The Endocannabinoid Anandamide Neither Impairs *In Vitro* T-Cell Function Nor Induces Regulatory T-Cell Generation

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Abstract. *Background: The cannabinoids have been proposed in the treatment of cancer. Generally, the cannabinoids are believed to be useful only in the palliative therapy of cancer-related symptoms, namely pain, anorexia and cachexia. However, preliminary experiments would also suggest an inhibitory effect of cannabinoids on cancer growth, whereas their influence on anticancer immunity is still controversial. The present study aimed to evaluate the influence of the endogenous cannabinoid anandamide (AEA) on T-cell phenotype and function. Materials and Methods: The in vitro effects of AEA were evaluated at different concentrations on lymphocyte proliferation, cytotoxicity and differentiation, and in particular on T-regulator generation. Results: AEA did not modify lymphocyte proliferation, neither under basal conditions, nor after IL-2 stimulation. Moreover, AEA did not induce the generation of regulatory T-lymphocytes nor the production of the immunosuppressive cytokine, IL-10. Conclusion: The direct antitumor activity of AEA together with the absence of negative effects on T-cell functions might provide new insights into the potential use of cannabinoid agents in cancer immunotherapy.*

Cannabinoid agents constitute promising molecules, at least in the supportive care of cancer. However, the optimal utilization of cannabinoids in the palliative and curative therapy of cancer requires a better definition of the effect of cannabinoids, not only on cancer cell proliferation and on antitumor immunity, but also on the whole psychoneuroendocrine system.

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Opioid (1) and endocannabinoid (2) systems represent the two major brain psychoneuroendocrine units responsible for the modulation of the interactions between psychological behaviour and immunological status (3, 4). The opioid system is activated in stress, pain, anxiety and depressive conditions (3, 5), whereas the endocannabinoid system is involved in mediating the amplification of the perception of pleasure and the expansion of consciousness, the so-called psychedelic activity (4, 6).

The opioid substances have been proven to stimulate cancer cell proliferation (7, 8) and to inhibit the anticancer immune response (9). On the contrary, the cannabinoid molecules appear to inhibit the proliferation of several tumor histotypes (10, 11), to have antiangiogenic role (12) and to inhibit tumor cell migration (13). Several studies have demonstrated a significant antitumoral action of cannabinoid ligands in animal models (14). Thus, cannabinoid administration to nude mice curbs the growth of different tumors, including gliomas, lung adenocarcinomas, thyroid epitheliomas, lymphomas and skin carcinomas (14).

The cannabinoid (CB) receptors are seven transmembrane domain proteins coupled to the Gi/o type of G-proteins (15, 16). CB1 receptors are found predominantly in the central nervous system but also in most peripheral tissues, including immune cells, the reproductive system, the gastrointestinal tract and the lung (17). On the other hand, CB2 receptors are found predominantly in the immune system, *i.e.* in tonsils, spleen, macrophages and lymphocytes (17). The psychotropic psychedelic action of cannabinoids is mediated by only the CB1 receptor, whereas the immunomodulatory effects of cannabinoids are mainly mediated by the CB2 receptor, even though the CB1 receptor is also involved, at least in part, in cannabinoid-induced neuroimmunomodulation (18, 19).

Δ-9-Tetrahydrocannabinol (THC), the major psychoactive component of marijuana, appears to inhibit anticancer immunity in mice by promoting the release of immunosuppressive cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)-beta (20). This effect has been proven to be mediated by the activation of CB2

receptor, since it may be blocked by the administration of specific CB2 antagonists (20, 21). The involvement of CB2 receptor in mediating the stimulatory effect of cannabinoids on the secretion of IL-10, which represents the most potent immunosuppressive cytokine of anticancer immunity (22), has been confirmed by other authors (23), even though an involvement of CB1 receptor has also been observed to mediate cannabinoid-induced immunosuppression (19).

As far as the endocannabinoid system is concerned, the main endogenous cannabinergic agent is represented by anandamide (arachidonylethanolamide, AEA), which is able to activate both CB1 and CB2 receptors and whose psychotropic and biological effects are identical to those described for the exogenous cannabinoid THC (2). AEA also appears to inhibit lymphocyte proliferation and to induce cell death by apoptosis (23). The inhibitory effect of cannabinoids on lymphocyte proliferation and their stimulatory action on IL-10 secretion have not been confirmed by other authors (24).

Unfortunately, most studies reported in the literature have been limited to the investigation of cannabinoid effects on the whole T-cell population (18, 20, 21, 24, 25), whereas there are very few data on the influence of cannabinoid agonists on the different lymphocyte subpopulations. In any case, most data suggest that cannabinoids may act by inducing a shift in the generation of T-helper Th-1 and Th-2 cells, with subsequent diminished production of cytokines released from Th-1, *e.g.* IL-2, and enhanced secretion of those produced by Th-2 cells, in particular IL-10 and IL-4 (25). The recent discovery of the existence of a subset of CD4/CD25 double-positive T-cells characterized by a very pronounced immunosuppressive activity, the so-called T-regulatory (T-reg) lymphocytes (26), makes necessary the investigation of cannabinoids on T-reg generation, which is under proliferative control by IL-2 (27). T-reg cells would in fact represent the most immunosuppressive immune cells in anticancer immunity through the release of IL-10 (27).

This background prompted us to investigate the effect of AEA on T-lymphocyte functions, by analyzing the proliferation of lymphocytes overall, T-reg generation, natural killer (NK)-mediated cytotoxicity and the production of the immunosuppressive cytokine IL-10, either in basal conditions or in response to IL-2.

Materials and Methods

Cells. Blood samples were collected at the Transfusion Center of the Desio General Hospital (Milan, Italy) under protocols approved by the board of the local Ethical Committee. Mononuclear cells were obtained after centrifugation on a density gradient using Ficoll-HyPaque (Pharmacia LKB, Uppsala, Sweden) and washed three times in phosphate-buffered saline (PBS) solution. Of these cells, 1×10^6 cells /ml were treated in RPMI-medium 1640 (Gibco, Invitrogen, Carlsbad, CA, USA) plus 10% fetal bovine serum (FBS) (Gibco, Invitrogen) with 30 nM or 3 μM of AEA (Sigma Aldrich, St. Louis, MO, USA) in the presence or absence of 100 U/ml of IL-2 (Proleukin, Aldesleukin; Chiron, BV Emeryville, CA, USA). Cultures were stopped after 48 h and lymphocytes were analyzed for phenotype and function.

Flow cytometric analysis. Aliquots of control or AEA-treated peripheral blood mononuclear cells were used to determine the expression of various surface markers on different $CD3^+$ T-cell subpopulations, using Peridinin-chlorophyll-protein Complex (PerCP)-anti-CD3 [Becton Dickinson Biosciences (BD) San Jose, CA, USA], PE-coniugated anti-CD4-FITC (BD), FITC-conjugated anti-CD8 (BD), PE-conjugated anti-CD56 (IQ Products, Groningen, the Netherlands), FITC-conjugated anti-CD45RA (BD) anti-CCR7 (Pharmingen San Diego, CA, USA), followed by incubation with anti-mouse biotin (Jackson Immunoresearch, West Grove, PA, USA) and PE-conjugated streptavidin (Pharmingen) with isotype-matched antibodies (BD) as controls.

Phenotyping of T-regs was performed by using anti-CD3-PerCP (BD), CD4-FITC (BD), CD25 APC (BD) and intracytoplasmic Foxp3-PE (eBioscience, San Diego, CA, USA), following manufacturer's instructions, after gating on CD3/CD4 double-positive cells. Ten thousand cells were acquired with a FACScan flow cytometer (BD).

Proliferation assay. Untreated or AEA-treated lymphocytes were cultured in the presence or absence of 100 U/ml of IL-2 in 96-well round-bottom microtest plates. $[^3H]$–Thymidine incorporation was measured on day 3 by 18 hours pulse (5 Ci/mmole; GE, Segrate, Italy).

^{51}Cr Release cytotoxicity assay. Cytotoxicity was measured in a 4-hour ^{51}Cr release cytotoxicity assay. K562 cells were labeled for 1 hour with ^{51}Cr and washed three times with RPMI+10% FBS. Untreated lymphocytes or AEA-treated lymphocytes cultured in the presence or absence of 100 U/ml of IL-2, were incubated with 5×10^3 K562 target cells at E:T ratios of 60:1, 30:1 and 10:1 in 200 μl of complete RPMI medium in 96-well U-bottomed plates. After 4 hours, 30 μl of supernatant were collected, 170 μl of scintillation liquid (Perkin Elmer Life Science, Boston, MA, USA) were added to the supernatant and the radioactivity was then detected by β-scintillation counter (PerkinElmer Life Science), as counts per minutes (CPM). The percentage of specific lysis was calculated using the following formula: [(cpm experimental release–cpm minimal release)/(cpm maximal release–cpm minimal release) ×100]. Minimum ^{51}Cr release was determined from wells containing target cells and medium only. Maximum ^{51}Cr release was determined from wells containing target cells with 100 μl of 5% Triton® X-100 (Sigma Aldrich, St. Louis, MO, USA).

Cytokine analysis. In two experiments, the capacity of untreated or AEA-treated lymphocytes to produce immunoregulatory cytokines was tested after activation or not with 100 U/ml of IL-2. Supernatant (50 μl) was collected after 48 hours and the cytokine content was then determined by Multiplex Fluorescent Bead Immunoassay (FBI; Dako Glostrup, Denmark) following the manufacturer's recommended protocol. The production of IL-10 was also evaluated by ELISA, as described elsewhere (28).

Statistical analysis. Student's *t*-test was used to evaluate the differences between groups. A p-value <0.05 was considered to be statistically significant.

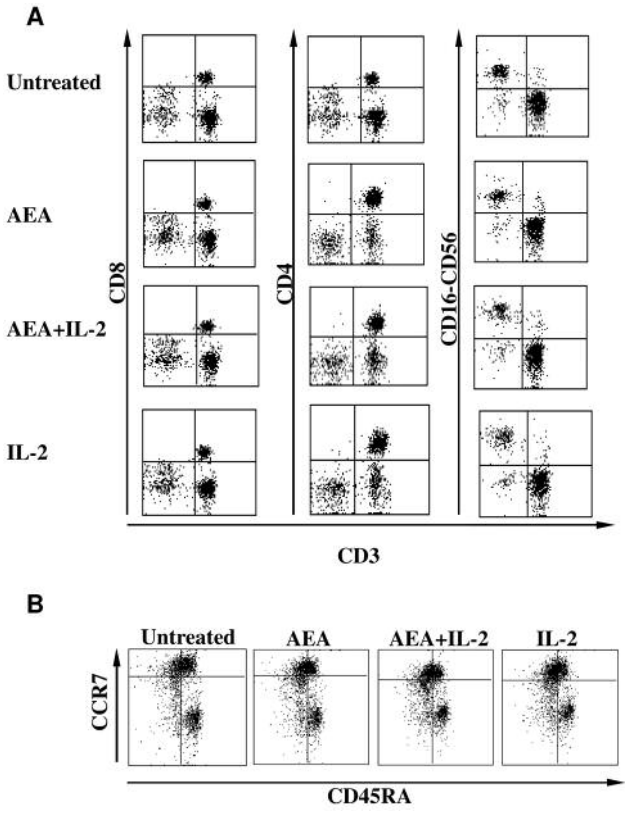

Figure 1. *Phenotypic analysis of anandamide (AEA)-treated CD3+ T-cells. Expression of surface markers was evaluated after 48 h of activation with 3 μM of AEA in the presence or absence of 100 U/ml of IL-2 . One representative experiment out of three is shown.*

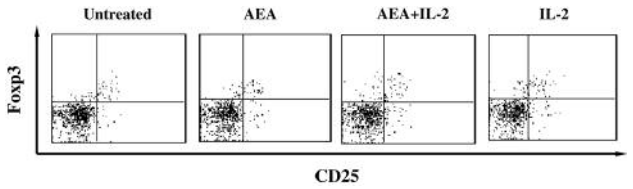

Figure 2. *Evaluation of T-reg population. Three selected donors were analyzed for expression of intracytoplasmic Foxp3 on gated CD4/CD25 double positive cells, after 48 h of activation with 3 μM of anandamide (AEA) in the presence or absence of 100 U/ml of IL-2. One representative experiment out of three is shown.*

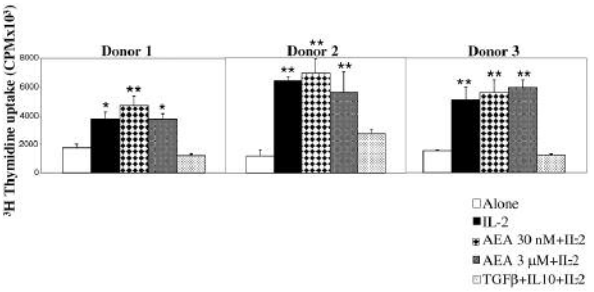

Figure 3. *Proliferation of anandamide(AEA)-treated lymphocytes. Lymphocytes were treated with 30 nM or 3 μM of AEA and their proliferation in response to 100 U/ml of IL-2 was evaluated in three different donors. Results show mean values ±SD of 3 replicates (*p<0.1 and **p<0.05 vs. untreated cells).*

Results

To evaluate the role of AEA on the regulation of the immune response, peripheral blood mononuclear cells were treated with AEA in the presence or absence of 100 U/ml of IL-2. At the end of incubation, the expression of different surface markers of $CD3^+$ T-cells, their proliferating activity, their cytolytic capacity and their ability to produce immunoregulatory cytokines were evaluated.

Immunophenotype of the AEA-treated $CD3^+$ T-cell subpopulation. The phenotypical analysis of untreated $CD3^+$ T-cells showed that 23.1% of cells were $CD3^+CD8^+$ (range=19.3%-27.4%; n=3), 48.6% were $CD3^+CD4^+$ (range=47.6%-50.6%; n=3) and 4.7% were $CD3^+CD56^+$ (range=2.7%-5.71%; n=3). The addition of 30 nM or 3 μM of AEA did not significantly alter the relative proportion of the $CD3^+$ cell subpopulation, as shown in Figure 1. After 48 h of incubation with IL-2, 23.0% $CD3^+CD8^+$ (range=20.2%-26.8%; n=3), 49.3% $CD3^+CD4^+$ (range=48.8%-50.0%; n=3) and 4.3% $CD3^+CD56^+$ (range=2.5%-6.3%; n=3) cells were present in the culture. Moreover, a high concentration of AEA (3 μM) did not modify the phenotype of IL-2-cultured cells, in fact 22.8% of the cells were $CD3^+CD8^+$ (range=20.0%-25.9%; n=3), 50.2% were $CD3^+CD4^+$ (range=49.4%-51.0%; n=3) and 4.4% were $CD3^+CD56^+$ (range= 2.4%-6.0%; n=3).

We also analyzed the memory phenotype of $CD3^+$ T-cells. Figure 1 showed that naïve ($CD45RA^+CCR7^+$), the central memory ($CD45RA^-CCR7^+$), effector memory ($CD45RA^-CCR7^-$) and effector memory RA^+ ($CD45RA^+CCR7^-$) T-cells were equally represented under all conditions tested.

Effect of AEA on T-reg cell generation. The percentage of $CD4^+CD25^+FOXP3^+$ T-reg cells was evaluated in 6 different donors. Figure 2 shows a representative experiment. The percentage of $CD4^+CD25^+FOXP3^+$ cells in the untreated $CD3^+$ T-cells was 1.8% (range=0.3%-3.3%; n=6) and, as expected, significantlyincreased in the presence of IL-2 (mean=3.5%, range=0.3%-5.2%; n=6; *p*-value<0.05). The percentage of T-reg cells did not change in the presence of 30 nM or 3 μM of AEA (mean=1.4%, range=0.3%-2.8%; and mean=1.8%, range=0.3%-3.7%, respectively; n=6). In addition, cells concomitantly treated with 3 μM of AEA and IL-2 showed a comparable percentage of $CD4^+CD25^+FOXP3^+$

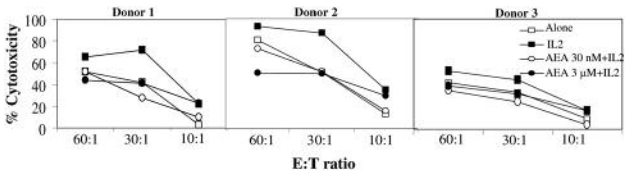

Figure 4. *Cytotoxic activity of anandamide (AEA)-treated lymphocytes. Lymphocytes were treated with AEA in the presence or absence of 100 U/ml of IL-2. The cytotoxic activity of lymphocytes was evaluated by a standard 4 h ^{51}Cr release assay against K562 target cells at different effect:target (E:T) ratios (60:1, 30:1, 10:1) in three different donors. Results show mean values ±SD of 3 replicates (**p<0.05 vs. IL-2 treated cells).*

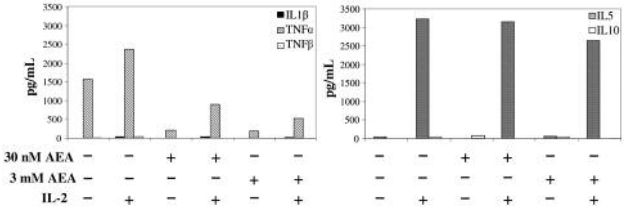

Figure 5. *Evaluation of helper cytokines produced by anandamide (AEA)-treated lymphocytes. Peripheral blood mononuclear cells were stimulated for 48 h with different concentrations of AEA in the presence or absence of 100 U/ml of IL-2. The release of Th1 and Th2 cytokines was quantified by Multiplex Fluorescent Bead Immunoassay.*

T-cells with those cultured in the presence of IL-2 (mean=2.4% , range=0.1% -3.9% ; and mean=3.2% range= 0.2% -4.2% respectively; n=6).

Proliferation of AEA-treated peripheral blood mononuclear cells. Three separate experiments assessed the effects of AEA on lymphocyte proliferation with or without 100 U/ml of IL-2.

While IL-2 was found to increase the proliferative response as compared to the control, there was no interaction between AEA and IL-2. Neither 30 nM nor 3 μM AEA significantly modified lymphocyte proliferation above the level achieved by IL-2 alone (Figure 3) (p>0.1). In contrast, the addition of immunosuppressive cytokines, such as IL-10 and TGF-β, totally abrogated the effect of IL-2 on lymphocyte proliferation.

Cytotoxicity of AEA-treated peripheral blood mononuclear cells. To evaluate the effect of AEA on mononuclear cell cytotoxic activity against the NK-susceptible target K562 cells, experiments were performed on cells from 3 different donors. As reported in the literature, we found that IL-2 strongly increased the NK-mediated cytotoxicity against K562 cells (mean=69.2% , range=45.3% -91.1% at E:T ratio 30:1) (Figure 4). In contrast, the addition of 30 nM or 3 μM of AEA to the culture significantly reduced the cytotoxic activity of cells compared with those activated with IL-2 alone. In particular, the cytotoxicity of cells treated with 30 nM or 3 μM of AEA was similar to or slightly less than that obtained by culturing in the absence of IL-2 (mean=36.0% , range=26.7% -43.2% ; mean=43.4% , range=32.0% -49.4% at E:T ratio 30:1, respectively; p-value >0.1). Surprising, at a low E:T ratio (10:1), cells treated with 3 μM of AEA showed a comparable cytotoxicity with those cultured with IL-2 alone.

Effect of AEA on stimulated cytokines. Cytokine production of lymphocytes is of major importance to initiate, amplify and orientate the antitumor immune response. To further investigate the effect of AEA on lymphocyte function, levels of Th-1 (TNF-α, IL-1β) and Th-2 (IL-5, IL-6 and IL-10) cytokines were measured in culture supernatants in two separate experiments. Figure 5 shows a representative experiment.

In the presence of IL-2, lymphocytes expressed high levels of IL-5 and TNF-α. The addition of AEA did not significantly modify IL-5 production, but did decrease TNF-α production in an apparently dose-dependent manner. In neither of the two donors tested did, IL-2 activated lymphocytes produce IL-10. After AEA treatment, IL-2 activated lymphocytes did not produce the immunosuppressive cytokine IL-10. The absence of IL-10 in the culture of the two donors tested was also confirmed in four additional donors by ELISA (data not shown).

Discussion

According to the results previously reported by Katona *et al*. (24) and in contrast to those given by most authors (18, 20, 21, 23), this study shows that the endocannabinoid agonist AEA does not inhibit lymphocyte proliferation in basal conditions, or after stimulation with IL-2. This study was thus able to demonstrate that AEA does not negatively influence IL-2-dependent lymphocyte activations, which is fundamental in the maintenance of natural resistance against cancer development (29). In particular, AEA does not induce T-reg generation when used alone or in combination with IL-2. Furthermore, this study clearly shows that the cannabinoid agonists do not stimulate the secretion of the immunosuppressive cytokine IL-10. On the contrary, in agreement with most authors (18, 20, 23), this study confirms the inhibitory effect of this cannabinoid agonist on NK-dependent cytotoxicity. However, the *in vivo* relevance of this effect needs to be better established because of its dependency on both the cannabinoid concentration and the E:T ratio, since at a low E:T ratio, a stimulation of NK-mediated cytotoxicity was observed in our experiments. Moreover, the cytotoxic activity against a standard cancer cell line, such as K562, could be different from that

obtained using fresh human cancer cells. It should be taken into consideration that lymphocyte response to both cytokines and immunomodulating neuroactive substances, whether in terms of proliferation or cytotoxicity, is under the modulatory control of macrophages (30).

In particular, it has been shown that macrophages were able to mediate the suppression of lymphocyte-dependent anticancer immunity (30). Therefore, to better establish what the *in vivo* influence of cannabinoid substances on lymphocyte proliferation and cytotoxicity may be, it is fundamental to also consider the influence of cannabinoids on macrophagic functions. On the contrary, the *in vitro* studies have generally been limited to the investigation of cannabinoid effects on single immune cell populations, whereas *in vivo*, the immune cells are linked by several reciprocal interactions. Cannabinoids have been proven to inhibit macrophage-mediated immunoinflammatory activities (30). Since macrophage-related inflammatory effects appear to suppress the anticancer cytotoxic immunity mediated by both T-lymphocytes and NK cells (30), the eventual cannabinoid-induced suppression of lymphocyte proliferation and cytotoxicity could be balanced and counteracted by the concomitant abrogation of macrophage-mediated negative influence on the anticancer immune response (30). Thus, the *in vivo* effects of cannabinoids on the anticancer immune response may be different from those separately observed *in vitro* on single immune cells and in particular on lymphocyte functions, which play a major role in the generation of an effective anticancer immunity (29, 30).

In any case, the apparent lack of relevant effects on T-cell function, as shown by our results, and the more evident action of AEA on NK cell activity are not surprising, since T-lymphocytes and NK cells have been proven to be the least and the more sensitive cells, to the immunomodulatory action of cannabinoids (18). Moreover, *in vivo*, cannabinoids could also indirectly influence T-lymphocyte functions by modulating macrophage immunosuppressive activity. In fact, it has been proven that the immunomodulatory effects of cannabinoids are mainly due to their action on macrophages rather than on lymphocytes (31), which are less sensitive to cannabinoids, because macrophage functions are inhibited by cannabinoid agonists at concentrations that do not influence lymphocyte activities.

The suppressive activity of AEA on NK cell functions might explain the reduction of TNF-α production observed in our experiments. In fact, as reported in the literature (32), among the mononuclear cell population, this cell represents the most relevant producer of this cytokine after activation by IL-2. In turn, AEA-mediated reduction of TNF-α might, at least in part, be correlated with the diminished functionality of NK cells. In contrast, we demonstrated that AEA did not affect the production of IL-5. It has been recently demonstrated that IL-5 plays a critical role in cancer elimination, by recruiting eosinophils (33). Several *in vivo* observations have found a strong link between tumor eradication and eosinophil recruitment. A number of earlier studies, in fact, showed that eosinophils, when recruited into tumors, can very effectively eradicate transplantable tumors by direct killing. Parmiani and colleagues analyzed the *in vitro* antitumor activity of eosinophils from cancer patients treated with subcutaneous administration of IL-2 (34). They demonstrated that significant cytotoxicity against tumor cells can be mediated by eosinophils after indirect IL-5-mediated *in vivo* activation by IL-2 and that eosinophils were involved in the antitumor response(s) induced *in vivo* by IL-2. The controversial data reported in the literature may be explained by taking into consideration that the immunomodulatory effects of cannabinoids may vary in relation to the different animal species, time of administration, dose administered and duration of treatment (18). In fact, T-lymphocyte proliferation appeared to be inhibited by high concentrations of cannabinoids, while it was stimulated in the presence of low concentrations (18). In addition, the CB2 receptor has been demonstrated to be differentially expressed on various immune cells. In fact, the expression of both CB1 and CB2 receptors is high on B-lymphocytes and NK cells, intermediate on monocytes and neutrophils, and low on T-lymphocytes, particularly on the $CD4^+$ T-lymphocytes. Representing the immune cells characterized by the lowest expression of both CB1 and CB2 receptors (18), $CD4^+$ T-lymphocytes would be the immune cells least responsive to the immunomodulatory effects of cannabinoids.

Conclusion

Considering that the anticancer properties of cannabinoids are due to several mechanisms, including antiproliferative and antiangiogenic effects (10, 12) and the inhibition of tumor initiation (35), this study, by excluding a negative influence on T-cell-mediated anticancer immunity, may allow us to suggest an anticancer activity of cannabinoids. Cannabinoids, in fact, might inhibit tumor cell proliferation (10, 12), without compromising the efficacy of the anticancer immune response, which is based on T-lymphocyte proliferation and killing activity (27). Obviously, even though the psychobiological effects of AEA are considered to be identical to those of THC (2), the results achieved by AEA cannot be automatically transferred to the exogenous cannabinoids derived from marijuana, since their immunomodulating action could, at least in part, be different from that described in the present study for AEA. Moreover, the *in vitro* results of this study need to be confirmed *in vivo*. Further studies in different *in vivo* models are required to analyze the effect of cannabinoids on macrophage function and their overall effects on IL-2-dependent anticancer immunity mediated by T-lymphocytes.

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