

# Differential microRNA (miRNA) expression could explain microbial tolerance in a novel chronic peritonitis model

Ziad Kanaan<sup>1</sup>, Rebecca Barnett<sup>2</sup>, Sarah Gardner<sup>2</sup>, Bobby Keskey<sup>2</sup>, Devin Druen<sup>2</sup>, Adrian Billeter<sup>2</sup> and William G Cheadle<sup>2,3</sup>

Innate Immunity  
19(2) 203–212  
© The Author(s) 2012  
Reprints and permissions:  
sagepub.co.uk/journalsPermissions.nav  
DOI: 10.1177/1753425912460557  
ini.sagepub.com



## Abstract

We observed persistent peritoneal bacteria despite a transient early innate immune response to intraperitoneal (IP) *Klebsiella pneumoniae*. Pretreatment with LPS prior to peritonitis induced a tolerant pattern of pro-inflammatory cytokine protein production over 72 h, but not at the mRNA level. MicroRNAs (miRNAs) regulate inflammatory cytokines and may explain this paradox. After pretreatment with IP LPS or saline, C57BL/6 mice were given  $10^3$  CFU of *K. pneumoniae* IP. Total RNA was isolated from peritoneal exudate cells (4 h, 24 h and 48 h following infection). mRNA and miRNA expression levels were detected and bioinformatics pathway analysis was performed, followed by measuring TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and High-mobility Group Box 1 (HMGB1) protein levels. Of 88 miRNAs studied, 30 were significantly dysregulated at all time points in the LPS-pretreated group, including *MiR-155*, *-146a*, *-142-3p*, *-299*, and *-200c* *-132* and *-21*. TNF- $\alpha$ , regulated by *miR-155* and *miR-146a*, was decreased in the LPS-pretreated group at all time points ( $P < 0.05$ ), as were HMGB1, a key alarmin regulated by *miR-146*, *-142-3p*, *-299* and *-200c* ( $P < 0.05$ ), and IL-1 $\beta$  and IL-6, both regulated by *miR-132* and *miR-21* respectively ( $P < 0.05$ ). Specific dysregulation of *miR-155*, *-146a*, *-142-3p*, *-299*, and *-200c* *-132* and *-21* with their corresponding effects on the TLR and NF- $\kappa$ B signaling pathways during inflammation, suggests a plausible mechanism for tolerance in this novel chronic model with persistent peritoneal infection.

## Keywords

Cytokine, microRNA, peritonitis, tolerance, Toll-like receptors

Date received: 19 March 2012; revised: 16 July 2012; accepted: 7 August 2012

## Introduction

Secondary bacterial peritonitis is an invasion of the normally sterile peritoneal cavity with microorganisms after mechanical breach of the gastrointestinal tract, which is typically caused by diverticulitis, appendicitis, bowel infarction or trauma.<sup>1</sup> Despite current treatment strategies involving antibiotic therapy combined with intensive care support and surgical intervention, patients with peritonitis are at high risk of developing sepsis, which can, ultimately, lead to organ damage and eventual mortality.<sup>2</sup> In response to severe infections, including peritonitis, there may be significant alterations in the immune response of circulating leukocytes, described as immune paralysis.<sup>3</sup>

Interestingly, endotoxin tolerance mirrors this clinical condition of immune paralysis. First described in

the late 1940s by Beeson, the subsequent administration of typhoid vaccine produced less of a fever response than the initial administration.<sup>4</sup> In another study, Greisman et al. injected bacterial endotoxin intravenously into healthy patients and reported diminished

<sup>1</sup>Department of Internal Medicine, Wayne State University, School of Medicine, Detroit, MI, USA

<sup>2</sup>Hiram C. Polk Jr MD Department of Surgery, University of Louisville, School of Medicine, Louisville, KY, USA

<sup>3</sup>Robley Rex Veterans Affairs Medical Center, Louisville, KY, USA

### Corresponding author:

William G Cheadle, Hiram C. Polk Jr MD Department of Surgery, University of Louisville School of Medicine, 550 South Jackson Street, Louisville, KY 40292, USA.

Email: wg.cheadle@louisville.edu

fever responses upon continuous or repeated endotoxin administration; this response was coined endotoxin tolerance.<sup>5</sup> Endotoxin tolerance is defined as a pre-exposure to bacterial endotoxin or LPS that leads to a diminished pro-inflammatory response upon subsequent stimulation.<sup>6</sup> Endotoxin tolerance can be divided into two stages. The first stage is an Ab-independent phase that occurs hours following LPS insult. This stage may last for several days, and is considered to be of great importance in septic patients who have impaired host defense against virulent and persistent pathogens. The second stage is Ab-dependent and appears weeks after the initial LPS administration.<sup>1</sup>

While the exact mechanism of LPS tolerance is unclear, it is clear that the innate immune system recognizes pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), including the TLRs, which are members of the IL-1 receptor superfamily.<sup>1</sup> LPS is recognized by TLR4, whereas other bacterial components are sensed by different TLRs. The interaction between TLRs and their ligands induces tolerance through transduction pathways controlling the expression of immune-response genes.<sup>7</sup> Multiple studies have shown that endotoxin tolerance is associated with impaired activation of the NF- $\kappa$ B and MAPK pathways.<sup>8</sup> This also includes a decrease in cytokines released by activated macrophages, including TNF- $\alpha$ , IL-10, IL-6, IL-1 and IL-12.<sup>1</sup> In addition, several reports have shown that diminished secretion of these cytokines is beneficial and decreases mortality in cases of LPS-induced endotoxic shock, acute infection and ischemia/reperfusion; however, this effect may be detrimental for bacterial clearance.<sup>9</sup>

We have previously reported microbial tolerance at the cytokine protein and mRNA levels in a novel *Klebsiella* model of persistent secondary murine peritonitis. In this model, mice were injected intraperitoneally (IP) with  $10^3$  CFU *Klebsiella pneumoniae*, a Gram-negative, anaerobic organism in secondary peritonitis.<sup>1</sup> This induced death in all animals within 3 d of infection. However, if animals received antibiotic treatment with gentamicin (5 mg/kg BID) subcutaneously (SC), survival was significantly enhanced and some mice survived the 14-d observation period.<sup>10</sup> The use of antibiotics protected animals from severe life-limiting bacterial overgrowth, but bacteria were never fully eliminated from the peritoneal cavity. Within 48 h of injection, peritoneal TNF- $\alpha$  and IL-6 concentrations diminished significantly despite peritoneal bacteria concentrations exceeding  $10^6$  CFU.<sup>10</sup> The relatively steady concentrations of peritoneal bacteria in our model induced microbial tolerance at the macrophage level, which likely accounted for diminished peritoneal cytokine concentrations and late mortality *in vivo*.<sup>1</sup> However, we showed previously that microbial tolerance could not

be attributed to dysregulation at the mRNA level in this model.<sup>11</sup> This is in line with previous studies, which found that mRNA expression correlates with sepsis and an increased pro-inflammatory response, but is less indicative of tolerance. As the regulation of endotoxin tolerance involves receptors, negative modulators and signaling molecules, it is speculated that gene reprogramming and immune modulation, rather than a global down-regulation of gene expression, could explain such phenomena. Post-transcriptional modifications of the expressed cytokines could possibly fit into a mechanistic framework that could explain the molecular basis of microbial tolerance.

Recent studies have shown that microRNAs (miRNAs), an evolutionarily conserved class of endogenous 22-nucleotide, non-protein-coding RNAs, contribute to the regulation of inflammation by repressing gene expression at the post-transcriptional level.<sup>12</sup> miRNAs are inhibitory and repress the translation of a complementary mRNA leading, ultimately, to a decrease in protein levels associated with the bound mRNA.<sup>13</sup> Wightman et al. demonstrated the importance of the complementary sites for regulation of lin-14 mRNA by miR-lin-4, also showing that this regulation reduces the amount of LIN-14 protein levels substantially without noticeable change in the levels of lin-14 mRNA.<sup>14</sup>

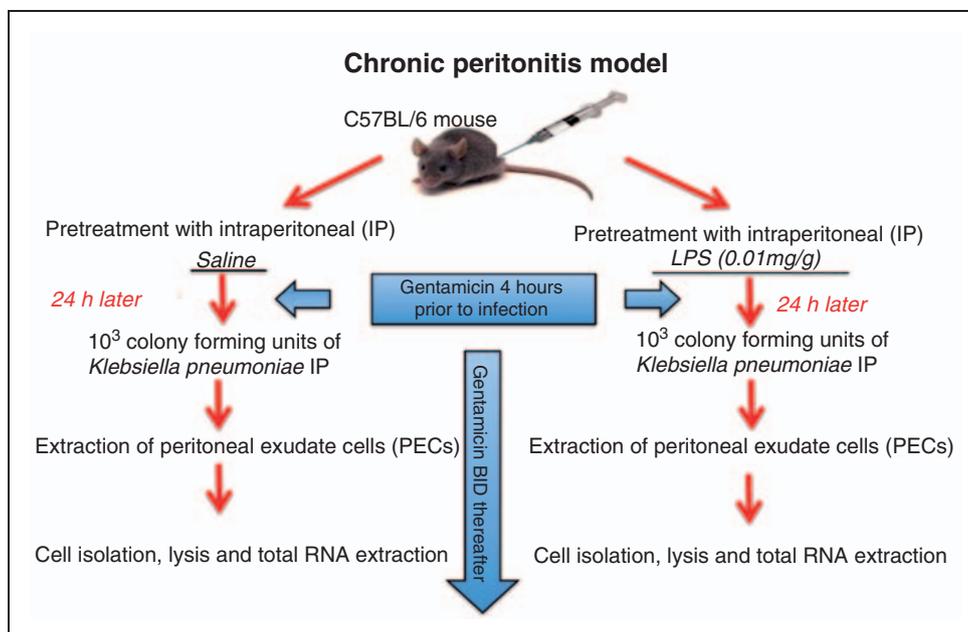
Based on our previous findings at the cytokine-protein and mRNA level, we sought to explain the mechanism behind microbial tolerance at the miRNA level using our previously-described model. We hypothesized that microbial tolerance was the result of dysregulation in the production of specific miRNAs related to inflammation leading to inhibition of cytokine production and, thereby, could account for the diminished immune response, bacterial persistence and, thus, late mortality.

Therefore, the aim of this study was to (i) investigate the differential expression of miRNAs by peritoneal exudate cells (PECs) during microbial tolerance and (ii) correlate the miRNA expression with the target mRNAs and cytokine levels indicative of microbial tolerance.

## Materials and methods

### Animals

Male 6–8-wk-old C57BL/6 animals were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care and were provided with food and water *ad libitum*. Studies were carried out according to the National Institutes of Health guidelines and under the supervision of a veterinarian. Mice were allowed to acclimate for at least 1 wk prior to experimentation.



**Figure 1.** Experimental protocol for chronic peritonitis model.

### Experimental protocol

Experimental mice were pretreated with saline (saline group) or LPS (LPS group) 24 h prior to inoculation with *K. pneumoniae*. Then, these mice received gentamicin BID until harvest at 4 h, 24 h and 48 h. Control mice were harvested without receiving pretreatment, *K. pneumoniae* inoculation or antibiotics (Figure 1).

### Pretreatment

Animals were pretreated 24 h prior to inoculation with IP 0.2 ml saline or 10 mg/kg LPS (Ultra Pure *Escherichia coli* LPS, 0111:B4 strain; InvivoGen, San Diego, CA, USA), in a final volume of 0.2 ml saline. Uninfected, non-pretreated animals served as controls. Twenty-four hours later, pretreated animals were inoculated with *K. pneumoniae*.

### *K. pneumoniae* inoculation

Mid-log growth phase of *K. pneumoniae* strain 43816 serotype 2 (American Type Culture Collection, Rockville, MD, USA) were obtained by growth in nutrient broth (Difco, Detroit, MI, USA) for 18 h at 37°C, followed by inoculation into Lennox L broth (Difco) for an additional 4 h growth. Bacterial concentration of the broth was quantified by measuring the absorbance at 655 nm and comparing it with a previously determined standard curve of concentrations. Bacteria were then diluted with endotoxin-free sterile saline to provide bacterial numbers of 10<sup>3</sup> CFU in a final volume of 0.2 ml. Animals were injected with a

final volume of 0.2 ml IP. Injected bacterial numbers were confirmed by colony counts of nutrient agar plate dilutions after a 24-h incubation period at 37°C.

### Antibiotic treatment

Animals received 5 mg/kg/d (BID) subcutaneous gentamicin (Sigma-Aldrich, St Louis, MO, USA) in 0.2 ml of sterile saline. The first dose was given 4 h prior to IP bacterial administration and antibiotic treatment was continued BID throughout the observation period.

### PEC collection

PECs were harvested at 4 h, 24 h and 48 h post-IP inoculation. PECs were recovered by peritoneal lavage with 4 ml of ice-cold heparinized RPMI 1640 medium (GIBCO/BRL, Bethesda, MD, USA). PECs were quantified manually using a hemocytometer with trypan blue exclusion to determine total PEC number and cell viability. PEC lavage media was diluted and plated for peritoneal bacteria determination. PEC samples were then centrifuged to pellet cells for RNA extraction and to collect supernatants. Supernatants were aliquoted and frozen at -80°C for cytokine analysis. The cell pellets were re-suspended in recommended volumes of RNeasy Lysis Buffer and frozen until RNA extraction. Resulting peritoneal CFU concentrations were multiplied by four to account for earlier dilution by the peritoneal lavage fluid and were expressed as log CFU/mouse. Limit of detection was peritoneal cavity 1.4 log CFU/mouse.

### Cell lysis, RNA extraction, mRNA and miRNA plate runs

Stabilized PEC cells were thawed and pelleted, and white cells were re-suspended in an appropriate lysis buffer. Total RNA extraction was performed using the Qiagen Kit Animal Spin protocol (Qiagen, Valencia, CA, USA). Total RNA was quantified using the Nanodrop N-1000 by Agilent Biosystems (Santa Clara, CA, USA). RNA quality control was maintained through measurements of RNA purity by UV spectrophotometry, and assessment of RNA quality and integrity. The first-strand mRNA-cDNA and miRNA-cDNA syntheses were performed on an Eppendorf Mastercycler with the appropriate cDNA Synthesis kit (SuperArray Bioscience, Frederick, MD, USA) and 100 ng of total RNA. cDNA was processed according to the manufacturer's protocol for mRNA and miRNA plates. The cDNA template was combined with RT<sup>2</sup> Real-Time™ SYBR Green/Rox Master Mix and RNase-free water (SuperArray Bioscience). A final reaction volume of 25 µl was added to each well of the mRNA TLR signaling Pathway PCR Array (SuperArray Bioscience) and the immunopathology RT<sup>2</sup> miRNA PCR Array (MAM-104 A) (SABiosciences). Finally, pathway-focused mRNA and miRNA plates were amplified on a 7300 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol.<sup>15</sup> The mRNA plates tested for 88 different mRNAs that have been shown previously to be involved in the TLR signaling pathways (supplementary Table S1).<sup>15</sup> The miRNA plates tested for 88 different miRNAs involved in the immune pathology process in mice (supplementary Table S2).<sup>15</sup> Housekeeping genes, as well as reverse transcription and positive controls, were included in both the mRNA and miRNA formats.

### RT-PCR data analysis

Alterations in mRNA and miRNA transcript levels at 4 h, 24 h and 48 h in both the saline and LPS groups were analyzed initially using SABiosciences webportal software. Fold changes were calculated and *P*-values were calculated using student's *t*-test for significant mRNAs. A *P*-value < 0.05 was considered significant.

### Cytokine assays

TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and HMGB1 levels were measured in peritoneal lavage fluid supernatants using ELISA. TNF- $\alpha$ , IL-1 $\beta$  and IL-6 ELISA murine Ready-Set-Go kits were purchased from e-Biosciences (San Diego, CA, USA) and a HMGB1 ELISA kit was purchased from IBL International Corp (Toronto, ON, Canada); these were then performed according to the manufacturer's instructions. Where necessary, samples were

diluted in assay diluent to achieve cytokine concentrations within the range of the standard curve. Cytokine concentrations were multiplied by four to account for dilution by lavage media and expressed in pg/mouse. Sensitivities for TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and HMGB1 were 8 pg/ml, 8 pg/ml, 4 pg/ml and 0.2 ng/ml respectively.

### Pathway analysis

In order to understand the genetic significance of mRNA and miRNA expression, fold changes and dysregulation, pathway analysis was done using Ingenuity Pathway Analysis (IPA 9.0; Redwood City, CA, USA). IPA is a software application used to identify biological mechanisms, dysregulation in signaling or metabolic pathways, and functions most relevant to their experimental data sets or genes of interest.<sup>16</sup> Official permission to use figures and analyses performed by this software was obtained. All RNA results were normalized against uninfected controls.

## Results

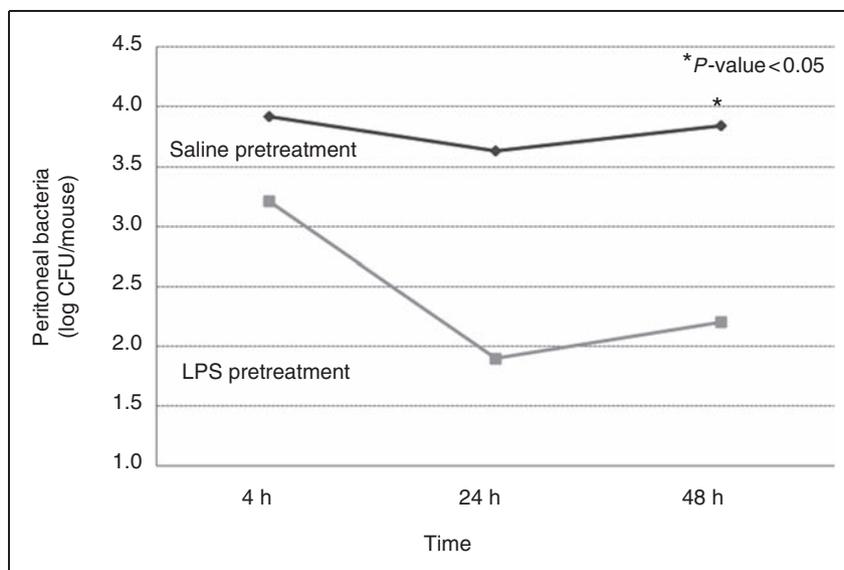
### Microbial tolerance in peritoneal macrophages

We first established that our selected pretreatments actually resulted in the induction of microbial tolerance. The animals appeared healthy at all time points—moving spontaneously around the cage, self-grooming, and had healthy coats and eyes. We have previously noted that they do experience a small percentage of mass loss post-*Klebsiella* inoculation which is regained after 48 h.

Following LPS pretreatment, peritoneal bacterial levels decreased significantly between 4 h and 24 h ( $P < 0.05$ ). At 48 h, bacterial numbers stabilized at  $10^2$ – $10^3$  CFU (Figure 2). Despite  $10^2$ – $10^3$  CFU bacterial levels at 48 h, PEC numbers decreased significantly in the LPS pretreated group compared with the saline pretreated one. Additionally, compared with the saline group we noticed in the LPS-pretreated group that TNF- $\alpha$  levels decreased at all time points ( $P < 0.05$ ), IL-1 $\beta$  levels decreased at 48 h ( $P < 0.05$ ) and IL-6 levels decreased at 24 h and 48 h ( $P < 0.05$ ). In summary, LPS-pretreatment led to a tolerant state with persistence of bacteria, reduced pro-inflammatory cytokines and reduced leukocyte counts.

### Significantly dysregulated miRNAs in the LPS-pretreated group compared with the saline-pretreated group

We first identified significantly dysregulated miRNAs in the LPS and saline pretreated groups compared with uninfected controls. Then, we identified the significantly dysregulated miRNAs in the LPS pretreated group compared with the saline pretreated group.



**Figure 2.** Peritoneal bacterial levels in the saline and LPS pretreated groups at 4 h, 24 h and 48 h.

At 4 h, 18 miRNAs were significantly upregulated, namely: *miR-155*, *miR-383*, *miR-19*, *miR-493*, *miR-147*, *miR-154*, *miR-370*, *miR-140*, *miR-142-3p*, *miR-205*, *miR-130*, *miR-132*, *miR-574*, *let-7f*, *miR-17*, *miR-146a*, *miR-200c* and *miR-150*. At 24 h, 24 miRNAs were significantly upregulated, namely: *miR-155*, *miR-383*, *miR-19*, *miR-493*, *miR-147*, *miR-154*, *miR-370*, *miR-140*, *miR-142*, *miR-205*, *miR-130*, *miR-132*, *miR-574*, *let-7f*, *miR-17*, *miR-146a*, *miR-150*, *miR-145*, *miR-363*, *miR-335*, *miR-17*, *miR-8*, *miR-9*, and *miR-299*. At 48 h, 12 miRNAs were significantly upregulated, including *miR-155*, *miR-146a*, *miR-574*, *miR-8*, *miR-9*, *miR-299*, *miR-21*, *miR-125*, *miR-183*, *miR-325*, *miR-27* and *miR-154*.

#### Pathway analysis of miRNA and mRNA data

According to IPA analysis, and based on our miRNA expression data, *miR-155*, *miR-146a*, *miR-142-3p*, *miR-299*, *miR-200c*, *miR-132* and *miR-21* were predicted to have the most significant direct effects on the TLR and NF- $\kappa$ B signaling pathways, and were thus evaluated along with their protein targets. After applying IPA analysis to our mRNA data, 6 main canonical pathways were constantly dysregulated and, in the same order of significance, in both the saline and LPS groups at 4 h, 24 h and 48 h (supplementary Figure S1). These were TLR and NF- $\kappa$ B signaling, hepatic cholestasis, IL-6- and LPS-mediated MAPK signaling pathways, and PRR of the bacterial pathway.

#### *miR-155* and *miR-146a* dysregulation, and TNF- $\alpha$ mRNA and protein levels

There was no difference in TNF- $\alpha$  mRNA expression between the saline and LPS pretreated groups at all

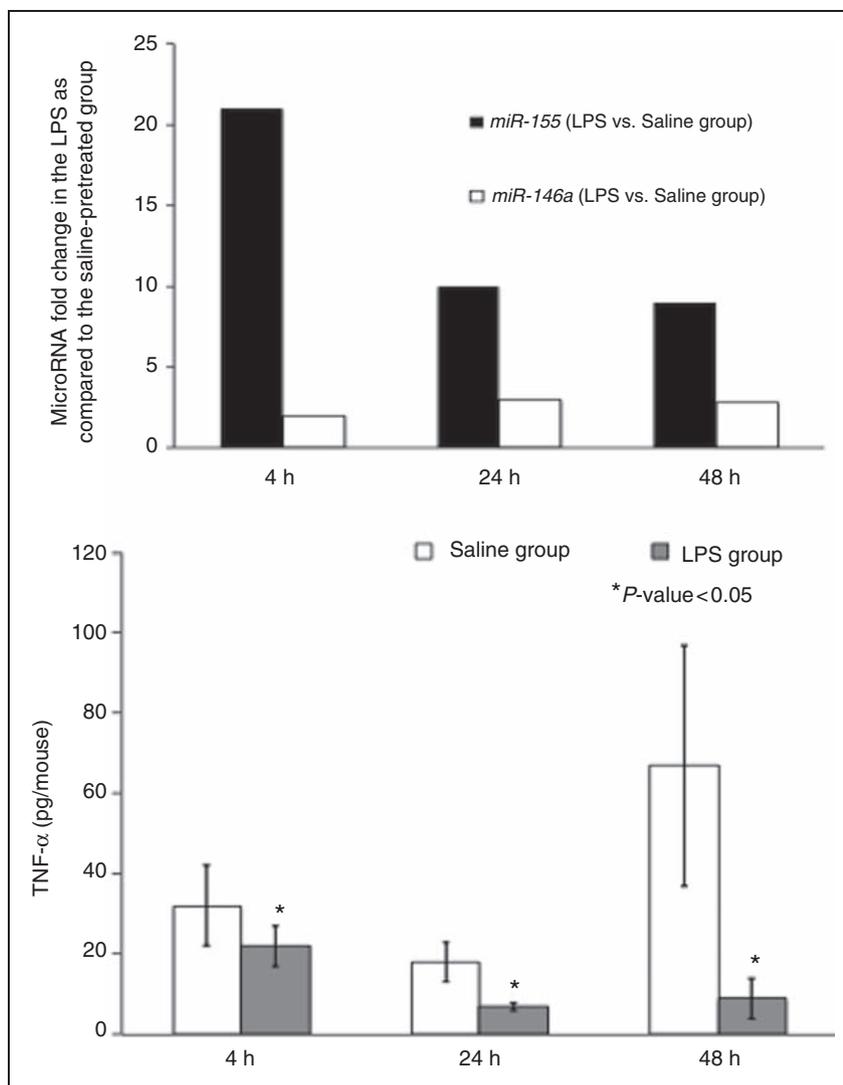
time points despite differences in TNF- $\alpha$  protein thus indicating a potential role for miRNAs as post-transcriptional fine tuners. TNF- $\alpha$  protein levels were significantly decreased at all time points in the LPS pretreated group (Figure 3). *MiR-155*, shown to target SHIP1/SOCS1, both inactivators of the TLR pathway, was significantly down-regulated at 24 h and 48 h in the LPS pretreated group, possibly explaining the decreased TNF- $\alpha$  levels at the same time points (Figure 3). *MiR-146a*, which targets key molecules of the TLR/IL-1 receptor pathway, such as IRAK 1/2 and TNF receptor-associated factor (TRAF) 6, and thereby inhibiting the inflammatory response, was significantly up-regulated at 24 h and 48 h. The inhibitory effect of *miR-146a* on TNF- $\alpha$  production would explain the decreased TNF- $\alpha$  levels (Figure 3).

#### *miR-146*, *-142-3p*, *-299* and *-200c*, and HMGB1 mRNA and protein levels

We found *miR-146*, *-142-3p*, *-299* and *-200c* to be significantly up-regulated in the LPS pretreated group. The protein levels of HMGB1, a key target of the aforementioned miRNAs and involved in inflammation after monocyte stimulation,<sup>17</sup> was decreased at 24 h and 48 h in the LPS-pretreated group in our study (Figure 4).

#### *MiR-132*, *miR-21*, and IL-1 $\beta$ and IL-6 mRNA and protein levels

*miR-132* and *miR-21*, predicted to target IL-1 $\beta$  and IL-6, respectively, were both up-regulated significantly in the LPS group. Compared with the saline pretreated group, IL-1 $\beta$  levels decreased at 48 h ( $P < 0.05$ ), whilst IL-6 levels decreased at 24 h and 48 h ( $P < 0.05$ ) (Figure 5). *miR-142-3p* also targets IL-6 and the



**Figure 3.** *miR-155* and *miR-146a* were up-regulated in the LPS group at all time points ( $P < 0.05$ ).  $\text{TNF-}\alpha$ , a common target of the aforementioned microRNAs (miRNAs), was, correspondingly, significantly downregulated at the protein level in the LPS group at 4 h, 24 h and 48 h as compared with the saline pretreated group.

up-regulation of *miR-142-3p* corresponds to the down-regulation of IL-6 in LPS pretreatment.

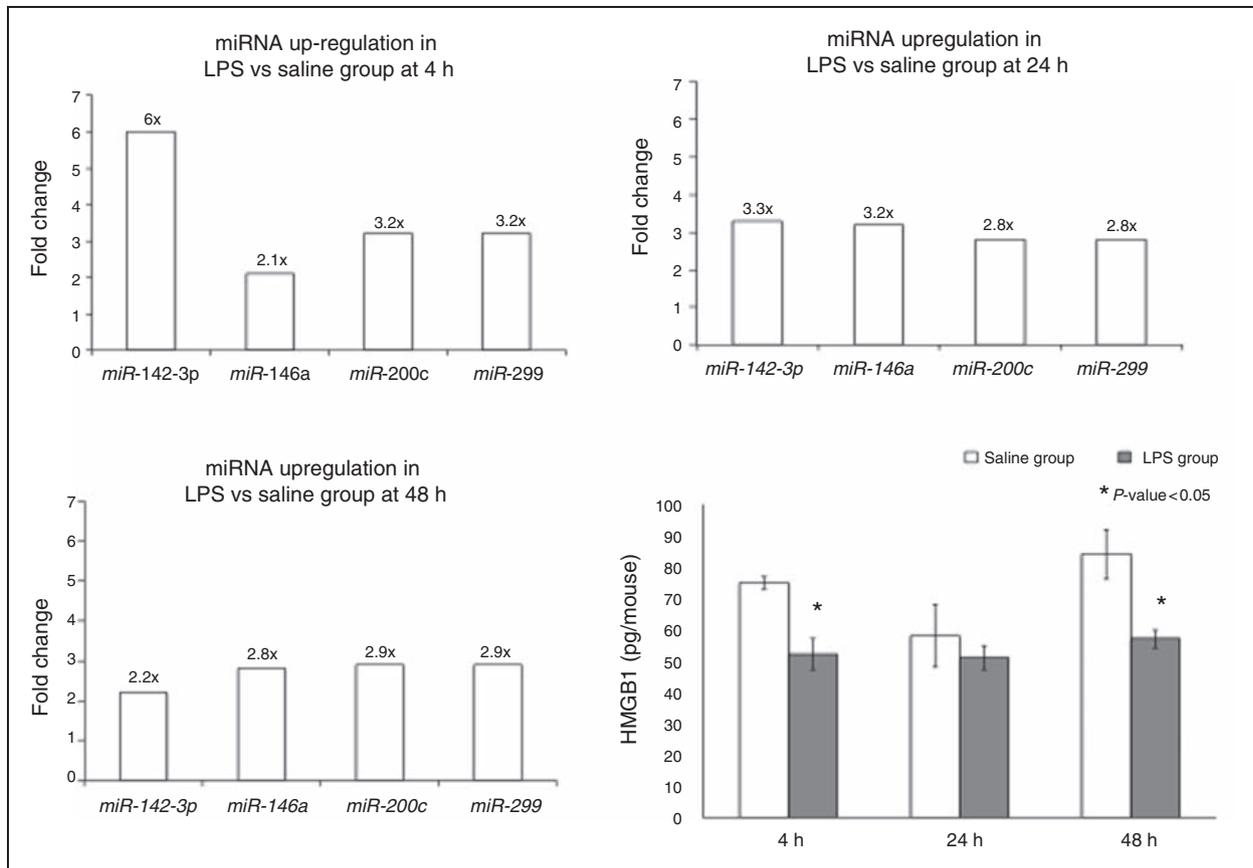
## Discussion

### *miRNAs and microbial tolerance*

miRNAs are non-protein coding RNAs that downregulate gene expression via their effect on mRNA. They play a crucial role in regulating the expression of proteins through specific interaction with complementary mRNA<sup>18</sup> and are now widely accepted as major regulatory gatekeepers of coding genes within the human genome. A single miRNA can bind to, and regulate, many different mRNA targets and, conversely, several different miRNAs can bind to and cooperatively control a single mRNA. The regulation of gene products by miRNA is therefore complex, with diverse roles

affecting many aspects of transcription and translation that can play a role in overall cellular homeostasis.

Whilst the role of miRNAs in cell fate decisions linked to proliferation, differentiation and apoptosis has been studied extensively and recognized, the significance of these non-coding small RNAs in immune system development and response has only recently been investigated in detail.<sup>19</sup> Several miRNAs have been attributed significant roles in the development of the immune system and during the formation of an immune response. For example, the activation of the innate immune response has been associated with changes in the expression of selected miRNAs (namely *miR-155*,<sup>20</sup> *miR-146a*,<sup>21</sup> *miR-142-3p*,<sup>22</sup> *miR-132*<sup>23</sup> and *miR-21*<sup>24</sup>). However, the role of most of these miRNAs in the development of an adequate immune response remains insufficiently elucidated. Herein, we report the profiles of miRNAs induced by



**Figure 4.** *miR-142-3p*, *-146a*, *-299* and *-200c* were up-regulated in the LPS group at all time points ( $P < 0.05$ ). HMGB1, a common target for all these microRNAs (miRNAs), was, correspondingly, significantly down-regulated at the protein level in the LPS group at 24 h and 48 h in the LPS pretreated group compared with the saline pretreated group.

inflammatory stimuli in PECs after LPS stimulation and during the development of microbial tolerance. We identify a significant synchronous correlation between *miR-155*, *miR-146a*, *miR-125b*, *miR-142-3p*, *miR-299*, *miR-200c*, *miR-132* and *miR-21* levels, and the development of a tolerant immune response through inhibition of specific targets in key pro-inflammatory pathways. These inhibit the inflammatory response and may lead, eventually, to the development of microbial tolerance.

The 48-h time point corresponds to the peak bacterial concentration observed in the animals, which would be similar to the clinical condition for generalized secondary bacterial peritonitis. Bacterial concentrations remain similar after this time point in all animals treated with antibiotics over at least 2 wk, as noted in previously-published experiments.<sup>25</sup>

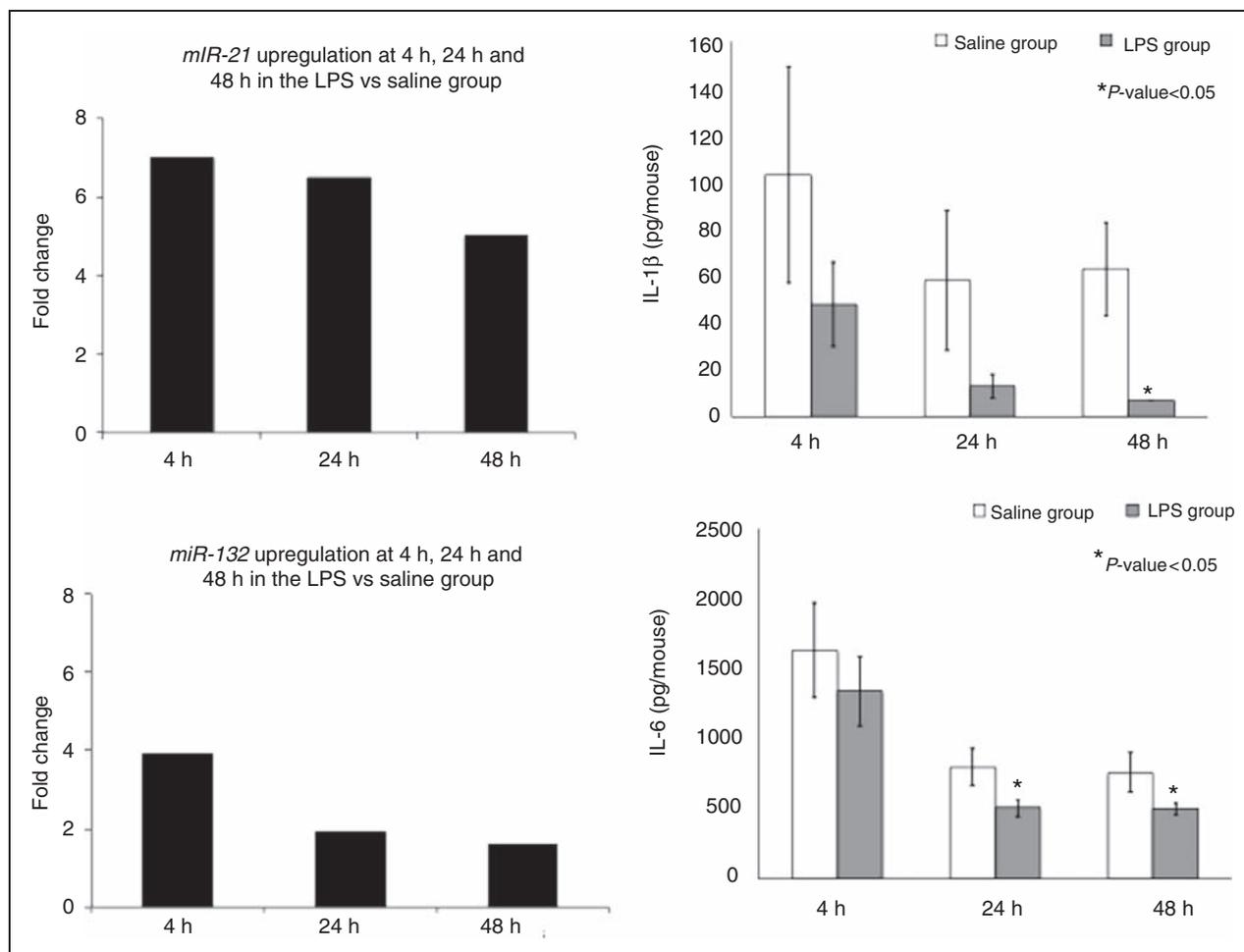
#### *miR-155* and *miR-146a* dysregulation correlates with $TNF-\alpha$ protein levels

O'Connell et al. initially demonstrated that LPS treatment in human monocytic cells augments the expression of two microRNAs, *miR-146a* and *miR-155*.<sup>20</sup> *miR-155* is up-regulated early in endotoxin shock

after TLR4 activation and is essential for a proper immune response.<sup>20</sup> Once up-regulated, *miR-155* targets negative regulator proteins of the inflammatory response, such as SOCS1 and SHIP1, which act on the signaling transducers of the TLR family and enhance the pro-inflammatory signals.<sup>26</sup> Later, *miR-155* attenuates TLR4 signaling by targeting IKK $\epsilon$  or MyD88, an essential signaling protein of all TLRs except TLR3,<sup>27</sup> directly; however, it was shown to increase  $TNF-\alpha$  production.<sup>28</sup> Costinean et al. further elucidated that *miR-155* knock-in mice are highly susceptible to LPS shock owing to high levels of  $TNF-\alpha$ .<sup>29</sup> The anti-inflammatory cytokine IL-10 downregulates *miR-155*, supporting the primary pro-inflammatory role of *miR-155*.<sup>30</sup>

In the LPS pretreated group in our study, the down-regulation of *miR-155* correlated with a significant  $TNF-\alpha$  downregulation at 24 h and 48 h, whilst both were up-regulated in the saline pretreated group at the same time points. The downregulation of *miR-155* and  $TNF-\alpha$  could contribute to tolerance in the LPS-pretreated group, despite persistent high peritoneal bacterial levels.

*miR-146a* is a well-described miRNA in the delayed anti-inflammatory phase in an inflammatory



**Figure 5.** *miR-132* was up-regulated in the LPS group ( $P < 0.05$ ). IL-1 $\beta$ , a target of the aforementioned miRNA, was, correspondingly, significantly downregulated at the protein level in the LPS group at 48 h compared with the saline pretreated group. *miR-21* was also up-regulated in the LPS group ( $P < 0.05$ ). IL-6, a target of the aforementioned microRNA (miRNA), was, correspondingly, significantly down-regulated at the protein level in the LPS group at 24 h and 48 h compared with the saline pretreated group.

response.<sup>31</sup> *miR-146* is first induced upon exposure of cells to LPS and a variety of other inflammatory stimuli, including IL-1 $\beta$  and TNF- $\alpha$ , and ligands for TLR-2 and TLR-5.<sup>32</sup> In addition, *miR-146a* production could be stimulated by IL-10 and seems to act as a major anti-inflammatory miRNA. The increased expression of *miR-146* decreases the expression of TNF- $\alpha$  production by inhibiting three of the central signaling proteins of the TLRs, IL-1 receptor-associated kinase 1 and 2, and TRAF 6. In addition, *miR-146a* is predicted to bind proteins of the MAPK signaling cascade and tune down this inflammatory pathway synergistically. Therefore, owing to its anti-inflammatory features, *miR-146a* plays a crucial role in endotoxin tolerance, where *miR-146a* is up-regulated after the first LPS exposure and continues to protect the host from the second LPS hit, and, subsequently, reduces TNF- $\alpha$  levels.<sup>23,24</sup> In concordance with the reported literature, *miR-146a* was up-regulated in the LPS pretreated group at 24 h and 48 h, and associated with a

downregulation of TNF- $\alpha$ , suggesting a possible role for *miR-146a* in downregulating TNF- $\alpha$  levels.

#### *miR-142-3p*, *-146a*, *-299* and *-200c* dysregulation correlates with HMGB1 protein levels

Alarmins have been described to serve as danger signals that promote activation of the innate immune system pathways and have been shown to participate in the activation of several immune receptors, including the TLRs.<sup>33</sup> HMGB1, an alarmin itself, is usually actively released by immune cells, such as monocytes, macrophages and dendritic cells.<sup>34</sup> The stimuli for secretion of HMGB1 from immune cells are diverse and include PAMPs, cytokines and certain states of cellular stress.<sup>35</sup> Normally, LPS-activated macrophages and monocytes secrete HMGB1 as a cytokine mediator of inflammation.<sup>17</sup> In our study, LPS pretreatment might have led to early secretion of HMGB1, which may have led to a perpetual activation of the miRNAs and,

eventually, a late downregulation of HMGB1 at the mRNA and protein level.

According to IPA, targets.org and microRNA.org, *miR-142-3*, *miR-146a*, *miR-299* and *miR-200c* are predicted to target HMGB1 (www.ingenuity.com, accessed 2 December 2011). In our study, these 4 miRNAs were significantly up-regulated at 24 h and 48 h in the LPS pretreated group compared with the saline pretreated group. In correlation, HMGB1, a common target for these 4 miRNAs, was significantly downregulated at the mRNA and protein levels at 24 h and 48 h.

### *miR-132 and miR-21 dysregulation correlates with IL-1 $\beta$ and IL-6 protein levels*

Taganov et al. identified, by means of expression profiling, several miRNA genes, including *miR-132*, among others, to be induced in the human monocytic cell line THP-1 by LPS, as well as other microbial components and pro-inflammatory mediators.<sup>23,36</sup> Early expression of *miR-132* with LPS-pretreatment in our study correlated with downregulation of IL-1 $\beta$  levels, a presumed target of *miR-132*, contributing to the development of microbial tolerance.

The tumorigenic function of *miR-21*, along with many of its predicted targets has been reported extensively and confirmed in the literature.<sup>36</sup> Recently, *miR-21* has been proven to affect the innate immune response by inducing IL-10 production via a complex negative feedback loop.<sup>36</sup> Mice deficient in PDCD4, a confirmed *miR-21* target,<sup>37,38</sup> exhibit lower LPS-induced mortality rates, lower IL-6 production and increased IL-10 protein levels. *miR-21* up-regulation, in our study, correlated with decreased IL-6 production and lower IL-1 $\beta$  levels at 24 h and 48 h compared with the saline pretreated group, contributing to the development of microbial tolerance. Furthermore, IL-6 is targeted by *miR-142-3p*. Sun et al. demonstrated convincingly that *miR-142-3p* is a major, if not the only, regulator of IL-6 production.<sup>22</sup> The up-regulation of *miR-142-3p* in our study correlated with the consecutive downregulation of IL-6 and might thereby contribute to the observed tolerance pattern.

This study has several limitations. We chose to use a monobacterial model for peritonitis instead of a polymicrobial model, which is clinically more relevant. However, the purpose of this model was to study the development of tolerance in an *in vivo* model and we believed that this question might be better addressed with a monobacterial, Gram-negative bacteria model rather than a polymicrobial one. Furthermore, we did not measure the level of downregulation of the target proteins of *miR-155* and *miR-146a*, which would strongly support the proposed action of these two miRNAs, nor did we transfect or knockdown any of the significant miRNAs to prove their action.

However, for most of the miRNAs mentioned in this study, their proposed effect has been demonstrated and proven by others. For example, *miR-155* and *miR-146a* have been shown to control TNF- $\alpha$  production in the macrophage response by several different groups.<sup>23,32,39</sup> The effect of *miR-142-3p* on IL-6 has been demonstrated thoroughly by Sun et al.<sup>40</sup>

Despite these limitations, we have shown that microbial tolerance can be induced *in vivo* and that miRNAs might play a pivotal role in the development of tolerance by fine-tuning the macrophage response, by targeting central inflammatory signaling pathways and by modulating cytokine production.

## Conclusion

The molecular mechanisms underlying microbial tolerance remain very complex. As discussed previously, microbial tolerance is a biologic phenomenon of gene reprogramming and immune modulation rather than a global downregulation of gene expression and function.<sup>8</sup> As the regulation of endotoxin tolerance involves multi-leveled components of the inflammatory pathway, we believe that post-transcriptional modification through miRNA regulation of pro-inflammatory cytokines could contribute to the molecular basis of microbial tolerance. According to our study, the specific dysregulation of miRNAs, including *miR-155*, *-146a*, *-142-3p*, *-299*, and *-200c* *-132* and *-21*, along with their effect on major inflammatory pathways like the TLR and NF- $\kappa$ B signaling pathway, but also on cytokine production directly, suggests a plausible mechanism for tolerance in this novel model with persistent peritoneal infection. Further *in vivo* studies using miRNA mimics and inhibitors could possibly be useful to further elucidate the effects of these miRNAs on survival in microbial tolerance.

## Funding

This research received funding from the Department of Veterans Affairs Merit Review Program.

## Acknowledgements

The authors would like to thank James C. Peyton for technical assistance with bacterial cultures and animal harvests.

## Conflict of interest

The authors do not have any potential conflicts of interest to declare.

## References

1. Lenz AM, Turina M, Alard P, Gardner SA and Cheadle WG. Microbial tolerance in secondary peritonitis is dose dependent. *Cell Immunol* 2009; 258: 98–106.
2. Evans HL, Raymond DP, Pelletier SJ, Crabtree TD, Pruett TL and Sawyer RG. Diagnosis of intra-abdominal infection in the critically ill patient. *Curr Opin Crit Care* 2001; 7: 117–121.

3. Volk HD, Reinke P, Krausch D, Zuckermann H, Asadullah K, Muller JM, et al. Monocyte deactivation—rationale for a new therapeutic strategy in sepsis. *Intensive Care Med* 1996; 22(Suppl. 4): S474–S481.
4. Beeson PB. Tolerance to bacterial pyrogens. I. Factors influencing its development. *J Exp Med* 1947; 86: 29.
5. Greisman SE, Young EJ and Woodward WE. Mechanisms of endotoxin tolerance. IV. Specificity of the pyrogenic refractory state during continuous intravenous infusions of endotoxin. *J Exp Med* 1966; 124: 983–1000.
6. Cavaillon JM and Adib-Conquy M. Bench-to bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. *Crit Care* 2006; 10: 233.
7. Akira S. TLR signaling. *Curr Top Microbiol Immunol* 2006; 311: 1–16.
8. Biswas SK and Lopez-Collazo E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends Immunol* 2009; 30: 475–487.
9. Lehner MD and Hartung T. Endotoxin tolerance-mechanisms and beneficial effects in bacterial infection. *Rev Physiol Biochem Pharmacol* 2002; 144: 95–141.
10. Lenz AM, Franklin GA, Fairweather M, McClintock ML, Jala VR, Peyton JC, et al. Endogenous IL-10 leads to impaired bacterial clearance and reduced survival in a murine model of chronic peritonitis. *Cytokine* 2007; 40: 207–215.
11. Kanaan Z, Gardner S, Carruba C, Mattingly J, Druen D and Cheadle WG. Macrophage genetic reprogramming during chronic peritonitis is augmented by LPS pretreatment. *J Surg Res* 2011; 175: 289–297.
12. Bazzoni F, Rossato M, Fabbri M, Gaudiosi D, Miolo M, Mori L, et al. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proc Natl Acad Sci U S A* 2009; 106: 5282–5287.
13. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; 116: 281–297.
14. Wightman B, Ha I and Ruvkun G. Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell* 1993; 75: 855–862.
15. SABiosciences Website (Help Manual). <http://www.sabiosciences.com> (accessed 10 January 2010).
16. Ingenuity Pathway Analysis Website (Help Manual). <https://analysis.ingenuity.com/pa/info/help> (accessed 14 December 2011).
17. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999; 285: 248–251.
18. Yang L, Belaguli N and Berger DH. MicroRNA and colorectal cancer. *World J Surg* 2009; 33: 638–646.
19. Pedersen I and David M. MicroRNAs in the immune response. *Cytokine* 2008; 43: 391–394.
20. O'Connell RM, Taganov KD, Boldin MP, Cheng G and Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci U S A* 2007; 104: 1604–1609.
21. Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM and Lindsay MA. Rapid changes in microRNA-146a expression negatively regulate the IL-1 $\beta$ -induced inflammatory response in human lung alveolar epithelial cells. *J Immunol* 2008; 180: 5689–5698.
22. Sun Y, Varambally S, Maher CA, Cao Q, Chockley P, Toubai T, et al. Targeting of microRNA-142-3p in dendritic cells regulates endotoxin-induced mortality. *Blood* 2011; 117: 6172–6183.
23. Taganov KD, Boldin MP, Chang KJ and Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A* 2006; 103: 12481–12486.
24. Roy S and Sen CK. MiRNA in innate immune responses: novel players in wound inflammation. *Physiol Genomics* 2011; 43: 557–565.
25. Woods DR, Heffley S, Peyton JC and Cheadle WG. Antibiotic modulation in a clinically relevant model of chronic intraabdominal infection. *Am Surg* 2006; 72: 655–660.
26. Wang P, Hou J, Lin L, Wang C, Liu X, Li D, et al. Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. *J Immunol* 2010; 185: 6226–6233.
27. McCoy CE, Sheedy FJ, Qualls JE, Doyle SL, Quinn SR, Murray PJ, et al. IL-10 inhibits miR-155 induction by toll-like receptors. *J Biol Chem* 2010; 285: 20492–20498.
28. Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, et al. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol* 2007; 179: 5082–5089.
29. Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, Heerema N, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci U S A* 2006; 103: 7024–7029.
30. Tang D, Shi Y, Jang L, Wang K, Xiao W and Xiao X. Heat shock response inhibits release of high mobility group box 1 protein induced by endotoxin in murine macrophages. *Shock* 2005; 23: 434–440.
31. Billeter AT, Druen D, Kanaan ZM, Polk Jr HC and MicroRNAs. new helpers for surgeons? *Surgery* 2012; 151: 1–5.
32. Baltimore D, Boldin MP, O'Connell RM, Rao DS and Taganov KD. MicroRNAs: new regulators of immune cell development and function. *Nat Immunol* 2008; 9: 839–845.
33. Apetoh L, Ghiringhelli F, Tesniere A, Criollo A, Ortiz C, Lidereau R, et al. The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. *Immunol Rev* 2007; 220: 47–59.
34. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999; 285: 248–251.
35. Klune JR, Dhupar R, Cardinal J, Billiar TR and Tsung A. HMGB1: endogenous danger signaling. *Mol Med* 2008; 14: 476–484.
36. Liu MF, Zeal JSL, and McQueen CA. Physiological and pathological functions of mammalian microRNAs. In: *Comprehensive toxicology*, 2nd ed. London: Elsevier Science, 2010, pp. 427–446.
37. Sheedy FJ, Palsson-McDermott E, Hennessy EJ, Martin C, O'Leary JJ, Ruan Q, et al. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. *Nat Immunol* 2010; 11: 141–147.
38. Lu Z, Liu M, Stribinskis V, Klinge CM, Ramos KS, Colburn NH, et al. MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene. *Oncogene* 2008; 27: 4373–4379.
39. Nahid MA, Pauley KM, Satoh M and Chan EK. miR-146a is critical for endotoxin-induced tolerance: Implication in innate immunity. *J Biol Chem* 2009; 284: 34590–34599.
40. Sun Y, Varambally S, Maher CA, Cao Q, Chockley P, Toubai T, et al. Targeting of microRNA-142-3p in dendritic cells regulates endotoxin-induced mortality. *Blood* 2011; 117: 6172–6183.