International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 11 (2014) pp. 606-616**
http://www.ijcmas.com

Original Research Article

Immunostimulatory RNA Induces cardiac transcriptional factors and modulates cytokine expression profiles

Farag Bleiblo[1,2*], Paul Michael[1], Danielle Brabant[1], Chilakamarti V.Ramana[3], T.C .Tai[1], Mazen Saleh[1], Joseph E. Parrillo[4], Anand Kumar[5] and Aseem Kumar[1]

[1]Department of Chemistry and Biochemistry and the Biomolecular Sciences Programme, Laurentian University, Sudbury, ON, Canada, P3E 2C6
[2]Department of Botany, University of Sirte, Sirte, Libya
[3]Department of Medicine, Yale University, New Haven, CT, USA, 06516
[4]Division of Cardiovascular Disease and Critical Care Medicine, Cooper University Hospital, Robert Wood Johnson Medical School, Camden, New Jersey, USA, 08103
[5]Section of Critical Care Medicine, University of Manitoba, Winnipeg, MB, Canada, R3A 1R9
**Corresponding author*

A B S T R A C T

Keywords

Cardiac myocytes, Immuno-stimulatory RNA, Transcription factors

Cardiac myocytes express pattern recognition receptors (PRRs) and can detect a diverse range of pathogenic moieties including nucleic acids. Inflammatory response is a central component of cardiac pathology associated with a number of heart diseases including sepsis, microbial infections, myocarditis, and myocardial infraction. We demonstrate that bacterial RNA induces activation of subset of transcription factor including NFκB, IRF1 and STAT1. Bacterial RNA was also found to differentially modulate the production of several numbers of cytokines involved in the regulation of inflammatory response. In addition to bacterial immunity, the results presented here may also have implications in RNA-based therapy and cardiac pathophysiology.

Introduction

Discriminating self from nonself is crucial to innate immunity that employs patterns recognition receptors (PRRs) to sense molecular patterns associated with pathogenic moieties including nucleic acids (Deane *et al.,* 2006; Kawai and Akira, 2007). Recent studies have indicated that other PRRs can recognize distinct subspecies of RNAs released from pathogenic organisms (Akira *et al.,* 2003; Alexopoulou and Kontoyiannis, 2005). Of considerable interest are the recent findings that bacterial RNA can induce antigen-specific immunity, secretion of proinflammatory cytokines and type I interferons (IFNs) in TLR-dependent manner (Lau *et al.,* 2005; Boyd *et al.,* 2006; Bourquin *et al.,* 2007). In addition to the TLRs, the NLRs have also been identified as additional quality control mechanism that detects bacterial and viral RNAs in a Nalp3-dependent manner (Kanneganti *et al.,* 2006). The RIG-I has been categorized as a specific

sensor for dsRNA, however recent reports have shown that ssRNA containing a 5'-triphosphate end can also function as a ligand for RIG-I (Hornung *et al.,* 2006; Pichlmair *et al.,* 2006; Takahasi *et al.,* 2008). Despite the difference in ligand specificity, localization and signaling cascades of these receptors, the above observations emphasized the unifying theme in innate immunity whereby the host could use the same immunreceptor to detect various subspecies of foreign RNA (Heil *et al.,* 2004; Sugiyama *et al.,* 2005).

Cardiac myocytes dysfunction has been clearly identified as a serious and frequent complication associated with patients suffering from microbial infections, septic shock and myocardial infarction (Frantz *et al.,* 2007). In addition to immune cells, cardiac myocytes also express PRRs and can detect a diverse range of pathogenic patterns including nucleic acids ((Frantz *et al.,* 1999; Boyd *et al.,* 2006; Frantz *et al.,* 2007). We reasoned that bacterial RNA could present a distinct pattern to be recognized as nonself and modulates the inflammatory response. To address this hypothesis, we examined whether RNAs derived from bacterial sources could induce the transcription factors NFκB, IRFs and STATs and modulate cytokine profiles.

Materials and Methods

Preparation of bacterial total RNA

Pathogenic isolates of *E. coli* (O18:K1:H7; ATCC #700973) and *S. aureus* [capsular serotype 8, non-toxic shock toxin (TSST-1) producer] were used for this study. Exponentially growing bacteria were harvested in ice cold 5% phenol/ethanol solution and then resuspended in a fresh lysis buffer of TE, 2 mg/ml lysozyme and 5 µg/ml lysostaphin (Sigma Alderish, St.Loius, USA). Samples were brought to 2% SDS and a volume of 850 µl water-saturated phenol was added to each tube and incubated for 5 minutes at 64ºC. Following centrifugation at 13000 X g, the aqueous phase was transferred to a fresh tube and equal amounts of chloroform were added and centrifuged for 10 minutes at 4ºC. The RNA was precipitated by 0.1 vol. 3M sodium acetate and 2.5 vol. ethanol. The integrity and purity of RNA species were determined by electrophoresis on agarose gels and A260/A280 ratios. Extracted crude RNA was treated with RNase-free DNase I (Roche, Indianapolis, USA) to remove contaminant genomic DNA and further purified by the Qiagen method (Qiagen, Valencia, USA) as per the manufacturer's instructions.

Preparation of mammalian total RNA

Total cellular RNA from human adult cardiac myocytes was prepared with extraction using a silica column-based method according to the manufacturer's instructions (Qiagen, Valencia, USA). A sample of about 2×10^6 human adult cardiac myocytes cells were washed three times with ice-cold PBS and then scraped with 1 ml buffer RLT. Prior to the first RPE wash buffer, DNase 1 incubation mix was added onto RNeasy silica-gel membrane and allowed to sit for 20 minutes to further eliminate any traces of genomic DNA contamination. RNA was quantified by UV spectrometry and electrophoresed on a 1% agarose gel to verify purity and integrity prior to use.

RNA digestion

Aliquots of RNA samples were incubated in the presence of a heterogeneous mixture of ribonucleases [(1 U per 2µl of RNA at 1µg/µl for 1 hr) (Roche, Indianapolis, IN)]

as described (Paladugu *et al.,* 2004). RNA samples were analyzed by denaturing agarose or polyacrylamide gel electrophoresis for quality assurance. Aliquots of digested total bacterial RNA were added to the media to yield a final concentration of 100 or 200 µg/ml.

Cardiac myocytes culture and stimulation

Human adult cardiac myocytes (HCMs) (ScienCell Research Laboratories) were grown in cardiac myocyte medium (CMM) supplemented with 10% fetal bovine serum, cardiac myocyte growth supplement (CMGS) and 1% of penicillin/streptomycin (ScienCell Research Laboratories) in 5% CO_2 at 37°C. Cells were grown to approximately 70% confluence, and then the growth medium was replaced with unsupplmented base CMM for 24 h before treatment. Poly I:C was resuspended in sterile distilled H_2O to a final concentration of 1.0 µg/µL. Approximately 2 x 10^6 cardiac myocytes were stimulated with 100 µg/ml or 200 µg/ml of bacterial total RNA, poly I:C, digested total RNA, or cardiac myocyte RNA for 12 h, 24 h, or 48 h or left untreated as a control.

Electrophoretic mobility shift assay (EMSA)

Cytoplasmic extracts were prepared and assayed for EMSA as described previously (Kumar *et al.,* 2005). Briefly, 6µg of proteins were subjected to EMSA in a binding reaction containing 300,000 cpm of [γ-P ^{32}] ATP-end labeled oligonucleotides probes to measure the binding activity for STAT1, NFκB, and IRF1.The oligonucleotides used were: the GAS sequence 5'-TTCCGGGAA-3' for binding to STAT1, hexamer sequence (5'-AAGTGA-3') for IRF1, and PRDII sequence 5'-TGGGGACTTTCCGC-3' for NFκB. For supershift experiments, the cellular extracts were incubated with 1µl of antibody against STAT1, IRF1 and p65 (Santa Cruz) for 10 min at room temperature before adding the probe

Human cytokine protein array

The human cytokine antibody arrays 3 (Ray Biotech, Inc) were used to compare the differential cytokine expression profiles between cells left unstimulated or stimulated with various RNAs. Each membrane contains protein pairs (double spotted) from 48 known human cytokines divided into different functional groups. Positive and negative controls are also included for hybridization specificity. Identical membranes were first blocked for 30 min with 1x blocking buffer and then probed with 1 ml serum free conditioned media derived from cells stimulated with *S. aureus* RNA, RNase- digested *S. aureus* RNA, cardiac myocyte RNA, or left unstimulated as a control. After incubation at 4°C for 24 h, the membranes were washed three times with wash buffer I and twice with wash buffer II. Biotin-conjugated anti-cytokine antibodies were incubated with membranes for overnight at 4°C and washed 5 times as directed to remove unbound antibodies. We next incubated the HRP-conjugated streptavidin with the membranes for 2 hrs at RT and washed as directed previously. The signals were directly detected from the membranes by multiple chemiluminescence exposures of variable lengths on Chemidoc imaging system. The intensity of each signal was quantified using the densitometry. Positive controls were used to normalize the results from different membranes being compared. Values were also corrected for differences in the probing efficiency between the membranes by dividing the average expression of all cytokines in the respective array (global normalization).

Construction of STAT, IRF and NFκB reporters and assays

For STAT, IRF and NFκB luciferase reporters, we used luciferase reporter vector pGL 4.26 and the background construct pGL 4.74 [hRluc/TK] for Renilla vector (Promega). These vectors contain enhancer restriction enzyme multicloning sites 5' to a functional TATATA RNA polymerase initiation binding site, which is 5' to the luciferase gene. We inserted the STAT regulatory element 5'-TTCCGGGAA-'3 using SmaI into the pGL 4.26 vector multicloning site and selected for two forward copies of STAT regulatory element and used this construct in our experiments. The NFκB pGL luciferase reporter construct is driven by the NFκB regulatory element 5' (TGGGGACTTTCCGC)'3 which was also purchased from Promega. We also cloned a promoter containing the IRF binding sequence 5'-AAGTGA-'3 upstream of the pGL4.26. GTE lysis buffer [50 mM glucose, 25 mMTris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), and 200ul/1.5 ml culture tube, 0.2 N NaOH / 1% SDS (300 ul per 1.5 ml culture tube)] was used for extraction of vector DNAs from gglycerol stocks of *E. coli* (DH5α) bearing reporter constructs. Vector DNA was precipitated by 0.6 volume of isopropanol and washed with 70% ethanol. Dissolved pellets were subjected to RNase A (sigma) digestion to remove any contaminant RNA and checked with agarose gels to verify the size of inserts. We used Qiagen purification kit (Qiagen, Hilden, Germany) to purify the reporters DNA as instructed by the manufacturer. Vectors DNA was quantified by BioRad fluorescence DNA quantification kit after constructing standard curves in triplicates. Fluorescence was measured using FluoStar detection machine. FuGene 6 (Roche, Germany) used to transfect cardiac cells seeded into 24 well plate at 20,000 cell/ well and grown in to serum and antibiotic free media at ration ratio 3:1 FuGene 6 to DNA for 6 hrs. The medium was then replaced with unsupplmented cardiac myocyte media and cells treated for 24 hrs. The dual luciferase reporter assay system (Promega) used to prepare the cellular lysates and measure the transcriptional activity following the recommendation by manufacturer. Ratios of the luciferase to renilla readings were measured by FluoStar Optima (BMG laboratories) for three separate experiments on triplicate samples. After normalization to Renilla, the SPSS student version software for statistical analysis used to determine the significance of the fold change in luciferase assay results of the RNA-treated cells over the untreated. Kruskal-Wallis one-way analysis of variance test was performed. A value of $p<0.05$ was considered significant and $p<0.005$ was considereid highly significant. All data are shown as mean ± SEM.

ELISA assays

Concentrations of IL-1β incardiac myocytes media stimulated with various RNA species were determined by ELISA according to the manufacturer's protocol (R&D Systems). The reagent diluents were optimized for the quantification of the cytokine.

Results and Discussion

To test the potential of bacterial RNA to activate transcription factors NFκB, IRF, and STAT family members, we performed luciferase reporter assay. Bacterial RNA-dependent activation of these transcription factors were normalized to the expression levels of Renilla construct cotransfected with the luciferase gene into cardiac cells (Figure 1). The NFκB regulatory element that used to drive the luciferase reporter has

been shown to be regulated by the family members p50, p52, RelA, RelB and c-Rel. We found that stimulation with bacterial RNA of cardiac cells transfected with the NFκB luciferase construct induced the reporter significantly by average of about four folds over the non-treated controls (Figure1 left panel). The efficiency of NFκB induction was comparable to that induced by poly I:C. As a positive control, TNF-α treated cell induced the NFκB activation to an extent comparable to that generated by bacterial RNA. We also found that treatment with bacterial RNA also activates the transcriptional activity of IRF construct driven by the IRF hexamer regulatory element which were comparable to that treated with poly I:C (Figure 1 right panel). Again, we found that treatment with IFN-γ as a positive control induced IRF activation to an extent comparable to that induced by bacterial RNA.

Because the regulatory elements used to drive the reporter constructs cannot distinct a specific family member or a specific subunit of the transcription factors NFκB, IRF, or STATs, therefore we used EMSA analysis to specify the transcription factor or subunit composition.We performed EMSAs using radio labeled PRDII regulatory elements specific for NFκB to examine its transcriptional induction by bacterial RNA. Consistent with luciferase reporters, we found that stimulation of cardiac cells with bacterial RNA activated transcription factor NFκB as compared with Poly I:C (Figure 2 left panel). The bacterial RNA-activated NFκB complex was identified as minimally containing the p65 subunit, since p65 antibody incubated in the reaction buffer ablated the complex. We also determined that treatment of cardiac myocytes with bacterial RNA resulted in activation of factors to multimeric hexamer element which was identified as IRF1 since IRF1 specific antibody abolished this complex from EMSA (Figure 2 middle panel). Similar results were obtained from EMSA performed with probed GAS regulatory element to assess DNA binding activity of transcriptional factor STATs to proteins from bacterial RNA-treated cells. Consistent with reporter assays, EMSA results shows that bacterial RNA induced STAT1 DNA binding activity (Figure 2 right panel).

Although the immunostimulatory potential of bacterial RNA is well documented, no comprehensive analysis of cytokine expression in response to naturally occurring bacterial RNA has been reported. Using human cytokine antibody array, we have analyzed the cytokine production profiles of human adult cardiac myocytes stimulated with various RNA including *S. aureus* RNA (SRNA), RNase-digested *S. aureus* RNA (DSRNA), cardiac myocytes RNA (CRNA), or left untreated (NT) as a negative control (Figure 3). The data presented in this work are the average of individual experiments repeated under the same experimental conditions and on identical cytokine membranes. The media that are used to probe the cytokine arrays were collected from three cardiac samples treated with or without RNA and mixed together to minimize the experimental variations of samples (Figure 3). Following detection, the images were subjected to densitometry using Bio-Rad's Quantity one software (v. 4.5.0). The intensities were corrected for background and then normalized to the average of the positive controls for each membrane from each exposure. The normalized data was imported to SPSS ver.11 and nonparametric test (Kruskal-Wallis test) was performed at significance level of 0.05. Where differences were shown to exist between groups from the Kruskal-Wallis test, a one way ANOVA was performed using Tukeys Post Hoc to

determine which groups were different from each other. The statistical analysis was done for the exposures 10, 30 60 and 120 seconds as these were the only exposures common to all treatments. The treatments were coded as follows for the analysis (1) NT (Figure 3 top left), (2) CRNA (Figure 3 top right), (3) DSRNA (Figure 3 bottom left) and (4) SRNA treated cells (Figure 3 bottom right). Our analysis has revealed changes in a number of cardiac proteins involved in regulating the adaptive immune responses including, proinflammatory cytokines, chemokines, and growth factors. The cytokines that were significantly different minimally between no treatment and bacterial RNA treated cells are GCSF, IGF1, IL-13, IL-1β, IL-7, MCP2, MCP3, MCSF, MDC, MIG, SDF1, leptin, PDGF-β, SCF and TNF-β. Collectively, the cytokine array data offer for the first time an insight into human cardiac myocytes response to immunostimulatory RNA such as bacterial RNA.

Our data also establish other definitive properties of human cardiac myocytes such as sensing and responding to danger signals with a complex molecular inflammatory response, thus analogous in some respect to immune cells. We next performed ELISA on the same samples of media to confirm the production of cardiac IL-1β by bacterial RNA. Consistent with the cytokine array data, we found that IL-β levels were significantly elevated by stimulation with bacterial RNA (Table 1). Caspase-1 which is present in cells in an inactive form undergoes proteolytic cleavage upon activation. Activated caspase-1 is essential for maturation of proinflammatory cytokines such as IL-1β and IL-18 and converts them to the active forms in response to infection or injury (Petrilli *et al.,* 2007). Thus, we next tested the ability of bacteria RNA to activate this inflammatory caspase. Western blot analyses shows that stimulation with bacterial RNA induces the production of active caspase-1 fragments (both 37p and 20p) suggesting the activation of this caspase (Figure 4).

Natural bacterial RNA and modified ssRNA are potent immunostimulatory agents characterized by the induction of proinflammatory cytokines such as IL-1β, IL-6, IL-12, TNF-α, type I interferon, and interferon-γ (Kariko *et al.,* 2005; Bourquin *et al.,* 2007). Although triggering the immune responses is generally considered as protective, the inappropriate response or the interplay between the cascades that are dysregulated could harm the host (Scheel *et al.,* 2004; Lan *et al.,* 2007). With regard to microbial nucleic acids, it has been shown that bacterial RNA significantly depresses cardiac myocyte cell contraction (Paladugu *et al.,* 2004).

Among cardiac cytokines, we have identified IL-1β and IL-13 levels to be elevated by bacterial RNA. IL-1 is thought to contribute to regulation of proliferation, inflammation, and contractility of cardiovascular cells. It has been shown that IL-1 stimulates proliferation of vascular smooth muscle and induces cytokine production of vessel wall or heart cells as well as expression of chemokines and adhesion molecules. Beside the above functions, IL-1 can also regulate levels of intracellular $Ca2^{+}$ of myocytes cells and induce apoptosis in cardiac myocytes (Maass *et al.,* 2002; Turner *et al.,* 2007). Previous studies have reported that DCs and TLR-expressing immune cells are potentially activated by bacterial or modified immunostimulatory RNA to release IL-1α or IL-1β.IL-13 was originally described as a T cell derived cytokine with pleiotropic effects.

Our analysis also revealed that stimulation with bacterial RNA also modulates a number of chemokines such as granulocyte colony stimulating factor` (G-CSF), granulocyte chemotactic protein 2(GCP-2), macrophage inflammatory protein (MIP) 1-α and MIP2-α. Chemokines constitute a large family of structurally related proteins that play important roles in host defences against pathogens by regulating the leukocyte signalling and differentiation (Van Damme *et al.,* 1997; Singh *et al.,* 2004). The elimination of bacterial and viral pathogens is accomplished primarily by the activity of professional phagocytes namely, the neutrophils, monocytes, and macrophges which are critical components of innate immune responses (Quinton *et al.,* 2002; Schaefer *et al.,* 2004). Proinflammatory cytokines such as G-CSF, GM-CSF, macrophage colony stimulatory factor (M-CSF), and INF-γ have been shown to upregulate the microbicidal activity in the effecter cells of innate immunity (O'Mahony *et al.,* 2008). G-CSF, a haematopoietic growth factor initiates proliferation and differentiation of bone marrow precursor cells into native neutrophil, can be produced in response to invading pathogens.

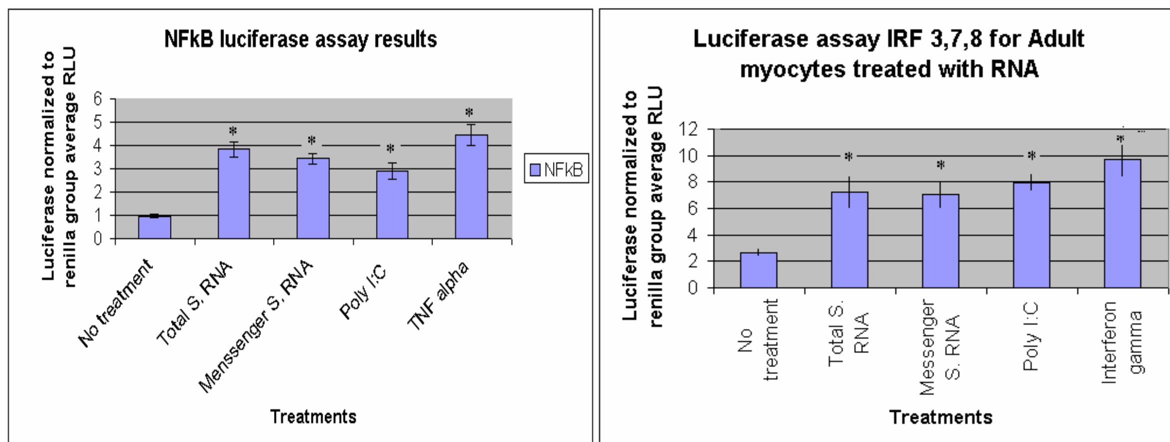
We also identified the growth factors insulin-like growth factor (IGF) 1, platelet derived growth factor (PDGF) -β, vascular endothelial growth factor (VEGF) and stem cells factor (SCF) to be modulated by bacterial RNA. Cardiac myocytes contain receptor on their surfaces for both types (IGF1-R and IGF2-R). The biologically relevant effects of IGF on cardiac cells are wide including promotion of cell growth, inhibition of apoptosis, and induction of cell differentiation (Su *et al.,* 2003; Lee *et al.,* 2006). In agreement with previous investigations, we suggest that the induction of cardiac IGF1 might be implicated in cardiac survival. Vascular endothelial growth factor receptor 1 (VEGFR1) and VEGFR2 are the protype of gene family encoding structurally related receptors and belongs to the receptor tyrosine kinase (RTK) (Rahimi, 2006; Shibuya*,* 2006). In addition to its central role in regulating angiogenesis, recent data have suggested that the VEGF may modulate various aspects of endothelial function leading to cardiac protection. Recent investigations have also shown that Flt1 is also implicated in response to bacterial and viral products. Administration of microbial products such as LPS in human and mouse models of infection and sepsis resulted in a time-dependent increase in VEGF concentrations (Yano *et al.,* 2006).

We have demonstrated that stimulation with bacterial RNA induces the transcriptional activity of NFκB, IRF1 and STAT1. Recognition of microbial components by PAMPs triggers a cascade of cellular ligands that culminates in the activation of NFκB and leads to inflammatory gene expression and clearance of the infectious agent (Doyle and O'Neill, 2006). The antiapoptotic functions of the NFκB family result from a direct interaction with tumor suppressor proteins, such as p53, or an imbalance in expression of proapoptotic and antiapoptotic proteins. The antiapoptotic function of NFκB also involves the intrinsic (mitochondrial) pathway or the extrinsic (receptor) pathway (Ghobrial *et al.,* 2005). Cells such as leukocytes, endothelial cells, cardiomyocytes, and fibroblasts therefore respond to pro-inflammatory cytokines by NFκB activation. This leads to or enhances expression of a host of genes, including those encoding several cytokines, leukocyte adhesion molecules, matrix metalloproteinases and iNO synthase. Although activation of NFκB induces pro-inflammatory genes, it has lately been indicated that the transcription factor is

involved in the signaling of endogenous myocardial protection evoked by ischemic preconditioning. Induction of cardiac NFκB 2 with bacterial RNA suggests that this transcription factor may be involved in the response to pathogen-associated molecules and signaling of myocardial protection IRFs are transcriptional mediators of IFN signaling pathways that are involved in antiviral defense, immune response, and cell growth regulation. Transcription factors of the IRF family have been shown to play an essential role in the regulated expression of type I IFN genes, IFN-stimulated genes (ISGs), and other cytokines and chemokines (Taki, 2002; Honda *et al.,* 2006). The promoter region of the IFN-β gene contains at least four regulatory cis elements, namely, the positive regulatory domains (PRDs) I, II, III and IV. PRD I and PRDIII are the binding sites for IRF family members, whereas PRD II and PRD IV elements are for NFκB and AP-1, respectively. We suggest that induction of cardiac IRF1 might be involved in cardiac apoptotic response induced by bacterial RNA and in agreement with several lines of evidence this transcription factor might play an essential role in host defense.

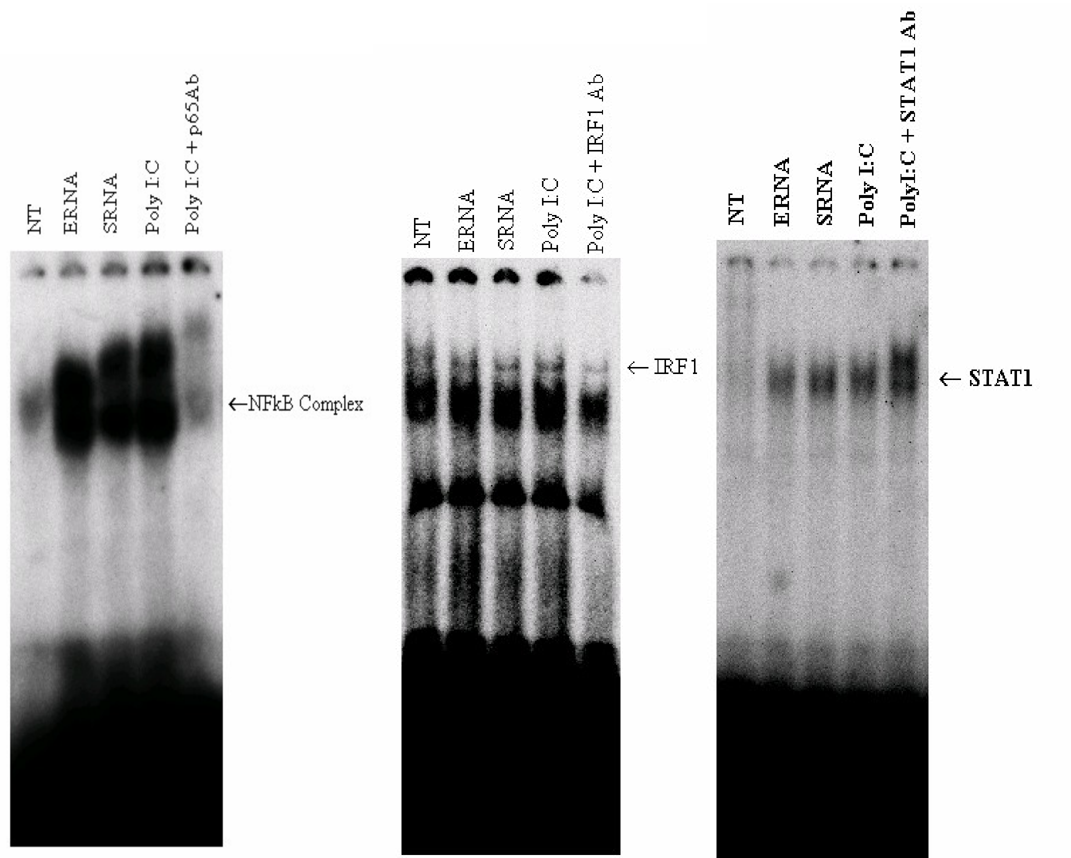
STATs are a family of transcription factors plays central roles in regulating diverse biological responses including cellular immunity, growth and proliferation, apoptosis, and tumorgenesis. Phosphorylation of STATs at a conserved tyrosine near the COOH-terminus is absolutely required for their dimerization, subsequent DNA binding, and IFN-dependent gene regulation (Shuai *et al.,* 1993). The induction of distinct sets of transcription factors by bacterial RNA may provide further insight into the mechanisms that innate immune system employs to responds to bacterial infection. Moreover, these observations may be useful for better understanding of the strategies used by pathogenic bacteria to escape immune surveillance. For example, it has recently shown that lysosomal recognition of bacterial RNA activates IRF1-TLR7 dependent pathway with a robust, host-protective interferon response (Mancuso *et al.,* 2009).

Figure.1 Transcription factors induction by bacterial RNA in human cardiac myocytes



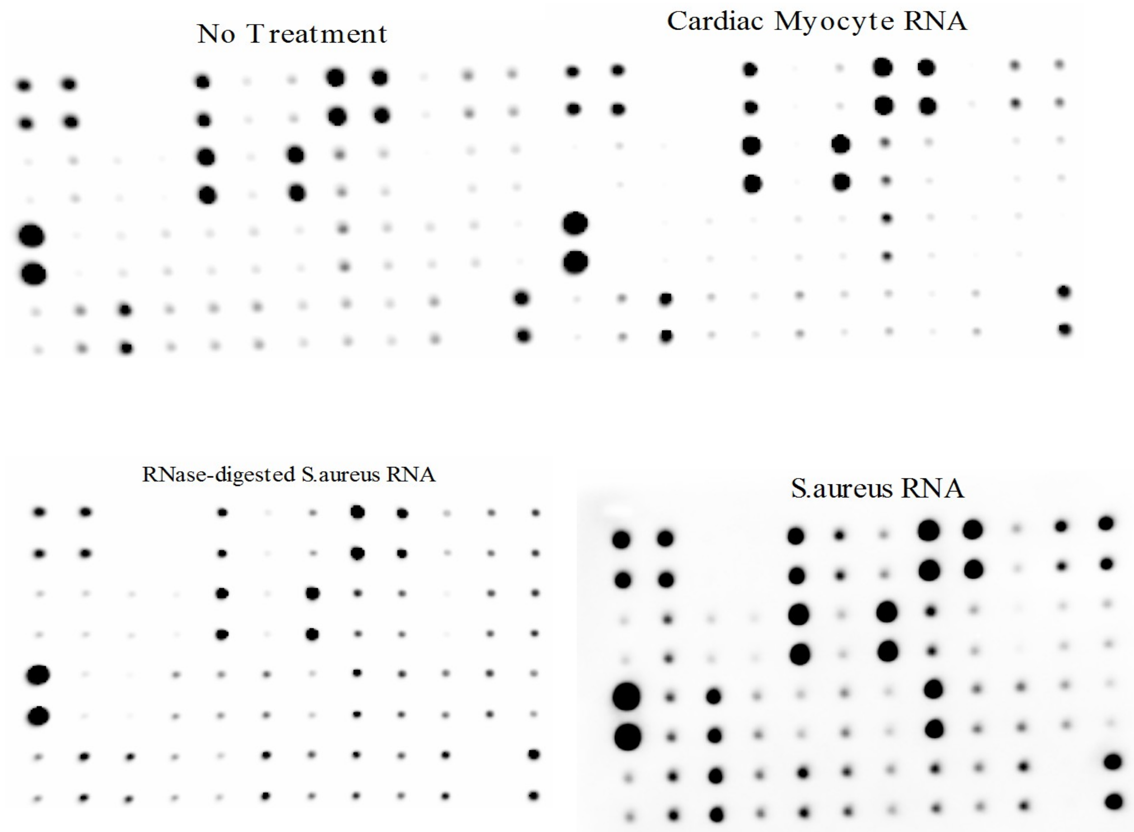
Note: NFκB (left panel) and IRF (right panel) reporter experiments were performed by transfecting NFκB- and IRF-luciferase constructs with cardiac cells and stimulated with various RNA for 24 hrs as indicated. Cells treated with TNF-α at 20 ng/ml or 500 units/ml IFN-γ for 24 hrs were used as positive controls for reporter studies.

Figure.2 Bacterial RNA activates transcription factors NFκB, IRF1, and STAT1



Note: EMSAs were performed on protein extracts prepared from cardiac myocytes treated with or without bacterial RNA. Transcriptional activities were determined by EMSA with P^{32} ATP-labeled PRDII regulatory elements specific for NFκB (left panel), hexamer regulatory elements for IRF (middle panel), or GAS regulatory elements for STATs (right panel). The transcription factors were identified by incubating extracts of cardiac cells treated with poly I:C with p65 antibody for NFκB,IRF1 antibody for IRF1 supershift, and STAT1 antibody for STAT1. NT: nontreated cells, ERNA: total RNA isolated from *E. coli*, and dERNA is total RNA isolated from *E. coli* and digested with RNase. Poly I:C was used as positive control for induction of these transcription factors.

Figure.3 Modulation of cardiac myocytes cytokines by RNA



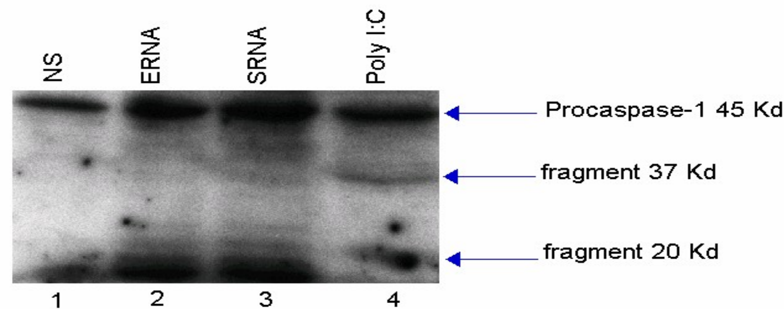
Note: The human cytokine antibody arrays 3 membranes were probed with 1 ml serum free conditioned media derived from cells stimulated with various RNA as indicated or left untreated (top left membrane). The signals were detected from the membranes by chemiluminescence exposures. These data are representative of separate repeated experiments

Table.1 Bacterial RNA stimulates the production of IL-1β

Hours	NT	ERNA	SRNA	Poly I: C
12	7.2 pg/ml	35.6* pg/ml	35.4* pg/ml	15.6* pg/ml
24	14.0 pg/ml	49.2* pg/ml	37.0* pg/ml	39.6* pg/ml

Note: Human adult cardiac myocytes were incubated in medium alone (NT), or stimulated with 100 µg/ml of *E.coli* RNA (ERNA), *S.aureus* RNA (SRNA) or dsRNA (poly I:C) for 12 h and 24 h. Culture media were collected and subjected to ELISA assay. Data represented in this table are the average of three separate experiments repeated with the same conditions. Star represents the samples were statistically significant ($p < 0.001$) when compared to their respective control (NT).

Figure.4 Bacterial RNA induces cleavage of caspase-1



Note: Protein extracts from cardiac myocytes challenged with 100 µg/ml RNA for 24 h were resolved on PAGE/SDS and subjected to Western blotting using antibodies to caspase-1. NT: nontreated cells, ERNA: cells challenged with *E. coli* RNA, SRNA: *S. aureus* RNA, and poly I:C used as a positive control. The caspase-1 and its cleaved fragments are indicated.

References

Akira, S., Hemmi, H. 2003. *Immunol. Lett.* 85: 85–95.

Alexopoulou, L., Kontoyiannis, D. 2005. *Cell Mol. Life Sci.,* 62: 1349–1358.

Bourquin, C., Schmidt, L., Hornung, V., Wurzenberger, C., Anz, D., Sandholzer, N., Schreiber, S., Voelkl, A., Hartmann, G., Endres, S. 2007. *Blood,* 109: 2953–2960.

Boyd, J.H., Mathur, S., Wang, Y., Bateman, R.M., Walley, K.R. 2006. *Cardiovasc. Res.,* 72: 384–393.

Deane, J.A., Bolland, S. 2006. *J. Immunol.,* 177: 6573–6578.

Doyle, S.L., O'Neill, L.A. 2006. *Biochem. Pharmacol.,* 72; 1102–1113.

Frantz, S., Ertl, G., Bauersachs, J. 2007. *Nat. Clin. Pract. Cardiovasc. Med.,* 4: 444–454.

Frantz, S., Kobzik, L., Kim, Y.D., Fukazawa, R., Medzhitov, R., Lee, R. T., Kelly, R.A. 1999. *J. Clin. Invest.,* 104: 271–280.

Ghobrial, I.M., Witzig, T.E., Adjei, A.A. 2005. *CA Cancer. J. Clin.,* 55: 178–194.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., Bauer, S. 2004. *Science,* 303: 1526–1529.

Honda, K., Takaoka, A., Taniguchi, T. 2006. *Immunity,* 25: 349–60.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K.K., Schlee, M., Endres, S., Hartmann, G. 2006. *Science,* 314: 994–997.

Kanneganti, T.D., Ozoren, N., Body-Malapel, M., Amer, A., Park, J.H., Franchi, L., Whitfield, J., Barchet, W., Colonna, M., Vandenabeele, P., Bertin, J., Coyle, A., Grant, E.P., Akira, S., Nunez, G. 2006. *Nature,* 440: 233–236.

Kariko, K., Buckstein, M., Ni, H., Weissman, D. 2005. *Immunity,* 23: 165–175

Kawai, T., Akira, S. 2007. *Semin. Immunol.,* 19: 24–32.

Kumar, A., Kumar, A., Michael, P., Brabant, D., Parissenti, A.M., Ramana, C.V., Xu, X., Parrillo, J.E. 2005. *J. Biol. Chem.,* 280: 42619–42626.

Lan, T., Kandimalla, E.R., Yu, D., Bhagat, L., Li, Y., Wang, D., Zhu, F., Tang, J. X., Putta, M.R., Cong, Y., Trombino, A.F., Sullivan, T., Agrawal, S. 2007. *Proc. Natl. Acad. Sci. U. S. A.,* 104: 13750–13755.

Lau, C.M., Broughton, C., Tabor, A.S., Akira, S., Flavell, R.A., Mamula, M. J., Christensen, S.R., Shlomchik, M.J., Viglianti, G.A., Rifkin, I.R., Marshak-Rothstein, A. 2005. *J. Exp. Med.,* 202: 1171–1177.

Lee, S.D., Chu, C.H., Huang, E.J., Lu, M.C., Liu, J.Y., Liu, C.J., Hsu, H.H., Lin, J.A., Kuo, W.W., Huang, C.Y. 2006. *Am. J. Physiol. Endocrinol. Metab.,* 291: E306–14.

Maass, D.L., White, J., Horton, J.W. 2002. *Shock,* 18: 360–366.

Mancuso, G., Gambuzza, M., Midiri, A., Biondo, C., Papasergi, S., Akira, S., Teti, G., Beninati, C. 2009. *Nat. Immunol.,* 10: 587–594.

O'Mahony, D.S., Pham, U., Iyer, R., Hawn, T.R., Liles, W.C. 2008. *Int. J. Med. Sci.,* 5: 1–8.

Paladugu, B., Kumar, A., Parrillo, J.E., Der, S., Osman, J., Mensing, J., Falvo, L., Xu, X., Kumar, A. 2004. *Shock,* 21: 364–369.

Petrilli, V., Dostert, C., Muruve, D.A., Tschopp, J. 2007. *Curr. Opin. Immunol.,* 19: 615–622.

Pichlmair, A., Schulz, O., Tan, C.P., Naslund, T.I., Liljestrom, P., Weber, F., Reis e Sousa, C. 2006. *Science,* 314: 997–1001.

Quinton, L.J., Nelson, S., Boe, D.M., Zhang, P., Zhong, Q., Kolls, J. K., Bagby, G. J. 2002. *J. Infect. Dis.,* 185: 1476–1482.

Rahimi, N. 2006. *Front. Biosci.,* 11: 818–829.

Schaefer, T.M., Desouza, K., Fahey, J. V., Beagley, K. W., Wira, C.R. 2004 *Immunology* 112: 428–436.

Scheel, B., Braedel, S., Probst, J., Carralot, J. P., Wagner, H., Schild, H., Jung, G., Rammensee, H. G., Pascolo, S. 2004. *Eur. J. Immunol.* 34: 537–547.

Shibuya, M. 2006. *Angiogenesis,* 9: 225–30 discussion 231.

Shuai, K., Stark, G.R., Kerr, I.M., Darnell, J. E., Jr 1993. *Science,* 261: 1744–1746.

Singh, U.P., Singh, S., Boyaka, P.N., McGhee, J.R., Lillard, J.W., Jr. 2004. *J. Leukoc. Biol.,* 76: 1240–1247.

Su, E.J., Cioffi, C.L., Stefansson, S., Mittereder, N., Garay, M., Hreniuk, D., Liau, G. 2003 *Am.J.Physiol. Heart Circ. Physiol.,* 284: H1429–40.

Sugiyama, T., Gursel, M., Takeshita, F., Coban, C., Conover, J., Kaisho, T., Akira, S., Klinman, D.M., Ishii, K.J. 2005. *J. Immunol.,* 174: 2273–2279.

Takahasi, K., Yoneyama, M., Nishihori, T., Hirai, R., Kumeta, H., Narita, R., Gale, M., Jr, Inagaki, F., Fujita, T. 2008. *Mol. Cell,* 29: 428–440.

Taki, S. 2002. *Cytokine Growth Factor Rev.,* 13: 379–391.

Turner, N.A., Mughal, R.S., Warburton, P., O'Regan, D.J., Ball, S.G., Porter, K.E. 2007. *Cardiovasc. Res.,* 76: 81–90.

Van Damme, J., Wuyts, A., Froyen, G., Van Coillie, E., Struyf, S., Billiau, A., Proost, P., Wang, J. M., Opdenakker, G. 1997. *J. Leukoc. Biol.,* 62: 563–569.

Yano, K., Liaw, P.C., Mullington, J.M., Shih, S.C., Okada, H., Bodyak, N., Kang, P.M., Toltl, L., Belikoff, B., Buras, J., Simms, B.T., Mizgerd, J.P., Carmeliet, P., Karumanchi, S.A., Aird, W.C. 2006. *J. Exp. Med.,* 203: 1447–1458.