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Title Page

Cannabinoid treatment suppresses the T helper cell polarizing function of mouse dendritic cells stimulated with *Legionella pneumophila* infection

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Running Title Page

a) Running title: THC suppresses the function of DCs

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d) Abbreviations: THC, Delta-9-tetrahydrocannabinol; DCs, dendritic cells; BM-DC, bone marrow-derived dendritic cell; Lp, *Legionella pneumophila*; Th1, type 1 T helper cell; Th2, type 2 T helper cell; IFN-gamma, Interferon gamma; IL-, Interleukin-; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; DMSO,

dimethyl sulfoxide; GM-CSF, granulocyte-macrophage colony stimulating factor; BCYE, buffered charcoal-yeast extract; HRP, streptavidin-horseradish peroxidase; TMB, tetramethyl benzidine; HBSS, Hanks balanced salt solution; PBS, phosphate buffered saline; CFU, colony forming units; RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

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Abstract

Marijuana cannabinoids, such as delta-9-tetrahydrocannabinoid (THC), suppress Th1 immunity in a variety of models including infection with the intracellular pathogen, *Legionella pneumophila* (Lp). To examine the cellular mechanism of this effect, bone marrow-derived dendritic cells (DCs) were purified from BALB/c mice and studied following infection and drug treatment. DCs infected *in vitro* with Lp were able to protect mice when injected prior to a lethal Lp infection; however, the immunization potential of the Lp-loaded cells along with Th1 cytokine production was attenuated by THC treatment at the time of *in vitro* infection. In addition, THC-treated and Lp-loaded DCs poorly stimulated in culture primed splenic CD4+T cells to produce IFN-gamma; however, this stimulating deficiency was reversed by adding recombinant IL-12p40 protein to the cultures. Moreover, THC treatment inhibited the expression of DC maturation markers such as MHC class II and co-stimulatory molecules CD86 and CD40 as determined by flow cytometry, as well as suppressed the Notch ligand, Delta 4, as determined by RT-PCR. However, THC treatment did not affect other DC functions such as intracellular killing of Lp determined by CFU counts of bacteria and Lp-induced apoptosis determined by annexin V staining. In conclusion, the data suggest that THC inhibits Th1 activation by targeting essential DC functions such as IL-12p40 secretion and the maturation and expression of co-stimulatory and polarizing molecules.

Introduction

Cannabinoid exposure increases susceptibility to infections (Klein and Cabral, 2006) and the mechanisms of this involve many factors including suppression of Th1 immunity (Newton et al., 1994; Klein et al., 2000; Klein, 2005). The mechanism responsible for suppressing Th1 immunity and polarizing to the Th2 response, however, remains elusive. In the current study, we postulated that the cannabinoids suppress adaptive immunity and Th1 polarization by inhibiting the function of dendritic cells (DCs).

DCs are professional antigen-presenting cells and able to induce and regulate innate and adaptive immune responses. DCs reside in peripheral tissues, capturing and processing pathogens and microbial products, and then migrating to lymphoid organs wherein DCs present these antigens to quiescent T cells (Kapsenberg, 2003). During this process, the phenotypic characteristics and functions of these cells change, including reduced phagocytic capacity and increased secretion of high levels of immunostimulatory cytokines and expression of MHC and co-stimulatory molecules (Kapsenberg, 2003). Furthermore, expression of the Notch ligands, Jagged and/or Delta, are increased which play a critical role in Th2 and Th1 polarization of naïve CD4 T cells (Amsen et al., 2004). We recently reported that THC treatment significantly suppresses IL-12p40 production in mouse bone marrow-derived DC (BM-DC) in culture (Lu et al., 2006). In the current study, we show that THC treatment of DCs impairs their immunizing and T helper polarizing function by inhibiting IL-12 production and the expression of essential co-stimulatory cell-surface proteins such as MHC class II, CD86, CD40 and the Notch ligand, Delta 4. This suggests that DCs might be a central target of THC-induced immune suppression.

Methods

Mice, Bacteria and Drugs. BALB/c mice, 7 wk of age, were obtained from NCI (Fredericksburg, MD) and housed and cared for in University of South Florida Health Sciences Center animal facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Serogroup 1, *L. pneumophila* (Lp; M124), is a virulent strain and obtained from a case of legionellosis at Tampa General Hospital (Tampa, FL) and cultured on BCYE medium (Difco, Detroit, MI) as described previously (Klein et al., 2000). After 48 hr, bacteria were adjusted spectrophotometrically to a working concentration. THC, obtained from the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD), was first diluted in dimethyl sulfoxide (DMSO) at 20 mg/ml and then in 5% fetal calf serum-RPMI1640 medium to a working concentration of 10 µM.

Preparation and treatment of bone marrow derived dendritic cells. Bone marrow cells were collected, as described before (Lu et al., 2006), from femurs and tibias of the BALB/c mice at 8 to 12 weeks age. Isolate cells were adjusted to1.0×10^6/ml in 6-well cell culture plates (GIBCO-Costar; Cambridge, MA) in RPMI1640 medium supplemented with 5 µM 2-mercaptoethamol, 2 mM L-glutamine, 1% antibiotic/antimycotic solution (Sigma; St. Louis, MO), 5% heat-inactivated fetal calf serum (HyClone; Logan, UT) and 10ng/ml granulocyte/macrophage colony-stimulating factor (GM-CSF) (BD-Pharmingen; San Diego, CA). After culture overnight, non-

adherent cells were removed and the adherent cells were re-incubated with fresh GM-CSF-containing medium for an additional 7-9 days, during which time the dendritic cells became nonadherent and were harvested. The purity of the cells was determined by flow cytometry staining using fluorochrome-conjugated monoclonal antibodies (mAbs) to CD11b and CD11c (BD-Pharmingen). The purity was about 100% $CD11b^+$ and greater than 75% $CD11c^+$ cells. The dendritic cells were either uninfected or infected with Lp at a ratio 10:1 for 30 min. Dendritic cells were then washed two times to remove non-internalized Lp and re-suspended to10^6 cells/ml and treated with either DMSO (vehicle control) or THC at 10 µM in the culture medium minus GM-CSF for 18 to 48 hr. In studies with CD4 T cells, cells were obtained from mice intravenously (iv) infected (primed) with a sub-lethal dosed of Lp (7×10^6) and the spleens were removed 5 days post-infection. The T cells were isolated from the splenocytes by mouse T cell Enrichment Columns (R&D system, Minneapolis, MN) and CD4+ T cells were negatively selected from the purified T cells with CD4 enrichment magnetic bead kits (BD-Pharmingen). Isolated CD4 T cells were then dispensed in 24-well cell culture plates (GIBCO-Costar) and co-cultured with DCs (CD4: DC = 10:1) in either the absence or presence of recombinant IL-12p40 (BD-Pharmingen) for 24 hr followed by cytokine analysis.

Animal injections and tissue sampling. Mice were immunized iv with $0.3\text{-}0.5 \times 10^6$ treated DCs suspended in PBS, and 7-9 days later spleens were isolated from mice. Single-cell suspensions of splenocytes (2×10^6 cells/ml) were cultured with formalin-killed Lp (10^7/ml) for 24 hr and supernatants collected for cytokine detection. Or, DC-treated mice were challenged iv with live Lp (sublethal dose, 7×10^6) diluted in pyrogen

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free saline. Spleens were obtained after 24 hr and CFUs of Lp were counted. In other experiments, mice were immunized iv with DCs (0.5×10^6) for two or three times at 7 day interval, challenged iv with a lethal dose of Lp ($1.7\text{-}2.0 \times 10^7$) and survival of mice was monitored.

Cytokine detection by ELISA. IL-12p40, IL-4, IL-23 and IL-10 were determined using a sandwich ELISA with antibody pairs from BD Pharmingen. In 96-well enzyme immunoassay plates (GIBCO-Costar), each well was coated with 50 µl of anti-murine antibody in 0.1 M NaHCO3, pH 8.2 (anti-IL-12 p40 for IL-12p40 and IL-23; 5 µg/ml) or in PBS (anti-IL-4 and anti-IL-10; 2 µg/ml) overnight at 4^{o}C. The wells of the plate were blocked with 150 µl of 3% BSA/0.05% Tween 20 in PBS (IL-12p40 and IL-23) or 0.5% BSA/0.05% Tween 20 in PBS (IL-4, IL-10) and incubated for 1 hr. The culture supernatants or serial dilutions of cytokine standards were added and incubated for 1-2 hr, followed by biotinylated detection antibodies (2ug/ml, 50ul) for 1 hr, and streptavidin-horseradish peroxidase (HRP) (1:1000 in 50ul) for 30 min. The plates were washed between each addition. The tetramethyl benzidine (TMB; Sigma) substrates were developed for 5-30 min; the reaction was stopped with 1 N sulfuric acid and read at 450 nm on an E_{max} microplate reader (Molecular Devices; Menlo Park, CA). The concentrations of sample cytokines were calculated from standard curves that were done for each plate. The levels of IFN-γ and IL-12p70 in supernatants were measured using BD OptEIA^{TM} Sets (BD Pharmingen) according to the manufacturer instructions.

Cell surface marker analysis by flow cytometry. To evaluate the effects of THC on MHC class II, CD86 and CD40 expression on DCs, cells, either uninfected or infected

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with Lp, were treated with DMSO or THC 10 μM for 48 hr. Following incubation, the cells were treated with fluorochrome-conjugated mAbs (BD-Pharmingen) at 4°C for 30 min, and then washed in PBS containing 2% BGS, and fixed in 1% paraformaldehyde. Cells were analyzed using flow cytometry. The following fluorochrome-conjugated mouse mAbs were used for DC surface marker staining: PE-conjugated anti-MHC class II; PE-conjugated anti-CD86; and PE-conjugated anti-CD40 (BD Pharmingen).

mRNA measurement by Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from DC cultures by standard techniques using TriReagent (Sigma) and quantitated using RiboGreen RNA Quantitation Kit (Molecular Probes; Eugene, OR). The extracted RNA was treated with DNase using DNA-free kit from Ambion (Austin, TX). Total RNA (1μg) was reverse transcribe with avian myeloblastosis virus reverse transcriptase (RT; Promega, Madison, WI). The cDNA product was amplified using TaKaRa Taq DNA polymerase (Fisher; Atlanta, GA). The primer pairs used were as follows: Jagged1, forward primer, 5'-AGAAGTCAGAGTTC

AGAGGCGTCC-3', reverse primer, 5'-AGTAGAAGGCTGTCACCAGCAAC-3' (113 bp product); Delta4, forward primer, 5'-AGGTGCCACTTCGGTTACACAG-3', reverse primer 5'-CAATCACACACTCGTTCCTCTCTTC-3' (123 bp product); and β-actin, forward primer, 5'-ATGGATGACGATATCGCT-3', reverse primer, 5'-ATGAGGTAGTCTGTCAGGT-3' (530 bp product). PCR was performed in a Mastercycler (Eppendorf, Westbury, N.Y.) for 35 cycles (Jagged1 and Delta4) or 28 cycles (β-actin) and 60^{o}C annealing. PCR products were visualized with ethidium bromide in 2% agarose gels.

Bacteria growth determined by CFU assay. After 24 hr infection, spleens from infected mice were homogenized in Hanks balanced salt solution (HBSS). In studies of Lp growth in DC cultures, cells were lysed by with 0.1% saponin (Sigma) and diluted in HBSS. Homogenized spleens or lysed DCs were plated on BCYE agar plates and incubated at 37°C for 72 hr. CFU counts were determined on an AutoCount apparatus (Dynatech Labs, Chantilly, Va.).

Cell viability and apoptosis detection. DC viability and apoptosis were detected using the Annexin V-FITC kit (BD-Pharmingen). Briefly, uninfected or infected cells (10^5), treated with DMSO (LpDC/DMSO) or THC 10 µM for 24 hr (LpDC/THC), were washed twice with PBS, and incubated with Annexin V-FITC (5 µl) and propidium iodide (5 µl) in binding buffer for 15 min. Early apoptotic cells (Annexin V positive and propidium negative) and late apoptotic or dead cells (Annexin V positive and propidium positive) were quantitated by flow cytometry.

Statistical analysis. Comparisons between groups were performed using the two-tailed Student's *t* test. A value of $p < 0.05$ was accepted as indicating significance.

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Results

THC impairs immunization potential of Lp-loaded DCs. Due to their pivotal role in stimulating T cells, DCs loaded with specific antigens have been utilized as immunizing vehicles in numerous studies of tumor therapies (Zitvogel et al., 1996; Celluzzi and Falo, 1998) and infectious diseases (Moll and Berberich, 2001). To test whether DCs loaded with Lp would induce an immune response to Lp or if THC would impair this ability, we treated Lp-infected DCs with THC (LpDC/THC) or drug vehicle DMSO (LpDC/DMSO) for 24 hr. The concentration of THC used in this study was 10 µM which we previously showed to be the lowest concentration exerting a significant effect on IL-12p40 production in Lp-infected DC cultures (Lu et al., 2006). DCs without infection and drug treatment were incubated for the same time as controls. DCs were injected iv into mice two or three times at 7 day intervals and seven days after the last injection, mice were challenged with a lethal dose ($1.7\text{-}2.0\text{x}10^7$) of bacteria and survival monitored. The results in Fig. 1 showed that uninfected DCs failed to induce protection as none of the mice survived; however, Lp-loaded DCs (LpDC/DMSO) induced significant with a survival ratio of 66 percent (6/9). However, mice receiving loaded DCs treated with THC (LpDC/THC) showed no survival after 25 hr indicating a lack of immunizing potential similar to mice injected with non-loaded DCs. In other experiments, mice were injected with DCs, LpDC/DMSO or LpDC/THC ($0.3\text{-}0.5\text{x}10^6$) and seven to nine days later challenged with a sublethal dose ($7\text{x}10^6$) of Lp. After 24 hr, spleens were isolated

and homogenized, and bacteria burdens measured by CFU analysis. The data showed that spleens from mice receiving Lp-loaded DCs had much lower CFUs than spleens from mice immunized with either unloaded DCs or loaded DCs treated with THC (Fig 2). These findings together demonstrated that mice immunized with Lp-loaded DCs were able to induce immunization against Lp infection and THC treatment significantly attenuated this effect.

THC treatment of Lp-loaded DCs inhibited the expression of Th1 type cytokines in splenocytes from immunized mice. Type 1 cytokines, including IL-12 and IFN-γ, are critical for the initiation and development of protective immunity against intracellular microbial infections (Pulendran, 2004). The cytokine profiles from splenocytes of immunized mice were analyzed to determine whether THC treatment of DCs suppressed an upregulation of Th1 activity in recipient mice. As in Fig. 2, mice were immunized with DCs only, LpDC/DMSO, or LpDC/THC and seven to nine days later, splenocytes were harvested and stimulated in vitro for 24 hr with specific antigen in the form of killed Lp. Supernatants from these cultures were collected and analyzed for type 1 cytokines by ELISA. As shown in Fig. 3, the splenocytes from mice receiving Lp-loaded DCs treated with DMSO produced 1.5-2 fold increases in IL-12p40 and IFN-γ as compared to splenocytes from mice treated with unloaded DCs. This suggested an upregulation of Th1 activity in the spleens of mice immunized with Lp-loaded DCs accounting for their enhanced resistance to Lp infection (see Fig. 1 and 2). However, THC treatment of the DCs inhibited this upregulation of Th1 activity suggesting an attenuation of the immunizing potential of these cells (Fig. 3 A and B). IL-4 production by splenocytes was also examined and we observed it was suppressed following

injection of Lp-loaded DCs either treated or not with THC (Fig. 3 C). This suggested that the increase in Th1 activity in the spleens coincided with a decrease in the Th2 cytokine, IL-4; furthermore, it suggested that THC suppressed Th1 cytokines by mechanisms other than the upregulation of IL-4. The data overall suggested that THC treatment of antigen-loaded DCs can suppress the immunizing and Th1 polarizing potential of these cells when subsequently injected into mice.

THC suppression of DC IL-12p40 production mediates loss of Th1 polarization of Lp-primed CD4+ T cells. We have previously reported that THC suppresses the production of IL-12p40 in Lp infected DCs *in vitro* (Lu et al., 2006). Therefore, to examine if this attenuation is responsible for the impaired Th1 polarizing potential of drug-treated and Lp-loaded DCs, co-cultures of DCs with T cells from both unprimed and Lp-primed animals were prepared to examine the reconstitution efficacy of exogenously added IL-12p40. Fig. 4A shows results from co-cultures of Lp-loaded DCs and unprimed CD4 T cells. As previously reported, Lp loading of DCs induces the production of IL-12p40 as detected in culture supernatants by ELISA and THC treatment of the cells suppressed this response. The addition of unprimed T cells had little effect on IL-12 production (Fig. 4A) and no IFN-γ was detected in these cultures (data not shown). We next examined the accessory cell potential of drug treated DCs in cultures containing Lp-primed T cells and supplied with various concentrations of IL-12p40. Fig. 4B shows that DCs plus primed T cells (LpCD4) produced little IL-12p40; however, when DCs were loaded with Lp (LpDC/DMSO), a robust IL-12p40 response was evident and this was significantly attenuated by THC treatment (LpDC/THC + LpCD4). Of interest was the finding that the addition of recombinant IL-12p40 protein to

the cultures increased the IL-12 supernatant concentrations above the amounts added (Fig. 4B). For example, addition of 0.5ng/ml recombinant IL-12p40 resulted in an increase of supernatant IL-12 from 2ng to 6ng/ml. Furthermore, in contrast to co-cultures containing unprimed CD4 T cells, cultures containing primed T cells, produced robust amounts of IFN-γ but only in the presence of Lp-loaded DCs (Fig. 4C) and this effect was attenuated by THC treatment of the DCs. However, as seen in Fig. 4C, the addition of recombinant IL-12p40 completely restored IFN-γ production suggesting a restoration of Th1 polarization by IL-12. In addition to IL-12p40, we also tested for the presence of IL-12p70, IL-23, and IL-10 in the culture supernatants. These cytokines were not detected suggesting that the suppression of IL-12p40 from DCs by THC treatment was primarily responsible for the reduced Th1 polarization.

THC suppressed the expression of DC maturation and polarizing markers. Upon exposure to microbes, DCs are activated to go through a maturation process characterized by an increase in surface expression of MHC class II and co-stimulatory molecules contributing to initiation of an effective adaptive immune response (Iwasaki and Medzhitov, 2004). To determine if THC modulated these markers, we treated infected and non-infected DCs with either DMSO or THC. After 48 hr, we observed by flow cytometry that Lp-loading increased the surface expression of CD86 and CD40; however, THC treatment significantly suppressed the expression of both markers (Fig. 5). Regarding MHC class II, we observed that although expression was high in all three groups, the mean fluorescence intensity (MFI) of the marker was enhanced in the Lp infected-DCs but was significantly decreased by THC treatment (Fig. 5). From these results, it is possible that drug suppression of T helper polarization is due in part to a

down-modulation of these markers. Other surface proteins such as Notch receptors are known to regulate T cell development (Radtke et al., 2004). Recently it was shown that the Notch ligands, Delta4 or Jagged1, on DCs, promote induction of either Th1 or Th2 activity, respectively (Amsen et al., 2004). We, therefore examined relative mRNA expression of these ligands in DCs loaded with Lp and treated with THC or vehicle for 18 hr. We observed that message for both ligands was increased in DCs after Lp infection (LpDC/DMSO group; Fig 6) but that the Delta 4 band intensity relative to β-actin intensity was decreased following THC treatment suggesting that the message level of the Th1 polarizing ligand was decreased by drug treatment.

THC treatment did not affect Lp survival in DCs or enhance apoptosis in Lp infected-DCs. THC has been observed to induce apoptosis in macrophages and lymphocytes (Zhu et al., 1998) and DCs (Do et al., 2004) and it could be argued that drug suppression of IL-12 production and marker expression could be due to a toxic effect on DCs. Also, antigen presentation to CD4 T cells by DCs requires internalization and procession of infectious agents by the DCs. In order to test the THC effect on other relevant DC functions, we studied the survival of Lp in DCs and the induction of apoptosis in Lp-infected DCs. DCs were infected with Lp for 30 min followed by washing to remove non-internalized bacteria. Infected cultures were then treated with THC or DMSO for 0, 24 and 48 hr and the cell-associated CFUs determined by cell lysis and viable bacteria plate counts. The results (Fig. 7) showed that the bacteria handling function as measured by intracellular survival was unaffected by THC treatment. Both drug-treated and vehicle treated cells restricted the growth of Lp in an equivalent manner over time. To determine if THC induced apoptosis in Lp-infected DCs, staining

with propidium iodide and annexin V in treated DCs was analyzed by flow cytometry. Compared with uninfected DCs, the percentage of apoptotic cells single positive for annexin V was enhanced after Lp infection (Fig 8), and treatment with THC did not increase the annexin positivity. Furthermore, analysis of propidium iodide staining as indicative of dead cells was similar in infected and infected plus THC treated cells. The data suggest THC treatment did not affect the degree of apoptosis or processing of bacteria in DCs following Lp infection.

Discussion

In the current study, we examined the THC effect on the Th1 polarizing potential of DCs in a model system involving adaptive immunity to *Legionella*. Loading of microbial antigens into DCs has been shown to immunize mice against infection with various pathogens (Ludewig et al., 1998; Su et al., 1998; von Stebut et al., 2000) and we showed that DCs loaded *in vitro* with Lp and injected into mice immunized and protected the mice to a subsequent lethal Lp infection (Fig. 1). Immunization was confirmed because the number of splenic CFUs in vaccinated mice was reduced after challenge injection with Lp (Fig. 2). The next question involved what effect THC treatment had on the immunizing potential of the loaded DCs. We showed exposure to THC decreased the capacity of these cells to protect against infection and to decrease the number of CFUs in the spleens of infected mice (Figs. 1 and 2).

Next, splenocytes from immunized mice were analyzed for Th1 and Th2 cytokine production *in vitro* in response to Lp antigens. The results in Fig. 3 showed that, as expected, immunization with Lp loaded DCs caused an increase in the Th1 polarizing cytokines, IL-12p40 and IFN-γ; however, this was attenuated in splenocytes from animals immunized with THC-treated DCs suggesting drug treatment suppressed DC polarizing potential. The mechanism surrounding the regulation of Th1 activity by IL-4 is controversial (Swain et al., 1990) (Biedermann et al., 2001); therefore, we examined for IL-4 production by splenocytes from immunized mice. Fig. 3 shows that immunization suppressed IL-4 production; furthermore, immunization with THC-treated cells had no effect on this suppression. These findings suggest several things. First, as expected,

immunization by Lp led to a decrease in IL-4 producing splenocytes as confirmation of Th1 polarization in response to this agent (Newton et al., 1994). Second, it appears that immunization with drug-treated and Lp-loaded DCs, causes a decrease in Th1 activity with no concomitant increase in Th2 activity, at least as measured by IL-4 producing splenocytes. And finally the studies suggest that Lp immunization under these conditions results in primarily a Th1 response and that the suppression of this response by THC is mediated by mechanisms independent of IL-4 production.

The previous studies suggested that drug treatment suppressed the Th1 polarizing function of DCs and we wanted to explore this further using an *in vitro* paradigm. Because IL-12 is potent in directing Th1 cell differentiation (Manetti et al., 1993) and because we had shown THC suppressed IL-12p40 in Lp-infected DC cultures (Lu et al., 2006), we evaluated the Th1 promoting potential (as measured by IFN-γ production) of both Lp-loaded DCs and drug-treated DCs in co-culture with Lp-primed CD4+ T cells as well as the role of IL-12p40 in the response. The data showed that co-culturing Lp-loaded DCs with Lp-primed T cells led to enhanced IL-12p40 and IFN-γ production by the cultures compared to co-culture with unprimed T cells, and that THC treatment of the DCs attenuated cytokine production (Fig. 4). Furthermore, the adding IL-12p40 to the THC-treated cultures restored the robust production of both IL-12p40 and IFN-γ suggesting suppression of the p40 protein plays a major role in inhibiting Th1 polarization. However, suppression of p40 protein is not the only factor involved. For example, addition of IL-12p40 to cultures of purified Lp-primed CD4 T cells in the absence of DCs resulted in very little IFNγ production (<300 pg/ml) (data not shown) supporting previous findings that full Th1 polarization requires both stimulation

by cytokines such as IL-12 as well as co-stimulation by contact with DCs (Kapsenberg, 2003). We looked at cytokines other than IL-12p40 such as IL-12p70 and IL-23 that have been shown to polarize toward Th1 (Hunter, 2005); however, these cytokines were probably not involved because they could not be detected in the supernatants (data not shown). In addition, IL-10 has been shown to suppress Th1 polarization under various conditions (Yao et al., 2005); but again it was not detected in the supernatants so was probably not involved in the drug effect. Together, these results show that IL-12p40 is a major Th1 polarizing protein as reported by others (Holscher et al., 2001; Cooper et al., 2002; Brombacher et al., 2003), and that its suppression by THC is a key factor in the inhibition of Th1 cell development.

Besides IL-12 production, DCs also promote the maturation of Th1 cells by the production of helper cell-surface proteins such as MHC class II and co-stimulatory molecules (Kapsenberg, 2003). To further explore the basis of THC suppressing DC function, we examined THC effects on the expression of these surface markers. The results showed THC treatment markedly reduced the expression of MHC class II and the co-stimulatory molecules, CD86 and CD40 (Fig. 5). The mechanism for this is unclear at this time; however, signaling through cannabinoid receptors could be involved because ligation of similar receptors (i.e., G_i-linked) has been shown to modulate DC maturation from human PBMCs (Coutant et al., 2002) and we have reported that cannabinoid receptors are partly involved in the THC-induced suppression of IL-12p40 production by DCs (Lu et al., 2006). We are currently studying the role of these receptors in the drug effect on surface marker development. In addition to the above markers, Notch receptors and ligands have been reported to be critical for CD4 T

cell differentiation. Bone marrow-derived DCs, under different conditions, can be induced to express either of the two distinct classes of Notch ligands, Jagged1 (Th2 polarizing) or Delta4 (Th1 polarizing) (Amsen et al., 2004). Our data showed that both Notch ligand mRNAs, Jagged1 and Delta4, were induced in DCs after Lp infection; however, THC treatment significantly suppressed the expression of Delta4 but had little effect on Jagged1 (Fig. 6). These results coupled with the above findings that the THC-treated DCs are deficient in Th1 polarizing splenocytes, support previous findings that the Delta ligands induce Th1 cells (Amsen et al., 2004). The mechanism of this drug effect could involve cannabinoid receptors because G protein-coupled receptors activated through G_s have been shown to increase Jagged ligand expression and polarize to Th2 (Amsen et al., 2004); cannabinoid receptors are coupled to G_i, and it is possible that these types of receptors might suppress Th1 by inhibiting Delta ligands as seen in our study along with inhibiting IL-12 as shown by others (la Sala et al., 2005).

To initiate an immune response against Lp infection, DCs must take up and process the intact bacteria and then transition through cellular maturation, cytokine production, and pathogen-related antigen presentation. In contrast to macrophages, wherein intracellular growth of Lp was observed (Salins et al., 2001), we showed in the current report that DCs restricted Lp growth with moderate killing over time (Fig. 7). THC-treated DCs showed a similar growth pattern and time course suggesting Lp handling was the same in both groups. THC has been reported to induce apoptosis in mouse DCs (Do et al., 2004) and Lp infection has been shown to be apoptotic in macrophages and other cells (Abu-Zant et al., 2005; Fischer et al., 2006). Our results with Annexin V staining (Fig. 8) showed that Lp infection induces limited apoptotic

activity in DCs as observed in other cell types (Abu-Zant et al., 2005; Fischer et al., 2006); however, we also showed that THC did not increase apoptosis over infection only and in addition both treatments had no effect on actual cell death as measured by propidium iodide staining (Fig. 8). Our results with THC and apoptosis appear to be at variance with previous results wherein 10 µM THC was shown to induce Annexin V positivity in 80% of the cells (Do et al., 2004). However, these studies were done using serum-free medium while ours were done with medium containing fetal calf serum which is known to reduce the potency of the added cannabinoids (Klein et al., 1985). Because THC at concentrations of 15-30 µM induced apoptosis in splenocytes and macrophages in medium containing fetal calf serum (Zhu et al., 1998), we expect that apoptosis would have been induced in the current studies at these high drug concentrations. It is concluded that THC treatment under the current conditions is not suppressing DC function by either altering the intracellular life cycle of Lp or by causing enhanced apoptosis and death of the cells.

In conclusion, our results show that a major cellular target of THC-induced immune suppression of Th1 immunity is the dendritic cell and that the drug attenuates polarizing function by suppressing IL-12p40 production and the expression of MHC class II and co-stimulatory molecules. Although, THC might compromise the host's ability to fight infection it also might be of use in the treatment of chronic inflammatory diseases such as coeliac disease and Crohn's disease (Hart et al., 2005; Rimoldi et al., 2005), rheumatoid arthritis (Thomas and Lipsky, 1996; Walker et al., 2006) and systemic lupus (Hardin, 2005) and therefore might be in the class of anti-inflammatory

drugs recognized to interfere with earlier stages of immunity by suppressing DC activation (Hackstein and Thomson, 2004).

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Footnotes

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b) Send reprint requests to: Dr. Thomas W. Klein, Department of Medical Microbiology and Immunology, MDC 10, University of South Florida College of Medicine, 12901 Bruce B. Downs Blvd, Tampa, Florida 33612.

Legends for Figures

Fig 1. THC impairs immunization potential of Lp-loaded DCs. Mice were iv immunized with DCs ($0.5x10^6$ cells/mouse) two to three times at 7 day intervals prior to being challenged with a lethal dose of Lp ($1.7 - 2.0 x10^7$/mouse). The DCs were either not loaded with Lp (DC group) or loaded with Lp and treated for 24 hr with either DMSO (LpDC/DMSO group) or THC, 10 μM (LpDC/THC group). Mice were monitored for survival and the data represent 9 mice per group from 3 experiments.

Fig. 2. THC treatment of Lp-loaded DCs inhibited immunizing potential as evidenced by increased bacterial burden. Mice were iv injected with $0.3\text{-}0.5x10^6$ DCs loaded or not *in vitro* with Lp and treated with DMSO (LpDC/DMSO) or THC at 10 μM (LpDC/THC) for 24 hr. Then, mice were challenged 7-9 days later with a sub-lethal dose of Lp ($7x10^6$ Lp/mouse), spleens isolated 24 hr post-infection, and colony forming units (CFU) determined by plate counts. Data presented as the mean CFU +/- SEM for 4 mice per group. # and * ($p < 0.05$) versus the uninfected DC control and LpDC/DMSO group, respectively.

Fig. 3. THC treatment of Lp-loaded DCs inhibited the expression of Th1 cytokines in splenocytes from immunized mice. Mice were iv injected with control DCs ($0.3\text{-}0.5x10^6$), Lp-loaded and DMSO treated DCs (LpDC/DMSO), and Lp-loaded and THC treated DCs (LpDC/THC) as in Fig. 2. Seven to 9 days post-injection, splenocytes were harvested and stimulated *in vitro* with killed Lp (10^7/ml) for 24 hr and cytokines detected in supernatants by ELISA. Data represent the mean of 5 experiments +/- SEM. # and * ($p < 0.05$) versus the control DC and Lp/DMSO group, respectively.

Fig. 4. THC suppression of DC IL-12p40 production mediates loss of Th1 polarization of Lp-primed CD4+ T cells. Cytokines were measured in 24 hr supernatants of co-cultures containing DCs and either Lp-primed or unprimed CD4+ T cells. Primed T cells were obtained 5 days post-infection from the spleens of mice infected with a sub-lethal dose of Lp. A) IL-12p40 measured in co-cultures containing Lp-loaded DCs treated with DMSO or THC (LpDC/ DMSO or LpDC/THC) co-cultured with unprimed CD4+ T cells. B) IL-12p40 measured in co-cultures treated as in panel A and containing primed CD4+ T cells; recombinant IL-12p40 was added in increasing amounts. C) IFN-gamma measured in co-cultures as in panel B and treated with IL-12p40. Data are representative of 4 experiments.

Fig. 5. THC suppressed the expression of maturation markers on Lp infected-DCs. Cell surface markers were determined by flow cytometry on DCs treated for 48 hr in various ways: uninfected (DC); Lp-infected and DMSO treated (LpDC/DMSO); and Lp-infected and THC treated (LpDC/THC). Data are expressed as percent expression (%) of the surface marker and mean fluorescence intensity (MFI) of the population for the marker. Data are representative of 4 similar experiments.

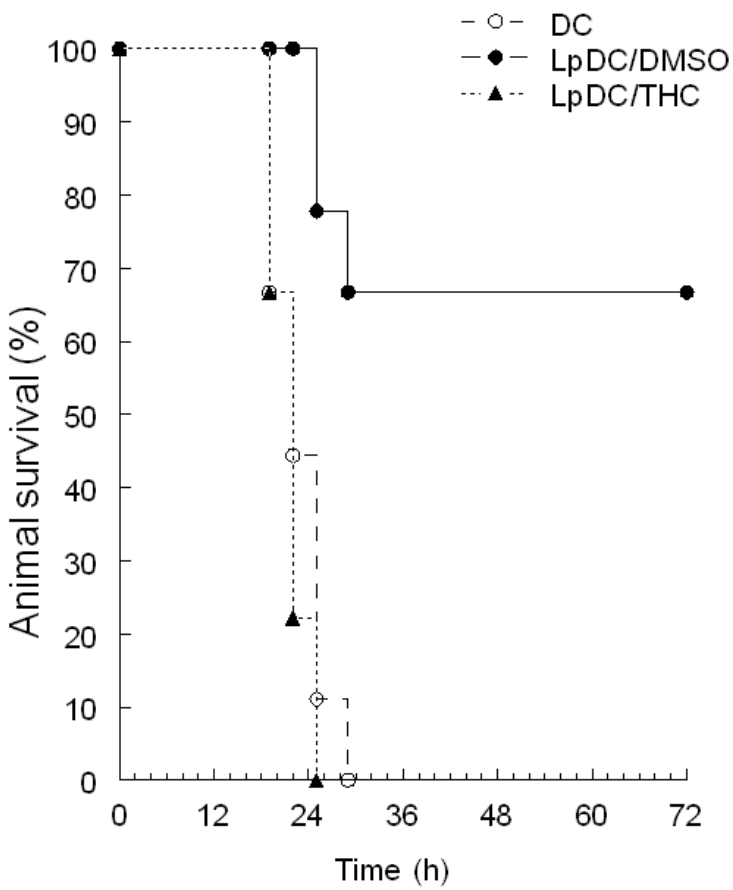
Fig. 6. THC suppressed the expression of Delta 4 in Lp-infected DCs (LpDC/THC) as compared to infected DCs treated with DMSO (LpDC/DMSO). DCs were uninfected or infected with Lp and treated with DMSO or THC for 18 hr. Jagged1, Delta4, and β-actin mRNAs were amplified by RT-PCR. Data are representative of 3 experiments.

Fig. 7. Lp uptake and survival in DCs was not affected by THC treatment. DCs were infected with Lp for 30 min, washed twice to remove non-internalized bacteria and treated with DMSO or THC for 0, 24, 48 hrs. At various times post-infection, cell lysates

were harvested and plated on agar culture medium, and CFUs/culture of Lp determined by plate counts at 72 hr. Data represent the mean of 3 experiments +/- SEM.

Fig. 8. Apoptosis and cell death were not affected by THC treatment. Cultures of DCs were untreated (DC) or infected with Lp and treated for 24 hr with either DMSO (LpDC/DMSO) or THC (LpDC/THC) and apoptosis and cell death were analyzed by staining with Annexin V and propidium iodide, respectively. A) Dot plot of propidium iodide and Annexin V staining; representative of 3 similar experiments. B) Percent of apoptotic cells (Annexin V+; propdium iodide-) and dead cells (propidium iodide+); mean +/- SEM , n=3. #, $p < 0.05$ versus DC control.

Fig. 1.



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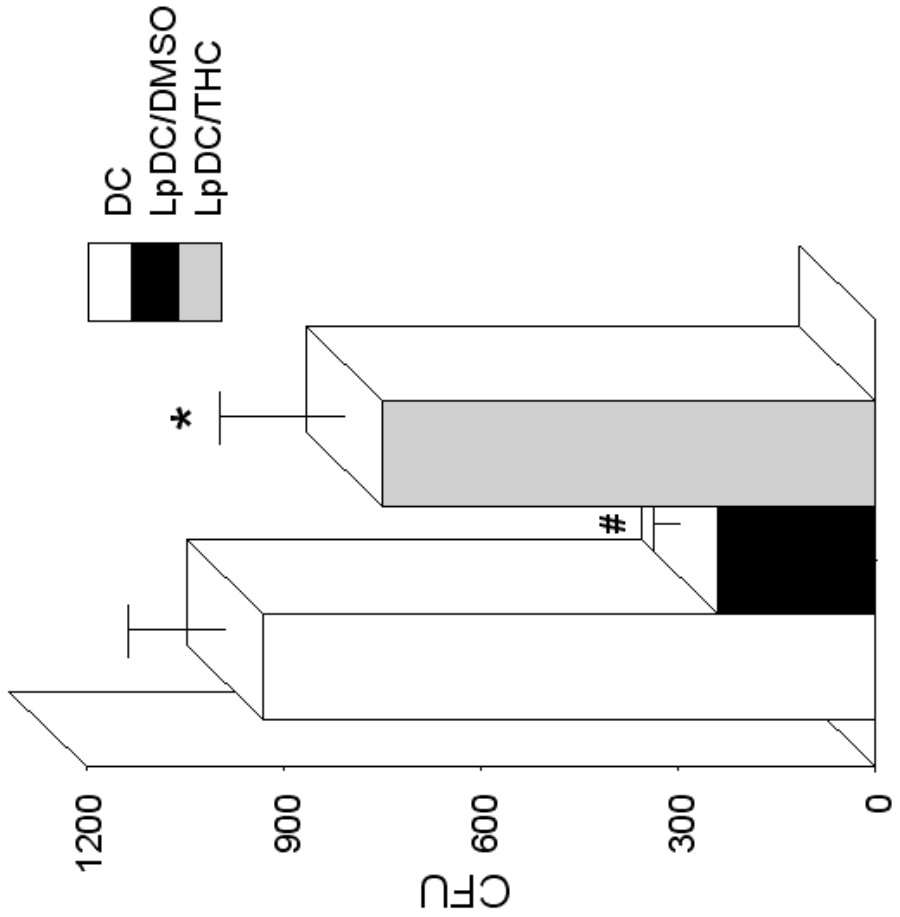

Fig. 2.

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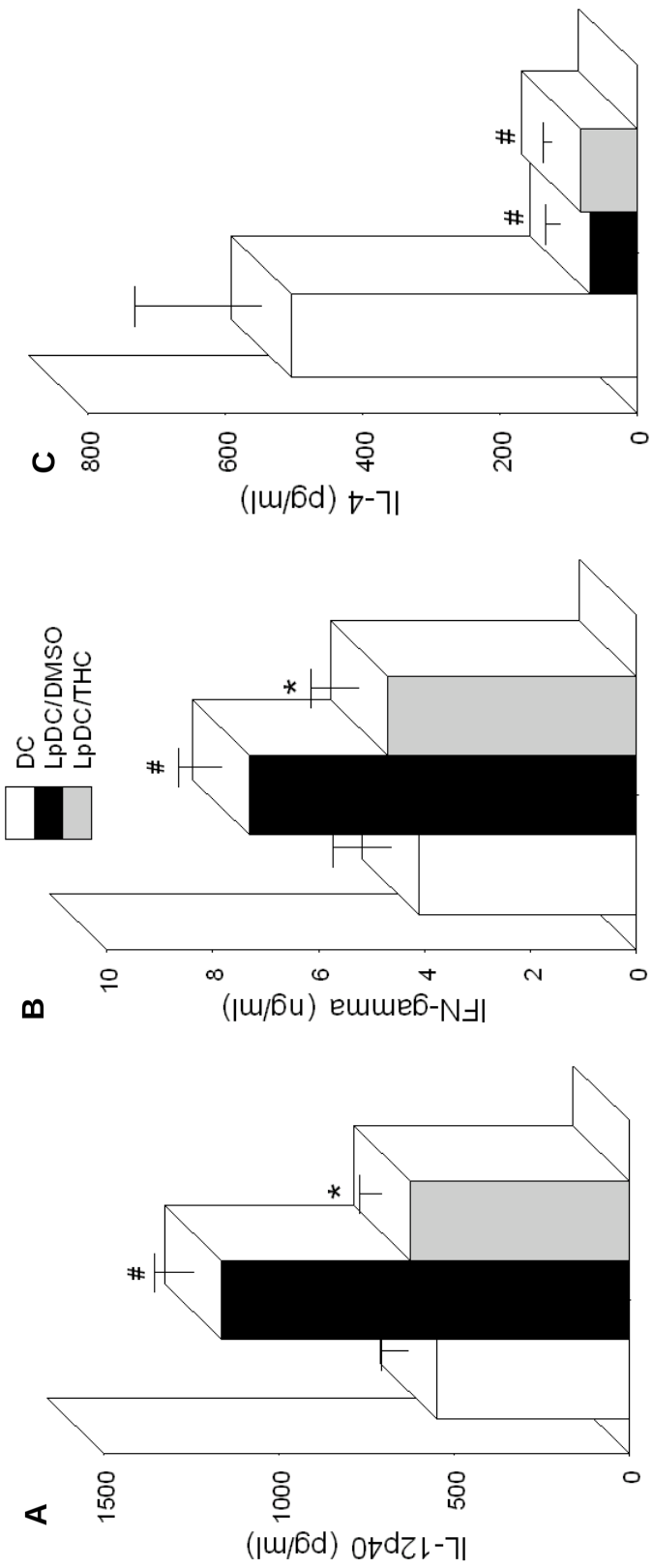

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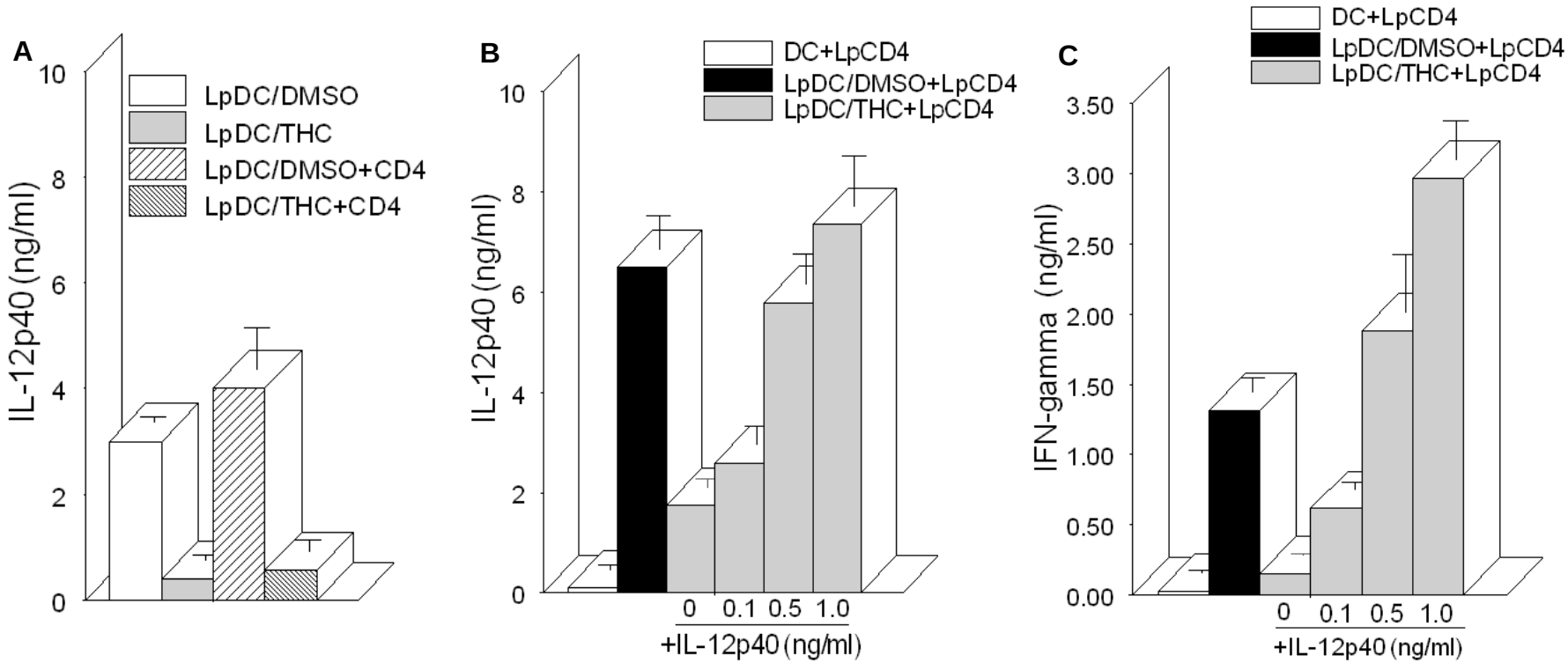

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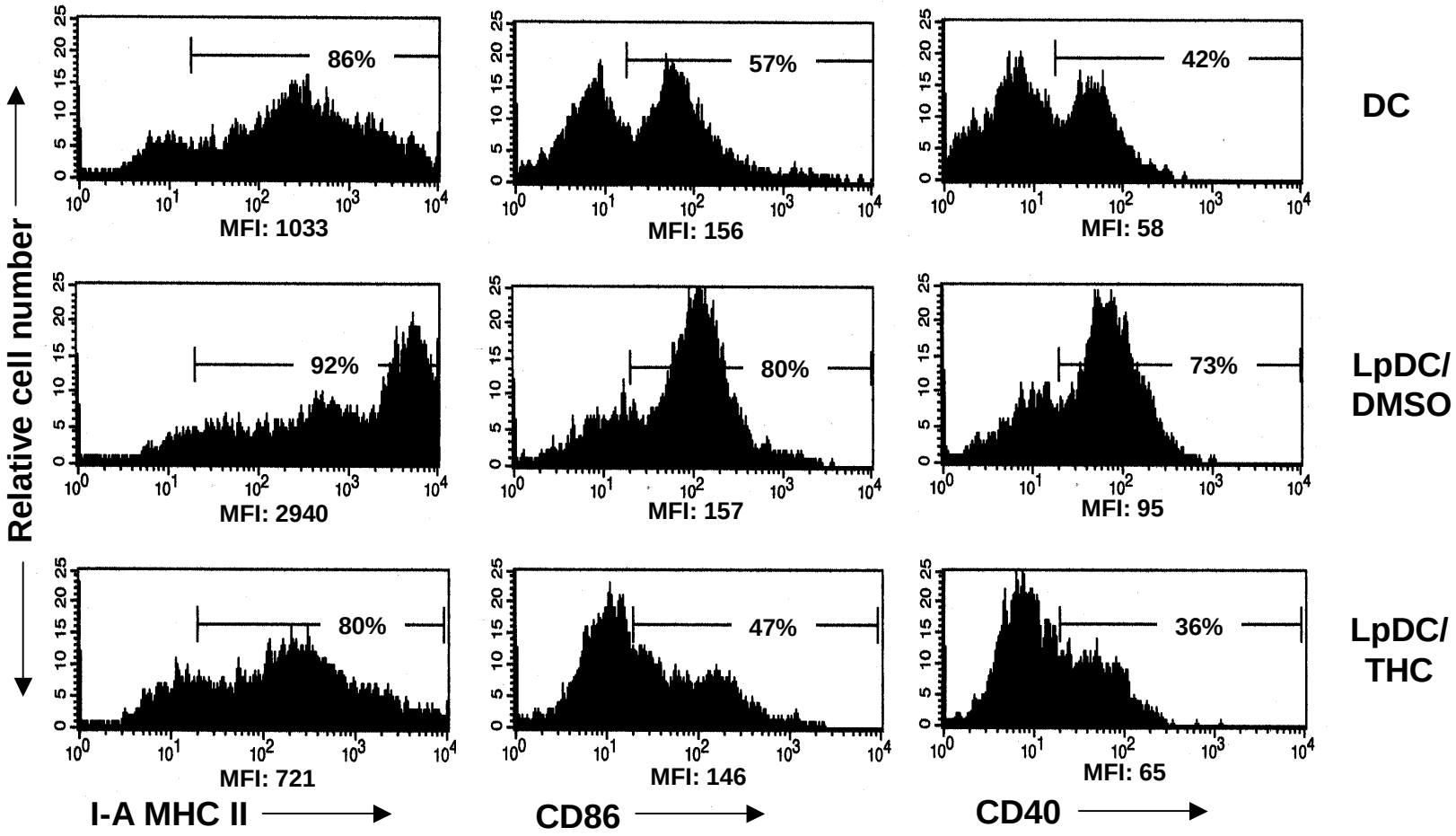

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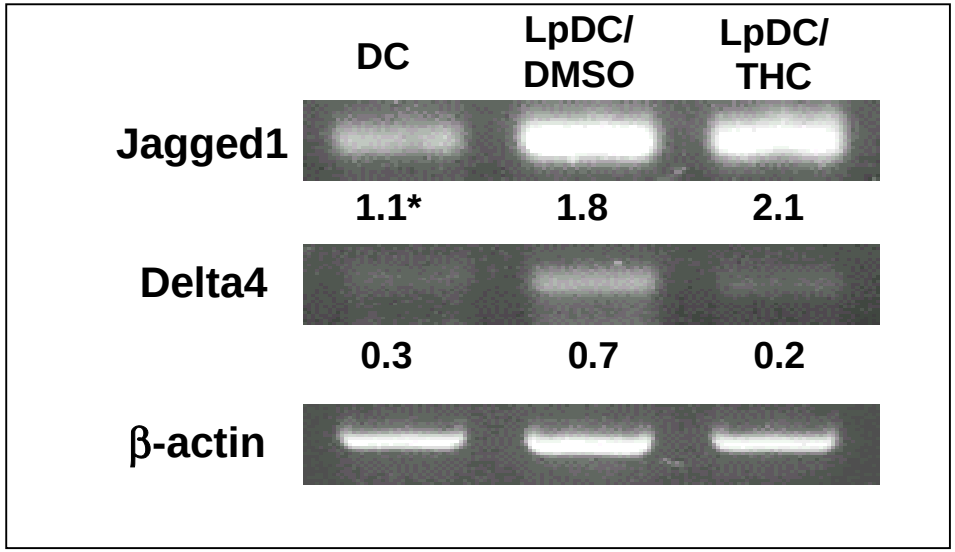

* Target to β –actin ratio

Fig. 7.

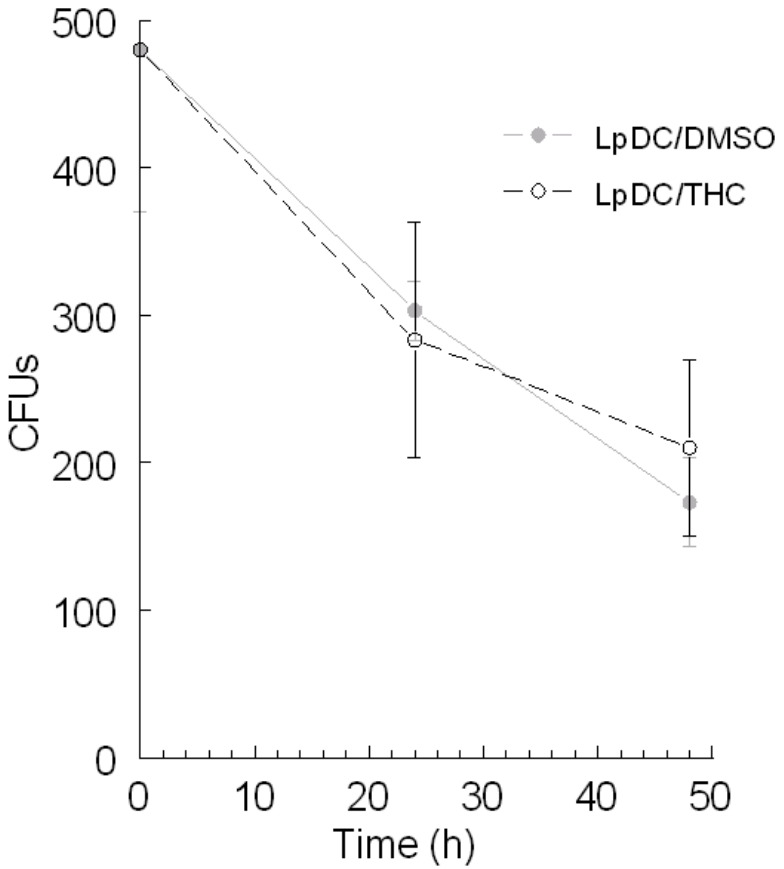

Fig. 8.