Formation of GW/P bodies as marker for microRNA-mediated regulation of innate immune signaling in THP-1 cells

Kaleb M Pauley1, Minoru Satoh2, Brad A Pauley1, Paul R Dominguez-Gutierrez1, Shannon M Wallet1,3, L Shannon Holliday4, Seunghee Cha1,5, Westley H Reeves2 and Edward KL Chan1

GW bodies (GB or P bodies) are cytoplasmic foci thought to result from microRNA (miRNA) regulation of messenger RNA (mRNA) targets and subsequent mRNA degradation. The purpose of this study is to examine the effects of lipopolysaccharide (LPS) stimulation of human monocytes on GWB formation, miRNA induction, miRNA target regulation and downstream cytokine and chemokine expression. In response to LPS stimulation, the number of GWB consistently increased by twofold at 8 h after stimulation and this increase was abolished when the miRNA-effector proteins Rck/p54 or argonaute 2 were depleted. As the level of miR-146a increased from 19-fold up to 100-fold during LPS stimulation, the transfection of a miR-146a mimic into THP-1 cells was examined to determine whether miR-146a alone can induce similar changes in GWB. The results showed transfected miR-146a could produce a comparable increase in the number of GWB and this was accompanied by a reduction in major cytokines/chemokines induced by LPS. These data show that the increase in size and number of GWB may serve as a biomarker for miRNA-mediated gene regulation, and miR-146a has a significant role in the regulation of LPS-induced cytokine production in THP-1 cells.

Keywords: cytokines; GW body; lipopolysaccharide; microRNA; monocytes; processing body
Further connections between miRNA and the mammalian immune system are becoming more evident with recent publications. One report showed that certain miRNA are upregulated in human monocytes in response to lipopolysaccharide (LPS). LPS is a component of the outer membrane of gram-negative bacteria that activates the production of pro-inflammatory cytokines in monocytes and macrophages. LPS stimulates toll-like receptor 4 and activates several intracellular signaling cascades that lead to the production of TNF-α, IL-6, monocyte chemotactant protein-1 (MCP-1) and other ro-inflammatory cytokines and chemokines. Taganov et al. reported that LPS stimulation of THP-1 monocytes resulted in increased expression of miR-146a, miR-155 and miR-132, and that TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase (IRAK-1), proteins in the toll-like receptor 4 signaling pathway, were targets of miR-146a. In 2008, Perry et al. reported an increase in miR-146a expression on IL-1β stimulation of lung alveolar epithelial cells, and showed that the increased miR-146a negatively regulated the production of IL-8 and RANTES. In addition, miR-155 has also been linked to several other immune functions including the germinal center response, normal lymphocyte functions and regulation of immunoglobulin class-switched plasma cells.

Taken together, these reports show an important role for miRNA in normal immune functions. The aim of this study is to analyze the effect of innate immune signaling and related miRNA on GWB because the latter has been linked with miRNA biogenesis in our earlier report using HeLa cells and reproduced by other investigators in insect cells.

RESULTS

GWB increase in size and number in response to LPS stimulation

Lipopolysaccharide stimulation of human monocytes/macrophages is a well-characterized model of innate immune signaling. To determine whether there were any changes in GWB associated with innate immune signaling, THP-1 human monocytes were treated with 1 μg ml⁻¹ LPS for 0, 4, 6, 8, 12 and 24 h and GWB were monitored by indirect immunofluorescence (Figure 1). Within 4 h of LPS stimulation, the number and size of GWB significantly increased compared with untreated cells. The number of GWB was highest at 8 h of LPS stimulation. This observation was quantified using CellProfiler image analysis software to count the number of GWB per cell. As shown in Figure 1b, the average number of GWB increased almost twofold with 8 h of LPS treatment compared with untreated cells cultured in parallel (18.6 in LPS-treated versus 10.5 in untreated cells). After 8 h, the number of GWB gradually declined as observed at 12 and 24 h. At the 6, 8, 12 and 24 h time points, there was a significant increase in the number of GWB in LPS-treated cells compared with untreated cells. The number of GWB per cell in untreated cells or LPS-treated cells for 0 to 24 h after LPS incubation (n>150 cells analyzed for each treatment). Asterisks (**) indicate P<0.0001 or (*) P<0.008 compared with paired untreated control as determined by Mann-Whitney.

Next, the expression levels of some of the protein components of GWB were examined by western blot to determine whether the GWB forming in response to LPS were assembled from de novo proteins or pre-existing proteins in the cytoplasm. THP-1 cell extracts were prepared from cells treated with 1 μg ml⁻¹ LPS for 4, 8 or 24 h together with paired untreated cells harvested at the corresponding time point (Figure 2). The western blot data showed that there were only slight changes in the expression levels of Ago2 and Rck/p54 in LPS-treated cells compared with untreated cells (lanes (+) versus (-)) for each time point analyzed. No significant difference was shown in western blot data from three independent replicate experiments that were prepared from cells treated with 1 μg ml⁻¹ LPS for 0, 4, 6, 8, 12 and 24 h and GWB were monitored by indirect immunofluorescence (Figure 1). Within 4 h of LPS stimulation, the number and size of GWB significantly increased compared with untreated cells. The number of GWB was highest at 8 h of LPS stimulation. This observation was quantified using CellProfiler image analysis software to count the number of GWB per cell. As shown in Figure 1b, the average number of GWB increased almost twofold with 8 h of LPS treatment compared with untreated cells cultured in parallel (18.6 in LPS-treated versus 10.5 in untreated cells). After 8 h, the number of GWB gradually declined as observed at 12 and 24 h. At the 6, 8, 12 and 24 h time points, there was a significant increase in the number of GWB in LPS-treated cells compared with untreated cells. The number of GWB per cell in untreated cells or LPS-treated cells for 0 to 24 h after LPS incubation (n>150 cells analyzed for each treatment). Asterisks (**) indicate P<0.0001 or (*) P<0.008 compared with paired untreated control as determined by Mann-Whitney.

Figure 1 Lipopolysaccharide (LPS) induces the assembly of GW bodies (GWB). (a) THP-1 cells treated with 1 μg ml⁻¹ LPS showed a time-dependent increase in the number and size of GWB. After LPS treatment, cells were fixed and co-stained with human anti-GWB serum (green) and rabbit anti-Dcp1a (red). Nuclei were counterstained by 4,6-diamidino-2-phenylindole (DAPI, blue). Images were acquired at 400× original magnification. Bar, 10 μm. (b) Bar graph showing representative data from the CellProfiler image analysis software used to quantitate the average number of foci per cell in untreated cells or LPS-treated cells for 0 to 24 h after LPS incubation (n>150 cells analyzed for each treatment). Asterisks (**) indicate P<0.0001 or (*) P<0.008 compared with paired untreated control as determined by Mann-Whitney.

Figure 2 Expression of GW bodies (GWB) protein components argonaute 2 (Ago2) and Rck/p54 in THP-1 cells are unaffected by lipopolysaccharide (LPS) treatment. THP-1 cell extracts from LPS treated (+) or untreated (−) cells for each time point were compared using western blot detection of Rck/p54, Dcp1a, Ago2 and actin, which is shown as a loading control. Representative data from three independent experiments are shown.
quantified by densitometric analysis. In contrast, the levels of Dcp1 in LPS-treated cells were ~two- to fivefold increased compared with untreated cells at all three time points examined. As the highest number of GWB was observed at 8 h (Figure 1), the minimal changes in Rck/p54 and Ago2 protein levels at this time point (Figure 2) cannot reflect the almost twofold increase in number of GWB; therefore, these data are more consistent with the conclusion that LPS-induced GWB are formed from pre-existing Ago2 and Rck/p54 proteins in the cytoplasm rather than entirely from de novo proteins. However, by 24 h, there were obvious increases in levels of these proteins compared with the 4 and 8 h time points (Figure 2).

miR-146a, miR-132 and miR-155 are upregulated in THP-1 cells in response to LPS
On the basis of the work by Taganov et al.,23 on the upregulation of certain miRNA in response to LPS, and our earlier work showing the importance of miRNA for GWB formation,8 we speculated that the LPS-induced increase in the size and number of GWB could be the result of increased miRNA expression (and possibly miRNA activity) in the cells. It is interesting to note that Taganov et al.,23 showed that global miRNA levels were not affected by LPS stimulation but rather that three specific miRNAs (miR-146a, miR-155 and miR-132) were upregulated. To test this hypothesis, we first set out to confirm that in our experimental conditions, these miRNA were upregulated in response to LPS. The expression of miR-146a, miR-132, miR-155 and let-7a in THP-1 cells were examined after LPS stimulation by quantitative real-time PCR (qRT-PCR). As expected, the expression of let-7a was unaffected by LPS treatment and was used to normalize the expression of miR-146a, miR-155 and miR-132. Fold change in miRNA expression was calculated by comparing 4-, 8-, 12- and 24-h LPS treatments to untreated samples cultured in parallel. As shown in Figure 3a, miR-146a was increased an average of 19-fold after 4 h and 28-fold after 8-h LPS treatment. In addition, miR-132 expression was increased 3.8-fold after 24 h of LPS treatment (Figure 3b) and miR-155 expression was increased up to 2.6-fold after 8 h of LPS treatment (Figure 3c). The increased levels of these miRNA are generally consistent with those reported by Taganov et al.,23 although the level of increase for miR-146a at 8 h was ~threefold higher (28-fold versus 8-fold) than previous reported. These large increases may be due in part to the relatively low basal level of miR-146a in unstimulated THP-1 cells and this basal level may vary because of slight differences in culture conditions. A second potential reason for the experimental difference is that another miRNA (let7) was used as internal control for comparison whereas miRNA were compared with 5S RNA in the study by Taganov et al.23 In sum, our data show that LPS stimulation results in the upregulation of several miRNA, with miR-146a level increased by 28-fold at 8 h, coinciding with the highest increase in number of GWB at this same time point.

miR-146a, miR-132 and miR-155 are upregulated in THP-1 cells in response to LPS
On the basis of the work by Taganov et al.,23 on the upregulation of certain miRNA in response to LPS, and our earlier work showing the importance of miRNA for GWB formation,8 we speculated that the LPS-induced increase in the size and number of GWB could be the result of increased miRNA expression (and possibly miRNA activity) in the cells. It is interesting to note that Taganov et al.,23 showed that global miRNA levels were not affected by LPS stimulation but rather that three specific miRNAs (miR-146a, miR-155 and miR-132) were upregulated. To test this hypothesis, we first set out to confirm that in our experimental conditions, these miRNA were upregulated in response to LPS. The expression of miR-146a, miR-132, miR-155 and let-7a in THP-1 cells were examined after LPS stimulation by quantitative real-time PCR (qRT-PCR). As expected, the expression of let-7a was unaffected by LPS treatment and was used to normalize the expression of miR-146a, miR-155 and miR-132. Fold change in miRNA expression was calculated by comparing 4-, 8-, 12- and 24-h LPS treatments to untreated samples cultured in parallel. As shown in Figure 3a, miR-146a was increased an average of 19-fold after 4 h and 28-fold after 8-h LPS treatment. In addition, miR-132 expression was increased 3.8-fold after 24 h of LPS treatment (Figure 3b) and miR-155 expression was increased up to 2.6-fold after 8 h of LPS treatment (Figure 3c). The increased levels of these miRNA are generally consistent with those reported by Taganov et al.,23 although the level of increase for miR-146a at 8 h was ~threefold higher (28-fold versus 8-fold) than previous reported. These large increases may be due in part to the relatively low basal level of miR-146a in unstimulated THP-1 cells and this basal level may vary because of slight differences in culture conditions. A second potential reason for the experimental difference is that another miRNA (let7) was used as internal control for comparison whereas miRNA were compared with 5S RNA in the study by Taganov et al.23 In sum, our data show that LPS stimulation results in the upregulation of several miRNA, with miR-146a level increased by 28-fold at 8 h, coinciding with the highest increase in number of GWB at this same time point.

Rck/p54 and Ago2 are required for LPS-induced GWB assembly
To further show the relationship between LPS-induced miRNA and GWB formation, we examined the effect of depleting two independent protein components of GWB that are known to be important for miRNA-mediated function, Rck/p54 and Ago2.28 siRNA were used to knockdown Rck/p54 or Ago2 in THP-1 cells. Forty-eight hours after transfection, transfected cells were stimulated with LPS and the number of GWB were monitored by immunofluorescence. Figure 4a shows the knockdown efficiency for Rck/p54 (~90% reduction) and Ago2 (~70% reduction) under these experimental conditions. Mock-transfected cells stimulated with LPS for 4, 8 and 24 h showed significant increases in the average number of GWB per cell as expected (P<0.0001, not indicated). As shown in Figure 4b, the average number of GWB per cell in the Rck/p54-depleted cells was reduced to 4.7 compared with an average of 9.7 foci per cell in mock-transfected cells before LPS treatment. These data are consistent with previous reports showing that Rck/p54 depletion results in the loss of GWB.13,28 In addition, LPS stimulation of Rck/p54-depleted cells did not induce an increase in the number of GWB at all time points examined. At 8 h, when the maximum increase in GWB after LPS treatment of the mock-transfected cells was observed, Rck/p54-depleted cells had an average of 3.1 foci per cell compared with 23.5 foci per cell in mock-transfected cells (Figure 4b), representing an 87% reduction. Consistent with our previous report,13 in Ago2-depleted cells, the average number of GWB per cell was similar to that of mock-transfected cells before LPS stimulation (Figure 4b). Like the Rck/p54-depleted cells, there was no increase in the number of GWB in Ago2-depleted cells except after 4 h of LPS stimulation. We speculate
that this initial increase in GWB could be because of incomplete knockdown of Ago2 as shown in Figure 4a. Overall, this data showed that Rck/p54 and Ago2 were both required for the LPS-induced increase in GWB assembly, supporting our hypothesis that miRNA-mediated function is responsible for this phenomenon, therefore in the absence of these critical proteins for miRNA function, GWB are no longer induced by LPS stimulation.

miR-146a alone can induce an increase in the size and number of GWB

As LPS has been shown to induce the expression of only a few miRNA,23 and miR-146a was the most significantly elevated by LPS treatment in our experimental system, the next logical step was to show whether the introduction of miR-146a alone into THP-1 cells could induce an increase in the size and number of GWB similar to LPS-induced GWB assembly. Precursor miR-146a (miR-146a mimic) was transfected at a concentration of 30 nM into THP-1 cells and GWB examined by immunofluorescence 24 h later. To monitor transfection efficiency, THP-1 cells were transfected with a Cy3-labeled-pre-miR negative control that mimics an endogenous miRNA but has no endogenous target. Figure 5a shows the increase in the size and number of GWB in THP-1 cells transfected with miR-146a mimic compared with mock-transfected cells. As shown in Figure 5b, only the cells transfected with miR-146a mimic showed greater than twofold increase in the average number of GWB per cell similar to the increase observed with 8-h LPS treatment (Figure 1b). This experiment was repeated using 15, 7.5, 3.75, 1.8 and 0.9 nM concentrations of miR-146a mimic, and a similar increase in size and number of GWB can be observed with as little as 1.8 nM miR-146a mimic (data not shown). Total RNA was also isolated from the transfected cells and the mRNA levels of TRAF6 and IRAK-1, the putative targets of miR-146a,23 were analyzed by qRT-PCR. THP-1 cells transfected with miR-146a mimic showed an 85% reduction in the mRNA level of TRAF6 and a 51% reduction in the level IRAK-1 compared with mock-transfected cells (Figure 5c). These data indicate that miR-146a alone can induce a comparable increase in the size and number of GWB as in LPS-induced assembly of GWB and functionally can downregulate endogenous TRAF6 and IRAK-1 at the mRNA level as expected of cellular RNA interference activity.

To further show the role of miR-146a in LPS-induced GWB formation, THP-1 cells were transfected with miR-146a inhibitor and these cells were then transfected with LPS and monitored for the change in GWB by immunofluorescence. As expected, LPS-induced GWB formation at 4, 8 and 24 h was abolished in these miR-146a inhibitor transfected cells (Supplementary Figure 1a). In fact, after 4 and 8 h of LPS treatment, GWB formation was decreased in cells transfected with miR-146a inhibitor. The efficiency of the inhibition of miR-146a function was monitored by the expression of its known mRNA targets TRAF6 and IRAK-1 and their levels were increased threefold and fourfold, respectively compared with mock-transfected cells (Supplementary Figure 1b).

miR-146a has a role in the regulation of THP-1 cytokine and chemokine production

To examine the functional significance of miR-146a in LPS stimulation, cytokine and chemokine production in mock-transfected cells compared with cells transfected with miR-146a mimic were monitored using quantitative multiplex cytokine analysis. The miR-146a mimic and mock-transfected cells were treated with 2 μg ml⁻¹ LPS for 24 h and the culture supernatants were harvested and analyzed. Five detected cytokines/chemokines (IL-8, IP-10, IL-1β, IL-6 and MCP-1) showed decreased production in LPS-stimulated THP-1 cells transfected with miR-146a mimic compared with mock-transfected cells (Figure 6). The production of IL-8, IP-10 and MCP-1 was decreased by approximately 70%, whereas that of IL-1β and IL-6 was decreased 89 and 92%, respectively (Figure 6). In these experiments, the production of TNF-α was not affected by transfection of miR146a mimic, but this may be due to the low level of TNF-α produced 24 h after LPS stimulation (data not shown). The negative effect of transfected miR-146a mimic on the production of IL-1β, IL-6 and IP-10 was reproduced in an independent experiment when cells were stimulated with LPS for 4, 8 or 24 h (Supplementary Figure 2). These data show that the delivery of miR-146a before LPS stimulation inhibits LPS-mediated cytokine/chemokine production; the underlying mechanism may be that miR-146a regulates TRAF6 and IRAK-1 and ultimately results in the decreased production of these cytokines and chemokines.

To further analyze this mechanism, a similar experiment was performed using TRAF6 or IRAK-1-deficient THP-1 cells. Cells transfected with siRNA for TRAF6 or IRAK-1 were stimulated with...
IL-6/C0, MCP-1, IL-10 undetected. Mechanisms not yet understood.

TNF-α, IL-1, IL-12p40 undetected. IFN-γ production were slightly increased, MCP-1 production was not affected by TRAF6 depletion. In IRAK-1-depleted cells, IL-8 and IP-10 decreased production. MCP-1 and IP-10 production was not affected by IRAK-1 depletion whereas IL-10, IL-8, IL-6 and IL-1β showed up to 20% decreased production.

The miRNA pathway is illustrated in Figure 7. LPS binds to LPS-binding protein, which in turn is then transferred to CD14 on the cell surface (step 1).24 LPS–CD14 then interacts with toll-like receptor 4 and its accessory protein MD-2. Toll-like receptor 4 stimulation by LPS, activates signal transduction through MyD88 ultimately phosphorylating IkB (step 2). Phosphorylated IkB is then released from NFκB and degraded whereas NFκB translocates to the nucleus and activates the transcription of pro-inflammatory cytokines and miRNA including miR-146a, miR-155 and miR-132 (step 3) (Figure 3).23,24 Among these, miR-146a has the highest fold increase in expression in response to LPS (Figure 3).23

MicroRNAs are transcribed as pri-miRNA, which are then processed into pre-miRNA by the Drosha/DGCR8 microprocessor complex (not shown). The pre-miRNA are exported into the cytoplasm by exportin 5 and cleaved by Dicer into mature miRNA duplexes, which are loaded into the Ago/RNA-induced silencing complex in which they bind their target mRNA (step 4).29 In the case of miR-146a, TRAF6 and IRAK-1, proteins in the MyD88 signaling pathway, are known target miRNAs (Figure 5c).23,30–35

The miRNA/RNA-induced silencing complex is enriched in GWB, although it is not clear whether miRNA/RNA-induced silencing complex is formed in the cytoplasm or whether the miRNA is loaded into RNA-induced silencing complex inside GWB.3,4 The influx of miRNA duplexes from LPS stimulation results in ~twofold increase in the number of GWB in cells within 8 h of LPS exposure (step 5) as described in the current report. This expansion of GWB in the cytoplasm may allow, directly or indirectly, for more efficient inhibition of mRNA targets resulting in the ability of the cell to rapidly regulate signaling cascades and cytokine secretion. The increase in number and size of GWB may also be due to an accumulation of target miRNAs undergoing degradation by a limited amount of decay factors.34 An alternative interpretation may be that some of the miRNAs are being degraded independently of miRNA through a GWB-mediated pathway, but this seems unlikely based on the evidence shown in this report of miRNA involvement.

The target miRNAs are translationally repressed (or degraded) resulting in a reduction of mRNA and protein expression of factors in the signal transduction pathway (step 6). TRAF6 and IRAK-1 regulation by miR-146a, results in the subsequent blockage of NFκB activation leading to the downregulation of inflammatory cytokine production (Figure 6 and Supplementary Figure 3). Disruption of the miRNA pathway would result in a lack of regulation after exposure to LPS. This lack of regulation would lead to overproduction of inflammatory cytokines and may also affect other monocyte functions but this will need further experimental validation.

**DISCUSSION**

**Protein model for miRNA-mediated GW body assembly after LPS stimulation**

Our proposed model for the LPS-induced assembly of GWB through the miRNA pathway is illustrated in Figure 7. LPS binds to LPS-binding protein, which in turn is then transferred to CD14 on the cell surface (step 1).24 LPS–CD14 then interacts with toll-like receptor 4 and its accessory protein MD-2. Toll-like receptor 4 stimulation by LPS, activates signal transduction through MyD88 ultimately phosphorylating IkB (step 2). Phosphorylated IkB is then released from NFκB and degraded whereas NFκB translocates to the nucleus and activates the transcription of pro-inflammatory cytokines and miRNA including miR-146a, miR-155 and miR-132 (step 3) (Figure 3).23,24 Among these, miR-146a has the highest fold increase in expression in response to LPS (Figure 3).23

MicroRNAs are transcribed as pri-miRNA, which are then processed into pre-miRNA by the Drosha/DGCR8 microprocessor complex (not shown). The pre-miRNA are exported into the cytoplasm by exportin 5 and cleaved by Dicer into mature miRNA duplexes, which are loaded into the Ago/RNA-induced silencing complex in which they bind their target mRNA (step 4).29 In the case of miR-146a, TRAF6 and IRAK-1, proteins in the MyD88 signaling pathway, are known target miRNAs (Figure 5c).23,30–35

The miRNA/RNA-induced silencing complex is enriched in GWB, although it is not clear whether miRNA/RNA-induced silencing complex is formed in the cytoplasm or whether the miRNA is loaded into RNA-induced silencing complex inside GWB.3,4 The influx of miRNA duplexes from LPS stimulation results in ~twofold increase in the number of GWB in cells within 8 h of LPS exposure (step 5) as described in the current report. This expansion of GWB in the cytoplasm may allow, directly or indirectly, for more efficient inhibition of mRNA targets resulting in the ability of the cell to rapidly regulate signaling cascades and cytokine secretion. The increase in number and size of GWB may also be due to an accumulation of target miRNAs undergoing degradation by a limited amount of decay factors.34 An alternative interpretation may be that some of the miRNAs are being degraded independently of miRNA through a GWB-mediated pathway, but this seems unlikely based on the evidence shown in this report of miRNA involvement.

The target miRNAs are translationally repressed (or degraded) resulting in a reduction of mRNA and protein expression of factors in the signal transduction pathway (step 6). TRAF6 and IRAK-1 regulation by miR-146a, results in the subsequent blockage of NFκB activation leading to the downregulation of inflammatory cytokine production (Figure 6 and Supplementary Figure 3). Disruption of the miRNA pathway would result in a lack of regulation after exposure to LPS. This lack of regulation would lead to overproduction of inflammatory cytokines and may also affect other monocyte functions but this will need further experimental validation.

**GWB are biomarkers for miRNA activity**

Our previous report showed that miRNA biogenesis is required for GWB assembly under normal cellular conditions.8 Specifically, when Drosha or DGCR8 was knocked down using short hairpin RNA plasmids to inhibit miRNA maturation at the pri-miRNA level, GWB were disassembled after mature miRNA were depleted. Our data showed that mature miRNA are required for normal GWB assembly, and exogenously introduced siRNA can act as a surrogate for endogenous miRNA to reassemble GWB.8 Our more recent study also showed that GWB assembly is also linked to siRNA activity.13 The transfection of siRNA into mammalian cells led to an increase in...
number and size of GWB, and this siRNA-induced GWB assembly was dependent on the presence of an endogenous target mRNA. Comparable to the data reported here for LPS-induced GWB, Rck/p54 and Ago2 proteins were found to be required for the siRNA-mediated increase in GWB. These reports show that GWB can serve as biomarkers for miRNA and siRNA activity.

In the present report, a more natural condition is used with inducible miRNA from LPS stimulation showing a mechanistic relationship between GWB and miRNA activity (Figure 7). It is likely that significant increase in miRNA-mediated activities during many cellular processes would result in increased number and size of GWB. Our data extend the current knowledge of GWB and their relationship to innate immune signaling, and are consistent with previous reports showing GWB as biomarkers for miRNA and/or siRNA activity. Currently it is not possible to exclude the possibility that formation of GWB is influenced by other factors. For example, our earlier study showed that the size and number of GWBs increases in response to changes in the cell cycle, in response to release of serum starvation, and in response to stimulation by concanavalin A. However, our current data showing that the formation of GWB is correlated with miRNA activity in the LPS stimulation of THP-1 cells and transfection plus knockdown experiments indicated that these GWB are consistent with miRNA activity—formation of GWB requires Ago2, Rck/p54 and miR-146a. Our new data suggest that a re-interpretation of these early findings may be warranted. Given that miRNA are expected to regulate 30% or more of the human genes, it is entirely feasible to consider that many miRNA activities take place during cell proliferation, cell cycle, and possibly also during concanavalin A stimulation that could result in the observed increases in the size and number of GWBs during these processes.

**miR-146a regulates LPS-induced cytokine and chemokine production**

This study showed that transfection of miR-146a mimic resulted in decreased production of 5 cytokines/chemokines (IL-6, IP-10, IL-8, MCP-1 and IL-1β, Table 1) and this is consistent with a recent report describing the negative regulatory role of miR-146a on IL-1β production in human alveolar epithelial cells. Thus, miR-146a is shown here to regulate acute inflammatory response resulted from LPS stimulation by restricting cytokine production after the initial innate response. It is interesting that directly knocking down TRAF6 or IRAK-1 also resulted in decreased cytokine production. However, there are some discrepancies between these two sets of data. IP-10 and MCP-1 show decreased production in miR-146a transfected cells, but are not affected by TRAF6 depletion (Table 1). These data suggest that miR-146a may regulate cytokine/chemokine production through other TRAF6/IRAK-1-independent mechanisms not yet understood.

In summary, our report has shown that GWB can serve as biomarkers for miRNA activity during innate immune signaling, miR-146a was shown to have an important role in regulating human monocyte functions such as cytokine and chemokine production. These findings may be clinically significant for patients suffering from autoimmune diseases such as systemic lupus erythematosus, Sjögren’s syndrome and rheumatoid arthritis that are driven by the...
overproduction of inflammatory cytokines, and further investigations are needed to evaluate the therapeutic potential of these findings. It is intriguing to note that recent studies showed reduced miR-146a levels in peripheral blood leukocytes from patients with systemic lupus erythematosus and it was shown that this miRNA contribute to elevated level of Type I IFN in this disease.\textsuperscript{30} In contrast, elevated expression of miR-146a in rheumatoid arthritis was reported in peripheral blood leukocytes\textsuperscript{32} as well as in synovial fibroblasts and synovial tissues.\textsuperscript{36,37} Conceptually, this study provides novel insights in understanding the role of miRNAs in the regulation of GW bodies in peripheral blood leukocytes as well as in synovial fibroblasts and synovial tissues.\textsuperscript{36,37} It is noteworthy to mention that recent studies showed reduced miR-146a levels in synovial tissues and the potential role of examining the number of GW bodies in the progression of RA.\textsuperscript{40,41} However, acknowledged that more studies are needed to examine the general nature of our observation and whether the same can be applied to other cell systems.

**Methods**

**Cell culture and lipopolysaccharide/cytokine treatment**

THP-1 human monocyes obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI 1640 medium with 2 mg/l-glutamine adjusted to contain 4.5 g/l glucose, 10 mM HEPES and 1.0 mM sodium pyruvate and supplemented with 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum. THP-1 cells were seeded at 5 x 10^5 cells per well in a six-well plate and treated with 1 µg/ml\textsuperscript{1} LPS from *Salmonella enterica* serotype minnesota (Sigma, St Louis, MO, USA) unless stated otherwise. After designated treatment time, cells were harvested and washed once in phosphate-buffered saline before analysis.

**Immunofluorescence**

THP-1 cells were cytospun onto glass slides at 1000 revolutions per minute for 5 min. Cells were fixed in 3% paraformaldehyde for 10 min and permeabilized in 0.5% Triton X-100 for 5 min. GWB were detected with human anti-GWB sera (1:6000) and rabbit anti-Dcp1a (1:1000). Secondary antibodies used were Alexa Fluor 488 goat anti-human IgG (1:400) and Alexa Fluor 568 goat anti-rabbit IgG (1:400) from Molecular Probes (Carlsbad, CA, USA). Slides were mounted using Vectashield Mounting Medium with 4’,6-diamidino-2-phenylindole (DAPI) (VECTOR Laboratories, Burlingame, CA, USA). Fluorescence images were taken with Zeiss Axiovert 200 m microscope and a Zeiss AxioCam MRm camera using the 20 x 0.75 NA objectives (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). Color images were assessed using Adobe Photoshop version 7. GWB were counted using Cell-Profiler image analysis software.\textsuperscript{38}

**Western blots**

THP-1 cell extracts were fractionated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The following antibodies and dilutions were used: rabbit anti-Ago2 antibodies 1:500; rabbit anti-Dcp1a antibodies 1:1000; rabbit anti-Rck/p54 antibodies 1:500 (MBL International, Woburn, MA, USA); mouse anti-tubulin antibodies 1:5000; mouse anti-actin antibodies 1:10 000; rabbit anti-Dcp1a antibodies 1:1000; rabbit anti-Rck/p54 antibodies 1:10 000; rabbit anti-golgin-97 antibodies\textsuperscript{38} 1:500; rabbit anti-Ago2 antibodies 1:500 (MBL International, Woburn, MA, USA); and the rabbit anti-Ago2 antibody. We also thank Drs Anne E Carpenter and grants from the Lupus Research Institute. K.M.P. was supported by NIDCR and serological associations of autoantibodies to GW bodies and a novel cytoplasmic phosphorylated cytoplasmic autoantigen, GW182, associates with a unique population of human mRNAs within novel cytoplasmic speckles.\textsuperscript{19,20}

**Quantitative real-time PCR**

RNA isolates were prepared using the mirVana miRNA Isolation kit (Ambion, Austin, TX, USA) according to the manufacturer’s protocol. RNA concentrations were determined and equal amounts of each RNA sample were used for qRT-PCR. qRT-PCR was performed using the TaqMan MicroRNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix and TaqMan MicroRNA Assay primers for human miR-146a, human miR-132, human miR-155 and human let-7a (Applied Biosystems, Foster City, CA, USA). The cycle threshold values, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, were determined and the miRNA expression, relative to untreated controls, was calculated using the 2^-ΔΔCt method.\textsuperscript{41}

**siRNA and miRNA**

The small interfering RNAs targeting Rck/p54, Ago2, TRAF6, and IRAK-1 and miR-146a mimic (Ambion) were transfected into THP-1 cells at a final concentration of 40 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. To monitor the transfection efficiency, Cy3-labeled siRNA targeting lamin A/C or Cy3-labeled pre-miR-negative control (Ambion) were transfected into cells in parallel to all transfections, and at least 80% transfection efficiency was achieved. The siRNAs targeting Rck/p54 and Ago2 were purchased from Dharmacon (Lafayette, CO, USA) and dissolved in 1× Universal buffer (provided by Dharmacon), and the resulting 20 μM stock was stored in aliquots at –80 °C before use. The siRNAs targeting TRAF6 and IRAK-1 were purchased from Applied Biosystems, dissolved in molecular biology grade water, and stored at 20 μM aliquots at –80 °C. The sense and antisense strand sequences are listed below:

- hAgo2: 5’-GCA CGG AAG UCC AUG UGA A dTdT-3’ and 5’-UUC AGC UGU ACC GGU UAG GGU UUU-3’;
- hTRAF6: 5’-GGU UGG UUG AAC AAG G dTdT-3’ and 5’-UUC UCU GUG ACC CAA ACA U dTdT-3’ and 5’-UUC UUG GGG UGA CGA AAC C dTdT-3’.

**Multiplex analysis of cytokines**

THP-1 cells were transfected as described above, and then treated with 2 μg/ml\textsuperscript{1} LPS for 24 h in culture medium. The culture supernatant was then harvested and frozen at –80 °C for storage before multiplex analysis. The human cytokine/chemokine LINCOplex premixed kit (Millipore, Billerica, MA, USA) or Beadlyte human 22-plex multi-cytokine detection system (Millipore) were used according to the manufacturer’s protocol to quantitatively detect the following human cytokines/chemokines: IFN-γ, IL-1β, IL-6, IL-10, IP-10, IL-12p40, MCP-1, TNF-α and IL-8. Samples were analyzed on a Luminex 200 system (Luminex, Austin, TX, USA).

**Statistical analysis**

All values are shown as the mean ± s.e.m. or s.d. of data. Comparison between groups was made with the Student’s t-test or Mann–Whitney test as indicated in figure legends. P<0.05 was considered significant.

**Acknowledgements**

We thank Dr J Lykke-Andersen, University of Colorado, for providing the rabbit anti-Dcp1a antibody and Dr T Hobman, University of Alberta, for providing the rabbit anti-Ago2 antibody. We also thank Drs Anne E Carpenter and Thouis R Jones, Whitehead Institute for Biomedical Research, for providing the CellProfiler software and technical support. This work was supported in part by National Institute of Health grant AI47859 and AR051766 and grants from the Lupus Research Institute. K.M.P. was supported by NIDCR oral biology training grant T32 DE007200.

---

\textsuperscript{1} Eustathioy T, Chan EKL, Tenenbaum SA, Keene JD, Griffith K, Fritzler MJ. A phosphorylated cytoplasmic autoantigen, GW182, associates with a unique population of human mRNAs within novel cytoplasmic speckles. *Mol Biol Cell* 2002; 13: 1338-1351.

\textsuperscript{2} Eustathioy T, Chan EKL, Takeuchi K, Mohler M, Luft LM, Zochode DW et al. Clinical and serological associations of autoantibodies to GW bodies and a novel cytoplasmic autoantigen GW182. *J Mol Med* 2005; 81: 811-818.

\textsuperscript{3} Eulalio A, Behm-Ansmant I, Izaurralde E. P bodies: at the crossroads of post-transcriptional pathways. *Nat Rev Mol Cell Biol* 2007; 8: 9-22.

\textsuperscript{4} Jakymiw A, Pauley KM, Li S, Ikeda K, Lian S, Eystathioy T, Chan EKL, Takeuchi K, Mahler M, Luft LM, Zochodne DW et al. The role of GW/P-bodies in RNA processing and silencing. *J Cell Sci* 2007; 120: 1317-1323.

\textsuperscript{5} Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008; 9: 102-114.

\textsuperscript{6} Parker R, Sheth U. P bodies and the control of mRNA translation and degradation. *Mol Cell* 2007; 25: 635-646.
Supplementary Information accompanies the paper on Immunology and Cell Biology website (http://www.nature.com/icb)