# APPA (Apocynin and Paeonol) modulates pathological aspects of human neutrophil function, without supressing antimicrobial ability, and inhibits TNFα expression and signalling.

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**Abstract**

Neutrophils are key players in the pathophysiological process underlying inflammatory conditions by release of tissue-damaging cytotoxic enzymes, reactive oxygen species (ROS) and a range of chemokines and cytokines. Here we report on the effects of APPA, and its constituent components, apocynin (AP) and paeonol (PA) on a number of neutrophil functions, including effects on TNFα- expression and signalling. Neutrophils were treated with APPA (10-1000μg/mL) prior to the measurement of cell functions, including ROS production, chemotaxis, apoptosis and surface receptor expression. Expression levels of several key genes and proteins were measured after incubation with APPA and the chromatin re-modelling agent, R848. APPA did not significantly affect phagocytosis, bacterial killing or expression of surface receptors, while chemotactic migration was affected only at the highest concentrations. However, APPA down-regulated neutrophil degranulation and ROS levels, and decreased the formation of neutrophil extracellular traps. APPA also decreased cytokine-stimulated gene expression, inhibiting both TNFα- and GM-CSF-induced cell signalling. APPA was as effective as infliximab in down-regulating chemokine and IL-6 expression following incubation with R848. Whilst APPA does not interfere with neutrophil host defence against infections, it does inhibit neutrophil degranulation, and cytokine-driven signalling pathways (e.g. autocrine signalling and NF-κB activation), processes that are associated with inflammation. These observations may explain the mechanisms by which APPA exerts anti-inflammatory effects and suggests a potential therapeutic role in inflammatory diseases in which neutrophils and TNFα signalling are important in pathology, such as rheumatoid arthritis.

## Introduction

Advances in identifying the pathophysiological processes underlying rheumatoid arthritis (RA) have led to the development of targeted therapies and enhanced outcomes for many patients (Nikiphorou et al. 2017). However, no single drug is effective for all patients and each is associated with significant risks of adverse effects demonstrating a need for novel, safe and effective therapies. A greater understanding of neutrophil biology has led to an appreciation that these cells play a significant role in rheumatoid arthritis (RA) and other inflammatory conditions (Wright et al. 2014), but targeting neutrophils therapeutically is challenging, as host defence must not be compromised. Neutrophils contribute to inflammatory diseases via the release of cytokines, chemokines, reactive oxygen species (ROS) and proteases (Jaillon et al. 2013) that are activated via distinct but sometimes overlapping agonist:signal transduction pathways. Targeting neutrophils in inflammatory diseases must therefore block tissue-damaging processes (e.g. secretion) but not interfere with opsono-phagocytosis or microbial killing.

TNFα is a key molecule in the pathology of RA and therapeutic targeting of this molecule, e.g. with biologics such as TNFi, can lead to dramatic improvements in many, but not all patients (Hyrich et. 2006; Emery et al. 2014). Neutrophils contribute to abnormal TNFα signalling in RA, by both responding to and expressing this cytokine during active disease (Wright et al. 2010). Membrane bound (m)TNFα is elevated on the surface of blood neutrophils in RA patients with active disease, and NF-κB activation levels (which may be triggered via TNFα signalling) are elevated, but both surface mTNFα levels and NF-κB activation levels return to healthy control levels during successful TNFi therapy (Wright et al. 2010). NF-κB is a key regulatory protein involved in inflammatory processes in conditions such as RA and

69 osteoarthritis (OA) (Pilichou et al. 2008). It regulates the functions of all cell types involved in
70 joint physiology and pathology, including synoviocytes, chondrocytes, osteocytes, endothelial
71 cells, vascular smooth muscle cells, fibroblasts and leukocytes (Bonizzi and Karin 2004) and so
72 targeting its over-activity may be beneficial in such diseases (Gilmore and Herscovitch 2006).
73 This transcription factor plays a central role in the regulation of a number of neutrophil
74 functions, and it is constitutively activated in many patients with active RA (Muller-Ladner et
75 al. 2002), likely via TNFα signalling (Kanbe et al. 2008). More recently, a new mechanism of
76 gene activation in neutrophils involving either endogenously-expressed or exogenously-
77 added TNFα has been identified following incubation of neutrophils with the TLR8
78 agonist/chromatin remodelling agent, R848 (Zimmerman et al. 2015). Human neutrophils do
79 not normally express IL-6 because the promoter region of this gene is transcriptionally "silent"
80 and in an inactive configuration. However, R848 alters chromatin structure at this locus to
81 enable its transcription. Both endogenously-expressed and exogenously-added TNFα are
82 required for this IL-6 expression by prolonging the synthesis of IκBζ co-activator and
83 sustaining C/EBPβ recruitment and histone acetylation at *IL-6* regulatory regions
84 (Zimmerman et al. 2015). In view of the importance of IL-6 and TNFα in the pathology of
85 RA, this mechanism of endogenously-expressed TNFα on expression of IL-6 on re-modelled
86 chromatin could be extremely important in understanding disease mechanisms. Targetting
87 these processes could have therapeutic benefits.
88
89 APPA, a synthetic combination of two anti-inflammatory molecules, apocynin (AP) and
90 paeonol (PA), has shown efficacy in canine models of OA (Glasson and Larkins 2012; Larkins
91 and King 2017A; Larkins and King 2017B) and is currently under clinical development for use
92 in human OA. Its efficacy is thought to lie predominantly in its effects on regulation of the

93 transcription factor, NF-κB as well as other signalling pathways (Muller-Ladner et al. 2002).
94 AP is a strong ROS scavenger (Nam et al. 2014; Stefanska and Pawliczak 2008; Impellizzeri et
95 al. 2011A) and inhibits the expression and release of several inflammatory cytokines and
96 matrix metalloproteinases, while PA, an isomer of apocynin, down-regulates activation,
97 nuclear translocation, and DNA binding of NF-κB (Su et al. 2010). These combined activities
98 of APPA inhibit many of the molecular events triggered during inflammatory activation.
99 However, the effects of APPA and its constituent components on neutrophil function, many
100 of which are regulated by TNFα, are completely unknown. Given the proposed mechanism of
101 actions of this drug, it might be predicted to down-regulate inflammatory responses in
102 neutrophils that are regulated by NF-κB. The aims of this research were to investigate the
103 effects of APPA, PA and AP on neutrophils *in vitro*, especially on functions that regulate host
104 defence against infections. We also investigated the ability of these molecules to modulate
105 R848-induced IL-6 expression via inhibition of endogenous TNFα activity and show that it is
106 as effective as TNFα-blocking antibodies in this action.
107
108 **Materials and Methods**
109 **Isolation of neutrophils**
110 Blood was collected into lithium-heparin vacutainers from healthy controls, after giving
111 informed consent: this study was approved by the NHS Health Research Authority
112 (Inflammatory Signalling Pathways; Ref 11/NW/0206: IRAS project ID 75388). Neutrophils
113 were isolated following sedimentation in HetaSep and centrifugation on Ficoll-paque (Wright
114 et al. 2016): contaminating erythrocytes were removed by hypotonic lysis. Neutrophils were
115 examined for purity by Romanowsky staining and microscopic analysis of cytospins, and
116 viability by trypan blue exclusion; these were >97% and >98%, respectively in freshly-isolated

cells. Neutrophils were incubated at $10^6$ or $5x10^6$ cells/mL (as described in the text) in RPMI media (Thermo-Fisher) plus 10% human AB serum (Sigma) and incubated at 37°C and 5% $CO_2$ for up to 20h. Cytokines were added as follows: IL-8 (100ng/mL, Sigma); GM-CSF (5ng/mL, Roche); TNFα (10ng/mL, Merck); IL-1β (10ng/mL, Source Bioscience); IFNγ (10ng/mL, Source Bioscience). R848 (Sigma) was used at a concentration of 5μM (Zimmerman et al. 2015). APPA (a 2:7 ratio of AP:PA) was dissolved in DMSO and was initially tested over a concentration range of 10-1000μg/mL (final concs). AP and PA were also used individually at the concentrations equivalent in the APPA mixture used at 100μg/mL.

**Measurement of apoptosis**

Neutrophils ($1x10^5$) were removed from culture (at the indicated times), diluted with 100μL of HBSS (Thermo-Fisher) containing 0.5μL annexin V-FITC (Thermo-Fisher), and incubated in the dark at room temperature for 15min. The total volume was then made up to 500μL with HBSS, and propidium-iodide added (final concentration 1μg/mL, Sigma) before analysis immediately on a Dako Cyan ADP flow cytometer. 10,000 events/sample were analysed.

**Degranulation**

Neutrophils ($5x10^6$/mL) were pre-incubated for 10min with APPA (100μg/mL), before priming with GM-CSF (5ng/mL) for 30 min and then stimulated to degranulate with cytochalasin B (5μg/mL) plus fMLP (1μM, both from Sigma) for 15min. Cells were pelleted gently, washed and analysed by flow cytometry, while supernatants were removed for SDS-PAGE after adding concentrated Laemmli protein sample buffer. After electrophoresis, proteins were transferred to PVDF membranes and probed with antibodies to myeloperoxidase (MPO) (R&D Systems), lactoferrin (Abcam), MMP9 (R&D Systems) and elastase (Abcam). Secondary antibodies were anti-rabbit IgG (GE Healthcare) and anti-mouse IgG (Sigma) HRP-linked

antibodies (1:10,000). Bound antibodies were detected using the ECL system (Merck) and film (Amersham).

**Antibody staining and flow cytometry**

Antibody staining was carried out on freshly-isolated neutrophils incubated for up to 1h, as described above. Neutrophils ($1x10^5$) were resuspended in PBS (+0.2% BSA). Antibody-binding was carried out at 4°C in the dark for 30min with conjugated antibodies added as follows: CD62L-FITC (R&D systems); CD11b-PE (R&D systems); CD16-PE (R&D systems); CD18-PE (R&D systems); CD63-APC (Thermo-Fisher); CD64-FITC (R&D systems); IL-8R (CXCR1)-FITC (R&D systems); CD66b-FITC (R&D systems); isotype controls (Santa Cruz). Fluorescence was measured immediately on a Dako Cyan ADP flow cytometer. 10,000 events/sample were analysed.

**Western blotting**

Proteins from $5x10^5$ cells, extracted in Laemelli buffer containing protease- and phosphatase-inhibitors, were separated by SDS-PAGE using a 12% gel and transferred onto PVDF membranes (Merck). Primary antibodies were: NF-κB (1:1000, Cell Signaling); IκBα (1:1000, Cell Signaling); Erk1/2 (1:1000, Cell Signaling); p38-MAPK (1:1000, Cell Signaling) and GAPDH (1:10,000, Abcam). Secondary antibodies were anti-rabbit IgG (GE Healthcare) and anti-mouse IgG (Sigma) HRP-linked antibodies (1:10,000). Bound antibodies were detected using the ECL system (Millipore) on carefully exposed film (Merck) to avoid saturation.

**Chemotaxis assay**

Chemotaxis was performed in 24-well tissue culture plates (coated with 12mg/mL poly-hema (Sigma)) using hanging cell inserts (Merck) with a 3µm-pore membrane separating media in the upper and lower chambers. Standard neutrophil chemotactic agents were added to 800µL RPMI media in the lower chamber (fMLP at $10^{-8}$M and IL-8 at 100ng/mL, final concs,

both from Sigma). Neutrophils ($10^6$) were added to the upper chamber and the plates incubated for 90min at 37°C and 5% $CO_2$. The number of migrated neutrophils in the lower chamber after 90min was measured using a Coulter Counter Multisizer3 (Beckman Coulter).

**Respiratory burst measurements**

Neutrophils ($5x10^6$/mL) were pre-incubated for 10min with APPA (10-1000 µg/mL), before incubation with GM-CSF (5ng/mL) or TNFα (10ng/mL) for 30min. Cells ($5 \times 10^5$) were then added to wells of a 96-well plate and diluted in HBSS containing luminol (10µM) and the respiratory burst stimulated with fMLP (1µM, Sigma) or PMA (100ng/mL, Sigma). Luminescence was measured using a Tecan GENios Plus Luminescence plate reader measuring continuously for 30min.

**Opsonisation and Phagocytosis of bacteria**

*Staphylococcus aureus* (Oxford strain) were heat-killed by incubation at 60°C for 30min, washed twice, and then resuspended in PBS containing 30µM propidium iodide (PI). The suspension was incubated in the dark at 4°C for 2h and then washed. Opsonization, using pooled human AB serum from healthy donors (stored in aliquots at -20°C), was achieved by incubating bacteria ($5x10^8$/ml) with 10% heat-inactivated human serum (v/v, final concentration) for 30min at 37°C before washing. Freshly-isolated neutrophils ($10^6$/mL) from healthy controls were incubated for 30min with PI- labelled, opsonised heat-killed *S. aureus* (SAPI) in a ratio of 1:10 and incubated in the dark for 30 min at 37°C with gentle agitation. Neutrophils were then pelleted by centrifugation, washed twice, and suspended in PBS containing 5mM EDTA, 3mM sodium azide and 1% paraformaldehyde followed by analysis using flow cytometry.

**Bacterial killing**

Freshly-grown *S. aureus* were harvested and washed, and suspended at $5x10^8$/ml in HBSS and opsonised as described above. Freshly-isolated neutrophils ($10^6$/mL) were incubated for 1h at 37°C with gentle agitation with opsonised bacteria at a ratio of 1:10. Neutrophils were then lysed to release live bacteria by serial dilution in distilled water and vigorous vortexing, before being plated onto LB agar plates and incubated overnight. Colonies were counted and results calculated as percentage of bacteria killed compared to bacteria only (no neutrophils) samples.

**NET formation**

(a) Quantitation of DNA release**:** Neutrophils ($5 \times 10^5$/500µL media containing 2% (v/v) FBS) were seeded into wells of a 24-well culture and incubated for 1h at 37°C. APPA (100µg/mL) was then added and incubated for 10min before stimulation with 100nM PMA solution for 3h at 37°C. After incubation, NET DNA was isolated using Micrococcal nuclease (500mU, Sigma) and quantified utilizing picogreen (Promega) and a DNA calibration curve. (b) microscopic visualisation: neutrophils were seeded and incubated as described above. Following incubation cells were fixed on cover slips, stained with neutrophil elastase antibody and DAPI (Thermo-Fisher) before being viewed microscopically on a Leica TCS SPE (Papayannopoulos et al. 2010).

**Gene expression using qPCR**

$1x10^7$ neutrophils ($5x10^6$/mL) were pre-incubated for 10min with APPA (10-1000µg/mL), before incubation with GM-CSF (5ng/mL), TNFα (10ng/mL) or IFNγ (10ng/mL) alone or in combination for 1h. Cells were then immediately pelleted and RNA extracted using Trizol (Thermo-Fisher) and stored at -20°C. RNA was cleaned with RNeasy kit (which included a DNAse step, Qiagen) before cDNA synthesis, which was amplified using primers for: TNFα (forward CAGAGGGCCTGTACCTCATC, reverse GGAAGACCCCTCCCAGATAG); CCL3 (forward

GCTCTCTGCAACCAGTTCTCT, reverse TGGCTGCTCGTCTCAAAGTAG) AND CCL4 (forward GCTGTGGTATTCCAAACCAAAAGAA, reverse AGGTGACCTTCCCTGAAGACT). IL-6 was amplified using a Bio-Rad pre-validated primer pair. GAPDH was used to normalise samples (forward CTCAACGACCACTTTGTCAAGCTCA, reverse GGTCTTACTCCTTGGAGGCCATGTG). Results were quantified by the Pfaffl method and are expressed as fold increase/decrease compared to untreated neutrophils.

**Statistical Analysis**

Statistical analysis was carried out using SPSS v24, using Student's t-test unless otherwise stated.

## Results

### Apoptosis is accelerated by high concentrations of APPA.

In initial experiments, neutrophils were pre-incubated with APPA (10-1000μg/mL) in the presence or absence (control) of anti-apoptotic cytokines (GM-CSF or TNFα) for 20h. While both GM-CSF and TNFα delayed neutrophils apoptosis (as described previously (Wright et al. 2014; Wright et al. 2010; Moulding et al. 2001) levels of apoptosis in APPA-treated cells at 20h were slightly increased above untreated (UT) control cells and while this effect was dose-dependent, these effects did not reach statistical-significance (Figure 1A). APPA had a greater effect on apoptosis of cytokine-treated neutrophils, and at the highest concentration used significantly inhibited GM-CSF- and TNFα-delayed apoptosis ($p<0.01$; Figure 1A).

### Neutrophil chemotaxis is impaired at high APPA concentrations.

Both IL-8 and fMLP are strong neutrophil chemottractants (Figure 1B **p<0.01, *p<0.05, respectively compared to no stimulus) and were used as positive controls to test the effects of APPA. Neutrophils were pre-incubated with APPA (10-1000µg/mL) for 10min, before measurement of chemotaxis for 90min toward IL-8 (100ng/mL, data not shown) or fMLP (0.01 µM, Figure 1B). Chemotaxis towards both IL-8 (not shown) and fMLP in APPA-treated neutrophils was only inhibited at high concentrations of APPA (500 and 1000µg/mL).

**APPA does not affect the ability of neutrophils to phagocytose and kill bacteria.**

Phagocytosis of PI-stained, serum-opsonised *S. aureus* was largely unaffected (>96% phagocytosis, compared to untreated control values) by treatment with APPA at all concentrations used (Figure 1C). Similarly, pre-incubation with APPA (10-1000µg/mL) for 10min did not impair killing of live, serum-opsonised *S. aureus*, with neutrophils killing ~40% bacteria over a 60min incubation period at all concentrations tested, which was not significantly different from untreated controls (Figure 1D).

**APPA does not alter surface receptor expression by neutrophils.**

Next, we examined the effect of APPA on expression of receptors that are important in chemotaxis and phagocytosis. Surface receptor expression of freshly-isolated neutrophils, and neutrophils incubated for 1h with GM-CSF and TNFα ± APPA (100µg/mL) was measured by flow cytometry. As previously reported, GM-CSF (Fossati et al. 1998) and TNFα (Lynn et al. 1991) resulted in small increases in expression of CD11b (Figure 1E), but APPA did not affect this up-regulation. Surface levels of CD18, CD16, CD32, CD64, L-selectin (CD62L) and CXCR1 (the IL-8 receptor) were unaffected by incubation with APPA (Figure 1E).

**Effects of APPA on the respiratory burst.**

Neutrophils were treated for 10min with APPA (10-1000µg/mL), before priming with GM-CSF (5ng/mL) for 30min. The respiratory burst was then stimulated via receptor-dependent or receptor-independent mechanisms with either fMLP (1µM) or PMA (100ng/mL), respectively. APPA decreased both the the fMLP-stimulated (Figure 2A) and PMA-stimulated ROS levels (Figure 2B,C) in a dose-dependent manner, with statistically-significant inhibition evident at 10µg/mL. As AP (a constituent of APPA) is a reported scavenger of ROS, we then added APPA 5min after activation of the respiratory burst had been stimulated by PMA. Both concentrations of APPA used (10µg/mL and 100µg/mL) resulted in an immediate decrease in the chemiluminescence signal, as would be expected following addition of a ROS scavenging agent (Figure 2D). For example, the addition of sodium azide (an inhibitor of myeloperoxidase) decreased ROS levels as rapidly as APPA, whereas the addition of superoxide dismutase (which catalyses the conversion of $O_2^-$ into $H_2O_2$ and $O_2$) resulted in a a much slower decline in ROS levels in this experimental system (Figure 2E). Further experiments utilizing known reactive oxidant scavengers in a cell free system (Figure 2F) confirmed that ROS quenching/scavenging was largely responsible for the decrease in chemiluminescence signal by APPA. We then examined the effects of the individual components of APPA, namely AP and PA for their effects on neutrophil reactive oxidant scavenging. When these components were added 5min after stimulation of the respiratory burst by PMA, PA had little effect on levels of reactive oxidants, whereas AP addition resulted in rapid and extensive quenching ($p<0.01$), that was equivalent to the quenching effect seen by APPA (Figure 2G, H).

**Effects of APPA on degranulation and NET formation.**

The effects of APPA on degranulation was examined. Neutrophils from healthy controls were pre-incubated for 30min with APPA (100µg/mL) and then primed for 30min with GM-CSF before stimulating degranulation with fMLP and cytochalasin B. Degranulation of primary granules, as measured by CD63 expression using flow cytometry (Figure 3A) was decreased in APPA-treated cells ($p<0.05$). APPA also inhibited the release of key secretory molecules, namely MMP9, elastase, MPO and lactoferrin (Figure 3B) as assessed by analysis of cell free supernatants of stimulated cells by western blotting.

The generation of neutrophil extracellular traps (NETs) may enhance the trapping and killing of extracellular pathogens (Carmona-Rivera and Kaplan 2016; Brankz et al. 2014; Smith and Kaplan 2015; Knight and Kaplan 2012; Grayson and Kaplan 2016), but may also break immune tolerance by extracellular exposure of autoantigens, thus contributing to autoimmunity (Thieblemont et al. 2016). PMA stimulated NET production was inhibited by APPA (Figures 4A,B). This inhibition of NET formation was due to the inhibitory effects of AP ($p<0.05$) in the APPA mixture, as PA alone had no significant inhibitory effect on PMA-stimulated NET formation (Figure 4C).

**APPA inhibits key signalling pathways in neutrophils.**

Cytokine exposure of neutrophils results in activation of a number of intracellular signalling cascades that trigger events regulating inflammation. These include ERK1/2 and transcription factors such as STAT3 and NF-κB, which are dynamically-regulated after exposure of neutrophils to agents such as GM-CSF, IL-6 and TNFα (Mouzaoui et al. 2014; Wright et al. 2014; McDonald et al. 1997). Neutrophils were incubated in the absence (control) or presence of APPA (100µg/mL) for 10min before stimulation with GM-CSF, IL-6 or TNFα for 15min. While

IL-6 had only minor effects on neutrophil function, it did activate STAT3; GM-CSF activated STAT3 and pERK phosphorylation; TNFα activated NF-κB (p65 phosphorylation) and enhanced IκBα turnover (Figure 5A). APPA significantly inhibited IL-6 activation of STAT3 (p=0.03: Figure 5B), GM-CSF activation of Erk 1/2 (p=0.03: Figure 5C) and TNFα-mediated activation of NF-κB (p=0.008: Figure 5D).

**Effects of APPA on neutrophil gene expression.**

In addition to their ability to prime neutrophils, GM-CSF, TNFα and IFNγ can also rapidly activate neutrophil gene expression. Therefore, we determined if APPA had any effect on the expression of several key neutrophil genes, particularly those regulated by NF-κB. Neutrophils from healthy controls were pre-incubated with APPA (100μg/mL) for 10min before stimulation with cytokines (GM-CSF, TNFα or IFNγ) for 1h. Gene expression was measured using qPCR to quantify transcripts for TNFα, IL-8 and IL-1β. Expression of Nrf2 was also measured as this transcription factor regulates the expression of antioxidant proteins that protect against oxidative stress (Niture et al. 2020; Murakami and Motohashi 2015). Figure 6 shows that cytokine treatment of neutrophils resulted in enhanced expression of IL-1β, (Figure 6A), IL-8 (Figure 6B), Nrf2 (Figure 6C) and TNFα itself (Figure 6D), and levels of expression of these genes were greater after TNFα treatment than were observed after incubation with either GM-CSF or IFNγ. Pre-treatment of neutrophils with APPA (100μg/mL) for 30 min resulted in down-regulation of TNFα-activated expression of IL-1β, IL-8 and TNFα, in line with its ability to inhibit NF-κB (Figure 6). However, APPA enhanced TNFα- and GM-CSF-induced expression of Nrf2 suggesting it is able to induce an anti-oxidative stress response.

The recent discovery that neutrophil chromatin can be re-modelled by agents likely to be important in inflammation, to enable transcription of normally silent genes (Zimmerman et al. 2015) has transformed our understanding of the transcriptional repertoire of neutrophils in disease. We therefore incubated neutrophils with the chromatin re-modelling agent, R848 for 7h and measured the effects of APPA on expression of the chemokines, CCL3 and CCL4, and the pro-inflammatory cytokine IL-6. Previous work has shown that endogenous TNFα is important for this R848-induced IL-6 expression (Zimmerman et al. 2015) and so we also incubated R848-treated neutrophils with the neutralising TNFα antibody, infliximab. R848 stimulated the expression of CCL3, CCL4 and IL-6 under these experimental conditions and this expression was significantly decreased in cultures co-incubated with infliximab (Figure 7 A-C), confirming the role of endogenous TNFα in this gene expression. When we measured the effects of the individual componts of APPA on this gene expression, both APPA and AP significantly decreased expression of IL-6 and CCL3, but PA further decreased expression levels to unstimulated, control values (Figure 8A,B).

**Discussion**

APPA and its constituent components, apocynin (AP) and paeonol (PA), have therapeutic effects in several inflammatory settings, explained in part by inhibition of the NF-κB signalling pathway and in part on its ability to scavenge ROS (Glasson and Larkins 2012; Impellizzeri et al. 2011B; Rigant et al. 2008). APPA is beneficial in canine OA (Glasson and Larkins 2012; Larkins and King 2017A; Larkins and King 2017B) and may have potential as a therapeutic in human inflammatory conditions. However, its mechanisms of action and possible effects on the immune system must be established before it can be considered as a novel therapeutic for human disease. This report details, for the first time, the *in vitro* effects of APPA on several

key elements of host defence and other relevant functions of human neutrophils. A major challenge in the design of new anti-inflammatory agents is to balance efficacy with safety, particularly ensuring that host defence to infection is protected.

In addition to their role in recognition, uptake and killing of pathogens, human neutrophils can express a variety of immuno-regulatory molecules such as chemokines, cytokines, growth factors and angiogenic factors (Wright et al. 2014; Jaillon et al. 2013; Cassatella 1995). These molecules regulate the function of other immune- and tissue-cells, and their inappropriate release by neutrophils also plays a role in inflammatory diseases by prolonging or sustaining inflammatory responses (Cassatella 1995). Many neutrophil functions are regulated by rapid activation of kinase cascades that control enzyme activity and/or result in changes in the affinities/surface expression levels of receptors that control opsono-phagocytosis (Cross et al. 2006; Fossati et al. 2002). These functions do not generally require changes in gene expression. However, other neutrophil functions, such as expression of certain chemokines/cytokines require activation of transcription factors, and perhaps chromatin remodelling, to control *de novo* gene expression (Wright et al. 2013).

We show here that APPA has little or no effect on neutrophil functions such as receptor expression, uptake and killing of osponised bacteria or chemotaxis. Moreover, APPA did not interfere with cytokine-mediated regulation of these functions under the experimental conditions employed in this study. Some inhibitory effects of high concentrations of APPA were noted (≥500µg/mL), but such concentrations are unlikely to be reached therapeutically (unpublished data, Professor Ian Clark, University of East Anglia). At the lowest concentration used here (10µg/mL), APPA decreased ROS levels following neutrophil activation by fMLP or

PMA. The assay used to detect these oxidants, namely luminol-enhanced chemiluminescence, requires the combined activities of the NADPH oxidase and myeloperoxidase (Edwards 1987), and can be modified experimentally to either measure the production of oxidants or the scavenging effects of anti-oxidants. We show here that the effects of APPA on ROS production are largely via its ability to scavenge oxidants, rather than by preventing their generation. In spite of the fact that the NADPH oxidase is required for the efficient killing of a large spectrum of micro-organisms (Elson et al. 2006; Zicha et al. 1997), some patients with autosomal recessive chronic granulomatous disease have decreased (but not absent) NADPH oxidase activity, and yet do not always have recurrent infections (Liese et al. 1996). Our experiments described here would support this observation: 10μg/mL APPA significantly scavenged ROS but did not impair killing of *S. aureus*. An alternative explanation is that APPA did not access the phagolysosome at concentrations sufficient to impair killing. It is also important to note APPA is a scavenger of ROS, rather than an inhibitor of the NADPH oxidase. Therefore, the ion-pumping activities of the NADPH oxidase, necessary for generating optimal protease activity within the phagolysosome (Reeves et al. 2002) will be unaffected by APPA and hence microbial killing can still occur, even though ROS may have been quenched. Based on these observations, we conclude that therapeutic doses of APPA are likely to have minimal impact, if at all, on neutrophil-mediated host defence against infection.

ROS production by neutrophils may also activate signalling networks such as MAPKs and NF-κB, to regulate expression of molecules such as IL-8, IL-1β and TNFα (Ndengele et al. 2005). Other groups have shown the importance of the ROS sensitive MAPKs/NF-κB signalling pathway in the induction of IL-8 in lung epithelial cells (Boots et al. 2012). ERK, STAT3 and NF-κB are suppressed by pre-treatment with PA, indicating that the beneficial therapeutic effect

of APPA may be mediated through its antioxidant activity and inhibition of ROS-sensitive inflammatory signalling (Liu et al. 2014). Here we show that APPA interferes with TNFα-mediated activation of NF-κB and GM-CSF activation of Erk1/2.

It is noteworthy that APPA decreased TNFα-activated expression of IL-8, TNFα itself and IL-1β, although the inhibitory effect on expression of the latter did not reach statistical significance. APPA was also an effective inhibitor of IL-6, CCL3 and CCL4 expression triggered by the TLR8 agonist and chromatin re-modelling agent, R848. This agonist triggers the expression of these genes following chromatin re-modelling via endogenous expression of TNFα, and we show here that APPA (and AP and PA) were as effective as the therapeutic agent, infliximab in the inhibition of this autocrine signalling process. This indicates that APPA could have anti-inflammatory potential, in clinical scenarios in which neutrophils and TNFα-signalling play a significant role in pathology e.g. RA (Wright et al. 2014). APPA also enhanced expression of Nrf2, an anti-inflammatory regulator of anti-oxidant proteins that protects against oxidative stress (Kaspar et al. 2009). Further work is necessary to fully characterise this phenomenon and determine if APPA regulates the expression of other proteins that control cellular responses to stress in inflammatory disease.

**Conclusion**

We show that whilst APPA has no significant effects on host defence neutrophil functions such receptor expression, phagocytosis and bacterial killing, it significantly down-regulates TNFα-mediated expression of cytokines and chemokines by neutrophils. This suggests that APPA may have significant anti-inflammatory potential in diseases characterised by

dysregulation of cytokine expression or oxidative stress in inflammatory diseases such as RA, without suppressing host defence.

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**Compliance with ethical standards**

This study was approved by the University of Liverpool Committee on Research Ethics (CORE), RETH000956. The authors declare that there were no conflicts of interest.

*Authors' contributions*

Each author has contributed to the design of the study, interpretation of data, manuscript preparation, editing and has provided approval of the final version submitted for publication.

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**Figure legends**

Fig. 1 Effects of APPA on neutrophil apoptosis, chemotaxis, phagocytosis/killing and receptor expression. In (A) neutrophils ($10^6$/mL) from healthy controls were incubated for 20h in the absence (UT) or presence of APPA (10-1000μg/mL) in the absence (control ■) or presence of cytokines known to regulate neutrophil apoptosis. Following 10min pre-incubation with APPA, the following additions were made: GM-CSF (5ng/mL, ▒) or TNFα (10ng/mL, □) and incubation was continued for a further 20h (n=7). In B, neutrophils ($10^6$) from healthy controls were incubated in the absence (UT) or presence of APPA (10-1000 μg/mL) for 10min, then migration towards fMLP ($10^{-8}$M) or IL-8 (100ng/mL) was measured after a 90min incubation period. Untreated neutrophils migrating towards fMLP ($10^{-8}$M) and IL-8 (100ng/mL) are shown as positive controls (** $p<0.01$, * $p<0.05$). Values shown are means (± SEM, n=4)**.** In C and D, neutrophils were pre-incubated for 10 min with the indicated concentration of APPA (or DMSO vehicle control). In C they were then incubated with a 10:1 ratio of PI-stained, heat-killed serum-opsonised *S. aureus* and phagocytosis was determined by flow cytometery. Values shown are mean MFI values (normalised to untreated control values of 100%), ± SD (n=3). In D, neutrophils subsequently incubated with a 10:1 ratio of live, serum-opsonised *S.aureus* and after 1h incubation, bacterial viability was determined by plate counting. Values shown are mean values ± SD (n=3).
In E, neutrophils were isolated from healthy controls and expression of cell surface receptors was measured on freshly-isolated cells by flow cytometry. These levels of expression were compared with those on neutrophils pre-incubated with APPA (100μg/mL) and stimulated for 1h with either GM-CSF (5ng/mL) or TNFα (10ng/mL), as follows: ■ No additions; ▒ TNFα only; ▒ GM-CSF only; ▒ APPA only; □ TNFα + APPA; □ GM-CSF + APPA. Levels of CD11b, CD18, CD16, CD32, CD64 and CXCR1 (IL-8R) were measured. Inset shows effects of APPA with and without GM-CSF or TNFα on CD62L expression levels. There was no significant difference in surface marker expression following treatment with APPA. Values shown are means ± SD (n = 3).

Fig. 2 APPA decreases ROS production by activated neutrophils. Neutrophils ($5x10^6$) from healthy controls were incubated in the absence (UT) or presence of APPA (10-1000µg/mL) for 10 min perior to mesurements of luminol-enhanced chemiluminescence. In A APPA-treated neutrophils were then primed for 30min with 5ng/mL GM-CSF before stimulating with fMLP (1µM), n=3, ** $p<0.01$, while in B APPA-treated neutrophils were stimulated using PMA (100ng/mL), n=3, * $p<0.01$. C shows representative chemiluminescence traces of PMA-stimulated respiratory burst activity in the absence and presence of increasing concentrations of APPA: (●) untreated controls, while ○, ▲, △, ■, □, show APPA concentrations of 10, 10, 200, 500 and 1000µg/mL, respectively. In D PMA-induced respiratory burst activity was stimulated (●) and after 5min incubation, APPA (at 10µg/mL, ○ and 100µg/mL: ▲) was added as indicated by the arrow. In E PMA was used to stimulate ROS production by neutrophils. As indicated by the arrow, the following additions were injected into the cell suspension: ●, no additions; ○, catalase (2U/mL); ▲, superoxide dismutase (40µg/mL); △ sodium azide (1mM); ■ APPA (100µg/mL). In F APPA (10-1000µg/mL) or DMSO (as solvent control) were added to a cell-free luminol system utilizing hydrogen peroxide, as follows: ●, no additions; ○ , DMSO; ▲, 10µg/mL APPA; △, 100µg/mL APPA; ■ 200µg/mL APPA; □, 500µg/mL APPA. representative result of 3 separate experiments. In G, Neutrophils were stimulated with with PMA (●) and after 5min incubation APPA (100µg/mL, ▲), AP (22µg/mL, △ ) or PA (78µg/mL, ○) added, as indicated by the arrow. H shows replicate data of total chemiluminescence from G, ** p value < 0.01, n = 11.

Fig. 3 APPA decreases neutrophil degranulation. In (A) and (B), neutrophils ($5x10^6$) from healthy controls were incubated in the absence (UT) or presence of APPA (100µg/mL) for 10min. APPA-treated neutrophils were then primed for 30min with GM-CSF before stimulating degranulation with fMLP (1µM) plus cytochalasin B (5µg/mL). In (A) neutrophils were analysed for expression of CD63, a marker of degranulation using flow cytometry (* $p<0.05$, n = 7). In B, supernatants from above were collected, proteins separated using SDS-PAGE before western blotting and probed for expression of MMP9, MPO, elastase and lacioferrin, as indicated.

Fig. 4 APPA decreases formation of neutrophil extracellular traps (NETs). Neutrophils were treated with PMA for 4h in the absence and presence of 100µg/mL APPA. NET formation was measured by DNA release in A (n=4, *p=0.04) and in B by microscopy utilizing dual DAPI and neutrophil elastase staining. In C, DNA released into NETs was determined after incubation with PMA in the p[resence of 100µg/mL APPA, 22µg/mL AP and 78µg/mL PA (n=6, **p <0.05).

Fig. 5 Effects of APPA on activation of cytokine-regulated cell signalling. Neutrophils ($5 \times 10^6$) were incubated in the absence (UT) or presence of APPA (100µg/mL) for 10min. APPA-treated neutrophils were then stimulated for 15min with either IL-6, GM-CSF or TNFα at the concentrations described in Methods. Western blotting was used to detect activated (phosphorylated) forms of STAT3, NF-κB, IκBα and Erk1/2. A shows typical blot obtained from 3 separate experiments, while B-D show combined densitometric data (n=3), for IL-6 stimulated STAT3 activation B, GM-CSF-stimulated Erk1/2 activation C and TNF stimulated NF-κB activation, D (* , p=0.03, 0.03 and 0.008, respectively) after normalisation to GAPDH protein levels.

Fig. 6 APPA down-regulates TNFα-stimulated gene expression but up-regulates expression of NRF2. Neutrophils ($10^7$) from healthy controls were incubated in the absence (▮) or presence (▯) of APPA (100μg/mL) for 10min. APPA-treated neutrophils were then stimulated with GM-CSF, IFNγ or TNFα for 1h. qPCR was used to quantify transcript levels of IL-1β (A), IL-8 (B), NRF2 (C) and TNFα (D). Values shown are mean (± SEM), n=6, * p=0.012.

Fig. 7 APPA, AP and PA are as effective as infliximab in down-regulating chemokine and cytokine expression. Neutrophils were incubated with 5μM R848 for 7h in the absence (R848) or presence of 200μg/mL infliximab (IFX) or 100μg/mL APPA. Expression levels of mRNA for CCL3 (A), CCL4 (B) and IL-6 (C) (normalised to GAPDH mRNA levels) were then measured by qPCR. * $p < 0.05$, ** $p = 0.01$ ($n = 5$). In D and E, neutrophils were incubated in the presence of R848 (5μM), APPA (100μg/mL), Infliximab (IFX, 200μg/mL), AP (22μg/mL) and PA (78μg/mL). Levels of mRNA for IL-6 (in D) and CCL3 (in E) were measured by qPCR and normalised to GAPDH mRNA levels. Values shown are means ± SEM ($n = 5$). * $p < 0.05$. ** $p < 0.01$.

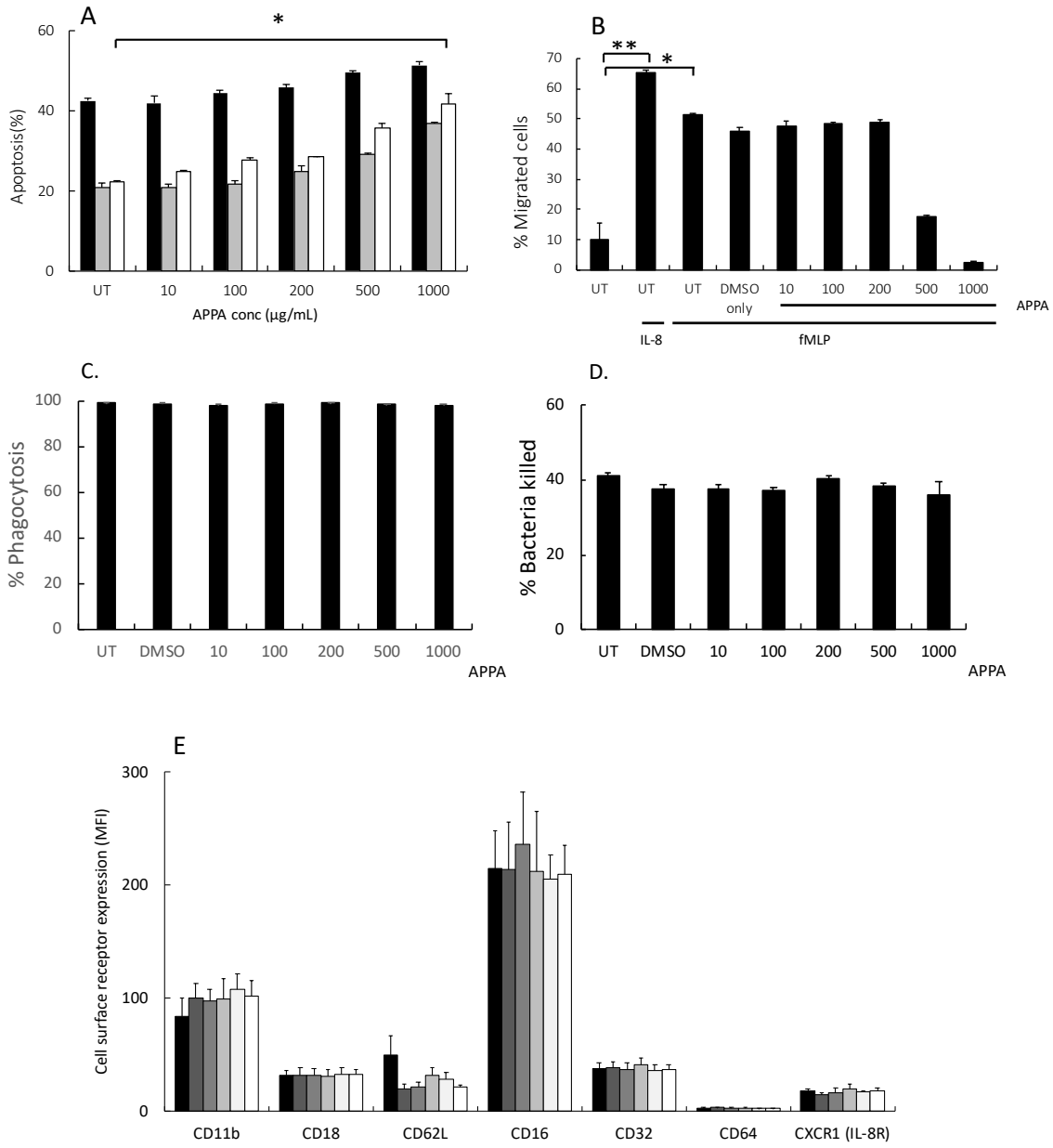
A
*
Apoptosis(%)
60
40
20
0
UT
10
100
200
500
1000
APPA conc (µg/mL)
B
**
*
% Migrated cells
70
60
50
40
30
20
10
0
UT
UT
UT
DMSO
only
10
100
200
500
1000
APPA
IL-8
fMLP
C.
% Phagocytosis
100
80
60
40
20
0
UT
DMSO
10
100
200
500
1000
APPA
D.
% Bacteria killed
60
40
20
0
UT
DMSO
10
100
200
500
1000
APPA
E
Cell surface receptor expression (MFI)
300
200
100
0
CD11b
CD18
CD62L
CD16
CD32
CD64
CXCR1 (IL-8R)

Figure 1

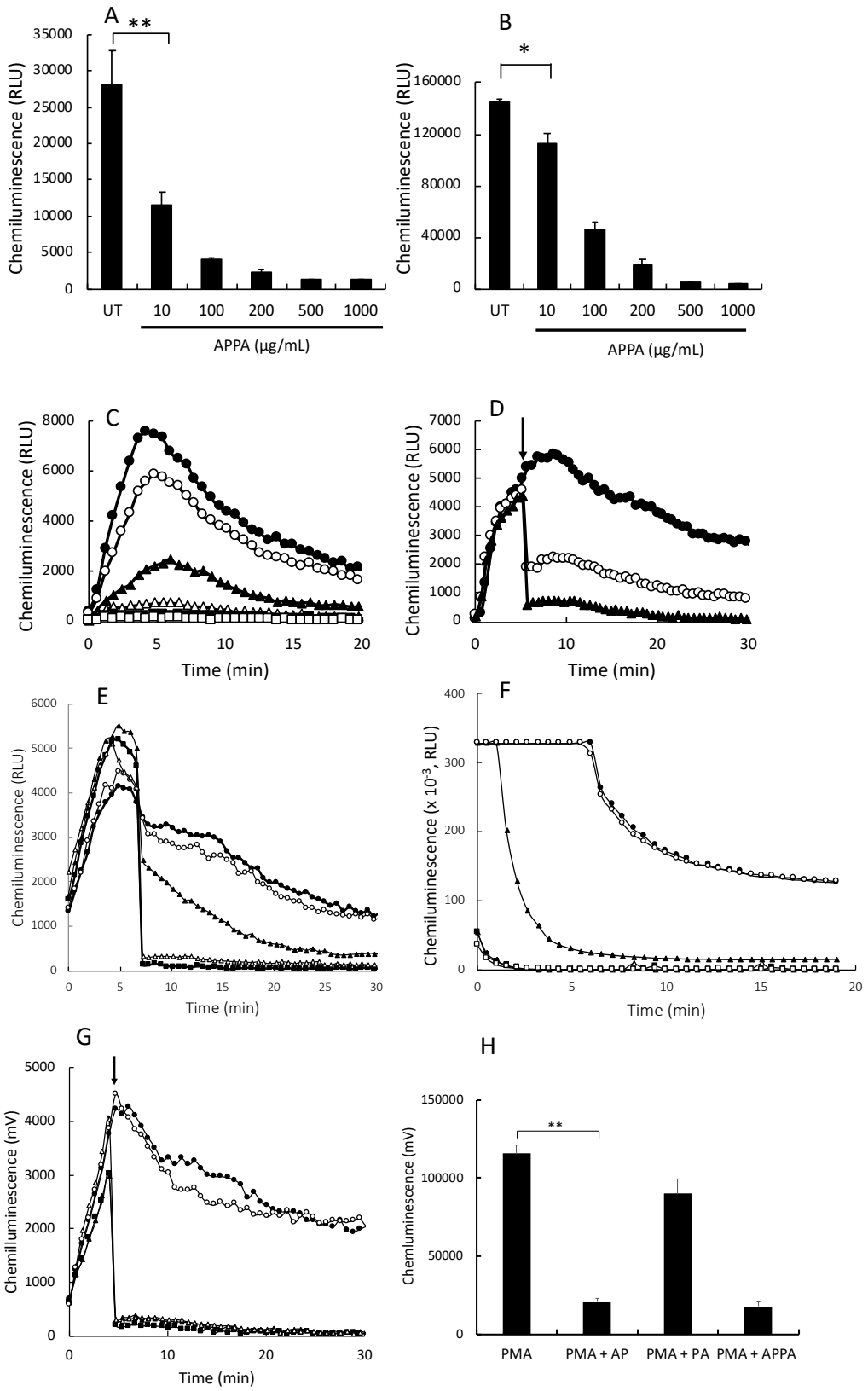
A
**
Chemiluminescence (RLU)
35000
30000
25000
20000
15000
10000
5000
0
UT
10
100
200
500
1000
APPA (μg/mL)
B
*
Chemiluminescence (RLU)
160000
120000
80000
40000
0
UT
10
100
200
500
1000
APPA (μg/mL)
C
Chemiluminescence (RLU)
8000
6000
4000
2000
0
0
5
10
15
20
Time (min)
D
Chemiluminescence (RLU)
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
Time (min)
E
Chemiluminescence (RLU)
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
Time (min)
F
Chemiluminescence (x 10-3, RLU)
400
300
200
100
0
0
5
10
15
20
Time (min)
G
Chemilluminescence (mV)
5000
4000
3000
2000
1000
0
0
10
20
30
Time (min)
H
Chemluminescence (mV)
150000
100000
50000
0
**
PMA
PMA + AP
PMA + PA
PMA + APPA

Figure 2

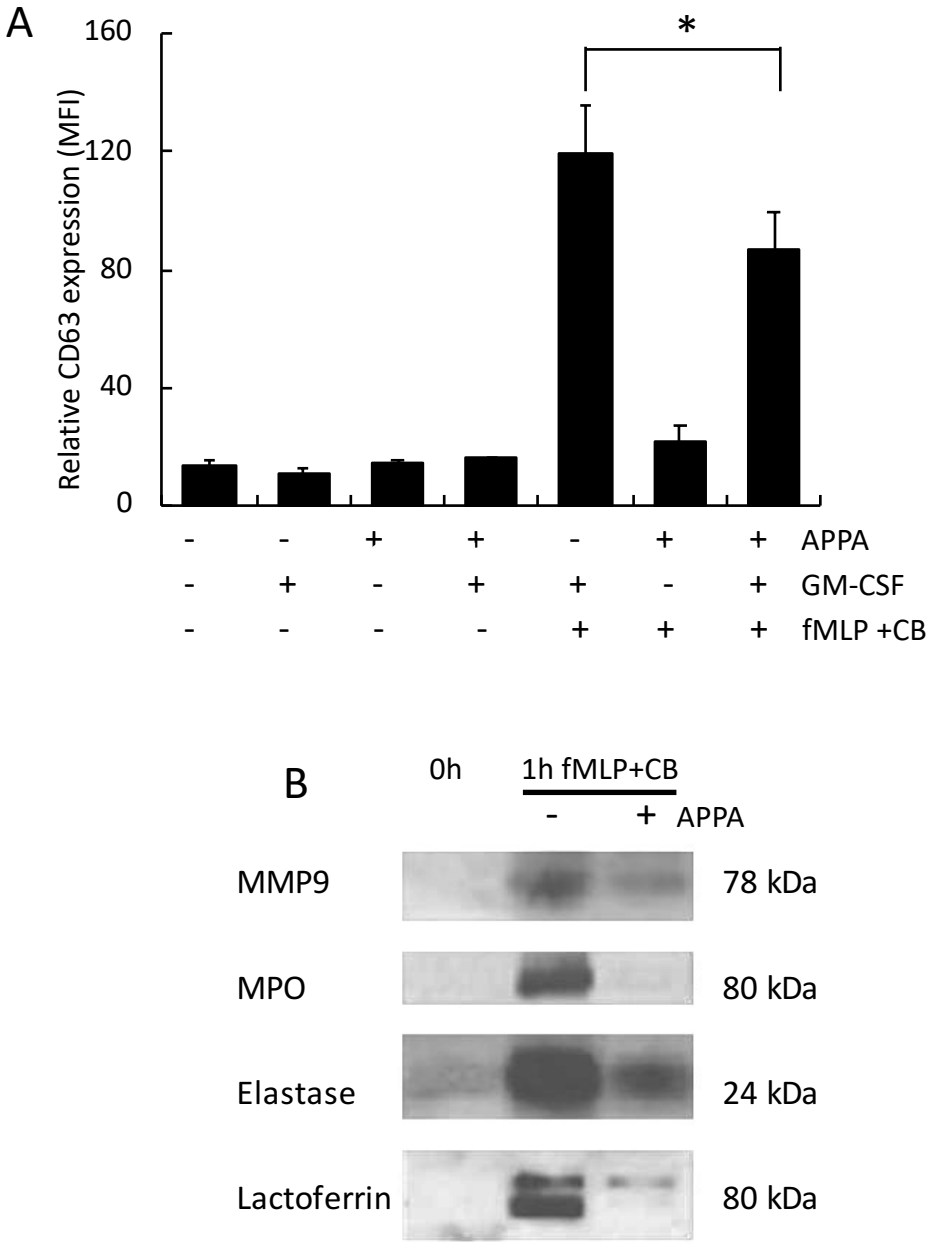
A
160
120
80
40
0
Relative CD63 expression (MFI)
*
- - + + - + + APPA
- + - + + - + GM-CSF
- - - - + + + fMLP +CB
B
0h
1h fMLP+CB
- + APPA
MMP9
78 kDa
MPO
80 kDa
Elastase
24 kDa
Lactoferrin
80 kDa

Figure 3

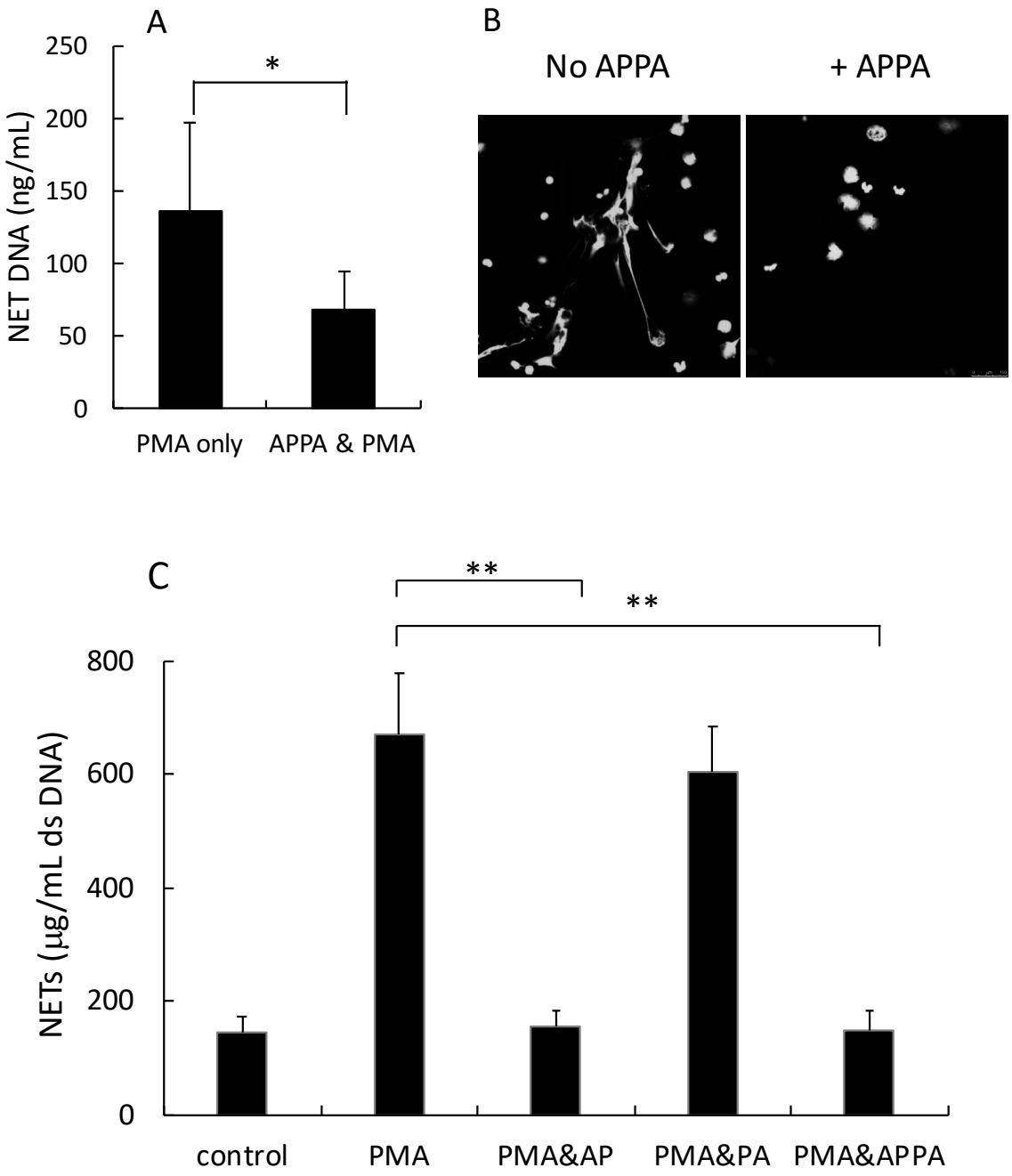
A
250
200
150
100
50
0
NET DNA (ng/mL)
*
PMA only
APPA & PMA
B
No APPA
+ APPA
C
**
**
800
600
400
200
0
NETs (μg/mL ds DNA)
control
PMA
PMA&AP
PMA&PA
PMA&APPA

Figure 4

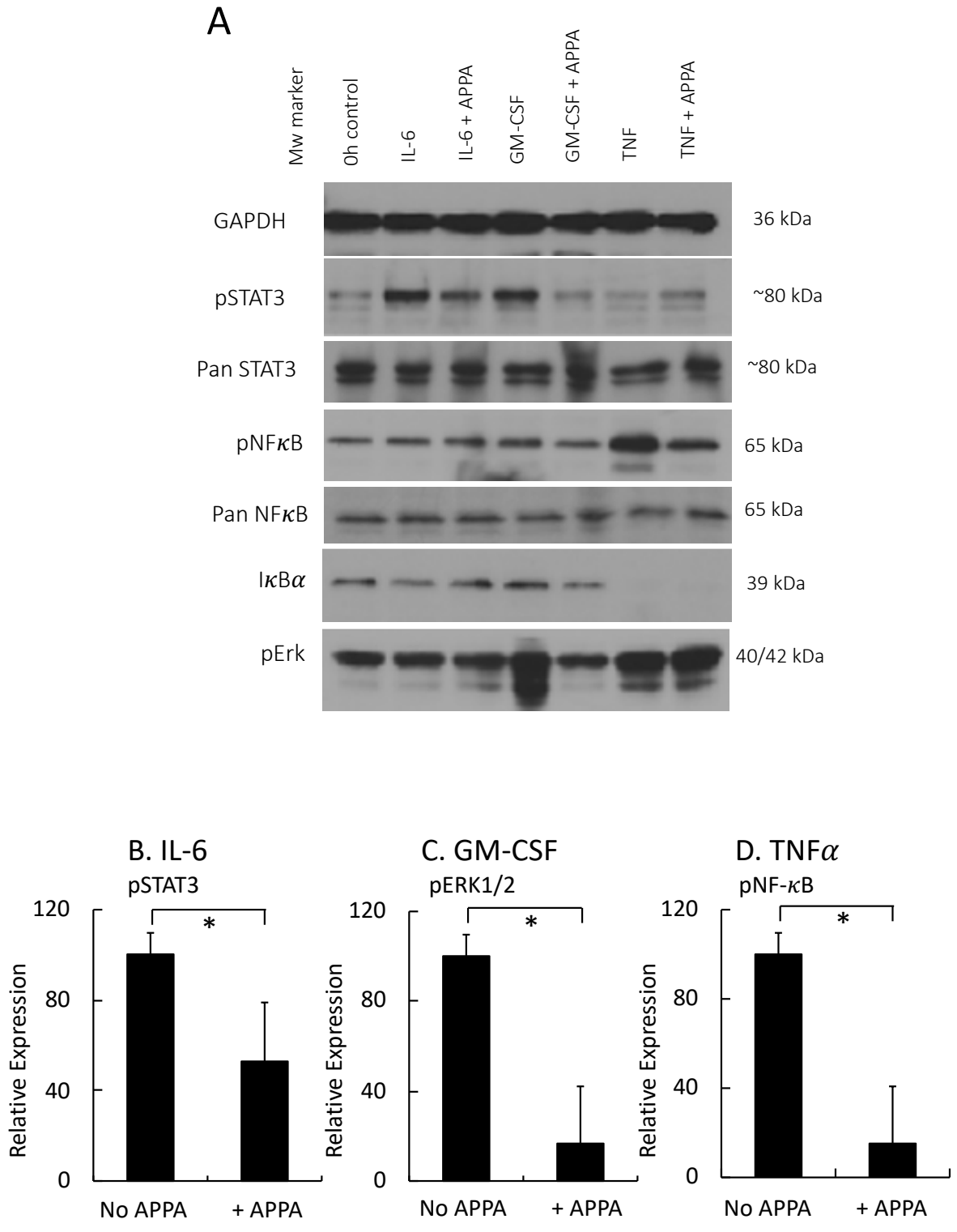
A
Mw marker
0h control
IL-6
IL-6 + APPA
GM-CSF
GM-CSF + APPA
TNF
TNF + APPA
GAPDH
36 kDa
pSTAT3
~80 kDa
Pan STAT3
~80 kDa
pNFκB
65 kDa
Pan NFκB
65 kDa
IκBα
39 kDa
pErk
40/42 kDa
B. IL-6
pSTAT3
C. GM-CSF
pERK1/2
D. TNFα
pNF-κB
Relative Expression
120
80
40
0
*
No APPA
+ APPA

Figure 5

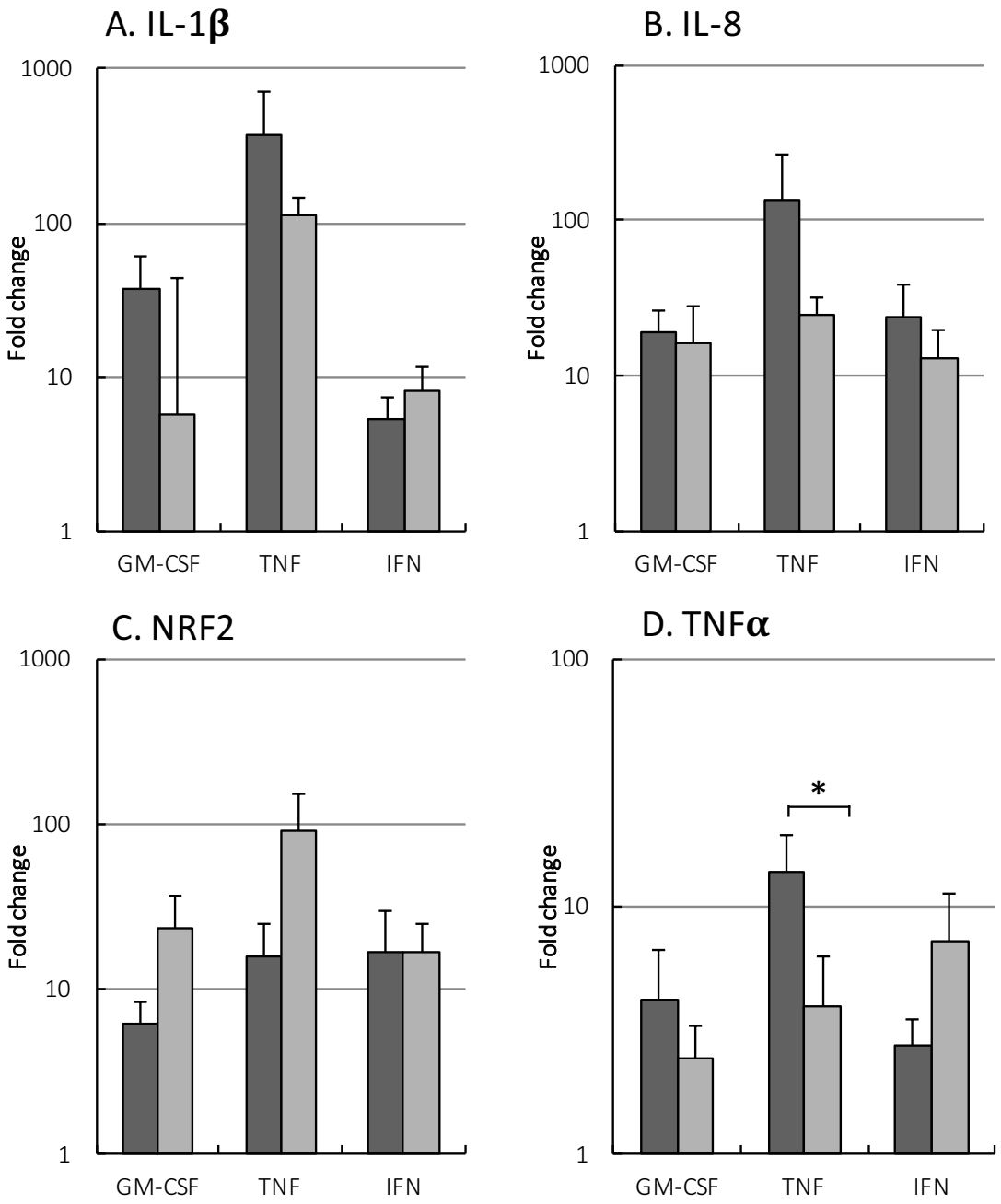
A. IL-1β
B. IL-8
C. NRF2
D. TNFα
Fold change
1000
100
10
1
GM-CSF
TNF
IFN
*

Figure 6

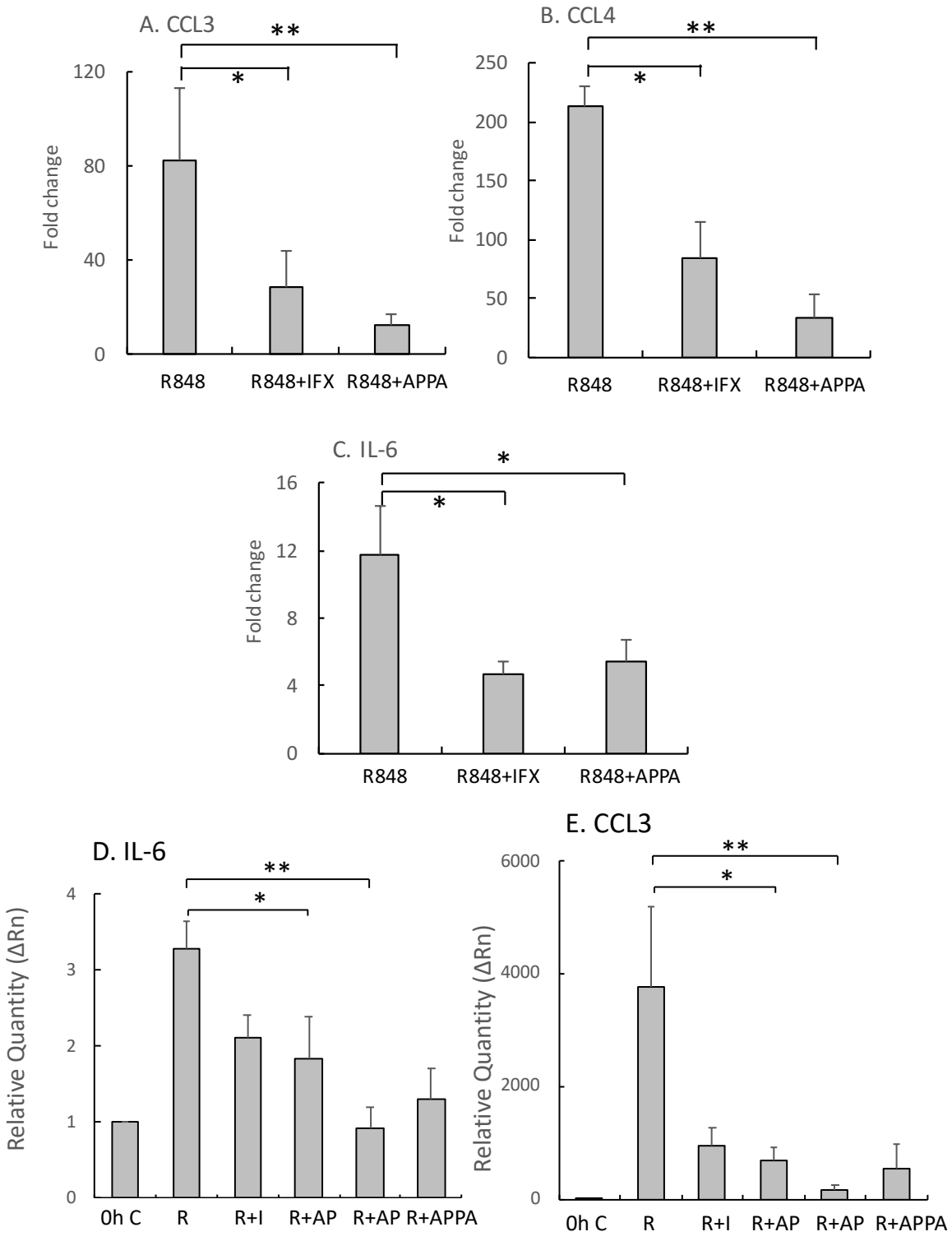
A. CCL3
Fold change
120
80
40
0
**
*
R848
R848+IFX
R848+APPA
B. CCL4
Fold change
250
200
150
100
50
0
**
*
R848
R848+IFX
R848+APPA
C. IL-6
Fold change
16
12
8
4
0
*
*
R848
R848+IFX
R848+APPA
D. IL-6
Relative Quantity (ΔRn)
4
3
2
1
0
**
*
0h C
R
R+I
R+AP
R+AP
R+APPA
E. CCL3
Relative Quantity (ΔRn)
6000
4000
2000
0
**
*
0h C
R
R+I
R+AP
R+AP
R+APPA

Figure 7