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Functional intersection of Human Defensin 5 with the TNF receptor pathway

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Abstract

Defensins are cationic antimicrobial peptides that contribute to regulation of host cell function also. Here, we report on the regulation of cell death by Human Defensin 5, the major antimicrobial peptide of ileal Paneth cells. We find that Human Defensin 5-mediated cellular effects depend on functional expression of Tumor Necrosis Factor receptors and downstream mediators of TNF signaling. Our data indicate the involvement of interactions between Human Defensin 5 and the extra-cellular domain of Tumor Necrosis Factor receptor 1. Human Defensin-5 also induces apoptosis intrinsically by targeting the mitochondrial membrane.

Keywords

Human Defensin 5; TNF receptor; interleukin 8; antimicrobial peptide, apoptosis

Introduction

Defensins are endogenous peptides produced by certain leukocytes and epithelial cells [1-3]. These peptides demonstrate antimicrobial, antiviral, toxin-neutralizing and immuno-modulatory properties [3-7]. They are small, cationic, beta-sheet, tri-disulphide peptides [8, 9] and, in humans, comprise genetically distinct alpha and beta subfamilies that evolved from a common ancestral gene [10, 11]. In humans, six -defensins have been identified to date, termed Human Neutrophil Peptide 1-4 and Human Defensin 5 and -6. Human Neutrophil Peptides (HNP1-3) differ from each other by an N- terminal amino acid residue and were originally isolated from azurophilic granules of neutrophils [12, 13]. HNP4, dissimilar to HNP1-3 and much less abundant, was found later in neutrophils [14-16], followed by the discovery of the two enteric α-defensins (HD-5 and HD-6), expressed in Paneth cell of the small intestine [17, 18].

Defensins also act in adaptive immunity by serving as chemoattractants and activators of immune cells [2, 19]. For example, HNP-2 and hBD2 are chemotactic to CD4+/CD45RA+

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naive T cells, CD8+ T cells, immature dendritic cells and monocytes/macrophages [20-22]. For hBD2, chemotaxis of immature dendritic cells and memory T cells results from its direct binding and activation of the chemokine receptor CCR6 who's only known chemokine-ligand is MIP-3α [23] . Members of the -defensin family have been further shown to interact with Toll-Like Receptor 4 [24] and more recently the melanocortin 1 receptor, causing black coat color in domestic dogs [25]. For most defensins, however, their cognate receptors in adaptive immune responses have yet to be identified. The only member of the human α-defensin family to date with reported receptor interactions is HNP1. Interaction of HNP-1 with the purinergic receptor P2Y on airway epithelial cells has been reported [26]. In addition, HNP-1 was shown to inhibit monocyte differentiation, partly due to interactions with the P2Y6 receptor [27]. More recently, the activation of macrophages by HNP-1 was shown to be insensitive to pertussis toxin, as well as independent of purinergic receptors, Toll-like Receptor and CD18 signaling [28] suggesting these effects may be cell type dependent.

We previously reported that HD-5 signaling is MAPK- and NF-$_{\kappa}$B-dependent and that HD-5 signals together with TNFα in a synergistic fashion [29, 30]. Here, we report on the involvement of TNFα receptors on HD-5-mediated cellular effects. We first examined the effects of HD-5 on murine cells lacking TNF receptor genes (TNFR1 and 2 knockout). More detailed involvement of receptors was examined in cells that express human TNF receptors carrying deletions or mutations in their extracellular domains. Cell viability, apoptosis, signaling, caspase activation and mitochondrial membrane activity was measured in cells deleted for TNF receptor downstream effectors RIP1 kinase and caspase-8.

Material and methods

Solid phase peptide synthesis

Chemical synthesis of HD-5 was carried out as described [31]. FAM-HD-5, a fluorescein-labeled form of HD-5 was prepared as follows: N-hydroxysuccinimide-activated FAM was added to HD-5 peptide at 3:1 molar ratio in 50% Di-Methyl Formamide (DMF), 0.1 M HEPES pH 7.3 at a final peptide concentration of 8 mg/ml. The reaction was carried out for 2 h and labeled peptide was re-purified by HPLC. The molecular mass of the peptides was verified by electrospray ionization mass spectrometry (ESI-MS) as described [31].

Cell culture

The Jurkat and the capsase-8 deficient Jurkat cell line I9.2 were obtained from the American Type and Tissue Culture (ATTC). The RIP1-deficient Jurkat cell line termed TM2 [32] was generously provided by Dr. Ting (Mount Sinai Hospital, New York). Wild-type and TNF receptor double knockout murine macrophages [33] were generously provided by Dr. Aggarwal (University of Texas MD Anderson Cancer Center). TNF receptor 1 and 2 double knockout murine macrophages expressing the extra-cellular domain of TNFR1 fused to FAS (TNFR1-FAS), the extra-cellular domain of TNFR1 with the first cysteine-rich domain deleted (ΔTNFR1-FAS) and the extra-cellular domain with the cysteine-rich domain of TNFR2 replacing that of TNFR1 (CRD1TNFR2-TNFR1-FAS) were generously provided by Dr. Scheurich (University of Stuttgart, Germany [34]). Jurkat TM2 cells were passaged in

Iscove's modified Dulbecco's medium with 10% iron- supplemented calf serum, 0.25 mg/ml xanthine, 0.1 mM hypoxanthine, and 5 ug/ml mycophenolic acid. For all experiments, the wild-type and KO12 murine macrophages and the Jurkat cell lines were maintained in RPMI 1640 medium (Gibco), supplemented with 10% FBS (Valley Biomedical, Winchester, Va), 2 mM L-glutamine (Quality Biological, Gaithersburg, MD), 20 mM HEPES, 1 × nonessential amino acids, 1 mM sodium pyruvate and 5% Penicillin/Streptomycin (Sigma) in a humidified incubator at 37 °C with 5% CO_2. The TNFR1-FAS, ΔTNFR1-FAS and CRD1TNFR2-TNFR1-FAS cells were maintained with 5% FBS RPMI 1640 medium (Gibco), supplemented with 10% FBS (Valley Biomedical, Winchester, Va), 2 mM L-glutamine (Quality Biological, Gaithersburg, MD), 20 mM HEPES, 1 × nonessential amino acids, 1 mM sodium pyruvate and 5% Penicillin/Streptomycin (Sigma) in a humidified incubator at 37 °C with 5% CO_2.

Cell viability

The effect of defensin peptides on cell viability was assessed by measuring the mitochondrial activity using MTS assays according to the manufacturer's instructions (Cell Titer 96 proliferation assay, Promega). The cells were cultured in 96-well plates in the presence of serum, washed once in culture medium without serum and subsequently exposed to the peptides at the indicated concentration for 16 hours. The number of viable cells was determined by measuring the absorbance at 450 nm on a microplate reader (Molecular Devices).

Evaluation of cytokine secretion

Jurkat cells were gently washed in serum-free medium and subsequently plated in 96-well plates at 1×10^6 cells/ml in serum-free medium. Cells were incubated for 16 hours in the presence of HD-5 peptide at indicated concentrations. Culture supernatants were collected for measurement of IL-8 using the Luminex-100 system (Bio-Rad).

Apoptotic assays

Caspase-8 and caspase-9 activity in cell lysates were measured using the fluorogenic Ac-VETDAMC peptide substrate (Sigma) or the caspase-9 colorimetric kit (Invitrogen) according to manufacturer's instructions. For these experiments, cells were cultured on 6-well tissue culture plates. Cells were washed with serum-free medium and incubated for 16 hours in serum-free medium containing the peptides at final concentrations as indicated. Following incubation, cells were washed with PBS and lysed in 200 μl of lysis buffer (20 mM HEPES, 50 mM NaCl, pH7.2 10 mM DTT containing 1% CHAPS, 1 mM EDTA, 2 mM PMSF, leupeptin (10 μg/ml; Sigma) and pepstatin A (10 μg/ml; Sigma) for 30 min on ice. After centrifugation (7,000 x*g*, 10 min), the protein concentration of the supernatant was determined using the BCA Protein assay reagent (Bio-Rad). Subsequently, 20 μg of each sample was diluted to a final volume of 200 μl in assay buffer (20 mM HEPES, 50 mM NaCl, pH7.2 10 mM DTT, 0.1% CHAPS containing either the caspase-8 or caspase-9 substrate) in a 96-well plate. For caspase-8, fluorescence was determined (excitation 405 nm, emission 538 nm) with a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, Ca). For caspase-9 activity, absorbance at 450 nm was measured.

Flow cytometry

Apoptosis in Jurkat cells was determined using the Annexin-FITC apoptosis kit from Calbiochem according to manufacturer protocol. Jurkat, Jurkat I9.2 or Jurkat TM2 cells (2×10^5 cells/ml) were exposed to peptide in serum-free medium for 16 h and subsequently analyzed by flow cytometry (FACSCalibur, BD Biosciences).

Confocal microscopy

Jurkat cells or murine macrophages were cultured on collagen-coated cover glass slips (Mylteni Biotech) as described above for 24 in the presence of serum. The cells were gently washed twice in serum-free medium and incubated at 4 °C for 90 min. Subsequently, cells were exposed to FAM-HD5 (10 μg/ml) and either fixed immediately with 4% formaldehyde or further incubated at 37 °C for 30 min before fixation. The localization and visualization of FAM-HD5 on the cells was analyzed using fluorescence confocal microscopy (Zeiss).

Cell membrane integrity and ATP release assays

Membrane integrity and ATP release was determined using the mitochondrial ToxGlo assay (Promega) according to manufacturer's instructions. For these experiments, cells were cultured on 96-well tissue culture plates, gently washed with serum-free medium and subsequently incubated for 16 hours in serum-free medium containing the peptides at final concentrations as indicated.

Results

Interaction of HD-5 with cells of the host

To study the functional interaction between HD-5 and TNF receptor-mediated events, we first determined the effects of functional expression of TNF receptors on the ability of HD-5 to induce cell death (Figure 1). Cells expressing the TNF receptor 1 and 2 or cells that do not express these receptors (KO12) were exposed to HD-5. While HD-5 induced cell killing in a concentration-dependent manner in wild-type murine macrophages, cell killing was abrogated in the absence of TNF receptors (Figure 1A). Caspase-8-dependent apoptosis mediated via TNF receptor 1 requires receptor internalization [35]. We thus examined the localization of fluorescently labeled HD-5 as well as its ability to induce caspase activity as a function of TNF receptor expression. Wild-type and TNFR1 and 2 knockout cells were incubated with labeled defensin at 4 °C to prevent endocytosis and peptide localization was visualized by confocal microscopy after shifting the cells to 37 °C (Figure 1B). In the absence of TNF receptor expression, HD-5 remained surface-associated, whereas the peptide was readily internalized in wild-type cells. Concomitant, HD-5 induced caspase-8 activity in wild-type, but not in TNF receptor knockout cells (Figure 1C). HD-5 did not induce the activity of caspase-9 in either cell line (data not shown). Thus, HD-5 mediated cell killing, internalization and induction of caspase-8 activity depends on functional TNF receptor expression.

We further examined the involvement of TNF receptors on the cellular interactions of HD-5 in more detail. First, we tested the possibility of interactions between HD-5 and extra-cellular domains of TNF receptors. For these experiments, we used TNF receptor knock-out

mouse fibroblast cell lines stably transfected with the following constructs: the extra-cellular domain of TNFR1 fused to FAS (TNFR1-FAS), the extra-cellular domain of TNFR1 with the first cysteine-rich domain deleted (ΔTNFR1-FAS) and the extra-cellular domain with the cysteine-rich domain of TNFR2 replacing that of TNFR1 (CRD1TNFR2-TNFR1-FAS) [34]. These cells were incubated in the presence of HD-5, TNFα or both at various concentrations and effects on cell survival were determined by MTT assays (Figure 2). TNFα reduced cell viability in a concentration-dependent manner only of the TNFR1-FAS cell line as described previously [34]. This is due to the observation that the CRD1 domain of TNFR1 alters the binding affinity of the receptor for its ligand without directly interaction with TNFα [34]. Exposure to5 μg/ml of HD-5 did not significantly reduced cell viability of these three cell lines. Exposure to 10 and in particular 25 μg/ml of HD-5 however, did reduce cell viability. HD-5 reduced cell viability of all three cell lines, however cell viability was significantly more reduced for the TNFR1-FAS cell line compared to the two other cell lines. The presence of 1 or 10 ng of TNFα greatly enhanced cell killing mediated by HD-5 of the TNFR1-FAS cell line only, even at 5 μg/ml concentration of the defensin peptide (Figure 2B). Only at the highest concentrations of TNFα and HD-5 tested (10 ng and 25 μg/ml respectively) was a reduction in cell survival observed of the ΔTNFR1-FAS and CRD1TNFR2-TNFR1-FAS cells. HD-5 was internalized by these cells (not shown).

Next, we examined the effects of TNF receptor downstream effectors mediating signaling or cell death as measures for involvement of TNF pathways in cellular effects of HD-5. For these experiments, we used Jurkat cells that are deleted for the central downstream TNF receptor kinase RIP1 (termed Jurkat TM2) or caspase-8 (termed Jurkat I9.2) respectively. We determined the ability of HD-5 to mediate cell death, induce secretion of interleukin-8 and activate caspase-8 in these cells compared to wild-type Jurkat cells (Figure 2). We find that HD-5 mediates cell killing of wild-type and RIP1-deficient Jurkat cells in a concentration-dependent manner (Figure 3A). Deficiency of caspase-8 however significantly negated cell killing. Deficiency of either caspase-8 or RIP1 did not alter the localization of HD-5, which was found intra-cellular in all Jurkat cells (Figure 3B). We next examine the ability of HD-5 to induce IL-8 secretion or activate caspase-8 by these cells (Figures 3C and D). Deletion of RIP1 abrogated the induction of IL-8 as well as caspase-8 activity observed in wild-type Jurkat cells exposed to the defensin. As expected, caspase-8 activity was absent in the caspase-8 deficient Jurkat I9.2 cells. Interestingly, IL-8 secretion by these cells was reduced compared to wild-type cells. Again, HD-5 failed to induce caspase-9 activity in these cell lines (not shown). Thus, HD-5 signaling and cell killing is severely diminished by deficiency in caspase-8. Deficiency of RIP1 negated induction of IL-8 secretion as well as induction of caspase-8 activity. Combined with the findings above, these results indicate that HD-5 affects TNF receptor activity extrinsically.

Deficiency in caspase-8 severely diminished, but not completely abolished cell death mediated by HD-5 (Figure 3A), suggesting that the defensin kills cells via a secondary mechanism. To investigate this, we first examined the ability of HD-5 to induce apoptosis by flow cytometry. Jurkat cells deficient in caspase-8, RIPK1 or wild-type were exposed to HD-5 and apoptosis was determined by Annexin V/Propium Iodide staining (Figure 4). Analysis of dead cells revealed that the majority of wild-type and RIP1-deficient Jurkat cells were in late stage apoptosis and/or necrosis. As expected, apoptosis was not detected in

caspase-8-deficient cells, and the majority of dead cells were necrotic when exposed to HD-5 (Figure 4, middle panel). HD-5 treated macrophages aggregated following de-adherence and could not reliably be analyzed by this approach.

Since the ability of HD-5 to induce cell death correlates with its intracellular location, we examined the possibility that the defensin mediates apoptosis intrinsically by affecting the integrity of the mitochondrial membrane. We examined the ability of HD-5 to affect mitochondrial function by measuring cellular ATP levels and cell membrane integrity simultaneously (Figure 5). HD-5 did not affect cell membrane integrity of either the wild-type or TNF receptor-deficient murine macrophages. In contrast, the cell membrane integrity of Jurkat cells was compromised by the defensin in a concentration-dependent manner, irrespective of caspase-8 or RIP1-deficiency (Figure 5A). Cellular ATP levels were significantly and concentration-dependently reduced by HD-5 in all cell lines, with the exception of the receptor knockout cell line (Figure 5B). Together, these data indicate that intracellular location of HD-5 is required for mitochondrial toxicity, suggesting an intrinsic mechanism-of-action of cell killing by the defensin, even in the absence of caspase-8.

Discussion

We previously reported that HD-5 acts synergistically with TNFα [29]. Here, we show the involvement of the TNF receptors and TNF-mediated signaling pathways in the cellular interactions of HD-5. Tumor Necrosis Factor α is a key regulator in the immune system with strong proinflammatory and immunomodulatory properties. It is also involved in controlling life or death of target cells and plays a critical role in many acute and chronic inflammatory diseases [36]. TNFα can be produced by many different cell types and exists in homo-trimeric transmembrane as well as soluble forms, both biologically active. Both forms of TNFα interact with the extracellular domain of either of two structurally distinct receptors, TNFR1 and TNFR2, activating distinct intracellular signaling pathways and gene transcription [35, 37]. The extracellular domains of TNF receptors are comprised of 3-4 cysteine-rich domains or CRDs. Trimers of receptor chains pre-assemble on the cell surface prior to ligand binding, owing to associations of the pre-ligand–binding assembly domain or PLAD [38]. These PLAD domains comprise of the first CRD domain plus an unstructured N-terminal sequence and are subtype-specific for TNF receptors. Native formation of ligand-independent trimers is necessary for ligand binding and death signaling [38, 39]. TNFR1 is expressed constitutively on almost every type of cell in the body except erythrocytes, whereas TNRF2 expression is generally inducible and more limited to endothelial cells and hematopoietic cells preferentially [40]. Although soluble TNFα binds to both receptors, it preferentially binds to TNFR1, whereas the trans-membrane form binds preferentially to TNFR2 [41, 42].

TNFR1 is capable of inducing cell death or anti-apoptotic signaling via NF-kB or MAPK pathways. TNFR1 receptor internalization is essential for transmitting the signals that lead to apoptosis [43]. Signaling initiated by soluble TNF via TNFR1 and TNFR2 is mediated by adapter proteins that bind to the cytoplasmic domains of the receptors upon extracellular ligand binding. The cytoplasmic region of TNFR1 contains a death domain that couples TNFR1 to either of 2 distinct signaling pathways via binding of the adapter protein TNFR-

associated death domain. The primary pathway leads to activation of nuclear factor kappa-B1 (NF-κB1), a family of transcription factors that control a large number of inflammatory genes, and a distinct signaling pathway leads to caspase-8– and caspase-3–dependent apoptosis. TNFR2 signaling generally promotes NF-κB1-dependent transcription of pro-survival genes [40]. Cross-talk between signaling pathways of TNFR1 and TNFR2 further determines the balance between cell death and survival [40].

We find that HD-5 interacts with the extra-cellular domain of TNF receptors. This interaction depended on the presence of the first cysteine-rich domain of TNFR1, a domain that does not directly bind to ligand, but does affect ligand binding affinity [34]. We further find that HD-5 exerts signaling and apoptotic events associated with those of TNF receptor 1 [35]: 1) HD-5 induces caspase-8 activity only upon internalization; 2) HD-5 signaling and caspase-8 activation is a RIP1-dependent; 3) deletion of the CRD1 domain of TNFR1 or its replacement with the CRD1 domain of TNFR2 significantly reduces cell-mediated killing by HD-5. Combined, these observations together with previously observed HD-5 signaling [30] and synergism of HD-5 and TNFα [29], our data suggest interactions between HD-5 and TNFR1. In addition, we find that HD-5 depolarizes the mitochondrial membrane only upon internalization. Mitochondrial membrane activity was observed in the absence of functional RIP1, and further, HD-5 did not induce caspase-9 activity. These findings indicate that, once inside the cell, the antimicrobial defensin peptide HD-5 kills the cells via a second mechanism, likely by directly targeting the bacterial-derived mitochondrial membrane.

A specific deficiency of HD-5 has been observed in patients with ileal Crohn’s disease, a chronic inflammatory disease [44, 45]. These findings have led to the notion that HD-5 as an innate immune effector molecule may play an important role in the maintenance of mucosal balance and that deficiency of HD-5, resulting in weakening of mucosal antibacterial capacity may contribute to pathogenesis [46]. Based on our findings, HD-5 may play additional roles in regulating cell death and differentiation by directly and specifically acting on cells of the host also. Interestingly, expression of both TNFα as well as defensins locates exclusively to Paneth cell granules in mice and humans in normal bowel [47, 48].

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- Human Defensin 5 functionally intersects with Tumor Necrosis Factor receptor pathways
- Cellular effects of Human Defensin 5 involve interactions with the extra-cellular domain of Tumor Necrosis Factor 1
- Human Defensin 5 induces cell death by targeting the mitochondrial membrane also

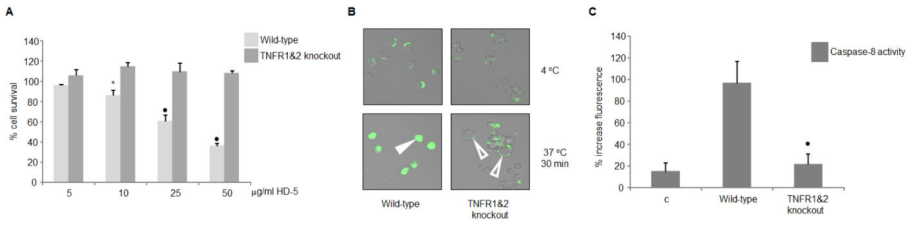

Figure 1.

Cellular interactions of HD-5 are TNF receptor-dependent. (**A**) wild-type or TNF receptor 1 and 2 (KO12) murine macrophages were exposed to the peptides at concentrations of 10, 25 or 50 µg/ml for 24 hours in serum-free medium. Mitochondrial activity was measured by MTS colorimetric measurement and plotted as percentage of untreated control cells. Data represent average of three experiments carried out in triplicate (**B**) Localization of FAM-HD-5 in murine macrophages determined by confocal microscopy. Panels show the bright field image with the fluorescence image superimposed. The fluorescence images represent a 1 µM optical section acquired approximately at the equator of the cells, which typically are ~15 µM. (**C**) Cells were exposed to HD-5 at a concentration (25 µg/ml) for 24 hours in serum-free medium. Caspase-8 activity was determined in cellular lysates by measuring the fluorescence of the Ac-VETD-AMC substrate conversion. Data represent average of three experiments carried out in sextuplet (* p 0.05; ●p 0.005).

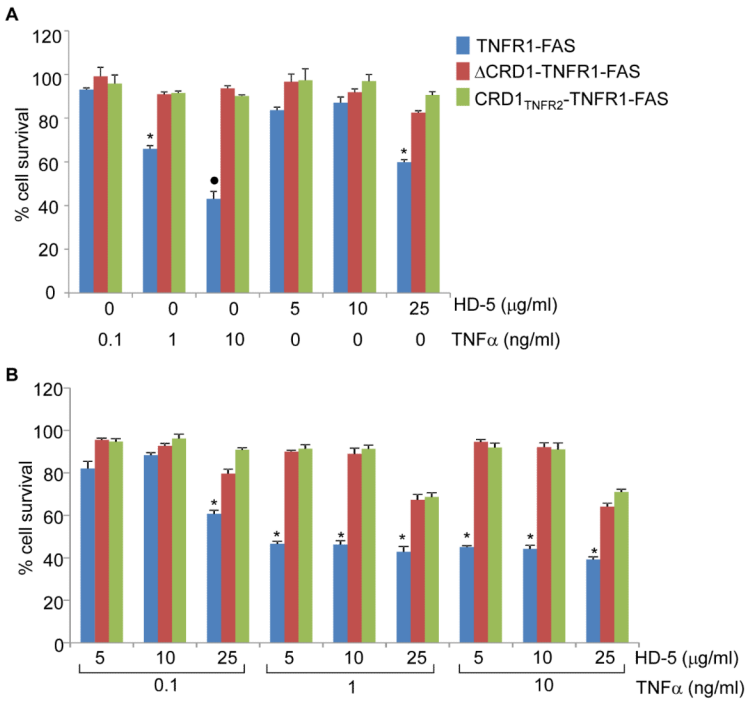

Figure 2.

Cellular interactions of HD-5 depend on the cysteine-rich domain 1 of TNFR1. TNFR1&2 double-knockout murine fibroblast cell lines stably expressing TNFR1-FAS, ΔCRD1-TNFR1-FAS or CRD1TNFR2-TNFR1-FAS were exposed to TNFα or HD-5 alone (**A**) or in combination (**B**) at peptide concentrations of 5, 10 or 25 μg/ml and/or TNFα concentrations of 0.1, 1 or 10 ng/ml as indicated. Data represent three experiments carried out in triplicate (* p 0.05; ●p 0.005).

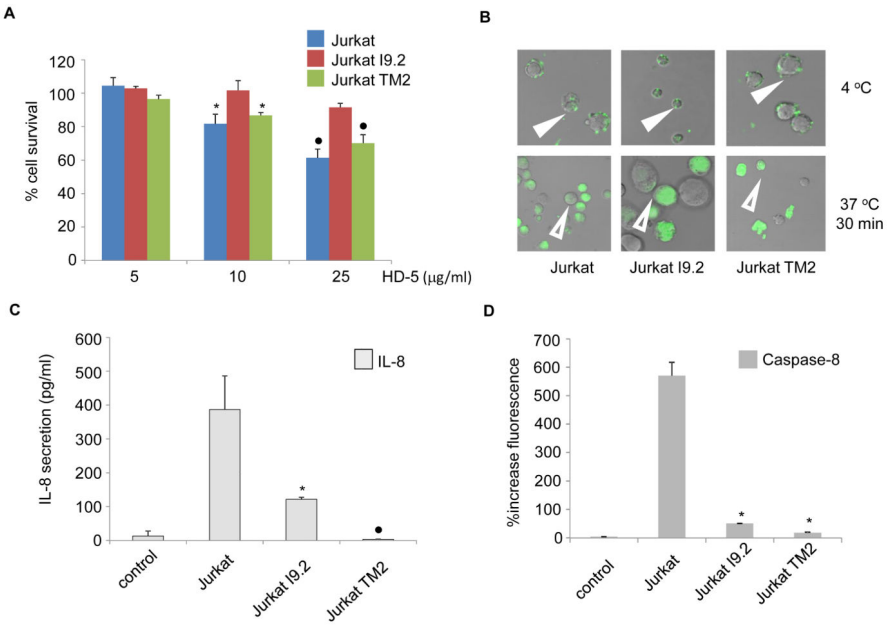

Figure 3.

Cellular interaction of HD-5 depends on functional expression of RIPK1 and caspase-8. (**A**) wild-type Jurkat, caspase-deficient Jurkat I9.2 or RIPK1-deficient Jurkat TM2 cells were exposed to the peptides at concentrations of 5, 10 or 25 μg/ml for 24 hours in serum-free medium. Mitochondrial activity was measured by MTS colorimetric measurement and plotted as percentage of untreated control cells (**B**) Localization of FAM-HD-5 in Jurkat cells determined by confocal microscopy. Panels show the bright field image with the fluorescence image superimposed. The fluorescence images represent a 1 μM optical section acquired approximately at the equator of the cells, which typically are ~15 μM. (**C**) Jurkat cell lines were exposed to HD-5 (25 μg/ml) for 16 hours. Interleukin-8 in the supernatant was detected by ELISA using the Luminex assay from Invitrogen. Means ±SEM for three independent experiments carried out in triplicate are shown. (**D**) Cells were exposed to HD-5 at a concentration of 25 μg/ml for 24 hours in serum-free medium. Caspase-8 activity was determined in cellular lysates by measuring the fluorescence of the Ac-VETD-AMC substrate conversion. Data represent three experiments carried out in triplicate (* p 0.05; ● p 0.005).

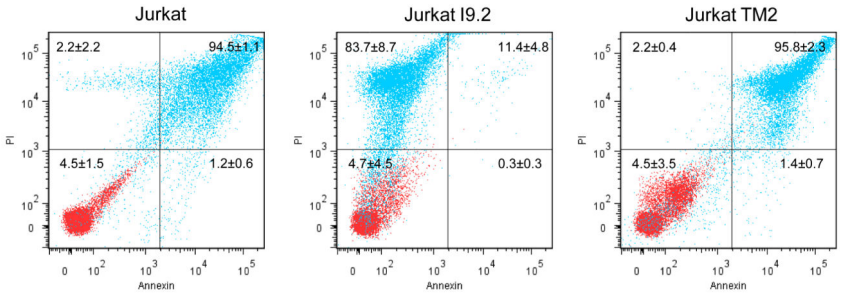

Figure 4.

HD-5 induces necrosis and apoptosis in a caspase-8-dependent manner. Jurkat (left panel), Jurkat I9.2 (middle panel) or Jurkat TM (right panel) cells were exposed to HD-5 (25 μg/ml) for 16 hours and assayed for apoptotic events by flow cytometry. Means ±SEM for three independent experiments are shown with results for one out of three representative experiments.

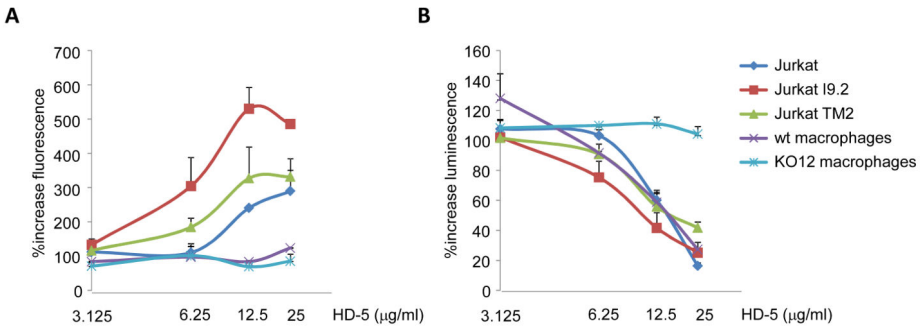

Figure 5.

HD-5 is a mitochondrial toxin. Wild-type and TNF receptor knockout macrophages, as well as wild-type, caspase-8- and RIPK1-deficient JURKAT cells were exposed to HD-5 at the indicated concentrations for 24 h in serum-free medium. Cell membrane integrity (A) and ATP release (B) were determined as described in Materials and Methods. Results shown represent three experiments carried out in triplicate.