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Chondroprotective and Anti-Inflammatory role of Melanocortin peptides in TNF-α activated human C-20/A4 chondrocytes.

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SUMMARY

Background and purpose: Melanocortin receptors, MC_1 and MC_3, mediate the anti-inflammatory effects of melanocortin peptides. Targeting these receptors could therefore lead to development of novel anti-inflammatory therapeutics. We investigated the expression of MC_1 and MC_3 on chondrocytes and the role of alpha-melanocyte-stimulating hormone (α-MSH) and the selective MC_3 agonist, [$DTRP^8$]-γ-MSH, in modulating the production of inflammatory cytokines, tissue-destructive proteins and induction of apoptotic pathway(s) in the human chondrocytic cell-line, C-20/A4.

Experimental Approach: The effects of α-MSH, [$DTRP^8$]-γ-MSH alone, or in the presence of the $MC_{3/4}$ receptor antagonist, SHU9119, on TNF-α induced release of pro-inflammatory cytokines, matrix metalloproteinases (MMPs), apoptotic pathway(s) and cell death in C-20/A4 chondrocytes were investigated, as well as their effect at inducing the release of the anti-inflammatory cytokine IL-10.

Key Results: C-20/A4 chondrocytes expressed functionally active $MC_{1,3}$ receptors. α-MSH and [$DTRP^8$]-γ-MSH treatment for 30 min prior to TNF-α stimulation resulted in a time and bell-shaped concentration dependent decrease in pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) release and increased release of the chondroproctective/anti-inflammatory cytokine, IL-10, while decreasing expression of matrix metallo-proteinases *MMP1, MMP3, MMP13* genes. α-MSH and [$DTRP^8$]-γ-MSH treatment also inhibited TNF-α-induced caspases 3/7 activation and chondrocyte death. The effects of [$DTRP^8$]-γ-MSH, but not α-MSH, were abrogated by the $MC_{3/4}$ receptor antagonist, SHU9119.

Conclusion and implications: Activation of MC_1/MC_3 receptors in C-20/A4 chondrocytes down-regulates the production of pro-inflammatory cytokines and cartilage destructive proteinases, inhibits initiation of apoptotic pathways and promotes release of chondroprotective/anti-inflammatory cytokines. Developing small molecule agonists to MC_1/

MC_3 receptors could be a viable approach for developing anti-inflammatory/chondroprotective therapies in rheumatoid and osteoarthritis.

Abbreviations: ACTH, adrenocorticotropin; DMEM, Dulbecco's Modified Eagle's Medium; ECM, extracellular matrix; FCS, Foetal Calf Serum; GPCR, G-protein coupled receptor; IBMX, isobutylmethylxantine; IL, interleukin; MC, melanocortin receptor; MMP, matrix metalloproteinases; OA, osteoarthritis; PGE_2, prostaglandin E_2; RA, rheumatoid arthritis; RT-PCR, reverse transcriptase polymerase chain reaction; α-MSH, alpha-melanocyte-stimulating hormone.

Key words: anti-inflammatory; apoptosis; caspases, chemokines, chondrocyte, chondroprotective, cytokines; GPCR; melanocortins; matrix metalloproteinases

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory systemic disorder (Getting *et al.,* 2009) whilst osteroarthritis (OA) is typically associated with obesity, age and abnormal joint loading (Recnik *et al.,* 2009) and was historically considered to be primarily a non-inflammatory arthropathy. However recent studies in both OA and RA patients and in animal models show a marked elevation in inflammation and pro-inflammatory cytokine levels, including TNF-α, IL-1β IL-6 and IL-8, in the cartilage which subsequently leads to progressive joint destruction (Fernandes *et al.,* 2002; Rai *et al.,* 2008, Koenders *et al.,* 2011). Chondrocyte activation can lead to increased expression and secretion of cartilage-degrading matrix metalloproteinases (MMP) (Shlopov *et al.,* 2000)), such as MMP1 and MMP13, which are highly elevated in OA cartilage (Reboul *et al.,* 1996) leading to degradation of the cartilage extracellular matrix (ECM). In addition to an increase in pro-inflammatory mediators, TNF-α also down-regulates the production of pro-resolving, anti-inflammatory proteins such as IL-10 (Iannone *et al.,* 2001) which may also contribute to an increased proportion of apoptotic cells in OA cartilage compared to healthy cartilage, resulting in further cartilage damage (Aigner *et al.,* 2001, John *et al.,* 2007).

Melanocortin peptides, including α-melanocyte stimulating hormone (α-MSH), are endogenous anti-inflammatory peptides (Gonzalez-Rey *et al.,* 2007) that exert their effects via activation of melanocortin receptors (MC) of which five have so far been identified (MC_{1-5}) (Getting *et al.,* 2009). Over the last three decades, melanocortin peptides and their receptors (MC_1 and MC_3) have been shown to be particularly important as modulators of innate immunity and therefore synthetic peptides such as [$DTRP^8$]-γ-MSH have been designed to evaluate these receptors' potential as targets for therapeutic intervention (Getting *et al.,* 2009, Holloway *et al.,* 2011). For example, melanocortin peptides display efficacy in pre-clinical models of arthritis (Patel *et al.,* 2010) and models of gout (Getting *et al.,* 2002) where

monosodium urate crystal–induced neutrophil migration and pro-inflammatory cytokines and chemokine release are significantly reduced. MC_1 and MC_3 receptors have been proposed to mediate these anti-inflammatory effects (Getting *et al.,* 2009, Holloway *et al.,* 2011) with the selective MC_1 agonist, BMS-470539, inhibiting leukocyte migration in the inflamed vasculature of mice (Leoni *et al.,* 2010), while pharmacological approaches have also highlighted a role for the MC_3 receptor as a possible anti-inflammatory target in models of gouty peritonitis (Getting *et al.,* 2006a), ischaemia-reperfusion injury (Leoni *et al.,* 2008) and RA (Patel *et al.,* 2010). In addition, deletion of the MC_3 gene in mice exacerbates the host inflammatory response in a model of ischaemia-reperfusion injury (Leoni *et al.,* 2008), whilst an increase of inflammatory arthritis is also seen in MC_3 null mice compared to wild type mice (Patel *et al.,* 2010), again confirming the importance of MC_3 receptor as a therapeutic target.

In spite of the substantial evidence suggesting a role of melanocortin peptides in numerous inflammatory pathologies, only a very limited number of studies have evaluated the therapeutic potential of melanocortin peptides in OA. For example, α-MSH down-regulates TNF-α induced expression of MMPs, by decreasing p38 MAPK phosphorylation and subsequent activation of NF-κB, in a human chondrosarcoma (HTB-94) cell line (Yoon *et al.,* 2008), whilst recent studies, using human articular chondrocytes, showed an α-MSH dependent decreases in IL-1β and TNF-α mRNA levels, mediated via the MC_1 receptor (Grassel *et al.,* 2009), whilst in rodent chondrocytes, adrenocorticotrophin (ACTH) treatment mediated via the MC_1 receptor promotes the development of the chondrocyte phenotype (Evans *et al.,* 2004).

In this present study, we have demonstrated the expression and functionality of MC_1 and MC_3 receptors on human C-20/A4 chondrocytes and the role of melanocortins in modulating

TNF-α induced pro-inflammatory cytokine production, MMPs release, caspase-driven chondrocyte apoptosis and chondro-protection.

MATERIALS & METHODS

The C-20/A4 human chondrocyte cell-line was a kind gift of Dr M.B. Goldring, (Hospital for Special Surgery, New York, USA) (Goldring *et al.,* 1994) and is derived from juvenile costal chondrocytes by immortalization via transfection with origin-defective simian virus 40 large T antigen (SV40-Tag) (Finger *et al.,* 2004). Briefly, C-20/A4 cells were cultured in monolayers in complete media (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated Foetal Calf Serum (FCS), Penicillin (100.0 U/ml) and Streptomycin (100.0 μg/ml)), in a humidified atmosphere supplied with 5% CO_2 and 95% air, until 80% confluent, as previously described (Goldring *et al.,* 1994). Prior to experimentation, FCS concentration was reduced to 1% (v/v) for 24 h and all subsequent experiments were performed under these conditions.

In vitro chondrocyte stimulation.

C-20/A4 chondrocytes were treated for 30 mins with either PBS (control), α-MSH (1.0 – 30.0 μg/ml; Sigma-Aldrich, Poole, Dorset, UK) or the selective MC_3 receptor agonist, [$DTRP^8$]-γ-MSH (1.0 – 30.0 μg/ml) (synthesised by Dr. P. Grieco, University of Naples, Italy, (Grieco *et al.,* 2000, Getting *et al.,* 2006b)) and stimulated with human recombinant TNF-α (0.0 – 80.0 pg/ml) (Kaneva *et al.,* 2010) and cell-free supernatants collected and stored at –20°C. In some experiments (as indicated), cells were also pre-treated for 1 h with the $MC_{3/4}$ receptor antagonist, SHU9119 (10.0 μg/ml) (Getting *et al.,* 2006a), prior to addition of α-MSH or [$DTRP^8$]-γ-MSH.

cAMP accumulation in C-20/A4 chondrocytes

To assess receptor functionality, a cAMP accumulation assay (RPN225, GE Healthcare, Amersham, UK) was used as previously described (Getting *et al.,* 2006a, 2008). Briefly, C-20/A4 chondrocytes (2.0 x 10^5 cells/well) were seeded in 96-well-plates in 100 µl complete media and incubated for 2 h to allow cell adhesion. Cells were then incubated for 30 mins in serum-free medium in a 37°C humidified atmosphere of 5% CO_2 and 95% air, in the presence of the direct adenylate activator, forskolin (3.0 µM, positive control), α-MSH (1.0 – 30.0 µg/ml) or [$DTRP^8$]-γ-MSH (1.0 – 30.0 µg/ml) alone or in the presence of the $MC_{3/4}$ antagonist, SHU9119 (10.0 µg/ml), all in the presence of 1.0 mM isobutylmethylxantine (IBMX). A negative control (cells incubated alone) was incubated under identical conditions. Cell supernatants were removed and adhered cells lysed as per the manufacturer's instructions and intracellular cAMP concentration determined (Getting *et al.,* 2006a, 2008).

Molecular Analysis

RT-PCR analysis

C-20/A4 chondrocyte RNA was extracted and isolated using the NucleoSpin® RNA II Kit (Macherey-Nagel, Duren, Germany) and RNA concentrations were determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer ($A_{260nm/280nm}$: 1.9-2.1). cDNA was synthesized by taking 1.0 µg of DNase-treated total RNA, PolyT, Random Primers and RNase inhibitor in a total volume of 20.0 µl using the Improm II Reverse Transcription System (Promega, UK). 2.0 µl aliquots of the cDNA (in a final volume of 25 µl) were used as a template for PCR amplification using specific primer pairs (see below) for *MMP1*, *MMP3*, *MMP13* using the GoTaq® Green Mastermix system (Promega, UK). The oligonucleotide primer sequences were as follows:

MMP1_FWD: 5′-CGACTCTAGAAACAGAAGAGCAAGA-3' and

MMP1_REV: 5′-AAGGTTAGCTTACTGTCACACACGCTT-3’;

MMP3_FWD: 5′-GGAAATCAGTTCTGGGCTATACGAGG-3’ and

MMP3_REV: 5′-CCAACTGCGAAGATCCACTGAAGAAG-3’;

MMP13_FWD: 5′-GTGGTGTGGGAAGTATCATCA-3’ and

MMP13_REV: 5′-GCATCTGGAGTAACCGTATTG-3’.

The PCR parameters were as follows: initial denaturing for 5 min at 95°C, followed by 25 cycles of denaturation (95°C for 60 s), annealing (55-59°C- depending on the primers used for 90 s) and extension (72°C for 90 s), with a single final extension of 72°C for 10 min. Oligonucleotide primers for human β-actin (FWD: 5′-GTCCCGGCATGTGCAA-3’; REV: 5′- AGGATGTTCATGAGGTAGT-3’) were used as a control. Amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide. Densitometry analysis was performed using Image J software (NIH, Bethesda, Maryland, USA).

Western blotting

C-20/A4 chondrocyte expression of MC_1 and MC_3 receptors was determined as previously described (Getting *et al.,* 2008). Following electrophoresis in a 10% SDS-polyacrylamide gel, proteins were transferred onto nylon membrane by electroblotting, blocked overnight in 5% non-fat milk solution in Tris-HCl buffered saline, pH 7.5 (TBS) containing 0.1% (v/v) Tween-20 and then incubated with either specific anti-MC_1 or anti-MC_3 (1:2000 dilution M9193 and M4937, Sigma-Aldrich, Dorset, UK) rabbit antibodies in blocking solution. Blots were washed in TBS prior to the addition of a secondary goat anti-rabbit HRP-conjugated antibody (1:2000 dilution) and specific antibody binding was detected by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA). Following detection, bound antibodies were removed by incubating the membranes in 100 mM Glycine-HCl, pH 2.5 for 30 mins and the blot re-probed to detect of α-tubulin as described previously (Getting *et al.,*

2008). Densitometry analysis was performed using Image J software (NIH, Bethesda, Maryland, USA).

Biochemical and cell viability analyses

MTT cytotoxicity assay

Cell viability was determined using an MTT assay (Lam *et al.,* 2006). Briefly, C-20/A4 cells plated at 2.0 x 10^5 cells/well in 96-well plates (in 200µl of complete medium) and allowed to adhere, prior to treatment as described above (see *In vitro* chondrocyte stimulation section). Following stimulation, cell culture medium was aspirated and a 1:10 dilution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium) solution added for 2 h at 37°C in a humidified chamber with 5% CO_2 and 95% air at 37°C. Following incubation, the supernatant was replaced with DMSO (100 µl/well), incubated for 15 min and the absorbance determined at 570 nm.

Cytokine quantification by ELISA

Human IL-1β, IL6, IL-8 and IL-10 concentrations in cell-free supernatants were determined using commercially available ELISA kits (R&D Systems Europe Ltd, Oxford, UK). These ELISAs showed negligible (<1%) cross-reactivity with other cytokines and chemokines (data furnished by manufacturer).

Caspase-Glo 3/7 apoptosis assay

Apoptosis was determined by measuring Caspase 3 and 7 activity following cell stimulation as detailed above (see *In vitro* chondrocyte stimulation section). Briefly, C-20/A4 chondrocytes were plated at 2.0x10^5 cells/well in 96-well plates and 100µl of Caspase-Glo 3/7 Reagent (Promega, Southampton, UK) was added to each well and incubated at room

temperature for 1 h after which luminescence was measured as per the manufacturer's instructions.

Statistics

All data are reported as mean ± SEM of *n* observations, using at least 3 experiments with 4 determinations per group. Statistical evaluation was performed using analysis of variance ANOVA (Prism GraphPad Software) incorporating either Dunnets' or Bonferroni's Multiple Comparison tests to allow for *post-hoc* analyses with a probability *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ taken as significant. Receptor nomenclature for melanocortin receptors was used in accordance with the "Guide to receptors and Channels (GRAC)" (Alexander *et al.,* 2009)

RESULTS

TNF-α up-regulates pro-inflammatory cytokine release from C-20/A4 chondrocytes

C-20/A4 chondrocytes were stimulated with different concentrations of TNF-α (0.0 – 80.0 pg/ml) to evaluate its ability to promote the release of IL-1β, IL-6 and IL-8 over a time course. TNF-α stimulation at all concentrations led to a significant release of IL-1β, IL-6 and IL-8 over basal levels at all time points evaluated (Figure 1A-C). IL-1β synthesis increased in a concentration dependent manner with a maximal release at 6 h, with a plateau observed between 60.0 pg/ml (27.4 ± 5.7 pg/ml) and 80.0 pg/ml TNF-α (28.6 ± 3.3 pg/ml), while IL-6 and IL-8 increased in a concentration-dependent manner peaking at 24 h, with 60.0 pg/ml TNF-α causing maximal stimulation (154.3 ± 10.3 pg/ml IL-6 and 482.8 ± 21.5 pg/ml IL-8) (Figure 1A-C).

C-20/A4 chondrocytes express functionally active MC_1 and MC_3 receptors.

Western blotting showed the presence of both MC_1 and MC_3 proteins on C-20/A4 human chondrocytes with product sizes of 35 and 40 kDa being observed (Figure 2A), α-MSH and [$DTRP^8$]-γ-MSH were used to test for functional receptors on these cells by measuring cAMP production. Addition of the pan-melanocortin receptor agonist, α-MSH (1.0 – 30.0 μg/ml), and the selective MC_3 receptor agonist, [$DTRP^8$]-γ-MSH (1.0 – 30.0 μg/ml), both provoked a significant accumulation of intracellular cAMP. The direct adenylate cyclase stimulator, forskolin (FSK, 3.0 μM), caused a 9-fold increase in cAMP (2230.0 ± 74.0 fmol/well) over control (249.0 ± 10.6 fmol/well), with α-MSH treatment causing a significant increase in cAMP accumulation at all concentrations tested, with a maximal increase being observed at 10.0 μg/ml (639.0 ± 41.6 fmol/well, $p \leq 0.001$) (Figure 2B). The selective MC_3 agonist, [$DTRP^8$]-γ-MSH, also caused a marked increase in cAMP accumulation with a maximal effect observed at 3.0 μg/ml (801.0 ± 30.0 fmol/well; $p \leq 0.001$) (Figure 2C). Higher concentrations of both α-MSH and [$DTRP^8$]-γ-MSH caused a less pronounced cAMP accumulation. (Figure 2 B and C).

The selectivity of responses to α-MSH and [$DTRP^8$]-γ-MSH on cAMP production was also evaluated by co-stimulating the chondrocytes with the peptides alone or in the presence of the $MC_{3/4}$ receptor antagonist SHU9119 (10.0 μg/ml). SHU9119 treatment caused no significant inhibition of α-MSH-induced cAMP production (Figure 2B) but in contrast caused a ~84 % reduction in cAMP accumulation, as elicited by [$DTRP^8$]-γ-MSH, at all concentrations tested ($p \leq 0.01$; Figure 2C).

α-MSH and D[TRP^8]-γ-MSH inhibit TNF-α induced cytokine but not PGE_2 release from C-20/A4 chondrocytes

Since C-20/A4 chondrocytes express functionally active MC_1 and MC_3 receptors and TNF-α (60.0 pg/ml) stimulation caused significant release of the pro-inflammatory mediators IL-1β, IL-6 and IL-8, we tested the effects of α-MSH and [DTRP^8]-γ-MSH (0.1 – 30.0 μg/ml) on the release of these cytokines and PGE_2.

α-MSH and [DTRP^8]-γ-MSH inhibited IL-1β release in a bell-shaped manner, with a maximal reduction of 84.8% and 72.7%, respectively observed at 3.0 μg/ml (Figure 3A and B), whilst higher concentrations did not sustain this level of inhibition. IL-6 release was inhibited by α-MSH with 3.0 μg/ml being the most effective concentration causing a 72.1% reduction (Figure 3C), whilst [DTRP^8]-γ-MSH (1.0 and 3.0 μg/ml) caused a similar maximal degree of inhibition of approximately 60.0% (Figure 3D), with higher concentrations not sustaining this level of inhibition. IL-8 levels were reduced in a bell-shaped fashion for α-MSH, with a maximal inhibition of 60.2% observed at 3.0 μg/ml (Figure 3E), whilst [DTRP^8]-γ-MSH caused a similar reduction in IL-8 release with a maximal inhibition of 75.9% detected at 10.0 μg/ml [DTRP^8]-γ-MSH, higher concentrations of either peptides did not sustain this level of inhibition (Figure 3F). In order to further elucidate the effects of the melanocortin peptides, α-MSH and [DTRP^8]-γ-MSH, on TNF-α stimulated cytokine production, the specific $MC_{3/4}$ receptor antagonist, SHU9119, was used to selectively block the function of the MC_3 receptor. As expected, SHU9119 did not significantly block α-MSH (3.0 μg/ml) mediated inhibition of IL-1β, IL-6 and IL-8 release (Figure 4A-C), did abrogate the effect of [DTRP^8]-γ-MSH (3.0 μg/ml) (Figure 4A-C). Following identification that α-MSH and [DTRP^8]-γ-MSH inhibited cytokine release, their effect on TNF-α (60.0 pg/ml) induced PGE_2 release was determined, since PGE2 has been shown to inhibit MMP 1 and 13

expression in chondrocytes (Nishitani *et al.,* 2010). TNF-α (60.0 pg/ml), led to a significant increase in PGE_2 release, compared to control cells. However, in contrast to their effects on pro-inflammatory cytokine release, neither α-MSH or [$DTRP^8$]-γ-MSH (3.0 μg/ml) significantly altered TNF-α stimulated PGE_2 levels with only a ~20% and ~14% reduction respectively (Table 1).

α-MSH and D[TRP^8]-γ-MSH inhibit *MMP1, MMP3 and MMP13* gene expression in TNF-α activated chondrocytes

Given the role matrix metalloproteinases *MMP1, MMP3 and MMP13* play in the pathogenesis of OA (Lawyer *et al.,* 2011), we determined the effects of α-MSH and D[TRP^8]-γ-MSH on *MMP1*, *MMP3* and *MMP13* gene expression. TNF-α treatment caused significant increases in *MMP1*, *MMP3* and *MMP13* gene expression 6 h post-challenge, while pre-treatment with α-MSH (3.0 μg/ml) led to a significant 5-fold reduction in mRNA levels for *MMP1* as well as a1.9-fold reduction in *MMP3* and a 3-fold reduction in *MMP13* gene expression compared to control (Figure 5A-D). Similarly, [$DTRP^8$]-γ-MSH (3.0 μg/ml) treatment also dramatically reduced *MMP1*, *MMP3* and *MMP13* gene expression by 9-, 4.2- and12.5-fold, respectively compared to control (Figure 5A-D). Treatment of cells with the $MC_{3/4}$ receptor antagonist, SHU9119, (10.0 μg/ml) synergistically enhanced the effect of α-MSH (3.0 μg/ml) in down-regulating *MMP13* gene expression (Figure 5A and D) with an 8.3-fold decrease compared to control ($p \leq 0.001$). In contrast, it had no significant effect on α-MSH's ability to reduce *MMP1* and *MMP3* gene expression, whilst SHU9119 completely abrogated the inhibitory effects of [$DTRP^8$]-γ-MSH on *MMP1*, *MMP3* and *MMP13* gene expression (Figure 5A-D).

α-MSH and [DTRP^8]-γ-MSH induce IL-10 release from chondrocytes

Following identification that α-MSH and [DTRP^8]-γ-MSH inhibited the release of both pro-inflammatory cytokines and metalloproteinases, we evaluated their ability to promote release of the anti-inflammatory cytokine IL-10.

α-MSH and [DTRP]-γ-MSH (0.1 – 30.0 μg/ml) both significantly increased IL-10 release compared to untreated chondrocytes, with α-MSH at 1.0 μg/ml causing a maximal 29-fold increase (48.42 pg/ml; Figure 6A) and [DTRP^8]-γ-MSH at 3.0 μg/ml causing a maximal 21-fold release (34.9 ± 2.2 pg/ml; Figure 6B), although higher concentrations of both peptides did not sustain the same levels of increase. These increase in IL-10 release were antagonised by the $MC_{3/4}$ receptor antagonist, SHU9119 for [DTRP^8]-γ-MSH but not α-MSH (Figure 6C).

α-MSH and [D-TRP^8]-γ-MSH inhibit caspase-3/7 activation and cell death in TNF-α-activated chondrocytes

Since melanocortin peptides both inhibited production of pro-inflammatory IL-1β, IL-6, IL-8 and induced release of the anti-inflammatory and chondroprotective cytokine IL-10, we examined their effects on chondrocyte death and apoptosis. TNF-α (60.0 pg/ml) caused a 25.9% reduction in chondrocyte viability and increased Caspase-3/7 activity 5.7-fold, compared to unstimulated (control) cells (Figure 7A). α-MSH and [DTRP^8]-γ-MSH alone had no effect on chondrocyte viability as detected *via* MTT assay, or caspase-3/7 activity (data not shown). However, each peptide inhibited TNF-α-induced chondrocyte death, with a maximal protection of 25% observed at 3.0 μg/ml α-MSH (Figure 7A), with a similar protective effect observed for 3.0 – 30.0 μg/ml [DTRP^8]-γ-MSH ($p \leq 0.01$; Figure 7B). TNF-α stimulation caused a 25.4% ($p \leq 0.05$) increase in the production of cleaved Caspase-3, with α-MSH and [DTRP^8]-γ-MSH (3.0 μg/ml) reducing activated caspase-3 by 50% and 42%

respectively ($p \leq 0.01$; Figure 7C). The $MC_{3/4}$ receptor antagonist, SHU9119, failed to antagonise the anti-apoptotic properties of α-MSH but reduced the effectiveness of the selective MC_3 agonist, [$DTRP^8$]-γ-MSH (Figure 7C).

DISCUSSION

In this study we have determined the chondroprotective and anti-inflammatory properties of the selective MC_3 receptor agonist D[TRP^8]-γ-MSH and pan-agonist α-MSH on TNF-α induced pro-inflammatory cytokine release, MMP gene expression, caspase 3/7 activity and cell viability in C-20/A4 chondrocytes. The data produced indicates that α-MSH and [$DTRP^8$]-γ-MSH reduced pro-inflammatory cytokine release and MMP gene expression whilst preventing C-20/A4 chondrocyte death via reduction in caspase-3 and caspase-7 activity.

Melanocortin peptides have been shown to possess potent anti-inflammatory, anti-pyretic (Getting *et al.,* 2009) and pro-resolving properties (Patel *et al*., 2010, Montero-Melendez *et al*., 2011) in a a number of experimental models of inflammation, including cell-lines (Lam *et al.,* 2006), primary human cells (Capsoni *et al.,* 2009) and rodent models of both rheumatoid (Patel *et al.,* 2010) and gouty (Getting *et al.,* 2002, 2006a) arthritis. These peptides down-regulate the host-inflammatory response by inhibiting both leukocyte migration and the release of pro-inflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6, and IL-8 (Getting *et al.,* 2002, Grässel *et al.,* 2009). To date, two melanocortin receptors, MC_1 and MC_3, have been shown to mediate these anti-inflammatory effects *via* the adenylate cyclase-PKA pathway (Lam *et al.,* 2006, Getting *et al.,* 2006a, 2008), leading to a reduction in pro-inflammatory cytokine production (Delgrado *et al.,* 1998) as well as an induction of anti-inflammatory and pro-resolving proteins IL-10 and heme-oxygenase 1 (Lam *et al.,* 2005, 2006) which aid in the resolution of inflammation (Montero-Melendez *et al.,*

2011). However, to date only two studies have evaluated the role of melanocortin peptides on chondrocytes (Evan *et al.,* 2004, Grässel *et al.,* 2009), which is somewhat surprising given the role that chondrocytes play in the development of OA and the anti-inflammatory and pro-resolving effects of melanocortin peptide treatment in other models of arthritis (Getting *et al.,* 2002, Patel *et al.,* 2010).

In this study we have tested the hypothesis that targeting melanocortin receptors in C-20/A4 chondrocytes may provide a novel therapeutic approach to inhibit pro-inflammatory cytokine production, MMP expression and reduce cell death associated with activation of apoptoic pathways. Current development of therapeutic strategies that prevent cartilage matrix degradation and allow cartilage repair largely depend on the accessibility of human cell culture models. However, primary articular human chondrocytes are difficult to obtain and lose chondrocytic phenotype when expanded in monolayer cultures (Shakibaei *et al.*, 1997), with upregulation in *COL1A1* and decreases in *COL2A1*expression observed (Benya and Shaffer, 1982; Zwicky and Baici, 2000). The C-20/A4 human chondrocyte cell-lines display stable expression of the *COL2A1* gene similar to human primary chondrocytes (Loeser *et al.,* 2000). Therefore, for the purposes of this research the stably differentiated chondrocytic cell line C-20/A4 was used as a tool for the characterisation and further validation of the role of melanocortin peptides in chondrocytes.

Intital experiments showed expression of MC_1 and MC_3 receptors in C-20/A4 chondrocytes confirming previous studies highlighting gene expression of MC_1 in the human chondrosarcoma cell line HTB-94 and primary articular-chondrocytes (Grässel *et al.,* 2009), although MC_3 receptor expression could not be detected in these cells (Grässel *et al.,* 2009). It is conceivable that the differences between our findings and those of the other groups might be due to variations in the cell-lines used, differences in experimental conditions or in the origin of the cells (primary chondrocytes extracted from healthy or OA articular

cartilage). It is also plausible that melanocortin receptor expression may be altered depending on resting state or following stimulation as a recent study (Muffley *et al.,* 2011). has demonstrated that MC_1 and α-MSH protein levels are upregulated in human burn wounds and hypertrophic scars compared to uninjured human skin where receptor and ligand were absent (Muffley *et al.,* 2011).

TNF-α and IL-1β play a pivotal role in the pathogenesis of OA and RA upregulating MMPs gene expression and pro-inflammatory cytokine production (Fernandes *et al.,* 2002, Lawyer *et al.,* 2011). In this study, TNF-α was selected as it is one of the major cytokines produced by chondrocytes and it activates effector caspases driving apoptosis (Stanic *et al.,* 2006). TNF-α stimulation led to a marked increase in IL-1β, IL-6 and IL-8 release in a time-dependent fashion as well as an upregulation in *MMP1*, *MMP3* and *MMP13* gene expression, which are established as important mediators involved in catilage degradation in OA (Grässel *et al.,* 2009). These data are confirmed by previous findings in primary chondrocytes, whereby TNF-α triggered a marked upregulation of pro-inflammatory cyokines (Rai *et al.,* 2008) and cartilage destruction (Kobayashi *et al.,* 2005), thereby validating the choice of C-20/A4 chondrocytes as a system for evaluating the effects of melanocortin peptides on these parameters.

The pan-agonist α-MSH (Getting *et al*., 2008), selective MC_3 receptor agonist [$DTRP^8$]-γ-MSH (Grieco *et al.,* 2000, Getting *et al.,* 2006a, b) and $MC_{3/4}$ receptor antagonist SHU9119 (Getting *et al.,* 1999) were used to confirm receptor functionality. α-MSH and [$DTRP^8$]-γ-MSH both caused an accumulation of intracellular cAMP in C-20/A4 chondrocytes confirming previous findings seen with α-MSH in human primary chondrocytes (Grässel *et al.,* 2009). The MC_3 agonist, [$DTRP^8$]-γ-MSH, previously shown to induce cAMP production in murine peritoneal and alveolar macrophages (Getting *et al.,* 2006a, 2008) also caused increases in cAMP in C-20/A4 chondrocytes suggesting the presence of functionally

active MC_3 receptors. These increases by [DTRP^8]-γ-MSH in cAMP levels were abrogated by the $MC_{3/4}$ receptor antagonist, SHU9119, in agreement with findings in murine macrophages (Getting *et al.,* 2006a), again confirming the presence of MC_3 on these C-20/A4 chondrocytes.

α-MSH and [DTRP^8]-γ-MSH inhibited IL-1β, IL-6 and IL-8 release from C-20/A4 chondrocytes, with the anti-inflammatory properties of D[TRP^8]-γ-MSH antagonised by SHU9119. This ability of melanocortin peptides to reduce cytokine release has been well documented in both *in vitro* and *in vivo* murine studies (Getting *et al.,* 2006a, 2008) and effect observed here agrees with the previous data obtained using human articular chondrocytes (Grassel *et al.,* 2009) in which IL-1β was reduced by α-MSH. However, in contrast to Grassel and colleagues (Grassel *et al.,* 2009), we also demonstrated that α-MSH and D[TRP^8]-γ-MSH inhibit IL-6 and IL-8 release. Interestingly a recent study using zymosan-stimulated macrophages showed that a synthetic α-MSH analogue (AP214) caused a 40% reduction in IL-6 (Montero-Melendez *et al.,* 2011) supporting the data observed here. A potential reason for the differences in IL-6 release between our study and the one of Grassel (2009), could be loss of phenotype in human chondrocyte monolayers (Shakibaei *et al.*, 1997) or that in this study and that of Montero-Melendez (Montero-Melendez *et al.,* 2011) the cells were stimulated with an inflammogen rather than freshly isolated.

Cytokine induced up-regulation of matrix metalloproteinases MMP1, MMP3 and MMP13 play a pivotal role in the pathogenesis of OA (Lawyer *et al.,* 2011, Goldring *et al.,* 2011) and have been shown to be reduced by calcitonin in articular chondrocytes *via* upregulation of cAMP-PKA pathway (Karsdal *et al.,* 2007). Following the inhibition of cytokine release by α-MSH and [DTRP^8]-γ-MSH, their effects on *MMP1*, *MMP3* and *MMP13* gene expression were determined. Both peptides strongly inhibited *MMP1, MMP3, MMP13* gene levels, this effect is novel for [DTRP^8]-γ-MSH and confirms previous studies with α-MSH in the human

chondrosarcoma cell line, HTB-94 (Yoon *et al.,* 2008) and in human chondrocytes (Grassel *et al.,* 2009) with respect to inhibition of *MMP13* expression. The inhibitory effect of [DTRP^8]-γ-MSH was lost following co-treatment with the $MC_{3/4}$ antagonistSHU9119, suggesting again an involvement of MC_3 receptors in regulating MMPs expression. α-MSH inhibited all MMP's evaluated; however co-stimulation with SHU9119 caused a synergistic inhibition of *MMP13* gene expression, thus possibly suggesting a compensatory role for the MC_1 receptor following antagonism at MC_3. At present it is not possible in this model to determine whether melanocortins have a direct effect on MMP expression or via their ability to inhibit cytokine-induced MMP expression (Lawyer *et al.* 2011). Since both [DTRP^8]-γ-MSH and α-MSH inhibited pro-inflammatory cytokines and MMP 1, 3 and 13 expression, their effect on the prostanoid PGE_2 was determined since PGE_2 inhibits IL-1β induced MMP1 and MMP13 expression (Nishitani *et al.,* 2010). A non-significant reduction in PGE_2 was observed following treatment with both melanocortin peptides. This modulatory effect is interesting since increased PGE_2 has been proposed to be chondro-destructive (Nah *et al.,* 2008), whilst other studies show long-term use of NSAIDs reduces PGE_2 leads to accerlerated progression of OA (Reijman *et al.,* 2005). Therfore peptides that modulate PGE_2 expression as opposed to abrogating it may be beneficial in the long-term managment of this pathology, thus suggesting activation of melanocortin receptors may play an important role in maintaining cartilage integrity.

Previous studies have shown a role for IL-10 in mediating the anti-inflammatory effects of α-MSH in murine models of eosinophil migration (Grabbe *et al.,* 1996) and RAW264.7 macrophage cell-lines (Lam *et al.,* 2006), while studies in both TNF-α stimulated OA and healthy primary human chonodrocytes show that IL-10 is chondroprotective by reducing *MMP1* and *MMP13* gene expression (Gonzalez-Rey *et al.,* 2007, Muller *et al.,* 2008). We therefore addressed the question of whether α-MSH and [DTRP^8]-γ-MSH could induce IL-10

production in C-20/A4 chondrocytes. Our results indicate that both α-MSH and [DTRP^8]-γ-MSH significantly increase IL-10 production, with SHU9119 antagonising the effect of [DTRP^8]-γ-MSH but not that of α-MSH. These data propose the exciting idea that melanocortin peptides (in part) exert a homeostatic control over chondrocyte physiology, with an ability to induce chondro-protective and pro-resolving cytokines, and a possible role in resolving chondrocyte-borne inflammation.

Chondrocyte death has also been shown to play an important role in cartilage degradation and progression of diseases such as OA and RA (Aigner and Kim, 2002; Oppenheimer *et al.,* 2011) with TNF-α shown to promote apoptosis (Stanic *et al.*, 2009) *via* caspase-3 and caspase-7 activation (Luthi and Martin, 2007, Lee *et al.,* 2011, Facchini *et al.,* 2011). In this study, TNF-α significantly increased the production of cleaved caspase-3 and caspase -3 and -7 activities by 30%. Treatment of cells with both α-MSH and [DTRP^8]-γ-MSH led to an inhibition of executioner caspases -3 and -7, with their effects largely mediated by MC_1 and MC_3, as co-administration with the $MC_{3/4}$ antagonist, SHU9119, prevented [DTRP^8]-γ-MSH but not α-MSH from inhibiting activated caspase-3 production. These pro-survival effects of melanocortin peptides has also been demonstrated by Chai and colleagues, who showed that the non-selective melanocortin peptide, NDP-MSH, inhibited caspase-3 activation in the neuronal cell line GT1-I (Chai *et al*., 2006), while α-MSH has also been shown to prevent LPS/INF-γ-induced astrocyte apoptosis (Caruso *et al*., 2007). However, to our knowledge, this is the first demonstration that α-MSH and [DTRP^8]-γ-MSH can prevent the production and activation of executioner caspases and initiation of chondrocyte death.

Conclusion

This study demonstrates that the anti-inflammatory, chondro-protective and anti-apoptotic effects of melanocortin peptides in C-20/A4 chondrocytes are largely mediated *via* the MC_1

and MC_3 receptors. This data provides a rational for the further investigation of these peptides as chondro-protective agents in OA and RA.

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Conflict of Interest

PG holds a patent on the MC_3 agonist.

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Figure Legends

Figure 1. TNF-α stimulates IL-1β, IL-6 and IL-8 release from C-20/A4 chondrocytes.

C-20/A4 chondrocytes were stimulated with TNF-α (0.0 – 80.0 pg/ml) and cell-free supernatants collected 0–24 h post-stimulation and analysed for IL-1β (Panel A), IL-6 (Panel B) and IL-8 (Panel C) levels by ELISA. Data are presented as mean ± SEM of n=4 independent experiments repeated in triplicate, *$p \leq 0.05$, **$p \leq 0.01$.

Figure 2. Endogenous expression of MC_1 and MC_3 functionally active receptors in C-20/A4 chondrocytes.

Western blotting was used to determine MC_1 and MC_3 protein levels using rabbit anti-MC_1 and rabbit anti-MC_3 mAbs (1:2000). Bands with sizes corresponding to MC_1 (35 kDa), MC_3 (40 kDa) and α-tubulin (55 kDa) were detected and densitometrically quantified (Panel A). C-20/A4 chondrocytes were stimulated with 1.0 -30.0 μg/ml α-MSH (Panel B) or [$DTRP^8$]-γ-MSH (Panel C) alone or in the presence of SHU9119 (10.0 μg/ml) for 30 min before measuring cAMP concentration by EIA. Dotted lines indicate basal cAMP accumulation in PBS-treated cells whilst dashed lines indicate maximal accumulation of cAMP in FSK-treated C-20/A4 cells. Data are presented as mean ± SEM of 3 independent experiments for Western blot analysis and n=6 samples for cAMP accumulation, *$p \leq 0.05$, ***$p \leq 0.01$ vs. PBS-treated control cells.

Figure 3. [$DTRP^8$]-γ-MSH and α-MSH inhibit IL-1β, IL-6 and IL-8 release from C-20/A4 chondrocytes.

C-20/A4 chondrocytes were pre-treated for 30 min with PBS, α-MSH or [$DTRP^8$]-γ-MSH (0.1 – 30.0 μg/ml) prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected 6 h post-stimulation and analysed for IL-1β (Panel A and B), IL-6 (Panel C and D)

and IL-8 (Panel E and F) concentration by ELISA. Data are presented as mean ± SEM of n=4 independent experiments repeated in triplicate, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Figure 4. SHU9119 antagonises [DTRP^8]-γ-MSH but not α-MSH inhibition of IL-1β, IL-6 and IL-8 release from TNF-α stimulated C-20/A4 chondrocytes.

C-20/A4 chondrocytes were left untreated or were pre-incubated for 1 h with SHU9119 (10.0 μg/ml) prior to α-MSH or [DTRP^8]-γ-MSH (3.0 μg/ml) treatment for 30 mins. Cells were then stimulated with TNF-α (60.0 pg/ml) and cell-free supernatants collected 6 h post-stimulation and analysed for IL-1β (Panel A), IL-6 (Panel B) and IL-8 (Panel C) levels by ELISA. Data are presented as mean ± SEM of n=4 independent experiments repeated in triplicate, *$p \leq 0.05$, **$p \leq 0.01$.

Figure 5. SHU9119 antagonises the inhibitory effect of [DTRP^8]-γ-MSH but not α-MSH on *MMP1, MMP3* and *MMP13* mRNA expression in C-20/A4 chondrocytes.

C-20/A4 chondrocytes were left untreated or pre-treated for 1 h with SHU9119 (10.0 μg/ml) prior to α-MSH or [DTRP^8]-γ-MSH (3.0 μg/ml) treatment for 30 mins. Cells were then stimulated with TNF-α (60.0 pg/ml) and total RNA was extracted at 6 h post-stimulation. Oligonucleotide primers specific for *MMP1, MMP3* and *MMP13* were used to detect and quantify gene expression by PCR followed by analysis on 2% agarose gels in triplicates, with β-actin used as an internal control (Panel A). Comparison of densitometrically quantified *MMP1, MMP3* and *MMP13* gene expression levels for α-MSH, [DTRP^8]-γ-MSH ± SHU9119 (Panel B, C and D) are shown in arbitrary units, each value normalized to the respective β-actin expression. Data is presented as mean ± SEM of n=4 independent experiments *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Figure 6. α-MSH and [DTRP^8]-γ-MSH stimulate IL-10 release from C-20/A4 chondrocytes.

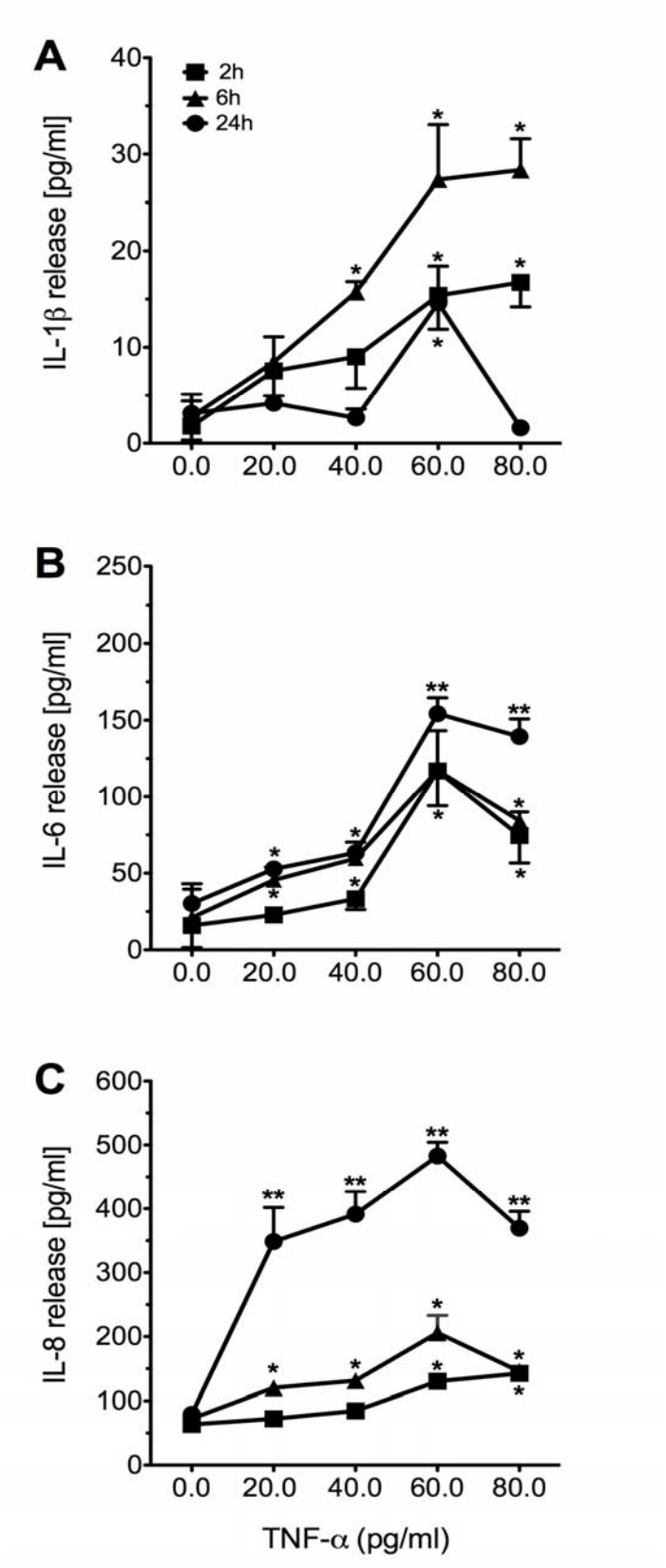
C-20/A4 chondrocytes were pre-treated for 30 min with PBS, α-MSH or [DTRP^8]-γ-MSH (0.1 – 30.0 μg/ml) prior to stimulation with TNF-α (60.0 pg/ml) and cell-free supernatants collected 6 h post-stimulation and analysed for IL-10 (Panel A and B) levels by ELISA. In separate experiments, C-20/A4 chondrocytes were left alone or pre-treated for 1 h with SHU9119 (10.0 μg/ml) prior to α-MSH or [DTRP^8]-γ-MSH (3.0 μg/ml) treatment for 30 mins. Cells were then stimulated with TNF-α (60.0 pg/ml) and cell-free supernatants collected 6 h post-stimulation and analysed for IL-10 (Panel C) concentration by ELISA. Dotted line indicates control levels and dashed line is TNF-α (60.0 pg/ml) treated cells alone. Data are presented as mean ± SEM of n=4 independent experiments repeated in triplicate, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Figure 7. α-MSH and [DTRP^8]-γ-MSH modulate Caspase-3/7 activity and protein levels and cell viability in human C-20/A4 chondrocytic cell line.

C-20/A4 chondrocytes were treated for 6 h with α-MSH or [DTRP^8]-γ-MSH (0.1 – 30.0 μg/ml) alone or in the presence of SHU9119 (10.0 μg/ml) for 30 min prior to TNF-α (60.0 pg/ml) stimulation. Caspase-3/7 activity was determined by Caspase-3/7 Glo Assay and cell viability determined by the MTT reduction assay (Panel A and B). The dashed line represents control sample cell viability (i.e. untreated cells as determined by MTT (100%). The dotted line shows Caspase 3/7 activity following DMEM treatment (control) (Panel A and B). Cleaved caspase-3 (Asp175; 17, 19 kDa) and α-tubulin (55 kDa) were detected by Western blotting, the image is representative of 4 individual experiments (Panel C). Comparison of densitometrically quantified cleaved Caspase-3 (Asp175) expression in human C-20/A4 cell-lines is shown in arbitrary units (au, Panel C). Dotted line indicates

control levels of caspase 3/7 activity and dashed line is control cell viability. Data are presented as mean ± SEM of n=4 experiments, assessed in triplicate. *$p \leq 0.05$, **$p \leq 0.01$, vs. TNF-α-treated controls.

Figure 1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80.0
B
IL-6 release [pg/ml]
0
50
100
150
200
250
C
IL-8 release [pg/ml]
0
100
200
300
400
500
600
TNF-α (pg/ml)

Figure 2

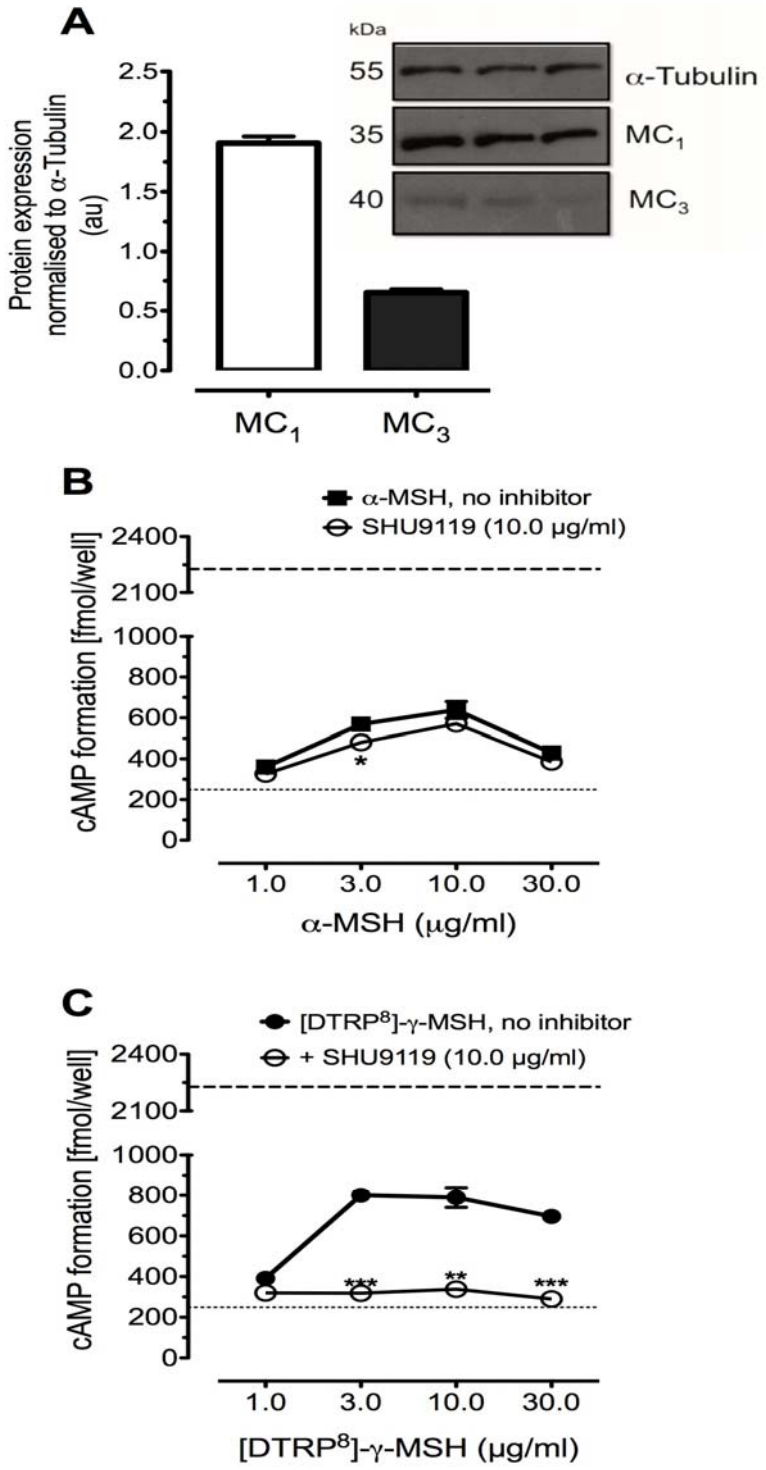
A
kDa
55
35
40
α-Tubulin
MC1
MC3
Protein expression normalised to α-Tubulin (au)
2.5
2.0
1.5
1.0
0.5
0.0
MC1
MC3
B
α-MSH, no inhibitor
SHU9119 (10.0 µg/ml)
cAMP formation [fmol/well]
2400
2100
1000
800
600
400
200
0
*
1.0
3.0
10.0
30.0
α-MSH (µg/ml)
C
[DTRP8]-γ-MSH, no inhibitor
+ SHU9119 (10.0 µg/ml)
cAMP formation [fmol/well]
2400
2100
1000
800
600
400
200
0
***
**
***
1.0
3.0
10.0
30.0
[DTRP8]-γ-MSH (µg/ml)

Figure 3

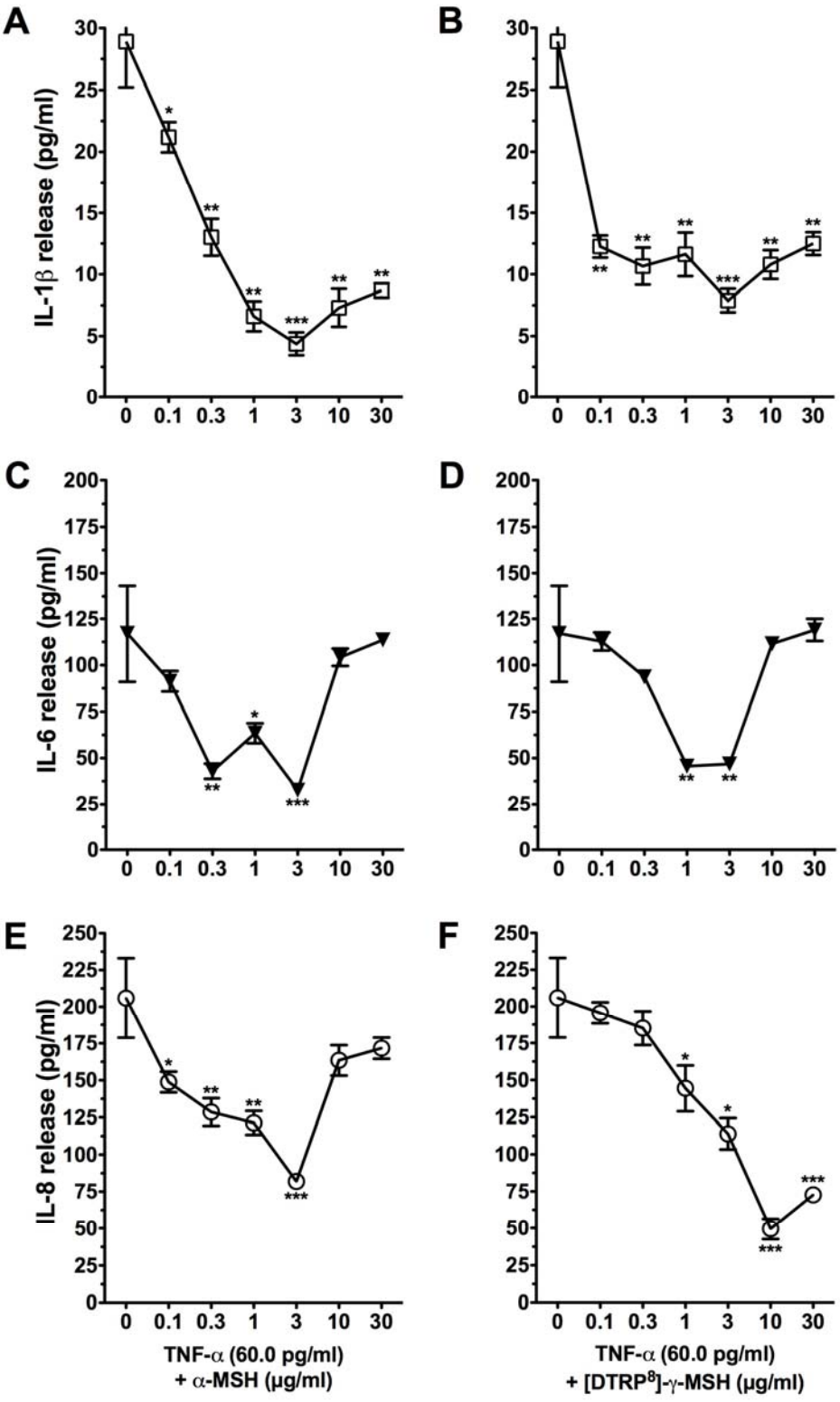
A
B
C
D
E
F
IL-1β release (pg/ml)
IL-6 release (pg/ml)
IL-8 release (pg/ml)
0 0.1 0.3 1 3 10 30
TNF-α (60.0 pg/ml)
+ α-MSH (µg/ml)
TNF-α (60.0 pg/ml)
+ [DTRP8]-γ-MSH (µg/ml)

Figure 4

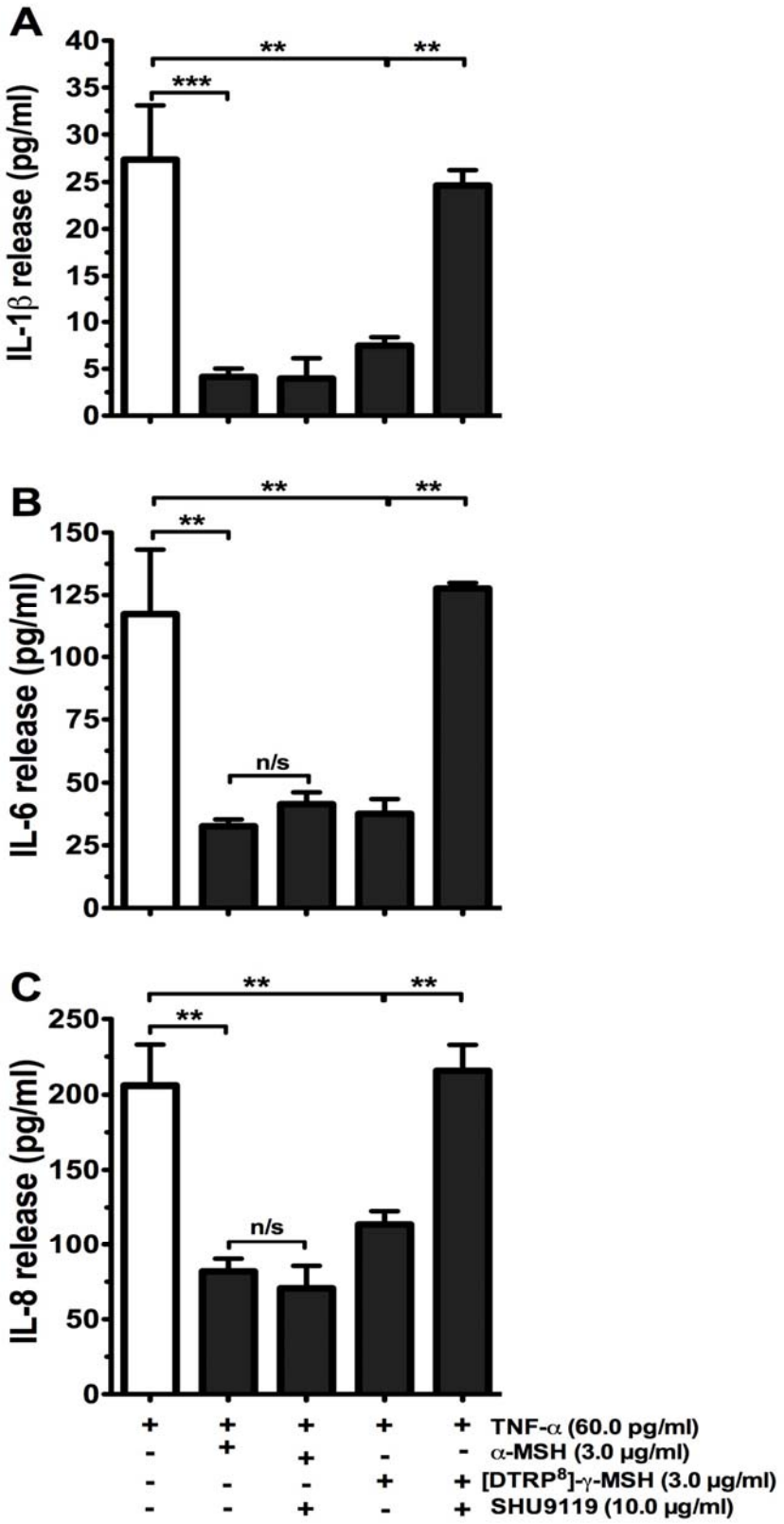
A
IL-1β release (pg/ml)
40
35
30
25
20
15
10
5
0
**
**
***
B
IL-6 release (pg/ml)
150
125
100
75
50
25
0
**
**
**
n/s
C
IL-8 release (pg/ml)
250
200
150
100
50
0
**
**
**
n/s
+ + + + + TNF-α (60.0 pg/ml)
- + + - - α-MSH (3.0 µg/ml)
- - - + + [DTRP8]-γ-MSH (3.0 µg/ml)
- - + - + SHU9119 (10.0 µg/ml)

Figure5

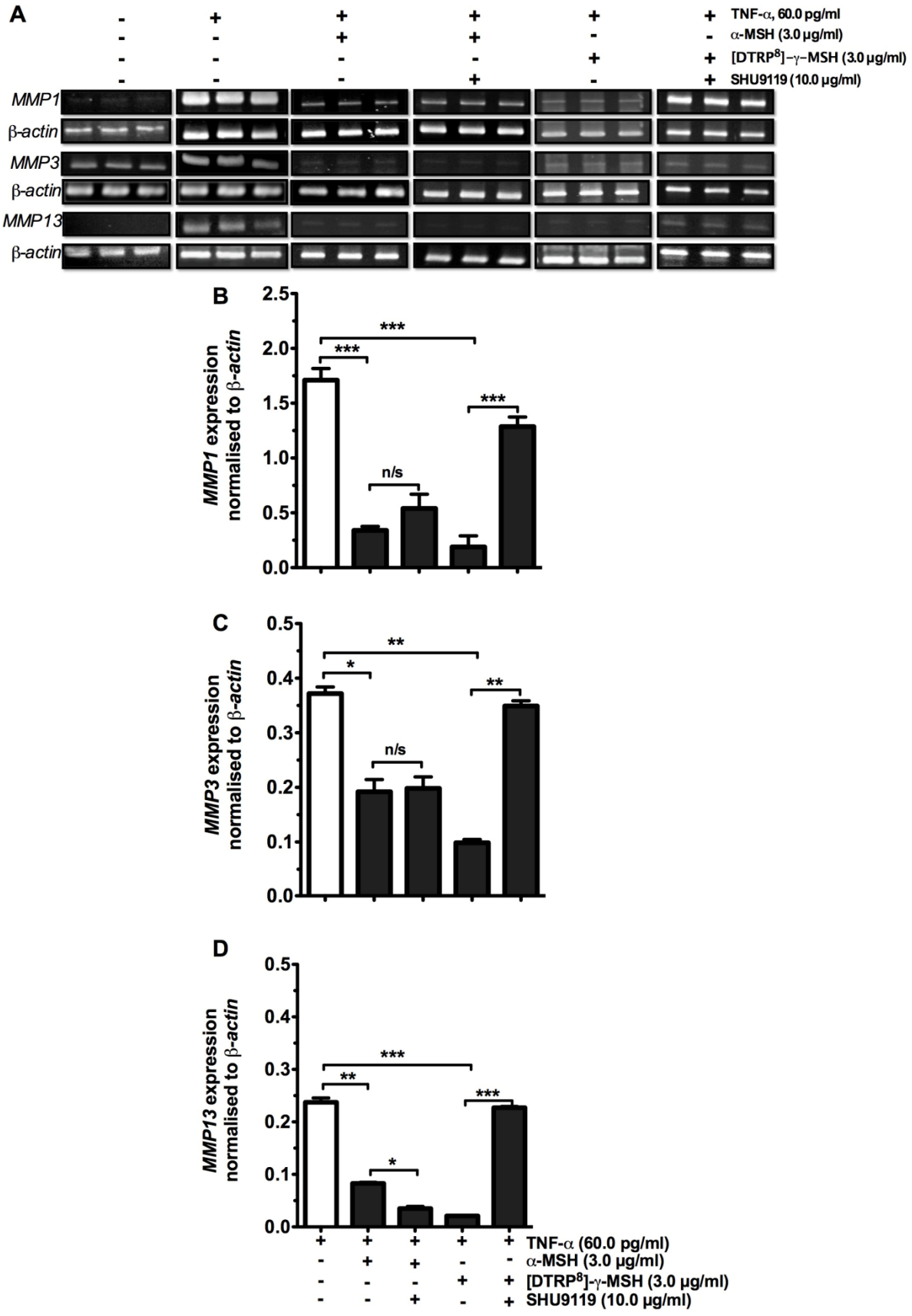
A
TNF-α, 60.0 pg/ml
α-MSH (3.0 μg/ml)
[DTRP8]-γ-MSH (3.0 μg/ml)
SHU9119 (10.0 μg/ml)
MMP1
β-actin
MMP3
β-actin
MMP13
β-actin
B
MMP1 expression normalised to β-actin
C
MMP3 expression normalised to β-actin
D
MMP13 expression normalised to β-actin
n/s
TNF-α (60.0 pg/ml)
α-MSH (3.0 μg/ml)
[DTRP8]-γ-MSH (3.0 μg/ml)
SHU9119 (10.0 μg/ml)

Figure 6

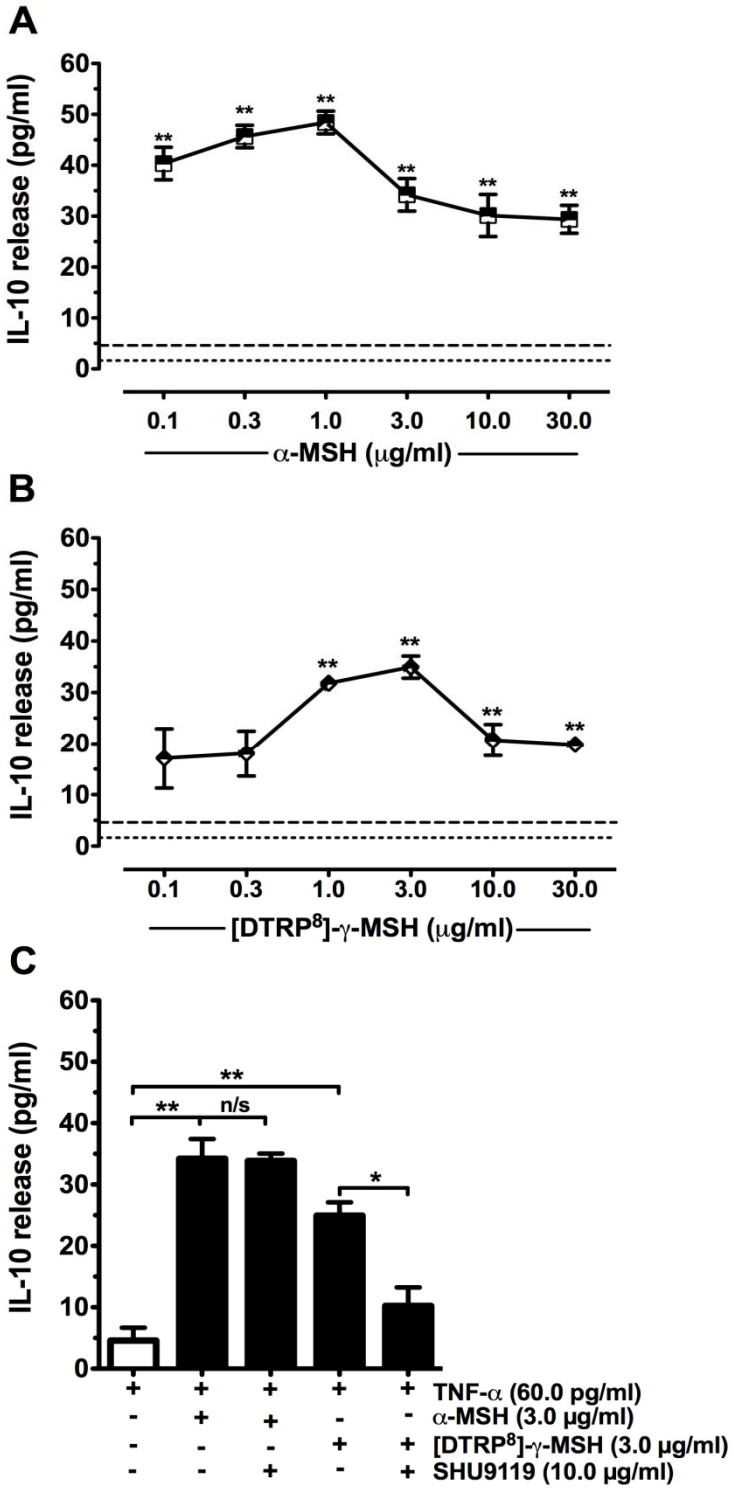
A
IL-10 release (pg/ml)
60
50
40
30
20
10
0
**
0.1
0.3
1.0
3.0
10.0
30.0
α-MSH (μg/ml)
B
IL-10 release (pg/ml)
[DTRP8]-γ-MSH (μg/ml)
C
IL-10 release (pg/ml)
n/s
*
TNF-α (60.0 pg/ml)
α-MSH (3.0 μg/ml)
[DTRP8]-γ-MSH (3.0 μg/ml)
SHU9119 (10.0 μg/ml)

Figure 7

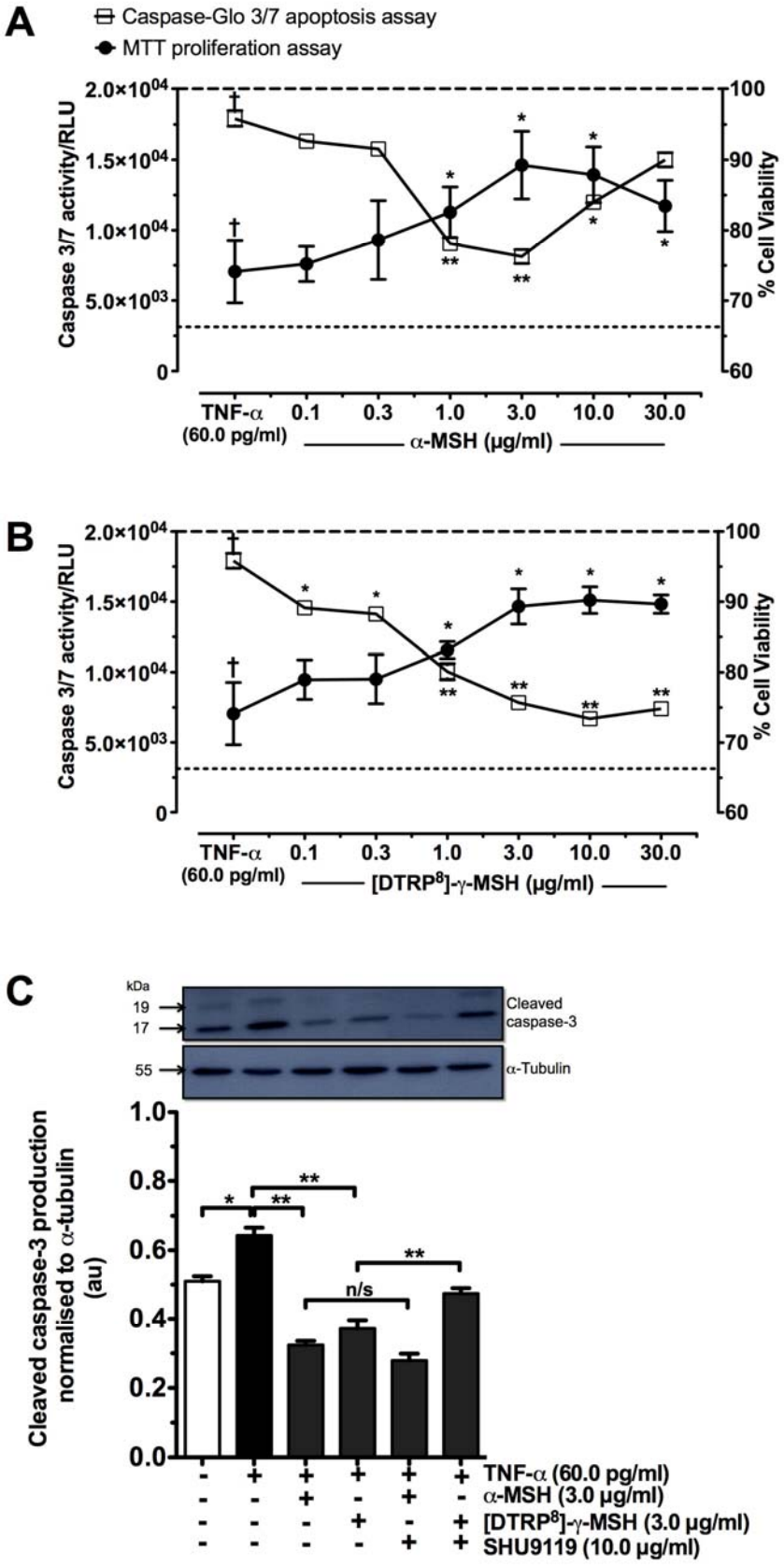
A
Caspase-Glo 3/7 apoptosis assay
MTT proliferation assay
Caspase 3/7 activity/RLU
% Cell Viability
TNF-α (60.0 pg/ml)
α-MSH (μg/ml)
B
[DTRP8]-γ-MSH (μg/ml)
C
kDa
19
17
55
Cleaved caspase-3
α-Tubulin
Cleaved caspase-3 production normalised to α-tubulin (au)
n/s
TNF-α (60.0 pg/ml)
α-MSH (3.0 μg/ml)
[DTRP8]-γ-MSH (3.0 μg/ml)
SHU9119 (10.0 μg/ml)

Table 1: Lack of effect of α-MSH and D[TRP^8]-γ-MSH on TNF-α induced PGE_2 release

Pre-Treatment	Stimulus	PGE_2 release (pg/ml)
None (control)	None	93.12 ± 12.58
None	TNF-α (60.0 pg/ml)	180.38 ± 6.21*****
α-MSH (3.0 μg/ml)	TNF-α(60.0 pg/ml)	145.38 ± 27.6$^{n/s}$
[DTRP^8]-γ-MSH (3.0 μg/ml)	TNF-α(60.0 pg/ml)	153.89 ± 32.52$^{n/s}$

Data are Mean ± SEM of n=4 of three determinations; *****$p \leq 0.05$ compared to control cultures. *P<0.05 vs. Control, non-significant (ns) compared to TNF-α-stimulated cells (One-way ANOVA, Dunnet's multiple comparison test). PGE2, Prostaglandin E_2.