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Interactions between octaarginine and U-937 human macrophages: Global gene expression profiling, superoxide anion content, and cytokine production

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ABSTRACT

Cell penetrating peptides such as octaarginine (R8) have been widely used as intracellular delivery vectors to import biologically active membrane-impermeable molecules. However, before using these peptides clinically, human immune responses to them must be fully understood. Because macrophages are important for immune responses, we evaluated the interactions between R8 and a human U-937 cell line. Cytotoxicity, binding, internalization, genome-wide profiling of gene expression, intracellular superoxide anion content, and cytokine release were assessed after U-937 cells had been incubated with different amounts of R8. Cytotoxicity was limited for up to 40 μM of R8 and 24 h of incubation. Kinetic analysis of the binding and uptake of cells treated with fluorescein-5-isothiocynate-R8 showed time- and concentration-dependent increases. Microarray analysis identified 4386 genes time-dependently regulated when U-937 macrophages were exposed to 10 μM of R8 for 0.5 h and 4 h; the majority of these genes were upregulated for each time point. Thirty-five upregulated genes responded to the stimuli with immune functions, and, using real-time quantitative reverse transcriptase-polymerase chain reaction analysis, five genes – FOS, OSM, C1R, TNF, IL1R1 – were confirmed. R8 induced superoxide anion production after 0.5 h, but not after longer incubations. Incubating U-937 cells with R8 for up to 24 h did not release the proinflammatory cytokines TNF-α, IL-1β, and IL-6. In summary, exposing U-937 macrophages to R8 did not induce proinflammatory cytokine release; however, it generated superoxide anion and affected gene expression.

1. Introduction

Interest in using cell penetrating peptides (CPPs) or protein transduction domains as carriers for intracellular delivery is increasing [1–3]. Cell penetrating peptides, alone or coupled with various cargo molecules, can cross biological membrane barriers without significantly damaging the membranes and with little cytotoxicity to the cells [4–7]. Of the cell penetrating peptides used, octaarginine (R8), a member of the synthetic peptide family of arginine-rich peptides, carries various cargos for intracellular delivery [8,9]. Arginine-rich peptides share efficient cellular uptake and an abundance of basic arginine amino acids with the most notable cell penetrating peptide, human immunodeficiency virus (HIV)-1 TAT-derived peptide (48–60) [3,8]. It has been suggested [10,11] that the internalization mechanisms of arginine-rich peptides may depend on various factors, such as the type of peptide, the nature of the cargo, and the linker between them.

Despite the highly efficient intracellular delivery of arginine-rich peptides, their immune responses when injected into the human body must be fully understood because they are derived primarily from non-human proteins, and very few studies have focused on the activity of immune cells after the uptake of these peptides [12]. Immune cells, especially macrophages, are crucial for directing the host's foreign-body reactions and producing various proinflammatory mediators [12]. Although up to 10 μM of oligoarginine peptides were non-toxic to murine RAW 264.7 macrophages for 24 h [13], these peptides induced intracellular responses that have not previously been explored. It has long been recognized that macrophages produce oxygen- and nitrogen-reactive metabolites during phagocytosis or when stimulated by a variety of agents [14]. Reactive oxygen species (ROS) such as superoxide anion (O_2^-) are widely investigated as signaling mediators of both protection and destruction in macrophages. Most studies, however, have been limited to functional gene expression profiles in cellular responses induced by arginine-rich peptides. Therefore, we used a whole-genome analysis by microarray to explore global gene expression profiles from U-937 human macrophages treated with R8. We also evaluated the generation of O_2^- and the production of proinflammatory cytokines in R8-treated macrophages. The results

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may offer a variety of valuable insights into the molecular mechanisms of R8-induced immune responses in macrophages.

2. Materials and methods

2.1. Peptide synthesis

The octaarginine (RRRRRRRR; R8) and fluorescein-labeled octaarginine used in this study were purchased from Genemed Synthesis, Inc. (San Antonio, TX, USA). They were prepared using solid phase peptide synthesis. Fluorescein-5-isothiocynate (FITC) (Sigma-Aldrich Co., St Louis, MO, USA) was coupled to the N-terminus of R8 (FITC-R8) for fluorescent labeling. The quality of the peptides was ascertained using reverse-phase high performance liquid chromatography (HPLC) and time-of-flight mass spectrometry. The purity of all peptides used was >95%. The fidelity of the synthesized peptides was ascertained using electrospray ionization mass spectrometry (ESI-MS) as follows: 1267.25 [expected for $(M+H)^+$ 1267.53] for R8 and 1625.57 [expected for $(M+H)^+$ 1625.85] for FITC-R8.

2.2. Cell culture and incubation protocol

A human macrophage-like U-937 large-cell lymphoma cell line was maintained in RPMI 1640 medium (Gibco BRL, Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 100 U/mL of penicillin/100 μg/mL streptomycin (Sigma-Aldrich) in a humidified atmosphere of 5% CO_2 at 37 °C. The U-937 cell line was chosen because it is a well-established human cell line with many monocytic characteristics and has been extensively used as an *in vitro* model for macrophage stimulation. For microarray analysis, the U-937 cells (1×10^9) were treated with 10 μM of R8 for 0.5 h (T1) and 4 h (T2). After they had been incubated, R8 was removed from the medium and the cells were washed with phosphate-buffered saline (PBS) for further analysis. Negative control cells (T0) contained no peptides. Each experiment was done twice.

2.3. Cytotoxicity assay

Dehydrogenase activity (an indicator of cell viability) in the treated and untreated cells was simultaneously assessed. Negative control cells contained no peptides. Ten microliters of a cell-counting kit (Cell Counting Kit-8; Dojindo Laboratories, Japan) solution, a tetrazolium salt that produces a highly water-soluble formazan dye when biochemically reduced in the presence of an electron carrier (1-methoxy PMS) (Dojindo Laboratories), was added to 100 μL of culture medium and incubated for 4 h. The absorbance at 450 nm was obtained using an ELISA reader with a reference wavelength of 595 nm. Results are reported as the cell-viability percentage (average optical density (OD)/average negative control OD) ± standard deviation (SD).

2.4. Flow cytometric detection of R8 binding and uptake

The cells (1×10^6/mL) were treated with various doses of FITC-R8 for the entire incubation period, as indicated. After the cells had been incubated, the FITC-R8 was removed from the medium and washed with PBS for further analysis. The cells were then incubated with trypsin (1 mg/mL) (Sigma-Aldrich) for 15 min at 37 °C to remove membrane-bound peptides. The cells were washed once more with PBS, and then cell fluorescence was measured using flow cytometry (FACScan; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). A 15-mM air-cooled argon-ion laser was used to excite fluorescent FITC at 488 nm, and the emitted fluorescence was measured using a 530/30-nm band-pass optical filter. Samples were run using 10^4 cells per test sample. Data were analyzed using the CELLQUEST programs. Negative control cells contained 20 μM of FITC only, but no FITC-R8. In some experiments, the trypsinization step was omitted in the washing protocol.

2.5. RNA purification and microarray analyses

Total RNA was extracted using a reagent (Trizol; Invitrogen Corp., Carlsbad, CA, USA) and then a kit (RNeasy Mini; Qiagen GmbH, Hilden, Germany). The purified RNA was quantified by increasing the optical density to 260 nm (OD 260 nm) using a spectrophotometer (ND-1000; Nanodrop Technologies, Inc., Wilmington, DE, USA) and qualitatively analyzed using a bioanalyzer (Bioanalyzer 2100; Agilent Technologies, Inc., Santa Clara, CA, USA). Microarray experiments were done following the manufacturer's protocols. Briefly, 0.5 μg of total RNA was amplified using a kit (Fluorescent Linear Amplification Kit; Agilent) and labeled with Cy3-CTP (CyDye, Perkin-Elmer, Fremont, CA, USA) during the *in vitro* transcription process. Two micrograms of Cy-labeled cRNA was fragmented to an average size of about 50–100 nucleotides by incubating it with fragmentation buffer at 60 °C for 30 min. Correspondingly fragmented labeled cRNA was then hybridized to a 4×44-k microarray (Whole Human Genome Oligo Microarray Kit; Agilent) at 60 °C for 17 h, and then scanned at 535 nm (Microarray Scanner; Agilent) at 535 nm. The scanned images were analyzed using commercial software (Feature Extraction 9.5.3; Agilent), as were the microarray data (GeneSpring GX 7.3.1; Agilent). Chip intensities of six microarray data from three duplicate time points (T0, T1, T2) were normalized to equal signal distribution using per-chip normalization at the 75th percentile. Per-gene normalization to time point 0 h (T0) was also used. The resulting 28,060 genes were selected for differential expression; 4386 differentially expressed genes were selected using 1.5-fold changes at one of three time points and the Significance Analysis of Microarray (SAM) method with a false-discovery rate (FDR) threshold of 0.05. The differentially expressed genes were further analyzed with cluster analysis. The gene ontology (GO) and gene annotations of each cluster were classified with gene ontology annotation (GOA) and proteome slim, which contains a list of general GO terms.

2.6. Real-time quantitative RT-PCR (Q-PCR)

The same RNA isolated for the microarray was used for RT-PCR. To prepare a cDNA pool from each RNA sample, total RNA (5 μg) was reverse transcribed using reverse transcriptase (Moloney Murine Leukemia Virus Reverse Transcriptase; Promega, Madison, WI, USA) and the resulting samples were diluted 40 times using a column with nuclease-free water. The specificity of each primer pair was tested using a template (Universal Rat Reference RNA; Stratagene, La Jolla, CA, USA), for a real-time PCR reaction, and then a DNA 500-chip run on the bioanalyzer to check the size of the PCR product. Real-time PCR reactions were done on a thermal cycler (LightCycler Instrument 1.5; Roche Diagnostics Australia Pty. Ltd., Castle Hill, Australia) using a kit (LightCycler FastStart DNA MasterPlus SYBR Green I, Cat. 03 515 885 001; Roche). Each sample was run in triplicate. The real-time PCR program steps were 95 °C for 10 min, 50 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 10 s. Five genes (FOS, OSM, C1R, TNF, IL1R1) were investigated using the following primer sequences: FOS sense CTACCACTCACCCGCAGACT and antisense AGGTCCGTGCAGAAGTCCT; OSM sense GTGGATGAGAGGAACCATCG and antisense GTAGCAGAGGGGAACAGGTTT; CIR sense TGCTCAACTACGTGGACTGG and antisense GATTCGAACCTAGTGAATTCTGG; TNF sense CAGCCTCTTCTCCCCTTCGAT and antisense GCCAGAGGGCTGATTAGAGA; IL1R1 sense ATTGTGCTTTGGTACAGGGATTCC and antisense ATGCGTCATAGGTCTTTCCATCTG. Human TBP (TATA box binding protein) was used as the reference gene.

2.7. Intracellular O_2^- content

Cell suspensions (1×10^6/mL) with or without R8 treatment were incubated with 10 μM of hydroethidine (HE) incubated for 15 min at 37 °C. HE is oxidized primarily by O_2^- and forms ethidium bromide (EB), which emits red fluorescence [15]. The cells were then analyzed on the flow cytometer. Samples were run using 10,000 cells per test

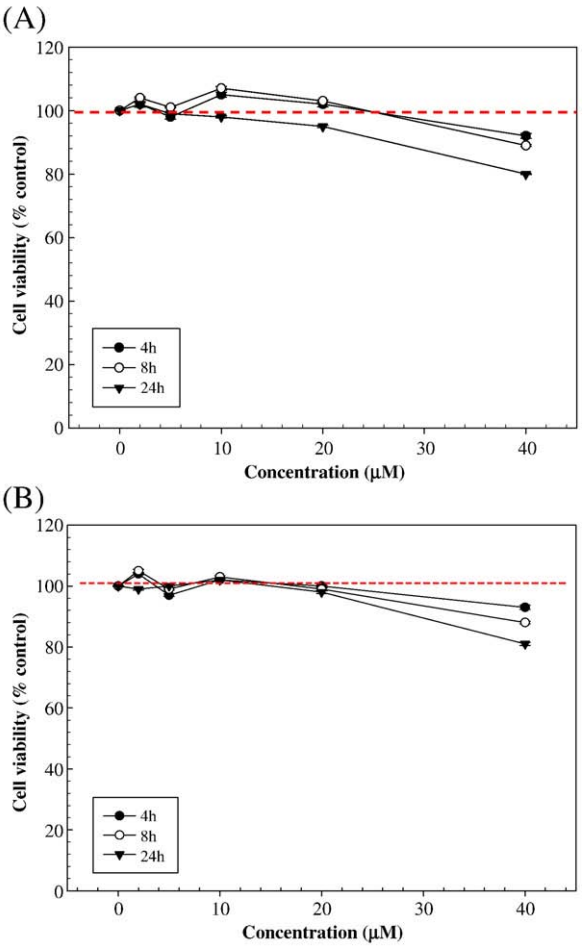


Fig. 1. Cytotoxicity assays of R8 (A) and FITC-R8 (B) on U-937 macrophages by measuring generated dehydrogenases. Negative control cells were grown without adding R8 and FITC-R8. Results are reported as cell viability percentage (average OD/average negative control OD) ± SD ($n = 3$).

sample. Negative control cells contained no R8. Positive control cells were treated with 1 mM of menadione (Sigma-Aldrich). Results are reported as the average fluorescence intensity ± SD.

2.8. Cytokine detection

Cells were seeded in 96-well plates (1×10^4 cells/well) at 37 °C in a 5% CO_2 atmosphere and incubated with different concentrations of R8. Lipopolysaccharide (LPS, from *E. coli*, Serotype 055:B5) (Sigma-Aldrich) at a concentration of 1 μg/mL was used as a positive control; the negative control consisted of medium alone. The concentrations of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in the supernatants were determined using a specific ELISA kit (eBioscience, San Diego, CA, USA).

2.9. Statistical analysis

Statistical analyses were done using a one-way analysis of variance (ANOVA) with a significance level of 0.05. The data from R8-treated cells at different dosages were compared with data from untreated cells at each corresponding incubation time.

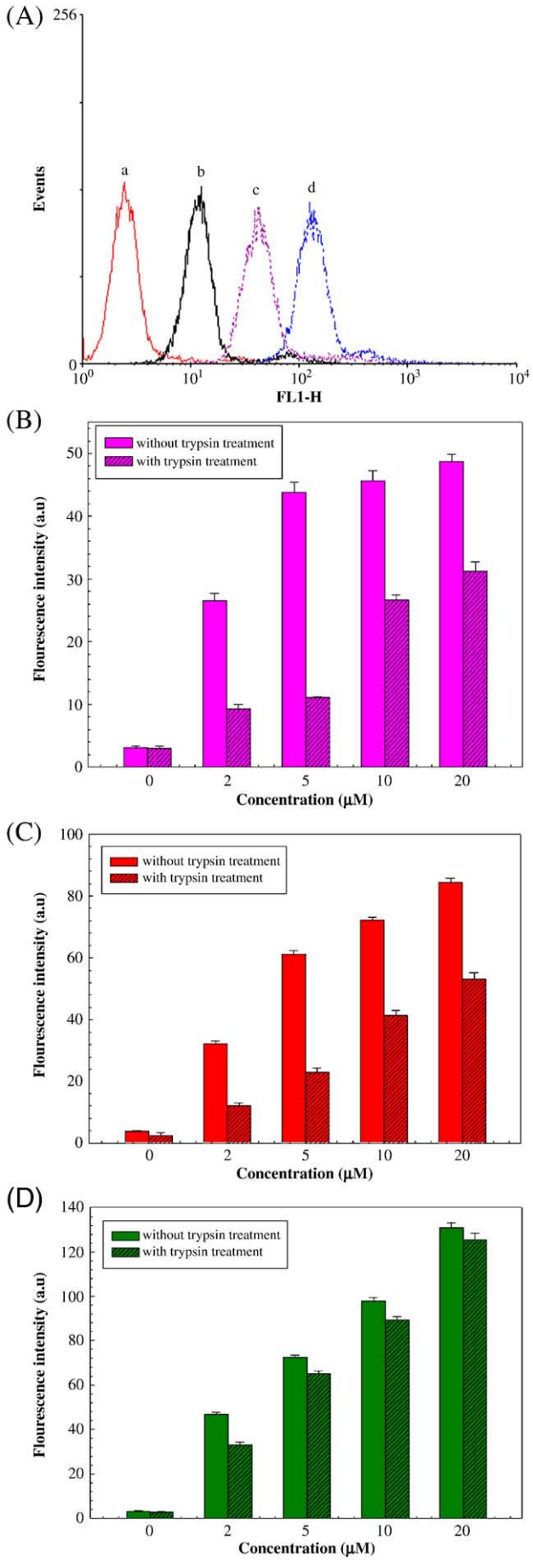


Fig. 2. Flow cytometric analysis of kinetics of R8 binding and uptake. A representative histogram (A) showing the changes in the FITC fluorescence intensity in untreated cells (curve a), cells treated with 20 μM of FITC (curve b), and cells treated with 20 μM of FITC-R8, with (curve c) and without (curve d) trypsin treatment, for 0.5 h. FITC fluorescence intensities in U-937 cells treated with FITC-R8 with and without trypsin treatment at different doses and for different times: 0.5 h (B), 4 h (C), and 8 h (D).

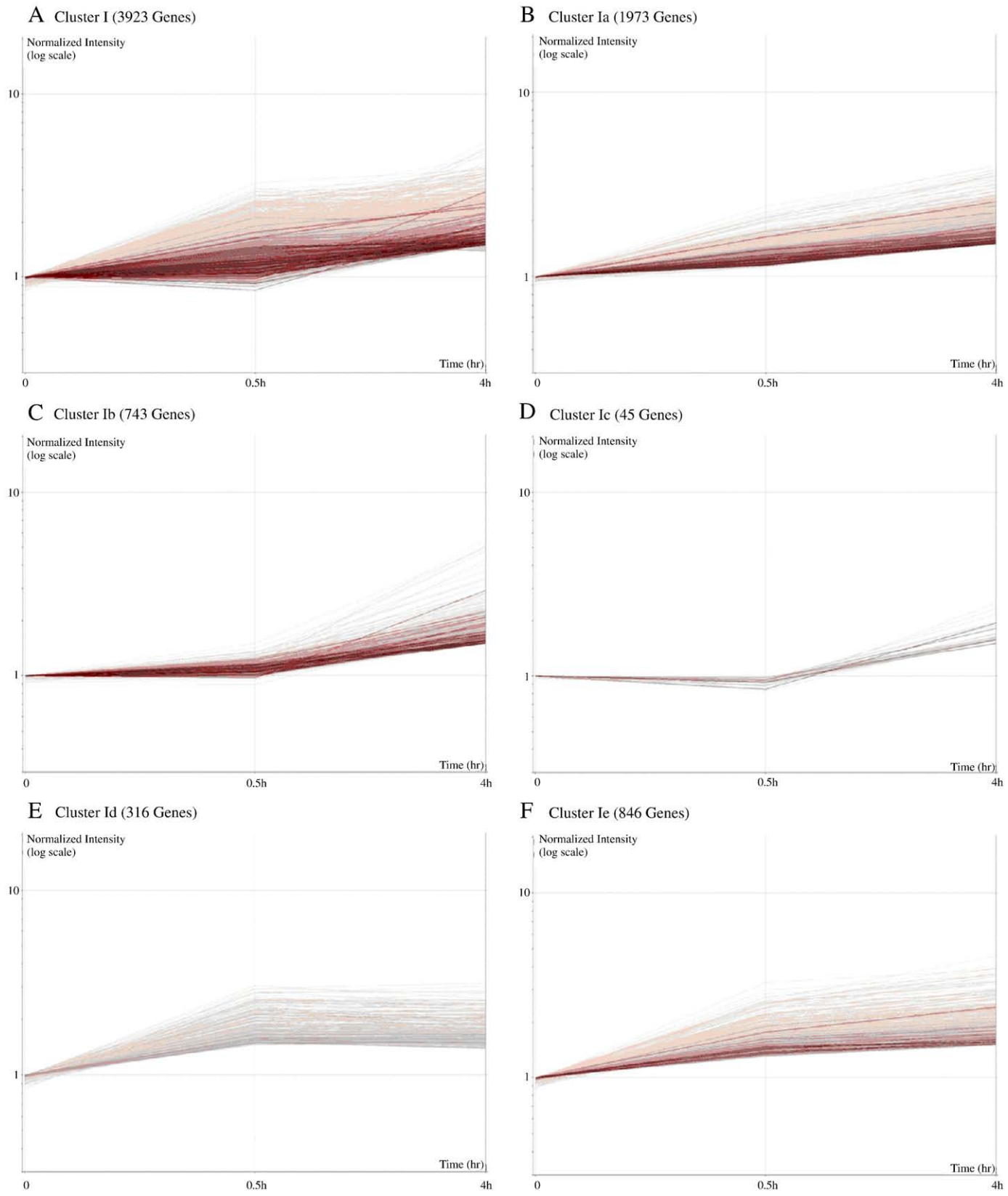
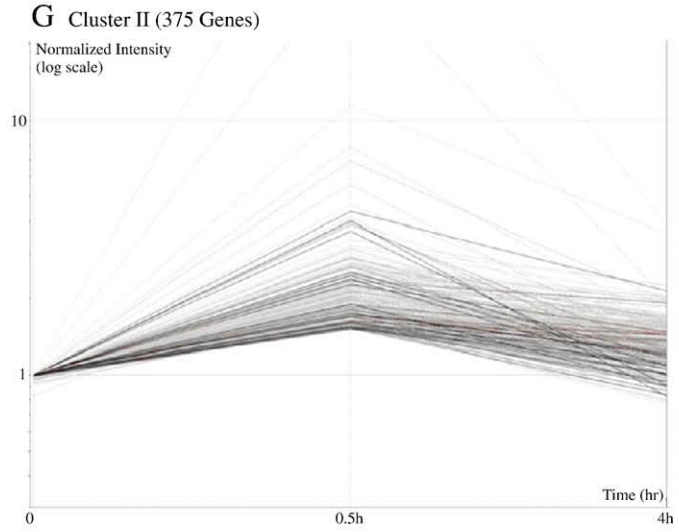
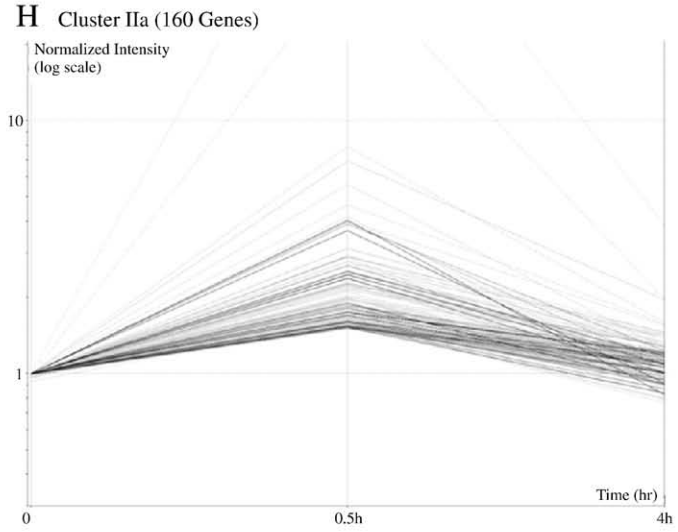
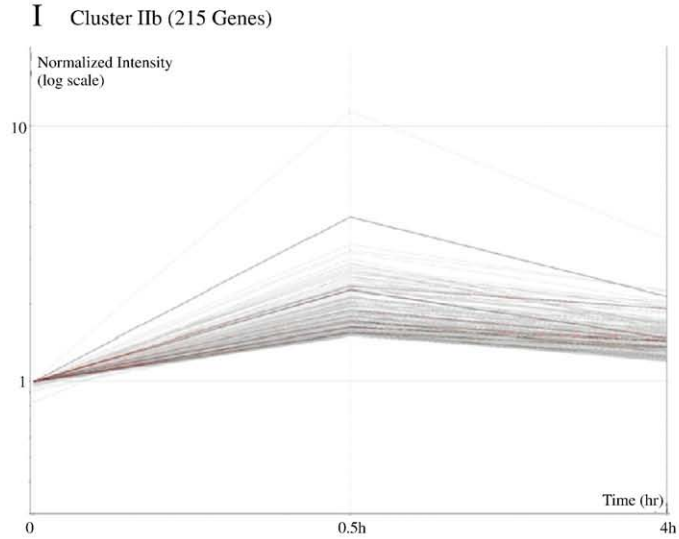
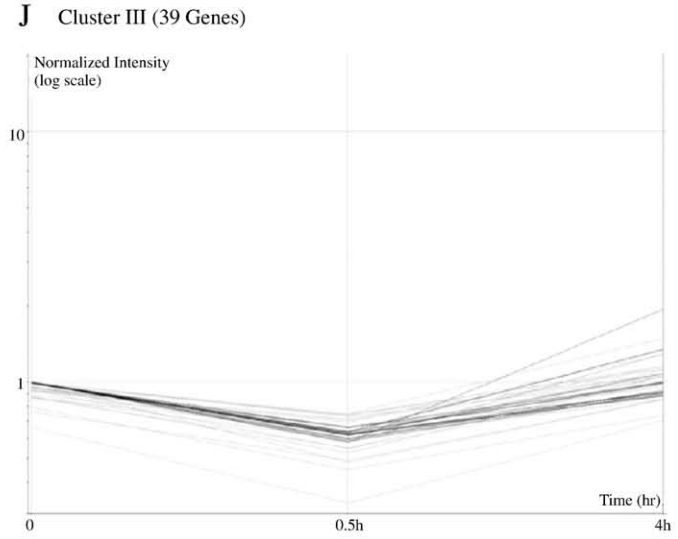


Fig. 3. Cluster analysis for gene responses in human U-937 macrophages treated with 10 μM of R8 after they had been incubated for 0.5 h and 4 h. The number of genes in each cluster is shown in parentheses. The genes categorized in each cluster can be found in Supplemental materials.









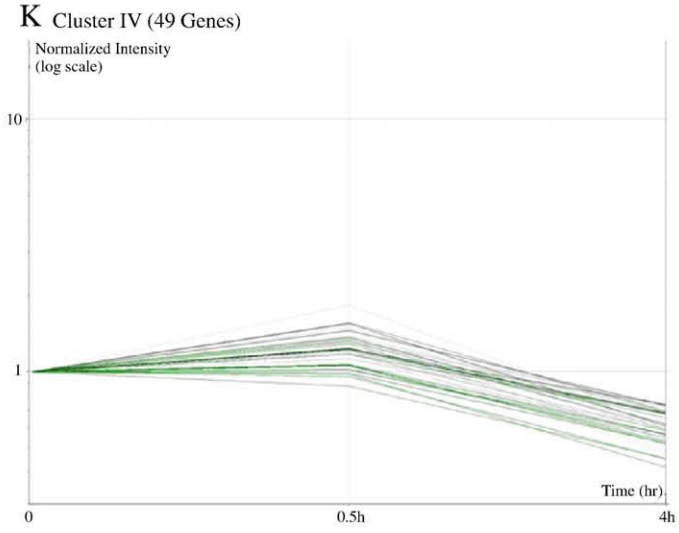


Fig. 3 (*continued*).

Table 1
The number of genes with significant gene expression changes (fold change >1.5).

	Number of upregulated genes	Number of downregulated genes
0.5 h	1276	25
4 h	3967	29

3. Results

3.1. Cytotoxicity assay

To assess the cytotoxic effect of R8, we incubated U-937 cells for 4, 8, and 24 h with various concentrations of R8 (2, 5, 10, 20, and 40 μM). Cell viability was above 80% at the concentrations used (Fig. 1A). The limited cytotoxicity of R8 was consistent with a previous report [13] on R8-treated RAW 264.7 macrophages. U-937 cells were also treated with increasing concentrations of FITC-R8, after which they were analyzed using cytotoxic assays (Fig. 1B). FITC-R8 also showed limited toxicity, which confirmed that FITC labeling did not increase the cytotoxicity of R8.

3.2. R8 binding and uptake

Flow cytometry does not distinguish between cell membrane-bound and internalized fluorochrome. Therefore, we treated the cells with trypsin to remove surface-bound peptide before a FACS analysis of the cellular uptake of FITC-R8. Cells incubated with FITC-R8 but without trypsin treatment showed higher fluorochrome levels than those with trypsin treatment (Fig. 2). Also, the binding and internalization were not due to the dissociation of FITC from R8, which is shown in cells incubated with FITC only (Fig. 2A, curve b). The binding and uptake of FITC-R8 were rapid, as the increases in fluorescence for 0.5 h of exposure show (Fig. 2B). The kinetic analysis of the binding and uptake of cells treated with FITC-R8 showed time- and concentration-dependent increases in cell fluorescence (Fig. 2B–D). The differences in the fluorescence of cells with and without trypsin treatment were smaller after 8 h of exposure to FITC-R8 (Fig. 2D), which indicated that the uptake of FITC-R8 was nearly completed.

3.3. Global gene expression profiles of U-937 macrophages treated with R8

A common characteristic of cell penetrating peptide delivery is that cells quickly uptake it. Therefore, we obtained global gene expression profiles for U-937 human macrophages incubated with and without 10 μM of R8 for 0.5 h and 4 h. We used 1.5-fold changes at one of three time points (0 h, 0.5 h, and 4 h) to select 4386 differentially expressed genes from 28,060 identified genes; cluster analysis was then used for additional analysis. We found 11 clusters with significant gene expression changes (Fig. 3). The majority of these genes with significant gene expression changes at each time point (0.5 h and 4 h) were upregulated (Table 1); 35 upregulated genes responded to the stimuli with immune functions in all 11 clusters and were also selected (Table 2). These genes included mostly cytokines, cytokine binding proteins, cytokine receptors, receptors, histocompatibility molecules, chemokines, complement components, CD8 antigens, pore-forming proteins, oncogenes, proteases, and phospholipases.

Table 2
Genes belong to the functional categories of responses of stimulus with immune functions.

GenBank	Synonym	Description	Fold change	
			0.5 h	4 h
NM_005252	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog(FOS)	41.821	1.964
NM_000594	TNF	Tumor necrosis factor (TNF superfamily, member 2)	6.918	1.965
NM_020530	OSM	Oncostatin M (OSM)	2.901	1.439
NM_002983	CCL3	Chemokine (C–C motif) ligand 3 (CCL3)	3.414	2.233
NM_002190	IL17	Interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8) (IL17)	1.950	2.221
NM_000912	OPRK1	Opioid receptor, kappa 1 (OPRK1)	1.556	2.019
NM_001185	AZGP1	Alpha-2-glycoprotein 1, zinc (AZGP1)	1.262	1.997
		1C7 precursor	1.570	2.077
NM_003811	TNFSF9	Tumor necrosis factor (ligand) superfamily, member 9 (TNFSF9)	1.918	2.313
BX640624	MGC27165	mRNA; cDNA DKFZp686K18196	1.251	2.064
NM_002000	FCAR	Fc fragment of IgA, receptor for (FCAR), transcript variant 1	1.336	2.291
NM_173044	IL18BP	Interleukin 18 binding protein (IL18BP), transcript variant D	1.654	2.132
NM_001733	C1R	Complement component 1, r subcomponent (C1R)	2.053	2.223
NM_001242	TNFRSF7	Tumor necrosis factor receptor superfamily, member 7 (TNFRSF7)	1.971	2.044
NM_133280	FCAR	Fc fragment of IgA, receptor for (FCAR), transcript variant 10	2.880	1.650
NM_003485	GPR68	G protein-coupled receptor 68 (GPR68)	1.129	2.070
BC034142	GKV1–5	Immunoglobulin kappa variable 1–5	1.865	3.592
NM_005515	HLXB9	Homeo box HB9 (HLXB9)	1.546	2.073
		cDNA FLJ39978 fis, clone SPLEN2029380	2.761	3.294
NM_139011	HFE	Hemochromatosis (HFE), transcript variant 11	2.636	2.031
NM_002258	KLRB1	Killer cell lectin-like receptor subfamily B, member 1 (KLRB1)	1.349	2.008
NM_005118	TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15 (TNFSF15)	1.695	2.393
NM_001622	AHSG	Alpha-2-HS-glycoprotein (AHSG)	1.762	2.123
NM_000877	IL1R1	Interleukin 1 receptor, type I (IL1R1)	2.065	1.456
NM_001768	CD8A	CD8 antigen, alpha polypeptide (p32) (CD8A), transcript variant 1	1.591	2.238
NM_001712	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) (CEACAM1)	1.953	3.026
NM_182549	HLA-DQB2	Major histocompatibility complex, class II, DQ beta 2 (HLA-DQB2)	1.299	2.516
NM_000715	C4BPA	Complement component 4 binding protein, alpha (C4BPA)	1.199	2.431
NM_006274	CCL19	Chemokine (C–C motif) ligand 19 (CCL19)	1.696	2.319
NM_012400	PLA2G2D	Phospholipase A2, group IID (PLA2G2D)	1.550	2.308
NM_153758	IL19	Interleukin 19 (IL19), transcript variant 1	1.759	1.995
NM_002121	HLA-DPB1	Major histocompatibility complex, class II, DP beta 1 (HLA-DPB1)	1.642	2.056
NM_005508	CCR4	Chemokine (C–C motif) receptor 4 (CCR4)	1.918	2.064
J00231	IGHG3	Human Ig gamma3 heavy chain disease OMM protein mRNA	1.334	2.195
NM_006610	MASP2	Mannan-binding lectin serine protease 2 (MASP2), transcript variant 1	1.095	2.117
NM_130441	CLEC4C	C-type lectin domain family 4, member C (CLEC4C), transcript variant 1	1.248	2.010
NM_005041	PRF1	Perforin 1 (pore-forming protein) (PRF1)	1.108	2.086

3.4. Confirming microarray results using real-time quantitative RT-PCR

To compare the gene expression results obtained from the microarray with the results from RT-PCR, five genes (FOS, OSM, C1R, TNF, IL1R1) at each time point (Table 2) were tested. We found that the regulation patterns of the five genes, as measured by RT-PCR, were consistent with those from the microarray (Fig. 4).

3.5. Intracellular O_2^- content

Superoxide production decreased time- and concentration-dependently relative to the increase of cellular uptake of R8 (Fig. 5).

3.6. Cytokine release

Exposing U-937 cells to R8 (2–40 μM) for 4, 8, 16, or 24 h of incubation did not stimulate the release of any proinflammatory cytokines (TNF-α, IL-1β, or IL-6) (data not shown). However, LPS (positive control) induced the release of proinflammatory cytokines after 8 h of incubation.

4. Discussion

After uptaking or binding foreign matter, macrophages may induce various molecular responses such as ROS production and cytokine release. A greater understanding of these cell penetrating peptide-induced responses in macrophages may help prevent harm when using cell penetrating peptides for delivering intracellular substances. We showed that R8 upregulated in macrophages a variety of immunoresponsive genes, 5 of which were confirmed using real-time quantitative RT-PCR. C1R is a modular serine protease and the autoactivating component of the C1 complex of the classical pathway of the complement system. C1R leads to various biological events that provide a vital component of the host immune system [16]. OSM is a cytokine in the IL-6 family, which consists of IL-11 (interleukin-11), LIF (leukemia inhibitory factor), CNTF (ciliary neurotrophic factor), and CT-1 (cardiotrophin-1) [17]. OSM is important for regulating the growth of both tumor and non-tumor cells, and the progression of inflammatory diseases. OSM is produced in activated monocytes, macrophages, and T lymphocytes [18]. IL1R1 belongs to the interleukin-1 receptor/Toll-like receptor (TLR) superfamily; it bridges innate and adaptive immune responses. Signal transduction pathways used by IL1R1 activate AP-1, NF-κB (a rel-type transcription factor), and p38 and JNK (stress-related MAP kinases) [19]. The v-Fos FBJ murine osteosarcoma viral oncogene homolog (Fos, c-Fos) is an immediate early gene, and FOS proto-oncoprotein is a transcription factor that contributes to the formation of the AP-1 complex [20]. Various biological processes, such as cell proliferation and differentiation, organogenesis, and apoptosis, are regulated by the AP-1 complex. FOS interacts with the Jun family members within AP-1 and is rapidly and transiently induced by a variety of stimuli in most cell types [21]. Finally, TNF (tumor necrosis factor) is the major cytokine in the pathogenesis of chronic inflammatory disease, and neutralizing TNF is potent treatment for some inflammatory diseases [22,23]. Although our results showed that TNF was upregulated

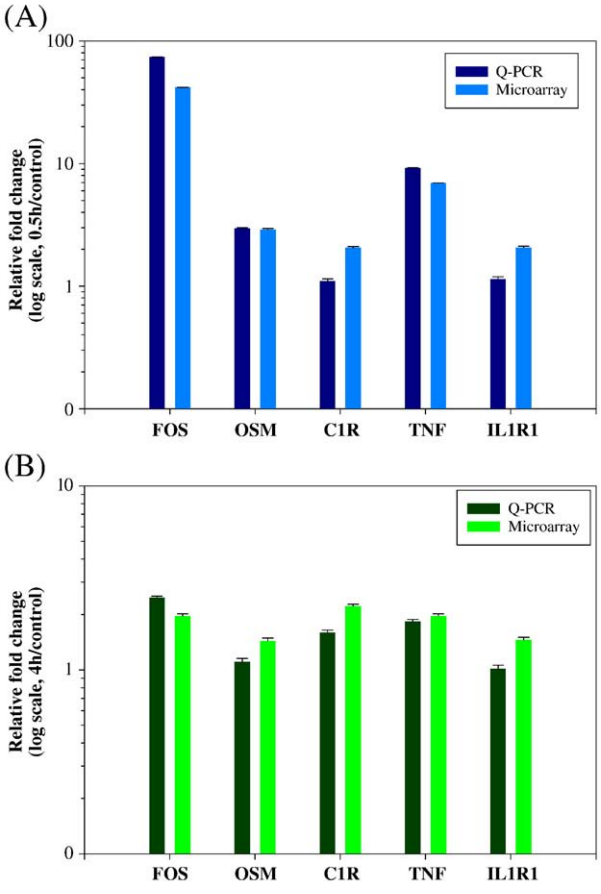


Fig. 4. Confirmation of microarray results with real-time quantitative RT-PCR. Relative expression levels of 5 genes (FOS, OSM, C1R, TNF, IL1R1) in macrophages incubated with 10 μM of octaarginine for (A) 0.5 h and (B) 4 h incubations are shown. Data are expressed as means ± standard deviations ($n = 3$).

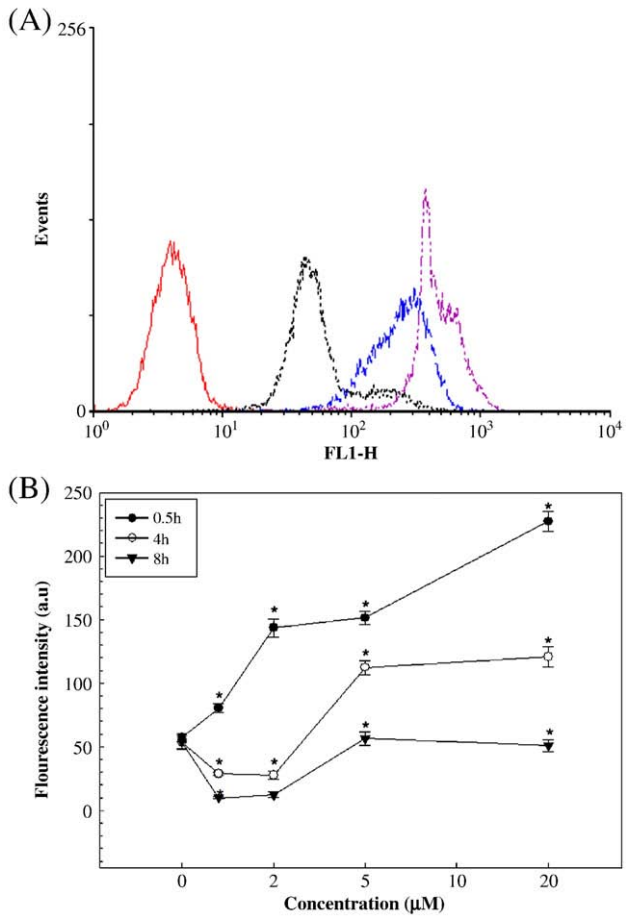


Fig. 5. Flow cytometric analysis of intracellular O_2^- content in U-937 macrophages treated with R8. (A) A representative histogram showing the change in ethidium bromide (EB) fluorescence intensity in untreated cells (curve a) and cells stained with 10 μM of hematoxylin and eosin (HE) (curve b, negative control), 20 μM of R8 (curve c), and 1 mM of menadione (curve d, positive control) for 0.5 h. (B) EB fluorescence intensities in cells treated with R8 at different dosages and for different incubation times. HE-only-treated cells were controls for each corresponding incubation period. Data are means ± standard deviations of three experiments carried out in duplicate. $^*p < 0.05$, compared with controls.

in R8-treated macrophages, TNF receptors are downregulated by nona-arginine in HeLa cells [24]. Our results showed that the gene regulation of TNF receptors by arginine peptides was dependent on cell-type specificity and the length of the peptide used. Also, the proinflammatory cytokines TNF-α, IL-1β, and IL-6 were not released after the uptake of R8. Therefore, these proinflammatory genes did not fully convert into final protein products after longer incubation times (8 h to 24 h). Our study also showed that the increase of intracellular O_2^- production in R8-treated macrophages occurred primarily after 0.5 h of incubation. Microarray analysis showed that no genes related to superoxide production had been upregulated in R8-treated cells, but that two genes – NCF1 (neutrophil cytosolic factor 1; GenBank #: NM_000265) and SOD3 (superoxide dismutase 3; GenBank #: NM_003102) – related to superoxide metabolism had been upregulated after 4 h of incubation (data not shown). This reflected the delicate balance between the production and destruction of superoxide in R8-treated macrophages. Polyarginine with a molecular weight less than 5000 does not induce O_2^- content in macrophages, while polyarginine with a molecular weight more than 10^4 does [25]. In the present study, however, we showed that R8 with a molecular weight less than 5000 stimulated transient production of O_2^- in macrophages after 0.5 h of incubation. Finally, flow cytometric analysis cannot determine whether internalized R8 is intact or degraded. Proteases on the cell membrane may degrade R8. Although FITC was used to evaluate the fluorescence of intracellular R8 uptake, it acted as a cargo attached to R8. The effect of attached cargo on the profiling of immune responses to macrophages must be further investigated.

5. Conclusion

We described the interaction of R8 with a phagocytic cell line (U-937) that is representative of a target in the body during drug delivery. Despite low cytotoxicity, R8 induced various changes in gene expression in macrophages after it had bound with and was internalized by them. Exposing U-937 cells to R8 did not stimulate the release of any proinflammatory cytokines (TNF-α, Il-1β, or IL-6). After U-937 macrophages had been incubated with R8 for 0.5 h, intracellular O_2^- production was detected, but this did not lead to cell death. Our study may provide helpful information about the molecular action of cell penetrating peptides in macrophages for delivering therapeutic substances.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2009.07.006.

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行政院國家科學委員會補助出席國際學術會議報告

98 年　7 月　10 日

報告人姓名	郭榮華 嘉南藥理科技大學藥物科技研究所		
時間 會議 地點	06/21/2009-06/24/2009 Seattle, Washington USA		
會議名稱	（中文）2009 美國藥學科學家協會國家生物技術年會暨展覽會 （英文）2009AAPS National Biotechnology Conference		
發表論文題目	（中文）甘露醇對 PEI-DNA 複合體於冷凍乾燥中凝聚現象之保護效果 （英文）Protective effect of mannitol on the aggregation behavior of polyethylenimine-DNA complexes during lyophilization		

報告內容應包括下列各項：

一、參加會議經過

本屆 2009 年的美國藥學科學家協會國家生物技術年會暨展覽會(2009 AAPS National Biotechnology Conference)為期四天，在美國西雅圖會議與貿易中心舉行。此次會議為生技製藥領域中最為重要的會議之一，今年有超過一千位的相關研究領域學業界參與這場盛會，並且安排至相關研究公司進行實驗室參觀。來自全世界各大生技製藥公司的研發人員都聚集於此，一方面發表最新的研究成果，另一方面可以了解未來生技製藥與未來創新應用方向。本次 plenary session 所邀請的是來自西雅圖生技公司的執行長及生技創技公司的創始人，以業界的角度討論如何在研究、製程、財務上能充分緊密的發展，並隨時回應 FDA 的臨床結果，因此對於學界在研發一項研究成果如何轉換成兼具實用與價值的產品，都可以在這個會議中獲益無窮。而本次會議涵蓋的主題非常廣泛及實用，如生技製藥分析技術扮演角色(Follow-on Bioloyics：The Role of Analytical characterization in Determining Equivalence and clinical Relevance)；生技藥品之控制釋放(Controlled Release Drug Delivery strategies for Biotech-derived Compounds)；蛋白質之交互作用(Drug-drug Interactions of Therapeutic proteins)；生物標記(Utility of Biomarkers for Drug Development (Qxology))；生技藥品 (Biotechnology drugs)；FDA 規範 (FDA regulation)；抗體藥品之發展 (Antibody drugs development)；電腦模式預測生技藥品 (Rational protein design via computational modeling)；體外-體內關係模式探討 (In vitro In vivo correlations)；生技藥品製程(New technology introduction into biotechnology drug product manufacturing)；基因製劑發展 (Gene pharmacutics)等。

二、與會心得

這次我報告的壁報論文，被大會安排在第二天，整天的基因製劑壁報論文場次中張貼並與其他有興趣的研究學者一同討論，在壁報張貼期間許多相關領域的專家都前來參觀壁報，也提出許多有用的意見及討論，使我能知道自己研究的盲點何在及本實驗在生技製藥界的價值及重要性，因此受益匪淺。此外在會議進行期間也有許多出版社、廠商設攤展示，進行一連串示範及演講活動，對於往後在研究中所使用相關材料及儀器亦十分有所幫助。

三、攜回資料名稱及內容

大會相關論文手冊、廠商 DM

Protective effect of mannitol on the aggregation behavior of polyethylenimine-DNA complexes during lyophilization

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Purpose. The purpose of this study was to systematically evaluate the protective effect of mannitol on the aggregation behavior of polyethylenimine -DNA complexes during lyophilization.

Methods. The pDNA (pSG5*lacZ*, 8 Kb), which encodes the *lacZ* gene for β-galactosidase, was driven by a SV40 promoter to assess gene expression. Aliquots of the 1 mL solutions containing both PEI (Mw=25 kDa)-DNA complexes (w/w 1/1; zeta potential = 22 mV; based on the optimal gene expression in the murine macrophage-like cell line, RAW 264.7) and excipients described in the text were prepared in this study. Vials were frozen by immersion in liquid nitrogen to minimize freezing damage and placed on the shelf of a freeze-drier (DC 1000, Panchum Scientic Corp., Taiwan).The mannitol crystallinity in freeze-dried formulations was analyzed by a powder X-ray diffractometer. The analysis of the aggregation behavior of PEI-DNA complexes after lyophilization has been investigated by light scattering measurements and in vitro transfection assay.

Results. Without lyoprotectants added, a significant increase of particle aggregation and a dramatic decrease in transfection efficiency of polyethylenimine (PEI)-DNA complexes were observed after freeze-drying. Mannitol did provide some protecting effect on the retention of particle size and transfection efficiency of PEI-DNA complexes after lyophilization or freeze-thawing. However, the crystallization of mannitol negatively affected size stability and transfection efficiency of PEI-DNA complexes during lyophilization and freezing-thawing. Co-lyophilization with disaccharides (sucrose or trehalose) resulted in avoiding particle aggregation and preserving transfection efficiency of PEI-DNA complexes during lyophilization or freeze-thawing. As compared with disaccharides, co-lyophilization of lyoprotectants with higher molecular weight (polyvinyl pyrrolidone and dextran) resulted in higher particle aggregation and lower transfection efficiency of PEI-DNA complexes after freeze drying or freeze-thawing. Disaccharides not only reduced the crystallization of mannitol of freeze-dried DNA formulations, they also protected stabilization of DNA during lyophilization or freeze-thawing. Except for lowing mannitol crystallinity, the characteristics of the excipient may influence the ability of the preservation of PEI- DNA complexes after lyophilization or freeze-thawing. This study also demonstrates that lyophilization process resulted in more aggregation than freeze-thawing.

Conclusions. The results allow us to gain some additional insight into the aggregation mechanism of cationic polymer-based gene delivery systems during freeze-drying.