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RahU: An inducible and functionally pleiotropic protein in *Pseudomonas aeruginosa* modulates innate immunity and inflammation in host cells

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Abstract

The aim of this study was to define the functional role of a recently identified RahU protein from *Pseudomonas aeruginosa* in macrophages and its role in bacterial defense. Recombinant (r)-RahU had no significant effect on cell apoptosis or cell viability in human monocytic THP-1 cells. Gene expression array of murine macrophage cells (RAW 264.7) stimulated with LPS showed modulation of common transcripts involved in inflammation. Functional cellular analysis showed RAW cells incubated with r-RahU at 1.0–10 μg/ml (0.06–0.6 μM) inhibited accumulation of nitric oxide (NO) in the presence of LPS by 10 to 50%. The IC_{50} of r-RahU (0.6 μM) was distinct from the known inhibitors of NO production: prednisone (50 μM) and L-NMMA (100 μM). rRahU also significantly inhibited chemotactic activity of THP-1 cells toward CCL2 or chemotactic supernatants from apoptotic T-cells. These reports show previously unknown pleiotropic properties of RahU in modulating both microbial physiology and host innate immunity.

Keywords

Innate immunity; Nitric oxide; Chemotaxis; Inflammation; Inflammation resolution

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1. Introduction

Aegerolysins (PF06355), which have been previously discovered in fungi, bacteria and plants, are highly similar proteins with different biological properties. These include ostreolysin (*Pleurotus ostreatus*), Asp-hemolysin (*Aspergillus fumigatus*), hemolysins from the basidiomycete (*Moniliophthora perniciosa*), and terrelysin from *Aspergillus. terreus* [1–8]. Although the putative biological role of fungal aegerolysins is suggested to be similar to bacterial aegerolysins [1;8;9], the functional role of microbial aegerolysin-like proteins in eukaryotic systems remains elusive [1]. A gene (PA0122) product of *Pseudomonas aeruginosa* belongs to the aegerolysin family of proteins [10] and is designated as RahU in this and adjoining manuscript. The present reports further investigated the role of recombinant (r)-RahU to understand host-bacteria interactions at the functional genomics and cellular level. The rationales of these studies include: (a) Surface plasmon resonance revealed that r-PA0122 (r-RahU) binds with high affinity 1.36 x 10^{-9} M to oxidized-low density lipoprotein (Ox-LDL) and to synthetic C:6 lysophosphatidylcholine (lysoPC) at 2.94 x 10^{-5} M, a major subcomponent of Ox-LDL, but not to the LDL [10]; (b) Oxidation of LDL is promoted mainly by macrophages and endothelial cells within the subendothelial extracellular matrix; (c) RahU protein was associated with inner membrane and also secreted into the extracellular medium [10], and we believe that it may form a modified ligand after combining with free or cellular bound Ox-phospholipids and may affect the cellular event; and (d) RahU or the modified ligands may bind and signal *via* scavenger and/or pattern recognition receptors including TLRs on macrophages. In the accompanying report we show that RahU functions as a sensing apparatus in *P. aeruginosa* to distinguish different forms of host-derived Ox-phospholipids, which may also interact with host cells during host-bacterial interactions in the microenvironment. Together, these observations and other reports provide several examples of ligands or classes of ligands and bacterial products that share seemingly dissimilar components of the innate immune system, and they raise intriguing questions about functional interactions between these molecules. In the present report we focused on the functional impact of r-RahU on model cell lines of macrophages and human monocytes.

Host innate immune systems are well conserved in evolution [11]. They consist of intracellular and extracellular signaling mechanisms that identify, respond to, and defend the host from a broad range of infections. The major functions of the vertebrate innate immune system include, but are not limited to: (a) activation of inflammatory cells and complement; production of cytokines, chemokines, nitric oxide and other reactive oxygen molecules; promotion of clearance of dead and apoptotic cells [12]; and (b) removal of foreign substances or pathogens; chemotaxis of inflammatory cells; and activation of adaptive immune system [13]. *P. aeruginosa* is an opportunistic pathogen causing chronic lung infections in cystic fibrosis (CF) patients, those hospitalized with urinary tract infections, in wounded or immune compromised patients, and in burn victims [14–17]. *P. aeruginosa* expresses numerous virulence factors, such as flagellum, pilus, LPS and secretory factors including extracellular products such as type III secretary proteins, quorum sensing molecules and alginate [18].

The host response to *P. aeruginosa* infections involves cells in the local environment, such as airway epithelial cells, macrophages and monocytes, neutrophils and lymphocytes, which release mediators that enable mounting of an attack on the invading bacteria [13;19;20]. These include macrophages that engulf and destroy the bacteria through the generation of a “respiratory burst”, causing the release of reactive oxygen species such as nitric oxide and hydrogen peroxide [21;22]. However, Vishwanath *et al.,* 1988, suggested that inhibition of phagocytosis by the leukocytes may be contributed by a defect in uptake and /or destruction of mucin-coated bacteria [23]. It should be noted that macrophages have roles in both the

innate and adaptive immune response to infection [24]. This is well documented in depletion of lung macrophage in mice, delayed neutrophil recruitment and chemotactic production, and delayed bacterial clearance as compared to controls [25]. Furthermore, macrophages have been reported to restrict *P. aeruginosa* growth, regulate neutrophil influx and balance pro and anti-inflammatory cytokines in BALB/c [13]. Activation of macrophages promotes the recruitment of other cells such as T cells to the site of inflammation and/or infection [26].

The experiments in the present study show a global impact of RahU on macrophage gene expression that is shared with "oral anti-inflammatory compounds" such as prednisone. r-RahU from *P. aeruginosa* also interferes in innate immunity by inhibiting nitric oxide production and chemotaxis of monocytes and/or macrophages. Together, these studies demonstrate a dual role of RahU (in host and bacteria), which also bridges and positions itself to cross-communicate multiple functions in host-bacterial interactions.

2. Materials and Methods

2.1. Cell Lines and Reagents

RAW 264.7 cells, a mouse macrophage cell line, human monocytic (THP-1) cells, and the human Jurkat T cell line (clone E6-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Isopropyl β-D-1-thiogalactopyranoside (IPTG), Griess kit, and CellTiter-Glo™ were procured from Promega (Madison, WI). BD Talon His-tag protein purification kit was purchased from BD Bioscience (Bedford, MA). iScript One-step-RT-PCR kit, Criterion SD-PAGE and blotting systems, iQ5 Real-Time system, Coomassie Brilliant Blue R-250, Immuno-Star HRP Chemiluminescence kit, and Bradford reagent were acquired from Bio-Rad (Hercules, CA). Lipopolysaccharide (LPS; L3024), which was extracted from *Escherichia Coli* 0111:B4 and purified by ion-exchange chromatography, was purchased from Sigma Aldrich (St. Louis, MO). Arachdoinc acid, Prednisone, Sodium deoxycholate, Proteinase K–Acrylic Beads, *Tritirachium album* and L-NMMA were purchased from Sigma-Aldrich. All the tissue culture media, fetal bovine serum (HyClone), L-glutamine solution, Phosphate Buffered Saline (PBS) pH 7.4, Detoxi-Gel™ Endotoxin Removing Gel, tissue culture cellBIND coated multi-well plates and tissue culture antibiotics were procured from Lonza Biowhittaker/Costar from (Thermo-Fisher) (Pittsburgh, PA). Recombinant human MCP-1/MCAF (CCL2) was purchased from PeproTech, (Rocky Hill, NJ). NanoDrop was from Thermo-Fisher, and 1450 Microbeta Trilux luminometer was acquired from Wallac (Ramsey, Minnesota). The RNAeasy kit from Qiagen (Valencia, CA) was used for RNA isolation and purification.

2.2. Isolation and purification of recombinant-RahU protein (r-RahU) from E. coli

The cloning and expression of recombinant-RahU protein in *E. coli* has been previously described [10]. *E. coli* was transformed with pET28b-RahU-His cloned plasmid to express r-RahU, with his-tag at C-terminus (data not shown). Briefly, a single colony of NovaBlue (DE3) containing pET28b-RahU-his was inoculated into Luria-broth (LB) and grown to OD_{600} = 0.5. The cells were induced with 1 mM IPTG, harvested after 4 h upon reaching OD_{600} = 1.0 and frozen at −80°C until further use. Purification of his-tagged r-RahU protein was performed twice using a BD TALON immobilized metal affinity chromatography (IMAC) resin, as recommended by the manufacturer. Briefly, the cell lysate was mixed with IMAC, loaded onto a column and washed extensively with the buffer. The r-RahU-his protein was eluted with imidazole and each fraction was analyzed with Coomassie Brilliant Blue R-250 staining on SDS-PAGE gels. The 16 kDa purified r-RahU-his protein was quantified by Bradford assay. Fraction(s) having a single 16 kDa protein were further purified and reassessed by repeating the above affinity purification protocol and SDS-

PAGE-Coomassie Brilliant Blue R-250 staining. All purified fractions representing a single 16 kDa r-RahU band were pooled and were also subjected to Western blot analysis using a specific anti-r-RahU (r-PA0122) antibody [10]. Pre-stained Kaleidoscope molecular weight markers were used in the SDS PAGE gels and Western blot analysis.

2.3. Preparation of endotoxin-free r-RahU protein

Immobilized polymixin B-agarose (Detoxi gel) was used according to manufacturer's recommendation and of which removed 99.99% EU (endotoxin units). Briefly, one ml pre-loaded column was washed with 5 volumes of 1% sodium deoxycholate followed by 10 volumes of PBS (pH 7.4). One ml of affinity purified r-RahU at ~1 mg/ml was loaded onto a Detoxi column and incubated at room temperature for 1 h. The column was then eluted with PBS to exclude residual contaminated endotoxin. The fractions were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Pooled fractions were quantified by Bradford assay. Only the fraction which showed a 16 kDa r-RahU band was pooled for further studies.

2.4. SDS-PAGE and Western blotting

Ten μg of r-RahU protein was loaded on 8–16% SDS-PAGE using the Criterion gel system from Bio-Rad. Separated strips were probed with polyclonal anti-r-RahU antibody at 1:2000 dilutions. A secondary antibody, peroxidase conjugateanti-mouse IgG antibody, was used at 1:5000 dilutions [10] for detection. The blots were developed using the Immuno-Star HRP chemiluminescence reagents and quantitated with a Chemi-Doc XRS which was procured from Bio-Rad.

2.5. Treatment of r-RahU protein with Proteinase K and absorption with Talon beads

Proteinase K (PK) conjugated beads (0.01 mg) or Talon beads were equilibrated in sterile PBS and incubated with 10 μg of r-RahU protein in 50 μl PBS for 12 h at 30°C with light agitation. r-RahU protein was incubated in a similar fashion without addition of beads. All the samples were centrifuged at 16.1x*g* for 5 min and the supernatants (SN) were used for the biological assays. The Proteinase K-degraded or talon-his-tag bead absorbed r-RahU protein was subjected to SDS-PAGE-Coomassie Brilliant Blue R-250 staining.

2.6. Cell culture

RAW 264.7 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine (1%), 100 units/ml penicillin, and 100 μg/ml streptomycin (1%). Two hundred microliters per well of RAW 264.7 cells (~$1.5x10^5$/ml) were seeded into a 96-well culture plate and incubated at 37°C in 5% CO_2 - 95% air for 18–24 h. When the cells reached a 60–75% confluence, they were induced with LPS (1 μg/ml) with fresh media in presence or absence of r-RahU protein (1–10 μg/ml) for 18 – 24 h. Cells were also incubated with 1 μg/ml of LPS in the presence of 50 μM Prednisone, 100 μM L-NMMA, and supernatants (SNs as described above) from 10 μg/ml r-RahU protein treated with Proteinase-K or talon beads. Some cells were pre-incubated for 24 h with 10 μg/ml of r-RahU, followed by stimulation in media containing 1 μg/ml of LPS. The experiments were terminated at 18–24 h of incubation. Nitrite accumulation in the medium was assessed by the modified Griess method [27]. Large scale experiments (in flasks) were performed for RNA and protein isolation.

2.7. Apoptosis and Viability Assays

To examine the cytotoxic effect of r-RahU protein on eukaryotic cells, we selected Human monocytic (THP-1) cells as a model to examine viability and apoptosis. THP-1 cells at $2x10^6$/mL were incubated in presence of 2 μM r-RahU at 37°C, in 5% CO_2 for 1 h. The cells

were stained with propidium iodide and annexin V according to the manufacturers' instructions (eBioscience, San Diego, CA), and analyzed by flow cytometry. Furthermore, to determine the extent of potential cell lysis in the presence of r-RahU, 50 μL of cell supernatants from r-RahU-treated THP-1 cells were examined using the CytoTox-Glo™ cytotoxicity assay (Promega) according to the manufacturers' instructions. The statistical analysis was performed using the Student's *t* test.

2.8. Chemotactic Assay

THP-1 cell migration assay was performed as previously reported [28]. Briefly, the assay contained endotoxin-free r-RahU protein added at a final concentration of 2 μM in RPMI-1640 medium with 5% FBS, 10 mM HEPES, 2 mM L-glutamine, 10 μg/ml of penicillin and streptomycin. Two separate chemoattractants which promote THP-1 chemotaxis were added to the assay: CCL2 at 10 ng/mL and apoptotic Jurkat T cell supernatants (prepared from Jurkat T cells exposed to 100mJ UV irradiation and incubated at 37°C with 5%CO^2 for 4 h). Three hundred and fifteen microliters of the control or chemoattractant-containing medium were then loaded into triplicate wells in a 96-well chemotaxis plate (NeuroProbe, Gaithersburg, MD). Thirty microliters of pre-warmed THP-1 cells (2 x 10^6/mL) were added to the top of the chemotaxis plate and incubated at 37°C, in 5% CO_2 for 1 h. We used either Proteinase-K treated r-RahU or Talon-absorbed supernatant from r-RahU as negative controls. The quantification of cell migration was performed by transferring 50 μl of medium from the lower chamber into an opaque 96-well plate with equal volume of CellTiter-Glo™ reagent (Promega), and analyzed using the 1450 Microbeta Trilux luminometer according to the manufacturer's protocol [29]. The statistical analysis was performed using the Student's *t* test.

2.9. RNA analysis and Quantitative Reverse Transcriptase (qRT)-PCR

Cells that were treated with various modulators with their respective controls were harvested at 18 h. The total RNA was purified using the Qiagen RNAeasy kit (QIAGEN) and quantitated by NanoDrop. RNA quality was assessed using Experion Automated Electrophoresis system (Bio-Rad). Each lane was loaded with 30 ng of total RNA according to the manufacturer's instruction. Real-Time quantitative PCR reactions were carried out on the iQ5 Bio-Rad Real-Time PCR system with iScript one-step-RT-PCR kit with SYBR Green mix as a fluorescent reporter. GAPDH and iNOS cDNA yields were monitored up to 40 cycles using equal amounts of (100 pg) of total RNA in triplicate for each sample. The average threshold cycle number was determined using the iQ5 Optical System software. Levels of inducible Nitric Oxide (iNOS) mRNA expression were normalized to GAPDH levels, and data was expressed as relative fold expression in the bar representation with ±SD. Sequences of primers used for iNOS were: Forward 5'-3':TCTATCAGGAAGAAATGCAGGAGAT, Reverse-5'-3':TGAACGTAGACCTTGGGTTTGC, and GAPDH: Forward-5'-3' GTGAAGGTCGGAGTCAACG, Reverse-5'-3': TGAGGTCAATGAAGGGGTC.

2.10. Affymetrix Gene Chip Analysis

Differential gene expression was performed using the Affymetrix gene chip at Virginia Bioinformatics Institute Core Laboratory Facility. Briefly: RAW 264.7 cells were stimulated with 1 μg/ml of LPS in the presence of 10 μg/ml of r-RahU or 50 μM of prednisone for 18–24 h. The total RNA was extracted and quantified as described above under qRT-PCR. Ten μg of total RNA was labeled and probed as recommended by the manufacturer (https://www.affymetrix.com/analysis/netaffx/). Gene expression analysis was performed with the Affymetrix Mouse 430 Plus 2.0 whole genome microarray on one control un-induced sample, one sample treated with LPS+Prednisone, one sample treated with LPS+r-RahU and two samples treated with LPS alone at 18 to 24 h, respectively. Gene expression

levels were determined using the probeset and signal values calculated using the Affymetrix MAS 5.0 software.

Our analysis focused on all of the genes that were upregulated by an average of 4-fold or more in the cells treated with LPS alone compared to control. The r-RahU and Prednisone effects were then evaluated by identifying expression inhibition (by at least 2-fold below LPS activated levels) of these selected genes. Expression of 135, 65 and 55 of the LPS upregulated genes was reduced by prednisone, r-RahU and both, respectively. The characteristics of these genes were further reviewed for common biological functions and enriched gene ontology categories using the online GO state software [30]

2.11. Statistical analysis

Statistical analyses performed using the Student's *t* test were employed to assess the significance of the differences between the mean values of control and experimental groups for each *in vitro* culture using a minimum of three samples as described above. The data, presented as mean ± standard deviation, are obtained from one experiment each, performed in triplicate.

3. Results

The accompanying paper describes the role of *P. aeruginosa* derived *rahU* gene (PA0122) in regulation of biofilm formation in presence of oxidized phospholipids [31]. In this paper we examine the biological role of r-RahU-protein on two host cells.

3.1. Purification of recombinant (r)-RahU

Recombinant r-RahU was purified as described in Materials and Methods to examine the role of this protein in host cells. The recombinant RahU protein was expressed in *E. coli* and purified using affinity Talon purification method as described elsewhere [10]. r-RahU was subjected to a polymixin B-agarose column to remove traces of endotoxin. Ten μg of purified r-RahU was loaded onto the SDS PAGE gel. The purification process generated a single 16 kDa immuno-reactive band, which was identified by Western blotting as shown in Fig. 1. No detectable contaminating proteins were observed upon Coomassie blue R staining.

3.2. Effect of r-RahU on apoptosis and cell viability

In view of the previous reports of cytotoxic effects of bacterial aegerolysin-like proteins in eukaryotic systems [1], we examined if r-RahU protein induced cell-apoptosis and/or cytotoxicity. As shown in Fig 2A, the distribution of percent viability and apoptotic or necrotic cells remained unaffected by r-RahU at a final concentration of 2 μM. The results were similar to PBS treated control cells or cells incubated with proteinase K inactivated r-RahU. To further confirm that r-RahU did not initiate cytotoxicity, the percentages of necrotic and apoptotic cells were plotted from the total number of viable cells as shown in Fig. 2B and Materials and Methods. Fig. 2C shows r-RahU treatment with corresponding negative controls [proteinase-K treated r-RahU or PBS treated cells without r-RahU]. The positive control included cells exposed and lysed with 0.01% Triton X-100 in the same experiment. It should be noted that concentration 2 μM (or less) of r-RahU protein was used in these cell viability/apoptosis assays and other physiological experiments reported in this manuscript.

3.3. Effect of r-RahU on Gene expression array in LPS activated macrophages

Macrophages are known to be activated by LPS during infections utilizing the CD14, TLR2, TLR4 and TLR9 receptors for signal transduction [11;32]. Previous studies have reported

the regulation of various inflammatory mediators involved in innate immunity, such as iNOS and COX-2, by LPS in RAW 264.7 cells [11;24]. Furthermore, LPS has been reported to modulate mRNA expression in RAW 264.7 cells between 1 to 36 h [26;28;33–36]. The effects of corticosteroids on innate immunity, inflammation and gene expression in various cell types prompted us to use prednisone as a control [37]. Furthermore, long term exposure to prednisone has been reported to induce the incidence of bacterial infections [38]. We hypothesized that there may be common denominators between r-RahU and prednisone with respect to gene expression in RAW 264.7 cells. RAW 264.7 cells were stimulated with LPS in the presence and absence of 10 μg/ml r-RahU or 50 μM prednisone for 18–24 h to identify the common transcript(s) that are modulated between r-RahU and prednisone.

3.4. Total RNA was isolated and analyzed using Affymetrix Gene Chip

Gene expression studies showed modulation of various transcripts in LPS stimulated cells as previously reported [24;32;33;39–42]. Two hundred fifty eight transcripts were upregulated by ≥four-fold as compared to the control un-stimulated cells. Among these, 133 and 65 were downregulated by ≥twofold by prednisone and r-RahU, respectively (Supplemental Tables 1 and 2). The Venn diagram displays inhibition of transcripts by r-RahU and prednisone (Fig. 3). Fifty five transcripts were commonly inhibited by both the modulators (Table 1). These include transcripts involved in angiogenesis, inflammation, chemotaxis and cytokine activity. These experiments demonstrated a global impact of r-RahU in LPS stimulated macrophages.

3.5. Regulation of Nitric Oxide by r-RahU in LPS Stimulated Macrophages

Previous studies have shown that Ox-LDL specifically impairs endothelium-dependent, NO-mediated dilation of coronary arterioles [43]. RahU's ability to bind to Ox-LDL and our preliminary experiments with gene expression arrays showing downregulation of iNOS mRNA by r-RahU prompted us to further investigate the regulation of iNOS by r-RahU. Furthermore, nitric oxide produced by iNOS endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi and tumor cells [12;24;44–48]. RAW 264.7 cells were incubated with purified r-RahU at 1.0, 2.0 and 10 μg/ml in the absence of LPS. Nitric Oxide levels in the medium were undetectable when compared to control cells (medium and r-RahU alone) by the methods described in this study as represented in Fig. 4, Experiment 1. These experiments confirmed that the recombinant protein (as expected) was devoid of endotoxins that may activate macrophages towards NO production.

Macrophages were incubated with 1 μg per ml of LPS and various concentrations of r-RahU which showed 10 to 50% inhibition of NO accumulation (data not shown). The IC_{50} of r-RahU was approximately 10 μg/ml (0.6 μM) as compared to 50 μM for prednisone and 100 μM for NMMA with respect to their ability to inhibit LPS-induced NO accumulation (Fig. 4). Pre-incubation of r-RahU with macrophages for 1 h followed by LPS stimulation also inhibited NO production by about 50% (Fig. 4, lane 9). To further ascertain the biological activity of RahU (and exclude the potential contribution by contaminating endotoxins), the recombinant protein was degraded by Proteinase K (Fig. 4B) before it was tested for its ability to inhibit NO production. Fig. 4, experiment 2 showed that Proteinase K treated r-RahU failed to inhibit NO production in LPS stimulated cells. It may be noted that to further rule out the possibility of any contaminating material that may contribute to the NO inhibitory activity in the r-RahU purified fraction, we subjected the purified r-RahU fraction to affinity absorption to deplete "RahU activity" in the test sample. Fig. 4, experiment 3 shows the lack of NO inhibitory activity in the r-RahU-absorbed fraction.

3.6. Mechanism of action of r-RahU on iNOS expression

We examined the mechanism of action of r-RahU (0.6 μM) by assessing its effect on iNOS mRNA and protein accumulation in LPS stimulated macrophages. Cells were stimulated with LPS in the presence of r-RahU or prednisone for 18 h. Total RNA was isolated and its purity and integrity were ascertained as described in Materials and Methods (data not shown). Fig. 5 shows the Real Time PCR results, expressed in relative fold expression (iNOS/GAPDH) after normalizing the values with GAPDH in the presence of RahU and prednisone in LPS, stimulated RAW 264.7 cells in the same experiment. Western blot analysis was also performed using a similar experimental setup. Briefly, LPS and r-RahU (0.6 μM) treated cell extracts showed inhibition of iNOS protein accumulation by more than 50% with no significant changes in the housekeeping protein β-actin at 24 h [49;50] (data not shown). This was consistent with the inhibition of iNOS mRNA expression as described above. In summary, the data shows about 75–85% inhibition of iNOS mRNA expression by r-RahU and prednisone, suggesting that one of the mechanisms of action of r-RahU was suppression of iNOS mRNA accumulation and/or synthesis.

3.7. Regulation of Chemotaxis by r-RahU with monocytic THP-1 cells

Gene expression studies showed inhibition of various chemokine (C-C or CXC motif) ligands (Table 1). In view of the previous observation that: (a) CCL2 “knock-out’ mice showed an early bacterial growth in their lungs and sepsis starting by day 2 after infection, finally resulting in ~50% decreased survival compared with wild-type mice; and (b) that monocyte chemoattractant protein-1, MCP-1 (CCL2) exhibits major importance in inflammatory monocyte recruitment to the lungs in response to bacterial infection, we were prompted to investigate the ability of r-RahU to interfere with cell migration {Winter, 2009 1501 /id}. We utilized the THP-1 induced chemotaxis model *in vitro* employing two different chemotaxis agents. THP-1 cells were incubated in a trans-well chemotaxis assay in the presence of MCP-1 (CCL-2) or 10 – 30 μg/ml of r-RahU. Although 10 – 20 μg/ml r-RahU showed no significant effect on chemotaxis in separate experiments (data not shown), 30 μg/ml (2 μM) of r-RahU (but not Proteinase K treated r-RahU) inhibited 70–80% of THP-1 cell chemotaxis induced by CCL-2 (Fig. 6).

Additionally, we also utilized a different chemoattractant to examine the role of r-RahU in the same assay. The generation of apoptotic cells is a key feature of inflammation [28;52;53]. The subsequent phagocytic removal of apoptotic cells involves chemotaxis by various cell types to the area of inflammation. Apoptotic cells are known to release chemoattractants for macrophages and monocytes to aid in this clearance process [28;52;53]. Therefore we prepared a chemo-attractant supernatant by inducing apoptosis in Jurkat cells given UV irradiation. Thus, we generated a “physiologically relevant chemotactic supernatant” as previously reported [28]. This chemoattractant demonstrated significant chemo attraction of THP-1 cells. This chemoattractant was incubated with THP-1 in the presence or absence of r-RahU. Fig. 6, experiment 2 represents chemotaxis of THP-1 cells induced with UV treated Jurkat cell apoptotic supernatants. Thirty μg/ml r-RahU inhibited the apoptotic-cell induced supernatant chemotaxis by 70–80%. These experiments confirm yet another function of r-RahU in the complex process of inflammation and innate immunity [35;41;42].

4. Discussion

Native aegerolysin proteins from fungal origin were reported to be hemolytic and cytotoxic in experimental animal models and various cell lines [1;54]. The LD_{50} of Asp-hemolysin for mice and chickens was 750 and 350 μg/kg respectively [1]. Asp-hemolysin also demonstrated cytotoxic activity in human leukocytes (ED_{50} 500 μg/ml) and macrophages

(ED_{50} 25–60 μg/ml) [1]. Several reports in the literature suggested the differential functional effects of native and recombinant aegerolysins [1]. Among these, several recombinant aegerolysins were found to be non-hemolytic and non-toxic in nature [55]. A His_6-tagged r-Rahu protein from *P. aeruginosa*, successfully expressed in *E. coli*, was found to be non-hemolytic [10] and showed no significant effects on viability and or cytotoxicity on monocytic THP-1 cells as shown in Fig. 2. In the present study we examine the role of r-RahU protein at concentrations that do not induce cytotoxicity in the host cells and that may be involved in inflammation, innate immunity and host-bacterial interactions [35;56], especially in the lung-related cells [57].

Many bacterial proteins and LPS are reported to interact with receptors in host cells [11;58]. Recent studies have also shown the upregulation of antimicrobial genes, caspase-1 and inflammasome by LPS or DNA molecules containing unmethylated CpG-dinucleotides (CpG-DNA) in the RAW 264.7 cells [35;36;41;42].

Kota et al. has shown that various groups of transcripts, which include pro- and anti-inflammatory genes and chemokines, are upregulated by LPS or IFN-gamma within 4 h of treatment of macrophages [40–42;59]. Many of these transcripts also remain upregulated at 18–24 h and are designated as “late transcripts”. Late transcripts were also observed during LPS-stimulation of RAW 264.7 cells. Our experiments showed that common transcripts that were modulated by r-RahU and prednisone may be involved in angiogenesis, inflammation, chemotaxis and cytokine activity. Furthermore, the “immunosuppressive” character of prednisone may be shared by RahU from Pseudomonas as shown in earlier reports that *P. aeruginosa* also induces localized immunosuppression during pneumonia [60].

Previous studies have shown that Asp-hemolysin-induced pro-inflammatory mRNA (such as TNF, IL-1, IL-6, IL-8 and others) in macrophages and may influence immune responses [1]. The present experiments indicate that RahU shares much of the anti-inflammatory properties exhibited by oral prednisone. Microarray analysis showed two main clusters of transcripts that were modulated by r-RahU in the presence of LPS, namely inflammation and chemotaxis. Our observations also suggest that the *P. aeruginosa* derived r-RahU may be involved in interfering with and/or damping the LPS-induced inflammatory response mounted by host cells. We examined the above hypotheses by evaluating the role of r-RahU on iNOS expression in macrophages, since it is well established that iNOS is modulated by NFkβ [61] and also plays a critical role in controlling intracellular pathogens [44–46]. Nitric oxide provides macrophages with cytostatic or cytotoxic activity against viruses, bacteria, and fungi and tumor cells [12;24;44–48]. Western blot analysis confirms that the inhibition of iNOS mRNA translated to decreased iNOS protein accumulation.

Our experiments demonstrate the ability of r-RahU to block a critical function of macrophages in innate immunity. Microbial systems have evolved to neutralize NO production by different mechanisms. For example, *Mycobacterium* inhibits nitric oxide synthase-recruitment to phagosomes during macrophage infection [62]. *Salmonella* possesses multiple enzymes that utilize NO as a substrate, and resist “nitrosactive” killing by macrophages [63]. *Salmonella* also generates flavorubredoxin that reduces NO to nitrous oxide [63]. The ability of RahU to decrease NO activity via inhibition iNOS expression defines an alternative mechanism by a microbial protein. Nathan and coworkers have previously demonstrated the critical role of NFkβ in iNOS transcription in murine macrophages [61]. Mice compromised for hepatocyte NFkβ activation showed high susceptibility to bacterial infections [64]. Glucocortiocoids such as dexamethasone also inhibit iNOS gene expression by inhibiting NFkβ activity [65]. Direct or indirect inhibition of NFkβ by r-RahU may promote a global inhibitory effect of various inflammatory mediators as suggested in the gene expression data. An intact protein structure of r-RahU

was essential to exhibit its action on iNOS expression. The IC_{50} of r-RahU (0.6 μM) was distinct from the known inhibitors of NO production: prednisone (50 μM) and L-NMMA (100 μM). It can be assumed that the multiple targets of r-RahU may contribute to the low IC_{50} of r-RahU. It should be noted that r-RahU also inhibited IL-10, which is known to be involved in anti-inflammatory activity [66].

Macrophages in areas of inflammation and/or infections generate MCP-1 and 2, followed by generation of other chemokines that recruit T cells, monocytes and neutrophils; this activity also combines the cellular and hormonal arms of immunity [67]. Monocytes as members of the human phagocyte system and precursors of dendritic cells and macrophages play crucial roles in the innate and adaptive immune responses [52]. There is compelling evidence to suggest that macrophages initiate the regulation of inflammation during *P. aeruginosa* infections [68]. At the same time, Pseudomonal proteases act both to destroy host physical barriers and to compromise host immune effectors [69]. In this report we show r-RahU interfered in expression of chemokine mRNAs known to be involved in chemotaxis of immune cells. Among these were chemokine (C-X-C and C-C motif) and chemokine ligands (Table 1). CC and CXC chemokines are reported to be co-induced in fibroblasts and leukocytes by microbial agents which also determines the number of phagocytes infiltrating into inflamed tissues [70]. Furthermore, C-C and C-X-C, which function synergistically in monocytes, are reported to facilitate adhesion of monocytes to vascular endothelium during inflammation [70;71]. In the present study we show r-RahU significantly inhibited the chemotactic activity of human acute monocytic leukemia cells towards CCL2 or chemotactic supernatants from apoptotic T-cells. These observations are similar to the role of various other bacterial endotoxins or products to inhibit IL-8 induced chemotaxis of neutrophils [72]. Recent studies have also shown that human cytomegalovirus exposed monocytes were unresponsive to inflammatory and homeostatic chemokines, although the basal cell motility and responsiveness to *N*-formyl-Met-Leu-Phe were unaffected [73]. Our data further highlight the complex level of interference that may be exerted by r-RahU on cells of the host immune system.

The results presented in this manuscript demonstrate two additional roles for r-RahU in monocytes and or macrophages in regulating inflammation and/or inflammation resolution. These studies suggests that RahU may function among the several virulence determinants of *P. aeruginosa* by not only sensing and signaling in bacteria based on the inflammatory environment (host Ox-phospholipids), but also by simultaneously modulating molecular events in cells involved in inflammation and host-bacterial interactions. These observations demonstrate that RahU shares some immunosuppressive properties with corticosteroids which includes inhibition of the mRNA of nitric oxide in murine macrophages [39;48;50] and chemotaxis in monocytes [74].

In summary, these two reports identify a previously unknown pleiotropic function of a single bacterial derived protein: RahU in *Pseudomonas* and macrophages. The data show that RahU potentially disrupts host defenses leading to increased bacterial virulence. The data are consistent with a role for the protein in pathology during host-bacterial interactions. It is tempting to speculate that RahU, which can participate in a hypothetical “innate signaling and communication apparatus”, may modulate “opportunistic behavior” in a pathological condition as shown in Fig. 7. Thus RahU underscores itself as a candidate target for therapeutics and biomarkers involved in inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

LysoPC	lysophosphatidylcholine; 1-hexanoyl-2-hydroxy-sn-glycero-3-phosphocholine
PAPC	1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine
Ox-PAPC	oxidized-1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine
PLA_2	Phospholipase A_2
AA	arachidonic acid
Gm	gentamicin
Tc	tetracycline
Sp	spectinomycin
Cb	carbenicillin
TMB	3,3´,5,5´-tetramethylbenzidine
TSA	Tryptic soya agar plate
BA	Blood agar plates
LB	Luria–Bertani medium
RahU	A Sanskrit word for “the seizer.”
Asp-hemolysin	*Aspergillus*-hemolysin protein
BHT	Butylated hydroxytoluene
VIGF C	Vascular endothelial growth factor
PIGF	Placental Growth Factor
PHSCA	*Pseudomonas*/Host Signal and Communication Apparatus

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Research highlights

- Pseudomonas protein: RahU is involved in bacterial defense
- RahU inhibits nitric oxide production in macrophages
- RahU inhibits in chemotactic human THP1 monocytic cells
- RahU modulates microbial physiology and host innate immunity

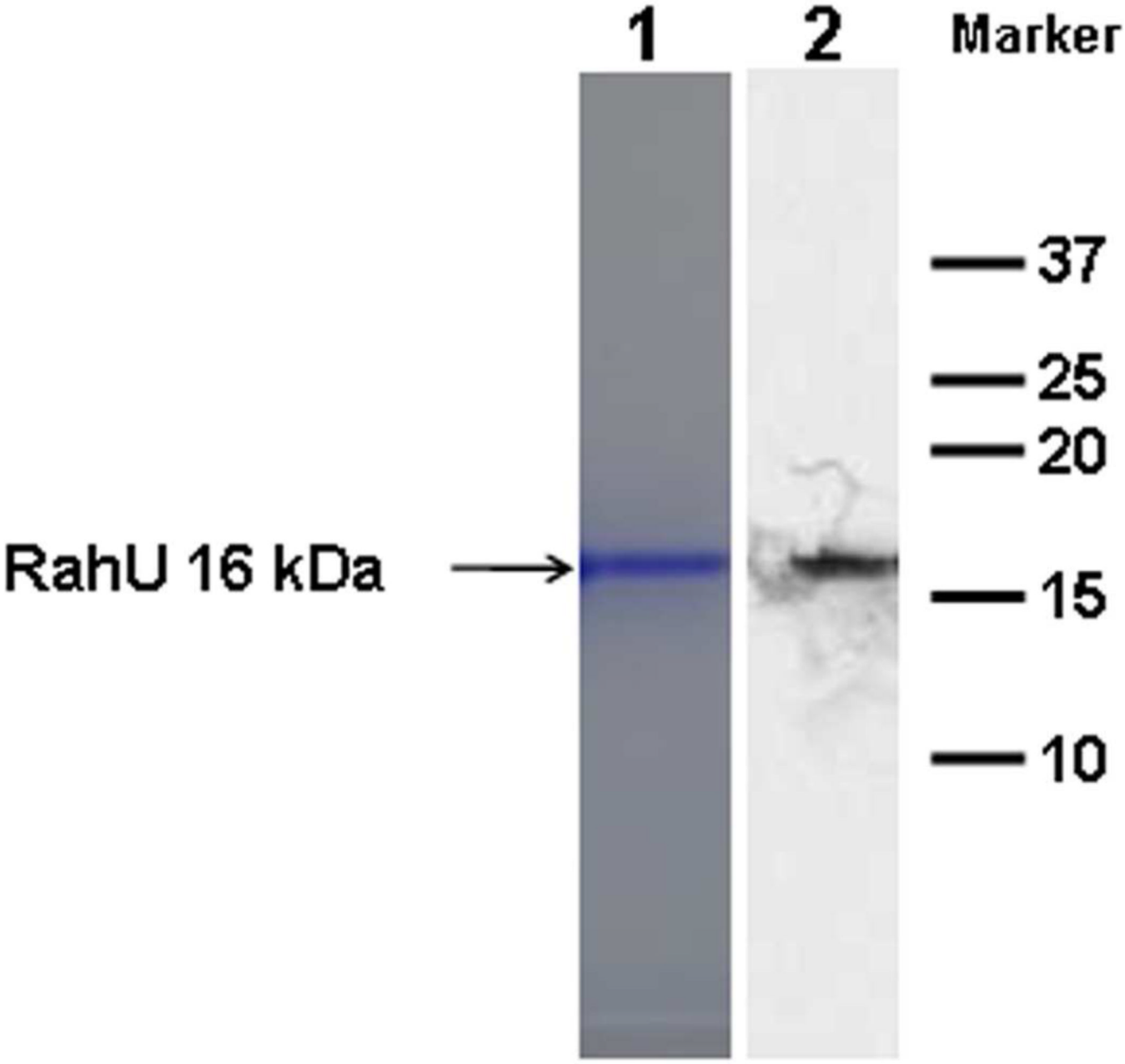

Figure 1.

Purification of recombinant RahU from *E. coli* and preparation of endotoxin free r-RahU. *rahU* cDNA was amplified from *P. aeruginosa* strain 383 and cloned into pET28^{b} plasmid. Recombinant RahU-his tagged protein was expressed in *E. coli* as described in Materials and Methods [10]. The recombinant protein was purified using a His-tag on a Talon agrose column. Endotoxin was removed from his-tagged r-RahU protein by using immobilized polymixin B-agarose. Lane 1 shows Coomassie Blue staining of 10 μg of purified 16 kDa r-RahU which ran between the 15 kDa and 20 kDa molecular weight standards. Lane 2 shows the Western blot of a duplicate lane (obtained from the SDS-PAGE gel) probed with a specific anti-r-RahU antiserum. This experiment represents one of four.

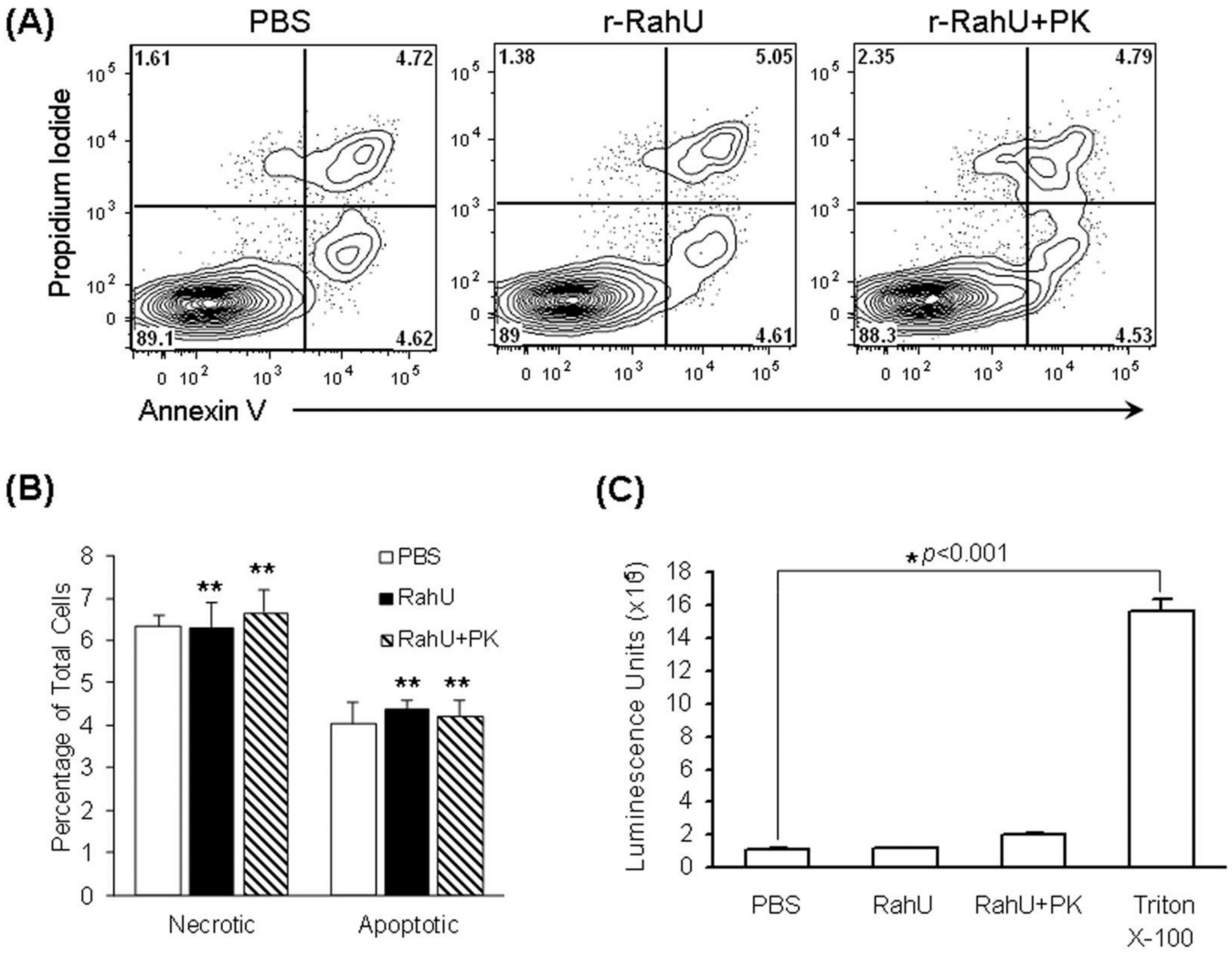

Figure 2.

Effect of r-RahU protein on apoptosis and cell viability of monocytic THP-1 cells in *in vitro*. Purified, endotoxin-free r-RahU protein was incubated with monocytic THP-1 cells at a final concentration of 2 μM as described in Materials and Methods. The viability of THP-1 cells was measured by flow cytometric analysis using propidium iodide and annexin V. (A) shows the distribution of subpopulations of viable cells (lower left quadrant), and small numbers of apoptotic (lower right quadrant), late apoptotic and necrotic cells (upper right quadrants). THP-1 cells after treatment with r-RahU and respective negative controls are designated as proteinase-K treated or PBS treated cells. Bars in (B) represent percentages of necrotic and apoptotic cells plotted from the total number of viable cells. The cytotoxic release assay was carried out using the CytoTox-Glo reagent on supernatants from THP-1 cells incubated for 1h with PBS, 2 μM r-RahU, 2 μM proteinase K-treated r-RahU or 0.01% Triton X-100 as a positive control (C). The bars represent the luminescence units. TX-100 treated cells represent a positive control. The values are represented as Mean ±SD as determined by Student's *t*-test where n=3–4. The *P* values are obtained by comparison with PBS and r-RahU incubated cells. ** represents non-significant values between PBS treated cells with RahU treated cells.

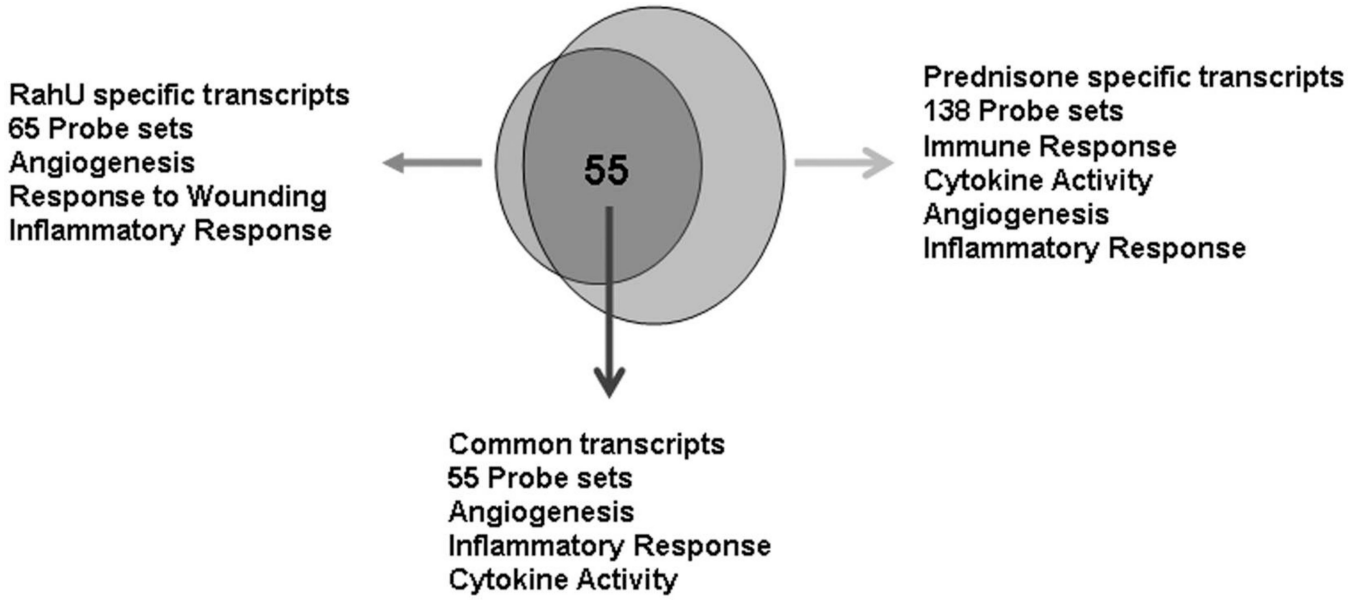

Figure 3.

Modulation of mRNA transcripts by r-RahU and prednisone in LPS activated RAW 264.7 cells identified by gene expression arrays. RAW 264.7 cells were grown *in vitro* as described in Materials and Methods. The cells were incubated with endotoxin free r-RahU (10 μg/mL) or 50 μM prednisone in presence and absence of LPS (1 μg/mL) for 18–24 h. Total RNA was purified and assessed for gene expression using mouse whole genome arrays. Transcripts that were upregulated by four fold in LPS stimulated cells as compared to normal unstimulated cells were selected by bioinformatics analysis. The Venn diagram shows the specific class of transcripts that were further down-regulated by two fold in r-RahU or prednisone treated cells with LPS. The list of these transcripts is detailed in supplemental data 1 and 2 and Table 1. The figure also shows the 55 downregulated transcripts shared commonly between r-RahU and prednisone treated LPS-stimulated cells.

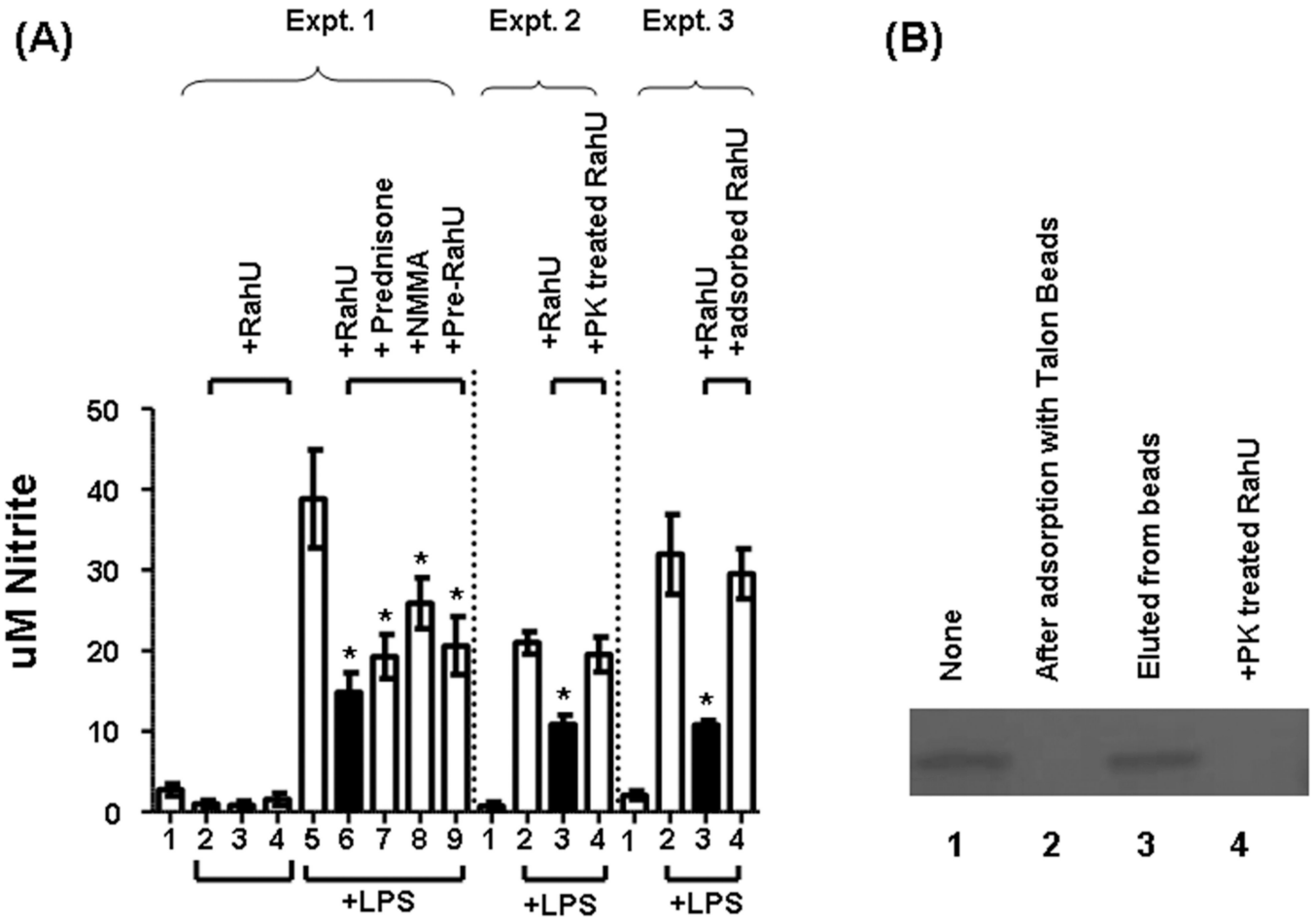

Figure 4.

r-RahU inhibits nitrite accumulation in LPS stimulated murine macrophages. Murine macrophage cells (RAW 264.7) were incubated with purified r-RahU (10 μg/mL) in the presence and absence of 1 μg/mL LPS as shown in Experiments 1, 2 and 3. Experiment 1: cells incubated with r-RahU alone at concentrations of 1, 2 and 10 μg/mL in lanes 2, 3 and 4 respectively. Lane 1 represents untreated control. Cells were also incubated with 1 μg/mL of LPS (lanes 5–9), in the absence of r-RahU (lane 5) and in the presence of 10 μg/mL r-RahU (lane 6), 50 μM prednisone (lane 7) and 100 μM NMMA (lane 8). Cells were pre-incubated for 1 h with 10 μg/mL of r-RahU, followed by stimulation with 1 μg/mL LPS (lane 9). Experiment 2: production of NO in untreated cells (lane 1) and cells stimulated with 1 μg/mL LPS alone (lane 2) or in the presence of 10 μg/mL RahU (lane 3) and Proteinase K treated RahU (lane 4). Experiment 3: nitrite levels in untreated cells (lane 1), and in cells treated with LPS (lane 2), LPS + 10 μg/mL r-RahU (lane 3) and LPS+ 10μg/mL. Talon agarose beads absorbed-r-RahU (lane 4). All the experiments were terminated at 16 – 18 h of incubation. The medium was used to estimate nitrite accumulation by the modified Griess method [27]. Fig. 3*B* shows the r-RahU that was utilized in various experiments described in Fig. 3*A*. Lane 1 shows SDS-PAGE analysis of the original purified r-RahU used in the experiments. r-RahU after absorption with Talon agarose beads was loaded in Lane 2 and bead elute was loaded in Lane 3. Lane 4 shows r-RahU after it was subjected to Protinase K treatment. Nitrite values are represented as Mean ±SD of the mean as determined by Student's *t*-test where n=3–4. The *P* values are obtained by comparison with LPS-stimulated cells (* p≤0.01).

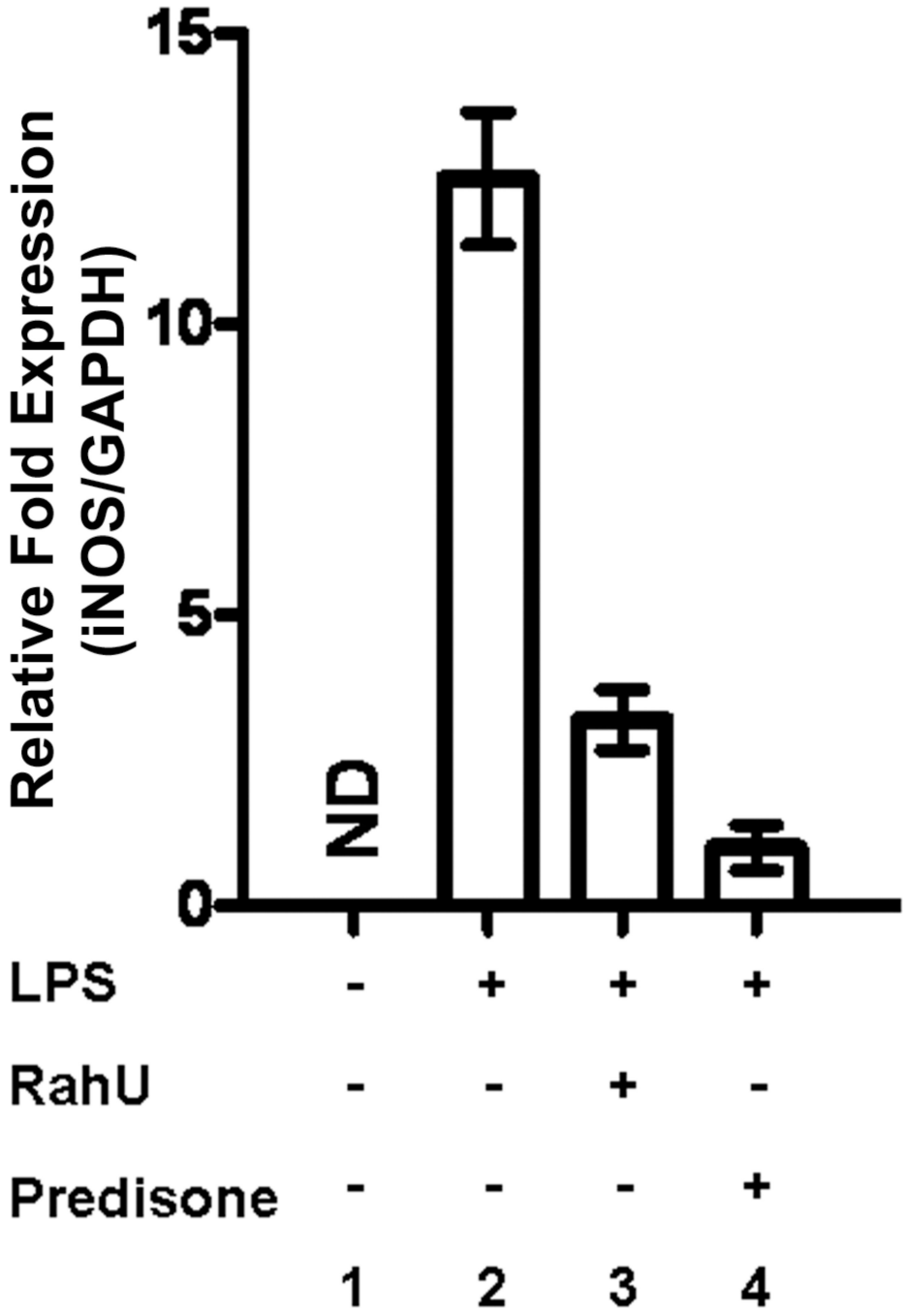

Figure 5.

Quantitative Real Time RT-PCR of mRNA of iNOS in RAW 264.7 cells treatment with LPS in the presence and absence of purified r-RahU and Prednisone. RAW 267.4 cells were incubated in the presence of 1 μg/ml LPS in the presence and absence of 10 μg/mL (0.6 μM) RahU or 50 μM prednisone for 16 – 18 h. Total RNA was isolated and quantitated by Experion Automated Electrophoresis. Total RNA from control untreated cells (lane 1); and cells treated with LPS (lane 2); LPS+r-RahU (lane 3); LPS+predisone (lane 4) was examined. The open bar represents the relative fold expression of iNOS mRNA after normalizing the values with GAPDH mRNA (house-keeping gene) as calculated by the BioRad IQ5 2.0 Standard Edition Optical System Software version 2. The treatment regime

is shown below the graph. The data represent one of the two similar experiments. ND: Not detectable.

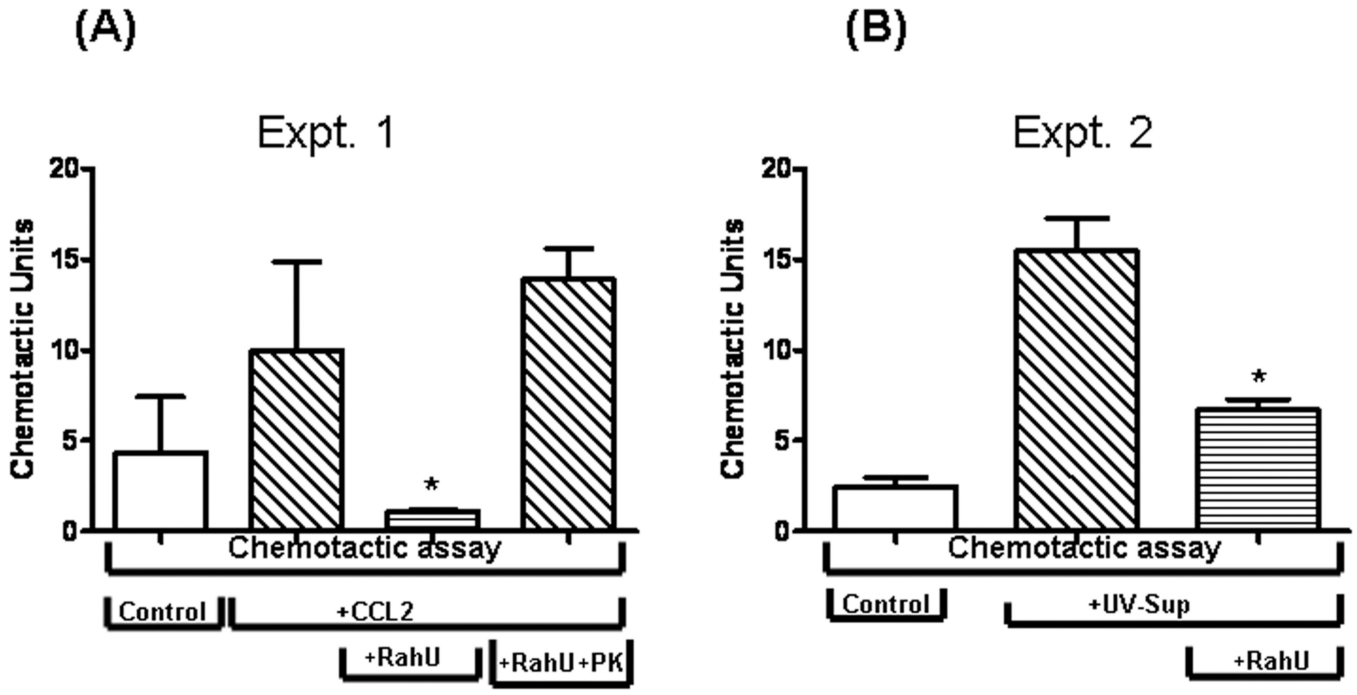

Figure 6.

r-RahU inhibits monocyte chemotactic protein (CCL-2) and apoptotic cell supernatant induced chemotaxis of human monocytes (THP-1). The monocyte trans-well chemotactic migration assay was established as described in Materials and Methods. Experiment 1: chemotaxis of THP-1 cells in medium alone which is described as control. The cells were exposed to CCL-2 (100 ng/mL) in the presence of 30 μg/mL of purified r-RahU and equivalent amounts of Proteinase K treated r-RahU. Experiment 2: chemotaxis of THP-1 cells induced with UV treated Jurkat T cell apoptotic supernatants as described in Materials and Methods. Three hundred and fifteen μl of Jurkat T cell apoptotic supernatants was incubated in the presence of 30 μg/mL of r-RahU. Chemotactic units are calculated as percent inhibition of migration. Chemotactic Units are representative of means ±SD values, as determined by Student's *t*-test. The *p* values indicate comparison of chemoattractant induced cells with r-RahU treated cells, n=3 (* $p \leq 0.01$). The data represent one of three similar experiments.

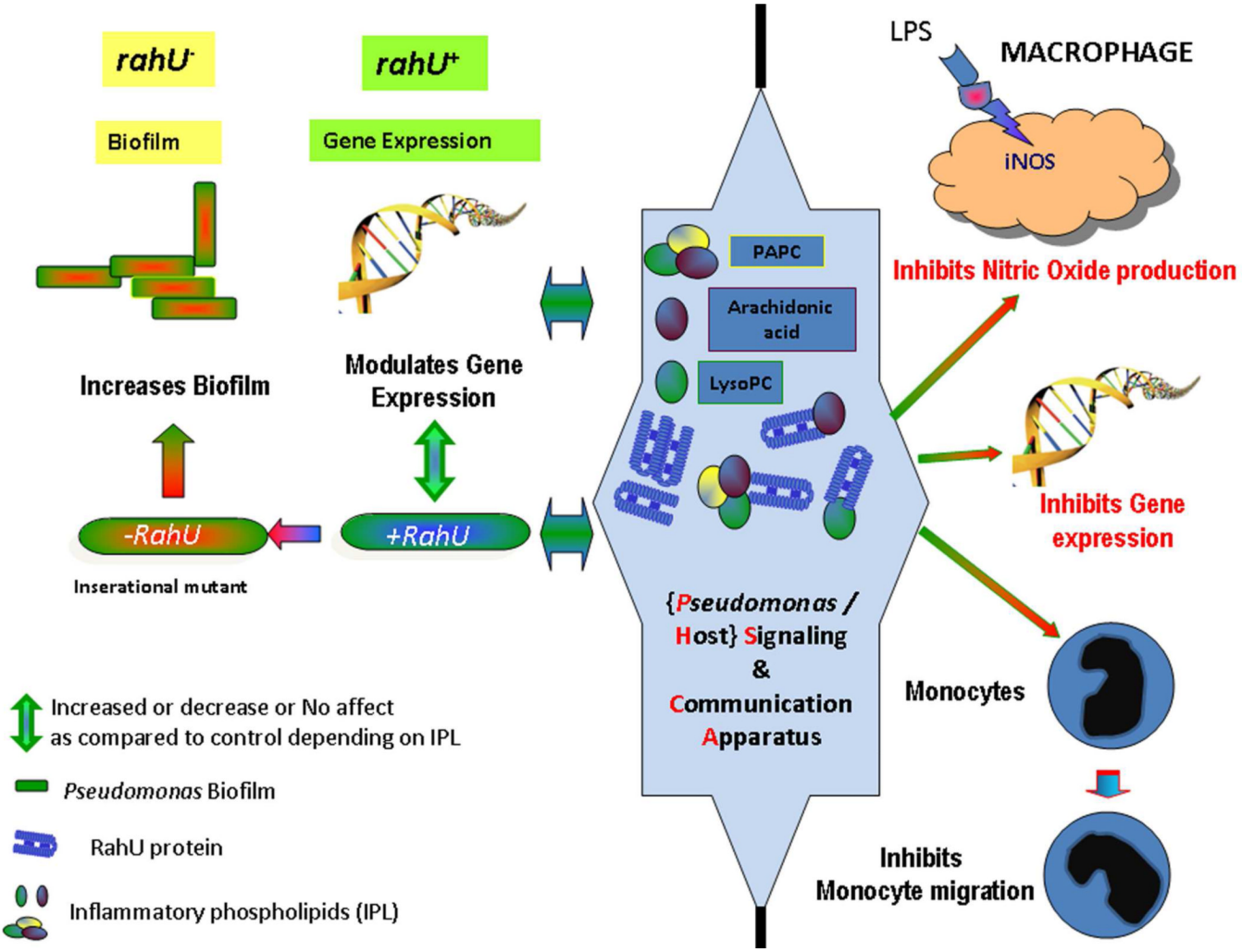

Figure 7.

Hypothetical representation of Pseudomonas/Host Signaling and Communication Apparatus (PHSCA) and its multifunctional-role/action in host-bacterial interactions. Inflammation and microbial sensing are two aspects of a single process as hypothesized by Beutler [11]. In the present study, Rao et. al., collectively shows how host phospholipids and *P. auroginosa* derived RahU may function in unison during inflammation, inflammation resolution and microbial sensing. r-RahU, at relatively high concentrations do not induce cytotoxicity as tested in this study. We propose that RahU functions as a common denominator in host-bacterial interactions as it regulates gene expression and microbial physiology in *Pseudomonas* and interacts and/or binds with various Ox-phospholipids released by host cells [19].

This common denominator may form a hypothetical structure: Pseudomonas/Host Signaling and Communication Apparatus (PHSCA) in the microenvironment, serving as one of the common ligands for host-bacterial interactions at the cellular level. *P. aeruginosa* senses Ox-phospholipids and regulates its own biofilm and gene expression *via* PHSCA as described in the companion manuscript. PHSCA also regulates signal transduction in host cells by inhibiting an array of genes induced by LPS, which are known to regulate inflammation, innate immunity and angiogenesis as described in this report. These genes include iNOS, which is known to be involved in the killing process of bacterial cells. r-RahU also inhibited {CCL2 or apoptotic supernatant} induced monocyte (THP-1) - chemotaxis in trans-well

assays. Although the receptors for RahU or (RahU + phospholipids) remain elusive, the scavenger or other pathogen-associated molecular patterns (PAMPs) recognizing receptors may be potential candidates. In summary, RahU is a multifunctional microbial protein participating in host-bacterial interactions.

Table 1

Common transcripts down regulated by r-RahU and Prednisone (Pred) in LPS stimulated macrophages

Number of transcripts	Probe ID	Ratio LPS *vs.* Cont.	Ratio LPS +RahU *vs.* Cont.	Ratio LPS +Pred. *vs.* Cont.	Accession	Gene Name
1	1434484_at	24.6	3.3	4.1	AV006463	RIKEN cDNA 1100001G20 gene
2	1429646_at	4.7	1.5	1.2	AK007173	RIKEN cDNA 1700112C13 gene
3	1450716_at	6.5	1.3	2.7	D67076	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1
4	1415818_at	8.0	3.6	1.4	NM_013472	annexin A6*
5	1429246_a_at	8.6	3.8	1.1	AK013026	annexin A6*
6	1419549_at	7.7	1.1	1.2	NM_007482	Arginase, liver
7	1418489_a_at	6.4	3.0	1.4	AB015595	calcitonin receptor-like
8	1415975_at	4.5	1.8	0.8	AU080787	calcium regulated heat stable protein 1*
9	1415976_a_at	5.0	2.0	1.2	AU080787	calcium regulated heat stable protein 1*
10	1419282_at	41.9	5.7	1.2	U50712	chemokine (C-C motif) ligand 12
11	1420249_s_at	16.1	1.4	5.8	AV084904	chemokine (C-C motif) ligand 6
12	1427357_at	21.1	8.9	3.9	AK008793	cytidine deaminase
13	1420627_a_at	4.9	1.9	1.3	NM_007784	Casein alpha s1
14	1419697_at	6.6	3.3	0.6	NM_019494	chemokine (C-X-C motif) ligand 11
15	1449368_at	4.4	1.9	1.4	NM_007833	Decorin
16	1419816_s_at	6.0	1.8	2.8	AI788755	ERBB receptor feedback inhibitor 1
17	1450779_at	11.9	4.2	1.0	NM_021272	fatty acid binding protein 7, brain
18	1429310_at	4.1	1.7	1.1	BE945486	fibronectin leucine rich transmembrane protein 3
`9	1440342_at	9.5	2.6	2.2	BB276544	RIKEN cDNA G530011O06 gene
20	1428815_at	16.7	3.5	1.1	BF319534	glutathione S-transferase, theta 4
21	1422155_at	4.6	2.0	1.5	BC015270	histone cluster 2, H3c2
22	1425895_a_at	4.7	0.8	0.9	U43884	inhibitor of DNA binding 1
23	1422537_a_at	5.8	2.8	1.6	NM_010496	inhibitor of DNA binding 2
24	1450330_at	20.1	8.4	3.6	NM_010548	interleukin 10
25	1422177_at	11.4	1.6	0.8	BC003723	interleukin 13 receptor, alpha 2
26	1418609_at	18.8	2.2	1.5	AF206697	interleukin 1 family, member 6

Number of transcripts	Probe ID	Ratio LPS *vs.* Cont.	Ratio LPS +RahU *vs.* Cont.	Ratio LPS +Pred. *vs.* Cont.	Accession	Gene Name
27	1448207_at	4.6	1.9	1.8	BC010840	LIM and SH3 protein 1
28	1421180_at	23.5	5.7	1.7	NM_025681	limb expression 1 homolog (chicken)
29	1456060_at	12.7	2.2	0.3	AV284857	avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog
30	1417256_at	4.4	1.1	1.2	NM_008607	matrix metallopeptidase 13
31	1420671_x_at	13.1	5.4	1.3	NM_029499	membrane-spanning 4-domains, subfamily A, member 4C
32	1450291_s_at	27.0	9.7	1.2	NM_022429	membrane-spanning 4-domains, subfamily A, member 4C
33	1427201_at	4.1	0.9	1.0	AJ277212	musculoskeletal, embryonic nuclear protein 1
34	1422002_at	8.8	4.3	1.8	L38926	MAX dimerization protein 1
35	1420393_at	7.0	2.1	2.5	AF065921	nitric oxide synthase 2, inducible, macrophage
36	1435529_at	4.8	2.2	1.2	BM245961	predicted gene, OTTMUSG00000016644
37	1421957_a_at	6.0	2.4	1.2	NM_009981	Phosphate cytidylyltransferase 1, choline, alpha isoform
38	1418471_at	6.2	1.8	1.3	NM_008827	placental growth factor
39	1417216_at	6.8	1.1	1.2	NM_138606	proviral integration site 2
40	1431046_at	4.8	1.4	1.7	BG962793	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 3
41	1455840_at	5.8	2.5	1.5	BM230524	Rap guanine nucleotide exchange factor (GEF) 5
42	1421111_at	4.3	1.9	1.4	NM_019743	RING1 and YY1 binding protein
43	1415943_at	8.2	3.6	1.0	BI788645	syndecan 1
44	1425906_a_at	4.1	1.8	1.7	AF034744	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E
45	1427673_a_at	4.1	2.1	1.6	Z93948	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E
46	1416521_at	4.1	1.7	1.7	NM_009156	selenoprotein W, muscle 1
47	1448025_at	8.1	2.4	0.7	AI662854	signal-regulatory protein beta 1
48	1460302_at	6.9	1.2	3.4	AI385532	thrombospondin 1
49	1416342_at	12.1	5.0	1.0	NM_011607	Tenascin C
50	1439739_at	4.4	2.2	1.9	BB295899	Testes-specific protease 50
51	1460667_at	7.4	2.3	1.4	NM_020562	cDNA sequence U90926
52	1419417_at	16.1	3.8	1.6	NM_009506	vascular endothelial growth factor C *
53	1439766_x_at	18.0	5.3	1.8	BB089170	vascular endothelial growth factor C *
54	1440739_at	23.2	5.5	1.7	AW228853	vascular endothelial growth factor C *

Number of transcripts	Probe ID	Ratio LPS *vs.* Cont.	Ratio LPS +RahU *vs.* Cont.	Ratio LPS +Pred. *vs.* Cont.	Accession	Gene Name
55	1443930_at	4.1	1.7	1.4	AI644578	zinc finger protein 811

RAW 264.7 cells were treated with and without LPS in the presence of r-RahU and/ or prednisone. mRNA was analyzed by gene chips and bioinformatics analysis as described in Materials and Methods. The controls (cont) represent gene expression in untreated cells. The table summarizes the common transcripts that were upregulated by LPS as compared to control. Columns 4 and 5 represent transcripts that were downregulated by r-RahU and /or prednisone in LPS stimulated cells. The data is represented as ratios * for respective signals of the same transcript with different probes sets on the chip. Probe sets from the gene chip and accession numbers from the gene bank are shown in Columns 2 and 7 respectively.