Melanocortin peptides protect chondrocytes from mechanically induced cartilage injury

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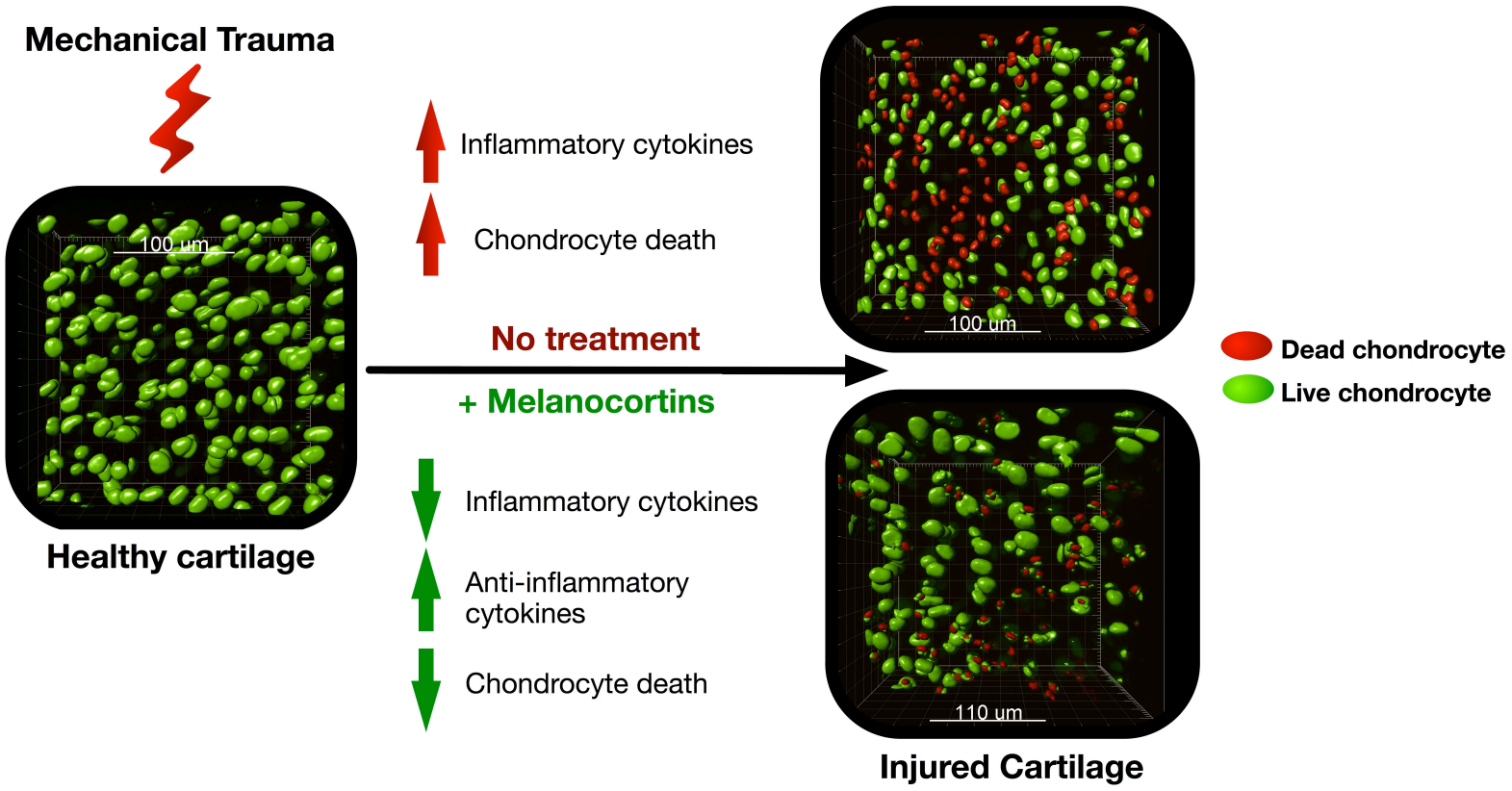
Mechanical Trauma
100 um
Healthy cartilage
Inflammatory cytokines
Chondrocyte death
No treatment
+ Melanocortins
Inflammatory cytokines
Anti-inflammatory cytokines
Chondrocyte death
100 um
110 um
Injured Cartilage
Dead chondrocyte
Live chondrocyte

Melanocortin peptides protect chondrocytes from mechanically induced cartilage injury

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ABSTRACT

Introduction: Mechanical injury can greatly influence articular cartilage, propagating inflammation, cell injury and death – risk factors for the development of osteoarthritis. Melanocortin peptides and their receptors mediate anti-inflammatory and pro-resolving mechanisms in chondrocytes. This study aimed to investigate the potential chondroprotective properties of α-MSH and [DTRP^8]-γ-MSH in mechanically injured cartilage explants, their ability to inhibit pro-inflammatory and stimulate anti-inflammatory cytokines in *in situ* and in freshly-isolated articular chondrocytes.

Methods: The effect of melanocortins on *in situ* chondrocyte viability was investigated using confocal laser scanning microscopy of bovine articular cartilage explants, subjected to a single blunt impact (1.14N, 6.47kPa) delivered by a drop tower. Chondroprotective effects of α-MSH, [DTRP^8]-γ-MSH and dexamethasone on cytokine release by TNF-α-activated freshly-isolated articular chondrocytes/mechanically injured cartilage explants were investigated by ELISA.

Results: A single impact to cartilage caused discreet areas of chondrocyte death, accompanied by pro-inflammatory cytokine release; both parameters were modulated by α-MSH, [DTRP^8]-γ-MSH and dexamethasone. Melanocortin pre-treatment of TNF-α-stimulated freshly-isolated chondrocytes resulted in a bell-shaped inhibition in IL-1β, IL-6 and IL-8, and elevation of IL-10 production. The $MC_{3/4}$ antagonist, SHU9119, abrogated the effect of [DTRP^8]-γ-MSH but not α-MSH on cytokine release.

Conclusion: Melanocortin peptide pre-treatment prevented chondrocyte death following mechanical impact to cartilage and led to a marked reduction of pro-inflammatory cytokines, whilst prompting the production of anti-inflammatory/pro-resolving cytokine IL-10. Development of small molecule agonists towards melanocortin receptors could thus be a viable approach for preventing chondrocyte inflammation and death within cartilage and represent an alternative approach for the treatment of osteoarthritis.

Keywords: Cartilage injury, Mechanical trauma, Chondrocyte death, Melanocortin peptides, Osteoarthritis.

Abbreviations: ACTH, adrenocorticotropic hormone; CLSM, confocal laser scanning microscopy; DMARDs, drug modifying anti-rheumatic drugs; ECM, extracellular matrix; GPCR, G-protein coupled receptor; IL, interleukin; MC, melanocortin receptor; MMP, matrix metalloproteinases; NSAIDs, non-steroidal anti-inflammatory drugs; OA, osteoarthritis; POMC, pro-opiomelanocortin; RA, rheumatoid arthritis; α-MSH, alpha-melanocyte-stimulating hormone.

Table 1. Structure of melanocortin peptides used in this study.

Melanocortin peptide	Chemical structure
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
[DTRP^8]-γ-MSH	H-Tyr-Val-Met-Gly-His-Phe-Arg-**ᴅTrp**-Asp-Arg-Phe-Gly-OH
SHU9119	Ac-Nle-cyclo(Asp-His-DNal(2′)-Arg-Trp-Lys)-NH_2

1. Introduction

Osteoarthritis (OA) is a disease affecting load-bearing joints, characterised by self-perpetuating low-grade inflammation and degradative processes within the articular cartilage of affected joints. It is a leading cause of disability affecting almost every age group, with prevalence increasing dramatically over the age of 50, affecting ~60% of people in this age group. With increasing age, obesity and longer life spans, OA represents an ever-increasing socio-economic burden [1], for which at present there is no cure.

Traumatic joint injuries are a major risk factor for the development and progression of OA [2] and increase the risk of arthritis 5 to 17-fold [3, 4]. Knee traumas, in particular, represent over 40% of all sports injuries [5-7] and often result from traffic accidents with surgical restoration of joint stability not preventing future arthritis development [8-11]. The incidence of post-traumatic arthritis is therefore high – creating insistent demand for pharmacological intervention, directed at limiting the progression and propagation of destructive processes taking place in the early stages post-injury [2, 12].

In a healthy joint, the smooth surface provided by articular cartilage promotes near frictionless joint movement allowing the joint to withstand tensile and compressive forces arising from movement [13]. Mechanical loading within physiological limits is an essential stimulus for chondrocytes to produce extra-cellular matrix (ECM), capable of withstanding normal levels of stress and is responsible for triggering the synthesis, exportation and degradation of ECM components – collagen and proteoglycans [14]. However, when the joint/cartilage experiences mechanical stresses above the normal physiological range and/or frequency, such as in impact trauma, this results in significant chondrocyte death attributed to mechanical necrosis [15] and apoptotic processes [16, 17] that could trigger the development of OA [18].

The effect of impact trauma on the functionality and metabolism of chondrocytes is receiving increasing attention [16, 17, 19, 20], because within mature articular cartilage, chondrocytes do not generally undergo cell division [21, 22]. Additionally, OA is featured by reduced cellularity [21, 23-29], a fact that is thought to contribute to the inability of the remaining chondrocytes to maintain normal matrix synthesis, thereby contributing to cartilage degradation [30].

Impact injury is associated with increased production of pro-inflammatory cytokines by affected chondrocytes [31]. Abnormal mechanical forces cause adult chondrocytes to initiate production of a large number of pro-inflammatory mediators including the cytokines TNF-α and IL-1β [18, 32], which in combination with reactive oxygen species and lipid-derived

inflammatory stimuli (including prostaglandins and leukotrienes) increase the catabolic activity of and ultimately kill chondrocytes distant from the impact-injury site [33-35]. This eventually leads impaired ECM synthesis, cartilage degradation and, ultimately, development of OA [36, 37].

Currently there is no treatment for OA, capable of reducing the degradation of cartilage or improving its function. Current treatment relies largely on conservative pain management strategies (analgesics and non-steroidal anti-inflammatory drugs (NSAIDs)). These are only temporarily effective with numerous side effects, and if unsuccessful leave expensive joint replacement surgery as the last resort. To find highly effective drugs with an enhanced safety profile for OA treatment is imperative. Development of compounds displaying both anti-inflammatory effects along with pro-resolving/chondroprotective properties represents an exciting therapeutic strategy.

Unravelling the mediators that provide tissue protection and developing peptide-based drugs targeted at the resolution phase of inflammation is an exciting concept [38, 39]. Amongst a host of such mediators are the melanocortins. The melanocortin peptides have long been shown to display anti-inflammatory effects from the early seminal studies by Lipton demonstrating their anti-pyretic effects [40] and their potency – they are 25,000x more potent than paracetamol [41]. Over the last four decades a substantial body of evidence has exposed their beneficial effects in models of asthma [42-44], inflammatory bowel disease [45-47], cardiovascular disease [48-53], and neuroprotection [54-56] to name just a few areas.

Within the arthritic field, the melanocortin system has been evaluated in patients with RA and juvenile chronic arthritis; increased α-MSH levels were detected in synovial fluid, with a correlation suggesting that higher levels of α-MSH decrease the level of inflammation observed [57]. These important findings highlighted the prospect of harnessing the anti-inflammatory effects of α-MSH for arthritic diseases and soon after the beneficial effects of the peptide were proven in a model of adjuvant-induced arthritis [58], while more recently, it was found to be beneficial in models of gouty and rheumatoid arthritis (RA) [59-62]. However, only a handful of studies have evaluated their effects in chondrocytes and OA [63],[64]. This surprising lack of interest in evaluating the potential of these molecules as a treatment for OA may stem from the fact that although inflammation is considered causal to both RA and gouty arthritis, OA has historically been perceived as simply a condition of natural ‘wear and tear’, with inflammation not regarded as a major contributor in the development of the pathology. Nevertheless, this initial viewpoint is currently changing [32, 64].

Melanocortin peptides are derived from the larger, pre-cursor pro-opiomelanocortin (POMC) protein [65, 66] and exert their effects *via* the activation of melanocortin receptors/adenylate cyclase/cAMP signalling pathway [65]. Although five melanocortin receptors (MC) have been identified, all positively coupled to adenylate cyclase *via* Gs and activate cAMP pathways, the anti-inflammatory effect of melanocortin peptides has been found to be mediated primarily via MC_1, MC_3 and MC_5 [65, 66].

Here, we have demonstrated for the first time the ability of melanocortins to limit the progression of mechanical impact-induced chondrocyte death. In addition, the peptides inhibited the resulting production of pro-inflammatory cytokines in both *in situ* and TNF-α-stimulated freshly-isolated articular chondrocytes, while promoting the release IL-10, thereby aiding in the resolution of inflammation and conferring further protection against cartilage damage.

2. Materials & Methods

Unless specified otherwise, all reagents were purchased from Sigma-Aldrich Inc., (Poole, UK).

2.1. Cartilage impact studies

2.1.1. *Cartilage dissection*

Four metacarpophalangeal joints from different 18–24 month old cows (obtained from local abattoir) were skinned, rinsed in water, and joint capsules opened under aseptic conditions within 12h of slaughter. Full-depth, healthy cartilage, excluding the subchondral bone, was harvested from the flat, load-bearing articular surfaces between the condylar ridges of each joint. Cartilage explants were cultured individually in HEPES-buffered high-glucose Dulbecco's Modified Eagle's Medium (DMEM, 280 mOsm/kg:H_2O, abbreviated to 'mOsm', pH 7.4; Gibco®, Life Technologies, Paisley, UK), supplemented with Penicillin (50.0 U/mL) and Streptomycin (50.0 µg/mL) at 37ºC, and 5% CO_2 in the absence of foetal calf serum (FCS; Invitrogen, Paisley, UK) and cultured within 24h or used for impact studies [67].

2.1.2 *Mechanical loading of tissue*

A vertical drop tower previously shown to cause impact damage to cartilage explants in aseptic conditions [30], was used to deliver a single defined impact (137g weight dropped from a height of 10 cm), equivalent to 1.14 N, 6.47 kPa (assuming linear acceleration), to individual bovine articular cartilage explants [30]. The Isolated, pre-weighed articular cartilage explants (~5mm^2) were incubated (within 24h of dissection) in 1.0 mL serum-free DMEM media with or without the melanocortin peptides α-MSH and [DTRP^8]-γ-MSH (3.0 µg/mL), or dexamethasone (10^{-6} M), for 1h prior to impact. Individual cartilage explants were then positioned with the synovial (articular) surface uppermost on the drop tower base and exposed to single impact. Samples exposed to accidental multiple impacts were discarded. Explants were then immediately returned to the same media, containing the melanocortin peptides (α-MSH or [DTRP^8]-γ-MSH) or dexamethasone for 6h, the reaction was then terminated and cell culture supernatants collected and stored at -20ºC prior to cytokines analysis by ELISA (R&D Systems Europe Ltd, Oxford, UK) as previously described [64]. Following treatment, the cartilage explants were transferred to dry 10 cm^2 plastic dishes, positioned flat on the dish and viewed perpendicular to the synovial surface. The tissue was immobilized in the center of the dish using a small drop of cyanoacrylate glue (Bostik, UK) [30], and incubated on a heated microscope stage (37ºC) for 30 min in fresh DMEM media containing Calcein-AM (5.0 µM; Anaspec Inc. Freemont, USA) and Propidium Iodide (PI; 1.0 µM; Cambridge Bioscience, Cambridge, UK). For determination of the viability of the *in situ* chondrocytes the articular cartilage explants were visualized using an upright Leica SP2

confocal laser scanning microscopy (CLSM) was used (Leica Microsystems, Milton Keynes, UK).

2.2 Confocal Analysis

2.2.1 *Microscopy and determination of chondrocyte cell viability in situ*

Images were acquired using an upright microscope attached to a Leica SP2 CLSM and associated Leica software. Calcein and PI excited using a 488 nm (argon laser), with an emission measured at a bandpass of 510-535 nm and PI excited using a 543nm (He-Ne laser) with an emission recorded at a bandpass of 660-709nm. Chondrocytes in cartilage explants were viewed with a low power magnification (x10 air objective) for *in situ* chondrocyte viability measurements. Z-series of individual images of the chondrocytes were taken at 10 μm z-steps. Scanning speed was 0.6 Hz with double frame integration, double line averaging for 512 x 512 pixel image with viable cells appearing green and dying cells – red [30].

2.2.2 *Confocal data analysis – in situ chondrocyte viability measurements*

Cell viability prior to and post impact was evaluated using Imaris 7.1.1 Spots feature (Bitplane AG, Zurich, Switzerland). The Spots feature models point-like structures in the data, and can be used for detecting cells. It automatically detects chondrocytes in the cartilage, and allows for manual correction of detection errors (such as mistaking background noise for a cell), visualizes the cells as spheres, and its statistics output, provides accurate count of the cells (spheres) in a given field of view. For the software to work properly, a measurement of the average size of the cells needed to be entered in the beginning of the analysis. As the diameter of viable cells (green) and “dying” cells (red) in the cartilage explants varies significantly, the diameter was measured for both entities within several areas of observation and the averaged parameters were entered into the Imaris Spot Analysis software. Viable cells had a diameter of (~10 μm), whilst dead cells were much smaller (~6 μm). The software utilised these values, differentiating between background noise and cells (separately for both laser channels), and then assigned a sphere to each of the detected cells. However, not all the cells in the image have the same intensity (>0 and <255) – threshold too high and dimmer cells will be missed, threshold too low and background noise will be counted as cells. Therefore, for accurate measurements of cell number, the threshold values for both Calcein and PI were calculated by selecting a smaller area with a known number of cells and threshold values from 0 – 100% were applied in increments of 10%. Using the resultant linear regression equation, the correct cell number was found at a threshold percentage of 20% for Calcein-AM-stained cells (viable cells) and 60% for PI-stained cells (dead cells), respectively and these parameters were kept constant throughout all subsequent analysis to avoid data skewing.

2.3 Primary articular chondrocyte isolation

Articular cartilage explants were incubated in serum-free DMEM adjusted to 380 mOsm with sterile-filtered NaCl (Fisher Scientific, Leicestershire, UK), containing Collagenase Type I (1 mg/mL; Gibco®, Life Technologies, Paisley, UK), for >18 h at 37ºC, 5% CO_2. Tissue digests were filtered through a 40 μm Falcon Cell Strainer (BD Biosciences, Oxford, UK), chondrocytes were washed twice by centrifugation (10 min, 20ºC, 500*g*) in serum-free DMEM (380 mOsm) and cultured at a density of $1.0x10^5$ cells/cm^2 in DMEM (380 mOsm; ascorbic acid (50.0 μg/mL), Penicillin (50.0 U/mL) and Streptomycin (50.0 μg/mL); pH 7.4) for 10 days prior to experimentation [67]. All experiments were performed with freshly isolated chondrocytes (at passage 0) in serum-free conditions as described above.

2.4 *In vitro* cell stimulations

Following the 10 days of culture the chondrocytes were released following 10 min incubation in 0.05% Trypsin-EDTA (Invitrogen, Paisley, UK), plated in 96-well plates at density $0.2x10^6$ cells/well and allowed to attach. Chondrocytes were stimulated for 6h with recombinant TNF-α (0–80 pg/mL) at 37ºC, 5% CO_2. In separate experiments, chondrocytes were treated for 30 min with either DMEM (untreated control), dexamethasone (Dex; 10^{-6} M), or 1-30 μg/mL α-MSH, (purity ≥97%; Sigma-Aldrich, Poole, Dorset, UK), or [$DTRP^8$]-γ-MSH [68] or the $MC_{3/4}$ antagonist SHU9119 (purity ≥97%; Bachem AG, Bubendorf, Switzerland) [69], prior to 6h stimulation with TNF-α (60.0 pg/mL). Cell-free supernatants were collected and analysed for cytokine release as described above. In some experiments (as indicated), cells were pre-treated for 1h with SHU9119 (10.0 μg/mL) prior to addition of α-MSH or [$DTRP^8$]-γ-MSH to scrutinize peptide selectivity.

2.5 Statistics and Receptor Nomenclature

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 Bonferroni or Dunnet's Post-tests to allow for *post-hoc* analyses, with a probability *p* value < 0.05 taken as significant. Receptor nomenclature for melanocortin receptors was in accordance with the "Guide to receptors and Channels (GRAC)" [70].

3 Results

3.1 Single impact to cartilage explants causes choncrocyte death and increase production of pro-inflammatory cytokines

Areas of cartilage/chondrocyte injury caused by impact force are shown in Figure 1A (non-impacted) versus Figure 1B (impacted). Spot analysis indicated a 4.5-fold increase of cell death in articular cartilage explants subjected to a single blunt impact (13.5±1.7%) compared to 2.95% cell death in non-impacted explants (p<0.05).

Single blunt impact injury to bovine articular cartilage explants caused a dramatic increase in the release of the potent pro-inflammatory cytokines IL-1β from the resident chondrocytes (Figure 1). The cartilage injury initiated a 19.5-fold upsurge in production of IL-1β from 18±5 pg/mL/g to 351±24 pg/mL/g in impacted explants (p<0.0001; Figure 1D). Levels of IL-6 rose 7.5-fold to 448±20 pg/mL/g above basal levels (59±9 pg/ml/g; p<0.0001) and IL-8 production was increased by 3-fold to 294±14 pg/mL/g, as compared to non-impacted control tissue (97±11 pg/mL/g; p<0.0001; Figure 1F).

3.2 α-MSH and [DTRP^8]-γ-MSH prevent articular chondrocyte death caused by impact injury of cartilage explants and reduce pro-inflammatory cytokines production

Previously, we have suggested that melanocortins have a chondroprotective potential due to their ability to reverse TNF-α-induced chondrocyte death [64]. As an addition to this work, we have now investigated the chondroprotective properties of α-MSH and [DTRP^8]-γ-MSH in a model of impact trauma of *in situ* chondrocytes (Figure 2).

Pre-treatment with α-MSH or D[TRP]^8-γ-MSH (3 μg/mL), or dexamethasone (10^{-6}M), significantly diminished chondrocyte death in the injured explants, with improved cell viability of 62% (p<0.05), 66% (p<0.05; Figure 2 A,B) respectively for α-MSH and D[TRP]^8-γ-MSH. The glucocorticoid dexamethasone, used throughout this study as a control, triggered 50% decline in cell death in impacted cartilage explants, compared to untreated impacted counterparts (p<0.05). Impacted tissue, however, benefitted from pre-treatment with α-MSH, [DTRP^8]-γ-MSH and Dex, with marked reductions of IL-1β production by 41%, 50% and 58% respectively (p<0.0001; Figure 2C). Congruently, IL-6 production was also modulated by α-MSH, [DTRP^8]-γ-MSH and Dex, whereby the drugs led to 65%, 71% and 84% reduction in the cytokine production (p<0.0001; Figure 2D) and IL-8 levels were inhibited by 53%, 54% and 65%, respectively (p<0.0001; Figure 2D).

Notably, treatment of non-impacted articular cartilage with α-MSH, D[TRP]^8-γ-MSH or Dex had no detectable effect on chondrocyte viability (Figure 3A, B, E) and did not alter the

production of key pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) within the time point selected for these experiments (Figure 3C-E).

3.3 TNF-α stimulates cytokine and chemokine release from bovine chondrocytes

Stimulation of freshly-isolated primary bovine chondrocytes with TNF-α (0–80 pg/mL) led to significant increases in cytokine release (Table 2). TNF-α caused a concentration-dependent increase in IL-6 with a maximal release observed at 80 pg/ml TNF-α of 347±30 pg/mL (p<0.001), while a bell-shaped response was observed for both IL-1β and IL-8, with 60 pg/mL TNF-α causing a near maximal or maximal release with concentrations of 46±3 pg/mL IL-8 (p<0.01) and 22±0.2 pg/mL IL-1β (p<0.05), compared to untreated controls (0.82±0.06 pg/mL and 2.81±1.46 pg/mL, respectively; Table 2).

3.4 α-MSH and [DTRP^8]-γ-MSH modulate pro-inflammatory cytokine release from TNF-α stimulated primary bovine chondrocytes

Production of IL-1β (Figure 4A) was potently modulated by α-MSH the melanocortin receptor pan-agonist; α-MSH (1-10 μg/mL) reduced the release of the potent pro-inflammatory cytokine by approximately 40-% (p<0.05), when compared to levels released by TNF-α treated chondrocytes; higher concentrations of the peptide (30 μg/mL) were less active, resulting in ~20% reduction (Figure 4A). The MC_3 agonist, [DTRP^8]-γ-MSH (at 3, 10 and 30 μg/mL) potently reduced the production of IL-β, triggering a bell-shaped decrease of 61%, 76% and 39%, respectively (p<0.05). In contrast, the $MC_{3/4}$ antagonist / MC_1 agonist, SHU9119, elicited just a modest inhibition of IL-1β release at 3 μg/mL (23% compared to TNF-α treated controls; Figure 4A).

Markedly, IL-6 levels were significantly modulated by α-MSH with 1 μg/mL causing IL-6 production to be abrogated by 93% (from 174±15 pg/mL to 13±1 pg/mL; p<0.001), with higher concentrations of α-MSH, (3, 10 and 30 μg/mL), prompting a reduced but still significant reduction in the release of IL-6 (87%, 76% and 48%, respectively), compared to TNF-α-treated controls. Similar results were observed following pre-treatment with [DTRP^8]-γ-MSH (1 – 10 μg/mL) with maximal inhibition of 72% at 3 μg/mL, while 1 and 10 μg/mL caused 40% and 48% reduction, respectively (p<0.001). Higher concentrations of [DTRP^8]-γ-MSH (30 μg/mL) produced only a small, statistically insignificant, reduction in cytokine production, whilst SHU9119 failed to inhibit IL-6 release at all concentrations tested (Figure 4B).

[DTRP^8]-γ-MSH attenuated TNF-α-induced IL-8 release (Figure 4C) in a bell-shaped manner, with maximal effect at 3 μg/mL, causing a 69% reduction in IL-8 secretion (p<0.01). At 10 and 30 μg/mL, the peptide reduced the chemokine release by 57% and 45%,

respectively. A similar observation was made for α-MSH (1, 3 and 10 μg/mL), with a 67%, 69% and 56% reduction in IL-8 secretion (p<0.01), whilst at higher concentrations (30 μg/mL) it was largely ineffective (Figure 4C).

3.5 SHU9119 attenuates the inhibitory effects of D[TRP]^8-γ-MSH but not α-MSH on pro-inflammatory cytokine release in TNF-α-activated bovine primary chondrocytes

Freshly-isolated bovine chondrocytes were pre-treated with either α-MSH or [DTRP^8]-γ-MSH (3 μg/mL) in combination with SHU9119 (10 μg/mL) for 30 min prior to 6h stimulation with TNF-α (60 pg/mL). Subsequent ELISA analysis for IL-1β, IL-6 and IL-8 revealed that both peptides potently inhibited the secretion of these mediators at the concentrations used in this study (Figure 5). Interestingly, co-stimulation of SHU9119 with [DTRP^8]-γ-MSH, but not α-MSH attenuated the release of IL-1β, IL-6 and IL-8 by the treated chondrocytes (Figure 5).

3.6 α-MSH and [DTRP]^8-γ-MSH enhance IL-10 production by activated primary chondrocytes

Production of IL-10 was undetectable in both non-stimulated and TNF-α-activated freshly isolated primary bovine chondrocytes (Figure 6). However, pre-treatment with both α-MSH (Figure 6A) and [DTRP^8]-γ-MSH (Figure 6B) enhanced the production of IL-10 above basal levels, with α-MSH (1, 3, 10 μg/mL) causing a release of 21±8 pg/mL, 34±1 pg/mL and 11±1 pg/mL of IL-10 respectively (p<0.05). [DTRP^8]-γ-MSH (1, 3, 10 μg/mL) caused a concentration-dependent increase in IL-10 production with 20±3, 29±3 and 32±5 pg/mL IL-10 detected, respectively (p<0.05). As previously observed the $MC_{3/4}$ antagonist SHU9119 failed to significantly inhibit the effect of α-MSH (Figure 6A) but completely blocked the induction of IL-10 release by [DTRP^8]-γ-MSH (p<0.001; Figure 6B).

4 Discussion

Although the exact pathogenesis of OA is not fully understood, abnormal mechanical stresses including sport injuries and trauma all play a role for development of OA [71]. Blunt trauma to articular cartilage, resulting from accidents or sport injuries are associated with high levels of chondrocyte death and is associated with local inflammatory reactions, thereby representing a major risk factor for the development of post-traumatic OA, which despite constant improvement of surgical techniques, still accounts for ~12% of all cases of OA [72].

Given the significance of trauma for the development of OA, the effect of a single blunt mechanical impact on chondrocyte viability and rates of pro-inflammatory mediator synthesis were determined. Cell death arising from the single impact has been previously revealed to be both temporal and spatial – an initial rapid phase rising from the mechanical trauma occurs at the tissue fissures and a slower wave of cell death takes place away from the impact lesion, thereby suggesting the release of soluble intercellular singalling molecules [73].

This study has taken a particular interest in the events occurring in areas of cartilage that are distant to the impact lesion and therefore not directly affected by the mechanical impact. In non-impacted cartilage, ~3% of chondrocytes were non-viable, possibly due to the excision of the explant from the bone and remained constant in the first 6h post-excision (data not shown). Single blunt impact to–cartilage explants triggered surface fissuring and stress-dependent loss of chondrocytes' viability along tissue cracks (data not shown) [30]. We detected significant 4.6-fold increase in chondrocytes, resident in the morphologically normal area of the impacted cartilage explant. In addition to loss of viability, chondrocytes responded to injury through increasing the production of IL-1β, IL-6 and IL-8 by and 17-, 8- and 3-fold, respectively.

These data clearly demonstrate that chondrocytes in injured cartilage explants are actively producing wide variety of pro-inflammatory cytokines well above basal levels, which may activate neighbouring chondrocytes in an auto and paracrine manner. It is well described that elevated levels of pro-inflammatory cytokines provoke resident chondrocytes to initiate pathological expression and secretion of inflammatory mediators [18, 32] and cartilage-degrading proteases [34, 74]. In addition, excess stimulation of chondrocytes with pro-inflammatory cytokines such as TNF-α and IL-1β induces apoptosis via activation of caspase-driven pathways [64, 75].

Impact injury has been directly associated with increased production of pro-inflammatory cytokines by affected chondrocytes. Abnormal mechanical forces appear to 'awaken' adult

chondrocytes from a state of low metabolic activity and stimulate the production of a large number of pro-inflammatory molecules including TNF-α, IL-1β [18, 32]

We have previously proposed that melanocortin peptides display potent chondroprotective, anti-apoptotic and anti-inflammatory effects through their ability to inhibit TNF-α-induced chondrocyte apoptosis and inflammation [64]. However, in spite of the potential of melanocortin peptides to limit inflammation, few studies have looked at their chondroprotective properties. We have recently demonstrated that both the melanocortin receptor pan-agonist α-MSH and [DTRP^8]-γ-MSH (structures shown in Table 1), which shows selectivity for MC_3, ameliorate TNF-α-induced chondrocyte apoptosis and the release of pro-inflammatory cytokines and MMP's [64]. Now, we describe the ability of the of melanocortins to: 1) prompt a homeostatic control over impact-induced inflammatory cytokine production, 2) confine chondrocyte death and cartilage damage to injury site, whilst 3) preserving the viability of chondrocytes in adjacent non-impacted areas, and 4) prompting the production of anti-inflammatory cytokine IL-10.

α-MSH and [DTRP^8]-γ-MSH improved significantly the survival rates of chondrocytes, resident in mechanically impacted articular cartilage. In fact, both α-MSH and D[TRP]^8-γ-MSH improved chondrocytes survival by approximately 65%, compared to 50% reduction of cell death by the glucorticoid Dex. These findings correlate with the effect of the peptides on cytokine production – the melanocortins potently modulated the production of IL-1β, IL-6 and IL-8 by injured cartilage explants, and these effects were akin to the effect of Dex, which almost completely inhibited IL-6 and IL-8. Whilst the ability of α-MSH and [DTRP^8]-γ-MSH to reduce cytokine relase is well documented both *in vitro* [63, 64] and *in vivo* models of inflammation [60-62, 76], this is the first time their protective properties have been studied in an *in situ* model of mechanically-induced cartilage injury, and the first indication that targeting the melanocortin receptor system for the development of potential treatment of mechanical/sports injuries and trauma is a viable option.

The sensitivity of primary bovine chondrocytes to inflammatory cytokines was further corroborated *in vitro* – significant increase in production of IL-1β, IL-6 and IL-8 by freshly-isolated articular chondrocytes was detected in response to TNF-α (Table 2). This finding has been previously reported in a human chondrocytic cell-line [64] and primary canine chondrocytes [77], whilst *in vivo* these changes lead to destruction of cartilage [78]. α-MSH and D[TRP]^8-γ-MSH, potently modulated the response of the chondrocytes to TNF-α, which is in agreement with the observed anti-inflammatory properties of the peptides in impacted cartilage explants.

In order to associate the observed effects to a specific receptor from the melanocortin family, the peptide SHU9119 was used, which antagonizes with high-specificity MC_3/MC_4, while displaying weak agonist profile towards MC_1/MC_5. Alone, SHU9119 caused a modest inhibition of IL-1β but had no effect on TNF-α-induced IL-6 or IL-8 production, consistent with its dual agonist/antagonist nature. Remarkably, while SHU9119 was unable to block the effect of α-MSH, it abridged the anti-inflammatory activity of D[TRP]^8-γ-MSH, thus suggesting a potential role for MC_3 in chondroprotection, which agrees with previous studies reporting MC_3 agonist activity of this peptide [51, 76, 79]. Moreover, at higher concentrations of α-MSH, the presence of SHU9119 synergistically reduced IL-6 release, thereby signifying that SHU9119 may be concerting efforts with α-MSH *via* either activation of MC_1 and/or MC_5. Since these effects were observed with the highest concentration of α-MSH only, a possibility exists that MC_1 sensitization is causing the receptor to internalize, a well-described feature of GPCRs such as the β_2-adrenergic receptor [80-82], while an upregulation in MC_5 may be a compensatory mechanism. However, the exact mechanism needs to be further elucidated.

An important feature of the melanocortin peptides that has captured the attention of academia and industry alike is their well-described ability to promote resolution of inflammation. They reduce the host's inflammatory response by modulating the production of pro-inflammatory mediators [59, 63, 64], and maybe even more importantly, by actively stimulating the resolution phase of inflammation through inducing IL-10 and heme oxygenase-1, which possess powerful anti-inflammatory and pro-resolving properties [83, 84]. Protective effects have been demonstrated extensively in murine models of gout [59-61] and RA [62]. In addition to these well reported effects in models of arthritis, the melanocortin peptides display a plethora of effects in other disease pathologies For example, in models of cerebral ischemia, α-MSH decreased TNF-α and IL-1 [85], ACTH-derived peptides have been shown to downgrade nitric oxide [86] and free radicals production [79, 87], whilst inducing IL-10 [55] and reducing apoptotic effects [54]. The ability of melanocortins to provoke similar responses in diverse disease models highlights their importance in restoring homeostatic balance to many tissues throughout the entire body. These findings are additionally supported by *in vitro* observations in primary human articular chondrocytes [63] and chondrocytic cell-lines [64].

α-MSH and [DTRP^8]-γ-MSH instilled markedly increased production of IL-10 by freshly-isolated bovine articular chondrocytes with the $MC_{3/4}$ antagonist SHU9119 abrogating the effect of [DTRP^8]-γ-MSH but not α-MSH. To our knowledge this is the first demonstration of melanocortin-induced IL-10 production in primary articular chondrocytes, a significant finding considering the previously reported chondroprotective properties of this cytokine brought about through reduction in *MMP1* and *MMP13* gene expression [74, 88] in TNF-α-stimulated

primary chondrocytes from both OA and healthy human cartilage. The pharmacological data shown emphasize a potential chondroprotective/anti-inflammatory role for MC_1, but could also implicate a role for MC_3 in primary bovine chondrocytes, akin to that observed in human C-20/A4 chondrocytes [64].

In this study, chondrocyte stimulation was conducted prior to impact as an initial attempt to assess the ability of the melanocortins to exert similar protective effects at tissue level (cartilage), as those observed at cellular level using the C20/A4 chondrocyte cell-line [64]. It is important to note that immediately after impact, cartilage explants were returned to media containing melanocortin peptides and left to bathe in it for the duration of the incubation (6h), therefore providing wider window for the peptides to exert effects. Nonetheless, evaluating their effects post-impact is crucial to fully appreciate the therapeutic potential of these compounds for trauma injury and studies aimed at addressing this point have already commenced.

Osteoarthritis resultant from traumatic joint injury is a serious complication, which leaves the injured individuals – frequently young people involved in sports accidents or car crashes – with lifetime of palpable pain, disability, leading to various degree of social isolation. Yet, current treatment options are inadequate, largely focused on pain management and often fail to address articular tissue degeneration. Since mechanical injury to joints leads to cartilage degeneration through chondrocyte death and matrix breakdown, prospective treatments targeting these pathways should be examined.

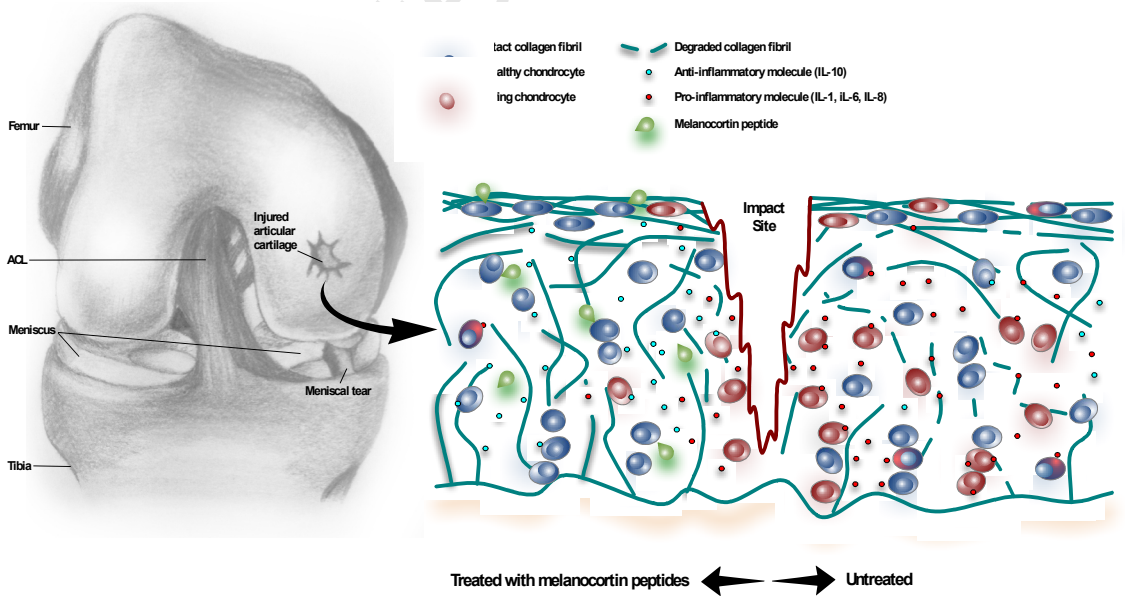

Figure 7.
A human knee joint with injured articular cartilage and meniscus (hand drawing for illustrative purposes only) – melanocortin peptides were able to prevent the progression of trauma-induced chondrocyte death and the consequential propagation of pro-inflammatory

cytokines into non-impacted areas of cartilage explants, all the while promoting the release of reparative pro-resolving molecule.

In this study we describe the rapid response of articular chondrocytes to mechanical trauma – the speedy propagation of cartilage inflammation and chondrocyte death, and accentuate on the ability of melanocortin peptides α-MSH and [DTRP^8]-γ-MSH to temper this response. We report that activation of both MC_1 and MC_3 receptor subtypes prevents the progression of trauma-induced chondrocyte death and the consequential propagation of pro-inflammatory cytokines into non-impacted areas of cartilage, all the while promoting the release of reparative pro-resolving molecules (Figure 7). Altogether, we propose that melanocortins could provide novel chondroprotective therapies for the prevention and treatment of post-traumatic osteoarthritis.

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Conflict of Interest

PG holds a patent on the MC_3 agonist.

Author Contributions

MKK, SJG, MJPK, ICL designed the concept of the study. MKK designed and performed all relevant experiments, collected, analysed and interpreted the data and wrote the manuscript. SJG and GPC provided conceptual advice, technical support and edited the manuscript at every stage. MJPK advised on confocal microscopy experimental design and provided conceptual support. All authors discussed the results and implications and commented on the manuscript at all stages. PG provided [DTRP^8]-γ-MSH.

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Figure Legends

Figure 1. Single blunt impact to cartilage explant causes chondrocyte death and increased production of pro-inflammatory cytokines.

Pre-weighted bovine articular cartilage explants were exposed to a single blunt impact delivered by a vertical drop tower. Impacted (A) and non-impacted (B) cartilage explants were stained with 5 μM Calcein-AM (green; viable cells) and 1 μM propidium Iodine (red; dying cells) for 30 min prior to CLSM. The projected images were used in Imaris 7.1.1 Spot Analysis software to determine the proportion of dead cells as a percentage of total cell number (C). Supernatants were collected and analysed for IL-1β (D), IL-6 (E) and IL-8 (F) Data are presented as Mean ± SEM of n=4 individual experiments under each condition, repeated in quadruplicate, *p<0.05, **p<0.01, ***p<0.0001 vs. non-impacted controls.

Figure 2. α-MSH and [DTRP^8]-γ-MSH prevent impact-induced articular chondrocyte death and inhibit pro-inflammatory cytokine release.

Pre-weighted bovine articular cartilage explants were stimulated with α-MSH or [DTRP^8]-γ-MSH (3.0 μg/mL), or dexamethasone (10^{-6} M) for 30 min prior to delivery of a single blunt impact and returned to incubate with the drugs for 6h. Explants were then stained with Calcein-AM (5 μM) and PI (1 μM) for 30 min prior to CLSM. Acquired series of images were used in Imaris 7.1.1 Spot Analysis software to determine the proportion of dead cells as a percentage of total cell number (A, B). Supernatants were collected and analysed for IL-1β (C), IL-6 (D) and IL-8 (E). Dashed line represents levels detected in non-impacted samples. Data are presented as Mean ± SEM of n=4 individual experiments under each condition, repeated in quadruplicate, *p<0.05, **p<0.01, ***p<0.0001 vs. impacted controls.

Figure 3. α-MSH and [DTRP^8]-γ-MSH does not affect viability of and cytokine production from chondrocytes in non-impacted cartilage explants.

Pre-weighted bovine articular cartilage explants were stimulated with α-MSH or [DTRP^8]-γ-MSH (3.0 μg/mL), or dexamethasone (10^{-6} M) for 6h. Explants were then stained with Calcein-AM (5 μM) and PI (1 μM) for 30 min prior to CLSM. Acquired series of images were used in Imaris 7.1.1 Spot Analysis software to determine the proportion of dead cells as a percentage of total cell number (A, B). Supernatants were collected and analysed for IL-1β (C), IL-6 (D) and IL-8 (E). Dashed line represents levels detected in non-impacted samples. Data are presented as Mean ± SEM of n=4 individual experiments under each condition, repeated in quadruplicate, *p<0.05, **p<0.01, ***p<0.0001 vs. impacted controls.

Figure 4. α-MSH and [DTRP^8]-γ-MSH inhibit pro-inflammatory cytokine release from TNF-α stimulated primary bovine chondrocytes.

Freshly isolated primary articular bovine chondrocytes were treated for 30 min with SHU9119, α-MSH or [DTRP^8]-γ-MSH (1 – 10 μg/mL) prior to 6h stimulation with TNF-α (60 pg/ml) and cell-free supernatants were collected and analysed for IL-1β (A), IL-6 (B) and IL-8 (C) concentration by ELISA. Dotted line represents untreated controls, whilst dashed line represents TNF-α stimulation alone. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p<0.05, **p<0.01, ***p<0.0001 vs. TNF-α alone.

Figure 5. SHU9119 antagonises [DTRP^8]-γ-MSH but not α-MSH inhibition of IL-1β, IL-6 and IL-8 release from TNF-α stimulated articular bovine chondrocytes.

Isolated primary bovine chondrocytes were left untreated or pre-incubated for 1h with SHU9119 (10.0 μg/mL) prior to α-MSH or [DTRP^8]-γ-MSH (3.0 μg/mL) treatment for 30 min. Cells were then stimulated with TNF-α (60 pg/mL) and cell-free supernatants collected 6h post-stimulation and analysed for IL-1β (A), IL-6 (B) and IL-8 (C) levels by ELISA. Dashed line represents TNF-α stimulated controls. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p<0.05, **p<0.01, ***p<0.0001 vs. TNF-α alone.

Figure 6. α-MSH and [DTRP^8]-γ-MSH stimulate IL-10 release from freshly isolated articular bovine chondrocytes.

Freshly Isolated primary bovine chondrocytes were left untreated or were pre-incubated for 1h with SHU9119 (10.0 μg/mL) prior to α-MSH or [DTRP^8]-γ-MSH (1-10 μg/mL) treatment for 30 min. Cells were then stimulated for 6h with TNF-α (60 pg/mL) and cell-free supernatants were analysed for IL-10 release by ELISA. TNF-α did not initiate production of IL-10. Data are presented as Mean ± SEM of n=4 independent experiments repeated in triplicate, *p<0.05, **p<0.01, ***p<0.0001 vs. TNF-α treated controls.

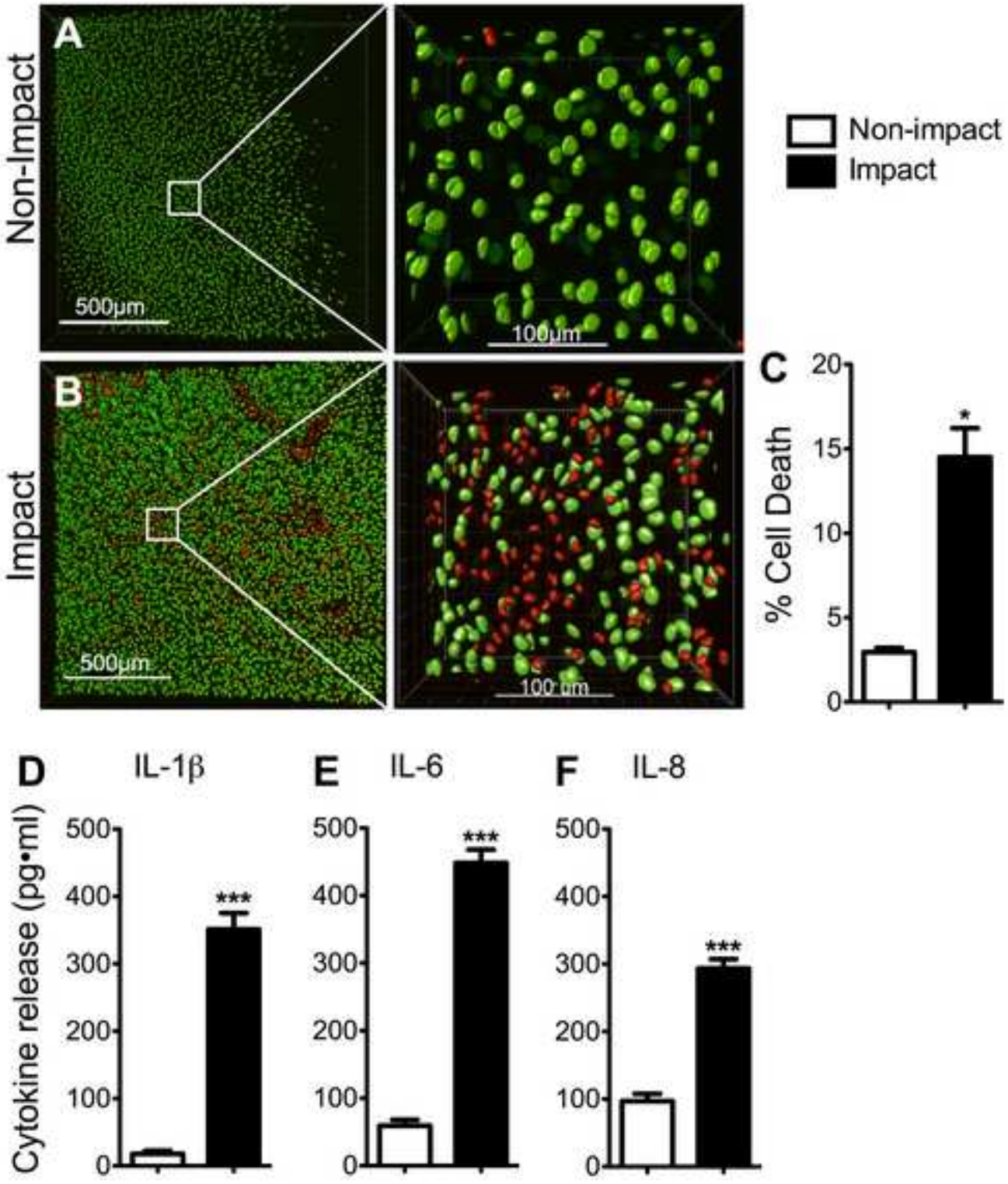
Figure 7.

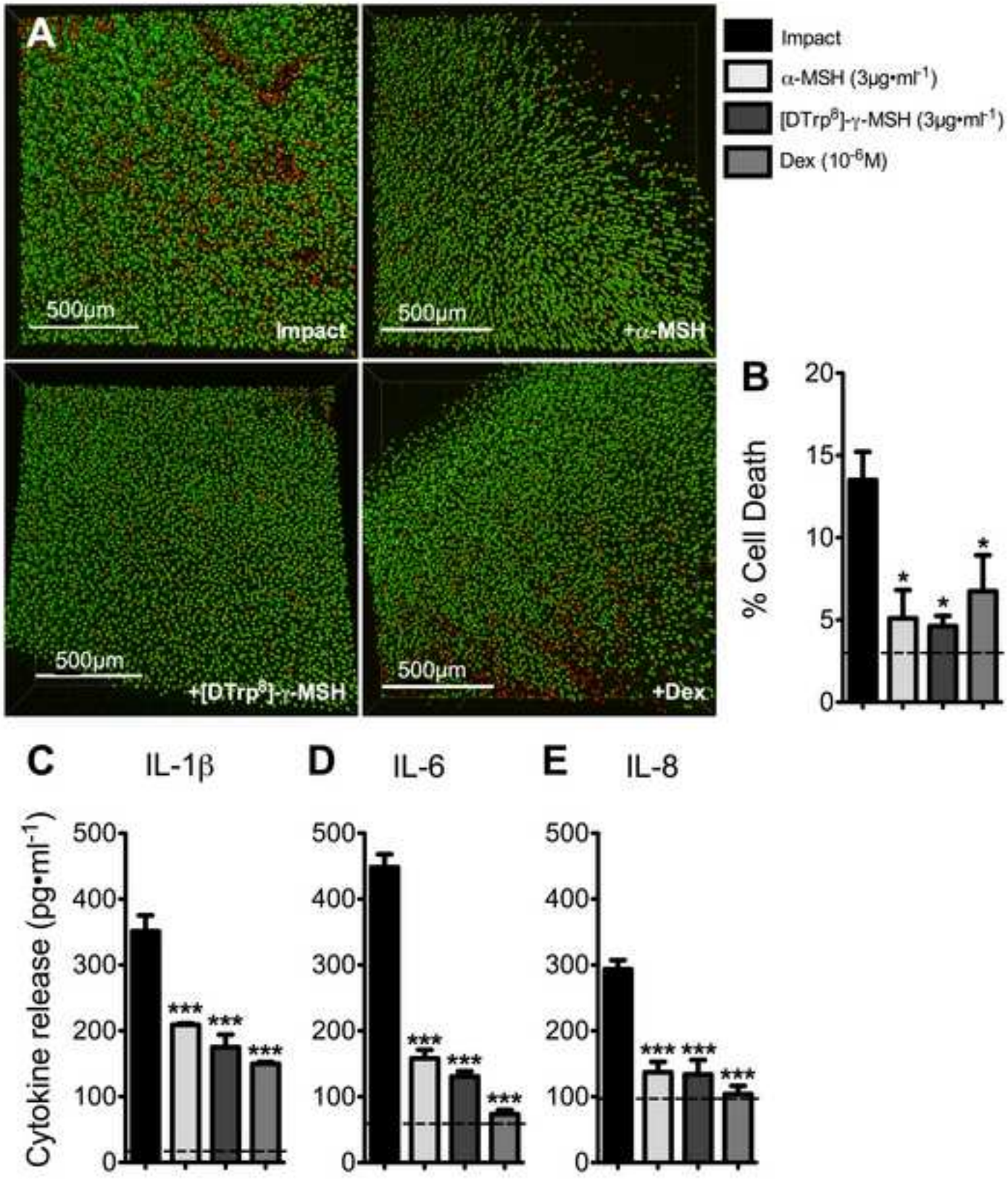
A human knee joint with injured articular cartilage and meniscus (hand drawing for illustrative purposes only) – melanocortin peptides were able to prevent the progression of trauma-induced chondrocyte death and the consequential propagation of pro-inflammatory cytokines into non-impacted areas of cartilage explants, all the while promoting the release of reparative pro-resolving molecule.

Table 2. TNF-α activates IL-6, IL-8 and IL-1□□release from freshly isolated bovine chondrocytes.

Stimulus	IL-1β release ($pg \cdot mL^{-1}$)	IL-6 release ($pg \cdot mL^{-1}$)	IL-8 release ($pg \cdot mL^{-1}$)
Untreated	0.82 ± 1.06	3.20 ± 1.38	2.81 ±1.46
TNF-α (20 $pg \cdot mL^{-1}$)	17.06 ±1.66*	38.54 ± 9.85*	4.44 ± 5.53
TNF-α (40 $pg \cdot mL^{-1}$)	17.40 ±1.23*	78.59 ± 13.68**	46.67 ± 13.65*
TNF-α (60 $pg \cdot mL^{-1}$)	21.87 ± 1.20*	174.33 ± 14.60***	45.55 ± 2.94**
TNF-α (80 $pg \cdot mL^{-1}$)	17.92 ± 1.26*	347.07 ± 29.79***	29.89 ± 3.91*

Isolated primary bovine chondrocytes were stimulated with TNF-α (0 – 80 pg/mL). Cell-free supernatants were collected 6h post-stimulation and analysed for IL-1β, IL-6 and IL-8 by ELISA. Data are presented as Mean ± SEM of n = 4 independent experiments repeated in triplicate, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ vs. untreated controls.





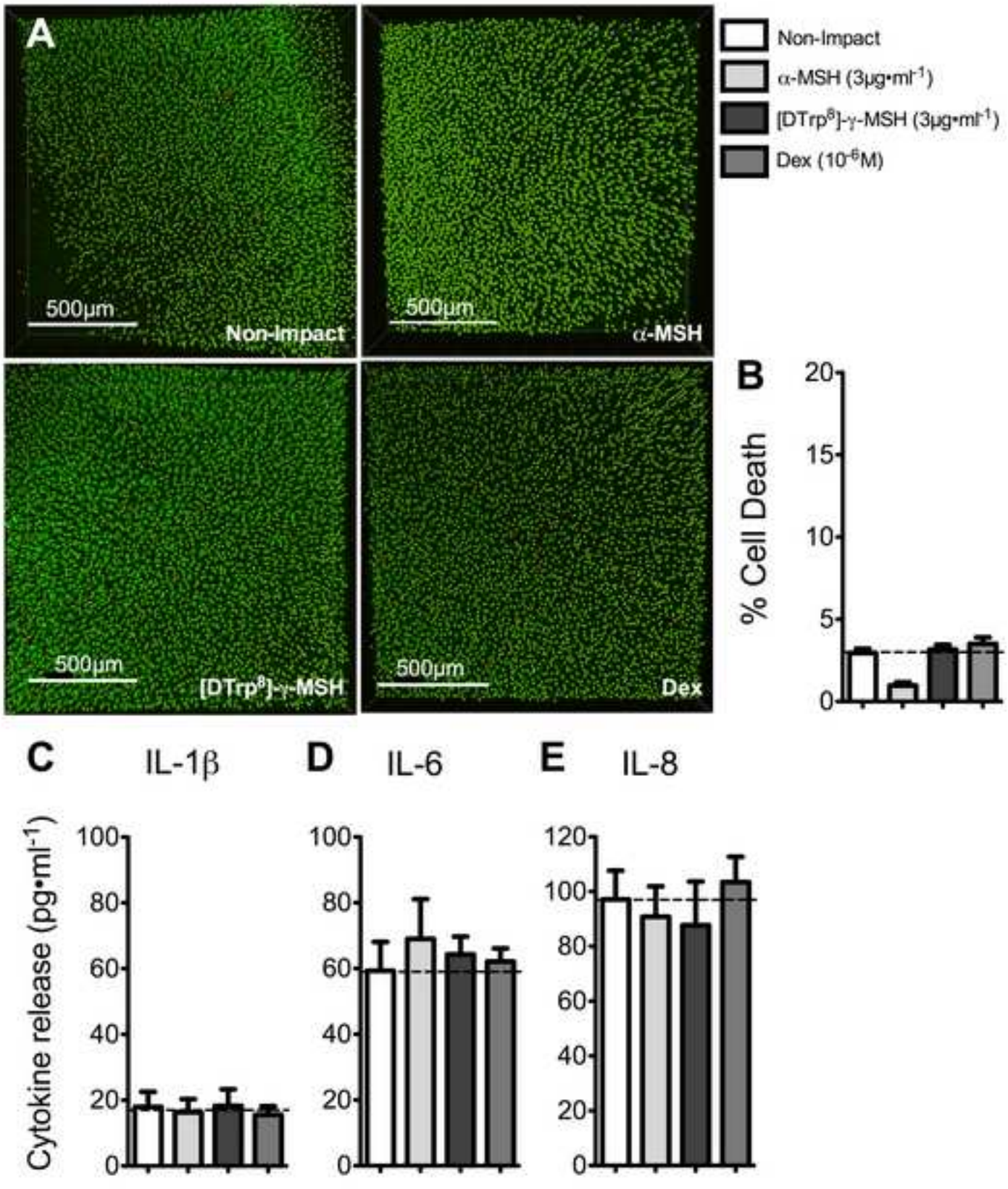
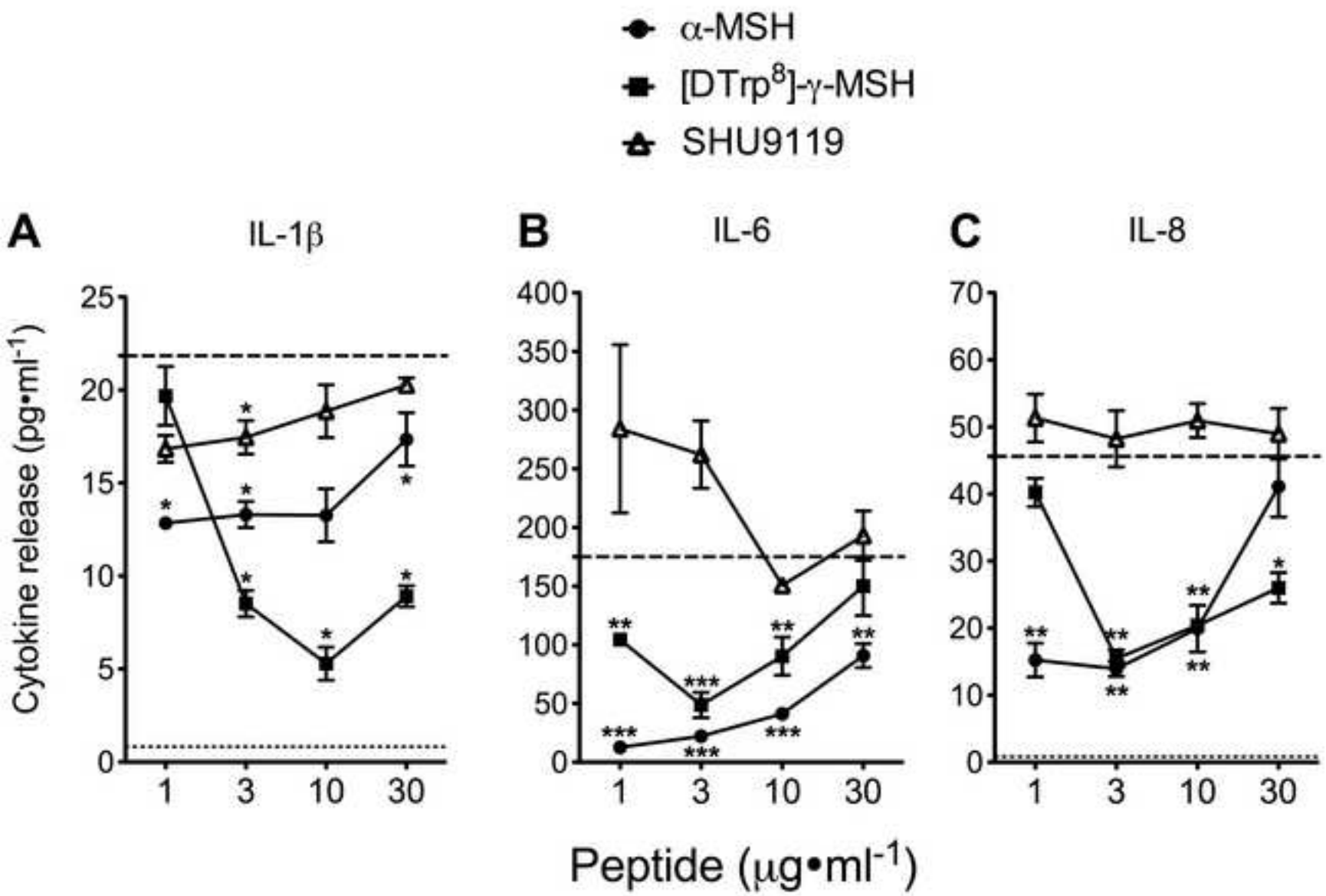


Figure 4


α-MSH
[DTrp8]-γ-MSH
SHU9119
A
IL-1β
B
IL-6
C
IL-8
Cytokine release (pg•ml-1)
Peptide (μg•ml-1)
0
5
10
15
20
25
50
100
150
200
250
300
350
400
10
20
30
40
50
60
70
1
3
10
30

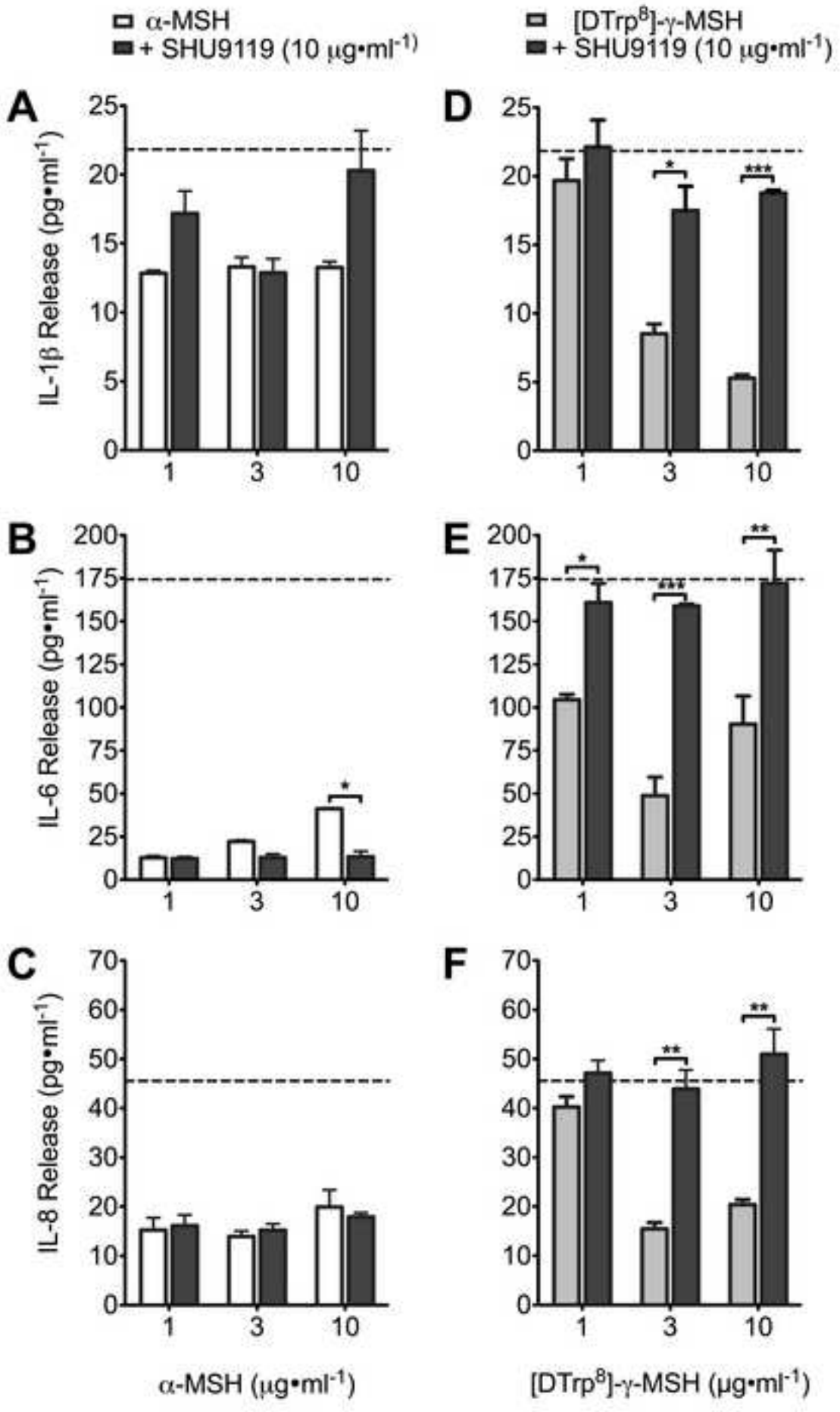


Figure 6

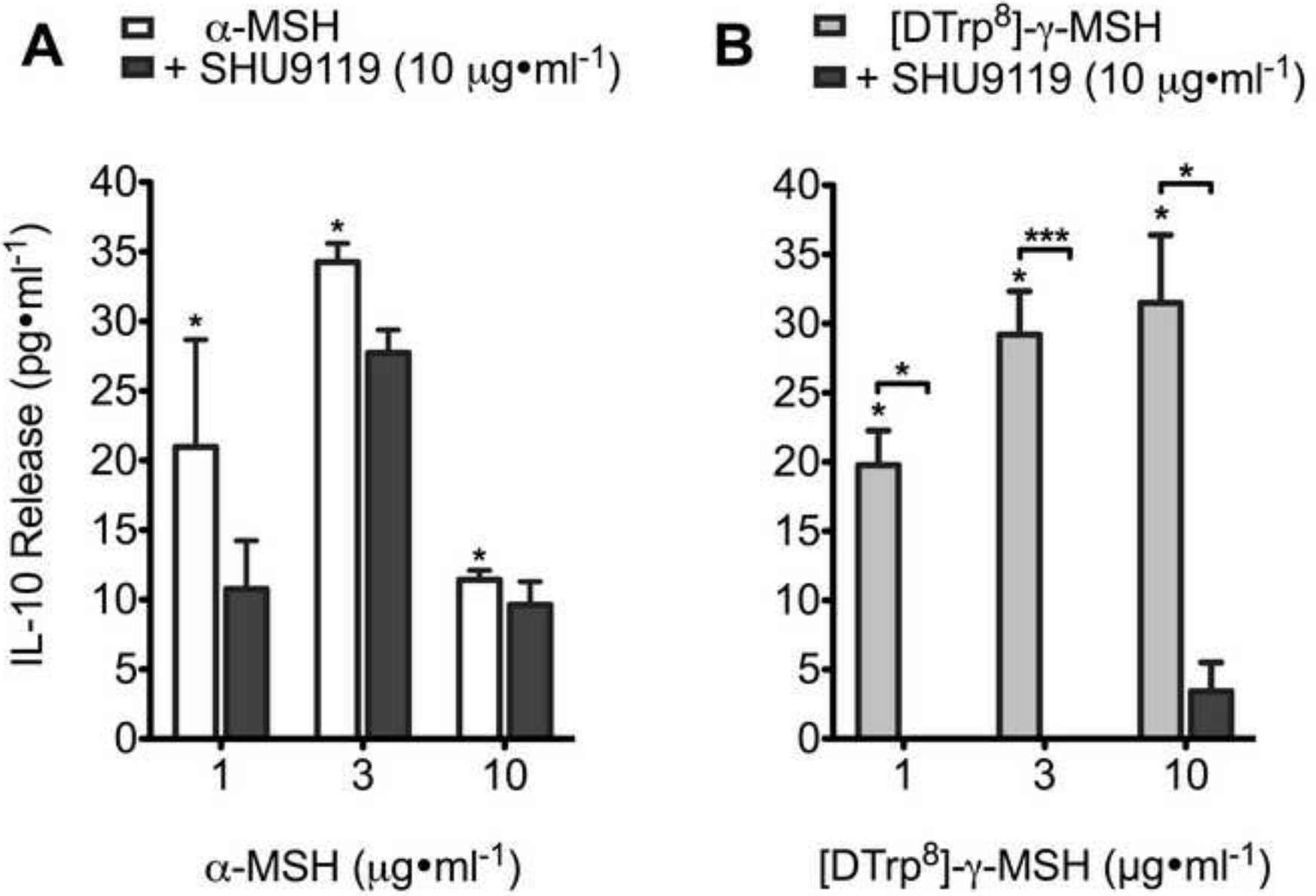
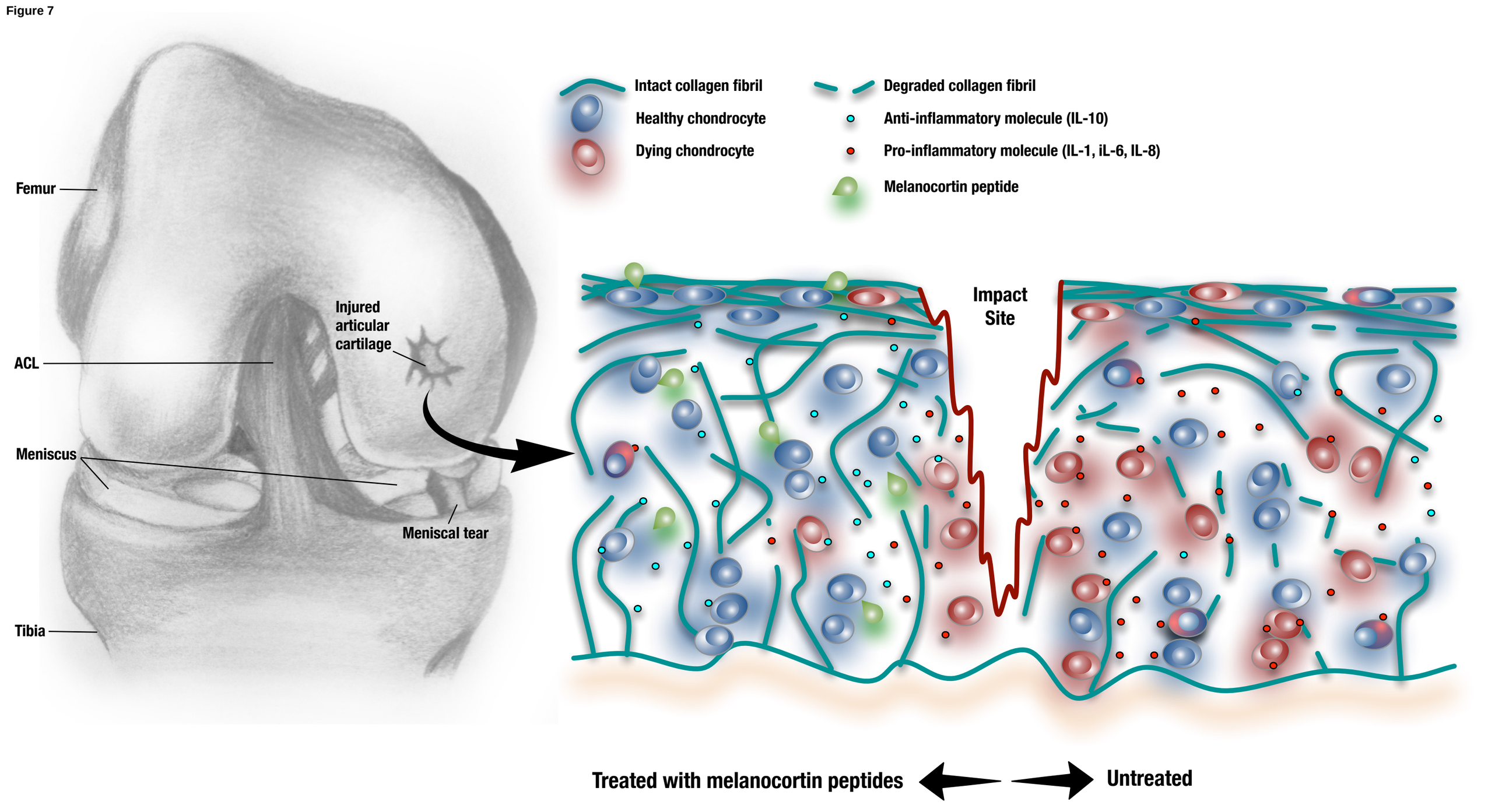
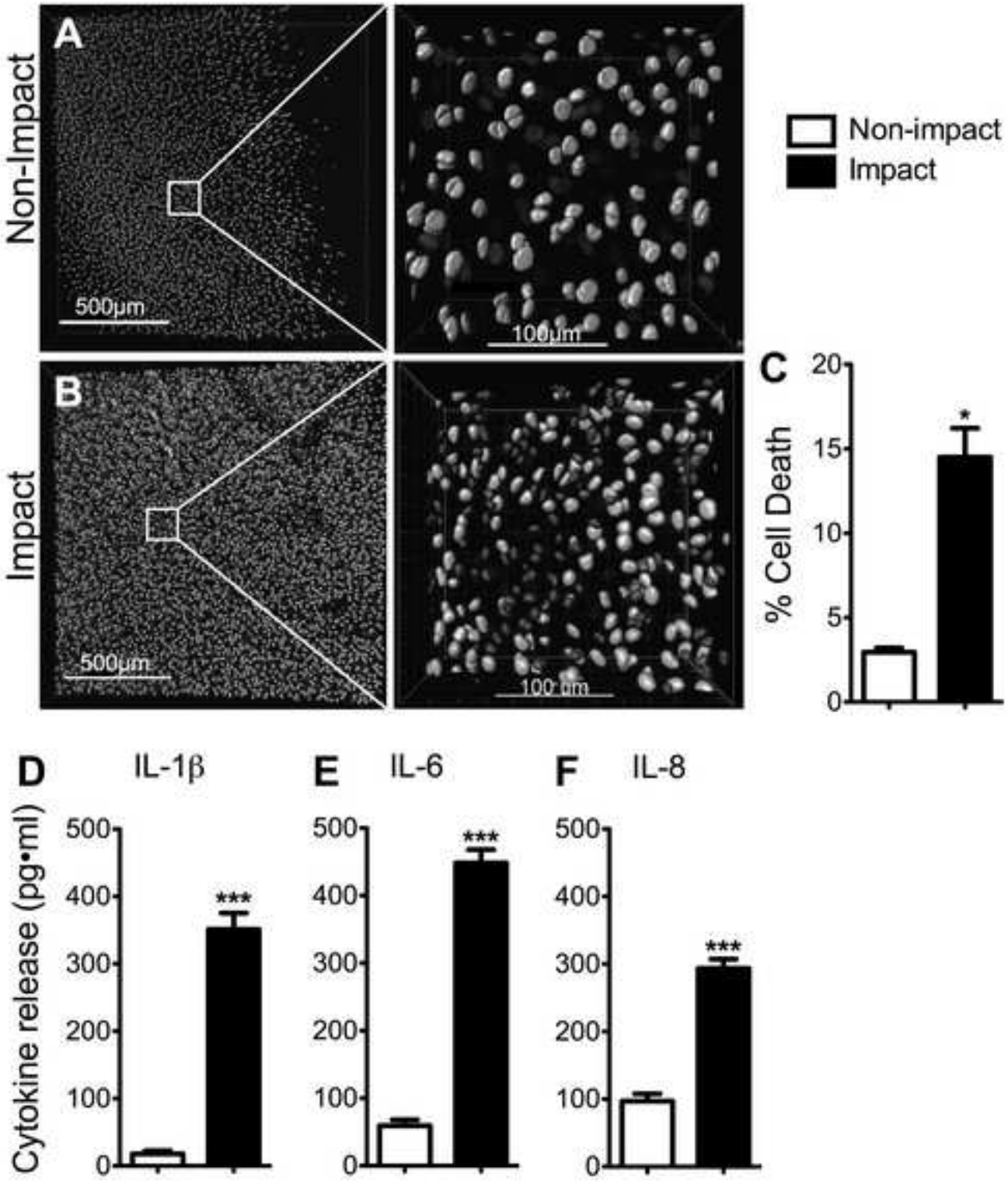
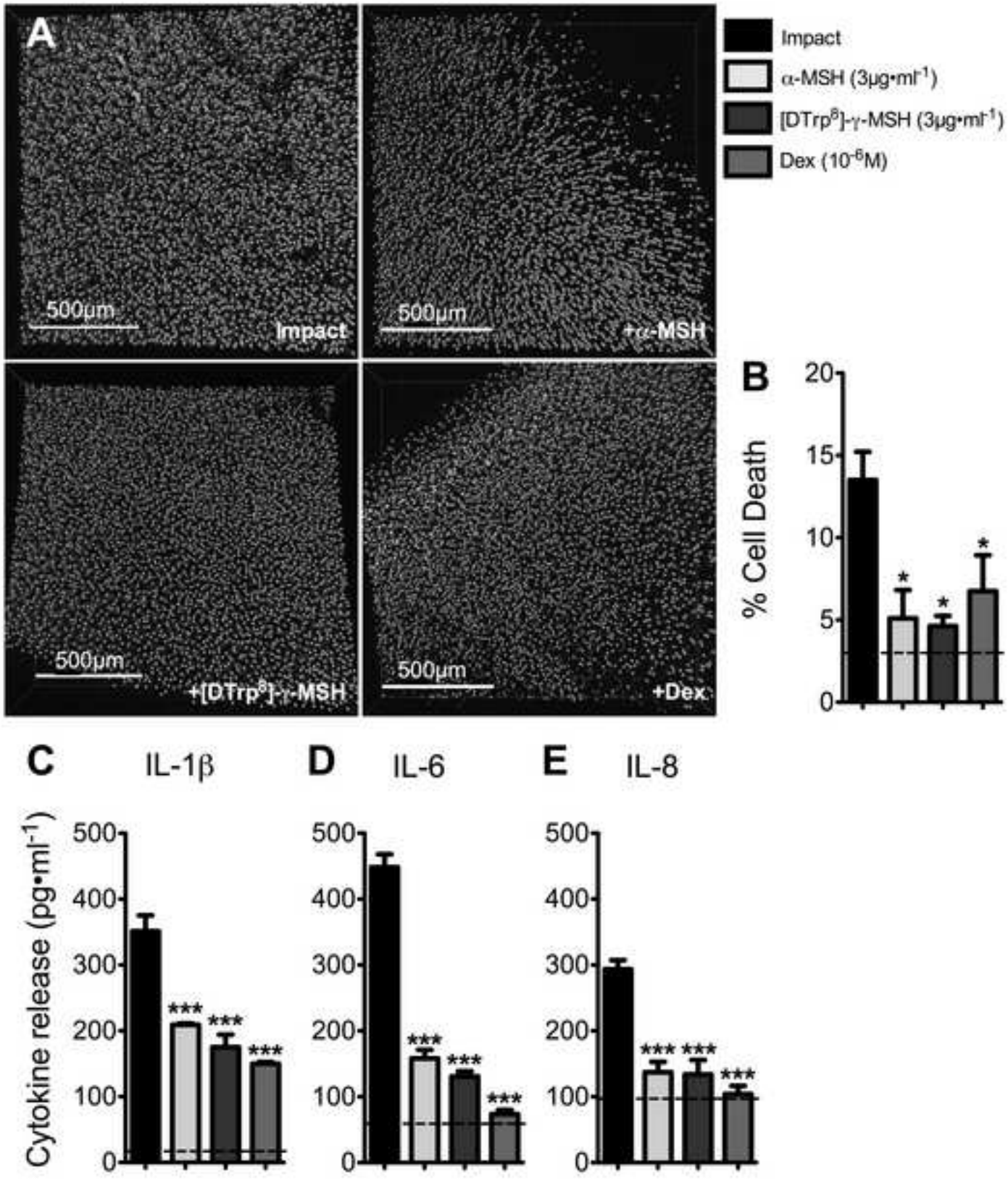

A
α-MSH
+ SHU9119 (10 μg•ml-1)
IL-10 Release (pg•ml-1)
40
35
30
25
20
15
10
5
0
1
3
10
α-MSH (μg•ml-1)
B
[DTrp8]-γ-MSH
+ SHU9119 (10 μg•ml-1)
***
[DTrp8]-γ-MSH (μg•ml-1)

Figure 7


Intact collagen fibril
Degraded collagen fibril
Healthy chondrocyte
Anti-inflammatory molecule (IL-10)
Dying chondrocyte
Pro-inflammatory molecule (IL-1, iL-6, IL-8)
Melanocortin peptide
Femur
Injured articular cartilage
ACL
Meniscus
Meniscal tear
Tibia
Impact Site
Treated with melanocortin peptides
Untreated




A
500µm
Impact
500µm
+α-MSH
500µm
+[DTrp8]-γ-MSH
500µm
+Dex
Impact
α-MSH (3µg•ml-1)
[DTrp8]-γ-MSH (3µg•ml-1)
Dex (10-6M)
B
% Cell Death
20
15
10
5
0
C
IL-1β
D
IL-6
E
IL-8
Cytokine release (pg•ml-1)
500
400
300
200
100
0
***

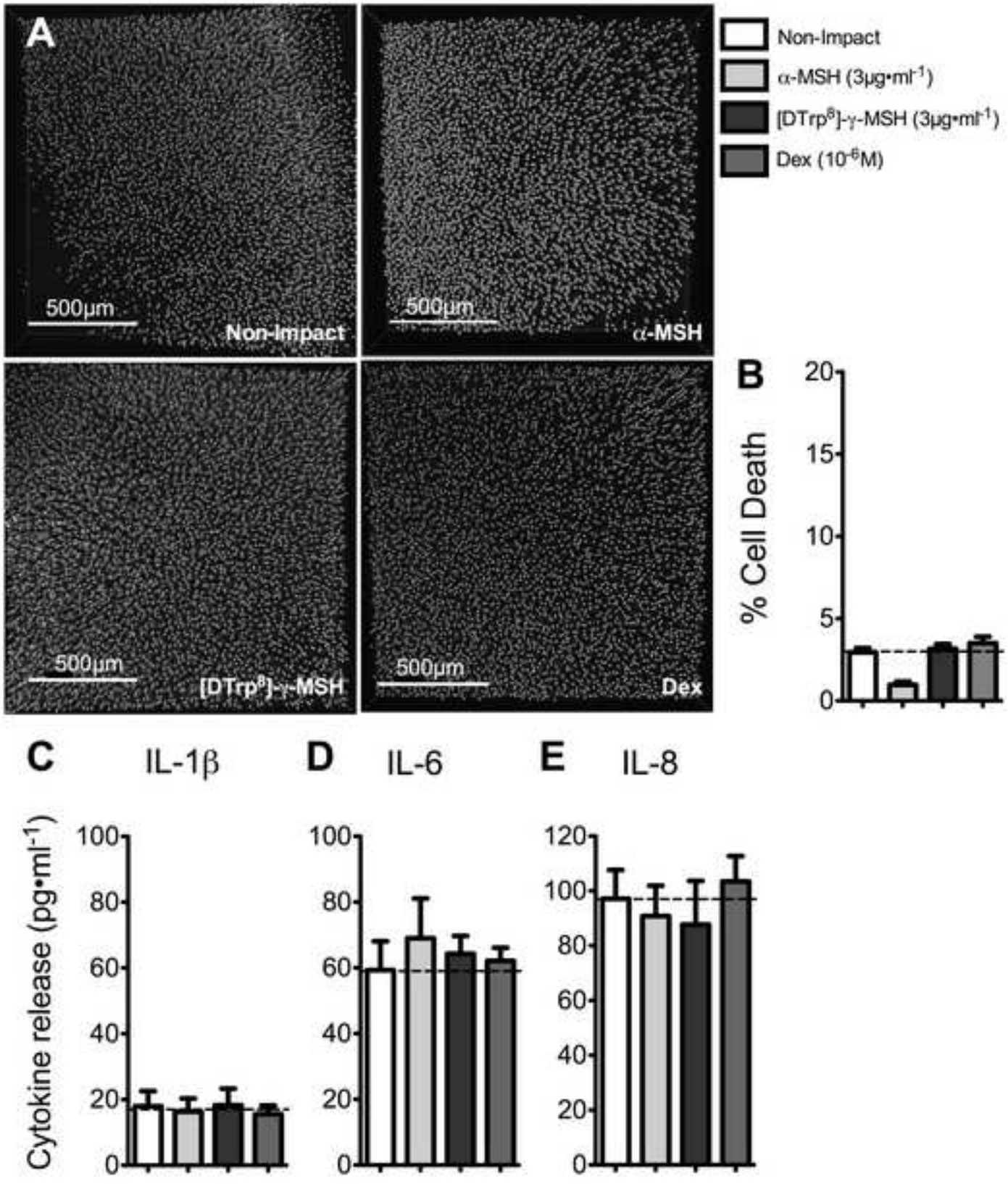

A
500µm
Non-Impact
500µm
α-MSH
500µm
[DTrp8]-γ-MSH
500µm
Dex
Non-Impact
α-MSH (3µg•ml-1)
[DTrp8]-γ-MSH (3µg•ml-1)
Dex (10-6M)
B
% Cell Death
0
5
10
15
20
C
IL-1β
D
IL-6
E
IL-8
Cytokine release (pg•ml-1)
0
20
40
60
80
100
120

Figure 7 Grayscale for printing