IL-10 inhibits miR-155 induction by Toll-like receptors

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IL-10 is a potent anti-inflammatory cytokine that is crucial for down-regulating pro-inflammatory genes which are induced by Toll-like Receptor (TLR) signalling. In this study we have examined whether modulation of micro-RNAs play a role in the inhibitory effect of IL-10 on TLR4 signalling. Analysing miRNAs known to be induced by TLR4, we found that IL-10 could inhibit the expression of miR-155 in response to LPS, but had no effect on miR-21 or miR-146a. IL-10 inhibited miR-155 transcription from the BIC gene in a STAT3 dependent manner. This inhibitory effect of IL-10 on miR-155 led to an increase in the expression of the miR-155 target gene, *SHIP1*. This is the first example of IL-10 playing a role in miRNA function and suggests that through its inhibitory effect on miR-155, IL-10 has the ability to promote anti-inflammatory gene expression.

IL-10 is a potent anti-inflammatory cytokine that is crucial for dampening the inflammatory response after pathogen invasion and acts to protect the host from excessive inflammation (1). For example, mice deficient in IL-10 have been shown to die from excessive inflammatory responses when exposed to bacterial pathogens (1). In addition, many inflammatory diseases in humans can be associated with poor IL-10 expression such as Ulcerative Colitis, Crohn's and asthma (2-3). One mechanism whereby IL-10 mediates its anti-inflammatory effect is through the down-regulation of pro-inflammatory genes induced downstream of Toll-like Receptor (TLR) signalling such as those encoding IL-1, IL-12, TNFα and IL-6. This is accomplished when IL-10 signals through the JAK1 - STAT3 pathway, resulting in the induction of as yet unknown STAT3 responsive genes, which are thought to be responsible for the inhibition of these pro-inflammatory proteins (1, 4). In this study we set out to examine if modulation of microRNAs (miRNA) might play a role in the inhibitory effect of IL-10 on signalling by TLR4, the receptor responsible for sensing the gram negative bacterial product, lipopolysaccaride (LPS).

The discovery of miRNAs has revealed an entirely new mechanism of negative regulation within the cell (5-8). miRNAs are small endogenous RNA molecules (~22nt) which have the ability to base-pair to mRNA sequences from protein coding genes, leading to partial or full degradation of the mRNA transcript (5-8). With the identification of over 500 miRNAs to date and the prediction that each miRNA may recognise several hundred target sequences, the current challenge is to identify these targets and understand how miRNAs are regulated within the cell. This is particularly important considering the mounting evidence demonstrating their contribution to disease and their roles in cellular

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mechanisms such as differentiation, metabolism and immunity (5-8).

A role for miRNAs in the innate immune response was demonstrated when miRNAs such as miR-146a, miR-155 and miR-21 were shown to become induced in response to TLR4 signalling in monocytes (9-11). miR-146a and miR-21 are both induced by LPS, where the former has been shown to target TRAF6 and IRAK1, two upstream signalling components within the TLR4 pathway, whereas miR-21 was shown to negatively regulate programmed cell death 4, a pro-inflammatory protein which promotes NFκB activation and suppresses IL-10 (9, 11). miR-155 is also induced by LPS, as well as other TLR ligands and pro-inflammatory cytokines (10). Numerous targets have been identified for miR-155 such as c-Maf, Bach1, PU.1, C/ebpβ and SHIP1, however their role in TLR signalling has never been extensively explored before (12-17). Mice deficient in miR-155 have defects in B-cell differentiation, as well as possessing severe deficiencies in immune responses when exposed to pathogens, thus highlighting the important role miR-155 plays in the immune system as a whole (12, 18-19).

In this study, we demonstrated that IL-10 could inhibit the expression of miR-155 in response to LPS but had no effect on miR-21 or miR-146a. This inhibition of miR-155 by IL-10 led to an increase in the expression of the miR-155 target gene, *SHIP1*. Since SHIP1 has been shown to limit TLR signalling (20), the ability of IL-10 to increase its expression via inhibition of miR-155 provides new insights into the complex signalling mechanism of IL-10. This finding also identifies a novel mechanism of control on miR-155, a miRNA which has been implicated in the innate immune response and cancer progression.

Experimental procedures

Reagents - LPS from *E.Coli*, Serotype 0111:B4 was from Alexis. Recombinant mouse and human IL-10 were from R&D Biosystems. Precursor-miR-155 (pre-155) oligonucleotide was obtained from Ambion.

Cell Culture-Immortalised bone marrow derived macrophages (BMDM), a kind gift from Doug Golenbock (UMASS, USA) and Raw264.7 cell lines, obtained from ECACC were maintained in DMEM. Wild-type and IL-10-deficient bone marrow obtained from Peter Murray (University of Memphis, USA) were isolated from the tibias and femurs of C57/Bl6 mice and primary BMDM were generated as previously described (11). Human peripheral blood mononuclear cells (hPBMC) were isolated from whole blood using a Ficoll gradient (21). Splenocytes from wild-type and Eμ-miR-155 transgenic mice, obtained from Carlo Croce (Ohio State University, USA) were maintained in RPMI and 50 μM β-mercaptoethanol. In all cases, DMEM and RPMI media were supplemented with 10 % FCS, 2 mM L-glutamine, 1 % pen/strep solution (v/v).

RT-PCR – Immortalised BMDM (I-BMDM) and differentiated primary BMDM or hPBMC were set up at 4×10^5 or 1×10^6 respectively, in 24-well plates one day prior to stimulation. Cells were stimulated with LPS and/or IL-10 as indicated in the figure legends. Total RNA was extracted using the RNeasy Kit (Qiagen), modified to obtain small RNA species. For miRNA analysis, miRNA TaqMan assays for miR-21, miR-146a, miR-155, miR-191 and RNU6B (Applied Biosystems) were used according to the maufacturers' instructions where 5 ng/ml of total RNA was used as starting material. For mRNA expression analysis cDNA was prepared from 20 – 100 ng/ml total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturers' instructions. mRNA expression was then monitored using SYBR-Green based chemistry (Invitrogen)

using the following primers: Gapdh 5'-gaa cgg gaa gct tgt cat caa_for, 5'-cta agc agt tgg tgg tgc ag_rev; Pri-mmu-155 5'-gac aca agg cct gtt act agc ac_for, 5'-gtc tga cat cta cgt tca tcc agc_rev; Pre-mmu-155 5'-gct aat tgt gat agg ggt ttt gg_for, 5'-gtt aat gct aac agg tag gag tc_rev; SHIP1 5'-ggt ggt acg gtt tgg aga ga_for, 5'-atg ctg agc ctc tgt ggt ct_rev; miRNA and mRNA expression were measured on the 7900 RT-PCR system (Applied Biosystems) and fold changes in expression were calculated by the Delta Delta Ct method using miR-191 (BMDM/ hPBMC) (22) or RNU6B (splenocytes) as an endogenous control for miRNA analysis and GAPDH as an endogenous control for mRNA expression. All fold changes are expressed normalised to non-stimulated control for each cell type.

ELISA-Murine IL-10 expression was measured from the supernatants of stimulated cells using an ELISA DuoSet kit (R&D Biosystems) according to manufacturers' instructions.

Luciferase assays - BIC luciferase plasmid along with the NF-κB, AP1 and Ets1 mutants were a kind gift from Eric Flemington (Tulane University, New Orleans, USA). Raw264.7 cells seeded at 2 x 10^5/ml in 24-well plates were transfected using 6 % GeneJuice with each plasmid, TK-Renilla and 25 nM murine si-control and si-STAT3 (Santa Cruz, sc-29494) for siRNA experiments. Cells were rested for 24-48 hrs prior to stimulation with LPS or LPS + IL-10 for 18 hrs. pMir-SHIP1 and SHIP1 mutant 3'UTR luciferase plasmids (kind gifts from David Baltimore, California Institute of Technology, USA) were co-transfected with TK-Renilla and increasing concentrations of pre-155 oligonucleotide into Raw264.7, or stimulated with LPS or LPS + IL-10 for 8 hrs. In all cases, cells were lysed in passive lysis buffer before being analysed for both luciferase and TK-Renilla activity as previously described (23). Data was normalised to TK-Renilla activity and represented as mean ± standard deviation for triplicate determinations where fold changes are expressed normalised to non-stimulated control.

Protein expression - Differentiated IL-10-deficient BMDM cells seeded at 4 x 10^5/ml in 6 well plates were stimulated with LPS and/or IL-10 as indicated in the figure legends. Cells were lysed in low stringency lysis buffer complete with protease inhibitors and protein concentration was determined using Coomassie Bradford reagent (Pierce, Rockford, USA). Lysates were resolved on 10 % SDS-PAGE gels and transferred onto PVDF membrane before being immunoblotted with anti-SHIP1 (P1C1, Santa Cruz) or anti-β-actin (AC-15, Sigma). Blots were developed by enhanced chemiluminescence (ECL) (Cell Signaling Technology Inc.).

RESULTS

IL-10 inhibits miR-155 expression in response to TLR4 stimulation. In an effort to determine if IL-10 could modulate any miRNA downstream of TLR4 signalling, we first decided to investigate the effect of IL-10 on miR-155, miR-146a and miR-21, miRNAs which are known to be induced downstream of TLR4 (9-11). As shown in Fig. 1a, in I-BMDM, LPS gradually induced the expression of miR-155 (upper graph), miR-21 (middle graph) and miR-146a (lower graph) over time where the expression of each miRNA was greatest at 24 hrs. The effect of LPS was particularly evident on miR-155, which was induced over 200-fold compared to non-stimulated control. When cells were treated with LPS in the presence of IL-10 the expression of miR-155 was inhibited, the effect being most evident at 24 hrs (upper graph). In contrast IL-10 had no effect on miR-21 or miR-146a (middle and lower graph). This indicated a specific effect for IL-10 on the sole expression of one miRNA, even though all three were induced by LPS. IL-10 alone appeared to have no effect on miR-155, miR-21 or miR-146a suggesting

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that IL-10 only works to inhibit miR-155 after TLR4 stimulation.

We next investigated the expression of miR-155 in response to LPS when pre-treated with varying concentrations of IL-10 or when pre-treated with IL-10 for various times (Fig. 1b). In response to LPS stimulation alone, miR-155 expression was induced 120-fold, however pre-treatment with increasing concentrations of IL-10 gradually decreased miR-155 expression, where the optimal inhibition occurred when IL-10 was used at 25 ng/ml (Fig. 1b, upper graph). miR-155 expression was then analysed in I-BMDM which were pre-treated with IL-10 for various times prior to the addition of LPS for 24 hrs (Fig. 1b, lower graph). miR-155 expression was induced 100-fold in response to LPS alone and interestingly irrespective of the length of time cells were pre-treated with IL-10, miR-155 expression was reduced. For these reasons cells were pre-treated for the minimum time of 5 min with IL-10 at a concentration of 20 ng/ml prior to the addition of LPS for all future experiments unless otherwise indicated.

We then set out to examine if IL-10 had the same effect on miR-155 in primary BMDM and hPBMC (Fig. 2a). LPS induced the expression of miR-155 40-fold in primary BMDM (upper graph), and 8-fold in hPBMC (lower graph) after 24 hrs stimulation. In both these cell types, IL-10 inhibited the expression of miR-155 from as early as 4 hrs, although the effect of IL-10 appeared to occur earlier in hPBMC (lower graph).

We next compared the effect of LPS on miR-155 expression in primary wild-type and IL-10-deficient BMDM. It is well known that in addition to LPS inducing pro-inflammatory cytokines, LPS also induces IL-10 to negatively feed-back on the pathway and switch off this pro-inflammatory response. As shown in Fig. 2b, upper graph, wild-type BMDM cells treated with LPS induced IL-10 protein expression, where the maximal amount

produced occurred at 8 hrs. As expected, LPS could not induce IL-10 in IL-10-deficient BMDM (Figure 2b, upper garph). The fact that LPS cannot induce IL-10 in IL-10-deficient BMDM predicts that miR-155 expression should be higher in these cells. LPS induced the expression of miR-155 approx 25-fold over non-stimulated control in wild-type BMDM and as predicted miR-155 expression was greater in IL-10-deficient BMDM where miR-155 expression had more than doubled to approx 55-fold over non-stimulated control (Fig. 2b, lower graph). As a control, a monoclonal IL-10 antibody was used to block the action of endogenous IL-10 in response to LPS in wild-type BMDM. Blocking IL-10 induction in wild-type BMDM relieved the inhibition of miR-155 and demonstrated that its expression could be increased to a level similar to that found in IL-10-deficient BMDM (Fig. 2b, lower graph). This demonstrated that the induction of endogenous IL-10 by LPS in wild-type cells can feed-back on the expression of miR-155 by LPS, thus keeping its expression in check.

IL-10 inhibits the transcription of miR-155 in a STAT3 dependent manner. We next addressed if IL-10 could inhibit transcription of the miR-155 gene. miR-155 is transcribed as a primary (pri-) transcript from the third exon of the non-protein coding gene BIC (B cell integration cluster) (24), after which it is sequentially processed by the enzymes Drosha and Dicer to form a precursor (pre-) and mature miR-155, respectively. We designed primers for the pri- and pre-miR-155 transcripts and stimulated IL-10-deficient BMDM with LPS alone or LPS in the presence of IL-10 (Fig. 3a). In response to LPS, pri-miR-155 was rapidly induced, where the highest expression was induced 50-fold at 4 hrs, after which its expression began to decline (Fig. 3a, top graph), most likely due to the processing of pri-miR-155 into the pre- and mature

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form. In contrast, in the presence of IL-10, generation of pri-miR-155 was inhibited, where pri-miR-155 expression was reduced to 15-fold at 4 hrs (Fig. 3a, upper graph). A similar trend was observed when the pre- miR-155 transcript was measured, where its highest expression in response to LPS was also observed at 4 hrs and IL-10 inhibited this expression from 27-fold down to 7-fold over non-stimulated control (Fig. 3b, middle graph). It could also be noted that the expression of pre-miR-155 in response to LPS appeared more gradual, indicating that pre-miR-155 generation occurs after pri-miR-155. Mature miR-155 was also measured demonstrating that IL-10 had the same inhibitory effect on miR-155 in IL-10-deficient BMDM as that observed in I-BMDM, primary wild-type BMDM and hPBMC (Fig. 3a, lowest graph). The observation that IL-10 inhibits the expression of both the pri- and pre-transcripts suggests that IL-10 acts upstream to inhibit the actual transcription of the BIC gene.

We further analysed the effect of IL-10 on transcriptional regulation of the BIC gene through the use of a luciferase reporter plasmid containing 1200 bp of the BIC promoter region (25). In addition, we investigated if any of the IL-10 effects were mediated through STAT3 by using siRNA targeted against STAT3. In the presence of control siRNA, LPS induced luciferase expression 3-fold from the BIC promoter and this expression was returned to near basal levels in the presence of IL-10 (Fig. 3b), validating that IL-10 appears to inhibit miR-155 at the transcriptional level. In the presence of si-STAT3, IL-10 could no longer suppress luciferase expression demonstrating that the effect of IL-10 on miR-155 transcription is dependent on STAT3.

The Ets1 binding site is required for the IL-10 mediated suppression of the BIC gene. In order to investigate how IL-10 acts to suppress BIC transcription, we analysed the effect of IL-10 when consensus transcription factor binding sites for NF-κB, AP1 and Ets1 found within the BIC promoter were mutated. In response to LPS, luciferase expression was induced 3-fold from the wild-type BIC promoter. A similar effect was observed when the NF-κB motif and Ets1 site were mutated (Fig. 3c). Luciferase activity was however reduced in a promoter with a mutated AP-1 motif, suggesting that this transcription factor plays a role in the LPS induction of miR-155 (Fig. 3c), supporting previous studies which show that AP1 is required for BIC gene induction in response to B cell stimulation (25).

In concordance with our previous results, IL-10 inhibited luciferase activity in the wild-type BIC promoter (Fig. 3c, bar 3 and 4). IL-10 was also able to reduce luciferase activity in both the NF-kB and AP1 promoter mutants. In contrast, IL-10 could no longer inhibit luciferase activity when the Ets1 site was mutated suggesting that this site is important in mediating the IL-10-driven suppression of miR-155.

IL-10 increases the expression of the miR-155 target, SHIP1. We next wanted to investigate a functional outcome for the IL-10 mediated suppression of miR-155. We therefore set out to examine the effects of LPS and IL-10 on the miR-155 target, SHIP1 (15, 16). We first verified that the 3'UTR of *SHIP1* is targeted by miR-155. The 3'UTR for *SHIP1* was cloned into the pMir luciferase reporter system. Under basal conditions, luciferase expression was present (Fig. 4a, white bars). However in the presence of increasing concentrations of pre-miR-155 (1, 10, 100 nM), luciferase expression was lost. In contrast, increasing concentrations of pre-miR-155 had no effect when the miR-155 seed sequence within the 3'UTR of *SHIP1* was mutated.

The fact that LPS potently induces mature miR-155 suggests that as miR-155 expression increases, its ability to bind to the 3'UTR of *SHIP1* should result in a decrease of *SHIP1* mRNA expression. As

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shown in Fig. 4b, the expression of *SHIP1* decreased over time upon LPS stimulation, reciprocal to the increase in miR-155 expression. However, in the presence of IL-10 when miR-155 expression is inhibited, the expression of *SHIP1* increased (Fig. 4b), particularly at 8 and 24 hrs when the effect of IL-10 on miR-155 suppression was greatest (Fig. 1a). This effect of LPS and IL-10 was also apparent on SHIP1 protein expression, where in response to LPS alone, SHIP1 expression was reduced (Fig. 4c, lane 3), whereas in the presence of IL-10, SHIP1 protein expression increased (Fig. 4c, lane 4).

We next wanted to examine if the increase in SHIP1 expression in response to IL-10 is dependent on miR-155. We first used the pMir-SHIP1 luciferase reporter system, where luciferase expression will only decrease in the presence of miR-155 (Fig. 4a). The fact that LPS can potently drive miR-155 would suggest that LPS also has the ability to decrease luciferase expression. As shown in Fig. 4d, LPS decreased SHIP1 luciferase activity whereas in the presence of IL-10, this inhibition was relieved, implicating that the suppression of miR-155 by IL-10 is mediating this effect.

This was further demonstrated by using transgenic (Tg) splenocytes which over-express miR-155 under the control of the Eμ-B cell promoter (26). We postulated that in cells where miR-155 is over-expressed, IL-10 would no longer be able to maintain its effect on miR-155 targets. As shown in Fig. 4e, left graph, miR-155 was over-expressed 35-fold in Tg-splenocytes compared to wild-type (WT) cells. IL-10 was able to decrease this expression somewhat, although substantial levels of miR-155 still remained in Tg cells. As expected IL-10 could increase the expression of SHIP1 in WT cells whereas in miR-155 Tg cells, SHIP1 expression was no longer detected, decreasing well below basal levels, and as predicted IL-10 could not rescue this effect (Fig. 4e, right graph).

DISCUSSION

This study highlights a novel mechanism of miR-155 regulation. We showed that IL-10 can inhibit the expression of miR-155, a miRNA induced downstream of TLR4 signalling but had no effect on miR-21 and miR-146a, two other miRNAs also induced by TLR4. This not only demonstrated a very specific effect for IL-10 but was also the very first example of IL-10 playing a role in miRNA function. miR-155 expression was doubled in response to LPS in IL-10-deficient cells, demonstrating that endogenous IL-10 can feed back on the system to keep miR-155 expression in check, highlighting an additional mechanism of IL-10 control on the pro-inflammatory response.

miR-155 was the first oncogenic miRNA to be discovered. It has been shown to be highly expressed in several types of B cell lymphoma in particular Hodgkin's lymphoma and diffuse large B cell lymphoma (27-28). In addition, transgenic mice over-expressing miR-155 succumb to B cell malignancies (26). miR-155 was also found over-expressed in patients with acute myeloid leukaemia and rheumatoid arthritis (14, 29). Together, this information demonstrates how miR-155 may provide a potential link between inflammatory diseases and cancer. It is therefore essential that miR-155 is tightly regulated in the cell and we propose that IL-10 is a likely candidate for this regulation.

We sought to investigate how IL-10 inhibits miR-155 expression. Measuring pri-mir-155 and pre-miR-155 expression we demonstrated that IL-10 could potently inhibit generation of both transcripts illustrating that IL-10 acts to inhibit miR-155 upstream of primary transcript generation. In addition, IL-10 reduced BIC promoter activity in a STAT3 dependent manner indicating that the IL-10 suppression of the BIC gene is

mediated through the canonical IL-10-STAT3 signalling pathway.

In an effort to further investigate the effect of IL-10 on miR-155 transcription, we analysed the effect of IL-10 when various transcription factor sites within the BIC promoter were mutated. IL-10 was able to suppress wild-type BIC promoter luciferase expression as well as suppressing luciferase activity when the NF-kB and AP-1 sites were mutated. In contrast, IL-10 could no longer suppress luciferase activity when the Ets1 site was mutated, demonstrating that the Ets1 site is required for mediating IL-10 suppression. To date, over 30 Ets family members exist which each have the potential to bind to the canonical Ets consensus sequence found in the BIC promoter (30). Etv3, an Ets family transcriptional repressor, was recently identified as a novel IL-10 induced gene (31). It is possible that the IL-10 suppression of miR-155 could involve recruitment of Etv3 and we are currently investigating this mechanism.

We also explored the functional outcomes for IL-10 suppression of miR-155. Taking SHIP1 as a well characterised miR-155 target, we were able to illustrate that over-expressing miR-155 decreased the expression of a SHIP1 3'UTR reporter. We went onto show that IL-10 could rescue the LPS driven down-regulation of SHIP1 at the mRNA and protein level and through the use of the SHIP1 3'UTR reporter and miR-155 tg-splenocyes

demonstrated that this effect was mediated by miR-155.

SHIP1 is an inositiol phosphatase that is known to convert the signalling molecule PIP3 back to PIP2, whereas PI3K is responsible for the opposite reaction. TLR signalling can promote the pro-inflammatory response through the activation of PI3K, resulting in generation of PIP3 and activation of MAPK and NF-κB (20). The induction of miR-155 by LPS supports this model, whereby expression of miR-155 can target SHIP1, decreasing its expression and promoting the conversion of PIP2 to PIP3 by PI3K. In this setting, IL-10 increases SHIP-1 and in this way acts to switch the pro-inflammatory response off by decreasing the levels of PIP3 (Fig. 5).

This study therefore sheds light on a novel role for IL-10 in miR-155 regulation. miR-155 has been shown to be directly involved in the regulation of more than 30 innate immune genes (32). Taking SHIP1 as an example, our data suggests that IL-10 could impact on these genes via its inhibitory effect on miR-155. It may therefore be possible that through the inhibition of miR-155, we may elicit some of the properties mediated by IL-10. With increasing studies performed on how to inhibit or increase miRNAs for therapeutic use *in vivo* (33), our study could provide new approaches in the effort to develop anti-inflammatory therapeutics.

REFERENCES

1. Murray, P. J. (2006) *Curr Opin Pharmacol* **6**, 379-386
2. O'Garra, A., Barrat, F. J., Castro, A. G., Vicari, A., and Hawrylowicz, C. (2008) *Immunol Rev* **223**, 114-131
3. Asadullah, K., Sterry, W., and Volk, H. D. (2003) *Pharmacol Rev* **55**, 241-269
4. Murray, P. J. (2006) *Biochem Soc Trans* **34**, 1028-1031
5. Taganov, K. D., Boldin, M. P., and Baltimore, D. (2007) *Immunity* **26**, 133-137
6. Gantier, M. P., Sadler, A. J., and Williams, B. R. (2007) *Immunol Cell Biol* **85**, 458-462
7. Lindsay, M. A. (2008) *Trends Immunol* **29**, 343-351
8. Kanellopoulou, C., and Monticelli, S. (2008) *Semin Cancer Biol* **18**, 79-88

9. Taganov, K. D., Boldin, M. P., Chang, K. J., and Baltimore, D. (2006) *Proc Natl Acad Sci U S A* **103**, 12481-12486

10. O'Connell, R. M., Taganov, K. D., Boldin, M. P., Cheng, G., and Baltimore, D. (2007) *Proc Natl Acad Sci U S A* **104**, 1604-1609

11. Sheedy, F. J., Palsson-McDermott, E., Hennessy, E. J., Martin, C., O'Leary, J., Ruan, Q., Johnson, D. P., Chen, Y., and O'Neill, L. A. (2009) *Nat Immunol*

12. Rodriguez, A., Vigorito, E., Clare, S., Warren, M. V., Couttet, P., Soond, D. R., van Dongen, S., Grocock, R. J., Das, P. P., Miska, E. A., Vetrie, D., Okkenhaug, K., Enright, A. J., Dougan, G., Turner, M., and Bradley, A. (2007) *Science* **316**, 608-611

13. Yin, Q., McBride, J., Fewell, C., Lacey, M., Wang, X., Lin, Z., Cameron, J., and Flemington, E. K. (2008) *J Virol* **82**, 5295-5306

14. O'Connell, R. M., Rao, D. S., Chaudhuri, A. A., Boldin, M. P., Taganov, K. D., Nicoll, J., Paquette, R. L., and Baltimore, D. (2008) *J Exp Med* **205**, 585-594

15. O'Connell, R. M., Chaudhuri, A. A., Rao, D. S., and Baltimore, D. (2009) *Proc Natl Acad Sci U S A* **106**, 7113-7118

16. Pedersen, I. M., Otero, D., Kao, E., Miletic, A. V., Hother, C., Ralfkiaer, E., Rickert, R. C., Gronbaek, K., and David, M. (2009) *EMBO Mol Med* **1**, 288-295

17. Costinean, S., Sandhu, S. K., Pedersen, I. M., Tile, E., Trotta, R., Perrotti, D., Ciarlariello, D., Neviani, P., Harb, J., Kauffman, L., Shidham, A., and Croce, C. M. (2009) *Blood* **114**, 1374-1382

18. Thai, T. H., Calado, D. P., Casola, S., Ansel, K. M., Xiao, C., Xue, Y., Murphy, A., Frendewey, D., Valenzuela, D., Kutok, J. L., Schmidt-Supprian, M., Rajewsky, N., Yancopoulos, G., Rao, A., and Rajewsky, K. (2007) *Science* **316**, 604-608

19. Faraoni, I., Antonetti, F. R., Cardone, J., and Bonmassar, E. (2009) *Biochim Biophys Acta* **1792**, 497-505

20. An, H., Xu, H., Zhang, M., Zhou, J., Feng, T., Qian, C., Qi, R., and Cao, X. (2005) *Blood* **105**, 4685-4692

21. Palsson-McDermott, E. M., Doyle, S. L., McGettrick, A. F., Hardy, M., Husebye, H., Banahan, K., Gong, M., Golenbock, D., Espevik, T., and O'Neill, L. A. (2009) *Nat Immunol* **10**, 579-586

22. Peltier, H. J., and Latham, G. J. (2008) *Rna* **14**, 844-852

23. McCoy, C. E., Carpenter, S., Palsson-McDermott, E. M., Gearing, L. J., and O'Neill, L. A. (2008) *J Biol Chem* **283**, 14277-14285

24. Eis, P. S., Tam, W., Sun, L., Chadburn, A., Li, Z., Gomez, M. F., Lund, E., and Dahlberg, J. E. (2005) *Proc Natl Acad Sci U S A* **102**, 3627-3632

25. Yin, Q., Wang, X., McBride, J., Fewell, C., and Flemington, E. (2008) *J Biol Chem* **283**, 2654-2662

26. Costinean, S., Zanesi, N., Pekarsky, Y., Tili, E., Volinia, S., Heerema, N., and Croce, C. M. (2006) *Proc Natl Acad Sci U S A* **103**, 7024-7029

27. Kluiver, J., van den Berg, A., de Jong, D., Blokzijl, T., Harms, G., Bouwman, E., Jacobs, S., Poppema, S., and Kroesen, B. J. (2007) *Oncogene* **26**, 3769-3776

28. Rai, D., Karanti, S., Jung, I., Dahia, P. L., and Aguiar, R. C. (2008) *Cancer Genet Cytogenet* **181**, 8-15

29. Stanczyk, J., Pedrioli, D. M., Brentano, F., Sanchez-Pernaute, O., Kolling, C., Gay, R. E., Detmar, M., Gay, S., and Kyburz, D. (2008) *Arthritis Rheum* **58**, 1001-1009

30. Oikawa, T., and Yamada, T. (2003) *Gene* **303**, 11-34

31. El Kasmi. K. C., Smith, A. M., Williams, L., Neale, G., Panopolous, A., Watowich, S. S., Hacker, H., Foxwell, B. M. J., and Murray, P. J. (2007) *J. Immunol.* **179**, 7215-7219

32. Gantier, M. P., (2010) *J. Interferon Cytokine Res.* **30,** *in press*

8

33. Liu, Z., Sall, A., and Yang, D. (2008) *Int J Mol Sci* **9**, 978-999

FOOTNOTES

FIGURE LEGENDS

Fig. 1. IL-10 inhibits miR-155 expression in response to TLR4 stimulation. a) I-BMDM were stimulated with LPS (100 ng/ml), LPS + IL-10 (20 ng/ml) or with IL-10 alone for the times indicated. Expression of miR-155, miR-21 and miR-146a were measured by RT-PCR. b) I-BMDM were pre-treated for 5 min with increasing doses of IL-10 or pre-treated for various times with IL-10 (20 ng/ml) prior to the addition of LPS (100 ng/ml) for 24 hrs. miR-155 expression was measured by RT-PCR. In both cases, results were normalised and represented as fold stimulation over the non-stimulated control and are representative of at least three separate experiments.

Fig. 2. IL-10 inhibits miR-155 in primary BMDM and hPBMC. a) primary BMDM and hPBMC were stimulated with LPS (100 ng/ml) or LPS + IL-10 (20 ng/ml) and measured for miR-155 expression by RT-PCR. b) Wild-type (WT) and IL-10-deficient BMDM (IL-10 KO) were untreated or pre-treated with monoclonal IL-10 antibody (5 μg/ml) for 1 hr prior to stimulation with LPS (100 ng/ml) for 24 hrs. Cells were analysed for IL-10 expression by ELISA and for miR-155 expression by RT-PCR. In all cases, graphs are representative of at least three separate experiments.

Fig. 3. IL-10 inhibits the transcription of miR-155. a) IL-10-deficient BMDM were stimulated with LPS (100 ng/ml) or LPS + IL-10 (20 ng/ml). pri-miR-155, pre-miR-155 and mature miR-155 were measured by RT-PCR. b) Raw264.7 cells were co-transfected with wild-type BIC promoter luciferase plasmid and either si-control or si-STAT3. Cells were stimulated with LPS (100 ng/ml) or with LPS + IL-10 (25 ng/ml) for 18 hrs. c) Raw264.7 cells transfected with wild-type BIC, NF-κB mutant, AP1 mutant or Ets1 mutant promoter luciferase plasmids were stimulated with LPS (100 ng/ml) or with LPS + IL-10 at two different doses (25, 50 ng/ml). Luciferase activity was measured where results were normalised for TK-Renilla activity and represented as fold stimulation over the non-stimulated control. In all cases, results are expressed as mean ± standard deviation for triplicate determinations and are representative of three separate experiments.

Fig. 4. IL-10 increases the expression of the miR-155 target, SHIP1. a) pMir-SHIP1 and pMir-SHIP1 mutant 3'UTR luciferase plasmids were co-transfected with increasing amounts of pre-155 (1, 10, 100 nM) in Raw264.7 cells. Luciferase activity was measured and results were normalised for TK-Renilla activity. b) IL-10-deficient BMDM were stimulated with LPS (100 ng/ml, white bars) or LPS + IL-10 (20 ng/ml, grey bars) for the times indicated. mRNA expression for *SHIP1* was measured by RT-PCR. c) IL-10-deficient BMDM were stimulated with IL-10 (20 ng/ml), LPS (100 ng/ml) or LPS + IL-10 for 24 hrs. SHIP1 and β-

actin protein expression were measured. d) pMir-SHIP1 3'UTR luciferase activity was measured in Raw264.7 cells after stimulation with LPS (100 ng/ml) or LPS + IL-10 (20 ng/ml) for 8 hrs. Luciferase activity was measured and results were normalised for TK-Renilla activity. e) Wild-type (WT) and transgenic (Tg) miR-155 splenocytes were stimulated with IL-10 (50 ng/ml) for 16 hrs. miR-155 and *SHIP1* expression was measured by RT-PCR. In all cases, results were represented as fold stimulation over non-stimulated control and expressed as mean ± standard deviation for triplicate determinations where each experiment is representative of three separate experiments.

Fig. 5. Schematic illustrating a possible role for miR-155 in TLR4 and SHIP1 signalling. In response to LPS, miR-155 expression is induced resulting in a decrease in SHIP1 expression, thus allowing PI3K activation of NF-κB and MAPK to proceed and promote the pro-inflammatory response. However, in the presence of IL-10, miR-155 expression is inhibited, allowing SHIP1 expression to recover and promote the conversion of PIP3 back to its inactive PIP2 state, switching off the pro-inflammatory response.
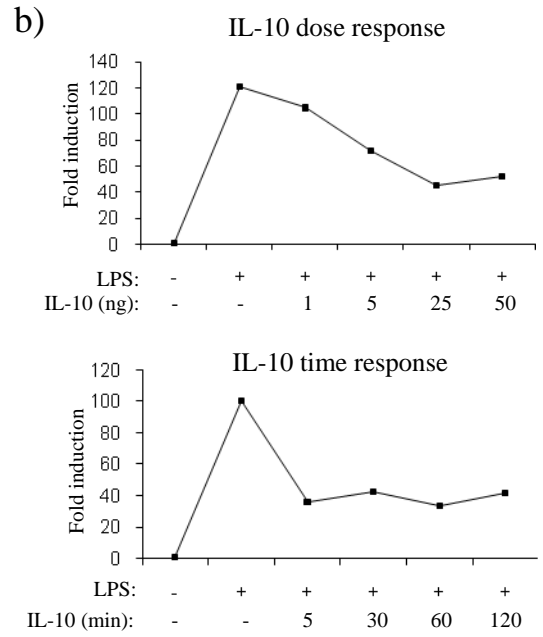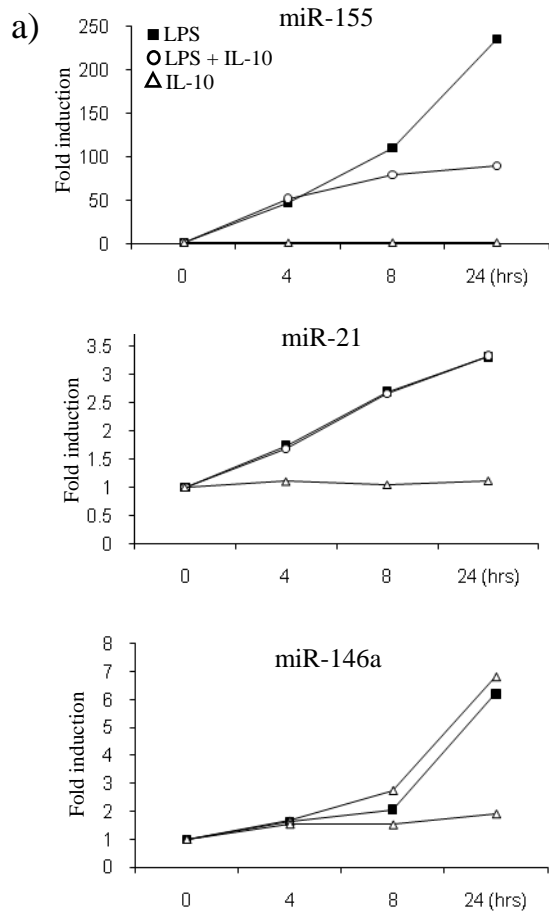
Figure 1

Figure 2

a)

Primary BMDM

- ■ LPS
- ○ LPS + IL-10

Fold induction

0   4   8   24 (hrs)

hPBMC

Fold induction

0   4   8   26 (hrs)

b)

IL-10 Expression

- ■ WT
- ○ IL-10 KO

pg/ml

0   4   8   24 (hrs)

miR-155

- □ Unstim
- ■ LPS

Fold induction

WT   IL10 KO   WT + mAb

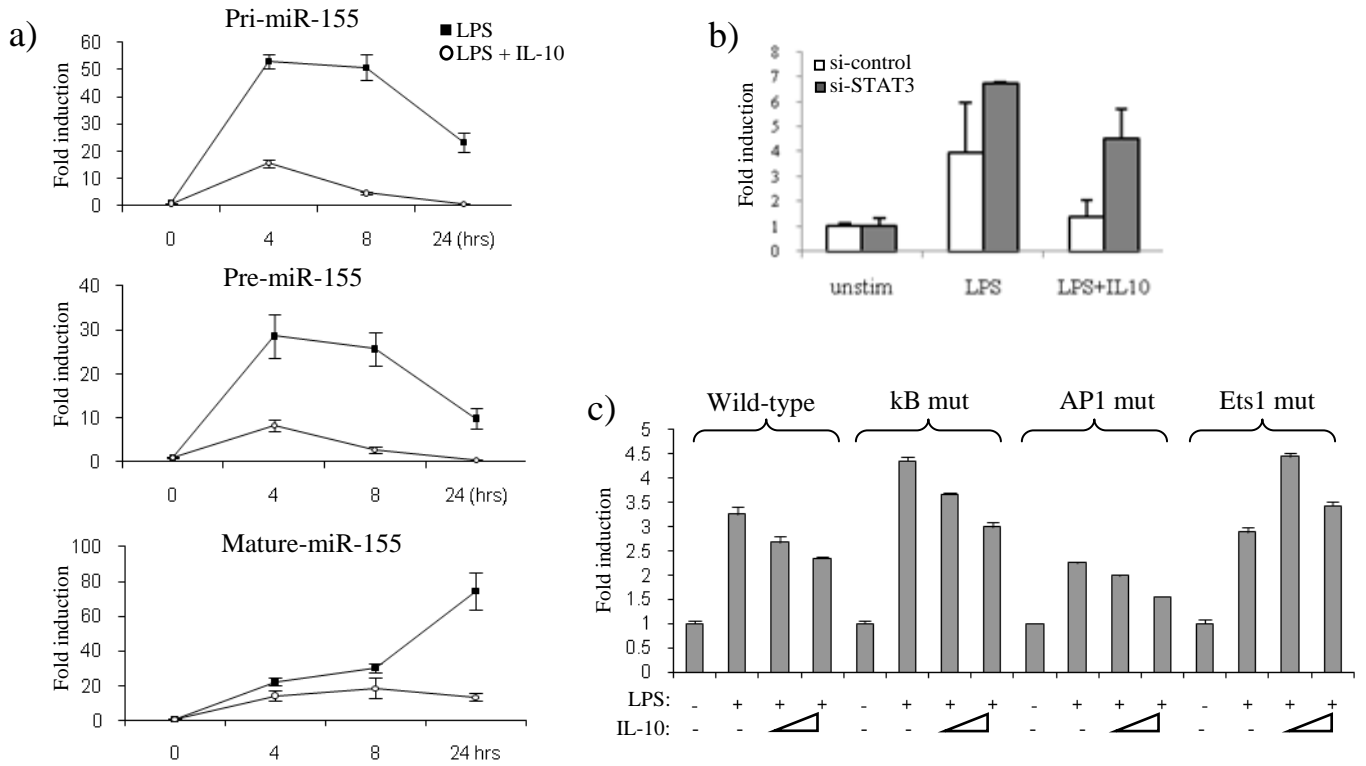# Figure 3

Figure 4

a)

pMir-SHIP1 luc expression

Legend:
- □ 0
- ▨ 1 nM
- ▨ 10 nM
- ▨ 100 nM

Y-axis: Fold induction (0.0 – 1.5)

X-axis categories: pMir-SHIP1, pMir-SHIP1 mut

c)

| LPS: | – | – | + | + |
| IL-10: | – | + | – | + |

SHIP1

β–actin

Lanes: 1 2 3 4

b)

SHIP1

Legend: □ LPS ▨ LPS + IL10

Y-axis: Fold induction (0.0 – 1.4)

X-axis: 0, 4, 8, 24 (hrs)

d)

pMir-SHIP1 luc expression

Y-axis: Fold induction (0 – 1.2)

X-axis categories: -, LPS, LPS+IL-10

e)

miR-155

Y-axis: Fold induction (0 – 40)

X-axis: WT, Tg-155

SHIP1

Legend: □ Unstim ▨ IL10

Y-axis: Fold induction (0.0 – 2.0)

X-axis: WT, Tg-155

Figure 5