the microbicidal activity of human and murine phagocytes against *B. suis* was in accordance with a receptor-mediated effect of the drug. SR141716A is a selective and potent CB1 receptor antagonist, its already well-documented specificity [21, 26, 40] agrees with a recent report claiming that CB1 receptor knockout mice behave exactly as SR141716A-induced mice when they are treated by THC [42]. The characterization of CB1 receptor protein on THP-1 cells by Western immunoblotting [38], the functionality of this receptor in VD3-THP-1 cells, and the SR144528 inefficiency on intra-macrophagic *B. suis* growth thus strongly suggests that in *Brucella* infection, SR141716A mediated a specific CB1 receptor process. Furthermore, the experiments which demonstrated that the CB1/CB2 receptor agonists CP55,940 or WIN55212-2 abrogated the SR141716A effect agree with this proposal. Alternatively, as mentioned for other CB1-mediated effects, SR141716A might also have acted as a partial agonist of CB2 receptors [43]. However, the effect of CP55,940 or WIN55212-2 on *Brucella* infection and the inability of SR144528 to reverse the SR141716A-induced phenomenon ruled out this possibility. Finally, even though they do not formally prove an interaction between CB1 macrophage receptors and SR141716A, the data altogether strongly support the possibility that such an interaction is responsible for the increased antimicrobial activity of phagocytes.

The development of a pathogen in a macrophage depends on its relative ability to activate or deactivate the phagocyte defenses that are normally triggered during infectious processes. The SR141716A-mediated signal thus appeared sufficient for preventing bacteria-induced macrophage deactivation. The data obtained with pretreated cells showed that SR141716A acted on at least two different levels, before and after phagocytosis. They demonstrated a direct effect of SR141716A on non-infected cells, which pre-activated the cells, rendering them more responsive to the drug. Different possibilities could explain this observation: (1) because the pre-activation step required a relatively long period (24 h), it could result from the synthesis of specific factor(s) regulating phagocyte differentiation and/or activation. CB ligands were known to modulate monokine production: THC inhibits the production of TNF-α [11, 12], and IL-12 [1], whereas it induces the secretion of IL-1 [14] and IL-10 [1]. (2) Exposure to SR141716A could also result in regulation of cell surface receptor expression that should lead to functional sensitization of the CB1 receptors. Such a complex phenomenon resulting in modulation of signal transduction pathways triggered through CB1 receptors was recently demonstrated in CB1 receptor-transfected Chinese hamster ovary cells [44].

CD14 and CD16 are membrane antigens expended on macrophage during bacterial infection [45] and inflammatory process [46], respectively, and are markers of macrophage activation. The synergistic effects of SR141716A on membrane antigen expression and the necessary presence of the drug after phagocytosis showed that SR141716A modulated phagocyte activation. This activation could involve different signals associated with CB1 receptor agonists, like adenylyl cyclase [47], ion channels [48], MAPkinase [49], and expression of the immediate-early gene *Krox 24* [50]. Moreover, in Chinese hamster ovary cells transfected with the CB1 receptor, SR141716A functions not only as an antagonist of CB1 receptors, but also as a selective inverse agonist for these receptors and delivers a biological signal that blocks the Gi protein [33]. It is thus possible that such a blockade triggers events that later on will result in prevention of *B. suis* infection.

The lack of SR141716A on bacterial phagocytosis, bacteria-induced formation of microbicidal ROS during this process, or intracellular bacteria number in the first hours after infection (Figs. 2 and 3) showed that the drug did not affect a very early step of *Brucella* installation. This observation was in line with the SR141716A-mediated inhibition of bacterial growth in *Brucella* and opsonized *Brucella*-infected cells: the inhibition similarly occurred in both types of infection, whereas the bacterial phagocytosis triggered different membrane receptors and different signals [30]. Together, these data show that the SR141716A-triggered biochemical events alter the bacteria development downstream from the signaling pathways occurring during phagocytosis. Moreover, the absence of SR141716A effect when it was added to infected cells during the exponential phase of *Brucella* growth suggests that the drug manipulates the adaptation process of bacteria before they impose the

conditions that allow their survival. This phenomenon could occur at (or after) the phagosome maturation level.

To date, no direct CB1 receptor-triggered immunomodulation has yet been reported and no SR141716A-induced reversal of leukocyte responses elicited by THC (or other CB agonists) has been observed. In our system, it is possible that SR141716A, as a selective inverse agonist for CB1 receptors [33], affects *B. suis* infection because it directly activates the macrophage anti-brucella properties. However, it was recently reported that SR141716A inhibits the activation of peripheral CB1 receptors in rats subjected to hemorrhagic shock by counteracting the effect of anandamide, an endogenous CB ligand produced by circulating monocytes [24]. These data and others [25] demonstrate that, besides many other neuromodulators [51], monocytic cells produce endogenous CB ligands. The endogenous CB ligand(s) released by infected macrophages are positioned for interactions with CB cell surface receptors, which may lead to macrophage deactivation and favor intracellular pathogen development, as observed in the presence of THC [2, 13]. The reversal of such an effect by a CB1 receptor antagonist could account for the anti-*Brucella* properties of SR141716A-treated cells. Our preliminary experiments showed that anandamide and 2-arachinoyl glycerol (2AG) were unable to reverse the effect of SR141716A. They thus counteracted this hypothesis, however, one cannot exclude that this observation should be due to the relative affinities of SR141716A and anandamide (or 2AG) or the instability of these endogenous ligands during the course of infection. Furthermore, it cannot be excluded that *Brucella* specifically modulated CB1 receptor expression (as in phorbol myristate acetate-induced THP-1 cells [52]) thus contributing by this mechanism to macrophage deactivation.

These findings demonstrate activation of host cell defense by a CB1 receptor antagonist and propose a possible way of impairing macrophage infection by an intracellular bacteria, *B. suis*, *in vitro*. In regard to these results, besides the effect on central nervous system [53], one can hypothesize that SR141716A (and perhaps some of its future congeners) might be a pharmaceutical compound useful for counteracting the development of intramacrophagic gram-negative bacteria. It will be necessary to determine whether the observed phenomenon could be generalized to other intracellular bacteria. Preliminary results showed that SR141716A inhibited macrophage cell infection by *Salmonella typhimurium* (not shown), another bacterium for which the macrophage is a possible host. In fact, *Salmonella typhimurium* and *Brucella* use very similar strategies to infect macrophages: they both inhibit the phagolysosome fusion [39, 54], and their development requires an acidification of bacteria-containing phagosomes [54, 55]. It is thus possible that the SR141176A-mediated effects should be closely linked to the strategy adopted by the pathogen to survive and develop inside its host. Analyses of the molecular mechanisms involved in SR141716A action will thus be necessary to specify the hypothetical application of this drug as a general non-antibiotic antimicrobial agent and the pathogenic bacteria concerned by its inhibitory effect. In addition, present and future studies on the microbicidal effect of SR141716A might help to clarify the role of the cannabinoid system in immune regulation and the relationship between endogenous CB ligands and bacterial infection.

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