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Anandamide inhibits IL-12p40 production by acting on the promoter repressor element GA-12: possible involvement of the COX-2 metabolite, prostamide E₂.

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Short title: Anandamide and IL-12p40 regulation.

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Abbreviations used: AEA, anandamide; CB, cannabinoids; CNS, central nervous system; eCBs, endocannabinoids; IFN γ , interferon-gamma; IL-12, interleukin-12; IL-23, interleukin-23; MS, multiple sclerosis; LPS, lipopolysaccharide; TMEV, Theiler virus.

The endocannabinoid system is being considered as a novel therapeutic target for immune disorders. Cytokines of the IL-12 family play essential functions in cell-mediated immunity. Here, we have addressed the mechanisms of action of the endocannabinoid anandamide (AEA) on the regulation of IL-12p40 by activated microglia/macrophages. We first showed that AEA inhibited the expression of the subunits p35, p19 and p40 which form the biological active cytokines IL-12 and IL-23 in microglia stimulated with LPS-/IFN- γ . Additionally, we provided evidence that AEA reduces the transcriptional activity of IL-12p40 gene in LPS-/IFN- γ -activated cells independently of the activation of cannabinoid (CB) or vanilloid receptors. Experiments performed with site-directed mutagenesis of the different elements of the p40 promoter showed that AEA- regulates IL-12p40 expression by acting on the repressor site GA-12. Prostanoid E₂, a product considered as a putative metabolite of AEA by COX-2 oxygenation, was also able to inhibit the activity of IL-12p40 promoter by acting at the repressor site. The effects of AEA and prostamide E₂ on p40 transcription were partially reversed by an antagonist of the prostanoid receptor EP2, raising the possibility that prostamide E₂ may contribute to AEA effects on IL-12p40 gene regulation. Accordingly, the inhibition of COX-2 by NS-398 partially reversed the inhibitory effects of AEA on IL-12 p40. Overall, our findings provide new mechanistic insights into the activities of AEA to manage immune-related disorders.

Key words: endocannabinoids, AEA, IL-12p40, prostamide E₂, microglia, multiple sclerosis

INTRODUCTION

Endocannabinoids (eCBs) are lipid signaling mediators that exert an important immunomodulatory role and that have also been shown to be useful in chronic inflammatory diseases [1, 2]. Microglial cells constitute a population of “facultative” macrophages adapted to neural environment which play a critical role in CNS immune surveillance and in the initiation and maintenance of intracerebral immune responses. Neurological disorders, such as multiple sclerosis (MS) involve activation and even dysregulation of microglial cells that, in addition to their phagocytic function, participate in the regulation of non specific inflammation as well as in adaptive immune responses [3]. Both exogenous and endogenous cannabinoids have been shown to be efficient in attenuating disease symptomatology and inflammatory reactions in chronic relapsing-remitting EAE and Theiler’s virus (TMEV) experimental models of MS [4, 5, 6, 7, 8, 9].

Interleukin-12 (IL-12) is a heterodimeric cytokine formed of p35 and p40 subunits, which regulates innate immunity and determines the type and duration of adaptive immunity [10]. IL-12 is produced mainly by monocytes, macrophages and dendritic cells as well as brain microglia [11]. Besides forming IL-12p70 heterodimer, p35 and p40 may dimerize with alternate partners to form distinct bimolecular complexes. For example, the subunit p19 which lacks biological activity *per se* combines with the p40 subunit to give the cytokine IL-23 which regulates the clonal expansion of T cells [12]. There is consistent evidence that dysregulation of IL-12 gene may contribute to the initiation and perpetuation of various autoimmune and chronic inflammatory diseases [13]. Despite the described reduction of IL-12 after the administration of the main psychoactive compound of *Cannabis sativa*, Δ^9 -tetrahydrocannabinol, in the case of

Legionella pneumoniae infection, the mechanisms underlying this response are still unclear [14].

The endocannabinoid family contains several structurally related lipids which may be synthesized and released by immune cells to act locally in an autocrine or paracrine manner [15]. Cannabinoids mainly act through of two distinct G-protein coupled receptors termed as CB₁ and CB₂. However these molecules have been described to display effects independently of the activation of these receptors. These effects have been proposed to be mediated through several, yet-to-be cloned, receptors or independently of any membrane receptor [1]. Within the CNS, neurons, microglial cells and astrocytes are able to synthesize the eCBs, anandamide (AEA) and 2-arachidonoylglycerol [16, 17, 18]. During neuroinflammation, eCBs are released mainly by microglial cells and exert neuroprotective actions. In particular, AEA has been shown to protect neurons from inflammatory damage [19]. However, despite the therapeutic relevance of eCBs, their mechanisms sustaining their actions on immune regulation remain unknown and studies on the role of AEA on microglia function are scarce. As a typical endocannabinoid, AEA is inactivated by a reuptake system and metabolized mainly by the fatty acid amidohydrolase (FAAH) [20]. In addition, AEA also may be oxygenated by cyclooxygenase-2 (COX-2) into prostaglandin E₂ ethanolamide (prostamide E₂) [21].

In the present study we investigated the role of AEA on the regulation of IL-12 and related cytokines in activated-macrophages/microglial cells. First, we assessed whether AEA modified mRNA expression of p19, p35 and p40 subunits of IL-12 and IL-23 as well as IL12p40 protein in LPS/IFN γ -activated microglial cells. We further analyzed the regulation of IL-12p40 promoter activity by AEA using RAW264.7 and BV-2 cells as cellular model. We identified the response element for AEA action on IL-12 p40

promoter. The involvement of CB and vanilloid receptors on AEA actions was also explored as well as the putative involvement of COX-2 pathway in the effects of AEA.

EXPERIMENTALS

Materials

Culture media and foetal bovine serum (FBS) were from Invitrogen (Barcelona, Spain). Anandamide (AEA) and LPS were from Sigma (Madrid, Spain). Murine recombinant interferon- γ (IFN γ) was from PreproTech (London, UK). Cannabinoid antagonists SR141716A (N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazol-carboxamide) (SR1) and SR144528 (N-[1S]-endo-1,3,3,-trimethylbicyclo [2.2.1]heptan-2-yl-5-(4-chloro-3methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) (SR2) were supplied by Sanofi Recherche (Montpellier, France). Prostaglandin E₂ ethanolamide (prostamide E₂) and AH6809 (6-isopropoxy-9oxoxanthene-2-carboxylic acid) were obtained from Cayman Chemical (Ann Arbor, USA). AH23848B was kindly supplied by GlaxoSmithKline (GSK; Herts, UK). Capsazepine ([N-(2-(4-Chlorophenyl) ethyl)-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothionamide]) was obtained from Alexis Biochemicals (Lausen, Switzerland). All other reagents were obtained from standard suppliers.

Animals

Balb/c mice from our in-house colony (Cajal Institute, Madrid) were used. Animals were housed in cages with filter tops in a laminar flow hood and maintained on food and water *ad libitum* in a 12-h dark-light cycle. Handling of animals was performed in compliance with the guidelines of animal care set by the European Union (86/609/EEC).

Cell Line Cultures

The murine macrophage cell line RAW 264.7 was purchased from Center of Biological Sciences (CIB, CSIC, Madrid; Spain). Cells were grown in Dubelcco's modified Eagle medium (DMEM) supplemented with 2mM glutamine, 10% FBS and 1% penicillin/streptomycin and passaged every 5 days for a maximum of 30 passages. Cells were grown in a humidified environment containing 5% CO₂ and held at a constant temperature of 37°C. BV-2 murine microglial cells were purchased from Interlab Cell Line Collection (ICLC) at the Istituto Nazionale per la Ricerca sul Cancro (Genoa, Italy). These cells exhibit morphological and functional properties comparable to primary microglia.

Microglia cultures

Primary mixed glial cultures were prepared as described previously [22]. Briefly, forebrains of newborn Balb/c mice were dissociated mechanically, filtered through a 150 µm nylon mesh, resuspended in DMEM containing 10% heat-inactivated FBS, 10% horse-serum and 1% penicillin/streptomycin and plated on poly-L-lisine (15 µg/ml) 75 cm² flasks (Falcon; Le Pont de Claix, France). After 15 days in culture the flasks were shaken at 230 rpm at 37°C for 3 h to remove loosely adherent microglia. The supernatant was plated on multiwell culture plates for 2 h. After this, medium was changed to remove non-adherent cells. Cells were grown in a humidified environment containing 5% CO₂ and held at a constant temperature of 37°C. The purity of microglial cultures was assessed by examining the characteristic cell morphologies under phase-contrast microscopy and was confirmed by immunostaining with Mac-1 anti-CD11b antibody (Serotec Ltd, Oxford, UK).

Cell Treatment

Prior to each experiment, medium containing serum was removed and replaced with fresh serum free DMEM. Microglia or cell lines were stimulated with LPS 50 ng/ml /

IFN γ 100 U/ml. The different treatments involved: AEA (1, 5 and 10 μ M), SR1 1 μ M, SR2 1 μ M, capsaizepine 10 μ M, prostamide E2 10 μ M (unless specified another concentrations), NS-398 (10 μ M), AH6809 10 μ M and/or AH23848B 30 μ M, according to each case.

Plasmids

Plasmid constructs for the luciferase reporter gen vector were generated as described previously for the p40/pGL2B [23] and for the p40/pXP1 [24]. Site-directed mutated p40/pXP1 plasmids were generated as described by [24]. Mutant sequences are as follow (two-bases mutation are underlined): ETSm : 5'-CCCAAAAGTCATTAACTCTTAGTTC-3'; NF κ Bm: 5'-GAACTTCTTGAAATTAGCCCAGAAGG-3'; GA-12m1: 5'-CTCGTTTTTCTACACACAC-3'; GA-12m3: 5'-CTCGTTTTGATACATCCAC-3'; C/EBPm : 5'-TGTTTTCAATGTTCTAACAAGTCAGT-3'.

Transfections and Reporter Gene Analysis

The p40/pGL2B or the p40/pXP1 firefly reporter gene vector (200 ng/cm²) along with a *Renilla* luciferase expression vector (Promega; Barcelona, Spain) were transiently transfected into RAW264.7 cells using LipofectamineTM Reagent (Invitrogen; Barcelona, Spain) according to the manufacturer's recommendation. After 18 h medium was removed and changed with fresh serum-free DMEM and 2 h later, cells were stimulated as described in each case. Stimulation was allowed to proceed for another 18 h before cells were harvested, washed with 0,1 M phosphate buffered pH 7.4 (PBS) and lysed in cell lysis buffer (Promega). Luciferase activity (both firefly and *Renilla* luciferase activity) were evaluated using the Dual-Luciferase[®] Reporter Assay System (Promega). Values were normalized to the *Renilla* luciferase activity (Promega). The Dual-Luciferase[®] Reporter Assay System refers to the simultaneous expression and

measurement of two individual reporter enzymes within a single system. Thus, the “experimental” reporter (firefly luciferase) is correlated with the effect of specific experimental conditions whereas the activity of the cotransfected “control” (*Renilla* luciferase) reporter provides an internal control. By normalizing the activity of the experimental reporter to the activity of the internal control we minimize experimental variability due to differences in cell toxicity, transfection efficiency and proliferation. Firefly and *Renilla* luciferase activity were measured as light emission over a period of 10 s each time with a standard luminometer (Sirius, Berthold Detection Systems, Pforzheim, Germany).

Decoy Oligonucleotides Assay

Decoy double-stranded oligonucleotides were transfected to RAW264.7 cells in an attempt to interfere with the binding of LPS/IFN γ -activated transcription factors to their *cis*-acting elements within the GA-12 sequence. The sequences of the oligonucleotides used are as follow (top strands): decoy GA-12 wild type (dGA-12 wt): 5'-CTCGTTTTGATACACACACAGAGA-3' and decoy GA-12m1 (dGA-12m1): 5'-CTCGTTTTTCTTACACACACAGAGA-3'. These oligonucleotides were cotransfected in the RAW264.7 cells together with the reporter constructs. After incubation of 18 h, cell lysates were prepared and assayed for dual-luciferase assay as described above.

Semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNAs from microglial primary culture were prepared using the RNeasy extraction columns (Qiagen; Hilden, Germany). Samples (1 μ g) of total mRNA were transcribed into cDNA using the Reverse Transcription System kit (Promega) according to manufacturer's instructions. cDNA libraries were amplified by PCR with oligonucleotides for Glyceraldehyde-3 phosphate dehydrogenase (GAPDH), IL-12p35 (p35) subunit, IL-12/23p40 (p40) subunit and IL-23p19 (p19) subunit (respectively 800,

300, 250 and 190 bp of PCR products). Specificity of PCR products was confirmed by direct sequencing showing 100% homology with the original sequence. The GAPDH primers sequences were: sense, 5'- CATTGACCTCAACTACATGGT-3'; antisense: 5'- CATTGACCTCAACTACATGGT-3'. The p35 oligonucleotides were: sense 5'- ATGACCCTGTGCCTTGGTAG-3'; antisense, 5'- CAGATAGCCCATCACCCCTGT-3'. The p40 oligonucleotides were: sense, 5'- AGGTGCGTTCCTCGTAGAGA-3'; antisense, 5'- AAAGCCAACCAAGCAGAAGA-3'. The p19 oligonucleotides were: sense, 5'- GACTCAGCCAACCTCCTCCAG-3'; antisense, 5'- GGCACCTAAGGGCTCAGTCAG-3'. The conditions for amplification of GAPDH were 94°C denaturation for 1 min, 60°C primer annealing for 1 min, and 72°C elongation for 1min, for 35 cycles. The conditions for p35 and p40 were 94°C denaturation for 1 min, 50°C primer annealing for 1 min, and 72°C elongation for 1 min, for 40 cycles. The conditions for p19 were 94°C denaturation for 1 min, 55°C primer annealing for 1 min, and 72°C elongation for 1min, for 35 cycles. PCR products were analyzed on 1.5% agarose gel.

IL-12p40 ELISA

Levels of IL-12p40 in microglia culture supernatants were quantified using specific ELISA kits purchased from Biosource Int. (CA, USA), according to the manufacturer's instructions. The assay detected > 2pg/ml and the intra and inter-assay coefficients of variations were between 3, 3-4, 5% and 5, 6-6, 4% respectively.

Statistical analysis

Results are presented as means \pm s.e. of at least three experiments performed with different cell preparations. Analysis of variance followed by the Tukey test for multiple comparison was used to determine statistical significance (95%; $P < 0.05$).

RESULTS

Anandamide negatively regulates the induction of both IL-12 (p40/p35) and IL-23 (p40/p19) in LPS/IFN- γ -activated microglia.

We first studied microglial responses to AEA treatment when this endocannabinoid is co-administered with LPS/IFN- γ (50ng/ml and 100 UI/ml respectively). The stimulation of murine microglial cells with LPS/IFN γ upregulated the levels of mRNA for IL-12 (p40/p35) and IL-23 (p40/p19) (Figure 1A), as shown by semi-quantitative RT-PCR. To assess the effects of AEA on the expression of these subunits we co-treated microglial cells with LPS/IFN γ and AEA. Experiments with different AEA doses (1, 5, 10 and 15 μ M) on IL-12p40 protein expression (Figure 1B) led us to select the dose of 10 μ M for the following experiments. None of the doses of AEA (1-15 μ M) exhibited cell toxicity effect as demonstrated by the LDH assay (not shown). In all cases, AEA (10 μ M) was able to reverse the induction of p40, p35 and p19 subunits caused by LPS/IFN γ (Fig. 1A).

Anandamide suppresses IL-12p40 promoter activity independently on activation of CB₁, CB₂ or TRPV₁ receptors.

Since p40 is the common subunit to both biologically active IL-12 and IL-23, we decided to focus our study on the effects of AEA on this particular subunit. To delineate the mechanisms underlying the inhibitory effects of AEA on the production of IL-12p40, its effects on the LPS/IFN γ activation of p40 promoter was explored. The RAW264.7 murine macrophage cell line has been extensively used as a cellular model to study the regulation of p40 promoter activity [23, 24, 25]. Besides, RAW264.7 cells express CB₁ and CB₂ receptors making them suitable for studying the regulation of IL-12p40 expression by eCBs [26]. Therefore, we used RAW264.7 cells as the main model for reporter gene experiments in the present study. Cells were transiently transfected

with a luciferase reporter gene construct driven by the wild type IL-12p40 promoter [23]. The stimulation with LPS/IFN γ caused a strong induction of IL12p40 transcriptional activity which was significantly reversed by the co-treatment with AEA at different doses, as shown in Figure 2A. AEA, when applied alone, did not induce any change on p40 promoter activity. We also confirmed the inhibitory effects of AEA on IL-12 p40 transcription in the cell line of microglial origin, BV-2 (data not shown). The involvement of the cannabinoid receptors CB₁ and CB₂ in mediating the inhibitory effects of AEA was next explored by assessing the ability of the CB₁ and CB₂ receptors antagonists respectively, SR141716A (SR1) and SR144528 (SR2), to regulate the inhibitory effect of AEA. As shown in Fig 2B, the presence of SR1 or SR2 had no influence on the inhibitory effect of AEA on IL-12 p40 transcription. This suggests a lack of involvement of the CB1 or CB2 receptor in mediating AEA effects. Because several studies have demonstrated that AEA also behaves as a full agonist and a modulator of the vanilloid receptor, namely TRPV₁ [27], we investigated whether the effects of AEA could be due to actions at TRPV₁ receptors. RAW264.7 cells transfected with the p40 promoter construct were treated with the TRPV₁ antagonist capzasepine at the effective dose of 10 μ M [27], prior to LPS/IFN γ and AEA treatment. As shown in Figure 1C, the pre-treatment with capzasepine was unable to prevent the effects of AEA on p40 promoter activity.

The GATA repressor promoter site is critical for the inhibitory actions of anandamide on IL-12p40 gene expression.

Studies on the regulation of IL-12p40 promoter activity indicate that IL-12p40 promoter contains several binding sites for transcription factors that contribute to gene regulation in LPS-stimulated macrophages. The three control elements that have been most extensively characterized bind NF- κ B, C/EBP β , and ETS transcription factor family

members. In addition, a control repressor element termed GA-12 (GATA sequence in the IL-12 promoter) has been characterized [24]. To investigate the molecular mechanisms controlling the transcription of IL-12p40 under AEA exposure, we performed a series of experiments to study the response elements contained in the IL-12p40 sequence (Figure 3A and B). Therefore, we transfected RAW264.7 cells with various constructs of the IL-12p40 promoter carrying two base-pair mutations in a luciferase reporter gene vector. These mutant promoter constructs have been described to induce loss of promoter activity in other models [24]. Site-directed mutagenesis of the C/EBP β , NF- κ B and ETS sites of the IL-12p40 promoter were unable to reverse the inhibitory effects of AEA on the reporter vector activity (Figure 3C, D and E). Interestingly, when RAW264.7 cells were transiently transfected with constructs carrying mutation on the GA-12 motif, AEA was unable to reverse LPS/IFN- γ induced activation of p40 promoter (Figure 3F). These results strongly suggest that the main mechanism of action of AEA is the activation of this repressor site on the IL-12p40 promoter. To confirm the critical role of GATA repressor element in the inhibitory effects of AEA, we performed decoy oligonucleotide assays. A decoy double stranded oligonucleotide which sequence corresponds to the GATA sequence was generated and transfected to the RAW264.7 cells in an attempt to interfere with binding of transcription factors modulated by AEA to their *cis*-acting elements within the IL-12p40 promoter sequence (Figure 4). The use of this decoy oligonucleotide completely blocked the effects of AEA over the IL-12p40 promoter activity (Figure 4). To further confirm that this was a specific effect of the decoy sequence we use a two point mutation on the GATA sequence decoy oligonucleotide as a control (Figure 4). Mutated decoy oligonucleotide was unable to reverse the effects of AEA on the down-regulation

of IL-12p40 promoter activity. These results strongly suggest that AEA reverses LPS/IFN γ induced IL-12p40 expression mainly by acting on the repressor site GA-12.

Interactions of anandamide and the COX-2 pathway: Inhibition of IL-12 p40 promoter activity by prostamide E₂ involves the activation of the prostanoid receptor, EP2.

Overall, our results show that the inhibitory effect of AEA on the regulation of IL-12p40 gene transcription is mediated by a mechanism independent on the activation of CB₁ or CB₂ receptors. Recent evidences [21, 28] point out that COX-2 is capable of oxygenating endocannabinoids to generate new types of prostanoids and thus, COX-2 may recognize AEA and catalyze its conversion to prostaglandin endoperoxidase ethanolamides, such as prostamide E₂ (Figure 5A). Therefore, the above findings prompted us to investigate the effects of prostamide E₂ in the regulation of IL-12 p40. First, we studied the effects of prostamide E₂ on the induction of p40, p35 and p19 subunits caused by LPS/IFN γ in primary microglial cells. As shown in Figure 5B, prostamide E₂ (10 μ M) markedly reversed the LPS/IFN γ -induced up-regulation of the p40 mRNA expression, as observed by semi-quantitative RT-PCR. Interestingly, induction of p35 and p19 genes was also reduced by prostamide E₂ (Figure 5B). In the following experiments we addressed the question of whether prostamide E₂ may be able to regulate IL-12p40 promoter activity. To analyze this, luciferase reporter gene assays were performed. As shown in Figure 5C, p40 promoter activity induced by LPS/IFN γ was decreased in a dose dependent manner (0.1 μ M, 1 μ M, 10 μ M) by prostamide E₂. In the next set of experiments we showed that prostamide E₂ also acts on the repressor site (GA-12) on the IL-12p40 promoter. Thus, when cells were transiently transfected with constructs carrying mutation on the GA-12 motif, prostamide E₂ was unable to reverse LPS/IFN- γ induced activation of p40 promoter (Figure 5D). It is well known that cells

of the myeloid lineage express different types of prostanoid receptors. Therefore, in an attempt to investigate whether AEA-induced inhibitory effects on IL-12p40 promoter activity may involve the generation of prostamide E₂, we used a pharmacological approach by treating the cells with AH6809, an antagonist of the prostanoid receptor - type 2 (EP2) which it is known to be activated by prostamide E₂ too [28]. AEA and prostamide E₂ were added alone or in the presence of AH6809, together with LPS/IFN- γ in RAW 264.7 cells. As shown in Figure 5E the presence of AH6809 partially reversed the inhibitory effect of both prostamide E₂ on the transcriptional activity of IL-12p40. Interestingly, the effect of AEA was reversed in the same manner when EP2 receptor was antagonized, strongly suggesting that part of the effects of AEA are mediated by EP2 activation. Prostanoid receptor type-4 (EP4) does not seem to be involved since the treatment with the antagonist of EP4, AH23848B, did not modify the inhibitory effects of AEA or prostamide E₂ on IL-12p40 gene transcription (Figure 5F). These results suggest that AEA could exert part of its effects on the regulation of IL-12p40 gene transcription by the generation of prostamide E₂.

Anandamide and prostamide E₂ down-regulate the induction of IL-12p40 protein by LPS/IFN- γ activated microglia.

Our next effort was to delineate AEA and prostamide E₂ actions at IL-12p40 protein level. AEA (10 μ M) inhibited LPS/IFN γ -mediated up-regulation of p40 protein in primary microglial cell cultures, as evaluated by ELISAs from bathing medium (Figure 6A). To explore whether AEA-induced inhibition of IL-12p40 protein synthesis may involve prostanoids, we co-treated microglial cells with LPS/IFN- γ and AEA or prostamide E₂ in the presence or absence of the EP2 antagonist AH6809. When we antagonized the EP2 receptor on AEA-treated microglia what we found was a partial reversion of AEA effects on the production of IL-12p40 protein stimulated by LPS/IFN-

γ , in a similar way as what was observed following the addition of prostamide E₂ (Figure 6A). Again, the treatment with the antagonist of the receptor EP4, AH23848B (Figure 6B), was unable to modify AEA or prostamide E₂ responses on IL-12p40 protein expression.

Inhibition of COX-2 pathway reverses the effects of anandamide on both p40 promoter activity and IL-12p40 expression.

Since both AEA and prostamide E₂ modulate IL-12p40 at protein and promoter levels, and taking into account that AEA can be oxygenated by COX-2 to generate prostamides, we postulated that the inhibition of COX-2 could result in a reversion of AEA effects on IL-12p40 promoter. We thus performed a set of experiments using the COX-2 specific inhibitor, NS-398 (10 μ M). In support to the contribution of COX-2 to AEA actions, the treatment of activated RAW 264.7 cells with NS-398 significantly reversed the inhibition of p40 promoter activity by AEA (Figure 7A). Interestingly, NS-398 lacked effects by itself and it did not modify the p40 promoter activity when co-applied with LPS/IFN γ . In addition, the presence of NS-398 was unable to reverse the effects of prostamide E₂, a downstream metabolite within the COX-2 pathway (Figure 7A). We further confirmed these results at the protein level in primary microglial cells, obtaining similar results to those of p40 promoter activity (Figure 7B). Again, COX-2 inhibition with NS-398 reversed the effects of AEA on IL-12p40 protein expression and it lacked effects by itself, in the presence of LPS/IFN γ and with prostamide E₂ (Figure 7B). Overall these results highlight the contribution of COX-2 to AEA actions.

DISCUSSION

Cannabinoid compounds were previously demonstrated to reduce the development of CREAE and TMEV models of MS [4, 5, 6]. Chronically activated microglia and

astrocytes produce proinflammatory molecules that may contribute to the loss of oligodendrocytes that are compromised in MS. Previously, we and others reported that cannabinoid agonists inhibit the production of proinflammatory molecules by CNS glial cells [7, 29, 30, 31]. Here, we addressed the question of whether the endocannabinoid AEA might be involved in the regulation of IL-12 and related cytokines by microglial cells that are capable of producing eCBs during CNS inflammation [18, 19]. This is of interest because of the role of these cytokines in the induction and maintenance phases of autoimmune inflammatory demyelination [32, 33]

Our results show that activated microglial cells express the p19, p35 and p40 subunits therefore, making possible the formation of the heterodimeric cytokines, IL-12 and IL-23, and then, raising the possibility that microglia may contribute to Th1-skewing within the CNS. In addition, we described for the first time that AEA is a potent inhibitor of the expression of mRNA for p19, p35 and p40 subunits and of IL-12p40 protein release by activated microglia. IL-12 (p35/p40) and IL-23(p19/p40) are known to play a crucial role in the development of EAE [34]. Thus, our results support the involvement of the eCB system in the modulation of neuroinflammatory processes, in line with its ability to limit the immune response in animal models of MS.

The mechanisms by which AEA inhibit the production of IL-12 family cytokines in activated microglia are unknown. Because the subunit p40 is shared by IL-12 and IL-23 we concentrated our studies from a mechanistic point of view on its regulation. In activated cells C/EBP, NFkB and ETS are some of the enhancer sites in p40 promoter that may up-regulate the expression of this subunit [24]. In contrast, in resting cells, there is a constitutively activated repressor site characterized as GA-12. In an approach towards the aim to understand p40 promoter regulation by AEA, we performed site-directed mutagenesis experiments in the IL-12p40 promoter. We observed that mutation

within the core region at GA-12 site abolished the ability of this construct to respond to AEA, strongly suggesting that the mechanism of action of AEA implicates the activation of this repressor site of the IL-12p40 promoter. Furthermore, decoy oligonucleotides experiments also support the idea that AEA down-regulates IL-12p40 expression by acting on the repressor site GA-12. Our data demonstrate for the first time that AEA-mediated down-regulation of p40 promoter activity implicates a mechanism in which the GA-12 repressor site is critical.

Studies on the regulation of IL-12p40 promoter indicated that AEA suppression of IL-12p40 promoter activity was independent of both CB₁ and CB₂ receptor activation. Although functional interactions between the eCB and vanilloid systems have been postulated [27], our results also indicate a lack of involvement of vanilloid system on AEA-induced suppression of IL-12p40. At this point, it is interesting to remark that prostamide E₂, an active metabolite of AEA through COX-2 activity [35], was able to down-regulate the transcriptional activity of IL-12p40 in a dose dependent manner. Although little is known about the mechanisms of prostamide E₂-induced biological actions, it is capable to activate the prostanoid receptor EP₂ [28]. Supporting this, prostamideE₂-induced reversion of IL-12p40 gene transcription by LPS/IFN- γ was attenuated by the pretreatment with the prostanoid EP₂ receptor antagonist. It is important to note that EP₂ receptor antagonist (AH3809) also caused a partial but significant attenuation of AEA-mediated inhibition of IL-12p40 transcriptional activity and protein secretion. Moreover, AH3809 was as efficient in reversing the effects of AEA as it was for prostamide E₂, result that would suggest that these compounds share signalling mechanisms. Therefore the production of prostamide E₂ through metabolisation of AEA by COX-2 could mediate part of the effects of AEA on IL-12p40 regulation. The pre-treatment with the antagonist of the prostanoid receptor EP₄

was ineffective indicating the specificity of the effects on EP₂ receptors. However, we cannot exclude the involvement of other mechanisms for AEA actions on IL-12 p40 regulation, including the existence of unknown CB receptors for AEA [36], or participation of lipids rafts. The up-regulation of COX-2 may be important in terms of favouring the oxygenation of AEA and then, the generation of prostamide E₂ which would contribute to the inhibition of IL-12p40. If COX-2 plays a role in the metabolism of AEA and generates prostamide E₂ which in turn contributes to abolish IL-12p40, then, the inhibition of COX-2 should influence positively the IL-12p40 production. In fact, we observed that the suppressive effects of AEA on IL-12p40 levels were attenuated after the treatment with the specific COX-2 inhibitor NS-398, but interestingly not the effects of prostamide E₂. Therefore, we suggest that during inflammation over-expression of COX-2 may drive AEA metabolism towards a derived prostanoid, prostamide E₂, which may contribute to the negative regulation of IL-12p40 production. Supporting this, the results by Kaminski's group [37] show that AEA caused a concentration-dependent inhibition of IL-2 by murine splenocytes through the generation of a COX-2 metabolite of AEA independently on CB₁/CB₂ activation.

In our study, AEA concentrations were used at the low micromolar range (1-15 μ M), in agreement with doses reported in other studies [37, 38, 39]. Even though AEA levels in rat and human plasma have been detected in the low nanomolar range, the local concentrations of AEA during pathological conditions could be higher. In addition, AEA has been usually measured in normal healthy tissue and plasma samples, whereas these levels differ substantially in inflamed or damaged tissues [19].

Endocannabinoid system is highly activated during CNS inflammation [19], microglial cells are capable of producing eCBs during CNS inflammation [18, 19], and the different components of eCBs are regulated in the brains from MS patients [40].

Activation of eCB system may represent a mechanism for controlling excessive inflammation. The inhibition of IL-12p40 may help to control and limit local immune response in order to prevent over-activation of cellular immunity. Our data also mean that excessive COX-2 resulting from inflammation will have significant impact on endocannabinoids-derived prostanoids signalling in immune reactivity.

Here, we present evidence that the repressor element GA-12 is critical for AEA-mediated down-regulation of IL-12p40 promoter activity. Although the physiological relevance of these findings needs to be established, *in vivo* production of prostamide E₂ has been described to occur after AEA administration, especially in the case of FAAH knockout mice [41]. The identification of the mechanisms underlying the limiting effects of endocannabinoids on the production of proinflammatory cytokines such as IL-12p40 may provide insights relevant for the consideration of endocannabinoid system as a novel and promising target for therapeutic treatment of autoimmune or chronic inflammatory diseases. Our observations in microglial cells point to the interest to study whether endocannabinoids can regulate IL-12 and IL-23, and then, coordinate Th1/Th2 responses on CNS autoimmune diseases, such as MS. Further studies are warranted to elucidate the above question.

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Legends to Figures.

Figure 1 AEA represses the expression of IL-12p35, IL-12p40 and IL-23p19 subunits in activated microglia.

(A) Microglial cells stimulated for 18h with LPS/IFN γ showed an induction in the expression of p35, p40 and p19 subunits mRNAs. This induction was downregulated by co-treatment with AEA (10 μ M). (B) Levels of the protein IL-12p40 were determined by ELISA after 18h stimulation with LPS/IFN γ in the supernants of microglial cultures. The augmented levels of p40 subunit in the supernatants from LPS/IFN γ -stimulated microglia were diminished with the co-treatment with AEA in a dose-response fashion. Results are mean \pm SEM of three independent experiments performed by triplicated, *p<0.05 vs control; #p<0.05 vs LPS/IFN γ ; +p<0.01 vs LPS/IFN γ .

Figure 2 AEA reduces IL-12p40 promoter activity independently of cannabinoid or vanilloid receptors.

(A) Means \pm SEM of the luciferase activity of RAW264.7 cells transiently transfected with a wild type p40/pGL2B promoter gene construct. Cells were left untreated or were stimulated for 18h with LPS/IFN γ in the presence or absence of different doses of AEA prior to luciferase measurement. Luciferase activity was normalized to Renilla luciferase activity (n=12) *p<0.02 vs control; #p<0.05 vs LPS/IFN γ ; +p<0.01 vs LPS/IFN γ . (B) Means \pm SEM of luciferase activity of RAW264.7 cells transiently transfected with a wild type p40/pGL2B promoter gene construct. Transfected cells were pre-treated with CB antagonists SR1 or SR2 and one hour later treated with LPS/IFN γ and AEA. Luciferase activity was measured 18 h after the last treatment. Neither SR1 nor SR2 were capable of reversing the effects of AEA on p40 promoter activity (n=12), *p<0.005 vs control; #p<0.002 vs LPS/IFN γ . (C) Means \pm SEM of luciferase activity of RAW264.7 cells transiently transfected with 400 ng/cm² of the wild type p40/pGL2B

promoter gene construct. Transfected cells were pre-treated with the VR1 antagonist capsazepine (10 μ M) for one hour and then treated for 18 h with LPS/IFN γ and AEA (10 μ M) (n=12) *p<0.001 vs control; #p<0.005 vs LPS/IFN γ .

Figure 3 AEA reduces the activity of the p40 promoter through the activation of the repressor site GA-12.

(A) Summary of consensus binding sites at -300 to +68 promoter region of the IL-12p40 gene. (B) Means \pm SEM of the luciferase activity of RAW264.7 cells transiently transfected with a reporter gene construct containing the wild type p40 promoter were stimulated for 18 h with LPS/IFN γ and AEA (10 μ M) prior to luciferase activity measurement (n=12), *p<0.001 vs control; #p<0.001 vs LPS/IFN γ . (C), (D) and (E) Means \pm SEM of luciferase activity of RAW264.7 cells transiently transfected with mutant p40/pXP1 reporter gene construct carrying 2-bp exchange at the indicated (underlined) promoter sites Ets, NF κ B or C/EBP as indicated in each case. In all of the three cases, the induction of p40 promoter activity was lower when compared to the wild-type promoter. In neither case the mutation was able to interfere with the actions of AEA in reducing the induction caused by the stimulus with LPS/IFN γ . (C) *p<0.001 vs control; #p<0.005 vs LPS/IFN γ . (D) *p<0.001 vs control; #p<0.001 vs LPS/IFN γ . (E) *p<0.002 vs control; #p<0.01 vs LPS/IFN γ . (F) Means \pm SEM of luciferase activity of RAW264.7 cells transiently transfected with a 2-bp mutated p40/pXP1 constructs in the GA-12 region. The mutation of the repressor element interfered with the actions of AEA, *p<0.001 vs control. In all cases n=12.

Figure 4 Decoy GA-12 wild type oligonucleotide is able to reverse the effects of AEA on the activation of the repressor site at the p40 promoter.

(A) Sequences of the wild-type (dGA-12wt) and mutated (dGA-12m) double-stranded oligonucleotides used in this study. Mutations are underlined. (B) Means \pm SEM of luciferase activity of RAW264.7 cells transiently transfected with wild-type p40/pXP1 reporter gene construct with or without double-stranded decoy oligonucleotides corresponding to wild-type or 2-bp mutated version of the GA-12 sequence. Co-transfection of dGA-12wt with p40/pXP1 completely blocked the effects of AEA on the reduction of the induction of p40 promoter activity induced by LPS/IFN γ , whereas co-transfection of dGA-12m1 with p40/pXP1 had no effects (n=12), *p<0.001 vs control; #p<0.001 vs LPS/IFN γ ; +p<0.001 vs LPS/IFN γ +AEA.

Figure 5 Prostaglandin E₂ reduces IL-12p40 promoter activity by activating the repressor site GA-12: involvement of the EP2 receptor.

(A) Scheme showing the metabolic pathway by which AEA is converted into prostamide E₂. (B) Microglial cells stimulated 18h with LPS/IFN γ showed an increase in the expression of p35, p40 and p19 subunits at mRNA levels. When co-treated with prostamide E₂ (10 μ M) this induction was reduced. (C) Means \pm SEM of luciferase activity of RAW264.7 cells transiently transfected with p40/pGL2B reporter gene construct. Cells were left unstimulated or treated with LPS/IFN γ in the presence or absence of different doses of prostamide E₂. Prostamide E₂ reduces p40 promoter activity in a dose-dependent fashion (n=12), *p<0.0001 vs control; #p<0.02 vs LPS/IFN γ ; ###p<0.01 vs LPS/IFN γ ; +p<0.005 vs. LPS/IFN γ . (C) Means \pm SEM of luciferase activity of RAW264.7 cells transiently transfected with wild-type p40/pXP1 reporter gene construct with or without double-stranded decoy oligonucleotides corresponding to wild-type or 2-bp mutated version of the GA-12 sequence. Co-transfection of dGA-12wt with p40/pXP1 completely blocked the effects of AEA on the

reduction of the induction of p40 promoter activity induced by LPS/IFN γ , whereas co-transfection of dGA-12m1 with p40/pXP1 had no effects on AEA downregulation of the p40 promoter activity (n=12), ***p<0.001 vs control; ###p<0.001 vs LPS/IFN γ ; +++p<0.001 vs LPS/IFN γ +AEA. **(D)** Means \pm SEM of luciferase activity of RAW264.7 cells transiently transfected with wild-type p40/pXP1 reporter gene construct. Transfected cells were left unstimulated or treated with LPS/IFN γ in the presence or absence of AEA (10 μ M) or prostamide E₂ (10 μ M). Pre-treatment with EP2 antagonist AH6809 (10 μ M) partially reverse the effects of AEA and prostamide E₂ on the p40 promoter activity (n=12), *p<0.001 vs control; #p<0.003 vs LPS/IFN γ ; +p<0.01 vs LPS/IFN γ +AEA; ++p<0.01 vs. LPS/IFN γ +prostamide E₂. **(E)** Means \pm SEM of luciferase activity of RAW264.7 cells transiently transfected with wild-type p40/pXP1 reporter gene construct Transfected cells were left unstimulated or treated with LPS/IFN γ in the presence or absence of AEA (10 μ M) or prostamide E₂ (10 μ M). Pre-treatment with EP4 antagonist AH23848B (30 μ M) did not modify the effects of AEA and prostamide E₂ on the p40 promoter activity (n=12) *p<0.02 vs control; #p<0.05 vs LPS/IFN γ .

Figure 6 Prostamide E₂ negatively modulates p40 protein levels in activated microglial cells.

(A) Microglial cells were treated overnight with LPS/IFN γ , AEA (10 μ), prostamide E₂ (10 μ M) and/or EP2 antagonist AH6809 (10 μ M). Antagonism of EP2 receptor partially blocked the effects of both AEA and prostamide E₂ on p40 protein expression. Supernatants were collected and p40 protein was measured by ELISA. Results are means \pm SEM of three independent experiments performed by triplicated, ***p<0.001

vs control; ^{###}p<0.001 vs LPS/IFN γ ; ⁺p<0.05 vs. LPS/IFN γ +AEA; ⁺⁺p<0.005 vs. LPS/IFN γ +prostamide E₂. **(B)** When pre-treated with EP4 antagonist AH23848B, it did not modify the effects of AEA and prostamide E₂ on the p40 protein expression measured by ELISA. Results are means \pm SEM of three independent experiments performed by triplicated, ^{**}p<0.002 vs control; ^{###}p<0.005 vs LPS/IFN γ ; [#]p<0.05 vs LPS/IFN γ .

Figure 7 Pharmacological inhibition of COX-2 reverses the effect of AEA both at protein and promoter levels.

(A) Means \pm SEM of the luciferase activity of RAW264.7 cells transiently transfected with a reporter gene construct containing the wild type p40 promoter. Cells were stimulated for 18 h with LPS/IFN γ and AEA (10 μ M) or Prostamide E₂ (10 μ M), in the presence or absence of the specific COX-2 inhibitor, NS-398 (10 μ M), prior to luciferase activity measurement (n=9). ^{*}p<0.05 vs control; [#]p<0.01 vs LPS/IFN γ ; ⁺p<0.05 vs LPS/IFN γ +AEA; ^{Δ} p<0.01 vs LPS/IFN γ . **(B)** IL-12p40 protein expression of microglia treated with NS398 (10 μ M). COX-2 inhibition significantly reduced the magnitude of AEA inhibition on IL-12p40 protein expression (measured by ELISA), whereas it has no effect on prostamide E₂ actions, supporting the involvement of this enzyme in AEA's effects. Results are mean \pm SEM of three independent experiments performed by triplicated, ^{*}p<0.05 vs control; [#]p<0.01 vs LPS/IFN γ ; ⁺p<0.05 vs LPS/IFN γ +AEA; ^{Δ} p<0.01 vs LPS/IFN γ .

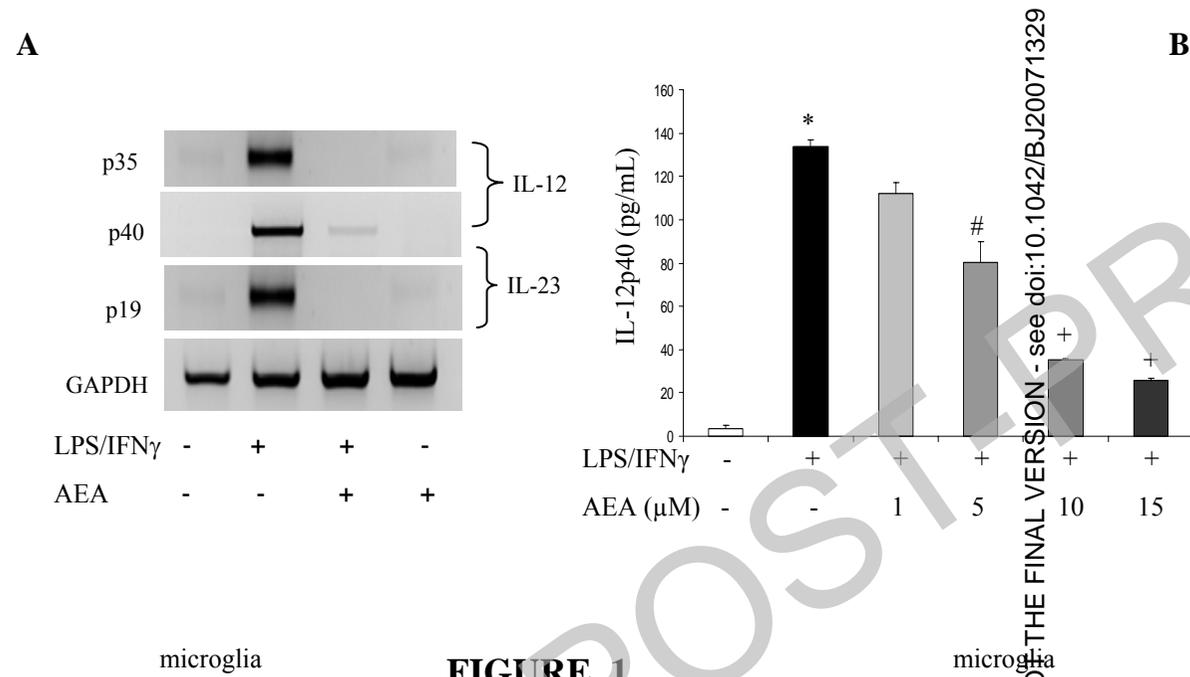


FIGURE 1

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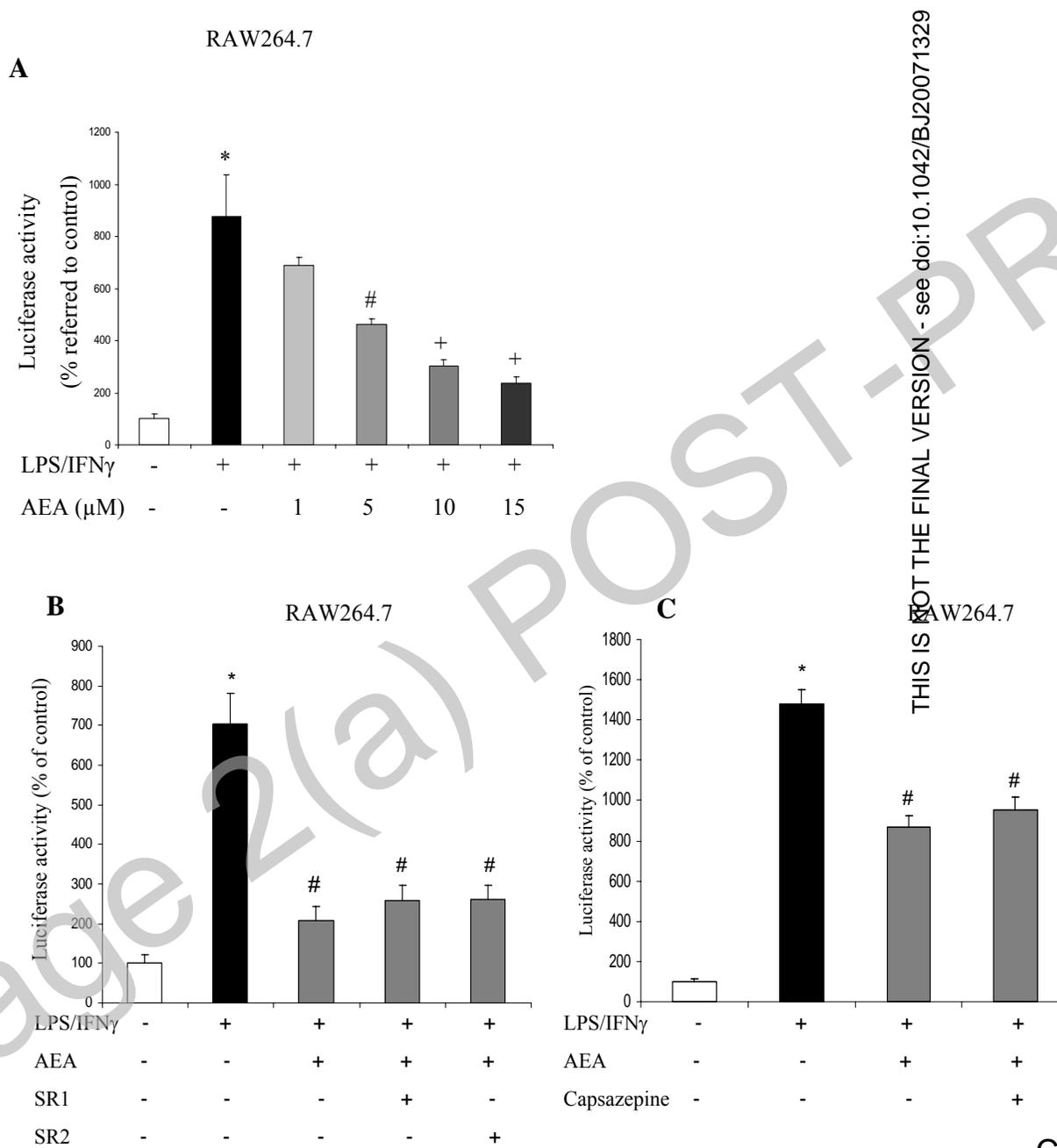


FIGURE 2

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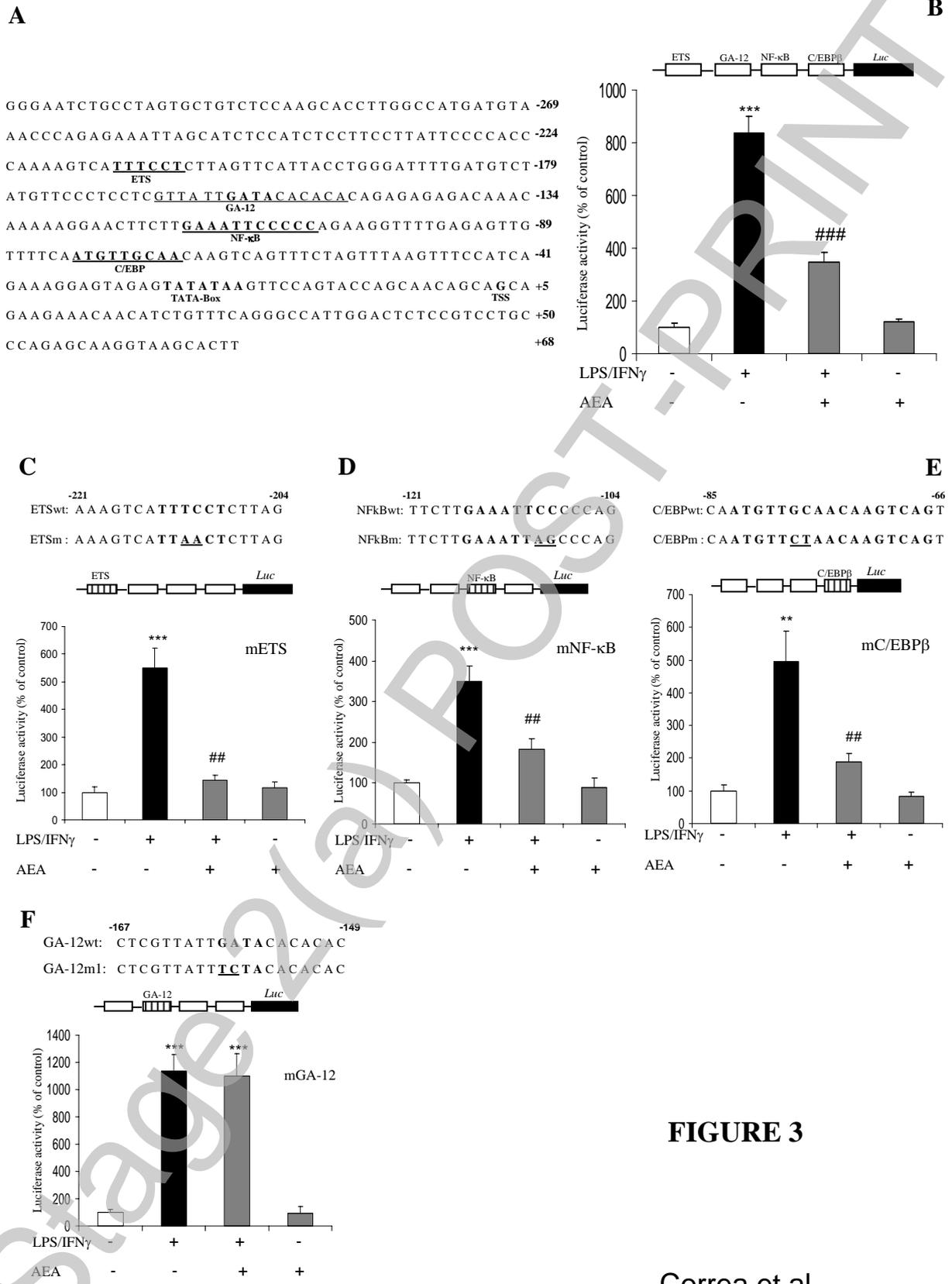


FIGURE 3

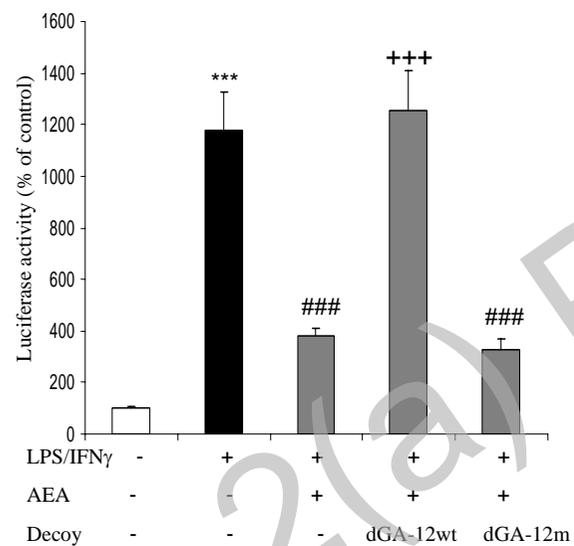
Correa et al

A

Decoy GA-12 wild type: 5'CTCGTTATTGATACACACACAGAGA3'
(dGA-12 wt)

Decoy GA-12m: 5'CTCGTTATTTCACACACACAGAGA3'
(dGA-12m)

B



RAW264.7

FIGURE 4

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Stage 2 (a) POST-PRINT

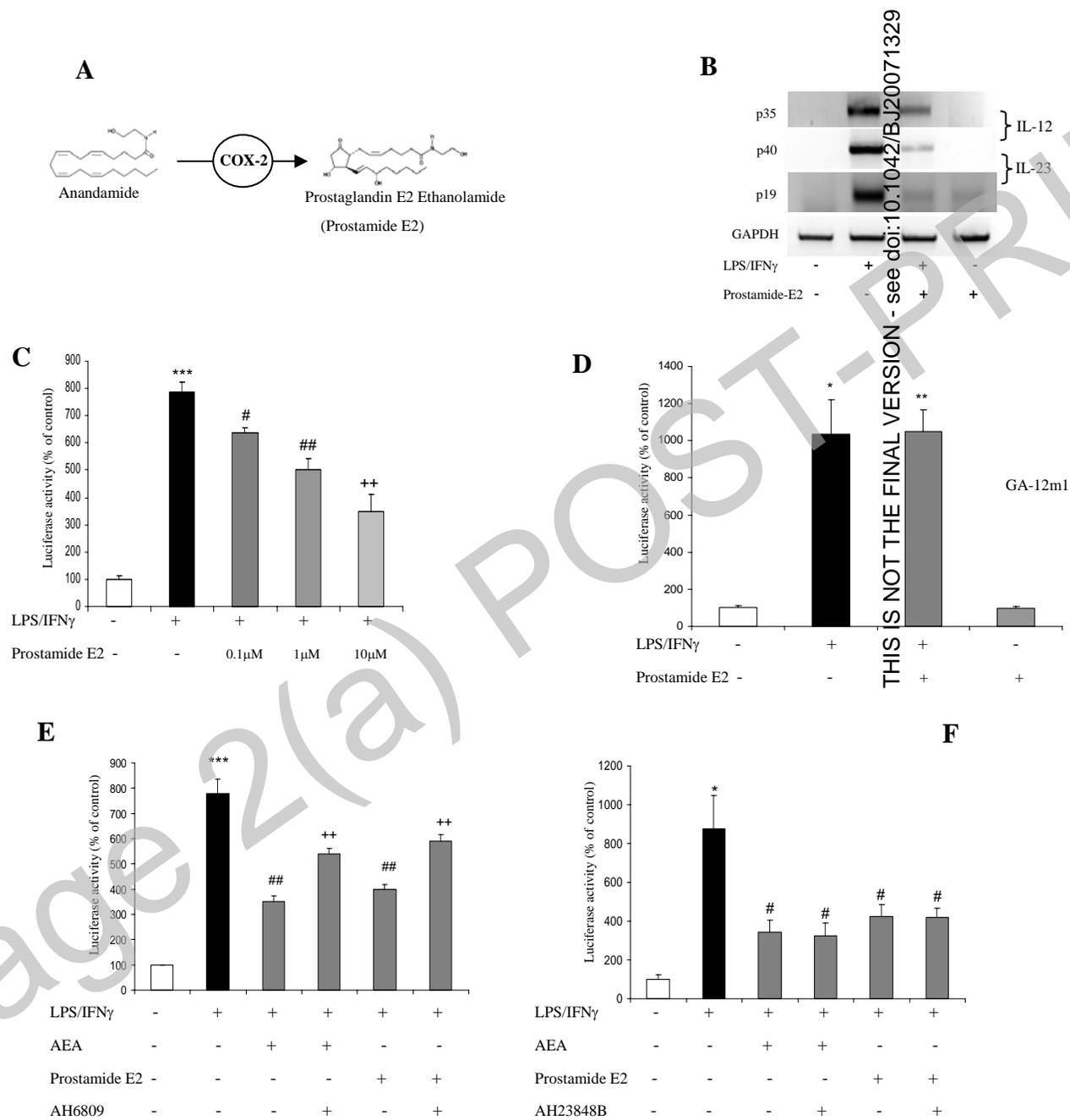


FIGURE 5

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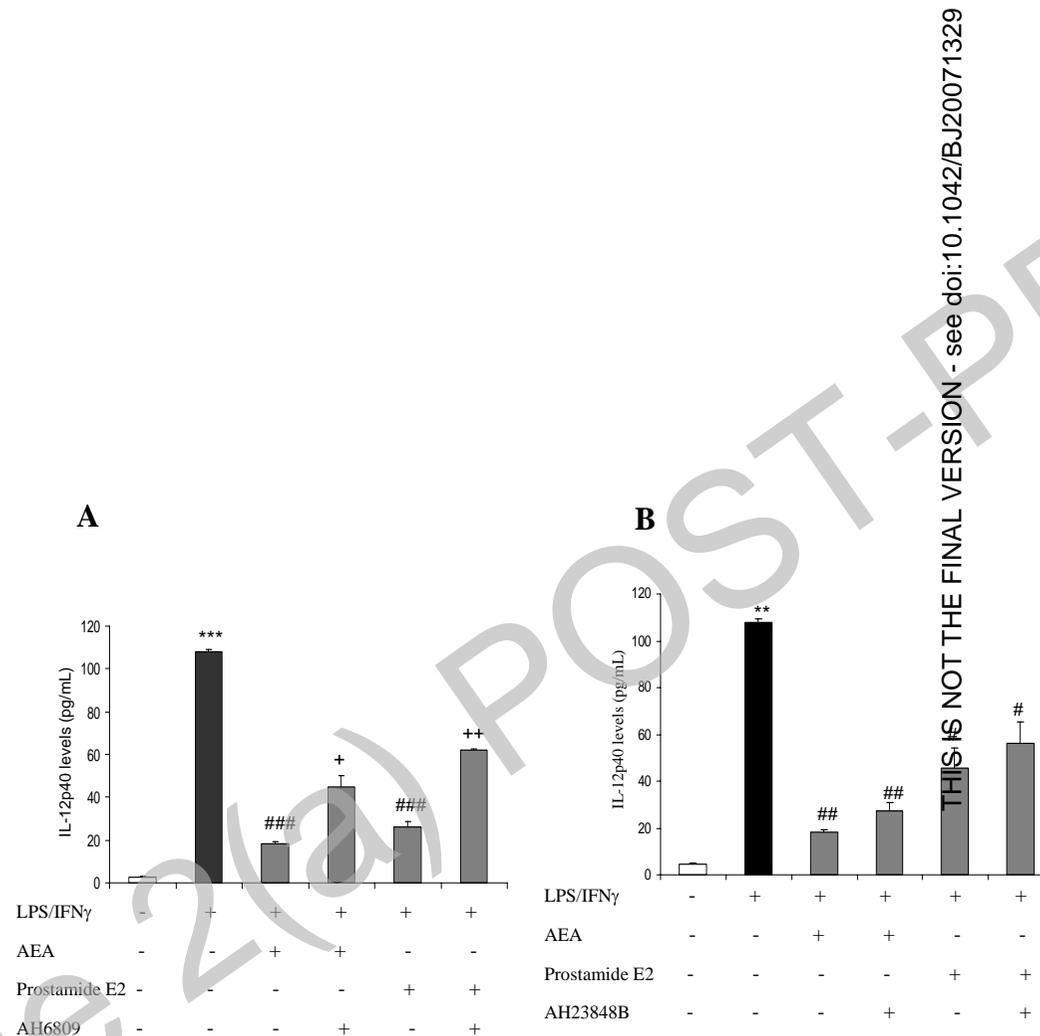


FIGURE 6

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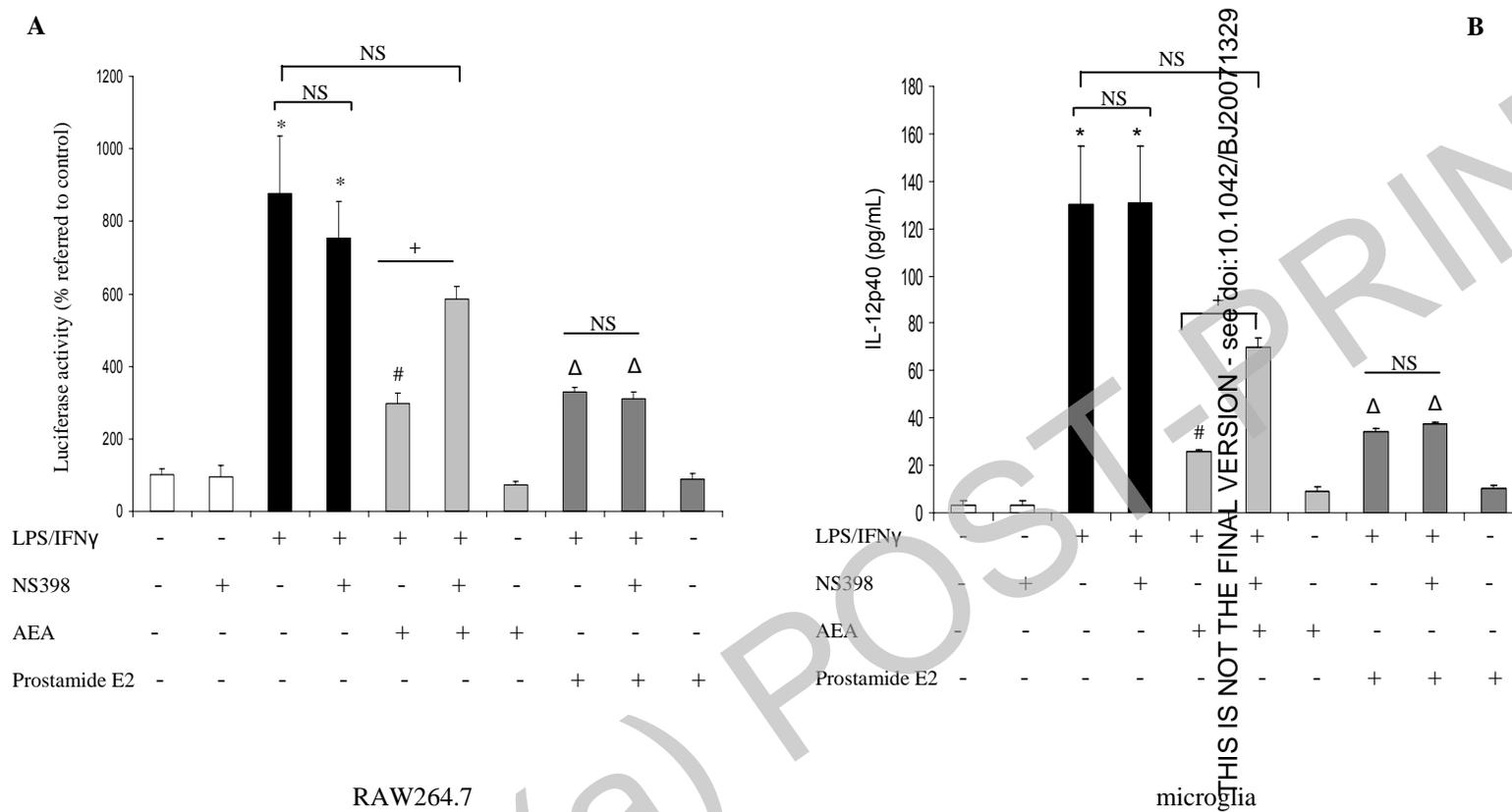


Fig. 7

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