Th17/Treg Imbalance in Opioids and Cannabinoids Addiction: Relationship to NF-κB Activation in CD4+ T cells

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Opioids are widely used for the treatment of severe pain. However, opioids, particularly morphine, is known to cause immunosuppression. This study investigated the impact of morphine and cannabinoids addiction on CD4+ T cell mediated immunity. We hypothesize that, accompanied immunosuppression is attributed to reduced T cell activation with an extent of affection to the cytoplasmic activity of the biologically active transcription factor nuclear factor-κB (NF-κB) which play crucial role in T-cell activation. A disturbance in cytokine balance, in particular, interleukin-17 (IL-17) / interleukin-10 (IL-10) production may also act as a mechanism of immunosuppression. Peripheral blood CD4+ T cells from 45 chronic morphine and cannabinoid addicts and 10 controls with no current or past history of drug abuse; were stimulated by anti-CD3 antibody plus phytoheamagglutinin (PHA). Activation of the NF-κB signaling pathway was examined by analyzing NF-κBp65 in a solid phase sandwich ELISA. IL-17/IL-10 balance was assessed using quantitative ELISA on cultured CD4+ T cells supernatants. We found that, morphine and cannabinoids inhibited NF-κB signaling in activated T cells of addicts, whereas it enhanced activated T cell apoptosis as measured by quantitative in vitro determination of cytoplasmic histone-associated DNA fragmentation following induced cell death. These effects of morphine and cannabinoids T cell suppression were accompanied by elevation of IL-10 level and concomitant reduction in IL-17 secretion from cultured CD4+ T cells. We concluded that Th17/Treg imbalance may be attributed to inhibited NF-κB activity in CD4+ T cells under the effect of morphine and cannabinoids addiction.

Little attention has been paid to the immunological abnormalities associated with drug addiction although number of studies have shown that drug abusers are more susceptible to infections. Opioids including morphine, and heroin (American psychiatric association 2000) are used by 16.4% of suicide victims (Hall et al., 2008). Even more, overdose associated with their use have shown an alarming increase in mortality in recent years (CDC 2006). It is evident that they are on the rise (Paulozzi et al., 2008). Additionally; cannabinoids are a group of compounds found in the marijuana plant (Cannabis sativa L.), Bhang is the dried mature leaves of the Cannabis sativa. Despite its current illegal status, the use of bhang became prevalent in Egypt and invaded all sectors of the society, especially university students, youth and manual workers (EL-Gohary et al., 2004).

Chronic opioid abuse has been documented to severely compromise the immune system (Roy et al., 2006). Morphine induced a decrease in splenic and thymic weight; leading to impaired T cell function (Bryant et al., 1991). It increases receptor-mediated production of transforming growth factor β (TGF- β), which is another possible indirect method by which opiates suppress immunity (Chao et al., 1992). Furthermore, morphine alteration of T cell apoptosis have been described (Roy et al., 2011). It modifies T cell differentiation and alters cytokine expressions. In human naïve T cells, suppression of interleukin 2 (IL-2) and its trans-activator, nuclear factor-κB (NF-κB) was observed (Borner et al., 2009, Mustelin et
al., 2003), and indirectly affects differentiation. Likewise, cannabinoids have been shown to act as potent immunosuppressive and anti-inflammatory agents (Raborn et al., 2010). Cannabinoid receptor 2 (CB2) is predominantly expressed on lymphocytes (Mackie et al., 2006) and the level of expression is dependent on the stimulus and the activation state of the cell (Lee et al., 2001). It was demonstrated that the expression and the release of IL-4 is induced by cannabinoids in T cells as well (Borner et al., 2006).

An area of great interest in T-cell-mediated immunity is the ability of naïve CD4+ T cells generated in the thymus to differentiate into various subsets including T-helper 1 (Th1), Th2, Th17, Th9, follicular helper T (Tfh), Th22, and regulatory T (Treg) cells, upon encountering different pathogens. Th17, a recently discovered CD4+ T-cell subtype, produces IL-17 (Weaver et al., 2006), which is a pro-inflammatory cytokine, expressed by CD4+ effector T helper cells, as well as by other immune cells, such as neutrophils and eosinophils (Weaver et al., 2007). Another unique T-cell subset that has attracted remarkable attention in recent years is the Treg cells, which are responsible for suppressing the immune response induced by effector T cells (Sakaguchi et al., 2008).

Several studies have focused on the influence of morphine on the T helper lineage bias towards Th2 phenotype (Qian et al., 2005), as morphine induced cAMP production has been shown to inhibit Th1 cytokine expression (Borner et al., 2009) and promote IL-4 production in vitro (Qian et al., 2005) and in vivo (Wang et al., 2007). Morphine-mediated inhibition of interferon gamma (IFN-γ) expression has been observed (Roy et al., 2005). Thus, the overall effects of morphine on T cells results in a robust inhibition of early pro-inflammatory response.

NF-κB family of transcription factors plays central roles in apoptosis and inflammation. NF-κB signaling is involved in immune cell development and function, and it is critical in modulation of the immune response through the transcriptional regulation of cytokine and chemokine expression (Hayden et al., 2006). The role of NF-κB pathway in the development and functional divergence of different helper T-cell subsets as well as in Treg was currently investigated. Notably, the NF-κB has been shown to regulate various aspects of T-cell development, activation, differentiation, and survival. Various genetic studies, employing inhibition or deficiency of specific NF-κB subunits in T cells, have implicated NF-κB activity in the protection from apoptosis and the production of IL-2 (Hayeden et al., 2011).

Quite a lot immunological defects can result from imbalanced differentiation of naïve CD4+ T cells to effector T cells, including Treg and Th17 cells (Dong et al., 2008). Thus, characterization of the factors that regulate T cell responses and differentiation is important for understanding the molecular mechanisms underlying immunosuppression observed in chronic morphine addiction.

Subjects and Methods

Subjects

A total of 45 morphine and cannabinoids addicts were consecutively enrolled from March 2012 to December 2013 from the department of Neurology and Psychiatry, Main Alexandria University Teaching Hospital, Egypt, without restrictions of age. All patients were confirmed for opiate and cannabinoid addiction through a routine urine screening test for drugs of abuse.

Group (1): included twenty five morphine addicts with no other drug addiction history including cannabinoids.

Group (2): included twenty cannabinoids addicts who did not use other drugs including morphine.
The control group encompassed 10 adults, with no current or past history of smoking or drug abuse. In addition, addicts with urine screening test positive for benzodiazepines, barbiturates or amphetamines were excluded from the study.

**Methods**

- Urinary screening test for identification of addicted drugs

This was done according to the method described by Hawks et al., (1986) employing the Multi-Drug One Step Screen Test Panel commercially available from Acon company. An immunoassay based on the principle of competitive binding of cannabinoids or opiates in the urine specimen against their respective drug conjugate for binding sites on their specific antibody. Test panel was immersed vertically into the urine specimen for at least 10-15 seconds and left for 5-10 minutes waiting for the color development at specific lines. The results were expressed as either negative or positive for any of the examined drugs (cannabinoids or opiates).

- Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Ficoll-Hypaque 1077 (Sigma, UK) according to the method described by Platsoucas et al., (1979). Briefly, blood was carefully over-layered onto the top of one volume of Ficoll-Hypaque and centrifuged in a swinging bucket rotor centrifuge at 1800 rpm for 30 minutes at room temperature. RBCs and granulocytes were allowed to sediment while PBMCs were prevented forming a thin film of cells directly over the Ficoll layer. The PBMC layer was carefully aspirated then washed 3 times by centrifugation at 1200 rpm for 5 min with sterile phosphate buffer saline (PBS). At the end of the last wash, PBMC pellet was re-suspended in a tissue culture medium composed of (RPMI-1640) (Biochrom KG Berline) supplemented with fetal calf serum (FCS) (Seromed, Germany), 2 m mol/L glutamine (Gibco, Scotland), Penicillin (100 IU/ml) and Streptomycin (100µg/ml) (Seromed, Germany).

- Viability and purity testing

Viability of the isolated cells was tested by dye exclusion technique (Castro-Concha et al., 2005) which is based on the impermeability of viable cells to Trypan blue 0.4% (0.4 gm in 100 ml redistilled water and stored at room temperature). 120 µl of the diluted suspension was added to 120 µl of Trypan blue solution, left for 5 minutes and examined microscopically using a haemocytometer. Subsequent cell culture assays were only applied if viability exceeded 95%. Four white squares were counted for viable cells. The number of viable cells per ml was calculated according to the following equation:

\[
\text{No. of viable lymphocytes /ml} = \frac{\text{No. of lymphocytes counted in 4 white squares} \times \text{dilution factor} \times 10^5}{4}
\]

- CD4+ T cell Purification

CD4+ T cells were isolated according to (Nascimbeni et al., 2004). PBMCs suspension was washed with PBS buffer by adding 10-20 × labeling volume and centrifuged at 300×g for 10 minutes. After pipetting off supernatant completely, cell pellet was re-suspended in 300 µl PBS. Column (Miltenyi Biotec USA) was placed in the magnetic field of MACS Separator, prepared by rinsing with 500 µL of buffer, and then cell suspension was applied onto the column.100 µl of Biotin-Antibody Cocktail (Cocktail of biotin-conjugated monoclonal antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and Glycophorin A) was added (Miltenyi Biotec Inc. USA). 500 µL of PBS buffer were added, mixed well and incubated for 10 minutes at 4−8 °C. After that, 100 µL of blocking Anti-Biotin Micro Beads were added, mixed well and incubated for an additional 15 minutes at 4−8 °C. Cells were allowed to pass through, and then collected as the fraction with unlabeled cells representing the enriched CD4+ T cell fraction. The column washed with buffer three times then collecting the entire effluent in the same tube. Retained cells eluted outside of the magnetic field. This fraction represents the magnetically labeled non-CD4+ T cells. The purity of the enriched CD4+ T cells was evaluated by fluorescence microscopy. Aliquots of the cell fractions were stained with a fluorochrome-conjugated antibody against CD4, (CD4-PE) (Miltenyi # 130-091-231) and then analyzed by fluorescence microscopy.

- Cell Culture

CD4+ lymphocytes adjusted to 2 × 10^6 cells/ml were maintained in a short-term (48 hours) culture for assessment of cell proliferation by MTT (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide). The tissue culture protocol was adopted from the standard method developed by Davis et al., (1995). Triplicate wells containing lymphocytes were used, and 100 µl of lymphocytes suspension (2 × 10^6/ml) were added to all wells. 100 µl of the supplemented RPMI-1640 tissue culture medium were dispensed in 96 wells microtitre tissue culture plate that were pre-coated with 1 µg/ml anti-CD3 mAb (PharMingen). Anti-Human CD3 mAb [OKT 3; eBioscience, Cat. No. 14-0037-82] diluted in carbonate buffer (32 mM Na2CO3/16 mM NaHCO3) from 100 ng/µl stock solutions directly
before use; Immuno™ Plate C96 Maxi Sorp™ [Nunc, Cat. No.: 430 341]. Two sets of three wells each; were then considered. CD4+ T cells were incubated in 96-well plates. Volumes of 10 µl (10 µg/ml) phytohaemagglutinin (PHA) were added only to the first set. Supernatants were taken from cultures at 48 h to determine the secreted levels. The other set was left without PHA stimulation as a control. The plate containing the stimulated and the non-stimulated cells was then incubated for 48 hours (5% CO2) at 37°C.

**MTT Assay**

This was done adopting the standard protocol of Bieback *et al.*, (2003) with minor modifications. For assessment of the state of T cell proliferation following PHA stimulation, 10 µl tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) (R&D systems, USA) were added to each well containing cultured lymphocytes as previously described. Then they were incubated for four hours at 37°C in a CO2 incubator at 37°C. MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals. After that, 100µl of crystal dissolving solution (R&D systems, USA) were added to each well and incubated for 2 hours, this solution dissolves the formazan crystals, producing a purple solution, so the absorbance can be read using a spectrophotometer with the use of an ELISA reader at 490 nm with a reference wavelength of >650 nm. The rate of tetrazolium reduction is proportional to the rate of cell proliferation. An increase in number of living cells results in an increase in the total metabolic activity in the sample. The absorbance was measured for each sample and stimulation indices were calculated as following:

\[
\text{Stimulation index (S.I) = } \frac{\text{Mean absorbance values in PHA-stimulated wells}}{\text{Mean absorbance values in unstimulated wells}}
\]

- **Assessment of Nuclear Factor kappa B (NF-κB) Activity**

  **1. In vitro Induction of NF-κB**

  CD4+ cells were *in vitro* induced for NF-κB by a short term culture according to the method described by Bieback *et al.*, (2003). Briefly, in 24 wells of flat-bottomed tissue culture plates, 1 ml of 2x10⁶ lymphocyte suspensions (CD4+) was dispensed in 2 separate wells. Volumes of 5 µl PHA at a final concentration of 5 g/ml were added to one of these wells representing the induced NF-κB activity well. The other well was left without stimulation representing the spontaneous NF-κB activity well. The preparation was incubated overnight at 37°C in CO2 incubator with an atmosphere of 5% CO2 and 95% air. At the end of the culture, the contents of all wells were aspirated and centrifuged at 1200 rpm for 5 min to separate cell pellets from supernatants. The supernatants were decanted and PBMC pellets were stored at -70°C until NF-κB assessments.

  **2. Extraction of cytoplasmic NF-κB fraction**

  Active cytoplasmic NF-κB dimmers were extracted from cell pellets collected from PHA-stimulated and un-stimulated cells. This was done following the standard instructions of NF-κB Immunooassay kit (Biosource, USA). Volumes of 100 µl hypotonic buffers, composed of 20 mMTris-HCl, pH 7.4, 10 and 3 mM MgCl2, were added to every pellet and were vortexed to mix, and then cell homogenates were incubated at 4-8 °C for 30 min. At the end of the incubation time, crude extracts were centrifuged for 10 minutes at 3000 rpm at 4°C. The supernatants containing the cytoplasmic fraction were isolated from the pellet representing the nuclear fraction of NF-κB.

  **3. Determination of NF-κB activity**

  NF-κB was assayed in cell pellets collected from PHA-stimulated wells. The concentration of active NF-κB dimmers was measured in cytoplasmic extracts of PBMCs by an enzyme linked immunosorbent assay (ELISA) employing commercial kits provided by Biosource, USA and according to the method described by Graff *et al.*, 2009. The Biosource NF-Bp65 kit is a solid phase sandwich ELISA, in which a monoclonal antibody specific for NF-Bp65 (regardless of phosphorylation state) has been coated onto wells of microtitre strips. 100 µl of samples (diluted 1:50 in Standard Diluent Buffer) are pipetted into these wells. NF-B p65 antigen binds to the coated antibody. After washing, a 100 µl rabbit antibody specific for NF-Bp65 was added to the wells and a 100 µl horseradish peroxidase-labeled anti-rabbit IgG (anti-rabbit IgG-HRP conjugate) was added, then a 100 µl of substrate solution was added. The absorbance of each well was read at 450 nm after blanking the plate reader against the chromogen blank well composed of 100 µl each of stabilized chromogen and stop solution. The absorbance of the standard wells was plotted on graph paper against different standard concentrations. Finally, the NF-κBp65 concentrations in pg/ml for unknown samples were estimated from this standard curve taking into consideration to multiply values obtained for samples by the dilution factor (50x) to correct for the proper concentration.

- **Assessment of PHA-induced PBMCs Apoptosis**

  The level apoptosis in CD4+ cells was determined through a photometric enzyme immunoassay
established for the quantitative in vitro determination of cytoplasmic histone-associated DNA fragmentation (mono- and oligo-nucleosomes) following induced cell death employing the method described by (Muppidi et al., 2004) and employing relevant commercial kits provided by Roche, Germany. 100 µl coating solution was pipetted into each well of the MP-modules, and incubated for 1 h at 15-25° C. 100 µl of Sample solution were added into each well for 90 min. Nucleosomes contained in the sample were then bound via their histone components to the immobilized anti-histone antibodies. 100 ml of anti-DNA peroxidase (POD) were then added which reacts with the DNA part of the nucleosome. The amount of peroxidase retained in the immune complex was determined by addition of its specific substrate. Finally the content of the wells were measured at 405 nm. The color developed was read spectro-photometrically where the Optical Density is proportional to the amount of fragmented DNA (nucleosome) present in the sample.

- Interleukin 17 and Interleukin 10
IL-17 and IL-10 levels were estimated in culture supernatants using commercial enzyme linked immunosorbent assay (Quantikine R&D system, ELISA kit, USA) according to manufacturer recommendation. The intensity of the color measured was in proportion to the amount of the cytokine bound. Using a microplate reader set to 450 nm, the optical density (OD) was measured comparing the O.D. of the samples to the standard curve.

**Statistical Analysis**

All data were presented as mean and SD (standard deviation of mean) they were compared with the tabulated probability value \( (P \text{ value}) \) as the 0.05 level using SPSS statistical package (SPSS Inc., Chicago, IL). \( P \) value was considered significant if it is 0.05. The following statistical tests were used: Mann-Whitney Rank-Sum test, Student t-test, Paired t-test, Wilcoxon signed ranks test and linear correlation coefficient \( \rho \) to examine the relationship between different parameters.

**Results**

**Subjects**

The present work was conducted on a total of 55 individuals; of them 25 were drug addicts for morphine. They were all males with age range from 20-31 years with mean \( \pm \) S.D of 25.5 \( \pm \) 3.02. In addition, 20 individuals diagnosed as abusers for cannabinoids were involved in the study; they were all males with age range from 16-46 years with mean \( \pm \) S.D of 30.40 \( \pm \) 8.21. Finally, a group of 10 age and sex matched healthy persons were included in the study as negative controls. They had age range of 25-43 years with mean \( \pm \) S.D of 32.0 \( \pm \) 5.64. Statistical analysis of demographic data is shown in table 1.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>Cannabinoids (n = 20)</th>
<th>Morphine (n = 25)</th>
<th>( P \text{ value} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Min. – Max.</td>
<td>25.0 - 43.0</td>
<td>16.0 - 46.0</td>
<td>20.0 - 31.0</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>32.0 ± 5.64</td>
<td>30.40 ± 8.21</td>
<td>25.94 ± 3.02</td>
<td>0.002(^2)</td>
</tr>
<tr>
<td>Median</td>
<td>29.50</td>
<td>29.50</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>( P_1 )</td>
<td>NS</td>
<td></td>
<td>0.012(^2)</td>
<td></td>
</tr>
<tr>
<td>( P_2 )</td>
<td>0.020(^2)</td>
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</table>

\( P_1 \): \( P \) value for Post Hoc test (Scheffe) for comparing between control and each other group
\( P_2 \): \( P \) value for Post Hoc test (Scheffe) for comparing between cannabinoids and morphine

*: Statistically significant at \( P \leq 0.05 \); NS= not significant.
PHA stimulated CD4+ T cells proliferation results (MTT)

Results of assessment of proliferation using MTT are summarized in Table 2 as S.I. Statistical analysis showed that, in morphine addicts, CD4+ T cells had the lowest proliferation (S.I mean = 1.04 ± 0.10) with a highly significant reduction in proliferation than control group (mean = 1.47 ± 0.35, P < 0.001). Cannabinoids addicted subjects showed also a marked reduction (mean = 1.14 ± 0.28, P = 0.001*) when compared to control as well. Nevertheless, statistical analysis showed a non-significant difference between the two addict groups, P = 0.283.

Table 2. MTT Stimulation Index in subjects under study

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>Cannabinoids (n = 20)</th>
<th>Morphine (n = 25)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min. – Max.</td>
<td>1.14 - 2.03</td>
<td>0.69 - 1.82</td>
<td>0.91 - 1.42</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.47 ± 0.35</td>
<td>1.14 ± 0.28</td>
<td>1.04 ± 0.10</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Median</td>
<td>1.35</td>
<td>1.05</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>P₁</td>
<td>0.001*</td>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P₂</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P: P value for F test (ANOVA) for comparing between the different studied group
P₁: P value for Post Hoc test (Scheffe) for comparing between control and each other group
P₂: P value for Post Hoc test (Scheffe) for comparing between cannabinoids and morphine
*: Statistically significant at P ≤ 0.05; SI= stimulation index; NS= not significant.

DNA fragmentation results (ELISA)

Apoptotic levels of CD4+ T cells were estimated as levels of DNA fragmentation measured by ELISA and expressed in mU/5 cells unit. They showed also a highly significant increase in morphine addicted group (mean ± S.D = 1.20 ± 0.31) and Cannabinoids addicted subjects (mean ± S.D = 0.88 ± 0.17) when compared to control (P < 0.001 for both). There was no significant difference between them, P = 0.219, Table 3.

Table 3. DNA fragmentation of CD4 T cells among subjects under study

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>Cannabinoids (n = 20)</th>
<th>Morphine (n = 25)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis (mU/5 cells)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Min. – Max.</td>
<td>0.50 - 0.83</td>
<td>0.58 - 1.23</td>
<td>0.82 - 1.91</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.71 ± 0.12</td>
<td>0.88 ± 0.17</td>
<td>1.20 ± 0.31</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Median</td>
<td>0.74</td>
<td>0.93</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>P₁</td>
<td>NS</td>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P₂</td>
<td>&lt;0.001*</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

P: P value for F test (ANOVA) for comparing between the different studied group
P₁: P value for Post Hoc test (Scheffe) for comparing between control and each other group
P₂: P value for Post Hoc test (Scheffe) for comparing between cannabinoids and morphine
*: Statistically significant at P ≤ 0.05; NS= not significant.
**NF-κB Activity**

The mean ± S.D of NF-κB activity in control subjects was 7.92 ± 2.74 pg/ml compared to 4.36 ± 0.40 pg/ml for cannabinoids addicts, and 5.60 ± 2.13 pg/ml for opiate abusers. The statistical analysis of these results is reviewed in table (4) and reveals a statistically significant reduction of PHA-induced NF-κB levels in cannabinoid and opiate addicts when compared to their corresponding controls \((P=0.001\) and 0.005 respectively), Table 4.

Table 4. NF-κB levels of cytoplasmic extract of CD4 T cells among subjects under study

<table>
<thead>
<tr>
<th></th>
<th>Control ((n = 10))</th>
<th>Cannabinoids ((n = 20))</th>
<th>Morphine ((n = 25))</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB levels (\text{pg/ml})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min. – Max.</td>
<td>5.10 - 13.90</td>
<td>3.70 - 5.10</td>
<td>3.40 - 11.40</td>
<td>&lt;0.001 †</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>7.92 ± 2.74</td>
<td>4.36 ± 0.40</td>
<td>5.60 ± 2.13</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>7.07</td>
<td>4.45</td>
<td>4.80</td>
<td></td>
</tr>
<tr>
<td>(P_1)</td>
<td>&lt;0.001 †</td>
<td></td>
<td>0.005 †</td>
<td></td>
</tr>
<tr>
<td>(P_2)</td>
<td>NS</td>
<td></td>
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</table>

\(P\): \(P\) value for F test (ANOVA) for comparing between the different studied group
\(P_1\): \(P\) value for Post Hoc test (Scheffe) for comparing between control and each other group
\(P_2\): \(P\) value for Post Hoc test (Scheffe) for comparing between cannabinoids and morphine

*: Statistically significant at \(P \leq 0.05\); NS= not significant.

**Interleukin 17**

The levels of IL-17 in the control group ranged from 150 - 300 pg/ml with a mean value ± SD of \((206.30 \pm 51.05)\). In Cannabinoids abusers, IL-17 ranged from 74.0 - 216 pg/ml with a mean value ± SD of \((129.05 \pm 44.24)\). In morphine abusers IL-17 ranged from 81.0 – 209 pg/ml with a mean value ± SD of \(131.11 \pm 35.28\). The means of IL-17 in both drug abusers were significantly reduced compared to the control group \((P<0.001\) for both). Table 5.

**Interleukin 10**

Table 6 shows that the levels of IL-10 in the control group ranged from 99 - 201 pg/ml with a mean value ± SD of 138.7 ± 38.11. In bhang abusers, IL-10 ranged from 188 - 485 pg/ml with a mean value ± SD of 258.10 ± 79.91. In morphine abusers IL-10 ranged from 126.0 – 499 pg/ml with a mean value ± SD 271.51 ± 91.46. The means of IL-10 in both drug abusers were significantly higher compared to the control group \((P=0.002\) and <0.001 respectively). Table 6.

**Correlation Studies**

Correlation studies were done among all studied parameters in all groups. Results are summarized in table (7) and figure (1) showing that IL-17 had a direct correlation with stimulation indices of T cells \((P=0.032)\) and a negative correlation with apoptosis (DNA fragmentation) \((P=0.010)\). As a reverse, IL-10 was negatively correlated to S.I of the proliferating cells \((P=0.030)\), while it was not correlated to apoptosis. The study revealed also a significant negative correlation between IL-17 and IL-10 \((P=0.027)\). When we studied these correlations among each group; there were not any significant correlations.
Table 5. Interleukin 17 of subjects under study

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>Cannabinoids (n = 20)</th>
<th>Morphine (n = 25)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Interleukin 17 (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min. – Max.</td>
<td>150.0 - 300.0 pg/ml</td>
<td>74.0 - 216.0 pg/ml</td>
<td>81.0 - 209.0 pg/ml</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>206.30 ± 51.05</td>
<td>129.05 ± 44.24</td>
<td>131.11 ± 35.28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median</td>
<td>200.0</td>
<td>116.50</td>
<td>122.0</td>
<td></td>
</tr>
<tr>
<td>( P_1 )</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( P_2 )</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P: P value for F test (ANOVA) for comparing between the different studied group
\( P_1 \): P value for Post Hoc test (Scheffe) for comparing between control and each other group
\( P_2 \): P value for Post Hoc test (Scheffe) for comparing between cannabinoids and morphine
*: Statistically significant at \( P \leq 0.05 \); NS= not significant.

Table 6. Interleukin 10 of subjects under study

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>Cannabinoids (n = 20)</th>
<th>Morphine (n = 25)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin 10 pg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min. – Max.</td>
<td>99.0 - 201.0 pg/ml</td>
<td>188.0 - 485.0 pg/ml</td>
<td>126.0 - 499.0 pg/ml</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>138.70 ± 38.11</td>
<td>258.10 ± 79.91</td>
<td>271.51 ± 91.46</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median</td>
<td>128.50</td>
<td>243.0</td>
<td>248.0</td>
<td></td>
</tr>
<tr>
<td>( P_1 )</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( P_2 )</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P: P value for F test (ANOVA) for comparing between the different studied group
\( P_1 \): P value for Post Hoc test (Scheffe) for comparing between control and each other group
\( P_2 \): P value for Post Hoc test (Scheffe) for comparing between cannabinoids and morphine
*: Statistically significant at \( P \leq 0.05 \); NS= not significant.

Table 7. Correlation between different parameters of subjects under study

<table>
<thead>
<tr>
<th></th>
<th>DNA fragmentation</th>
<th>S.I</th>
<th>IL-10</th>
<th>IL-17</th>
<th>NF-κB</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA fragmentation</td>
<td>r</td>
<td>-0.240</td>
<td>0.185</td>
<td>-0.319</td>
<td>-0.114</td>
<td>-0.226</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>S.I</td>
<td>r</td>
<td>-0.270</td>
<td>0.267</td>
<td>0.345</td>
<td>0.044</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.030</td>
<td>0.032</td>
<td>0.005</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>r</td>
<td>-0.274</td>
<td>-0.393</td>
<td>-0.176</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.027</td>
<td>0.001</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-17</td>
<td>r</td>
<td></td>
<td>0.192</td>
<td>0.220</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>r</td>
<td></td>
<td></td>
<td>-0.161</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

r: Pearson coefficient
*: Statistically significant at \( P \leq 0.05 \); NS= not significant.
MTT: (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).
Discussion

The current study revealed a significant impairment of the PHA-stimulated CD4+ T cell proliferation in opiate addicts as compared to their corresponding healthy controls (P = <0.001). Interestingly, there was not a significant difference in T cell proliferation in opiate abusers relative to their cannabinoid opponents. Cannabinoids have been revealed to act as potent immunosuppressive and anti-inflammatory agents (Raborn et al, 2010). Chen et al., (2006) suggested that, chronic use of opiates leads to a decrease in the proliferative capacity of lymphocytes. In addition, Tetrahydrocannabinol (THC) was noted by Klein et al., (2006) to suppress mouse T lymphocyte proliferation in response to the T cell mitogen PHA. Similarly, Kumar et al., (2004) during an analysis of peripheral blood CD4+ T cells from morphine-treated, HIV-infected animals revealed that massive CD4+ T cell loss was recorded; more pronounced in the morphine-treated than in the HIV-infected control group. Klein et al., (2000) showed that heavy marijuana smokers demonstrated also a suppressed lymphocyte proliferation and Th1 development by inhibiting the production of IFN-γ and IL-12. Sancho et al., (2002) stated that arvanil (a synthetic anandamide hybrid) inhibits lymphocyte proliferation. Lombard et al., (2007) demonstrated that JWH-015, a CB2 selective agonist, inhibited proliferation of splenocytes stimulated with either B cell or T cell mitogens in vitro and also found that the decrease in mice lymphocyte proliferation correlated with an increase in the level of apoptosis. This finding is supported by the results from Govitrapong et al., (1998) who demonstrated a decrease in the response of T cells to a wide range of concentrations of PHA in heroin addicts.

A portion of the proliferation defect could be related to a drug-induced inhibition of Ca""^{2+} mobilization that normally occurs following mitogen stimulation (Yebra et al., 1992). In addition, Condie et al., (1996) showed that, treatment of murine thymoma-derived T cell lines with cannabidiol disrupted the adenylatecyclase signaling cascade by inhibiting cAMP accumulation, that lead to a decrease in the binding of transcription factors to CREB consensus sequence.

Morphine affects the immune response directly through μ-, κ-, and δ-opioid receptors on immune cells (Mcarthy et al., 2001). The central opioid pathways were involved in immunosuppression of lymphocyte proliferation (Lysle et al., 1996).

Martin et al., (2008) suggested that, activation of opiate receptors on immune cells induced altered intracellular Ca""^{2+} levels, activation of cAMP-dependent pathways and changes in mitogen-activated protein (MAP) kinase induction. Roy et al., (2005) showed that, acute stimulation of opioid receptors operate in a way similar to cannabinoids where they induce acute inhibition of adenylatecyclase and reduction in cAMP production. In contrast, chronic opioid exposure leads to increase in GATA expression, which results in an increase in IL-4 induction and then a switch to the less proliferative T cell subset Th2 phenotype with subsequent inhibition of Th1 and the resultant reduction in T cell proliferation. Morphine induced cAMP production has been shown to inhibit Th1 cytokine expression (Bornert et al., 2009) and promote IL-4 production in vitro (Qian et al., 2005) and in vivo (Kelschenbah et al., 2005) via multiple mechanisms including cAMP dependent suppression of IFN-γ (Wang et al., 2003), modulation of the GATA-3/T-bet switch (Roy et al.,2004) and Fas/Fas ligand dependent activation-induced cell death of the Th1 cells (Greeneltch et al., 2005).

On the other hand, the cannabinoid receptor expression is dependent on the stimulus and
the activation state of the cell (Lee et al., 2001). Although these receptors are not constitutively expressed in immune effector cells, they are induced in response to various stimuli. For example, TNFα and IL-4 induce the expression of opioid receptors in T and B lymphocytes, monocytes, and granulocytes (Kraus et al., 2001, 2003) (Borner et al., 2004) and the expression and the release of IL-4 is induced by cannabinoids in T cells (Borner et al., 2006). McCoy et al., (1995) have demonstrated that THC can differentially affect macrophage processing and presentation of soluble protein antigens that is a necessary event for the activation of CD4+ T lymphocytes.

Likewise, the regulation of opioid receptor transcription is mediated by the transcription factors NF-κB, STAT6 and GATA3 (Kraus et al., 2001, 2003, Borner et al., 2004, 2006). It is well established that T cell receptor-mediated activation of T cells leads to activation of transcription factors including AP-1, NF-κB, and NFAT (Huang et al., 2004). NF-κB family is composed of five members, NF-κB1, NF-κB2, RelA, RelB, and c-Rel, which function as various hetero- and homo-dimers (Oh et al., 2013). The mature NF-κB proteins are normally sequestered in the cytoplasmic environment through physical association with a family of inhibitory proteins, IκBs. In response to various stimuli, NF-κB dimers translocate to the nucleus as a result of IκB degradation. The presence of different IκB members, with distinct properties in signal response, creates a level of complexity in NF-κB regulation (Hayden et al., 2011, Hsieh et al., 2004). IκB-like protein, p105 plays a critical role in regulating T cell homeostasis and differentiation and preventing T cell mediated inflammation. Roles of p105 in regulating T cell homeostasis and Th17 differentiation may involve regulation of the response of CD4+ T cells to Tregs. In contrast, regulation of Th17 cell differentiation appears to involve an indirect mechanism, because the loss of p105 in T cells has little effect on their commitment to the Th17 lineage. More significantly, p105 negatively regulates the induction of IL-6 gene. Biochemical and genetic evidence suggests that IL-6 is a critical cytokine that stimulates Th17 cell differentiation (Chang et al., 2009).

Treg cells either develop directly in the thymus or differentiate from CD4+ naive T cells in the periphery in response to specific environmental cues. TCR signaling in thymocytes induces downstream signaling pathways that elicit NF-κB and NF-AT activity. It has been suggested that TCR signaling in Treg development is different from the TCR signaling that occurs during the development of Tconv cells. Deficiency of several mediators of TCR signaling such as TAK1, BCL10, and IKKβ significantly reduced or abrogated the number of Treg cells generated in the thymus (Medoff et al., 2009). These molecules are known to be important for signal transduction upstream of NF-κB activation, which suggests a possible role for NF-κB in thymic Treg development. This demonstrates that, forced activation of NF-κB can bypass the requirement of self-antigen recognition via TCR engagement. This strongly suggests that that NF-κB activation is critical for thymic Treg development. The NF-κB subunit c-Rel binds to CNS3 and plays a major role in thymic and peripheral FoxP3 expression and Treg differentiation (Long et al., 2009). Recently, a possible role of NF-κB in the regulation of RORγt gene expression in the differentiation and function of Th17 cells was demonstrated (Ruan et al., 2011).

Our results showed an increased apoptosis in CD4+ T cells in addicts in comparison to controls and these results were in accordance with results of Schwarz et al., (1994) who suggested that an endogenous cannabinoid receptor ligand was shown to induce
apoptosis in human lymphocyte cultures. In addition, Zhu et al., (1998) showed that, THC treatment of cultured immune cells triggered apoptosis to murine T cells through the regulation of Bcl-2 and caspase activity. Similarly, McKallip et al., (2002) reported in in-vitro studies, that naive or mitogen-stimulated splenocytes, underwent apoptosis when cultured with THC. Furthermore, Lee et al., (2008) demonstrated that cannabidiol (CBD), induced apoptosis in murine splenocyte populations in a time- and dose-dependent manner, they showed that, apoptosis were due to the formation of reactive oxygen species (ROS), activation of caspase 8 and caspase 3. At optimal concentrations, cannabinoids induce apoptosis in immune cells alleviating inflammatory responses. Targeting the immune cells via CB2 agonists may trigger apoptosis and act as anti-inflammatory therapy. CB2 select agonists are not psychoactive and because CB2 is expressed primarily in immune cells, use of CB2 agonists could provide a novel therapeutic modality against autoimmune and inflammatory diseases (Sadiye et al., 2010). On contrary, Sancho et al., (2002) showed that the phenotype of activated CD4⁺ T cells treated with arvanil shows a down-regulation of T cell activation markers such as CD25, HLA-DR and CD134 in an addicting drug dose-dependent fashion. Arvanil and anandamide do not induce apoptosis for CD4⁺ T cells suggesting that arvanil blocks the G1/S phase transition of the cell cycle in stimulated peripheral blood mononuclear cells, inducing activation of cytoplasmic adaptor proteins and phosphorylation of relevant transcription factors and they also inhibit early and late events in T cell activation.

Bidinger et al., (2003) indicated that cannabinoids induces apoptosis of human T lymphocytes in a concentration and time-dependent manner. The precise mechanisms are not known, it may suppresses translocation of NF-κB from cytosol to nucleus where its translocation mediates several survival genes and prevents apoptosis. In fact, cannabinoinds reduce Bcl-2 gene expression and protein production (Zhu et al., 1998).

Cannabinoids alter the expression of cytokines, which cross-signal among immune cells and play a critical role in both T cell functional activities and induction of pro-inflammatory versus anti-inflammatory cytokines. In our study, morphine and cannabinoid addiction enhanced IL-10 and decreased IL-17 production. In accordance with that, in vitro studies have demonstrated opioid inhibition of IL-23/IL-17 production by murine macrophages (Ma et al., 2010) and their production by murine dendritic cells with significant suppression of antimicrobial proteins and further potentiated morphine anti-inflammatory functions (Wang et al., 2011). Other studies showed receptor-mediated increase in the production of another T reg secreted cytokine, transforming growth factor β (Chao et al., 1992) a decrease in IL-2 and IFN-γ and an increase in the production of IL-5 (Wang et al., 2008). It was shown that morphine inhibits the proinflammatory mediators IL-6 and TNF-α while induces the expression and the release of IL-4 from lymphocytes (Bonnet et al., 2008). Furthermore, it was reported that IL-4 itself up-regulates mu-opioid receptor gene expression in many immune effector cells (Greenelitch et al., 2005). Karus et al., (2009) suggested that, expression of mu-opioid receptors is restricted to Th2 cells.

In accordance with our results, Azarang et al., (2007) found that, immune cells from heroin addicts presented a significantly enhanced production of IL-10 and lower production of IFN-c after PHA or LPS stimulation. Neri et al., (2013) demonstrated that, heroin induces inflammatory response and cytokine release. In particular, oxygen-
regulated protein 150, cyclooxygenase-2, heat shock protein 70, IL-6 and IL-15. In addition, Smith et al., (2000) revealed that administration of the cannabinoid receptor agonists before exposure to mitogen resulted in decreased circulating levels of TNF-α and IL-12 concomitant with increased levels of the anti-inflammatory cytokine IL-10. This finding has been enforced by the data coming from the work of Friedman et al., (2003) who showed that, levels of the immune inhibitory Th2 cytokines, IL-10 and transforming growth factor (TGF-β) were augmented, whereas those of the immune stimulatory Th1 cytokine IFN-α were down-regulated, at both the tumor site and in spleens of THC-treated mice.

It is obvious, from our results, supported by findings of other studies, that, both opioids and cannabinoids significantly inhibit lymphocyte proliferation and nuclear factor kappa B stimulation as well as interleukin 17 secretion. It was shown also that this was accompanied by enhanced apoptosis and interleukin -10 in cultured lymphocytes. This may be the cause of suppressed immune reaction in subjects addicted to these drugs.

References


