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CANNABINOIDS INCREASE LUNG CANCER CELL LYSIS BY LYMPHOKINE-ACTIVATED KILLER CELLS VIA UPREGULATION OF ICAM-1

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Abbreviations: AM-251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; AM-630, (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl)(4-methoxyphenyl)methanone; CB₁, cannabinoid receptor 1; CB₂, cannabinoid receptor 2; CBD, cannabidiol, 2-[(1S,6S)-3-methyl-6-(prop-1-en-2-yl) cyclohex-2-enyl]-5-pentylbenzene-1,3-diol; FAAH, fatty acid amidohydrolase; ICAM-1, intercellular adhesion molecule 1; IL-2, interleukin-2; LAK cells, lymphokine-activated killer cells; LFA-1, lymphocyte function associated antigen 1; NSCLC, non-small-cell lung cancer; siRNA, small interfering RNA; THC, Δ^9 -tetrahydrocannabinol; TIMP-1, tissue inhibitor of matrix metalloproteinases-1; TRPV1, transient receptor potential vanilloid 1; WST-1, 4-[-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazo-lio]-1,6-benzene disulfonate

ABSTRACT

Cannabinoids have been shown to promote the expression of the intercellular adhesion molecule 1 (ICAM-1) on lung cancer cells as part of their anti-invasive and antimetastatic action. Using lung cancer cell lines (A549, H460) and metastatic cells derived from a lung cancer patient, the present study addressed the impact of cannabinoid-induced ICAM-1 on cancer cell adhesion to lymphokine-activated killer (LAK) cells and LAK cell-mediated cytotoxicity. Cannabidiol (CBD), a non-psychoactive cannabinoid, enhanced the susceptibility of cancer cells to adhere to and subsequently lysed by LAK cells, with both effects being reversed by a neutralizing ICAM-1 antibody. Increased cancer cell lysis by CBD was likewise abrogated when CBD-induced ICAM-1 expression was blocked by specific siRNA or by antagonists to cannabinoid receptors (CB₁, CB₂) and to transient receptor potential vanilloid 1. In addition, enhanced killing of CBD-treated cancer cells was reversed by preincubation of LAK cells with an antibody to lymphocyte function associated antigen-1 (LFA-1) suggesting intercellular ICAM-1/LFA-1 crosslink as crucial event within this process. ICAM-1-dependent pro-killing effects were further confirmed for the phytocannabinoid Δ^9 -tetrahydrocannabinol (THC) and R(+)-methanandamide, a stable endocannabinoid analogue. Finally, each cannabinoid elicited no significant increase of LAK cell-mediated lysis of non-tumor bronchial epithelial cells, BEAS-2B, associated with a far less pronounced (CBD, THC) or absent (R(+)-methanandamide) ICAM-1 induction as compared to cancer cells. Altogether, our data demonstrate cannabinoid-induced upregulation of ICAM-1 on lung cancer cells to be responsible for increased cancer cell susceptibility to LAK cell-mediated cytotoxicity. These findings provide proof for a novel antitumorigenic mechanism of cannabinoids.

Key words: Cannabinoids, intercellular adhesion molecule 1, lung cancer, immune surveillance, lymphokine-activated killer cells

1. INTRODUCTION

Cannabinoids have been demonstrated to exert anticarcinogenic effects via multiple mechanisms (for review see 1,2). However, despite increasing knowledge on cannabinoids' effects on cancer cell apoptosis, invasion, metastasis and cancer-associated angiogenesis, its role in immunological antitumor responses is barely investigated. Most of the work published in this field has focussed on the modulation of immune cell migration by cannabinoids. Accordingly, the endocannabinoid 2-arachidonylglycerol has been demonstrated to induce the migration of natural killer cells (3), splenocytes, B lymphoid cells as well as myeloid leukaemia cells (4). By contrast, Δ^9 -tetrahydrocannabinol (THC), the major psychoactive ingredient of marijuana, was associated with inhibition of antitumor immunity via a CB₂ receptor-mediated, cytokine-dependent pathway (5). Other work addressed the impact of cannabinoids on the induction of cell death or apoptosis of diverse immune cell populations (for review see 6). However, the susceptibility of cancer cells to the cytolytic action of lymphokine-activated killer (LAK) cells in response to cannabinoid treatment has not been studied as yet. In line with this notion, comprehensive in vitro studies addressing the mechanisms by which anticancer drugs elicit tumor-immune interactions have not been published so far.

Recently, our group has shown that the cannabinoids cannabidiol (CBD), THC as well as R(+)-methanandamide, a hydrolysis-stable anandamide analogue, induce the expression of the intercellular adhesion molecule 1 (ICAM-1) in several lung cancer cells as well as in metastatic cells of a lung cancer patient thereby conferring increased levels of the tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) and a subsequent decreased cancer cell invasiveness (7). In athymic nude mice the non-psychoactive CBD elicited an increase of ICAM-1 and TIMP-1 protein in A549 xenografts and an antimetastatic effect that was fully reversed by a neutralizing antibody against ICAM-1 (7). Apart from this and other evidence (8) pointing to a function for ICAM-1 in cellular signal transduction pathways, the glycoprotein containing 5 extracellular immunoglobulin-like domains, a transmembrane and a C-terminal

intracellular domain (9), is known to play a crucial role as an adhesion molecule in trafficking of inflammatory cells and in cell-to-cell interactions during antigen presentation (8).

In context with the endogenous tumor immune surveillance an increasing number of data suggests a functional role of ICAM-1 on the cancer cell surface. Accordingly, several studies revealed increased tumor susceptibility to lymphocyte adhesion and cell-mediated cytotoxicity following transfection or upregulation of ICAM-1 (10-13). Vice versa, downregulation of ICAM-1 by transforming growth factor β_1 has been demonstrated to decrease both lymphocyte adhesion to cancer cells as well as cytotoxicity on cancer cells (14). In line with this notion, ICAM-1 expression has been reported to be negatively correlated to metastasis of several cancer types in clinical studies (15-17).

In view of this data the present study addressed the impact of cannabinoids on tumor immune surveillance with special reference to the role of ICAM-1 in this response. Here we demonstrate that cannabinoid-induced upregulation of ICAM-1 on lung cancer cells is responsible for increased cancer cell susceptibility to LAK cell-mediated cytotoxicity. These findings provide a novel mechanism within the diverse antitumorigenic effects of cannabinoids.

2. MATERIALS AND METHODS

2.1. *Materials*

AM-251, AM-630 and leupeptin were bought from Biomol (Hamburg, Germany). Aprotinin, calcein-AM, capsazepine, p-coumaric acid, luminol, orthovanadate and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (Steinheim, Germany). (-)-CBD was supplied by Biotrend AG (Cologne, Germany). THC and R(+)-methanandamide were bought from Lipomed (Weil am Rhein, Germany) and Tocris Bioscience (Wiesbaden-Nordenstadt, Germany), respectively. Dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), glycerol, hydrogen peroxide (H₂O₂), sodium chloride (NaCl), Tris hydrochloride (Tris-HCl) and Tris ultrapure were obtained from AppliChem (Darmstadt, Germany). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was bought from Ferak (Berlin, Germany). Dulbecco's modified eagle medium (DMEM) with 4.5 g/l glucose and with L-glutamine and Roswell Park Memorial Institute medium (RPMI 1640) with L-glutamine were provided by Lonza (Cologne, Germany). Fetal calf serum (FCS) and penicillin-streptomycin were purchased from Invitrogen (Darmstadt, Germany) and phosphate-buffered saline (PBS) was provided by PAN Biotech (Aidenbach, Germany). Lymphocyte separation medium LSM 1077 was obtained from PAA (Cölbe, Germany) and recombinant human interleukin 2 (IL-2) was supplied by ReliaTech (Wolfenbüttel, Germany). Triton[®] X-100 was bought from Roth (Karlsruhe, Germany). Neutralizing ICAM-1/CD54 antibody and isotype control antibody were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). LEAF[™] Purified anti-human CD11a and LEAF[™] Purified Mouse IgG1, κ Isotype Control were supplied by BioLegend (London, UK).

2.2. *Cell culture*

The non-small-cell lung cancer (NSCLC) cell lines A549 and H460, the lung cancer patient's metastatic cells as well as the human bronchial epithelial cell line BEAS-2B (Sigma-Aldrich,

Steinheim, Germany) were maintained in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin.

A549 human lung carcinoma cells were purchased from DSMZ (Braunschweig, Germany; A549: DSMZ no.: ACC 107, species confirmation as human with IEF of MDH, NP; fingerprint: multiplex PCR of minisatellite markers revealed a unique DNA profile). H460 cells were purchased from ATCC-LGC (Wesel, Germany; ATCC™ Number: HTB-177™; cell line confirmation by cytogenetic analysis). Following resuscitation of frozen cultures none of the cell lines was cultured longer than 6 months.

Lung cancer patient's metastatic cells were obtained from resection of brain metastasis of a 47-year-old female Caucasian with NSCLC with the procedure of cell preparation described recently (7). The patient had been informed about the establishment of cellular models from its tumor and had given informed consent in written form. The procedure was approved by the institutional ethical committee. Experiments were performed using passages 2-9 of these cells.

Cells were grown in a humidified incubator at 37°C and 5% CO₂. All incubations with test substances were performed in serum-free medium. Test substances were dissolved in ethanol or DMSO and diluted with PBS to yield final concentrations of 0.1% (v/v) ethanol (for all cannabinoids) or 0.2% (v/v) DMSO (for AM-251, AM-630, AM-251 plus AM-630, capsazepine). As vehicle control PBS containing the respective concentration of ethanol or DMSO was used.

2.3. Generation of LAK cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors. A volume of 50 to 70 ml of buffy coat was diluted 1:2 with PBS, carefully poured over 20 ml of Lymphocyte Separation Medium (LSM 1077) and centrifuged at 1171 x g for 25 min; no brake was applied during deceleration. Following centrifugation lymphocytes concentrating in the interphase (white phase) were collected and washed twice with PBS.

Washing was performed by centrifugation at 300 x g for 10 min in the first step and 200 x g for 10 min in the second step. After centrifugation pellets were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Adherent cells were removed from PBMC suspensions (density of 2×10^6 cells per ml) by attachment to plastic at the flask bottom for 1-2 hours. This procedure was repeated once more before cells in the culture supernatant were subjected to further treatment. For generation of LAK cells the cell suspension was incubated with 10 ng/ml IL-2 for 6 days at a density of about 1.5×10^6 cells per ml. After 3 days the medium was changed and fresh IL-2 was added.

For some experiments fractions of LAK cells were treated with vehicle or CBD. In this case vehicle or CBD were added to LAK cells into the culture flask 48 h before starting the LAK cytotoxicity assay or lysing LAK cells for subsequent Western blot analysis.

2.4. Adhesion assay

Tumor cells (target cells) were plated into 96-well flat bottom plates at a density of 1×10^4 cells per well and were allowed to grow for 24 h. Subsequently, cells were washed with PBS and stimulated with vehicle or test substance in serum-free DMEM. After 48 h of incubation tumor cells were washed. Before starting the adhesion assay, generated LAK cells (effector cells) were labelled with 5 µM of calcein-AM for 30 min. LAK cells were washed twice with PBS and centrifuged at 500 x g for 10 min. Afterwards, cells were resuspended in serum-free RPMI 1640 and added to the stimulated target cells using an effector:target cell ratio of 4:1. Following a 1-h co-incubation, LAK cells not adhering to target cells were removed by gently washing the culture with PBS. Adherent calcein-labelled LAK cells were lysed with 2% (v/v) Triton® X-100 for about 20 min and fluorescence was measured at 485 nm using Tecan infinite pro200 plate reader (Tecan, Grailsheim, Germany). In parallel to the adhesion assay, viability of tumor cells was determined under equal conditions using the WST-1 assay (Roche, Grenzach-Wyhlen, Germany). The viability was detected by measuring the absorbance at 450 nm. The adhesion was calculated from the specific activity of each

calcein-labeled LAK cell preparation as described previously with slight modification (11). Due to toxic effects of CBD fluorescence data were normalized to the viability of tumor cells according to the following formula: % adhesion = (fluorescence of adherent cells / viability of cancer cells). Vehicle controls were defined as 100% for evaluation of changes of adhesion to stimulated cancer cells. Blank values for both fluorescence and viability were subtracted from experimental data. Before adhesion was calculated the raw data of the fluorescent measurement were analysed with Nalimov test and outliers were excluded.

To evaluate the effect of ICAM-1 on adhesion, the stimulated tumor cells were incubated with 1 µg/ml of ICAM-1 antibody or an isotype control antibody. Incubation of target with effector cells was performed following a 3-h incubation period, subsequent to rinsing of the supernatant and addition of LAK to target cells.

2.5. LAK cytotoxicity assay

The cytotoxicity of LAK cells on tumor or BEAS-2B cells was determined by the calcein-AM release assay. Tumor or BEAS-2B cells (target cells) were plated into 96-well flat bottom plates at densities of 5×10^3 to 1×10^4 cells per well and were allowed to grow for 24 h. Afterwards cells were washed with PBS and treated with vehicle or test substance in serum-free DMEM. Following a 48-h incubation period, target cells were washed and labelled with 5 µM of calcein-AM for 30 min. Subsequently, cells were washed with PBS and generated LAK cells (effector cells) were added to the target cells at an effector:target cell ratio of 8:1 (Fig. 4B, 7B, 8B) or 4:1 (all other experiments) in a final volume of 100 µl per well. After a 6-h incubation (37°C, 5% CO₂) supernatants were transferred to other wells of the 96-well plate and remaining target cells were lysed with 2% (v/v) Triton® X-100. The fluorescence of supernatants and lysed target cells was measured at 485 nm using a Tecan infinite pro200 plate reader. In preliminary tests different cell numbers and effector:target cell ratios were analysed. On the basis of these pre-tests a final number of cancer cells and a final ratio were chosen.

LAK-induced cytotoxicity was monitored by the release of calcein into the supernatant by cancer cells due to toxic effects induced by LAK cells in the co-culture. To account for a probable modulation of cancer cell viability by vehicle or test substances, the fluorescence of cancer cells in the absence of effector cells, referred to as spontaneous release of calcein, was subtracted from these values. Finally, values were normalised to the toxic range that can be achieved maximally by the effector cells. This value appearing as denominator in the formula was calculated as difference between fluorescence released from target cells completely lysed with Triton[®] X-100 and fluorescence emitted from vehicle- or cannabinoid-treated cancer cells in the absence of effector cells. Accordingly, the percentage of LAK cytotoxicity was calculated as follows: % LAK cytotoxicity = (fluorescence of supernatant of sample well with effector cells – fluorescence of spontaneous release) / (fluorescence of maximal release – fluorescence of spontaneous release) (10). Blank values for fluorescence were subtracted from the experimental data. Before LAK cytotoxicity was calculated the raw data of the fluorescent measurement were analysed with Nalimov test and outliers were excluded.

Experiments to assess the functional involvement of ICAM-1 in enhanced killing of tumor cells by LAK cells were performed using a neutralizing antibody against ICAM-1 as well as small interfering RNA (siRNA) targeting ICAM-1 mRNA in tumor cells. Experiments with the ICAM-1 neutralizing antibody were performed by incubation of target cells with 1 µg/ml of an ICAM-1 antibody or an isotype control antibody as negative control for 2 h. Following pre-incubation of cancer cells with the antibodies, supernatants were removed carefully and without washing the co-incubation with LAK cells was started. For analysis of the involvement of lymphocyte function associated antigen 1 (LFA-1) in LAK cell cytotoxicity against tumor cells a CD11a antibody or an isotype control antibody was used. Experiments were carried out by incubation of LAK cells with 1 µg/ml of the respective antibody for 2 h before starting cytotoxicity assay by adding the LAK cell suspension with the therein present antibody to the target cells. To investigate the role of cannabinoid receptors (CB₁, CB₂) and transient receptor potential vanilloid 1 (TRPV1), tumor cells were preincubated with the

respective antagonist (AM-251, AM-630, capsazepine) for 1 h before stimulation with test substances or vehicle.

2.6. siRNA transfection

Transfection of tumor cells was performed using siRNA targeting ICAM-1 mRNA. The target sequence of ICAM-1 siRNA (Qiagen, Hilden, Germany) was 5'-CGGCCAGCTTATACACAAGAA-3'. A BLAST search revealed that the sequence selected did not show any homology to other known human genes. Cells were transfected using RNAiFect as transfection reagent (Qiagen, Hilden, Germany) and nonsilencing negative control RNA (Eurogentec, Cologne, Germany), respectively. Tumor cells were plated into 6-well (for Western blot analysis) or 96-well plates (for cytotoxicity assays) and allowed to grow for 2-3 h. Afterwards cells were transfected with 1.25 µg/ml ICAM-1 siRNA or nonsilencing siRNA as negative control with an equal ratio (w/v) of RNA to transfection reagent for 21 h in 10% DMEM. Before starting incubation with cannabinoid or vehicle cells were washed with PBS and transfected again in serum-free DMEM to provide constant transfection conditions.

2.7. Western blot analysis

To analyze protein levels of ICAM-1, lung tumor or non-tumor cells were grown in 6-well plates at a density of 2×10^5 cells per well for 24 h and subsequently incubated with vehicle or test substance for 48 h. For analysis of LFA-1 protein levels LAK cells were incubated with vehicle or CBD for 48 h as described under "Generation of LAK cells". After incubation cells were washed with PBS, harvested and lysed in solubilization buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton[®] X-100, 10% (v/v) glycerol, 1 mM PMSF, 1 mM orthovanadate, 1 µg/ml leupeptin, 10 µg/ml aprotinin). Lysis was performed for at least 30 min on ice and frequently mixing on the vortex mixer. Subsequently, lysates were centrifuged at 10,000 x g for 5 min and supernatants were then used for Western blot analysis. Total protein of cell lysates was determined using the bicinchoninic acid assay (Pierce, Rockford, USA). Proteins were separated using 10% sodium dodecyl sulfate polyacrylamide gels and

then transferred to nitrocellulose membranes (Roth, Karlsruhe, Germany) that were blocked with 5% milk powder (BioRad, Munich, Germany). Membranes were incubated with primary antibody raised to ICAM-1 (Santa Cruz Biotechnology, Heidelberg, Germany) or LFA-1 (BioLegend, London, UK) at 4°C overnight. Subsequently, blots were probed with horseradish peroxidase conjugated anti-mouse IgG (New England Biolabs GmbH, Frankfurt am Main, Germany) and incubated for 1 h at room temperature. Antibody binding was visualized by a chemiluminescent solution (100 mM Tris-HCl pH 8.5, 1.25 mM luminol, 200 μ M p-coumaric acid, 0.09% [v/v] H₂O₂). Densitometric analysis of band intensities was achieved by optical scanning and quantifying using the Quantity One 1-D Analysis Software (Bio-Rad, Munich, Germany). To identify the band size of the Western blots, the prestained SDS-PAGE Standard (Broad Range; Bio-Rad, Munich, Germany) was used. A regression of the prestained standard revealed a band size of ICAM-1 at 90 kDa, of β -Actin at 42 kDa and of LFA-1 (CD11a) at 180 kDa. Vehicle controls were defined as 100% for evaluation of changes in protein expression. To ascertain equal protein loading in Western blots of cell lysates, membranes were probed with an antibody raised to β -actin (Sigma-Aldrich, Steinheim, Germany). All densitometric values were normalized to β -actin.

2.8. Statistical analysis

Comparisons between groups were performed with Student's two-tailed *t* test or with one-way ANOVA plus post hoc Bonferroni or Dunnett test using GraphPad Prism 5.04 (GraphPad Software, Inc., San Diego, USA).

3. RESULTS

3.1. *Role of ICAM-1 in CBD-induced adhesion of lung cancer cells to LAK cells*

In line with the data published recently by our group (7) CBD was found to induce the expression of ICAM-1 protein in both lung cell lines (A549, H460) as well as in metastatic cells derived from a lung cancer patient (Fig. 1A-C).

To investigate a possible proadhesive action of CBD and a potential involvement of ICAM-1 in this response, calcein AM-labelled LAK cells were added to cancer cells. Adherent LAK cells were quantified as calcein fluorescence released by remaining and thus adherent cells following washing of the co-cultures to remove non-adherent cells and subsequent lysis. According to Fig. 1A-C, CBD caused a highly significant increase of adhesion. To investigate a causal link between the CBD-induced ICAM-1 upregulation and the concomitant increase of adhesion by CBD, a neutralizing antibody to ICAM-1 was tested for its inhibitory action on adhesion. In all cell lines investigated the ICAM-1 antibody significantly suppressed the CBD-induced adhesion when compared to cells treated with vehicle and isotype control antibody, respectively (Fig. 1A-C).

Notably, adhesion data were normalized to the viability of cancer cells in the absence of LAK cells to account for the toxicity by CBD that has recently been reported by our group (18). In these and subsequent viability tests performed in parallel to the killing assays, incubation of cells with CBD at 3 μ M yielded viability rates as compared to vehicle (100%) that ranged between 64-88% for A549, 54-76% for H460 and 49-80% for lung cancer patient's metastatic cells. In these experiments the presence of antibodies left both basal as well as CBD-modulated viability rates virtually unaltered (data not shown).

3.2. *Role of ICAM-1 in CBD-induced LAK cell-mediated tumor cell killing*

To investigate the functional consequence of increased adhesion of LAK to cancer cells, LAK cell-mediated tumor cell lysis was investigated next. To this end, tumor cells were labelled

with calcein-AM, incubated with LAK cells and evaluated for release of dye subsequently. As shown in Fig. 2, CBD elicited a significant increase of tumor cell lysis.

In context with our experimental setting it is worthy to note that LAK cells have not been exposed to test substances as would be the case under in vivo conditions. To exclude the possibility that LAK cells exposed to CBD may exert a less active status, further experiments were carried out by use of the same protocols but with additional parallel exposure of LAK cells to CBD. According to Fig. 2 (A-C) this approach revealed CBD-treated tumor cells to be killed by LAK cells regardless of a pretreatment of LAK cells with CBD. Notably, in some cases lysis of cancer cells appeared to be higher in the absence of LAK cells resulting in negative values of calculated percental LAK cytotoxicities, which is in line with observations from other groups (19,20).

Further experiments with LAK cells focussed on LFA-1, the cognate ICAM-1 receptor on the cell surface of immune cells, which has been reported to be an important link to conjugate ICAM-1-bearing cells with natural killer cells (21) and to confer lymphocyte-induced tumor cell killing (22). In contrast to CBD-induced ICAM-1 expression in cancer cells, treatment of LAK cells with 3 μ M CBD left protein expression of LFA-1 virtually unaltered as compared to vehicle (Fig. 2D).

To address the impact of ICAM-1 in tumor cell lysis elicited upon LAK cell treatment, ICAM-1 was neutralized using a specific antibody. In line with the adhesion data, the ICAM-1 neutralizing antibody abolished the CBD-induced tumor cell killing by LAK cells (Fig. 3A-C).

To further confirm a causal link between the CBD-mediated ICAM-1 and the subsequent tumor cell lysis by LAK cells, the expression of ICAM-1 was blocked by transfecting cells with ICAM-1 siRNA. According to Fig. 4A-C, transfection of cells with ICAM-1 siRNA was associated with a complete reversal of cancer cell lysis by LAK cells without interference with basal cytotoxicity. Using a setup published recently (7) these findings were supported by Western blot analyses that demonstrated transfection of the respective cells with ICAM-1

siRNA to profoundly decrease CBD-induced ICAM-1 protein expression without altering basal ICAM-1 levels (Fig. 4A-C).

3.3. *Involvement of cannabinoid receptors and TRPV1 in CBD-induced LAK cell-mediated tumor cell killing*

Recently, a role of cannabinoid receptors 1 and 2 (CB₁ and CB₂) as well as transient potential vanilloid 1 (TRPV1) in ICAM-1 upregulation by cannabinoids in lung cancer cells was established (7). On the basis of these finding we next addressed the question whether the promoting action of CBD on LAK cell-mediated tumor cell killing shares the same upstream targets. To this end, cells were preincubated with the CB₁ receptor antagonist AM-251, the CB₂ receptor antagonist AM-630 or the TRPV1 antagonist capsazepine. All antagonists were used at a concentration of 1 μ M, which has been reported to be within the range of concentrations inhibiting CB₁-, CB₂- and TRPV1-dependent events (23-26). Antagonists to both cannabinoid receptors and TRPV1 suppressed the CBD-induced cytotoxic action of LAK cells on cancer cells (Fig. 5A-C, left). Western blot analysis revealed inhibitory effects of each of the antagonists (AM-251, AM-630, capsazepine) on CBD-induced ICAM-1 protein expression (Fig. 5A-C, right) which, in case of A549 and H460, is in line with recently published data (7).

3.4. *Role of LFA-1 in CBD-induced LAK cell-mediated tumor cell killing*

To evaluate LFA-1 as a potential receptor in LAK cell-mediated tumor cell killing, LAK cells were pretreated with 1 μ g/ml of an LFA-1 antibody. According to Fig. 6, the antibody significantly inhibited the CBD-induced killing of lung cancer cells by LAK cells suggesting the intercellular ICAM-1/LFA-1 crosslink to be involved in CBD-induced enhanced susceptibility of lung cancer cells to LAK cell-mediated cytotoxicity.

3.5. Role of ICAM-1 in LAK cell-mediated tumor cell killing by other cannabinoids

To determine whether induction of ICAM-1-dependent tumor cell killing was unique for CBD or shared by other structurally unrelated cannabinoids, additional experiments were performed with the phytocannabinoid THC as well as the hydrolysis-stable anandamide analogue, R(+)-methanandamide. In a recent study both cannabinoids were shown to upregulate ICAM-1 in lung cancer cells thereby conferring TIMP-1 induction and subsequent inhibition of cancer cell invasion (7). According to the results shown in Fig. 7 and 8, both cannabinoids elicited a significant increase of LAK cytotoxicity and concomitant upregulation of ICAM-1 protein with both effects being reversed by siRNA against ICAM-1.

3.6. Impact of cannabinoids on ICAM-1 expression and LAK cell-mediated killing of non-tumor bronchial epithelial cells

To investigate the impact of the cannabinoids CBD, MA and THC on ICAM-1 expression and LAK cell-mediated killing of non-tumor cells, the bronchial epithelial cell line BEAS-2B was used. As shown in Fig. 9, both CBD and THC only slightly influenced ICAM-1 expression of BEAS-2B cells. Accordingly, at concentrations of 3 μ M, CBD and THC increased ICAM-1 protein levels 1.5-fold and 1.4-fold, respectively. MA did not alter ICAM-1 protein level at any concentration tested (Fig. 9). In comparison, the same concentration of cannabinoids tested under comparable experimental conditions yielded upregulation of ICAM-1 protein levels in lung cancer cells (A549, H460, lung cancer patient's metastatic cells) by up to 6- (Fig. 5A), 11- (Fig. 4B) or 9.2-fold (Fig. 5C) for CBD, 3.3-, 5.4-, or 3.9-fold for THC (Fig. 8A-C) and 2.6-, 2.2- and 2.2-fold for MA (Fig. 7A-C). In line with the substantially lower or absent ICAM-1 induction, none of the cannabinoids led to a significant increase in cytotoxic lysis of BEAS-2B by LAK cells (Fig. 9).

4. DISCUSSION

Recently, ICAM-1 was identified as an essential link within the signal transduction pathway conferring the TIMP-1-dependent anti-invasive action of the cannabinoids CBD, THC and R(+)-methanandamide on human lung tumor cells (7). Using the same cell lines (A549, H460) as well as metastatic cells from a lung cancer patient, the present study analyzed the contribution of cannabinoid-induced ICAM-1 on cancer cell susceptibility to cytolytic LAK cells. As a major result the data from the present study suggest cannabinoids to enhance the susceptibility of lung cancer cells to cytolytic cell death by LAK cells via increase of ICAM-1.

There are several lines of evidence supporting this notion. First, the susceptibility of CBD-treated cancer cells to both adhesion to as well as subsequent lysis by LAK cells was reversed by a neutralizing ICAM-1 antibody. Second, post-transcriptional knock-down of CBD-induced ICAM-1 expression using an siRNA approach was likewise found to abrogate increased cancer cell lysis. Third, a reversal of the cytolytic action of LAK cells on CBD-treated cancer cells was achieved when cannabinoid receptors (CB₁, CB₂) or TRPV1 were blocked with specific antagonists which is in line with CBD's recently established cannabinoid receptor- and TRPV1-dependent upregulation of ICAM-1 expression (7). Fourth, key experiments with the phytocannabinoid THC and the stable anandamide analog R(+)-methanandamide confirmed a pivotal role of ICAM-1 in LAK cell-mediated tumor cell killing suggesting ICAM-1-mediated lung cancer cell lysis by LAK cells as an effect shared by diverse ICAM-1-inducing cannabinoids.

Altogether, these data are in agreement with several studies suggesting ICAM-1 overexpression on tumor cells to elicit a reduced tumor growth in vivo correlating with an increase of tumor cell lysis by tumor-infiltrating lymphocytes (27-29). In vitro LAK-induced cancer cell lysis was shown to be significantly inhibited by ICAM-1 antibody in melanoma (30) as well as gastric cancer cells (31). In line with this notion, melanoma cell killing by monocytes has been reported to increase as response to pre-treatment with ICAM-1-inducing cytokines (32). Vice versa, ICAM-1 downregulation was found to be associated with

enhanced liver and pancreatic metastasis in animal models (14) and to negatively correlate with lymph node metastasis in breast cancer (15) as well as gastric cancer patients (16). Finally, ICAM-1 has been found to be associated with relapse-free survival of patients with esophageal carcinoma (33).

Considering the low affinity of CBD to either CB₁ or CB₂ receptors it appeared rather surprising that cannabinoid receptor antagonists inhibited CBD's effect on LAK-mediated killing and ICAM-1 induction. However, in line with this and recent findings from our group (7,26), other authors found similar results. Accordingly, CBD has been reported to modulate cytokine release and macrophage chemotaxis (34) as well as antiproliferative (35) and proapoptotic properties (36) in a cannabinoid receptor-dependent manner. A possible reason for the obvious discrepancy between the low receptor affinity and the apparent involvement of cannabinoid receptors in CBD's effects may be given by its inhibitory action on fatty acid amidohydrolase (FAAH) activity that confers decrease of endocannabinoid degradation and thus a prolonged action of FAAH substrates at the CB₁ and CB₂ receptor (37-39). Interestingly, in H460 cells, the antagonist to CB₁, AM-251, elicited only a partial inhibitory effect on CBD-induced ICAM-1 expression which is in agreement with an earlier publication from our group (7). Considering the full inhibition of LAK-induced killing by AM-251 in H460 cells, it is tempting to speculate that ICAM-1 elicits a threshold rather than a concentration dependent effect on LAK cell-induced cancer cell killing.

Further experiments addressed the role of LFA-1, the natural ligand of ICAM-1, in CBD-mediated increased tumor cell killing. The LFA-1 heterodimer (CD11a/CD18) belongs to the β_2 -integrin family of adhesion molecules and is expressed on lymphocytes, monocytes, granulocytes, and bone marrow cells. The LFA-1 complex has been reported to be essential for cell-mediated cytotoxicity by cytotoxic T-lymphocytes and natural killer cells (40,41) and represents an important link to conjugate ICAM-1-bearing cells with natural killer cells (21) and to confer lymphocyte-induced tumor cell killing (22).

The present study found a reversal of enhanced killing of CBD-treated cancer cells by preincubation of LAK cells with an antibody to LFA-1 suggesting LFA-1 as a crucial counter-

receptor of ICAM-1 in conferring the CBD-induced enhanced susceptibility of lung cancer cells to LAK cell-mediated killing.

According to the data presented here an enhancement of tumor immune surveillance may be an essential mechanism of cannabinoids' action on cancer progression. Noteworthy, in context with a probable use of cannabinoids as systemic anticancer drugs it is of essential interest whether the immune system is influenced by these drugs. Previous *in vitro* studies suggest cannabinoids to induce immunosuppressive effects via induction of apoptosis of dendritic cells (42) and by induction of thymic and splenic atrophy (43). However, under the conditions presented here pre-treatment of LAK cells with cannabinoids did not exert reduced killing of tumor cells suggesting CBD to enhance the susceptibility of tumor cells to LAK cell-induced killing while sparing direct effects on LAK cell activity.

Clearly, more research is needed to understand the complete role of ICAM-1 in tumorigenesis. Thus, besides the antitumorigenic properties observed by us and others, some studies found ICAM-1 to be associated with increase of cancer cell invasiveness (44-46). Independent thereof, there is now substantial evidence that ICAM-1 poses an essential target for cannabinoids in exerting its antitumorigenic function. Thus, besides increasing cancer cell susceptibility to LAK cell-mediated cytolysis, we recently provided the first inhibitor-based evidence for ICAM-1 as intermediate link in CBD's antimetastatic action on lung cancer cells (7).

Another topic of interest concerns the impact of cannabinoids on ICAM-1 in healthy tissues. In fact, ICAM-1-mediated immunoreactivity also poses a mechanism that mediates several adverse effects such as vascular damage based on leukocyte endothelial interactions (47). In hitherto published studies, CBD was shown to inhibit ICAM-1 expression in high-glucose-treated human coronary artery endothelial cells (48) without altering basal ICAM-1 expression when tested at a final concentration of 4 μ M. In the present study experiments with BEAS-2B, a cell line established from normal bronchial epithelium of non-cancerous individuals and characterised as non-cancerous (49,50), were performed accordingly. Using these cells, ICAM-1 expression was found to be not (MA) or only marginally increased (CBD,

THC) by cannabinoids. In line with this notion, cannabinoids elicited no significant increase of cytotoxic lysis of non-tumor BEAS-2B by LAK cells supporting ICAM-1 induction by cannabinoids as integral component of their pro-killing properties. Considering ICAM-1 to be strongly induced by cannabinoids in lung cancer but much lesser or not at all in BEAS-2B cells, it is tempting to speculate that profound cannabinoid-induced ICAM-1 expression may occur specifically in tumor cells. However, additional studies addressing the concentration-dependent impact of diverse cannabinoids on ICAM-1 in other non-cancerous cells are advisable.

Collectively, the present study provides first-time proof for a contribution of cannabinoid-induced ICAM-1 on lung cancer cells to increased LAK cell-mediated tumor cell killing as a novel antitumorigenic mechanism of cannabinoids.

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FIGURE LEGENDS

Figure 1

Effect of CBD on ICAM-1 protein expression in human lung cancer cells and role of ICAM-1 in adhesion of LAK cells to cancer cells. Left panels: Concentration-dependent effect of CBD on ICAM-1 protein expression in A549 (A), H460 (B) and lung cancer patient's metastatic cells (C). Cells were incubated with CBD at the indicated concentrations for 48 h. Values above selected blots are means \pm SEM obtained from densitometric analysis of $n = 5$ (A) or $n = 7$ (B, C) blots. Right panels: Effect of a neutralizing ICAM-1 antibody on adhesion of LAK cells to cancer cells. Tumor cells (10,000 cells per well) treated with 3 μ M CBD or vehicle for 48 h were washed and pre-incubated with ICAM-1 antibody (1 μ g/ml) for 3 h before starting co-incubation (1 h) with LAK cells. An isotype control antibody (1 μ g/ml) was used as negative control. Values are means \pm SEM of $n = 36$ (A, 8 donors), $n = 44$ (B, 7 donors) and $n = 32$ (C, 5 donors) experiments. $**P < 0.01$, $***P < 0.001$; one-way ANOVA plus post hoc Bonferroni test.

Figure 2

Impact of CBD on cytotoxic activity (A-C) and LFA-1 protein expression (D) of LAK cells. Histograms (A-C) indicate LAK cell-mediated tumor cell killing as response to a 48-h pretreatment of cancer cells with vehicle (left triple bars) or 3 μ M CBD (right triple bars). The respective subgroups indicate killing of cancer cells when LAK cells were preincubated for 48 h in medium (white bars) or medium containing vehicle (grey bars) or 3 μ M CBD (black bars) prior to co-culturing with cancer cells. As LAK cell medium RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10 ng/ml IL-2 was used. Cytotoxicity was analyzed following a subsequent 6-h co-incubation of LAK cells with cancer cells (10,000 cells per well) at standardized conditions (37 $^{\circ}$ C, 5% CO₂). Values are means \pm SEM of $n = 24$ (A, 5 donors) or $n = 16-20$ experiments (B, C, 4 donors). $**P < 0.01$, $***P < 0.001$ vs. corresponding vehicle control; Student's t-test. (D) To analyze the impact of

CBD on LFA-1 expression, LAK cells were incubated with CBD (3 μ M) or vehicle for 48 h using RPMI 1640 with the above indicated supplements. Values above the selected blot are means \pm SEM obtained from densitometric analysis of $n = 4$ blots from 4 different donors.

Figure 3

Effect of a neutralizing ICAM-1 antibody on cytotoxic lysis of cancer cells by LAK cells. A549 (A), H460 (B) and lung cancer patient's metastatic cells (C) were incubated with vehicle or 3 μ M CBD for 48 h. Experiments were carried out with 10,000 cells per well. Before starting cytotoxicity assay cancer cells were pre-incubated with an ICAM-1 antibody (1 μ g/ml) for 2 h. An isotype control antibody (1 μ g/ml) was used as negative control. Values are means \pm SEM of $n = 24$ (A, 6 donors), $n = 32$ (B, 8 donors) $n = 20$ (C, 5 donors) experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; one-way ANOVA plus post hoc Bonferroni test.

Figure 4

Influence of ICAM-1 siRNA (si) on CBD-induced LAK cell cytotoxicity against cancer cells. Left panels: effect of ICAM-1 siRNA (1.25 μ g/ml) or non-silencing siRNA (non si; 1.25 μ g/ml) on cytotoxicity of LAK cells against CBD-treated A549 (A), H460 (B) and lung cancer patient's metastatic cells (C). Experiments were carried out with 10,000 cells per well (A549, lung cancer patient's metastatic cells) or 5,000 cells per well (H460). Values are means \pm SEM of $n = 32$ (A, 7 donors) or $n = 28$ (B, C, 6 donors) experiments. $*P < 0.05$, $***P < 0.001$; one-way ANOVA plus post hoc Bonferroni test. Right panels: Western blot analysis of the impact of ICAM-1 siRNA on CBD-induced ICAM-1 protein expression following a 48-h incubation period. Densitometric analyses refer to $n = 4$ (A, B) or $n = 5$ (C) experiments.

Figure 5

Involvement of cannabinoid receptors (CB₁, CB₂) and TRPV1 in CBD-induced cytotoxic lysis of tumor cells by LAK cells. Left panels: Effect of AM-251 (CB₁ antagonist, 1 μ M), AM-630 (CB₂ antagonist, 1 μ M) and capsazepine (TRPV1 antagonist, 1 μ M) on cytotoxic activity of

LAK cells against CBD-treated A549 (A), H460 (B) and lung cancer patient's metastatic cells (C). Cancer cells were pre-incubated with the antagonists for 1 h. Subsequently, cells were stimulated with 3 μ M CBD and the incubation was continued for 48 h. Experiments were carried out with 10,000 cells per well. Values are means \pm SEM of $n = 28-32$ (A, B, 5 donors) or $n = 28$ (C, 6 donors) experiments. $***P < 0.001$ vs. corresponding vehicle control; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. CBD; one-way ANOVA plus post hoc Bonferroni test. Right panels: Western blot analysis of the effect of the antagonists on CBD-induced ICAM-1 protein expression. Values above selected blots represent means \pm SEM obtained from densitometric analysis of $n = 6$ (a, B) or $n = 7$ (C) blots.

Figure 6

Effect of CD11a (LFA-1) antibody on cytotoxicity of LAK cells against cancer cells. A549 (A), H460 (B) and lung cancer patient's metastatic cells (C) were incubated with vehicle or 3 μ M CBD for 48 h. Experiments were carried out with 10,000 cells per well. Before starting cytotoxicity assay LAK cells were pre-incubated with a CD11a antibody (1 μ g/ml) for 2 h. An isotype control antibody (1 μ g/ml) was used as negative control. Values are means \pm SEM of $n = 28$ (A, C, 7 donors) or $n = 24$ experiments (B, 6 donors). $**P < 0.01$, $***P < 0.001$; one-way ANOVA plus post hoc Bonferroni test.

Figure 7

Influence of ICAM-1 siRNA (si) on R(+)-methanandamide (MA)-induced LAK cell cytotoxicity against cancer cells. Left panels: effect of ICAM-1 siRNA (1.25 μ g/ml) or non-silencing siRNA (non si; 1.25 μ g/ml) on cytotoxicity of LAK cells against MA-treated A549 (A), H460 (B) and lung cancer patient's metastatic cells (C). Experiments were carried out with 10,000 cells per well (A549, lung cancer patient's metastatic cells) and 5,000 cells per well (H460), respectively. Values are means \pm SEM of $n = 20-24$ (A, B, 5 donors) or $n = 16$ (C, 4 donors) experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; one-way ANOVA plus post hoc Bonferroni test. Right panels: Western blot analysis of the effect of ICAM-1 siRNA on MA-induced

ICAM-1 protein expression following a 48-h incubation period. Densitometric analysis refer to $n = 5$ (A, C) or $n = 4$ (B) experiments.

Figure 8

Influence of ICAM-1 siRNA (si) on THC-induced LAK cell cytotoxicity against cancer cells. Left panels: effect of ICAM-1 siRNA (1.25 $\mu\text{g/ml}$) or non-silencing siRNA (non si; 1.25 $\mu\text{g/ml}$) on cytotoxicity of LAK cells against THC-treated A549 (A), H460 (B) and lung cancer patient's metastatic cells (C). Experiments were carried out with 10,000 cells per well (A549, lung cancer patient's metastatic cells) and 5,000 cells per well (H460), respectively. Values are means \pm SEM of $n = 20-24$ (A, B, 5 donors) or $n = 16$ (C, 4 donors) experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; one-way ANOVA plus post hoc Bonferroni test. Right panels: Western blot analysis of the effect of ICAM-1 siRNA on THC-induced ICAM-1 protein expression following a 48-h incubation period. Densitometric analysis refer to $n = 4$ (A) or $n = 5$ (B, C) experiments.

Figure 9

Effect of CBD, R(+)-methanandamide (MA) and THC on ICAM-1 protein expression in human bronchial epithelial cells (BEAS-2B) and on cytotoxic lysis of BEAS-2B cells by LAK cells. Left panels: Concentration-dependent effect of CBD (A), MA (B) and THC (C) on ICAM-1 protein expression in BEAS-2B cells. Cells were incubated with CBD, MA or THC at the indicated concentrations for 48 h. Values above selected blots are means \pm SEM obtained from densitometric analysis of $n = 7$ (A) or $n = 6$ (B, C) blots. Right panels: Effect of CBD (A), MA (B) and THC (C) on cytotoxic lysis of BEAS-2B cells by LAK cells. BEAS-2B cells (10,000 cells per well) were incubated with CBD, MA or THC at the indicated concentrations for 48 h. Values are means \pm SEM of $n = 48$ (7 donors) experiments. One-way ANOVA plus post hoc Dunnett test revealed no significant effect of cannabinoids versus vehicle.

Figure

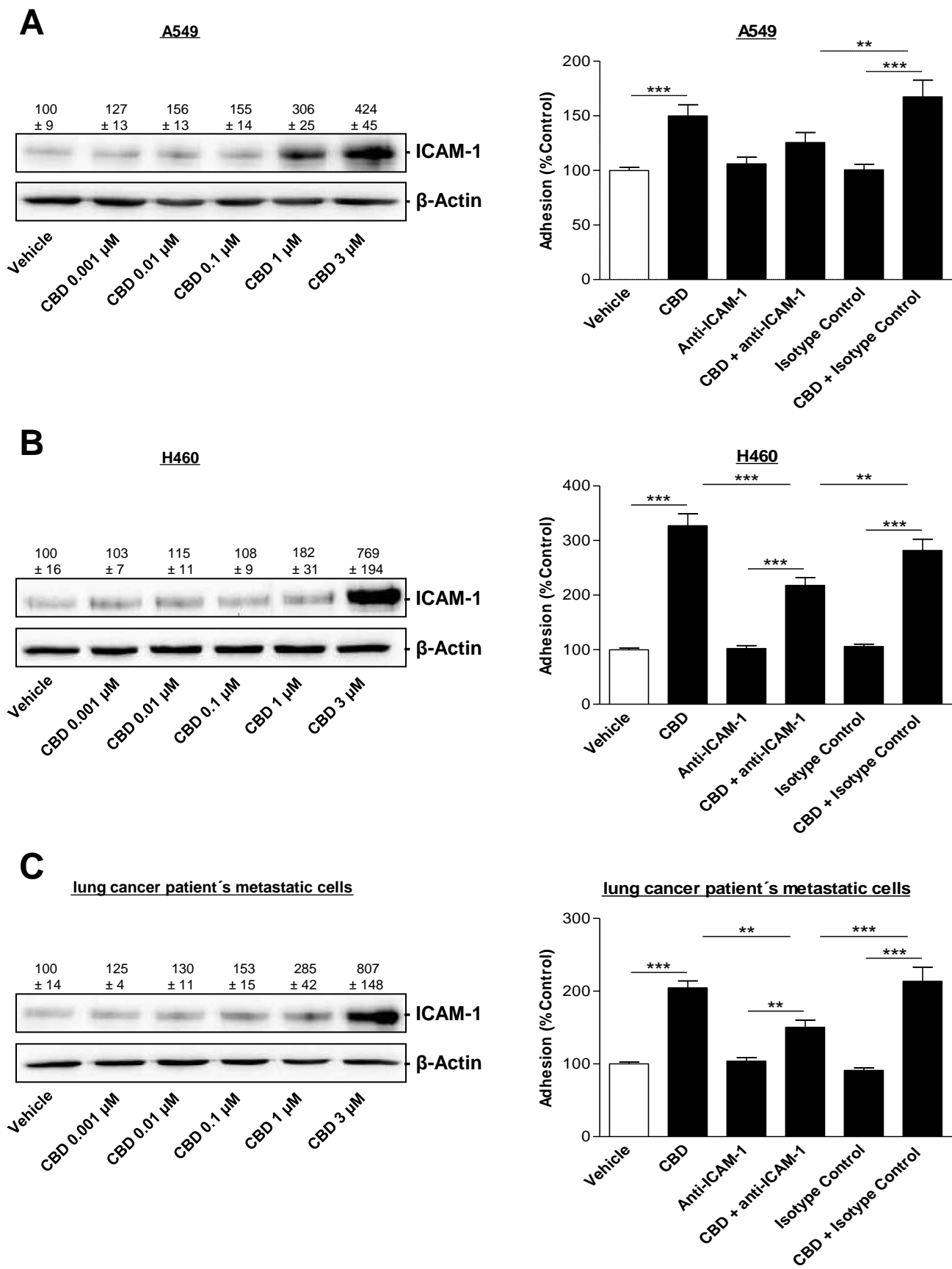


Figure 1

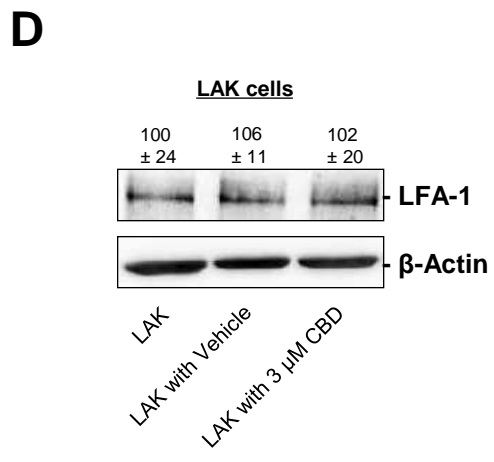
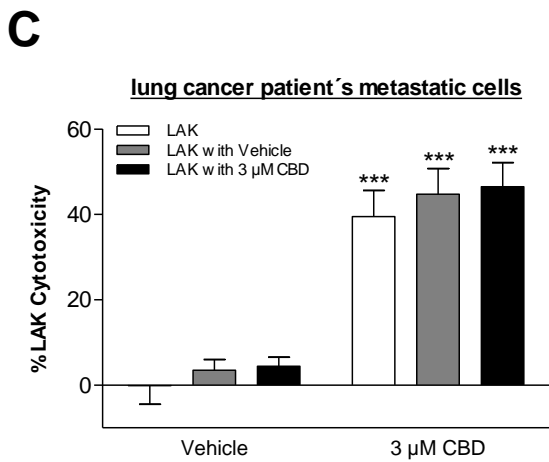
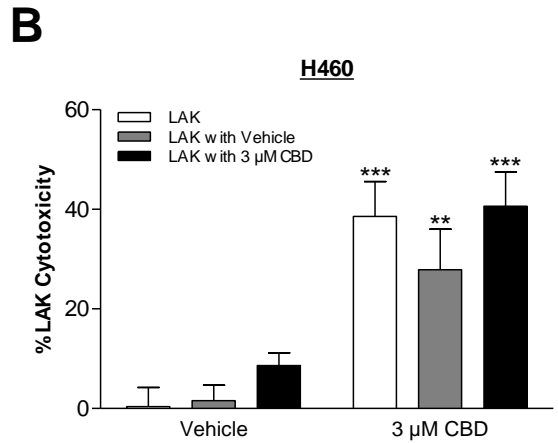
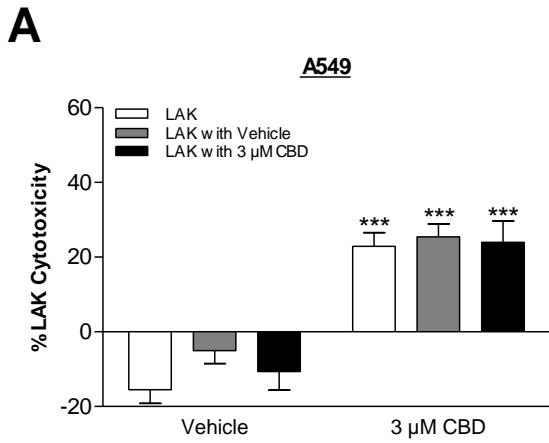


Figure 2

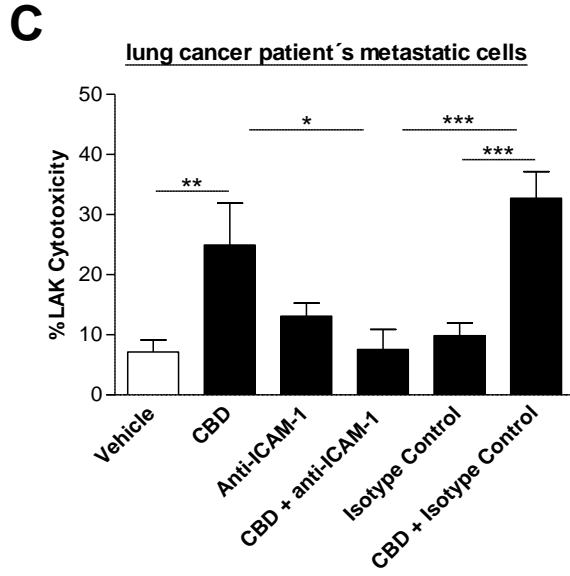
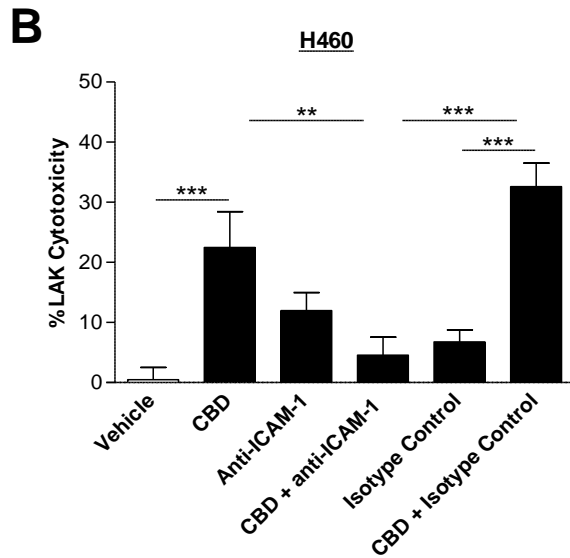
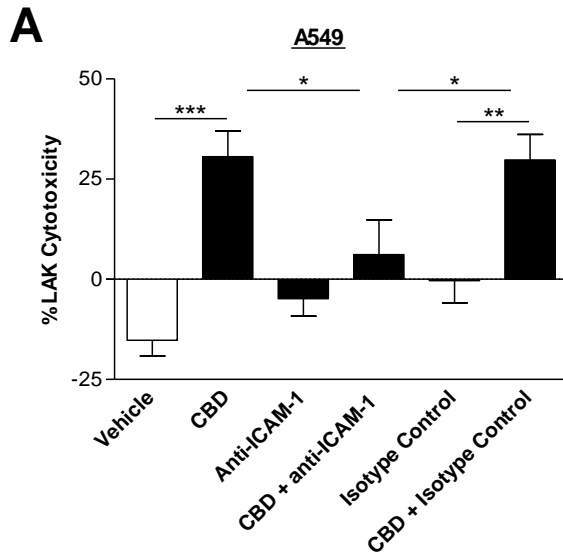
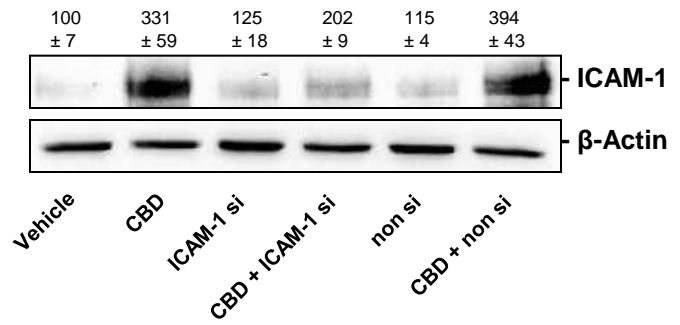
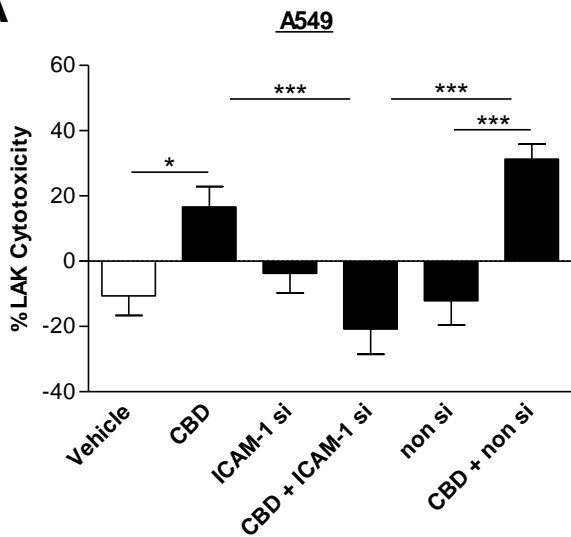
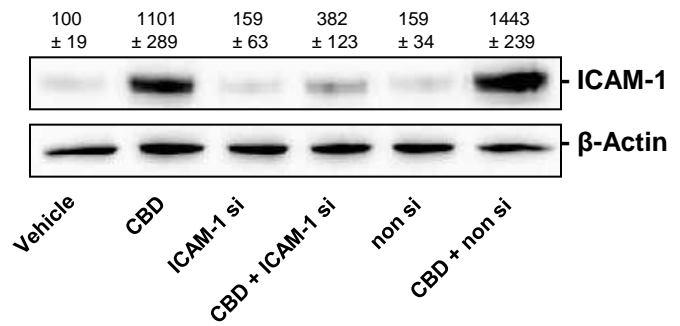
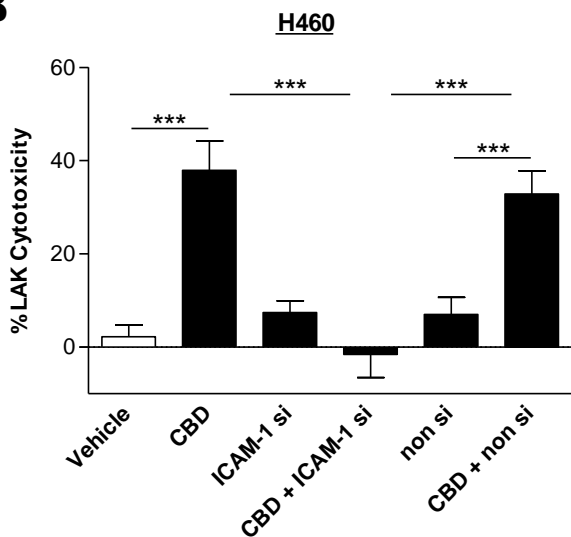


Figure 3

A**B****C**

lung cancer patient's metastatic cells

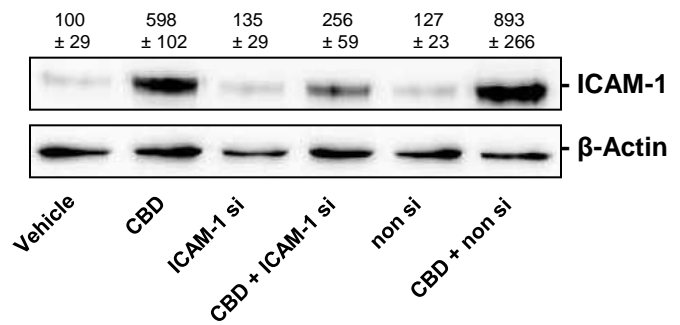
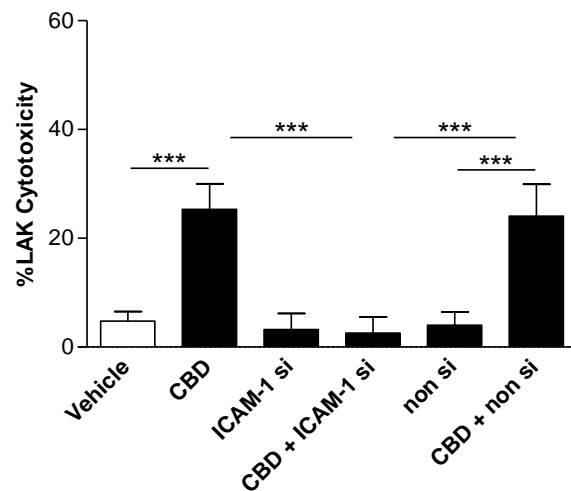


Figure 4

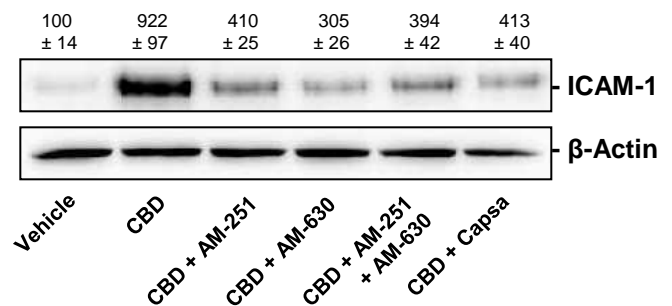
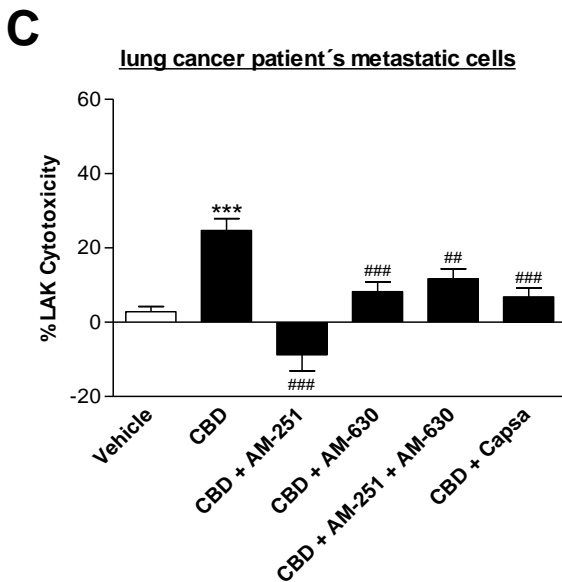
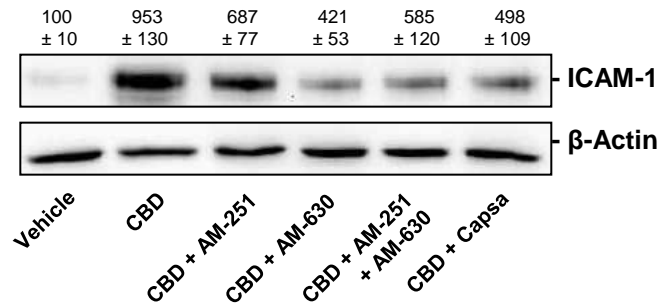
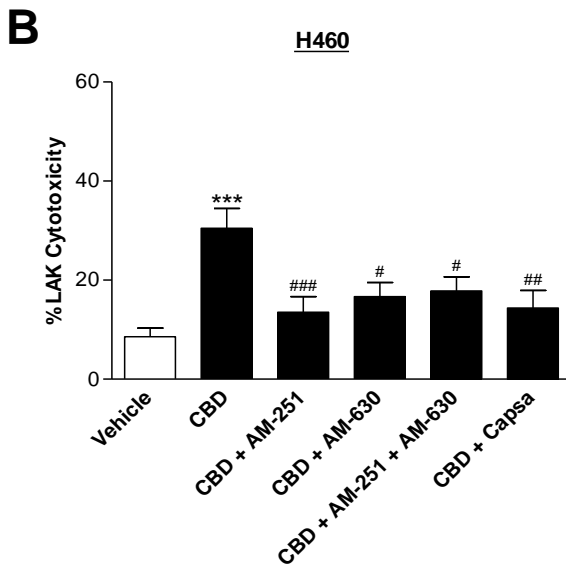
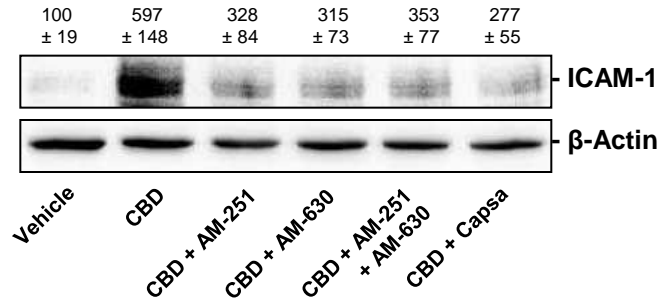
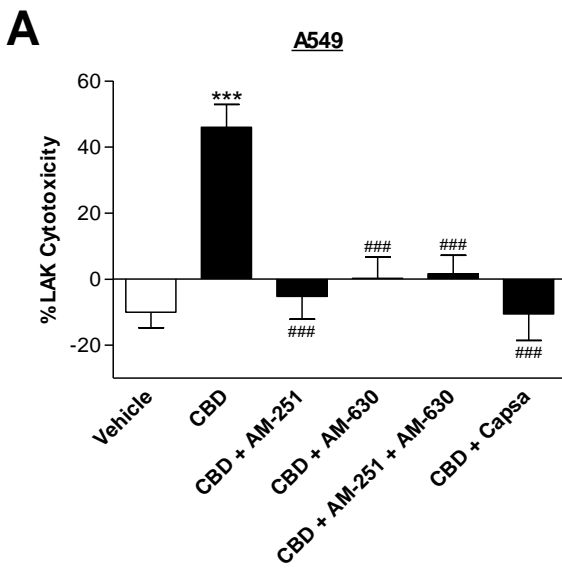


Figure 5

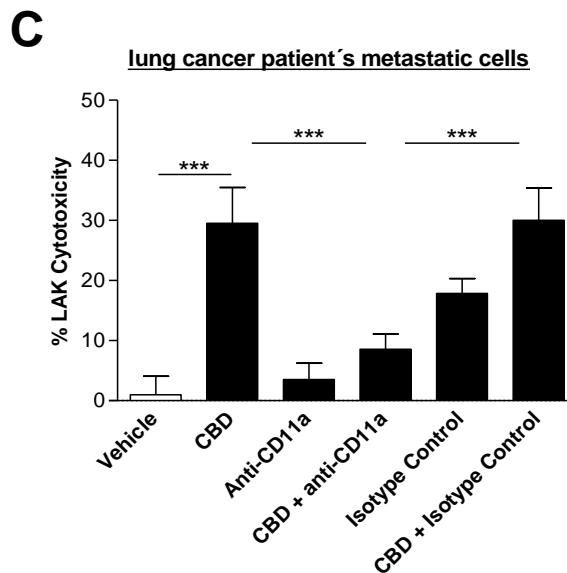
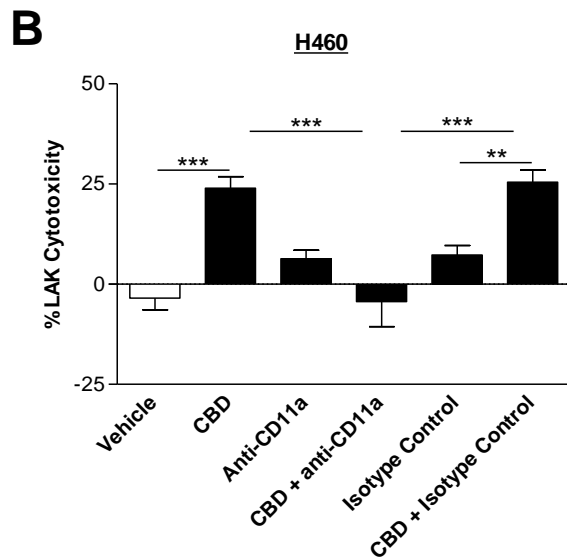
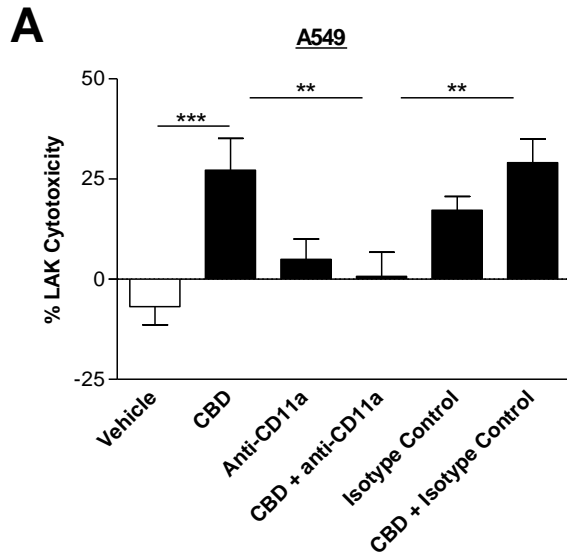
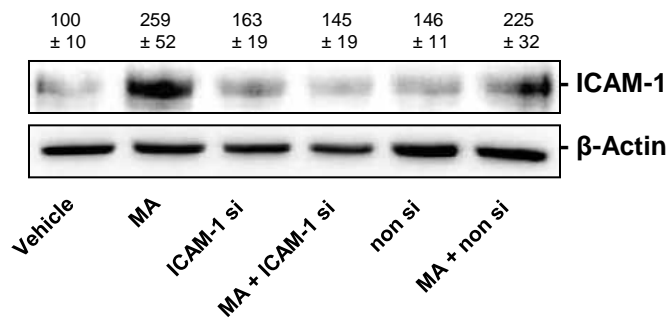
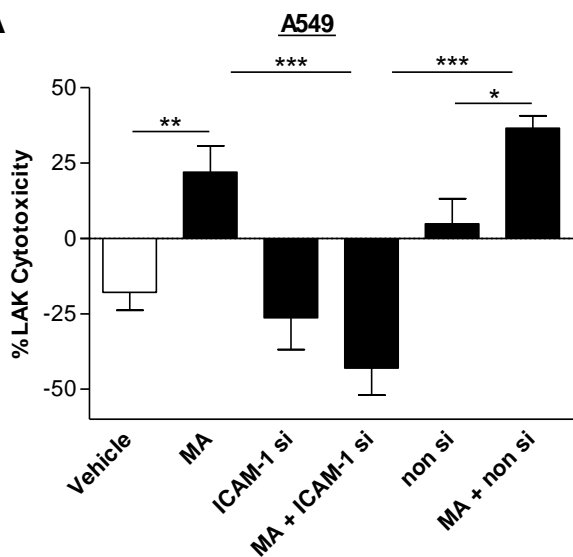
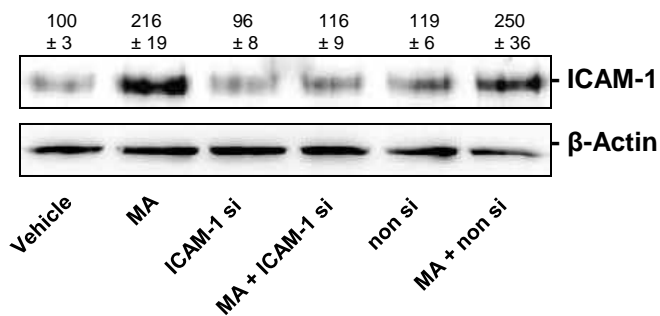
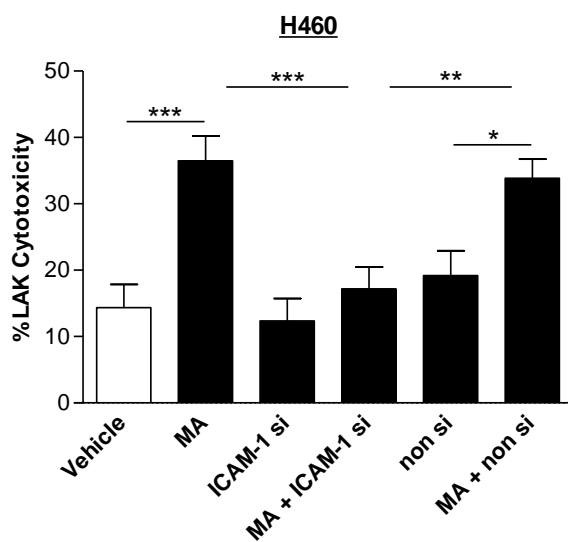


Figure 6

A**B****C**

lung cancer patient's metastatic cells

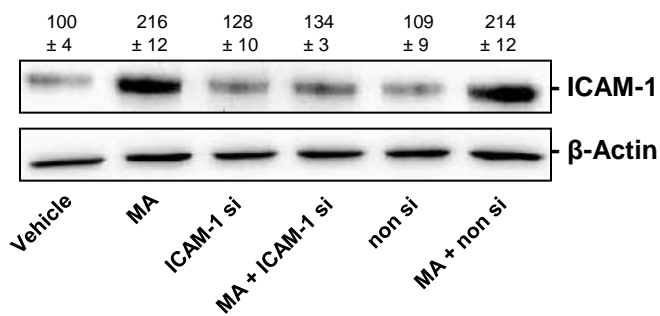
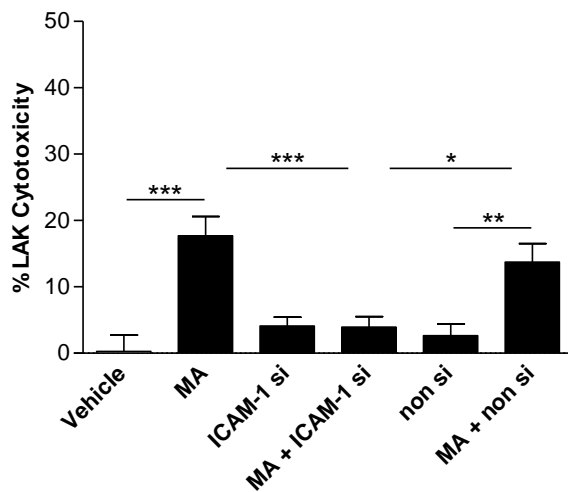


Figure 7

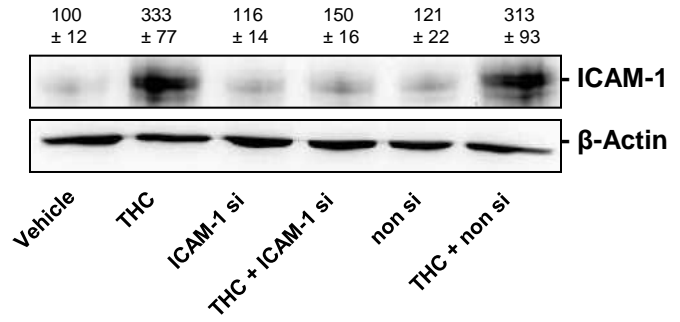
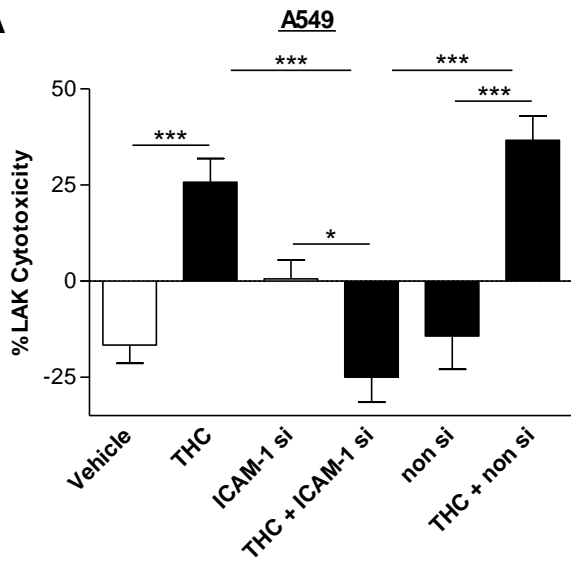
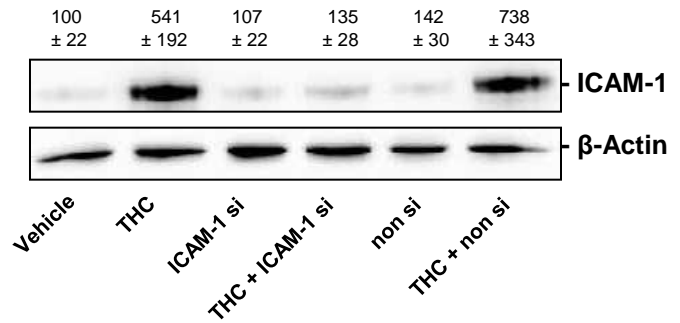
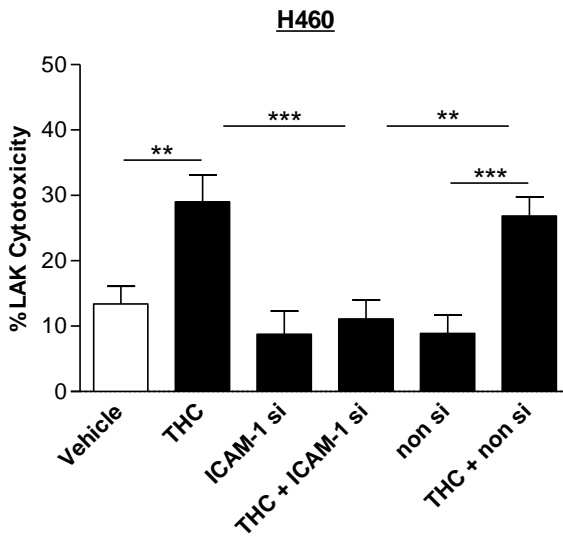
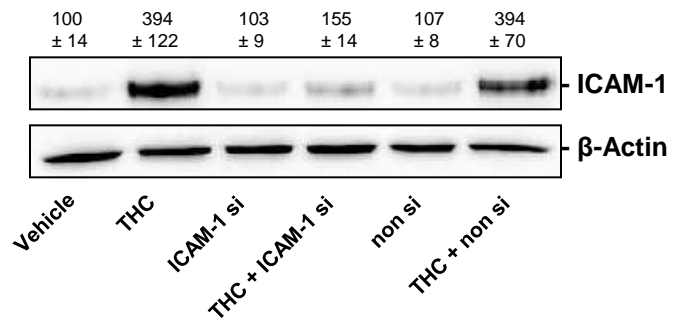
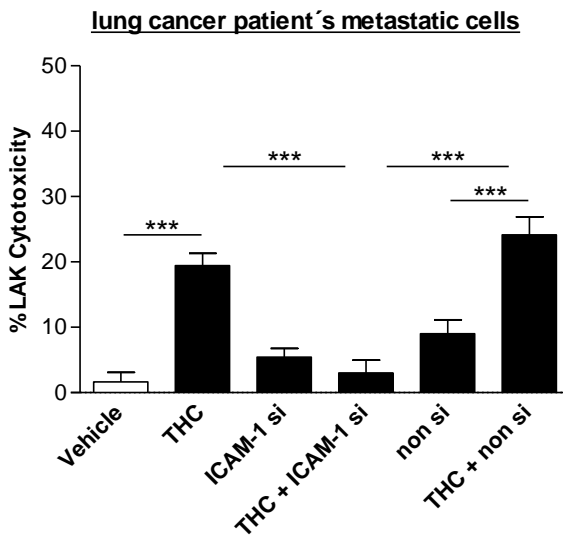
A**B****C**

Figure 8

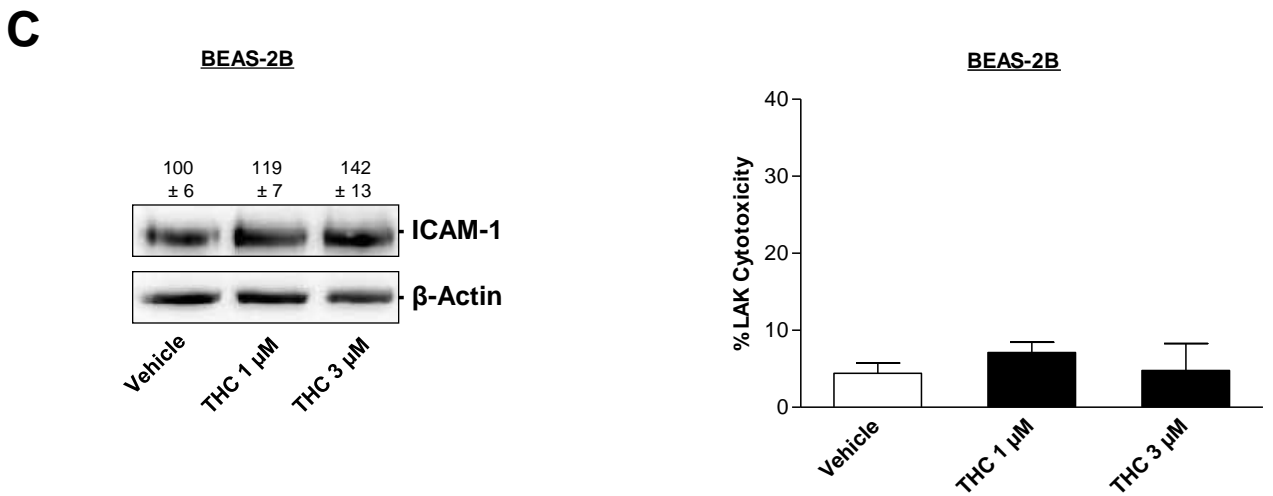
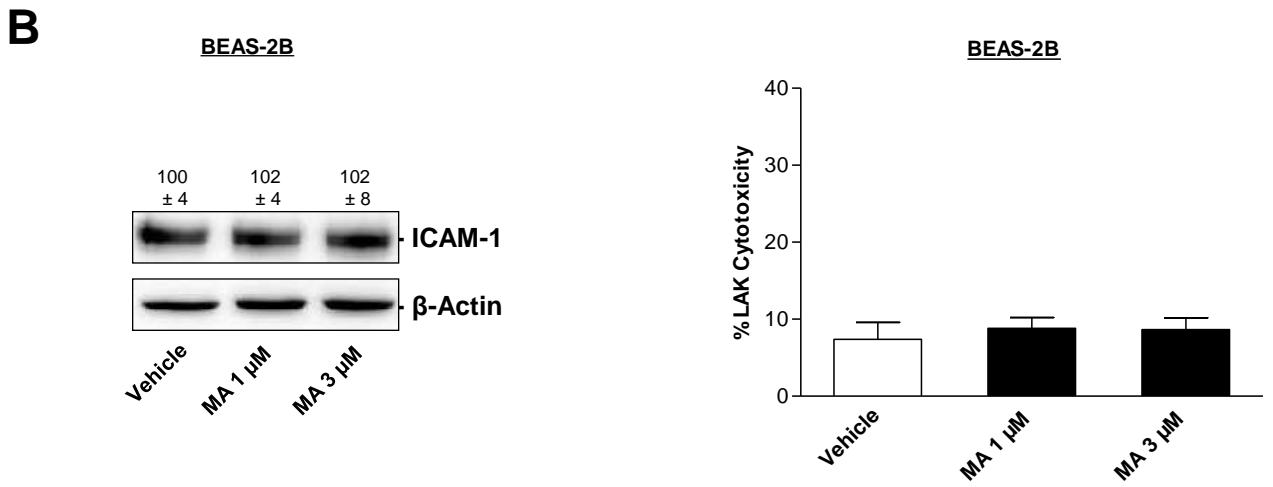
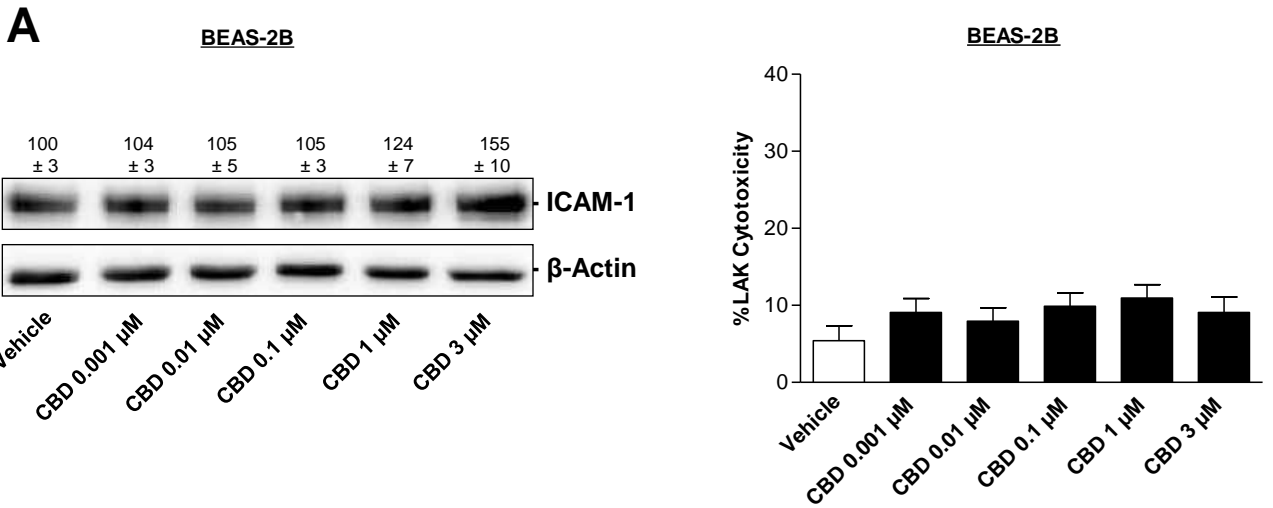


Figure 9

