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Neutrophil-derived Alpha Defensins control Inflammation by inhibiting Macrophage mRNA Translation.

Matthew Brook[2]*, Gareth Tomlinson[1]*, Katherine Miles[1]*, Richard W.P. Smith[2], Adriano G Rossi[1], Pieter S Hiemstra[3], Emily F.A. van 't Wout[3], Jonathan Dean[4], Nicola K Gray[2], Wuyuan Lu[5] and Mohini Gray[1]

1- MRC Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, Scotland, U.K. 2-MRC Centre for Reproductive Health, Queen's Medical Research Institute, 3- Department of Pulmonology, Leiden University Medical Center, The Netherlands. 4- Kennedy Institute of Rheumatology, University of Oxford, Oxford OX3 7FY 5- Institute of Human Virology and Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine

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Neutrophils are the first and most numerous cells to arrive at the site of an inflammatory insult and amongst the first to die. We previously reported that alpha-defensins, released from apoptotic human neutrophils, augmented the antimicrobial capacity of macrophages whilst also inhibiting the biosynthesis of pro-inflammatory cytokines. *In vivo*, alpha defensin administration protected mice from inflammation, induced by thioglychollate induced peritonitis or following infection with S. enterica serovar Typhimurium. We have now dissected the anti-inflammatory mechanism of action of the most abundant neutrophil α-defensin, Human Neutrophil Peptide 1 (HNP1). Herein we show that HNP1 enters macrophages and inhibits protein translation without inducing the unfolded-protein response or affecting mRNA stability. In a cell-free *in vitro* translation system, HNP1 powerfully inhibited both cap-dependent and cap-independent mRNA translation, whilst maintaining mRNA polysomal association. This is the first demonstration of an eobiotic peptide released from one cell type (neutrophils), directly regulating mRNA translation in another (macrophages). By preventing protein translation, HNP1 functions as a 'molecular brake' on macrophage driven inflammation; ensuring both pathogen clearance and the resolution of inflammation with minimal bystander tissue damage.

macrophages | α-defensins | mRNA translation | inflammation

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Introduction

Neutrophils, via the release of key inflammatory mediators, convey signals to practically all other immune cells, orchestrating both the innate inflammatory and subsequent adaptive immune responses (1). Through the *de novo* generation of lipid mediators they are also key players in the resolution of inflammation [reviewed in (2)]. Following neutrophil apoptosis, their subsequent uptake by human monocyte derived macrophages (HMDMs) induces complex phenotypic changes, including the release of the immunosuppressive cytokines IL-10 and TGF-β [reviewed in (3)]. We previously reported that the human antimicrobial peptides, α-defensins, [which are released following apoptosis, necrosis or NET-osis (4) of neutrophils] also inhibited the secretion of multiple cytokines from activated HMDMs, for up to 72 hours, with full recovery thereafter and no effect on cell viability (5). *In vivo,* in mice, neutrophil derived α-defensins, given at the time of inducing peritonitis led to a diminished inflammatory exudate (5). In addition mice infected with pathogenic S. enterica ser. Typhimurium showed a reduced bacterial load and serum TNFα levels upon administration of exogenous α-defensin. Hence neutrophil-derived α-defensins, were able to affect profound changes in the inflammatory environment whilst also serving as effective anti-microbial peptides.

α-Defensins are small (3-4 kDa) cationic peptides that form part of a larger family of defensins [that also includes beta and theta peptides]. Four structurally related peptides (HNP 1-4) exist within the azurophil granules of neutrophils, of which HNP1 is the most abundant (6-9). They share a similar triple-stranded β-sheet structure, which is critically held together by three intra-molecular disulphide bridges. Once the azurophil granules fuse with phagosomes they release high concentrations of α-defensins close to the pathogen surface, where their amphipathic nature allows them to rapidly gain entry to the cell's membrane (10). The permeabilization of membranes by α-defensins is believed to be crucial to their ability to kill microbes and host cells, elicited by membrane disruption and leakage of cellular contents (9, 11). Importantly however, α-defensins only kill proliferating *E. coli* and a simple model of 'death by pore formation' is inadequate to explain all their antibacterial properties (12). They have also been noted to inhibit bulk bacterial protein synthesis in *E. coli*, though this is thought to be a consequence of membrane disruption and is temporally associated with cell death (11, 12). Additionally following HIV-1 infection, α-defensins play a crucial role in inhibiting their life-cycle (13, 14), suggesting that they have at their disposal a number of different mechanisms to kill diverse pathogens (7, 15). In favour of this hypothesis is the observation that α-defensin dimerization (which requires a tryptophan residue at position 26) is vital for its ability to kill *S. aureus* (16), but has little effect on its ability to kill *E. coli* (17).

Significance

Neutrophils are the major effectors of acute inflammation responding to tissue injury or infection. The clearance of apoptotic neutrophils by inflammatory macrophages also provides a powerful pro-resolution signal. Apoptotic or necrotic neutrophils also release abundant amounts of the antimicrobial peptides, alpha defensins. In this report we show that the most abundant of these peptides, HNP1 profoundly inhibits protein translation. It achieves this without affecting mRNA stability or by preventing mRNA polysomal association. This is the first demonstration of a peptide released from one cell, a leukocyte, entering and directly modulating the translatome of another cell. It alludes to a novel mechanism, driven by dying neutrophils, that ensures the timely resolution of macrophage driven inflammation, without compromising antimicrobial function.

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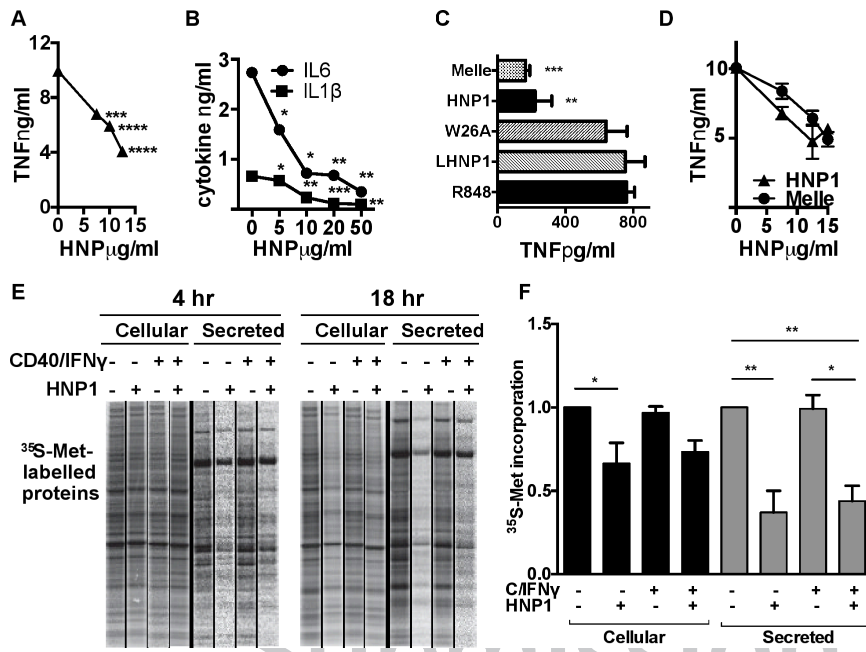

Fig. 1. HNP1 inhibits bulk protein synthesis, which is dependent on HNP1 tertiary structure.(A-B) HNP1 treated HMDMs were stimulated with the TLR7 ligand R848 (1μg/ml) **(A)** or with 3μg/mL CD40L + 5ng/mL IFNγ **(B)** for 18 hrs. TNFα (A), IL6 and IL1β (B) were assayed by ELISA. **(C-D)** HMDMs stimulated as for (A) and treated with 12.5 μg/ml of HNP1 or the mutant peptides LHNP, W26A or Melle at the same (C) or variable concentrations (D). TNFα assayed by ELISA after 18 hours. Representative of five independent experiments. One way ANOVA with Dunnett's multiple comparison tests. ** P<0.01, * P<0.05. **(E)** Methionine starved HMDMswere then cultured with 10μCi/mL ^{35}S-methionine +/- activation [with 3μg/mL CD40L and 5ng/mL IFNγ], and +/- addition of HNP1 (25μg/mL) for 4 or 18 hours. Secreted and intracellular proteins were resolved by SDS-PAGE. Phosphorimages of radiolabelled cellular and secreted protein gels show *de novo* protein synthesis. **(F)** *De novo* protein synthesis of ^{35}S-Methionine labeled proteins following 18 hours of culture, quantified by scintillation counting and normalised to untreated controls. N=3. Error bars represent the mean ± SEM; ** P<0.01, * P<0.05 (Tukey's *post hoc* test following a one-way ANOVA).

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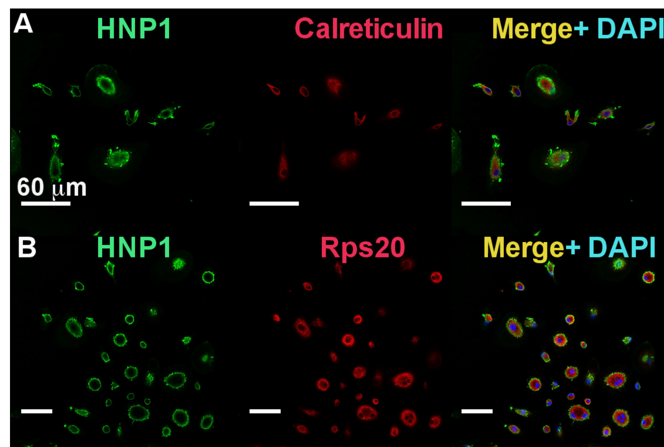

Fig. 2. HNP1 enters HMDMs. Confocal microscopy images of HNP1 treated HMDMs prior to visualization of anti-HNP1 (green) and DAPI (blue) seen on the merged images. In addition, red secondary staining indicates calreticulin (specific for the ER) in (A) and the ribosomal associated protein Rps20 in (B). Representative images from 1 of 6 independent experiments. White size bars indicate 60μm.

We wished to understand how α-defensins could simultaneously function as an effective antimicrobial antibiotic, whilst also inducing profound changes in HMDM gene expression. We report here that HNP1 enters HMDMs, where it profoundly inhibits protein translation in both resting and activated macrophages, without affecting mRNA stability or turnover. Instead it abrogates mRNA translation without affecting mRNA polysomal association.

Results

HNP1 inhibits the synthesis of proteins, which is dependent on HNP1 tertiary structure. We have previously shown that whilst alpha defensins augmented the macrophage's ability to kill intracellular *Pseudomonas aeruginosa,* these peptides simultaneously inhibited the production of multiple cytokines (TNFα, IL-6, IL-8 and IL-1β) (5). HNP1 also inhibited TNFα biosynthesis from HMDMs stimulated with the toll-like receptor 7/8 (TLR7/8) agonist R848 [Fig 1A]. The biosynthesis of IL-6 and IL-1β induced via the T cell surrogate stimulus CD40L/IFNγ, was also reduced [Fig 1B], confirming that disparate stimuli and multiple secreted proteins were susceptible to HNP1-mediated inhibition. The structure of HNP1 was crucial for its cytokine inhibitory potential. When the intra-molecular disulphide bonds that stabilize the triple-stranded beta-sheet structure of HNP1 was disrupted (L-HNP), or when dimerization was prevented by replacing the tryptophan residue at position 26 with the non-polar amino acid alanine (W26A) (16), a complete loss of cytokine inhibitory potential was seen [Fig 1C and (5)]. In contrast N-methylation of Ile20 (Melle), (which also prevents dimerization), had a minimal effect on the ability of HNP1 to inhibit R848-induced TNFα production by HMDMs [Fig 1C and 1D].

To test if HNP1 might inhibit protein synthesis *per se*, stimulated HMDMs were labelled with ^{35}S-methionine in the presence of HNP1. ^{35}S-methionine incorporation into proteins within cellular lysates (i.e. cellular proteins) and the culture media (i.e. secreted proteins) was visualised [Fig 1E] and quantified, following 18 hours of culture [Fig 1F]. Strikingly, HNP1 treatment significantly reduced the quantity of both ^{35}S-labelled cellular and secreted proteins in un-stimulated HMDMs and robustly inhibited the labelling of secreted proteins in CD40L/IFNγ stimulated HMDMs, possibly reflecting the highly secretory phenotype of the stimulated macrophage. As expected, secreted TNFα was significantly reduced by HNP1 [Fig S1A]. However the overall cellular protein levels were unchanged during the time-course of the experiment [Fig S1B], consistent with a lack of increased global protein turnover and with maintenance of cell number and viability, as previously reported (5). Taken together neutrophil-derived HNP1 profoundly inhibits global protein synthesis within the resting or activated macrophage.

Exogenous HNP1 accumulates in the macrophage. HNP1 gained entry to macrophages and was found within the membrane and cytoplasm. However there was no clear co-localisation of HNP1 (or the control peptide W26A) with the ER marker calreticulin [Figs 2A and Fig S2A and Fig S2C] or with ribosomes [stained with anti Rps20, Figs 2B, Fig S2B and Fig S2D]. Control experiments also showed no non-specific staining or cross reactiv-

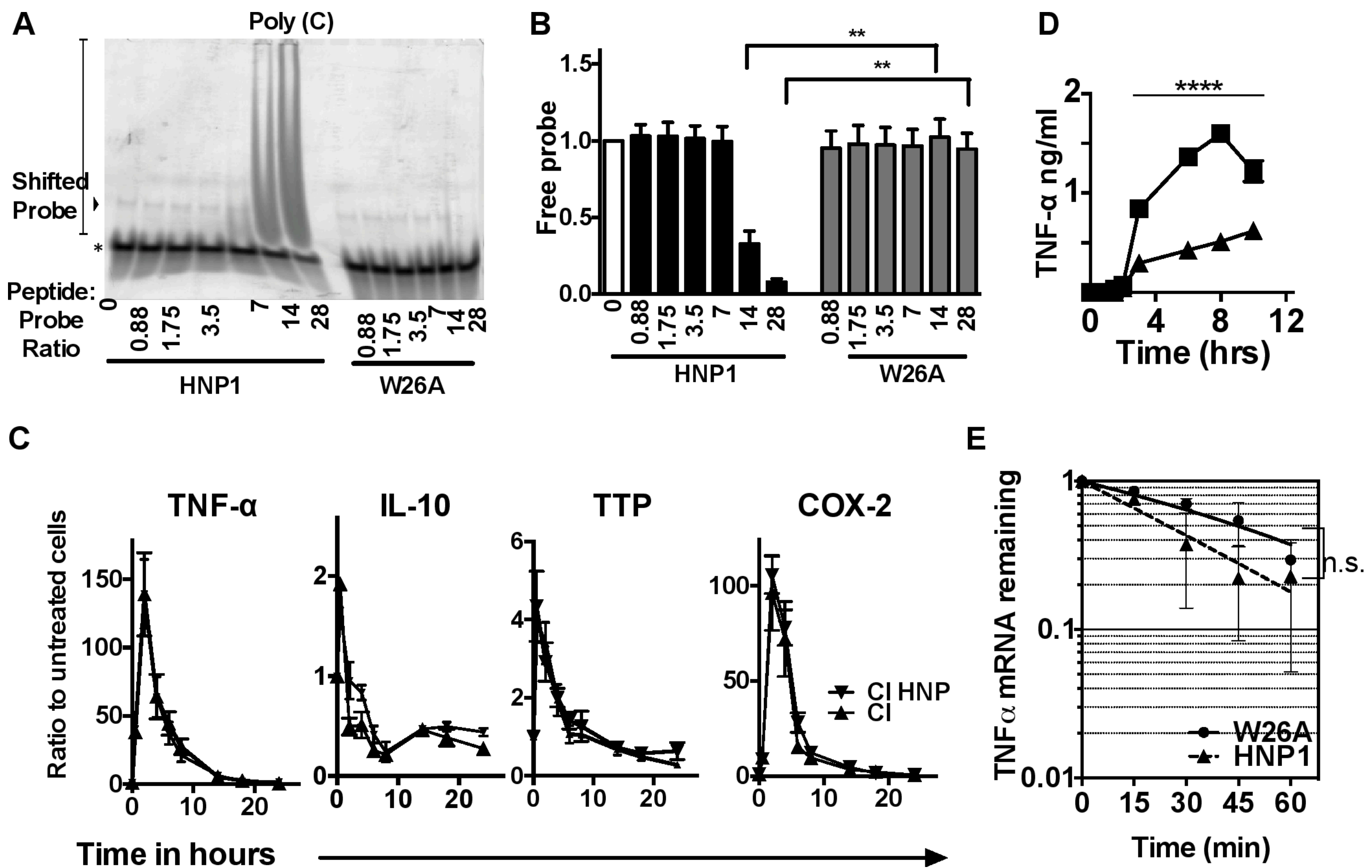

Fig. 3. . HNP1 binds to mRNA but does not affect mRNA stability. (A) Electrophoretic mobility shift assay. Poly(C)$_{25}$ RNA oligonucleotide probe [10 pmoles] incubated with molar ratios of HNP1 or W26A and RNA:peptide complexes resolved by non-denaturing acrylamide gel electrophoresis. * = free poly(C) probe, arrowhead = non-specific complex. Error bars represent mean ± SD. (B) Binding of HNP1 and W26A to poly(C)$_{25}$ RNA relative to total input RNA (where the relative amount of free probe is given in arbitrary units) (C) RNA was extracted from CD40L/IFNγ stimulated HMDMs and mRNA of TNF-α, IL-10, tristetraprolin (TTP) and cyclooxygenase 2 (Cox-2) was quantified by qRT-PCR and expressed as the ratio of mRNA from treated to untreated HMDMs. (D) Supernatants were collected for the first 10 hours from cells treated as in (C) and TNFα protein assayed by ELISA. (E) TNFα mRNA levels were quantified from HMDMs that had been treated with 12.5µg/mL of HNP1 or W26A, then stimulated with R848 (1µg/ml) for 1 hour before adding actinomycin D (5µg/mL).TNFα is expressed relative to T = 0 min. Error bars are mean ± SEM for each time point and line represents a non-linear 2-phase decay fit with R^2 values of 0.8667 and 0.8351 for W26A and HNP1 respectively. Results are derived from 3 separate experiments. A-D are representative of experiments repeated three times. (A-B) Tukey's *post hoc* test following a one-way ANOVA. ****P<0.0001 * P<0.03, (C-D) Tukey's *post hoc* test following a two-way ANOVA. n.s = not significant, p = 0.094.

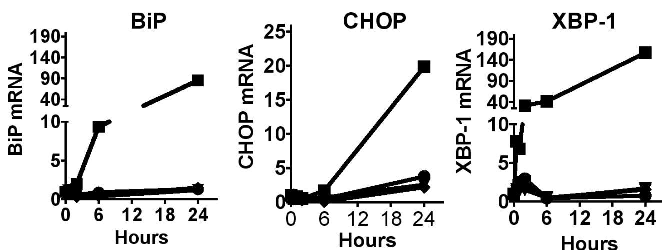

Fig. 4. . HNP1 does not cause ER stress. (A) R848 (1µg/ml) stimulated (u) HMDMs with either 12.5µg/mL HNP1 (filled ∇) , L-HNP1(n) or 1µM thapsigargin (<). Macrophage mRNA for CHOP, spliced XBP1 and BiP were quantified by qRT-PCR and expressed relative to the same mRNA in untreated control HMDMs. Hours represent time following stimulation N=3. Error bars= mean ± SD.

ity between HNP1 and the ER or ribosomal secondary antibodies [Fig S3].

HNP1 binds non specifically to RNA but does not alter mRNA transcription or stability. As HNP1 enters the macrophage it may, by reason of its positive charge and amphipathic nature (10, 18), bind to mRNA, so altering its turnover and inhibiting protein synthesis. This was tested using electrophoretic mobility-shift assays (EMSAs) with 25mer homopolymeric RNA oligonucleotides. In contrast to W26A, HNP1 showed concentration-dependent shifts of poly(C) [Fig 3A-B], poly(A) [Fig S4A-B] and poly(U) RNA [Fig S4C-D], which was observed both in the presence or absence of Mg^{2+} [Fig S4E]; a cation often required for nucleic acid binding by proteins. An antibody supershift EMSA also confirmed that HNP1 could bind to mRNA (coding for the firefly luciferase (fLuc) or β-galactosidase (β-gal) reporters) [Fig S4F].

To ask if HNP1 affected mRNA transcription, we quantified the steady-state mRNA levels generated by CD40L/IFNγ stimulated HMDMs. The mRNA levels of TNFα, IL-10, cyclooxygenase (Cox2) and tristetraprolin (TTP) were unaffected by HNP1 treatment of HMDMs over a 24hr time course [Fig 3C], despite a clear reduction in TNFα protein production [Fig 3D]. To assess mRNA decay, HNP1 or W26A treated HMDMs were stimulated (with R848) for 1 hour resulting in maximal TNF-α mRNA levels, prior to the addition of actinomycin D to arrest further transcription. The decay rate of TNF-α mRNA was not significantly modulated in HNP1 versus W26A-treated HMDMs over a further 1 hour time-course [Fig 3E]. As TNF-α mRNA stability is mediated in part by the zinc-finger protein TTP, which binds AU-rich sequences, we also assessed TNF-α protein secretion from activated mouse bone marrow derived macrophages (BMDMs), isolated from TTP deficient ($TTP^{-/-}$)

[Fig S5A]. Hence the profound inhibition of protein synthesis by HNP1 was not the result of an induced UPR.

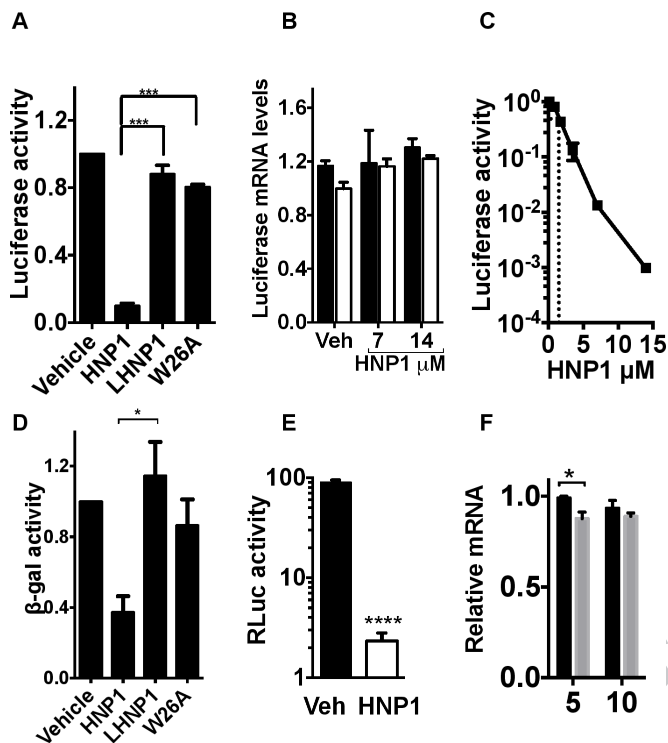

Fig. 5. HNP1 inhibits protein synthesis downstream of translation initiation. **(A)** 1 ng m^7G-fLuc-A_0 reporter mRNA, translated *in vitro* using the RRL with 25 μg/mL [7.3μM] HNP1, LHNP1, W26A or vehicle control (0.01% acetic acid). Translational output quantified as relative firefly luciferase activity (normalised to vehicle control-treated samples). Error bars= mean ± SEM (n=3) **(B)** As for (A) but relative m^7G-luciferase-A_0 reporter mRNA levels quantified by qRT-PCR. Black bars represent pre-translation levels and white bars the post translation levels. A representative experiment of n=3 experiments. **(C)** As for (A), 400 pg m^7G-fLuc-A_0 reporter mRNA translated in the presence of increasing concentrations of HNP1. The IC 50 (shown by the dotted line) is 1.6+/-0.02μM. Mean ± SEM from two independent experiments. **(D)** 1ng CSFV IRES-β-gal-A_0 reporter mRNA was *in vitro* translated as for (A). Values plotted relative to vehicle control. (n=3)Error bars represent mean ±SEM (n=3).for A and D: ***P<0.001, *P<0.05 (analysed by Tukey's multiple comparison *post hoc* test following one-way ANOVA). **(E)** 1ng CrPV IRES-β-gal-A_0 reporter mRNA translated as in (A). ****P<0.0001, analysed by unpaired T test. Values are plotted relative to vehicle control. **(F)** RRL was pre-treated with 150μg/mL cycloheximide and either 25 μg/mL HNP1 or vehicle control. 1ng ^{32}P-labelled m^7G-fLuc-A_0 reporter mRNA was then added for the indicated times (shown in minutes) prior to 15-30% sucrose density gradient fractionation. Graph depicts the relative amounts of mRNA sedimenting with initiating ribosomes, normalised to amount recruited at 5 min in vehicle control-treated RRL. Black bars are control and grey bars are HNP1 treated. Error bars represent mean ±SEM (n=3), *P<0.05 (unpaired t test).

mice or wild-type littermate controls. Again, HNP1 (but not L-HNP1) was still able to significantly inhibit the secretion of TNF-α from $TTP^{-/-}$ BMDMs [Fig S4G]. Taken together these data show that HNP1 can bind to RNA, likely in a sequence-independent manner, but does not affect mRNA stability or turnover.

HNP1 does not induce ER stress. We have previously shown that HNP1 does not inhibit the exocytosis of TNFα from HMDMs (5). We also wished to confirm that it did not prevent protein synthesis by inducing the unfolded protein response [UPR] [reviewed in (19)]. In contrast to the positive control thapsigargin (TG), we did not detect an increase in the synthesis of glucose-regulated protein 78 (Grp78), X box-binding protein (XBP1) or CCAAT/enhancer-binding protein homologous protein (CHOP) in HNP1 treated and stimulated HMDMs [Fig 4], despite a clear inhibition of R848-induced TNFα production at 6 and 24 hours [Fig S5A]. Hence the profound inhibition of protein synthesis by HNP1 was not the result of an induced UPR.

HNP1 does not block translation initiation. To ask if HNP1 affected translation directly, and to avoid the confounding effects of mRNA transcription, processing or nuclear export, we utilised the cell-free rabbit reticulocyte lysate (RRL) *in vitro* translation system. Translation of the canonical fLuc reporter mRNA, was profoundly inhibited in the presence of HNP1, but not by the mutant control peptides, L-HNP nor W26A [Fig 5A]. As with TNFα mRNA, HNP1 did not destabilise the reporter mRNA because input mRNA levels were maintained [Fig 5B]. The IC_{50} value for this effect was approximately 1.6μM (or 5.5μg/ml) [Fig 5C], a concentration that significantly reduces the production of pro-inflammatory cytokines from stimulated HMDMs *in vitro* [Fig 1].

Eukaryotic mRNA has a 5' monomethylated cap structure (m^7G) which is crucial for canonical translation initiation, the rate-limiting and primary node of translation regulation (reviewed in (20)). To interrogate the role of translation initiation in HNP1-mediated inhibition we employed reporter mRNAs that contained a viral internal ribosome entry site (IRES) in their 5' untranslated regions (5'UTR), bypassing some or all of the eukaryotic translation initiation factor (eIF) requirements and initiating translation cap-independently (reviewed in (21)). The Classical Swine Fever Virus (CSFV) IRES mRNA reporter initiates translation independently of the majority of eIFs but is dependent on the ternary complex (eIF2, GTP and tRNAi), whilst the Cricket Paralysis Virus (CrPV) IRES allows the direct assembly of the 80S ribosome at the start codon, bypassing all canonical initiation factor requirements (22). Remarkably, despite their diverse mechanisms of translation initiation, HNP1 was also able to prevent the synthesis of both the CSFV-driven translation of β-Gal [Fig 5D] and the CrPV-driven translation of Renilla luciferase (RLuc) [Fig 5E]. As HNP1 is able to prevent the translation of mRNAs utilising diverse mechanisms of translation initiation it is most likely that it is acting downstream of this point. To confirm this empirically, ribosomal recruitment onto a radiolabelled m^7G-capped fLuc reporter mRNA was quantified in the presence of cycloheximide, to halt the 80S ribosome at the start codon, preventing translation elongation. Whilst HNP1 weakly inhibited translation initiation at 5 minutes following mRNA addition, by 10 minutes similar maximal 80S recruitment to that seen in vehicle control-treated extracts was observed [Fig 5F], indicating only a small reduction in the rate of 80S recruitment in the presence of HNP1 and supporting the conclusion that HNP1 predominantly inhibits mRNA translation post-initiation.

HNP1 does not affect ribosomal association with mRNA. Finally to ask if luciferase mRNA was maintained on polysomes despite its significantly reduced translation, we assessed the steady-state ribosomal association of m7G-fLuc mRNA in the presence or absence of HNP1. Despite utilising a concentration of HNP1 that profoundly inhibited reporter protein synthesis [Fig 1E], we observed no change in the polysomal profile [Fig 6A] or the distribution of m7G-fLuc mRNA across the polysomal region of the density gradient (fractions 4-10) [Fig 6B]. In contrast, the presence of EDTA resulted in polysomal dissociation and depletion of the reporter mRNA from the fractions containing translating mRNA [Fig 6B and S5B]. We also wished to confirm if a similar mode of action was seen in HMDMs, that had been treated with HNP1 or vehicle control (for 18 hours). HMDMs so treated were then stimulated with R848 for two hours to upregulate the synthesis of TNFα. Again, the bulk polysome profile for HNP1 treated HMDMs was similar to that of control stimulated cells [Fig 6C]. Importantly, the polysomal association of TNFα mRNA in untreated or HNP1-treated stimulated HMDMs was not significantly altered [Fig 6D], despite the significant inhibition of TNFα protein synthesis [Fig S5C]. This data confirms

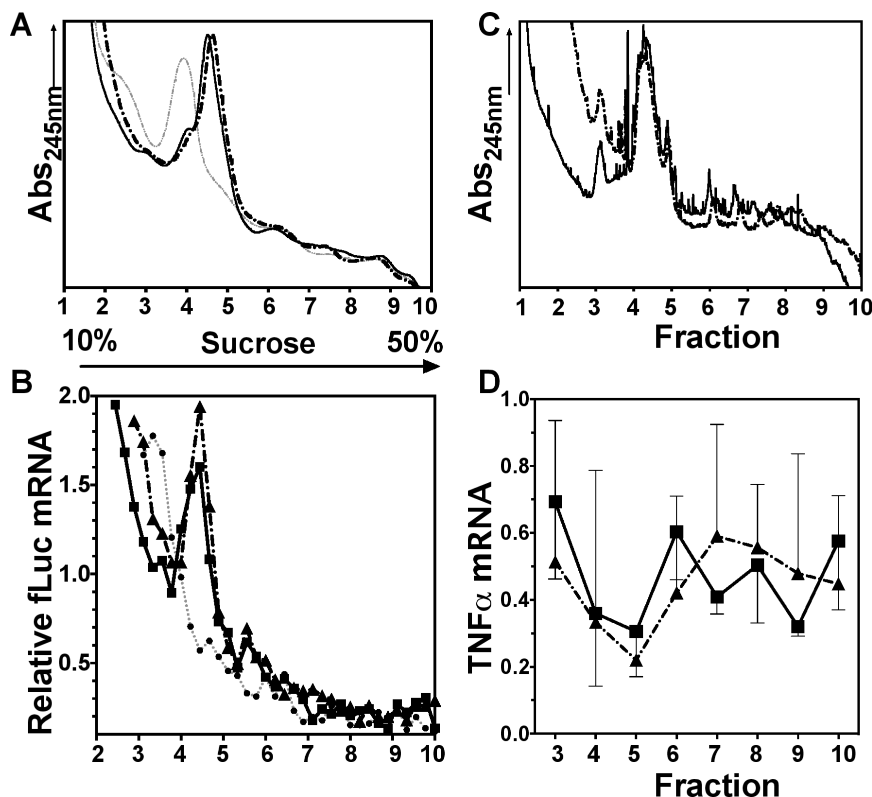

Fig. 6. HNP1 has no effect on polysome profile. **(A)** RRL pre-treated with 25 μg/mL HNP1 or vehicle control. 2ng ^{32}P-labelled m^{7}G-fLuc-A$_{0}$ reporter mRNA translated for 30 min prior to addition of 150μg/mL cycloheximide or 25mM EDTA and 10-50% sucrose density gradient fractionation. Solid black line = vehicle control-treated, broken black line = HNP1-treated, dotted grey line = EDTA-treated. **(B)** Relative reporter mRNA content of gradient fractions expressed as a percentage of the total input mRNA. Solid black line with squares = vehicle control-treated, broken black line with triangles = HNP1-treated, dotted grey line with filled circles = EDTA-treated. **(C)** HMDMs treated 25 μg/mL HNP1 or vehicle control prior to R848 stimulation for 2 hours. 150μg/mL cycloheximide added for 10 minutes prior to lysis and 10-50% sucrose density gradient fractionation. Abs$_{254nm}$ trace to determine sedimentation of 80S ribosome and polysomes. Solid black line = vehicle control-treated, dotted line = HNP1-treated. **(D)**TNFα mRNA content of gradient fractions expressed relative to maximal TNFα mRNA detected in fractions 3-10 (43S/60S to polysomal). Solid black line = vehicle control-treated, broken black line = HNP1-treated, error bars represent mean ±SD (n=4); paired t test, no significant differences detected.

that whilst HNP1 profoundly alters protein translation at a point after translation initiation, it does not prevent mRNA polysomal association.

Discussion

Cells of the immune system have developed tightly regulated systems to ensure the timely resolution of inflammation. The control of mRNA translation is emerging as a major mechanism that regulates the levels of proteins within leukocytes [reviewed in (23, 24)]. We have now identified a novel mechanism in which the most abundant neutrophil α-defensin, HNP1, [which is readily released as these cells die (5)], inhibits bulk protein translation within macrophages. Whilst the characteristic hydrophobic, amphipathic nature of α-defensins allows them to partition into the membrane lipid layer (25), it also ensures ready access to the cell's interior. Confocal imaging showed that HNP1 entered macrophages [Fig 2], without inducing an unfolded protein response [Fig 4] or affecting mRNA stability [Fig 3]. To our knowledge this is the first description of an eobiotic peptide released by one cell profoundly affecting the translational capacity of another, in the absence of a requirement for *de novo* transcription, and without compromising antimicrobial function.

HNP1 was able to inhibit translation initiated via diverse mechanisms. Both canonical cap-dependent [Fig 5] and non-canonical, cap-independent translation (driven by either a CSFV or CrPV IRES) were profoundly inhibited *in vitro*. However the small inhibitory effect of HNP1 on translation initiation [Fig 5F] was insufficient to explain the magnitude of the effects seen *in vitro* and within macrophages. Rather, the dramatic inhibition of CrPV IRES-driven translation, which dispenses with the initiation event implicates an HNP1-mediated inhibition, downstream of translation initiation. HNP1 could inhibit translation by binding non-specifically to mRNA or equally it could sequester factors essential for translation, such as tRNA or ribosomal protein and/or rRNA components. Previous reports point to several RNA-binding proteins that require a net positive charge and arginine side chains (18). α-Defensins also possess 4 positively-charged arginines, that might allow it to interact with RNA [Fig 3]. These side chains are important for its function, as the substitution of these amino acids for similarly charged lysine, significantly reduces its bactericidal activity [(17, 26) and reviewed in (10)]. Considering the ability of HNP1 to kill a diverse array of bacterial and viral pathogens, it will be of interest to determine whether HNP-1 can similarly prevent prokaryotic protein translation.

Since HNP1 binds non-specifically to RNA we asked if it could inhibit translation by modulating ribosome engagement with mRNA. However both reporter and cellular mRNAs remained polysome-associated [Figs 5 and 6] and the polysomal distribution of these mRNAs were similar in control and HNP1-treated RRL and HMDMs. Translational repression could be occurring via either elongation and/or termination (27) and we would speculate that HNP1 prevents translation elongation (22), which has recently been established as a major control point for protein synthesis (30).

Previous studies also allude to the greater importance of protein synthesis rate over degradation rate in determining overall protein levels (28, 29). However, the lack of a significant change in overall HMDM cellular protein level [Fig S1B] argues against an HNP1 mediated increase in non-specific cellular protein degradation. Further, HNP1 profoundly inhibits reporter protein synthesis in cell-free assays in which protein turnover pathways are fundamentally compromised and HNP1 itself has no known protease activity. Taken altogether we believe these data indicate that HNP1 affects *de novo* protein synthesis.

The tertiary structure of monomeric HNP1 is also clearly important for translational inhibition, as highlighted by the loss of efficacy observed for linearized HNP1 (L-HNP1) or W26A [Fig 1C]. However, the N-methylation of HNP1 Ile-20 (Melle), which prevents dimerization, does not alter the ability of Melle to inhibit TNF-α production, confirming that HNP1 dimerization is

not required to inhibit macrophage protein translation [Fig 1D]. The concentration of HNP1-3 in the synovial fluid of patients with rheumatoid arthritis is between 3 and 25 μg/ml, with an average of 12.4 μg/ml, suggesting that the concentration reached in tissues is similar to that used in our assays (5). Our previous studies have shown that HMDMs fully recover their pro-inflammatory potential within 72 hours following exposure to α-defensins; so whilst they clearly disable the macrophage protein translation machinery, they do not induce macrophage apoptosis (5). A previous study reported that α-defensins reduced the release of IL1β from activated monocytes, whilst not affecting the transcription of IL1β mRNA (30). Based on our findings, these observations can likely be explained by the translation of pro-IL1β being impaired.

In summary we have uncovered that neutrophil α-defensins abrogate the bulk mRNA translation of proteins within HMDMs, without affecting mRNA transcription or stability. In this way they prevent an excessive pro-inflammatory response that would create its own collateral damage, whilst still acting as powerful antimicrobial peptides. This is the first demonstration of an anti-microbial peptide that also has a translation-based anti-inflammatory role, acting as a 'molecular brake'. It opens the way forward to developing similar peptide-based therapeutics that would act as effective combined anti-inflammatory and antimicrobial agents.

Materials and Methods

All materials and the following protocols and are fully described in the SI Appendix. Briefly, synthetic HNP1 and mutant derivatives were prepared by solid-phase synthesis as previously described (31). Template plasmids pCSFV-lacZ (32), pT7-Luc (33) for reporter mRNA transcription were previously described and pSL200-CrPV-RLuc, Renilla luciferase downstream of a Cricket Paralysis Virus (CrPV) IRES, was a kind gift from Matthias Hentze (EMBL, Heidelberg). Healthy donor peripheral blood mononuclear cells (PBMCs) were purified from whole blood as previously described (5). Stimuli included 1μg/mL R848 (Invivogen), 3μg/mL CD40L (Peprotech) and 5ng/mL IFNγ (Peprotech). Cytokines were quantified by sandwich ELISA (R&D Systems). For assessment of protein synthesis HMDMs were incubated in L-Methionine-free DMEM (MP Biomedicals) for 2 hours at 37°C, 5% CO_2 followed by 10μCi/mL ^{35}S-Methionine (Perkin Elmer), stimulation with CD40L and IFNγ and defensin peptides. In vitro transcription was assessed by m^{7}G- or ApG-capped, nonadenylated, ^{32}P-UTP-labelled or non-labelled reporter mRNAs were synthesised as previously described (35). In vitro translation was assessed using the nuclease-treated rabbit reticulocyte lysate (RRL) *in vitro* translation kit (Promega) according to manufacturers' recommendations. For electrophoretic mobility shift assays 10 pmoles 5'-Cy5 labelled 25mer oligonucleotide (poly-Adenine, poly-Cytosine or poly-Uracil) (Eurogentec) was incubated with HNP1 or W26A peptide in 10μL binding and then resolved by electrophoresis. For immunocytochemistry HMDMs were grown on glass coverslips and stained with mouse monoclonal anti-human HNP1-3 antibody together with polyclonal rabbit anti-human ribosomal protein rps20 (Abcam, dilution 1:250) or polyclonal rabbit anti-human calreticulin (Abcam, dilution 1:250). ER stress and the UPR, the mRNA stability assay along with RNA quantitation and polysome analysis are fully explained in the supplemental Materials and Methods section.

Submission PDF

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