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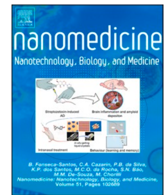

Original Article

Synthetic peptides of IL-1Ra and HSP70 have anti-inflammatory activity on human primary monocytes and macrophages: Potential treatments for inflammatory diseases

Alba Pensado-López, PhD [a], Aldo Ummarino, PhD Student [a,b], Sophia Khan, PhD [c], Anna Guildford, PhD [c], Iain U. Allan, PhD [c], Matteo Santin, PhD [d], Nathalie Chevallier, PhD [e], Elina Varaillon, MSc, PhD [e], Elizaveta Kon, MD [a,b], Paola Allavena, MD [b], Fernando Torres Andón, PhD [a,f,*]

[a] *Department of Biomedical Sciences, Humanitas University, 20072 Pieve Emanuele, Milan, Italy*
[b] *IRCCS Humanitas Research Hospital, Rozzano, 20089, Milan, Italy*
[c] *Tissue Click Ltd, The Knoll Business Centre, Old Shoreham Rd, Hove, BN3 7GS, UK*
[d] *Centre for Regenerative Medicine and Devices, School of Applied Sciences, University of Brighton, Huxley Building Lewes Road, Brighton BN 24GJ, UK*
[e] *IMRB, U955, INSERM, Unite d'Ingenierie et de Therapie Cellulaire-Etablissement Français du Sang, Universite Paris-EST Créteil, 94017 Créteil, France*
[f] *Instituto de Investigación Biomédica de A Coruña (INIBIC), Medical Oncology Unit, Complexo Hospitalario de A Coruña (CHUAC), 15006 A Coruña, Spain*

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ABSTRACT

Chronic inflammatory diseases are increasing in developed societies, thus new anti-inflammatory approaches are needed in the clinic. Synthetic peptides complexes can be designed to mimic the activity of anti-inflammatory mediators, in order to alleviate inflammation. Here, we evaluated the anti-inflammatory efficacy of tethered peptides mimicking the interleukin-1 receptor antagonist (IL-1Ra) and the heat-shock protein 70 (HSP70). We tested their biocompatibility and anti-inflammatory activity *in vitro* in primary human monocytes and differentiated macrophages activated with two different stimuli: the TLR agonists (LPS + IFN-γ) or Pam3CSK4. Our results demonstrate that IL-1Ra and HSP70 synthetic peptides present a satisfactory biocompatible profile and significantly inhibit the secretion of several pro-inflammatory cytokines (IL-6, IL-8, IL-1β and TNFα). We further confirmed their anti-inflammatory activity when peptides were coated on a biocompatible material commonly employed in surgical implants. Overall, our findings support the potential use of IL-1Ra and HSP70 synthetic peptides for the treatment of inflammatory conditions.

Abbreviations: ATP, adenosine triphosphate; CCL2, chemokine (C—C motif) ligand 2; CXCL8, chemokine (C-X-C motif) ligand 8; DAMPs, danger-associated molecular patterns; DCs, dendritic cells; DIPEA, N,N-diisopropylethylamine; DMF, dimethylformamide; FBS, fetal bovine serum; FTIR, fourier-transform infrared spectroscopy; HBTU, 2-benzotriazole-N,N,N0,N0-tetramethyl-uronium-hexafluoro-phosphate; HMDMs, human monocyte derived macrophages; hMSCs, human mesenchymal stem cells; hrIFN-γ, human recombinant interferon-gamma; hrM-CSF, human recombinant macrophage-colony stimulating factor; HSP, heat-shock protein 70; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; MS, mass spectrometry; MW, molecular weight; NPs, nanoparticles; NSAIDs, non-steroidal anti-inflammatory drugs; Pam3CSK4, Pam3CysSerLys4; PAMPs, pathogen-associated molecular patterns; PBMCs, peripheral blood mononuclear cells; PCU, polycarbonate urethane; PRRs, pattern recognition receptors; RA, rheumatoid arthritis; TGF-β, transforming growth factor beta; TLRs, toll-like receptors; TNFα, tumor necrosis factor-alfa.

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* Corresponding author at: Instituto de Investigación Biomédica de A Coruña (INIBIC), Medical Oncology Unit, Complexo Hospitalario de A Coruña (CHUAC), As Xubias s/n, 15006 A Coruña, Spain.

E-mail addresses: alba.pensadolopez@humanitasresearch.it (A. Pensado-López), aldo.ummarino@hunimed.eu (A. Ummarino), s.khan@tissueclick.com (S. Khan), a.guildford@tissueclick.com (A. Guildford), i.allan@tissueclick.com (I.U. Allan), m.santin@brighton.ac.uk (M. Santin), Nathalie.CHEVALLIER@efs.sante.fr (N. Chevallier), elina.varaillon@gmail.com (E. Varaillon), elizaveta.kon@humanitas.it (E. Kon), paola.allavena@hunimed.eu (P. Allavena), fernando.torres.andon@sergas.es (F. Torres Andón).

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Introduction

Background

Inflammation is a defensive immune response triggered by "danger signals", such as infection or tissue injury, which acts by removing harmful pathogens and initiating the healing process and tissue repair.[1] Acute inflammation is an indispensable mechanism to maintain and restore homeostasis.[2] Nevertheless, uncontrolled acute inflammatory responses can become chronic due to social, psychological, environmental and biological factors, leading to chronic inflammatory diseases: including autoimmune, neurodegenerative, metabolic and vascular diseases, as well as cancer.[3] Acute inflammation is typically initiated by tissue-resident macrophages. By the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) through pattern recognition receptors (PPRs), such as Toll-like receptors (TLRs),[4] macrophages acquire a M1 pro-inflammatory phenotype (classically activated) and release pro-inflammatory cytokines: including tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6), and chemokines such as chemokine (C—C motif) ligand 2 (CCL2) or chemokine (C-X-C motif) ligand 8 (CXCL8 or interleukin 8, IL-8).[5–7] Monocytes, originating in the bone marrow, migrate *via* the blood stream and are recruited by the pro-inflammatory cytokines to the site of inflammation, where they differentiate to macrophages with a M1 pro-inflammatory phenotype, thus contributing to local and systemic inflammation.[8,9] In "healthy" circumstances, acute inflammation is followed by its resolving phase characterized by a reduction in the amount of DAMPs and PAMPs, and the phagocytosis of "dying cells" and associated debris. Along the course of this phase, the phenotype of the macrophages switches from pro-inflammatory to anti-inflammatory (M2 macrophages or alternatively activated) resulting in the termination of pro-inflammatory mediator synthesis and the catabolism of the remaining mediators, accompanied by secretion of reparative molecules such as interleukin 10 (IL-10), interleukin 4 (IL-4) or transforming growth factor beta (TGF-β).[10,11] Nevertheless, the eventual failure of macrophages to polarize from a pro-inflammatory M1 phenotype towards an anti-inflammatory M2 one, or the high numbers of M1 *versus* M2 macrophages can lead to chronic inflammation.[12]

Current treatments for chronic and acute inflammation include steroidal and non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids or immunosuppressant drugs, although clinical outcomes are variable.[13] To improve anti-inflammatory efficacy and prevent side effects of these drugs, several nanotechnology approaches have been investigated for their conjugation or encapsulation into nanocarriers, offering a targeted delivery of the active agent to the site of inflammation.[14–16] Other approaches include the design of nanoparticles (NPs) to directly kill M1 macrophages, to inhibit their pro-inflammatory properties or their products and also to promote an anti-inflammatory reparative M2 phenotype.[17] Reprogramming macrophage phenotype from M1 towards M2 has been achieved by conjugating nanocarriers with DNA plasmids encoding anti-inflammatory cytokines (*e.g.* IL-10) or M2 markers (*e.g.* CD163),[18,19] or loading NPs with mRNAs to achieve cell specific expression of anti-inflammatory cytokines (*e.g.* IL-10),[20] or with miRNAs (*e.g.* miRNA-21) to improve the resolution of inflammation.[21] The production of pro-inflammatory cytokines has also been inhibited by NPs loaded with siRNAs (*e.g.* anti-TNFα) or miRNAs targeting macrophages.[22,23] Another strategy entails the generation of molecules mimicking the binding and/or functional sites of proteins involved in the inflammatory signaling. A few synthetic peptide complexes mimicking proteins have been manufactured with high reproducibility, while retaining bioactive or biomimicry properties[24] and reducing the risk of immunogenicity.[25] However, not many peptides mimicking natural proteins with anti-inflammatory activity have been investigated.[26–29]

The aim of our study was the *in vitro* evaluation of tethered peptides designed to mimic the IL-1 receptor antagonist (IL-1Ra) or the heat-shock protein 70 (HSP70). The inhibition of the IL-1R using antagonists has shown to alleviate inflammation by prevention of IL-1 release, and subsequent downregulated secretion of related cytokines (*e.g.* IL-6 or IL-8).[30,31] HSPs, and in particular HSP70, have been described as attenuators of inflammation *in vitro,* and also in rheumatoid arthritis (RA) models *in vivo*, through downregulation of pro-inflammatory cytokines secretion (*e.g.* TNFα, IL-6 or IL-8).[32–34] Interestingly, HSP70 overexpression has shown to inhibit the inflammasome activation, reducing production of IL-1β.[35] This scientific evidence encourages a dedicated study on the anti-inflammatory activity of peptides mimicking IL-1Ra or HSP70.

Therefore, we synthetized and investigated two tethered peptides mimicking IL-1Ra or HSP70, using three *in vitro* models to test their biocompatibility and anti-inflammatory activity directly towards primary human monocytes, primary human monocyte-derived macrophages (HMDMs) or during the differentiation of monocytes towards macrophages. Additionally, given the immunomodulatory activity of human Mesenchymal Stem Cells (hMSCs),[36] the interaction of the IL-1Ra and HSP70 peptides with these cells was also explored (Supplementary Data). Finally, as a proof-of-concept, to test the potential biomedical application of the IL-1Ra and HSP70 peptides, we performed a series of experiments to validate their anti-inflammatory activity upon coating a biocompatible material (Polycarbonate Urethane, PCU) widely used for prosthesis.[37,38]

Methods

Synthesis and characterization of IL-1Ra and HSP70 peptides

The peptides were developed based on solid phase peptide synthesis protocols as previously described.[39] Briefly, resin swollen in dimethylformamide (DMF, Fisher-Scientific, UK) was washed and coupled with rink amide linker (Sigma-Aldrich, UK) dissolved in DMF supplemented with 2-benzotriazole-N,N,N0,N0-tetramethyl-uronium-hexafluoro-phosphate (HBTU, Cambridge Reagents, UK) and N,N-diisopropylethylamine (DIPEA, Fisher Scientific, UK). Upon completion of coupling, piperidine was used to remove the protecting groups and the desired peptide sequence was added. The amino acid sequence of the IL-1Ra peptide is LPLAYPQWYYANSEEWTFPTE. The sequence of the HSP70 peptide is ITDAVPYFN. In both cases, the peptide sequences were covalently bound to a linear poly-L-Lysine through an NHS/EDC reaction, and the presentation of these sequences to the cells was maximized by interposing a spacer of two Glycine molecules in between the side amino group of the poly-L-Lysine and any of the two sequences. Poly-L-Lysine side amino groups were functionalized at 1 % mol/mol. Upon completion of synthesis, each peptide complex was dried and cleaved from the resin at room temperature. Before grafting to the modified backbone, the peptides were characterized by mass spectrometry (MS)[40] (see Supplementary Fig. S1). The final peptide modified polymers were characterized by FTIR (Fig. 1a–d).

Isolation of primary human monocytes from healthy blood donors

Human monocytes were isolated from whole blood of healthy donors (buffy coats) using the Ficoll-Percoll separation method, as previously described.[41] Monocytes were counted with Trypan Blue staining 0.04 % using a counting chamber (Kova International, BVS100H) and directly used as described in Section 2.4., or differentiated to macrophages as described in Section 2.5.

Pre-coating of wells with IL-1Ra and HSP70 tethered peptides

Lyophilized IL-1Ra and HSP70 peptides were hydrated in ethanol 75 % to a final concentration of 0.1 mg/ml and subsequently filtered with 0.22 μm filters (Sartorius, 17,597-K), according to manufacturer's indications. 24-well plates (24MWs) were pre-coated with 200 μl/ well of

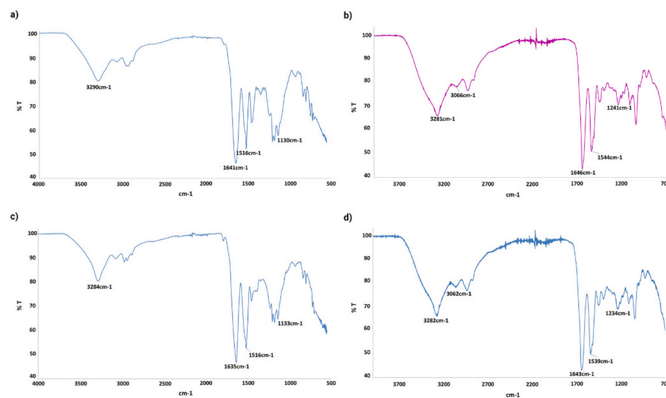

Fig. 1. FTIR of IL-1Ra and HSP70 peptides. Sample FTIR trace of a) the IL-1Ra peptide without the modified backbone; b) the complete IL-1Ra peptide complex; c) the HSP70 peptide without the modified backbone and; d) the complete HSP70 peptide complex.

IL-1Ra or HSP70 peptides and further set under UV irradiation in a flow cabinet for 3 h to allow the evaporation of ethanol in sterile conditions. The concentration of peptides used (0.1 mg/ml) allows the uniform and complete coating of the whole surface of the wells, without interfering in the correct attachment of the cells to the bottom of the well and their optimal confluence (Fig. S2).

Treatment of primary human monocytes with IL-1Ra and HSP70 tethered peptides for 24 h, with or without pro-inflammatory stimuli

The 24MWs were coated with IL-1Ra and HSP70 tethered peptides as described in 2.3. Once ethanol was evaporated, each well was washed twice with RPMI without FBS to equilibrate wells' surface. Isolated monocytes (described in 2.2) were resuspended in warm (37 °C) RPMI without FBS, containing 100 ng/ml lipopolysaccharide (LPS, Sigma, L4005) and human recombinant interferon-γ 50 ng/ml (hrIFN-γ, Peprotech, 300-02) or the synthetic triacylated lipopeptide Pam3CysSerLys4 100 ng/ml (Pam3CSK4, InvivoGen, tlrl-pms), as pro-inflammatory stimuli. $1*10^6$ cells were plated in each well of a 24 MW and incubated at 37 °C for 3 h to allow cell attachment to the bottom of the plate. Finally, 100 μl of FBS were added to each well and monocytes were incubated for 24 h. The schematic representation of this experiment is shown in Fig. 2a.

Differentiation of primary human macrophages during 6 days, under treatment with IL-1Ra and HSP70 tethered peptides, and exposure to pro-inflammatory stimuli

Isolated monocytes (described in 2.2) were resuspended in RPMI without FBS containing 25 ng/ml of human recombinant M-colony stimulating factor (hrM-CSF, Peptrotech, 300-25) to obtain human monocyte derived macrophages (HMDMs). $1*10^6$ monocytes were plated in each well of a 24 MW pre-coated with peptides, as above described. Cells were incubated at 37 °C for 3 h to allow their attachment to the plate, subsequently 100 μl of FBS were added to each well and cells were incubated until day 5. At day 5 media was replaced by RPMI with FBS containing LPS 100 ng/ml and hrIFN-γ 50 ng/ml or Pam3CSK4 100 ng/ml (used as pro-inflammatory stimuli), and macrophages were incubated for 24 h. The schematic representation of this experiment is shown in Fig. 3a.

Stimulation of macrophages with LPS + ATP for assessment of IL-1β secretion

To assess production and secretion of IL-1β, occurring as a consequence of inflammasome activation,[42,43] as an alternative to the pro-inflammatory stimuli described above (LPS + IFN-γ or Pam3CSK4), we tested macrophages primed with LPS + ATP (Adenosine Triphosphate).[42] ATP (Sigma, A6419) was weighted and dissolved in RPMI to generate a 100 mM stock, which was filtered with a 0.22 um filter (Sartorius, 17597-K) and aliquots were stored at −20 °C. Just immediately before cell stimulation, 10 mM, 3 mM, 1 mM and 0.3 mM dilutions of ATP were prepared from the stock. Then, isolated primary human monocytes were differentiated for 5 days towards macrophages with or without the peptides (IL-1Ra and HSP70) and further stimulated for 24 h with LPS (100 ng/ml) and ATP (0.3 mM, 1 mM, 3 mM and 10 mM). At day 6, cell viability was evaluated by Alamar Blue (see 2.9) and supernatant was collected for IL-1β quantification (see 2.10).

Treatment of primary human macrophages for 24 h with IL-1Ra and HSP70 tethered peptides

Isolated monocytes (described in 2.2) were resuspended in RPMI with FBS containing 25 ng/ml of hrM-CSF to obtain HMDMs. For this, $3*10^6$ cells were plated in each well of 6 ultra-low attachment-well plate and incubated at 37 °C to allow their differentiation for 5 days. At day 5, macrophages were carefully detached from the plates by up-down pipetting, centrifuged (830 rpm, 10 min), resuspended in warm RMPI without FBS and counted with Trypan Blue staining 0.04 % using a counting chamber (Kova International, BVS100H). $1.1*10^6$ cells in RPMI containing LPS 100 ng/ml + hrIFN-γ 50 ng/ml or Pam3CSK4 100 ng/ml were plated in each well of a 24 MW pre-coated with peptides (see 2.3), and incubated at 37 °C for 3 h to allow the attachment of cells to the bottom of the plate. Finally, 100 μl of FBS were added to each well and macrophages were incubated with or without peptides for 24 h. The schematic representation of this experiment is shown in Fig. 5a.

Towards biomedical application: anti-inflammatory implants

IL-1Ra and HSP70 peptides were linked to a biocompatible material (Polycarbonate Urethane, PCU) commonly used for implants. The maintenance of non-toxic profile and anti-inflammatory efficacy of peptide-coated PCU discs were tested using macrophages as described in 2.7.

Coating PCU discs with IL-1Ra and HSP70 tethered peptides

IL-1Ra and HSP70 peptides were prepared as described in 2.3. 15 × 15 mm PCU discs were placed in each well of 24 MW, coated with IL-1Ra or HSP70 peptides and further set under UV irradiation in a flow cabinet for 3 h to allow the evaporation of ethanol in sterile conditions.

Treatment of human primary monocyte derived macrophages for 24 h with IL-1Ra- and HSP70- PCU discs

HMDMs were prepared as described in 2.7. $1.1*10^6$ cells in RPMI containing LPS 100 ng/ml + hrIFN-γ 50 ng/ml were plated in 24MWs containing peptide-coated PCU discs, and incubated at 37 °C for 3 h to allow cell attachment to the bottom of the plate. PCU discs uncoated, IL-1Ra or HSP70-coated wells were used as controls. Finally, 100 μl of FBS were added to each well and macrophages were incubated with or without treatments for 24 h. At day 6, cell viability was evaluated by Alamar Blue (see 2.9) and supernatant was collected for TNFα quantification (see 2.10).

Toxicity/biocompatibility evaluation

To test cell viability, the Alamar Blue assay was used for both monocytes and macrophages (after 24 h or 6 days of exposure). At the end of each experimental protocol (2.4, 2.5. or 2.6.) the supernatants were collected for cytokine quantification (described in 2.8.), while 1 ml of AlamarBlue™ HS Cell Viability Reagent 10 % (Invitrogen, A50101) was added to the remaining cells in the bottom of each well (24MWs), following manufacturer's protocol. The plates were incubated for 3 h at

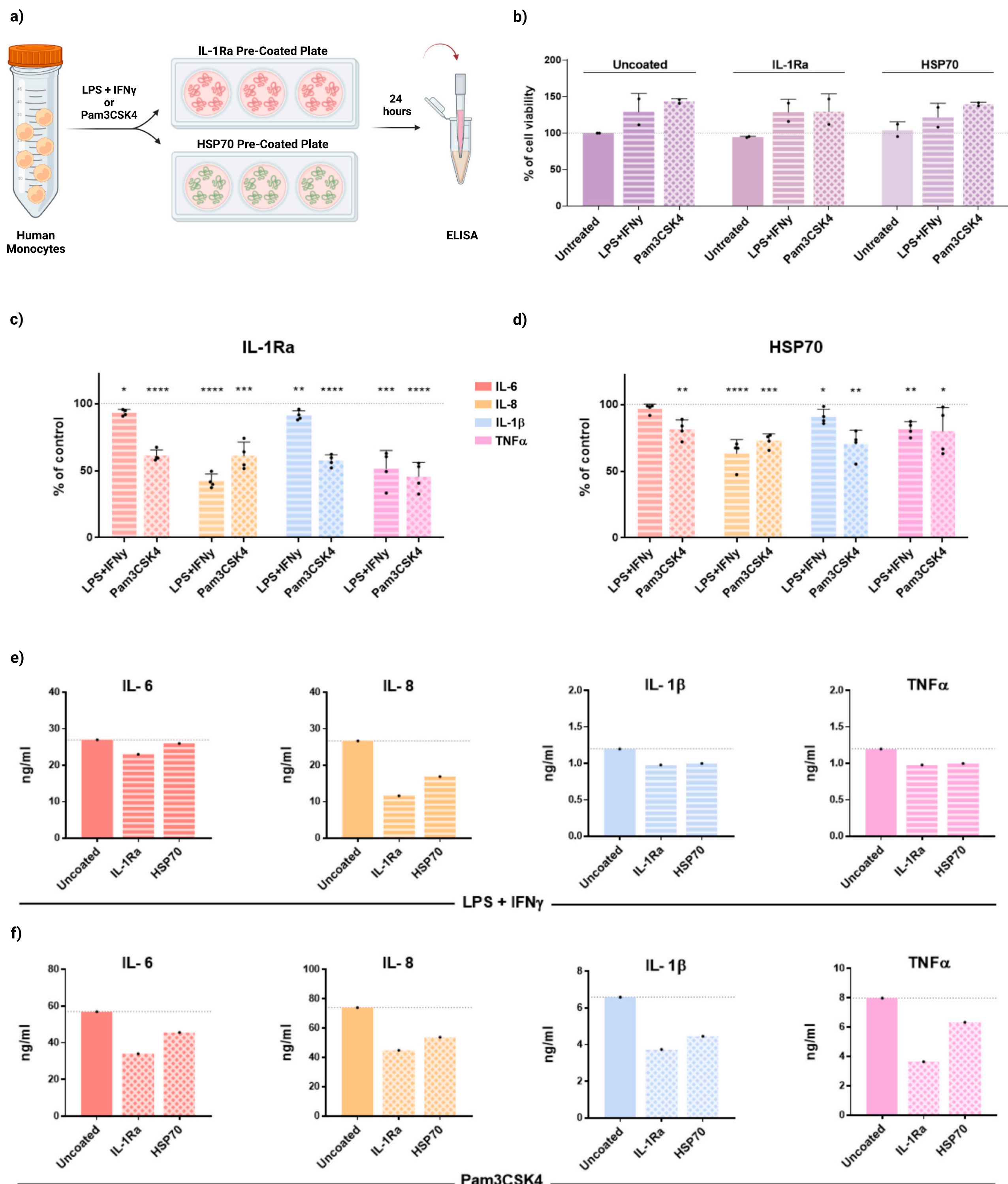

Fig. 2. Treatment of primary human monocytes with IL-1Ra and HSP70 tethered peptides for 24 h. a) Schematic representation of these experiments. b) Viability of monocytes seeded on the peptides, with or without pro-inflammatory stimuli (LPS + hrIFN-γ or Pam3CSK4), by Alamar Blue. c) Quantification of cytokines secretion (IL-6, IL-8, IL-1β and TNFα) compared to the uncoated controls (normalized to 100 %) by monocytes stimulated with the pro-inflammatory stimuli while treated with the IL-1Ra peptide; d) or with the HSP70 peptide for 24 h. e) Concentration of pro-inflammatory cytokines (IL-6, IL-8, IL-1β and TNFα) in representative samples (selected donors) from c) and d) experiments after stimulation e) with LPS + IFN-γ; or f) with Pam3CSK4.

37 °C protected from light. 100 μl from each well, in triplicate, were transferred to black 96-well plates and fluorescence was measured at 560–590 nm using the Synergy H4 Microplate reader (BioTek). Non-treated cells were used as controls and considered as 100 % cell viability. Cell viability was calculated according to the equation: % Cell viability = (Sample Fluorescence / Control Fluorescence) × 100.

Quantification of cytokine secretion

The anti-inflammatory activity of the tethered peptides was

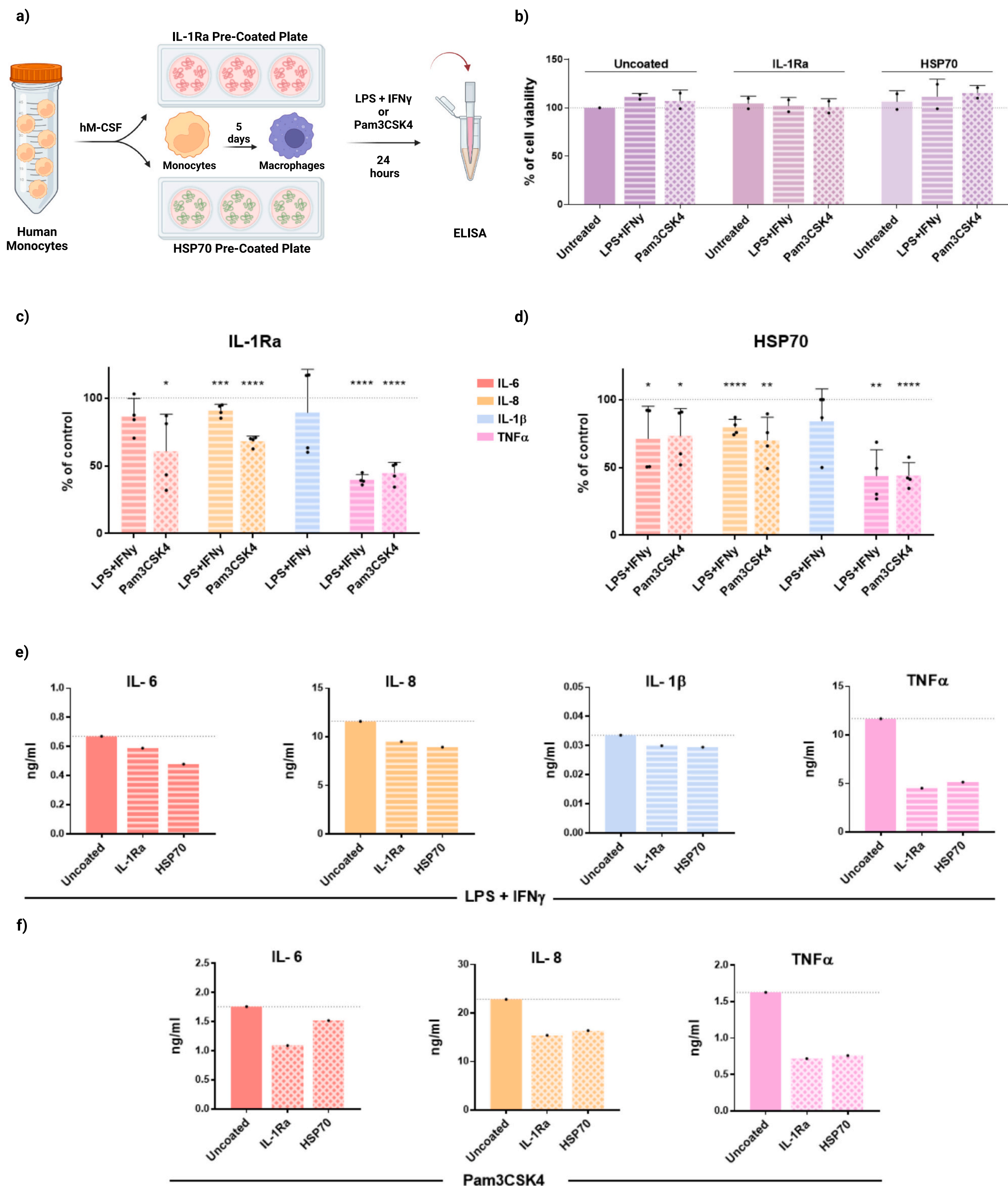

Fig. 3. Treatment of primary human monocytes during their differentiation towards macrophages (for 6 days) with IL-1Ra and HSP70 tethered peptides. a) Schematic representation of these experiments. b) Viability of cells seeded on peptides under differentiation for 5 days and then 24 h with or without pro-inflammatory stimuli (LPS + hrIFN-γ or Pam3CSK4). c) Quantification of cytokines secretion (IL-6, IL-8, IL-1β and TNFα) compared to the uncoated controls (normalized to 100 %) by cells stimulated with LPS + hrIFN-γ or Pam3CSK4 for 24 h, while treated with the IL-1Ra peptide; d) or with the HSP70 peptide for 6 days. e) Concentration of pro-inflammatory cytokines (IL-6, IL-8, IL-1β and TNFα) in representative samples (selected donors) from c) and d) experiments after stimulation e) with LPS + IFN-γ or f) with Pam3CSK4.

evaluated by quantification of cytokine secretion collected in the supernatant of cell cultures at the end of each protocol (2.4., 2.5., 2.6.) using ELISA. Assays were performed using the commercial kits DuoSet® ELISA Development Systems (Bio-Techne): IL-6 (DY206); IL-8 (DY208), IL-1β (DY201) and TNF-α (DY210), following the manufacturer's instructions.

Statistical analysis

Data were analyzed using GraphPad Prism version 7.02. Statistical comparison was performed using unpaired T-Test or One-way ANOVA followed by Dunnett's multiple comparison test. Statistically significant differences were considered when $p \leq 0.05$. Differences are represented in Figures as follows: $*p < 0.05$; $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Results

Synthesis and characterization of IL-1Ra and HSP70 peptides

Two different peptides were synthesized and characterized mimicking the IL-1 receptor antagonist (IL-1Ra) or the heat-shock protein 70 (HSP70). The aim was to develop a coating strategy of simple design and synthesis, which could be easily applied to biomedical implants to confer anti-inflammatory properties to their surface. Both peptides were characterized by FTIR before and after modification to the backbone structure (Fig. 1a–d. Before modification of the backbone structure the IL-1Ra peptide demonstrated three prominent peaks by MS, giving a combined total molecular weight (MW) of 2681.90 g/mol, compared to its theoretical 2701.91 g/mol (Supplementary Fig. S1a). Likewise, for the HSP70 peptide, the most prominent peaks identified from MS trace showed a total MW of 1721.19 g/mol, close to its theoretical 1707.85 g/mol (Supplementary Fig. S1b). After covalent bond coupling to the poly-L-lysine by an EDC/NHS reaction, final products were characterized by FTIR (Fig. 1b and d). The comparison between the free and bound IL-1Ra or HSP70 peptides shows significant shifts and peak ratios in the primary and secondary amine regions, in the range of 3500–2800 and 1800–700. Ninhydrin staining validated the uniform coating of the wells by film casting and evaporation (data not shown). The modified backbone of IL-1Ra and HSP70 peptides enabled their direct tethering to the substrate (*i.e.* for biomedical implants).

IL-1Ra and HSP70 tethered peptides induce anti-inflammatory activity in primary human monocytes, without affecting cell viability

Monocytes play a critical role in inflammatory responses as their recruitment to the inflammation site contributes to local and systemic responses,[44] so the modulation of their functions is an useful strategy for anti-inflammatory therapies. Therefore, the capacity of IL-1Ra and HSP70 peptides to modulate monocytes' phenotype was assessed.

The first requisite for the medical use of new molecules with pharmacological activity is their safety[45]; thus, to study the biocompatibility/non-toxic profile of IL-1Ra and HSP70 peptides, primary human monocytes, with the presence or absence of pro-inflammatory stimuli (LPS + hrIFN-γ or Pam3CSK4), were exposed for 24 h to the tethered peptides and cell viability was evaluated. As shown in Fig. 2b, the treatment with IL-1Ra or HSP70 peptides does not compromise the viability of monocytes, independently of the TLR agonist (LPS + hrIFN-γ or Pam3CSK4) used, indicating their satisfactory biocompatible profile. Using the same experimental protocol (Fig. 2a), to evaluate the anti-inflammatory efficacy of the peptides on monocytes, 24 h after incubation the cell culture supernatant was collected and levels of pro-inflammatory cytokines (IL-6, IL-8, IL-1β and TNFα) were measured. As shown in Fig. 2c–f and Table I, treatment with IL-1Ra or HSP70 peptides resulted in significant decreased secretion of cytokines. This reduction was more pronounced in monocytes treated with IL-1Ra, independently of the TLR agonist used (Fig. 2c, e, f and Table I). In particular, in monocytes treated with IL-1Ra we found a 40–45 % decrease in IL-6, IL-8 and IL-1β levels in cells stimulated with Pam3CSK4, whereas with LPS + hrIFN-γ we observed 10 % decrease of IL-6 and IL-1β and 60 % for IL-8 (Fig. 2c, e, f and Table I). The treatment with HSP70 resulted in a 20–30 % reduction of IL-6, IL-8 and IL-1β levels in cells stimulated with Pam3CSK4 whereas with LPS + hrIFN-γ we observed 5 %, 40 % and 10 % decrease for IL-6, IL-8 and IL-1β, respectively (Fig. 2d–f and Table I). The levels of secreted TNFα were reduced around 50 % or 20 % when using the IL-1Ra or the HSP70 peptide respectively, independently of the pro-inflammatory stimuli applied (Fig. 2c–f and Table I). Thus, a higher anti-inflammatory activity towards monocytes was observed for the IL-1Ra *versus* the HSP70 peptides, clearly demonstrated by the IL-8 levels in the cells stimulated with LPS + hrIFN-γ, by IL-6 and IL-1β levels in the cells stimulated with Pam3CSK4, and also by reduced secretion of TNFα regardless of the stimuli. Neither untreated (without IL-1Ra or HSP70) nor unstimulated (without LPS + hrIFN-γ or Pam3CSK4) cells presented significant secretion of cytokines.

Anti-inflammatory activity of IL-1Ra and HSP70 tethered peptides on M-CSF-differentiating macrophages

We tested the effect on cell viability of IL-1Ra or HSP70 tethered peptides along monocyte-to-macrophage differentiation during 6 days in the presence or absence of pro-inflammatory stimuli (LPS + hrIFN-γ or Pam3CSK4) by the Alamar Blue assay. As shown in Fig. 3b, the viability of macrophages was not compromised by the treatment with IL-1Ra or HSP70 peptides, independently of the TLR agonist (LPS + hrIFN-γ or Pam3CSK4) used, indicating a satisfactory biocompatible profile. Then, we evaluated the modulation of cytokine secretion induced by the tethered peptides. As shown in Fig. 3c–f and Table I, IL-1Ra and HSP70 peptides showed anti-inflammatory effects on cells treated for 6 days, preventing the secretion of IL-8 by 10 % (LPS + hrIFN-γ) or 30 % (Pam3CSK4), and around 30 % (LPS + hrIFN-γ or Pam3CSK4),

Table I
Percentage of reduction of secretion of pro-inflammatory cytokines (IL-6, IL-8, IL-1β, TNFα) by monocytes or macrophages stimulated with LPS + hrIFN-γ, Pam3CSK4 or LPS + ATP, and treated with IL-1Ra or HSP70 peptides for 24 h or 6 days. Cytokine reduction ≥30 % in bold.

			Monocytes (24 h)		Monocyte to Macrophage differentiation (5 days + 24 h)		Macrophages (24 h)	
			IL-1Ra	HSP70	IL-1Ra	HSP70	IL-1Ra	HSP70
IL-6	LPS + hrIFNγ		10 %	4 %	24 %	29 %	22 %	10 %
	Pam3CSK4		**39 %**	19 %	**40 %**	27 %	9 %	14 %
IL-8	LPS + hrIFNγ		**58 %**	**38 %**	10 %	21 %	24 %	25 %
	Pam3CSK4		**39 %**	28 %	**32 %**	**30 %**	25 %	24 %
IL-1β	LPS + hrIFNγ		11 %	11 %	11 %	26 %	**51 %**	**45 %**
	Pam3CSK4		**43 %**	**30 %**	–	–	18 %	4 %
	LPS + ATP	0.3 mM	–	–	**56 %**	**52 %**	–	–
		1 mM	–	–	**69 %**	**74 %**	–	–
TNFα	LPS + hrIFNγ		**49 %**	20 %	**61 %**	**57 %**	27 %	28 %
	Pam3CSK4		**55 %**	21 %	**56 %**	**57 %**	29 %	**37 %**

*LPS = lipopolysaccharide; IFN-γ = interferon-gamma; Pam3CSK4 = Pam3CysSerLys4; IL-6 = interleukin 6; IL-8 = interleukin 8; IL-1β = interleukin 1-beta; TNFα = tumor necrosis factor-alfa; ATP = adenosine triphosphate.

respectively. The secretion of TNFα was reduced around 60 % with both peptides, regardless of the stimuli. In the case of IL-6, although presenting a trend of inhibition, the anti-inflammatory activity of IL-1Ra was only significant when macrophages were stimulated with Pam3CSK4 (40 %) (Fig. 3c, e, f and Table I), while 30 % decrease was observed for the HSP70 peptide under both pro-inflammatory stimuli (Fig. 3d–f and Table I). Neither untreated (without IL-1Ra or HSP70) nor unstimulated (without LPS + hrIFN-γ or Pam3CSK4) cells presented significant secretion of cytokines.

The secretion of IL-1β was not detected after stimulation with Pam3CSK4, and very low with LPS + hrIFN-γ (0.0336 ng/ml), showing a non-significant reduction in cells treated with IL-1Ra or HSP70 peptides (Fig. 3c–f). Due to these low levels of IL-1β detected after the long-term exposure, we tried an alternative protocol of macrophage stimulation with LPS + ATP, well-known inducers of inflammasome activation and consequent secretion of IL-1β.[42,43] Monocytes were differentiated towards macrophages for 5 days in presence or absence of peptides and then exposed for 24 h to LPS + ATP. First, different concentrations of ATP, from 0.3 mM up to 10 mM were tested, showing very high toxicity above 3 mM while no effect on cell viability at 0.3 or 1 mM (Fig. 4a). Therefore, non-toxic doses of 0.3 and 1 mM ATP + LPS (100 ng/ml) were used, showing significant secretion of IL-1β (~10-times higher than LPS + hrIFN-γ) (Fig. 4b). As shown in Fig. 4b and c, cells exposed to IL-1Ra or HSP70 peptides showed an average inhibition of 54 % (LPS + ATP 0.3 mM) or 72 % (LPS + ATP 1 mM), respectively, in the secretion of IL-1β, demonstrating satisfactory anti-inflammatory activity.

As a whole, long-term exposure of monocytes to IL-1Ra or HSP70 tethered peptides during their differentiation towards macrophages results in cells with decreased pro-inflammatory properties, as revealed by reduced secretion of IL-8, IL-6, IL-1β and TNFα. In this case we only found a superior anti-inflammatory activity for IL-1Ra *versus* HSP70 peptides in terms of IL-6, when the cells were exposed to Pam3CSK4, whereas higher reduction was obtained for IL-6, IL-8 and IL-1β when cells were exposed to LPS + hrIFN-γ and treated with HSP70 peptides. In the case of TNFα, IL-1Ra and HSP70 peptides showed similar anti-inflammatory activity under both pro-inflammatory stimuli tested.

Additionally, the possible decrease in the secretion of IL-10, a well-known anti-inflammatory cytokine,[46] was evaluated in the same conditions (at 24 h). Our results showed that levels of IL-10 in cells treated with IL-1Ra or HSP70 are similar to the amount secreted by untreated controls, regardless the stimuli (LPS + hrINFγ or Pam3CSK4) (Fig. S3). Likewise, other researchers have also found alteration of the pro-inflammatory cytokines, but not the anti-inflammatory ones, by HSP70 targeting.[33] Therefore, we have continued our work focusing on the testing of the pro-inflammatory mediators.

IL-1Ra and HSP70 tethered peptides induce anti-inflammatory activity in primary human macrophages

We evaluated the effect of IL-1Ra or HSP70 tethered peptides on macrophage viability in the presence or absence of pro-inflammatory stimuli (LPS + hrIFN-γ or Pam3CSK4) for 24 h. As shown in Fig. 5b, the viability of macrophages was not compromised by their treatment with IL-1Ra or HSP70 peptides, independently of the TLR agonist (LPS + hrIFN-γ or Pam3CSK4) used, indicating a satisfactory biocompatible profile. Then, the ability of tethered peptides to reduce the secretion of pro-inflammatory cytokines by macrophages exposed for 24 h to the pro-inflammatory stimuli was investigated (LPS + hrIFN-γ or Pam3CSK4). In this case, unlike the previous observations in monocytes exposed for 24 h, macrophages displayed a nearly equivalent cytokine reduction pattern (IL-6, IL-8, TNFα), compared to controls, independently of the peptide or the TLR agonist used, but not for the secretion of IL-1β (Fig. 5c–f and Table I). In particular, IL-6 levels were slightly reduced (~11 %) with both peptides and stimuli, only presenting a significant decrease when macrophages were treated with IL-1Ra and stimulated with LPS + hrIFN-γ (22 %). On the contrary, a clear decrease up to 25 % or 30 % was observed for IL-8 or TNFα respectively, when macrophages were treated with any peptide and pro-inflammatory

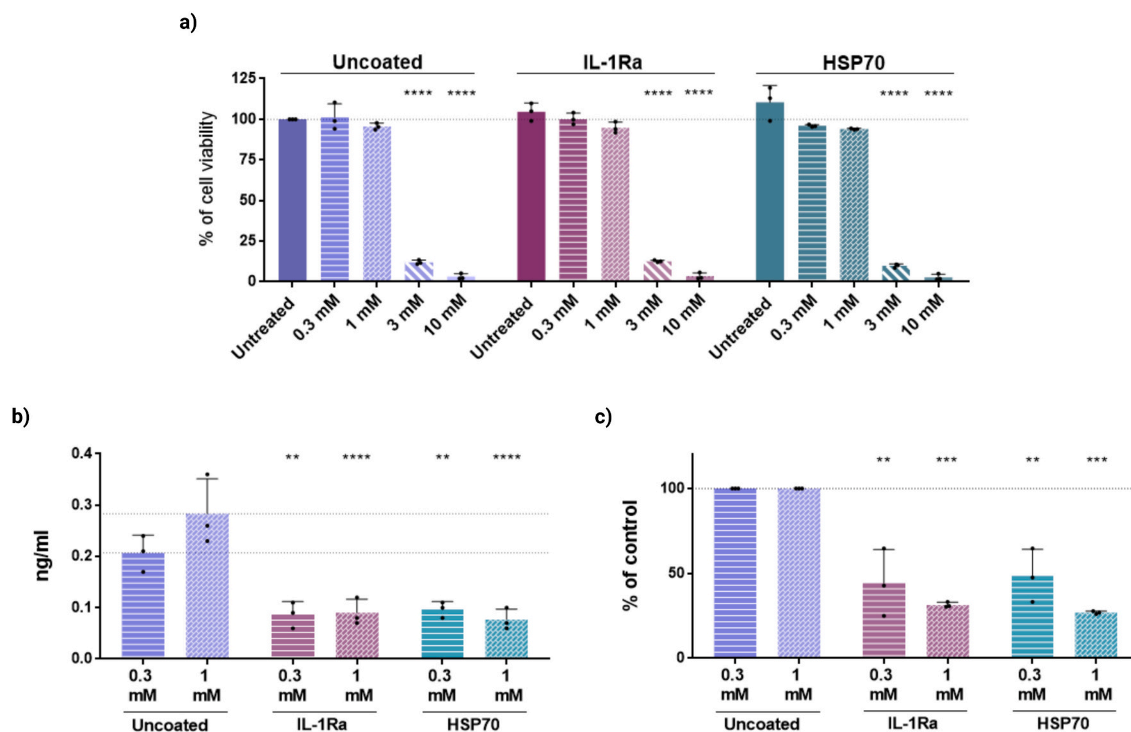

Fig. 4. Stimulation of primary human macrophages with LPS + ATP for induction of inflammasome activation and secretion of IL-1β. a) Viability of cells seeded on peptides for 5 days for monocyte-to-macrophage differentiation with M-CSF, and with or without the pro-inflammatory stimuli LPS (100 ng/ml) + ATP (at different concentrations: 0.3, 1, 3 and 10 mM). b) Concentration of IL-1β secreted by macrophages exposed to the IL-1Ra or HSP70 peptides and stimulated for 24 h with LPS (100 ng/ml) + ATP (0.3 or 1 mM), *versus* uncoated controls. c) Normalized representation to 100 %, compared to controls, of IL-1β levels.

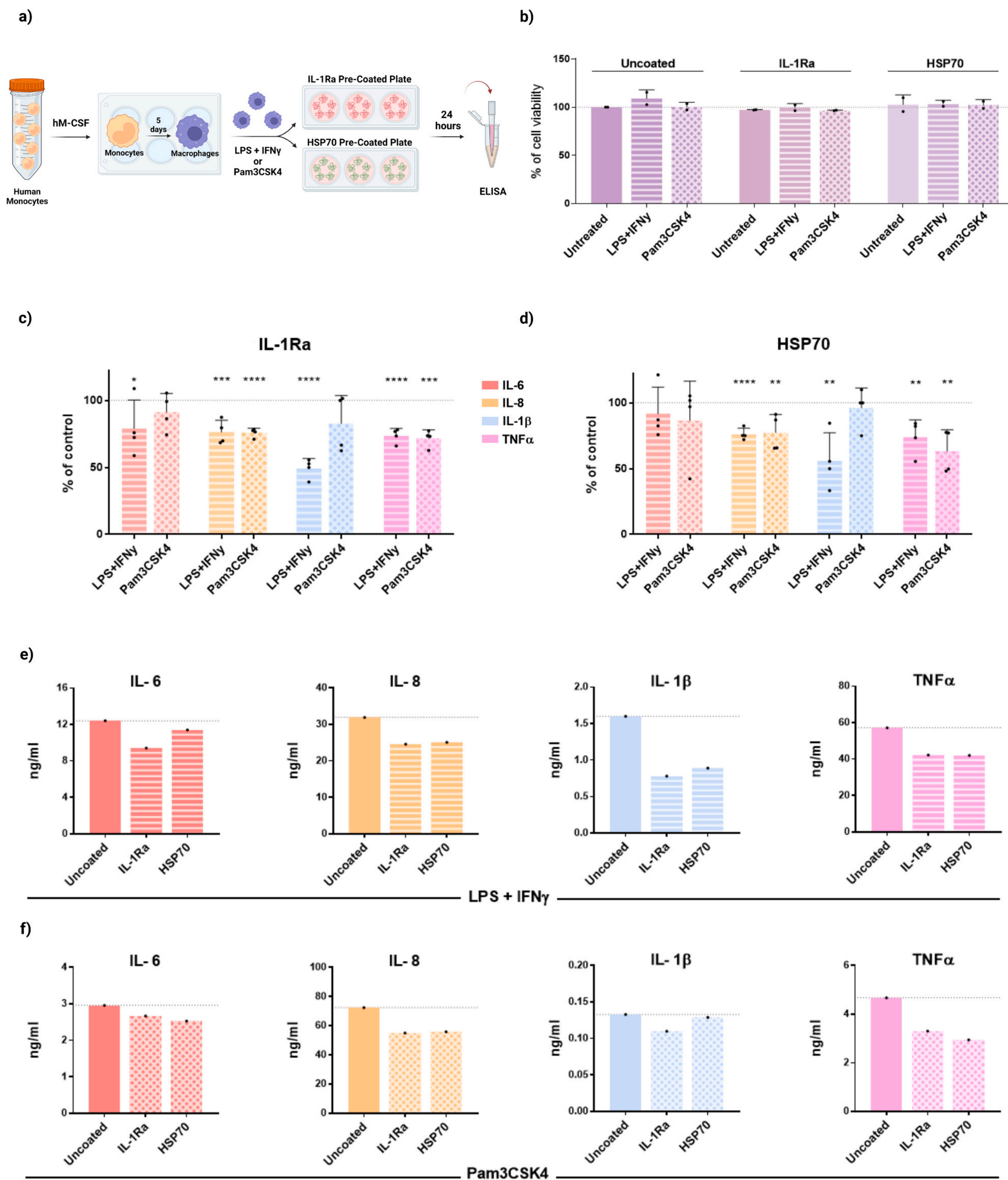

Fig. 5. Treatment of primary human macrophages with IL-1Ra and HSP70 tethered peptides for 24 h. a) Schematic representation of these experiments. b) Viability of macrophages seeded on the peptides, with or without pro-inflammatory stimuli (LPS + hrIFN-γ or Pam3CSK4), by Alamar Blue. c) Quantification of cytokines secretion (IL-6, IL-8, IL-1β and TNFα) compared to the uncoated control (normalized to 100 %) by macrophages stimulated with the pro-inflammatory stimuli while treated c) with the IL-1Ra peptide or d) with the HSP70 peptide for 24 h. e) Concentration of pro-inflammatory cytokines (IL-6, IL-8, IL-1β and TNFα) in representative samples (selected donors) from c) and d) experiments after stimulation with LPS + IFN-γ; f) or with Pam3CSK4.

stimuli. The levels of IL-1β were reduced by 50 % with both peptides, but only after LPS + hrIFN-γ stimulation.

As a whole, the highest anti-inflammatory activity in treated macrophages was observed for IL-8 and TNFα, regardless the tethered peptide or the stimuli; for IL-1β in macrophages stimulated with LPS + hrIFN-γ and treated with either peptide; and for IL-6 in macrophages treated with IL-1Ra and stimulated with LPS + hrIFN-γ. Neither untreated (without IL-1Ra or HSP70) nor unstimulated (without LPS + hrIFN-γ or Pam3CSK4) cells presented significant secretion of cytokines.

IL-1Ra and HSP70 coated PCU discs maintain their anti-inflammatory activity in primary human monocyte derived macrophages

Finally we evaluated whether the same anti-inflammatory properties were preserved for the tethered peptides when coating a biomaterial which can be used for surgical implants. Polycarbonate Urethane (PCU) discs were coated with the IL-1Ra or HSP70 peptides and macrophage viability was tested in the presence or absence of pro-inflammatory stimuli (LPS + hrIFN-γ) for 24 h. As shown in Fig. 6a, the viability of macrophages was neither compromised by their exposure to uncoated PCU discs, nor by their treatment with the IL-1Ra or HSP70 coated PCU discs. As previously observed for the tethered peptides alone, the PCU coated discs were non-toxic thus validating their satisfactory biocompatible profile. We also evaluated the modulation of TNFα secretion by macrophages exposed to the peptide-coated PCU discs. As shown in Fig. 6b and c, anti-inflammatory activity was found on macrophages treated for 24 h with IL-1Ra or HSP70 coated PCU discs, preventing the secretion of TNFα by 35 % or 20 % respectively. As expected, non-coated-PCU discs did not affect TNFα production, and cytokine levels secreted by macrophages treated with peptide-coated-PCU discs were equivalent to those observed for the cells treated with tethered peptides alone, thus validating their anti-inflammatory activity when coating a biocompatible material commonly used in the clinic.

Discussion

The use of anti-inflammatory peptides, both natural and synthetic, capable of mimicking the binding and/or functional sites of proteins involved in inflammatory signaling pathways represents a promising strategy to improve therapeutic outcomes.[24,47] As examples, short peptides mimicking the TGF-β1 binding domain resulted in potent anti-inflammatory activity both *in vitro* and *in vivo*[26]; a superoxide dismutase mimic functionalized with cell penetrating peptides improved its inherent anti-inflammatory activity in a cellular model[27]; Nrf2-activating bioactive peptides exerted anti-inflammatory effects by inhibiting the NF-κB pathway in human cells[28]; and protease activated receptor (PAR)-derived peptides displayed anti-inflammatory activity by suppressing macrophage NLRP3 inflammasomes.[29]

Following a similar approach, here we investigated new tethered peptides designed to mimic IL-1Ra or HSP70 with a focus on their influence on monocyte and macrophage inflammatory polarization. Previous studies investigating pharmacological molecules with potential anti-inflammatory activity have used human or murine myeloid leukemia cell lines, such as THP-1 or RAW264.7, which limit the reliability of the results as these cells present abnormal proliferation and their potential mutations might affect the molecular signaling mechanisms.[48,49] Instead, we used primary monocytes or macrophages, isolated from human "buffy coats", which are more sensitive to inflammatory stimuli and more reliable for the translation of the *in vitro* results to the clinic.[50,51] Firstly, the viability of monocytes or macrophages exposed to the IL-1Ra or HSP70 tethered peptides was tested after 24 h, or during 6 days of exposure along the differentiation of monocytes towards macrophages (Fig. 3). The pro-inflammatory stimuli LPS + hrIFN-γ or Pam3CSK4 for 24 h, mimicking inflammatory conditions, were applied to trigger the secretion of cytokines. No toxicity was found, regardless of the TLR agonists and/or the treatments, thus validating the satisfactory biocompatible profile of the tethered peptides in cell culture conditions and providing guarantee for their safe application in the clinic.[45] The anti-inflammatory efficacy of the IL-1Ra or HSP70 peptides on monocytes and macrophages after 24 h of exposure, was similarly tested with or without TLR stimulation (Figs. 2 and 5). As expected, in concordance to the pro-inflammatory M1 phenotype,[52] stimulation for 24 h with Pam3CSK4 (TLR1/2 agonist) or LPS (TLR4 agonist) + hrIFN-γ (type II interferon) promoted the secretion of IL-6, IL-8, IL-1β and TNFα by both monocytes and macrophages. Additionally, we confirmed that none of the peptides induce cytokine secretion by themselves, as no production

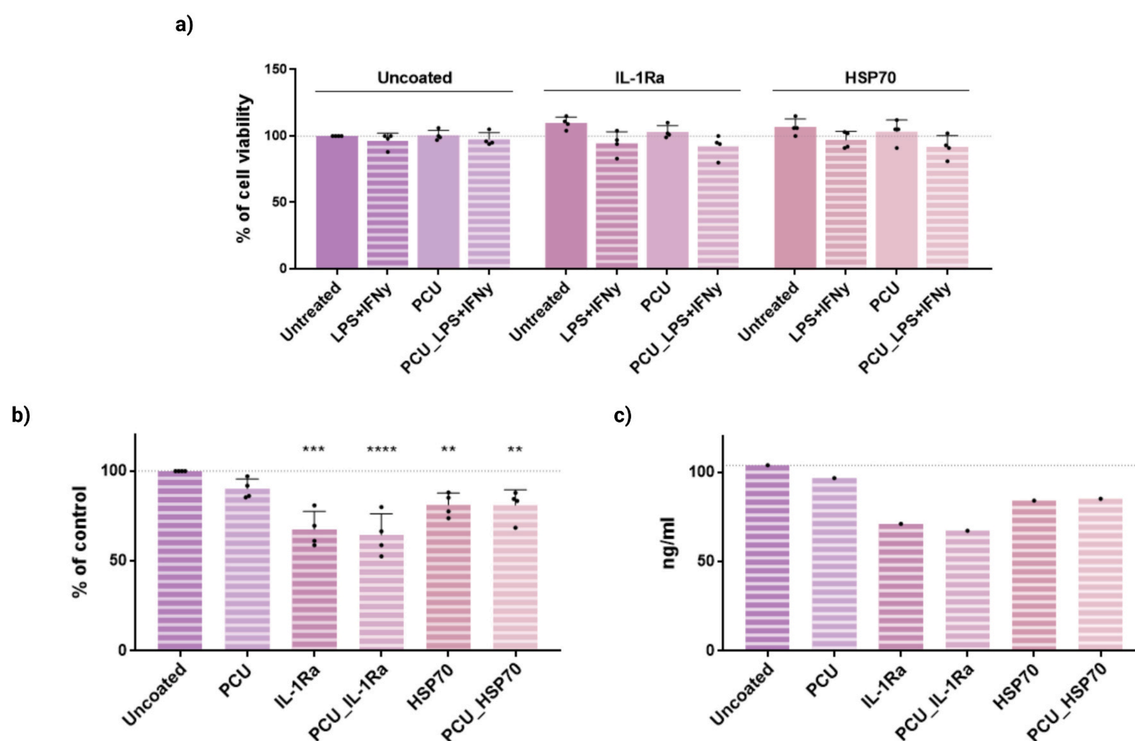

Fig. 6. Treatment of primary human macrophages with IL-1Ra or HSP70 tethered peptides *versus* IL-1Ra- or HSP70- coated PCU discs for 24 h. a) Viability of macrophages seeded on the peptides or coated discs, with or without pro-inflammatory stimuli (LPS + hrIFN-γ), by Alamar Blue. b) Quantification of TNFα secretion compared to the uncoated control (normalized to 100 %) by macrophages stimulated with LPS + hrIFN-γ while treated with tethered peptides or coated PCU discs for 24 h. c) Concentration of TNFα in a representative sample (selected donor) from b) experiments.

was detected neither by unstimulated monocytes or macrophages exposed to the tethered peptides for 24 h. As a result, we found that in the monocytes or macrophages stimulated with LPS + hrIFN-γ or Pam3CSK4 the treatment with the IL-1Ra and HSP70 peptides was able to revert the inflammation, showing a significant decrease in the production of all the tested cytokines (IL-6, IL-8, IL-1β and TNFα). Collecting all experimental data together (type of stimuli and evaluated cytokines), IL-1Ra or HSP70 peptides led, respectively, to an average decrease on the cytokine production of 38 % or 21 % by monocytes, and 26 % or 23 % by macrophages. Moreover, the influence of IL-1Ra or HSP70 peptides on monocyte-to-macrophage differentiation was also tested. After isolation, monocytes were exposed for 5 days to the tethered peptides while treated with M-CSF for their differentiation towards macrophages, and subsequently stimulated with LPS + hrIFN-γ or Pam3CSK4, with or without peptides. No significant influence was observed for the IL-1Ra or HSP70 peptides on the differentiation of the cells, and an average reduction in the secretion of cytokines of 37 % and 40 % was found, respectively. As a whole, we found that monocytes and differentiating macrophages were more sensitive to inflammatory inhibition by these peptides than fully differentiated macrophages. For monocytes, still immature cells, similar findings have been previously observed with other inhibitors. Instead for the differentiating macrophages this superior activity could be explained by their longer exposure (5 days) to the tethered peptides, resulting highly interesting for their application in implanted surgical devices which are likely to provide better outcomes.

To advance further, with the intention to generate knowledge on the potential biomedical application and facilitate their translation towards the clinic, we linked the IL-1Ra and HSP70 peptides to PCU discs, a biomaterial commonly used in different forms and sizes for prosthesis, including meniscus,[53] knee,[54] hip[55] or spinal implants.[56] PCU has shown superior biostability, resistance to hydrolysis, environmental stress cracking and metal ion oxidation when compared to other commonly used materials such as polyester or polyether urethanes.[57] *In vitro* and *in vivo* studies, by others, have demonstrated that PCU implants induce less inflammatory responses over long-term exposure.[58,59] In our experiments, uncoated, IL-1Ra- or HSP70-coated PCU discs showed no toxicity towards macrophages after 24 h of exposure, with or without LPS + hrIFN-γ stimulation, thus guaranteeing their safe biocompatibility (Fig. 6). Furthermore, the anti-inflammatory activity of the PCU-coated discs was similar to our previous experiments with the same peptides tethered to the surface of the cell culture plates, showing similar reduction in TNFα (25 % decrease *versus* the positive control of macrophages exposed to LPS + hrIFN-γ but without peptide treatment).

Moreover, we have also considered a possible activity of the IL-1Ra or HSP70 peptides on mesenchymal stromal cells (MSCs). MSCs can migrate to the site of injury with reparative purposes, not only by replacing expired or damaged tissues, but also by secreting trophic factors.[60–62] The IL-1Ra or HSP70 tethered peptides did not affect viability of MSCs (Fig. S2), supporting their use to modulate inflammation without impairing tissue repair).

In conclusion, our experiments using primary human monocytes or macrophages, have demonstrated that IL-1Ra and HSP70 tethered peptides do not present significant toxicity *in vitro*, thus guaranteeing their biocompatibility and encouraging their safe application in the medical practice. The IL-1Ra and HSP70 peptides presented ability to inhibit the secretion of the pro-inflammatory cytokines IL-6, IL-8, IL-1β or TNFα by monocytes or macrophages exposed to TLR agonists. Furthermore, we have confirmed that their anti-inflammatory activity remains well-preserved when the IL1-Ra and HSP70 peptides are linked to PCU discs. Altogether, our results encourage the further testing of these peptides *in vivo,* alone or linked to biocompatible polymers which could offer new solutions for patients with acute or chronic inflammatory disorders.

CRediT authorship contribution statement

FTA and EK decided the scientific concept for the design of the experiments. AP-L, AU and EV performed the *in vitro* experiments. AP-L made the figures and the table, and wrote the manuscript with contributions from PA, FTA, EK, AG, IUA, MS and NC. AP-L and FTA revised the manuscript. All authors agreed on the final version of the manuscript.

Declaration of competing interest

The authors declare that the research was conducted in absence of conflict related to personal scientific relationships. IL-1Ra and HSP70 peptides were provided by project partner Tissue Click Ltd. PCU discs were provided by Active Implants®.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2023.102719.

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