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Incensole Acetate, a Novel anti-inflammatory compound Isolated from *Boswellia* Resin, Inhibits Nuclear Factor (NF)-kappa B Activation.

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Running title page

a) A running title: Incensole Acetate Inhibits NF-κB Activation

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d) ABBREVIATIONS: IA, incensole acetate; IN, incensole; PE, petroleum ether; NF-κB, nuclear factor kappa B; IKK, IκB kinase; TNFα, tumor necrosis factor-α; LPS, lipopolysaccharide; PMA, phorbol-12-myristate-13-acetate; KA, Kinase Assays; EMSA, electrophoretic mobility shift assay; TAK, TGFβ (transforming growth factor) activated kinase; TAB, TAK-binding protein; JNK, C-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; WB, western blotting.

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ABSTRACT

Boswellia resin is a major anti-inflammatory agent in herbal medical tradition, as well as a common food supplement. Its anti-inflammatory activity has been attributed to boswellic acid and its derivatives. Here, we re-examined the anti-inflammatory effect of the resin, using IκBα degradation in TNFα-stimulated HeLa cells as a read-out for a bioassay-guided fractionation. We thus isolated two novel NF-κB inhibitors from the resin, their structures elucidated as incensole acetate (IA) and its non-acetylated form, incensole (IN). IA inhibited TAK/TAB-mediated IκB kinase (IKK) activation loop phosphorylation, resulting in the inhibition of cytokine and LPS mediated NF-κB activation. It had no effect on IKK activity *in vitro*, nor did it suppress IκBα phosphorylation in costimulated T-cells, indicating that the kinase inhibition is neither direct, nor is it affecting all NF-κB activation pathways. The inhibitory effect appears specific as IA did not interfere with TNFα-induced activation of JNK and p38 MAPK. IA treatment had a robust anti-inflammatory effect in a mouse inflamed paw model. Cembrenoid diterpenoids, and specifically IA and its derivatives may thus constitute a potential novel group of NF-κB inhibitors, originating from an ancient anti-inflammatory herbal remedy.

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Boswellia species are natives of Eastern Africa, where their resin, commonly known as ″Frankincense″ or "Olibanum" is used and exported as incense. It has been extensively used for many centuries for various medical purposes, especially for the treatment of inflammatory diseases, in European, Middle Eastern and African medical traditions. In India, *Boswellia* resin is widely used in the treatment of inflammatory conditions, including Crohn's disease, arthritic diseases and asthma; hence a considerable amount of work has been done on the anti-inflammatory properties of *Boswellia* (for examples, see Gerhardt et al., 2001; Gupta et al., 1998; Altmann et al., 2003). Numerous previous reports attribute the anti-inflammatory and cytotoxic properties of *Boswellia* resin solely to boswellic acid and its derivatives (e.g. Khanna et al. 2007; Gerhardt et al., 2001; Altmann et al., 2003; Xia et al., 2005).

NF-κB is an inducible transcription factor that plays a central role in the mammalian innate immune response and chronic inflammation (Karin, 2005; Perkins, 2007). Ubiquitously expressed and involved in the activation of a multitude of genes in response to various stress stimuli, NF-κB plays a pivotal role in immune and inflammatory responses (Karin and Ben-Neriah, 2000). This effect is exerted through the regulation of target genes that encode pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors and inducible enzymes (Lawrence et al., 2001). Inappropriate regulation of NF-κB is thus directly involved in a wide range of human disorders, including arthritis, asthma, inflammatory bowel disease, a variety of cancers, ataxia telangiectasia and neurodegenerative diseases (Karin and Ben-Neriah, 2000; Ben-Neriah and Schmitz, 2004). Hence, identification of drugs allowing modulation of the NF-κB transduction pathway is of considerable interest (Bremner and Heinrich, 2002; Calzado et al., 2007). In non-stimulated cells, NF-κB is normally sequestered in the cytoplasm and must be translocated into the nucleus for the

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exertion of its function. This sub-cellular localization is controlled by a family of inhibitory proteins, which bind NF-κB, inhibit its DNA-binding and prevent its nuclear accumulation, namely IκB proteins. Specific extra-cellular stimuli lead to the rapid phosphorylation, ubiquitination, and ultimately proteolytic degradation of IκB, which frees NF-κB to translocate to the nucleus, where it regulates gene transcription (Karin and Ben-Neriah, 2000; Perkins, 2007). Cytokines act through distinct signaling pathways that converge on the activation of IKK. IκB degradation, following its phosphorylation by the IKK complex at Ser-32 and Ser-36, is considered to be the major step in NF-κB regulation (Karin and Ben-Neriah, 2000). Thus, activation of IKK is a key event in canonical NF-κB activation (Hacker and Karin, 2006). The core IKK complex consists of the kinases IKKα and IKKβ and the regulatory IKKγ/NEMO protein. The activation of both IKKs depends on phosphorylation of serines at their activation loop. This process probably involves transautophosphorylation of IKKs and phosphorylation by upstream kinases such as TGFβ (transforming growth factor) activated kinase (TAK)1. TAK1 is recruited to the IKK complex via the ubiquitin-binding adaptor proteins TAK-binding protein (TAB)2 and TAB3 (Hacker and Karin, 2006).

We revisited the anti-inflammatory properties of *Boswellia* resin and examined the mechanism by which the active ingredients of the resin inhibit NF-κB activition. A bioassay-guided fractionation, testing the inhibition of IκBα phosphorylation/degradation, led to the identification and isolation of incensole acetate (IA) and its non-acetylated form, incensole (IN) as inhibitors of NF-κB activation. Although IA and IN have previously been identified in *Boswellia* species (Corsano and Nicoletti, 1967) and are considered to be biomarkers of these species (Hamm et al., 2005) their biological activities have not been studied so far.

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Materials and Methods

Extraction and Isolation of IA. *Boswellia carterii* resin (20 g., Pamir, Tel-Aviv, Israel) was extracted with petroleum ether (PE) (3 times with 150 mL). PE extract was washed with NaOH 5% solution (3 times with 200 mL). The non acid containing PE fraction was acidified with HCl (1M) and then washed with a saturated NaCl solution and dried over $MgSO_4$. After evaporation, the residue was chomatographed on a silica column. Fractions were assayed for their activity on IκBα degradation as described below. A fraction eluted with 3% diethyl-ether in PE, which contained IA, showed activity. Pure IA was obtained by chomatography on a semi preparative HPLC column (Spectra-physics applied bio systems 783 absorbance detector with a vydac C18 semi-preparative HPLC column – Valco). Acetonitrile and water were used as mobile phase for HPLC and the gradient consisted of 90-99% ACN for 30 mins.

Structure Elucidation. A Waters HPLC instrument: pump 600, PDA 996 detector 600 with an analytical C18 Symmetry column (4.6/250 mm) were used to analyze the purification process.

Electrospray ionization and high resolution mass spectral analyses (Bruker APEx3 ICRMS) as well as several NMR methods (^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HSQC, HMBC, TOCSY and NOESY) were used for the structure elucidation of the isolated active compounds. NMR spectra were recorded both in $CDCl_3$ and in C_6D_6 solutions using a Bruker avance spectrometer 400 MHz and repeated using a Varian Unity Spectrometer Varian Unity Inova spectrometer 500 MHz.

GC-MS Analysis was performed using a Hewlett-Packard G1800A GCD system with a HP5971 gas chomatograph with an electron ionization detector. An SPB-5 (30 m x

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0.25 mm x 0.25μm film thickness) column was used. The following method was used for analysis: The column was held at 70°C for 4 mins, after which, a temperature gradient was applied from 70°C to 280°C, at a rate of 50 degree/min. (Inlet temperature: 280°C; Detector temperature: 280°C; Splitless injection; gas – Helium, 1 mL/min).

Cell Lines. HeLa cells and 293T cells were grown in Dulbecco´s modified Eagle medium supplemented with 10% foetal calf serum and 1% (v/v) penicillin/streptomycin (all from Biological Industries, Kibbutz Beit Haemek, Israel) in a humidified incubator at 37°C.

The RAW 264.7 macrophage cell line derived from BALB/c mice was obtained from American Type Culture collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (Hyclone, Logan, UT), 1% (v/v) penicillin/streptomycin (Beit Haemek, Israel), nonessential amino acid (Sigma, St. Louis, USA), 1% glutamine (Beit Haemek, Israel) and 1% pyruvate (Beit Haemek, Israel). Cells were grown in a humidified incubator at 37°C.

Jurkat T leukemia cells were grown at 37°C in RPMI 1640 medium containing 10% (v/v) heat-inactivated foetal calf serum, 10 mM HEPES, 1% (v/v) penicillin/streptomycin (all from Life Technologies, Eggenstein, Germany) and 2 mM glutamine.

The 5.1 Jurkat and HeLa-Tat-Luc cell lines have been previously described (Sancho et al., 2004). 5.1 cells is a Jurkat derived clone stably transfected with a plasmid containing the luciferase gene driven by the HIV-1-LTR promoter, responsive to the NF-κB activator cytokine TNFα. The HeLa-Tat-Luc contains the luciferase gene

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driven by the HIV-1 LTR promoter and the Tat gene regulated by the CMV promoter. Therefore, the HIV-1 LTR is highly activated in this cell line as a consequence of high levels of intracellular Tat protein and the luciferase activity is in the order of 10^7 R.L.U./10^5 cells (considered 100% activation).

A549 cells (10^5/mL) were transiently co-transfected with the KBF-Luc reporter (0.2 μg/mL) together with empty vectors or over-expressing vectors for IKKα/IKKβ (0.5 μg/ml each), TRAF-2 (1 μg/ml) and TAK1/TAB2 (0.5 μg/ml each). The transfections were performed using Lipofectamine Plus™ reagent (Life Technologies) according to the manufacturer's recommendations for 24 h.

Isolation of Human Monocytes. Human peripheral monocyte*s* from healthy human donors were prepared following a standardised protocol (Ficoll gradient preparation, Amersham-Biosciences, Freiburg, Germany) using a completely endotoxin-free cultivation as previously described (Noble et al., 1968; English and Andersen, 1974). By using 50 mL tubes, 25 mL Ficoll were loaded with 25 mL blood of Buffy coats from healthy blood donors. The gradient was established by centrifugation at 1800 rpm, 20°C for 40 mins by using slow acceleration and brakes. Peripheral blood mononuclear cells in the interphase were carefully removed and resuspended in 50 mL pre-warmed phosphate buffered saline (PBS, Invitrogen, Karlsruhe, Germany) followed by centrifugation for 10 mins at 1600 rpm and 20°C. The supernatant was discarded and the pellet washed in 50 mL PBS and centrifuged as described above. The pellet was then re-suspended in 50 mL RPMI-1640 low endotoxin-medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% human serum (PAA, Coelbe, Germany).

Animals. Female mice – Sabra (Harlan, Israel, 15-20 weeks old) were used for *in vivo* anti-inflammatory assessments. Ten mice were housed in each cage. The

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animal care and protocols met the guidelines of the U.S. National Institutes of Health, detailed in the Guide for the Care and Use of Laboratory Animals, and were applied in conformity with the Institutional Ethics Committees. Temperature in the animal room was maintained between 20-22°C, the light cycle was 12 h lights on (8:00-20:00h); 12 h lights off (20:00-8:00h).

IκBα Phosphorylation and Degradation. HeLa cells were pre-incubated with tested compounds (dissolved in ethanol) for 2 h, and then stimulated for 20 mins with TNFα (20 ng/mL, Emeryville, CA, USA) or costimulated with phorbol-12-myristate-13-acetate (PMA; 20 ng/mL) and ionomycin 100 ng/mL for 15 mins. After removing the slides from plates for immunostaining (see below), proteins were extracted in NP-40 lysis buffer [50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, 10 mM NaF, 0.5 mM sodium vanadate, leupeptin (10 μg/mL), 1% (v/v) NP-40 and 10% (v/v) glycerol] from remaining cells in the plates. Total protein concentration was determined using the Bradford method. Lysates were then analyzed either by Western blotting (WB) or by *in vitro* kinase assays (see below). Boswellic acid mixture (α and β) was obtained from the laboratory of Dr. Gerald Culioli (France).

Kinase Assays (KA). The IKK complex was isolated from precleared NP-40 HeLa cell extracts (see above) by immunoprecipitation with 2 μg αIKKγ antibodies (Santa Cruz, California, USA) and 25 μl protein A/G sepharose. The precipitate was washed three times in the above NP-40 lysis buffer and two times in kinase buffer (20 mM Hepes/KOH pH 7.4, 25 mM ß-glycerophosphate, 2 mM DTT, 20 mM $MgCl_2$). The kinase assay was performed using glutathione S-transferase (GST) fusion proteins as substrates, in a final volume of 20 μl kinase buffer containing 2 μg of bacterially expressed GST-IκB-α (1-54), 20 μM ATP and 5 μCi γ-^{32}P-ATP. After

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incubation for 20 mins at 30°C, the reaction was stopped by the addition of 5 x SDS sample buffer. After separation by SDS-PAGE, the gel was fixed, dried and autoradiographed. The JNK assays were performed with a similar protocol, with the difference that the immunoprecipitation was done with αJNK1 and αJNK2 antibodies (Santa Cruz, California, USA) and that GST-c-Jun (5-89) was used as a substrate protein.

IKK phosphorylation assay. After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminiscence according to the instructions of the manufacturer (Amersham Lifescience).

p65 Subunit Immunostaining. HeLa Cells were preincubated with IA and then stimulated with TNFα, as described in the IκBα degradation assay above. Cells were then fixed with formaldehyde 1%, permeabilized with 0.25% Triton X-100, stained with rabbit anti-p65 (Santa Cruz, California, USA) and visualized with anti-rabbit Rhodamine Red-labeled secondary antibody (Jackson ImmunoResearch, Baltimore, USA). Cells were also stained with DAPI for nuclei location (data not shown). The cells were examined under an Axioscope Zeiss microscope with a plan-Neofluor x 60 lens.

Electrophoretic Mobility Shift Assay (EMSA). Cells were preincubated for 1 h with IA and stimulated for 15 mins as shown. The oligonucleotides were synthesized at MWG Biotech, Germany, and the single strand oligonucleotides were annealed according to standard procedure by heating and subsequent cooling down to 50°C in 10 mM Tris/HCl, pH 7.5 and 100 mM NaCl. Equal amounts of protein contained in TOTEX buffer [20 mM Hepes/KOH, pH 7.9, 0.35 M NaCl, 20% (v/v) glycerol, 1% (v/v) Nonidet P-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM

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phenylmethylsulfonyl fluoride] were incubated with a ^{32}P-labelled double stranded oligonucleotide containing a NF-κB recognition site for 15 mins. Bound and free oligonucleotides were separated by electrophoresis on a native 0.5 x TBE 4% polyacrylamide gel. The dried gel was then exposed to an X-ray film.

Luciferase Assays. The various cell lines were preincubated with the compounds and stimulated as specified in the figure legends. Cells were harvested, washed with PBS and then lysed in a luciferase lysis buffer (25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol). Luciferase activity was measured using an Autolumat LB 953 luminometer (EG&G Berthold, USA) following the instructions of the luciferase assay kit (Promega, Madison, WI, USA).

Inflamed Paw Model. Vehicle (isopropanol:Emulphor:saline = 1:1:18) or vehicle containing IA (50 mg/kg) was administered by intraperitoneal (*i.p.*) injection 30 mins before applying the inflammatory stimulus, . Emulphor is a commercial emulsifier. Hind paws were injected with 50 μl of saline (left or right alternatively) or λ-carrageenin (4%, right or left alternatively), using 26G needles. The resulting inflammatory swelling was measured by increase in foot volume in a plethysmometer (Ugo-Basile, Italy) as described before (Calhoun et al., 1987). Paw volume as well as redness (as a measure of erythema) and licking (as a measure of pain) were assayed before carrageenin application and every 60 mins until 4 h.

Data Analysis. Dose response data were plotted and analyzed using GraphPad Prism 4.01 software (San Diego, CA). Differencees were considered statistically significant if the *p* value was < 0.05.

Results

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Isolation of the Active Components of *Boswellia* Resin that Inhibit NF-κB Activation. The PE extract of *Boswellia carterii* inhibited the TNFα-induced degradation of IκBα in HeLa cells; solvent partition of the extract into acid and non-acid fractions resulted in the localization of the active components in the non-acid fraction. Further fractionation, guided by IκBα degradation assay led to the isolation of the active compounds.

Structure Elucidation of the Active Compounds. The major active component was found to exhibit a molecular ion at 349.2745 m/z on high resolution mass spectrometry (HRMS), indicating an elemental composition of $C_{22}H_{36}O_3$. IA (Fig. 1), which has the same elemental composition, is a known constituent of *Boswellia* species. We therefore compared the ^{13}C NMR spectrum of the active compound isolated by us with a published ^{13}C NMR spectrum of IA (Gacs-Baitz et al., 1978). The spectra were identical (Table 1 in Data Supplement). The ^{13}C NMR spectrum of the isolated IA, compared with the known spectrum of IA, together with proton NMR and several 2D NMR experiments (see Materials and Methods) and the HRMS analysis, fully elucidated the structure of the active compound. A full NMR assignment of IA is given as Table 2 in Data Supplement. A second active compound was isolated from *Boswellia carterii* resin and its structure was elucidated as IN, the non-acetylated form of IA. The ^{13}C NMR spectrum of the isolated IN was compared with the known spectrum of IN (Gacs-Baitz et al., 1978; see Table 1 in Data Supplement). Together with proton NMR and several 2D NMR experiments, (see Materials and Methods) these spectra confirmed the structure of the compound. For further validation of structure, we hydrolyzed IA (using $LiAlH_4$ as a reducing agent) and compared the GC-MS and ^{13}C-NMR spectra of the resultant IN to that of isolated

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IN. They were found to be identical (see Fig. 1 in Data Supplement for a GC-MS comparison).

IA and IN, but not Boswellic Acid, Inhibit IκBα Degradation. We compared the effect of the main component of *Boswellia carterii* resin that inhibited IκBα degradation, to that of a mixture of α and β boswellic acids. While the inhibition of IκBα degradation by IA was statistically significant, boswellic acid did not inhibit the degradation of IκBα (Fig. 2). We assayed IA and IN at different concentrations for their effect on IκBα degradation in TNFα-stimulated HeLa cells. Both compounds inhibited IκBα degradation in a similar dose dependent manner (Fig. 3).

IA Inhibits IκBα and p65 Phosphorylation by Impairment of IKK Activation. As IKK is also essential for the phosphorylation of p65 at serine 536 (Sizemore et al., 2002; Yang et al., 2003), we further analyzed whether IA affects the TNFα-induced phosphorylation of p65 in addition to its activity on the degradation of IκBα. IA inhibited the p65 subunit phosphorylation as well as the phosphorylation of IκBα and the phosphorylation by IKKs in TNF-stimulated HeLa cells (Fig. 4A, upper panel). The inhibitory effect of IA on IKK activity is apparently specific, as JNKs and p38 MAPK, which are also activated after treatment with TNFα (Kang et al., 2004; Rizzo and carlo-stella, 1996) were unaffected by IA (Fig. 4A, lower panel). In contrast to IκBα phosphorylation and subsequent degradation in TNF-stimulated HeLa cells, IA did not inhibit IκBα phosphorylation in human Jurkat T leukemia cells, costimulated by PMA in combination with ionomycin (Fig. 4B, upper panel). We examined the effect of IA on TNF-stimulated Jurkat cells and found a robust inhibition of IκBα phosphorylation and degradation (Fig. 4B, lower panel). The lack of IA-mediated IKK inhibition in costimulated T-cells raises the possibility that this compound does not directly target the IKKs (see Mattioli et al., 2004). Accordingly,

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in vitro phosphorylation experiments showed full functionality of IKKs in the presence of IA (Fig. 4C), suggesting that IA targets an upstream event. Collectively, these data suggest that IA inhibits the NF-κB pathway upstream of IKK. To address this possibility experimentally, we examined the effect of IA on TNFα-induced phosphorylation of IKK. These experiments showed inhibition of IKKα/IKKβ activation loop phosphorylation by IA (Fig. 4D), attributing its effect to an upstream event.

IA Interfers with TAK/TAB Mediated Phosphorylation of IKKα/β Activation Loop. To further examine the mechanism of the effect of IA on IKK activation, we assayed A549 cells, transfected with KBF-luc alone or in combination with IKKα/IKKβ, TRAF2, or TAK1/TAB2 expression vectors. Treatment with IA did not interfere with NF-κB activition triggered by TRAF2, while TAK1/TAB2 stimulated NF-κB activition was significantly and dose-depndently inhibited in the presence of IA (Fig. 5A). In contrast, the effect of IA on NF-κB activation in IKKα/β overexpressing cells was very mild, and can presumably be attributed to the interaction of IKKα/β with the endogenous TAK/TAB module. To determine whether IA can interfere with TAK/TAB induced phosphorylation and thus activation of IKKα/β, HA-TAK1 and Myc-TAB1 were overexpressed in 293T cells and IKK α/β phosphorylation was examined by immunoblotting. IA exerted a dose-dependent reduction of IKKα/β phosphorylation in TAK/TAB overexpressing cells, as displayed in Fig. 5B. These experiments demonstrate that IA interferes with a critical step relaying the TAK/TAB module with IKKα/β activation loop phosphorylation.

IA Inhibits NF-κB Accumulation in Cell Nuclei and DNA Binding. Immunostaining of the p65 subunit of NF-κB in TNF-stimulated HeLa cells illustrates

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the inhibition of the nuclear accumulation of NF-κB by IA (Fig. 6A).. IA also inhibited NF-κB DNA-binding in LPS-stimulated human peripheral monocytes, as examined by EMSA and depicted in Fig. 6B.

IA Inhibits Gene Expression by NF-κB. The HIV-1 promoter contains two high affinity binding sites for NF-κB and is highly responsive to both the TNFα-induced NF-κB pathway and the Tat/TAR-dependent pathway. Using stably transfected cell lines with a plasmid, in which the luciferase gene is driven by the HIV-1 LTR promoter, we found that IA inhibits TNFα-induced (Fig. 7A), but not Tat-mediated HIV-1-LTR trans-activation (Fig.7B) in a dose-dependent manner The lack of interference with Tat-induced transcription rules out potential effects of IA on the basal transcriptional machinery or other nonspecific effects. In contrast to IA, the CDK9 inhibitor 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole riboside (DRB) effectively inhibited luciferase activity in HeLa-Tat-Luc cells.

IA suppresses Inflammation in the Mouse Paw Model. Having established that IA inhibits the NF-κB pathway *in vitro*, we studied the anti-inflammatory properties of IA *in vivo* and found that IA significantly reduced inflammation in the inflamed paw model in mice (n = 5 per group) during a 4 h period. The decreased inflamed paw volume in the treated mice reflects a decrease in edema, which is a component of the inflammatory response. There were highly significant effects of treatment ($F = 11.7$, df = 3,64, $p < 0.001$), time ($F = 10.6$, df = 4,64, $p < 0.0001$) and interaction ($F = 3.9$, df = 12,64, $p < 0.001$) as seen in Fig. 8. IA also significantly reduced other inflammatory parameters: redness (scored by visualization) and pain, as measured by the paw licking frequency by the mouse (data not shown).

Collectively, these data show that IA inhibits NF-κB activation and exerts anti-inflammatory properties in the an *in vivo* model of inflamed paw.

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Discussion

In the current study, we demonstrate that the major NF-κB inhibitory components in *Boswellia* resin are IA and its derivative IN. IA inhibits IKK phosphorylation and activation *in vivo*, but not *in vitro*, implying that it exerts its action upstream of IKK. IA blocks NF-κB activation in response to TNFα and LPS, but does not inhibit IKK activition in costimulated Jurkat T cells. TAK1 plays a critical role in TNFα-induced NF-κB activation (Blonska et al., 2005). TAB2 and TAB3 are adaptors that link the kinase TAK1 to upstream regulators in the proinflammatory TNF signaling pathway (Hong et al., 2007). IA attenuates TAK/TAB-induced phosphorylation of the IKKα/β activation loop by interfering with a step that couples TAK to IKK phosphorylation and activation. However, this inhibition appears to be specific, as IA does not impair TNFα-induced activation of JNK and p38 MAPK. This specificity might suggest that IA can serve as a pharmacological tool in the intensive research conducted on the activation of IKK by upstream events.

The inhibition of IκBα phosphorylation and of subsequent degradation, as well as the inhibition of p65 phosphorylation at serine 536 can be attributed to the inhibitory effect of IA on IKK, as IKK plays a major role both in IκBα and p65 phosphorylation (Sizemore et al., 2002). Downstream of IκB, IA inhibits the accumulation of NF-κB in TNF-stimulated HeLa cell nuclei, NF-κB DNA Binding in LPS-stimulated human peripheral monocytes and the induction of NF-κB dependent gene expression.

The inhibition of IKK activation by IA resembles that of the anti-apoptotic protein embelin, recently demonstrated to be an NF-κB inhibitor. Like Embelin (Ahn et al., 2007), IA mediates its effects on IKK activation through impairment of a step

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connecting TAK to the IKK activation loop phosphorylation. TRAF2 apparently also recruits the IKK complex directly via the complex of TRADD, TRAF2, TRAF5 and RIP1 (Hacker and Karin, 2006). Thus, the lack of inhibitory effect by IA on TRAF2 overexpressing cells supports a specific IA intervention at the TAK1-IKK activation step. The effect of IA on IKK also resembles that of the tetracyclic kaurene diterpenes as shown by Castrillo et al., as both are signaling rather than direct inhibitors (Castrillo et al., 2001). However, it appears that IA's activity is more specific to the NF-κB pathway, as kaurenes also inhibit the phosphorylation of p38, ERK1, and ERK2 MAPK.

Diterpenoids are natural compounds with a backbone of 20 carbon atoms biosynthesized from geranylgeranyl pyrophosphate (Hanson, 2005). It is an important and chemically diverse group of natural products which share some common biosynthetic steps and are of considerable biological importance (Hanson, 2005). Interestingly, although members of this group of natural products share no common chemical moiety, a large arsenal of biologically active compounds has been identified among them (see Ojo-Amaize et al., 2002; Tempeam et al., 2005; Zhang et al., 2005 for some examples) and several diterpenoids are known as inhibitors of NF-κB activation (Castrillo et al., 2001; Leung et al., 2005; Yinjun et al., 2005). The mechanism by which terpenes impair IKK activation has so far been poorly characterized (Castrillo et al., 2001). IA's mechanism of action is, however, completely different from several other anti-inflammtory diterpenoids that inhibit the NF-κB pathway, for example, oridonin, ponicidin, xindongnin A, and xindongnin B. These diterpenoids, isolated from *Isodon rubescens*, directly interfere with the DNA-binding activity of NF-κB to its response DNA sequence (Leung et al., 2005), whereas IA inhibits IKK activation. Even with kaurene diterpenoids that inhibit IKK

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activation (Castrillo et al., 2001) there are differences, in the mechanism of action, such as the specificity of action and the effect on p65 phosphorylation. The observation that some members of a large group of natural products are a source of NF-κB modulators by a multiplicity of pathways implies that they can serve as a valuable tool for the examination of the NF-κB pathway, especially upstream of IKK, where this pathway is still to be unfolded.

Based on our findings, we attribute the main NF-κB inhibitory effect of *Boswellia* resin to IA and its derivatives. The resin of *Boswellia* species, containing IA derivatives, has been used to treat inflammatory conditions for many centuries in traditional medicine in Europe, Asia and Africa and is still in such use, besides its common religious use as incense. One interesting example of its use as an ingredient of an important anti-inflammatory remedy that has been common in Europe and Asia for hundreds of years is the Jerusalem Balsam (Moussaieff et al., 2005). *Boswellia* extracts are also marketed as food supplements for the treatment of arthritis in the US and Europe and in view of our current data we propose that these products should be standardized for IA and its derivatives as well as boswellic acids. Moreover, the possible synergistic effects of these compounds need to be investigated. We propose the GC-MS fingerprinting depicted in Data Supplement Fig. 1 as a simple method for the standardization of *Boswellia* resin for IA and its derivatives.

IA demonstrates a robust anti-inflammatory effect in a mouse inflamed paw model. This effect is within the range of drugs such as salicylates injected *i.p* (Siqueira-Junior, 2003), However, IA and IN are practically insoluble in water and poorly soluble in other solvents used for injection to animals or in cell systems. Hence, the actual active concentrations of these compounds are probably considerably lower. The identification of the NF-κB inhibitory effect of cembranoid diterpenes, an

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important group of common natural products, present in tobacco among other herbs and marine creatures, may further open these fields to the discovery of novel drugs for the treatment of diseases that pose unanswered challenges and affect a large segment of the population.

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Footnotes

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Legends for Figures

Fig. 1. The structures of IA (R = Ac.) and IN (R = H). Structures elucidation was done according to NMR (see Materials and Methods; Tables 1, 2 in Data Supplement) and MS data (see Materials and Methods; Fig. 1 in Data Supplement).

Fig. 2. Comparison of isolated IA with boswellic acid on IκBα degradation in TNFα stimulated HeLa cells. Cells were treated with non-toxic concentrations of IA (220 μM) and boswellic acid (280 μM) and stimulated with TNFα (20 ng/mL for 20 mins). Equal amounts of protein were separated by SDS-PAGE and further analyzed by immunoblotting. IκBα / β-actin ratio of untreated cells was considered as 100%. * $p < 0.05$; ** $p < 0.001$ by *t* test (vs. vehicle + TNFα stimulated cells). The error bars represent SEM

Fig. 3. IA and IN inhibit IκBα degradation in a dose-dependent manner. HeLa cells were pre-incubated with IA (upper) or IN (lower) at the indicated concentrations for 2 h prior to 20 mins exposure to TNFα (20 ng/mL). A representative experiment is shown.

Fig. 4. IA inhibits the phosphorylation and subsequent degradation of IκBα by impairment of IKK phosphorylation and activation. A, HeLa cells were stimulated with TNFα (20 ng/mL for 20 mins) in the absence or presence of IA (140 μM) as shown. Subsequently, whole cell extracts were prepared and aliquots thereof analyzed either for the stability and phosphoryation of the indicated proteins by WB or for IKK activity by kinase assays (KA). IKKγ/NEMO was immunoprecipitated from cell

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lysates and IKK activity was determined by immune complex kinase assays using recombinant GST-IκB-α (1-54) as substrate. An autoradiogram from a reducing SDS gel is shown. The lower panel shows the effect of IA on the phosphorylation of p38 and JNK1/2 and JNK *in vitro* kinase activity. The experiment shown is representative of three independent experiment sets. B, IA does not impair IκBα phosphorylation in costimulated T cells. Human Jurkat T leukemia cells were left untreated or incubated with IA (560 μM). T cells were costimulated by treatment with 20 ng/mL PMA in combination with ionomycin (100 ng/mL) as shown. After 15 mins, cell extracts were prepared and analyzed by immunoblotting for the phosphorylation of IκBα. TNF-stimulated Jurkat cells (lower panel) were left untreated or incubated with IA in the same manner as costimulated cells. C, IA does not inhibit IKK activity *in vitro*. HeLa cells were stimulated with TNFα as shown and the IKK complex was isolated by immunoprecipitation with αIKKγ/NEMO antibodies. Immune complex kinase assays using the GST-IκBα substrate protein were performed in the presence of IA (150 μM) or ethanol as a solvent control. An autoradiogram from a reducing SDS gel (upper) and a Coomassie staining of the GST-IκBα fusion proteins (lower) are shown. D, IA inhibits the phosphorylation of IKK activation loop. HeLa cells were stimulated with TNFα (20 ng/mL for 20 mins) in the presence of IA (140 μM) or ethanol as a solvent control. Whole cell extracts were prepared and analyzed for the phosphoryation of IKKα/β by WB.

Fig 5. IA Inhibits TAK/TAB-triggered IKK activation. A,. A549 cells were transiently transfected with KBF-luc alone or in combination with IKKα/IKKβ, TRAF2 or TAK1/TAB2 expression vectors. After 24 h of transfection, cells were treated with IA for 6 h and luciferase activity was assayed. In order to allow comparability, activation by IKKα/IKKβ, TRAF2 or TAK1/TAB2 in untreated cells were given a value of 100% and

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the IA inhibitory effect is represented as percentage of activation. Average values from three independent experiments are shown, error bars show SEM. The data represent the results of 5 independent experiments. *, $p < 0.05$; ** $p < 0.001$ compared to nontreated cells (Two way ANOVA, followed by a Bonferonni multiple comparison test). B, IA inhibits the phosphorylation of IKKα/β induced by TAK/ TAB overexpression. 293T cells were transfected with expression vectors for HA-TAK1 and Myc-TAB1 or with a control vector. 36 h post transfection, cells were treated for 6 h with increasing concentrations of IA as shown and lysed. Equal amounts of protein were examined for phosphorylation of IKKα/β activation loop by immunoblotting. Similar results were obtained when transfecting cells with TAK1/TAB2 (data not shown).

Fig 6. IA inhibits the accumulation of p65 in cell nuclei of TNFα-stimulated HeLa cells and the NF-κB DNA-binding of LPS-stimulated human peripheral monocytes. A, HeLa cells were stimulated with TNFα (20 ng/mL for 20 min) in the presence of IA (140 μM) or ethanol as a solvent control. Cells were fixed and then stained with rabbit anti-p65 followed by anti-rabbit Rhodamine Red-labeled secondary antibody and with DAPI for nuclear location (not shown). The cells were examined under an Axioscope Zeiss microscope with a plan-Neofluor * 60 lens. Results of one of three independent experiments are shown. B, Human peripheral monocytes extracts in the presence or in the absence of IA (100 μM) were tested by EMSA for NF-κB DNA-binding. The filled arrowhead indicates the location of the DNA-NF-κB complex; the circle indicates the position of a constitutively DNA-binding protein.

Fig. 7. IA inhibits NF-κB activation in TNFα-stimulated 5.1 Jurkat cells. A, 5.1 Cells were preincubated with IA for 30 mins at the indicated concentrations and stimulated with TNFα (2 ng/mL) for 6 h. The luciferase activity was then measured. There was no effect of solvent on cell viability or on the enzymatic activity of luciferase at the

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highest concentration (data not shown). B, HeLa-Tat-Luc cells were incubated with either DRB (50 μM) or IA at the indicated concentrations for 18 h. Tat-induced LTR-luciferase activity was measured.

Luciferase activity is given as fold induction or relative light units (RLU), standard deviations are given. ** $p \leq 0.01$ to fold values in the absence of IA by *t* test.

Fig. 8. IA inhibited inflammation in the inflamed paw model after injection of carrageenin. IA (50 mg/kg) or vehicle was injected *i.p.* to Sabra female mice (5 per group) 30 mins before induction of the inflammatory stimulus. Hind paws were then injected with 50 μl of saline or λ-carrageenin (4%). Ensuing inflammatory swelling was measured by increase in foot volume in a plethysmometer. IA also reduced paw redness (as a measure of erythema) and licking (as a measure of pain) (data not shown). There were highly significant effects of treatment ($F = 11.7$, df = 3,64, $p < 0.001$).

Vehicle + saline, open triangles; vehicle + carrageenin, closed triangles; IA + saline, open circles; IA + carrageenin, closed circles; *, different from IA + saline, $p < 0.05$; **, ***, different from vehicle + saline at $p < 0.01$, $p < 0.001$ respectively; #, different from vehicle + carrageenin, $p < 0.05$.

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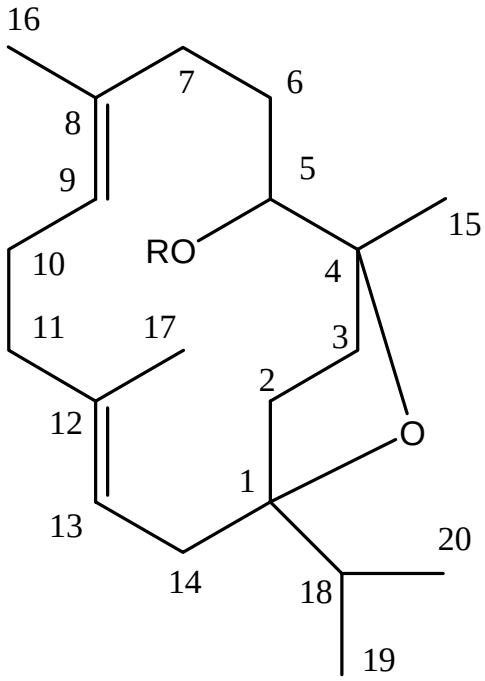
Fig. 1



R= Ac (IA), H (IN)

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Fig. 2

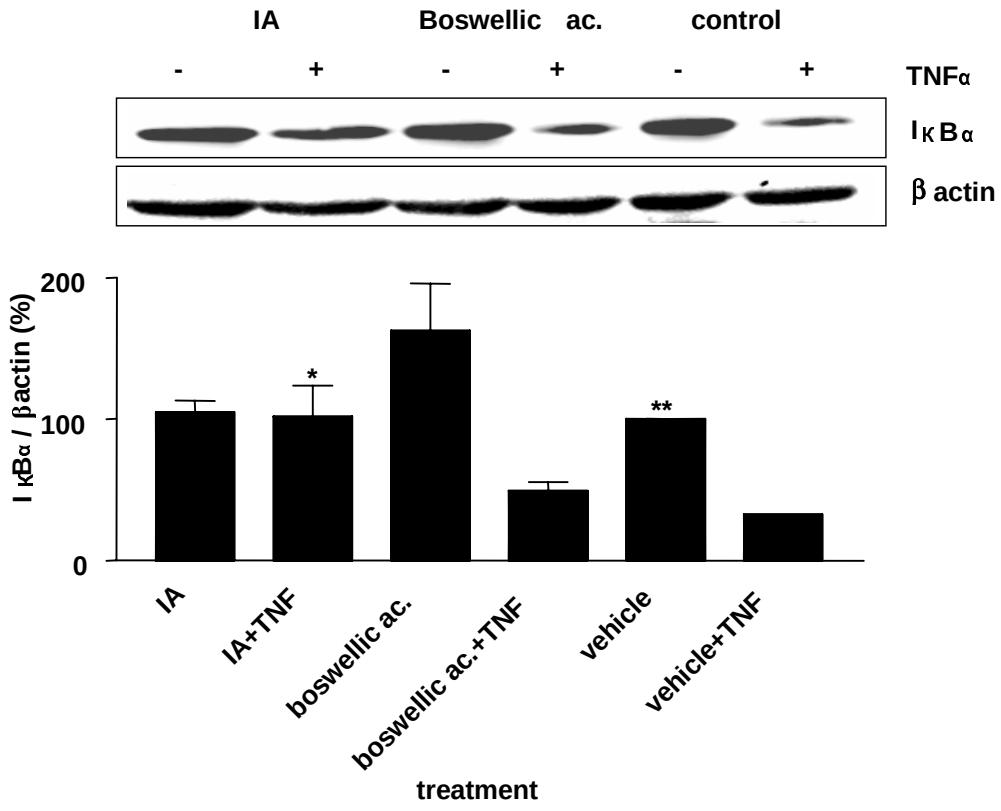

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Fig. 3

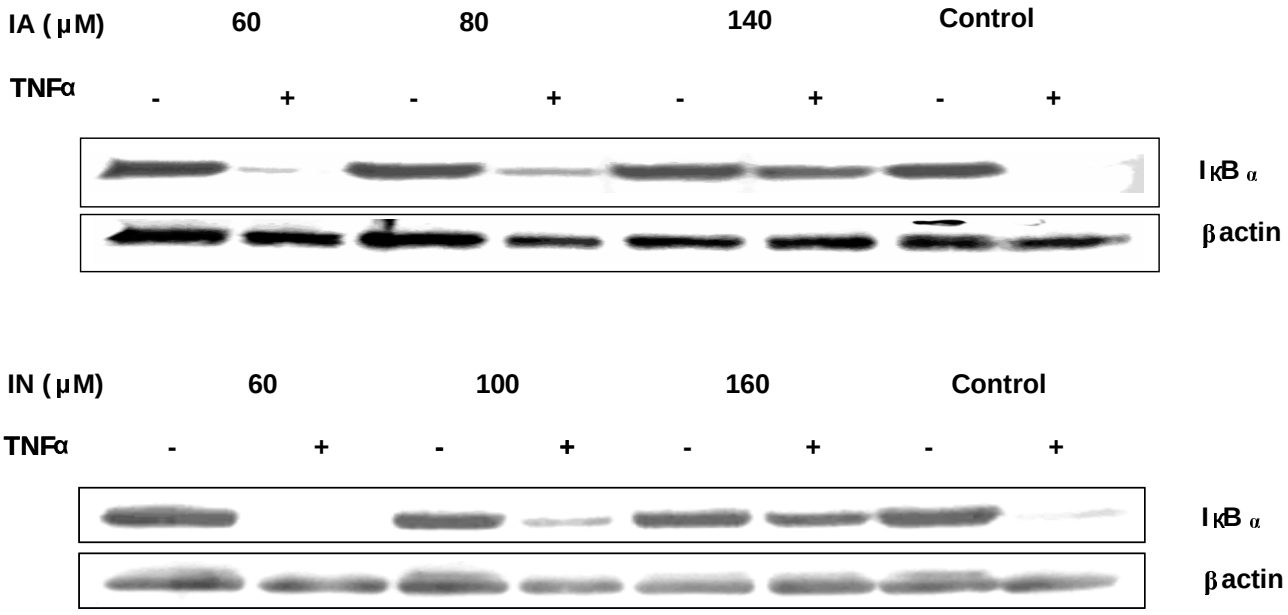

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Fig. 4

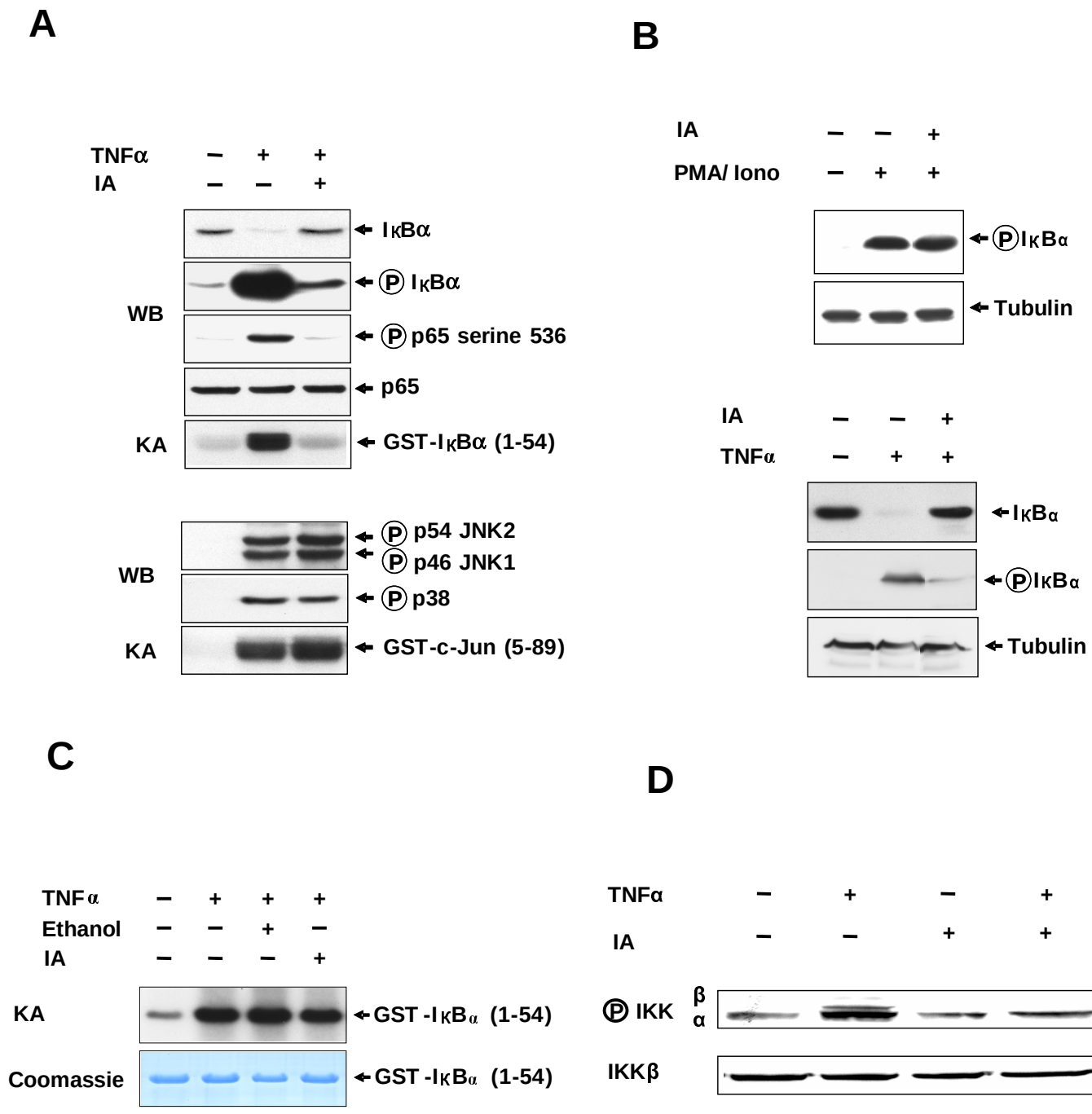

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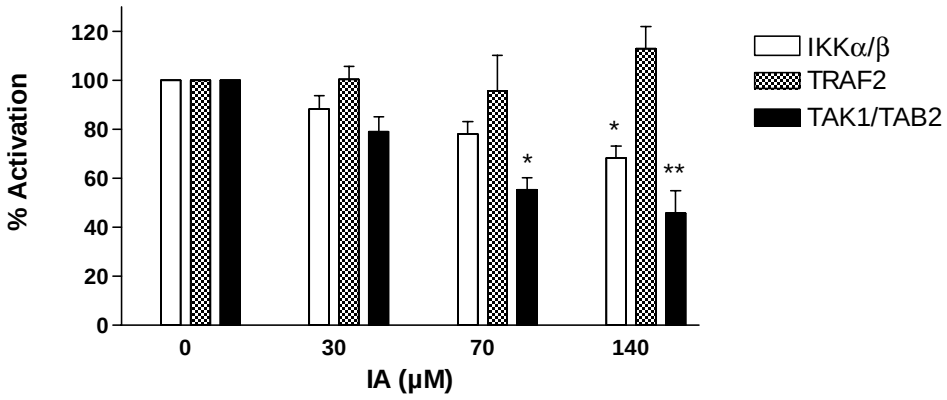
Fig. 5

A



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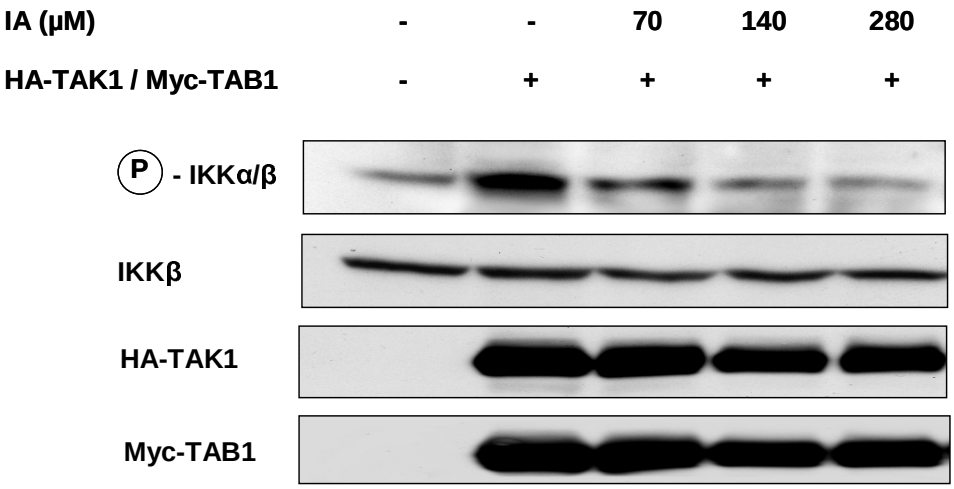

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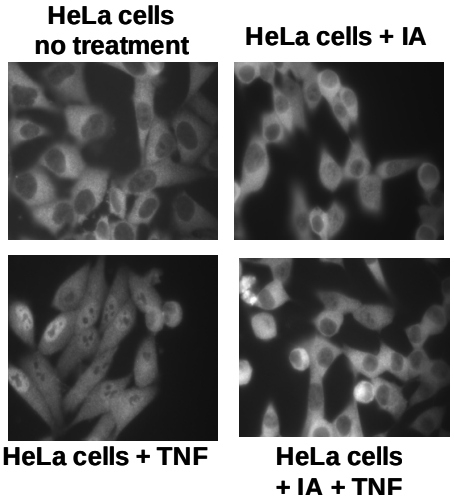
Fig. 6

A



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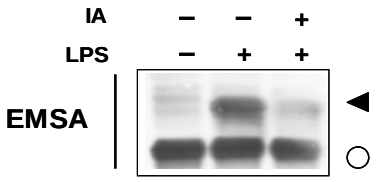

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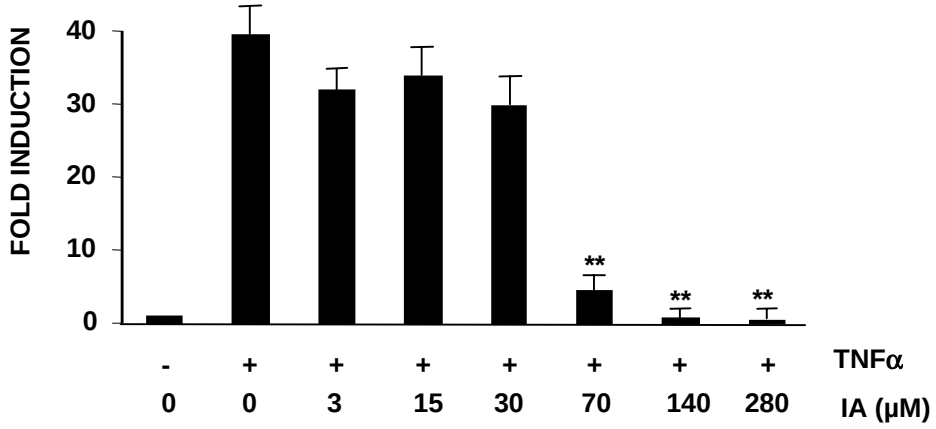
Fig. 7

A



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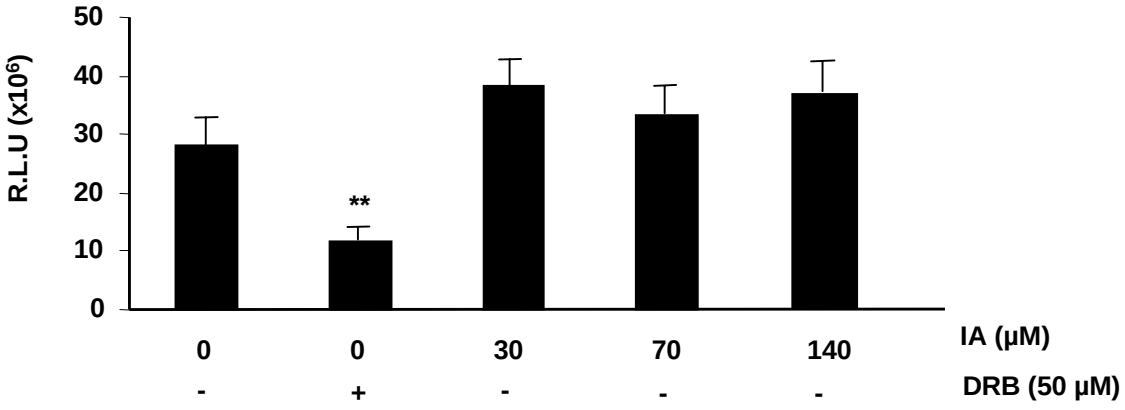

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Fig. 8