Enhancing arginase 2 expression using target site blockers as a strategy to modulate macrophage phenotype

Chiara De Santi,1 Frances K. Nally,1 Remsha Afzal,1,2 Conor P. Duffy,1 Stephen Fitzsimons,1 Stephanie L. Annett,1 Tracy Robson,1 Jennifer K. Dowling,1,2 Sally-Ann Cryan,1,3,4,5,6 and Claire E. McCoy1,2

1School of Pharmacy and Biomolecular Sciences, Royal College of Surgeons in Ireland, D02 YN77 Dublin, Ireland; 2FutureNeuro SFI Research Centre, Royal College of Surgeons in Ireland, D02 YN77 Dublin, Ireland; 3Tissue Engineering Research Group, Department of Anatomy and Regenerative Medicine, RCSI University of Medicine and Health Sciences, Dublin, Dublin, Ireland; 4SFI Centre for Research in Medical Devices (CÚRAM), RCSI University of Medicine and Health Sciences, Dublin, Ireland; 5SFI Advanced Materials and Bioengineering Research (AMBER) Centre, RCSI University of Medicine and Health Sciences and Trinity College Dublin, Dublin, Ireland; 6Trinity Centre for Biomedical Engineering, Trinity College Dublin, Dublin, Ireland

Macrophages are plastic cells playing a crucial role in innate immunity. While fundamental in responding to infections, when persistently maintained in a pro-inflammatory state they can initiate and sustain inflammatory diseases. Therefore, a strategy that reprograms pro-inflammatory macrophages toward an anti-inflammatory phenotype could hold therapeutic potential in that context. We have recently shown that arginase 2 (Arg2), a mitochondrial enzyme involved in arginine metabolism, promotes the resolution of inflammation in macrophages and it is targeted by miR-155. Here, we designed and tested a target site blocker (TSB) that specifically interferes and blocks the interaction between miR-155 and Arg2 mRNA, leading to Arg2 increased expression and activity. In bone marrow-derived macrophages transfected with Arg2 TSB (in the presence or absence of the pro-inflammatory stimulus LPS), we observed an overall shift of the polarization status of macrophages toward an anti-inflammatory phenotype, as shown by significant changes in surface markers (CD80 and CD71), metabolic parameters (mitochondrial oxidative phosphorylation) and cytokines secretion (IL-1β, IL-6, and TNF). Moreover, in an in vivo model of LPS-induced acute inflammation, intraperitoneal administration of Arg2 TSB led to an overall decrease in systemic levels of pro-inflammatory cytokines. Overall, this proof-of-concept strategy represent a promising approach to modulating macrophage phenotype.

INTRODUCTION

Macrophages are a sub-type of immune cells that play key roles in the pathogenesis and regulation of the inflammatory reaction. The development of inflammation is critical to fighting infections, and macrophages play a key role in this process by releasing pro-inflammatory cytokines and toxic mediators.1 However, if inflammation persists, macrophages can also become destructive, leading to the severe tissue damage observed in variety of chronic inflammatory diseases such as ulcerative colitis,2 rheumatoid arthritis,3 and multiple sclerosis.4 In contrast, macrophages can also produce anti-inflammatory mediators and potentiate cell proliferation, tissue repair, and the healing process.5 Therefore, a therapeutic strategy aimed to reprogram pro-inflammatory macrophages toward an anti-inflammatory phenotype could hold merit in the fight against inflammatory diseases.

Arginase 2 (Arg2) is a mitochondrial associated enzyme involved in L-arginine metabolism, hydrolyzing arginine to ornithine and urea.6 We have recently shown that Arg2 promotes an anti-inflammatory state in murine macrophages by modulating mitochondrial dynamics, enhancing oxidative phosphorylation (OxPhos) and regulating IL-1β secretion.7 Importantly, we have also shown that Arg2 is a target of miR-155 in macrophages.7 MicroRNAs (miRNAs) are evolutionarily conserved non-coding RNAs that negatively regulate gene expression by binding to the 3’ untranslated region (UTR) of the target mRNA and repressing translation or decreasing mRNA stability. MiR-155 over-expression is closely associated with various inflammatory disorders and miR-155 inhibition with an anti-miR-155 has shown encouraging results in terms of limiting disease progression in animal models.8–14 However, this strategy could be prone to off-target effects on unintended physiological miR-155 targets, as well as to co-inhibition of other miRNAs.15

TSBs are locked-nucleic acid antisense oligonucleotides that specifically compete with miRNAs for the binding to individual miRNA recognition elements (MREs) of a target mRNA, hence preventing them from gaining access to those sites and ultimately leading to increased levels of the target protein. Recently, a TSB targeting the interaction between miR-10a and ligand-dependent nuclear receptor corepressor (Lcor) was shown to decrease mitochondrial respiration...
in macrophages and increase atherosclerotic lesion formation in the aortic arch compared with control TSB. In non-macrophage related contexts, TSBs have been proposed as therapeutics for cystic fibrosis and cancer.

In this work, we designed a TSB that specifically blocked the interaction between Arg2 mRNA and miR-155 (henceforth called Arg2 TSB) in murine macrophages. We assessed whether Arg2 TSB, by increasing the expression of Arg2, could modulate macrophage phenotype in a variety of in vitro assays and we further evaluated the effect of Arg2 TSB in an in vivo model of acute inflammation.

**RESULTS**

**Arg2 TSB effectively blocks miR-155-mediated repression of Arg2**

To design and test a TSB for specific inhibition of miRNA targets, the MRE within the miRNA of interest needs to be identified and experimentally validated in the appropriate cellular context. *In silico* predictions (TargetScan v7.1) and the previous literature identified an MRE for miR-155 at position 30–37 of Arg2 3’ UTR (Figure 1A). Previous studies have shown that miR-155 mimic can repress luciferase activity in 293T cells when Arg2 3’ UTR is cloned into psiCHECK-2 reporter plasmid. We confirmed this work in macrophages using the RAW 264.7 murine macrophage cell line by illustrating that the over-expression of miR-155 using a miR-155 mimic can inhibit Arg2 3’ UTR luciferase activity in a pmir_Arg2_wt plasmid (Figure 1B, first and second bars), an effect that was lost when the miR-155 seed region is mutated (Figure 1B, fifth and sixth bars). We designed a sequence-specific TSB that binds to position 23–38 of Arg2 3’ UTR, which should prevent miR-155 from binding and having its desired effect. In Figure 1B, the Arg2 TSB was able to significantly rescue the miR-155-dependent inhibition of pmir_Arg2_wt plasmid (Figure 1B, third and fourth bars, compared with the first and second bars). However, this effect was lost in the mutant plasmid where the miR-155 binding site at position 30–37 was disrupted (Figure 1B, seventh and eighth bars, compared with the fifth and sixth bars), demonstrating that Arg2 TSB specifically blocks the interaction between miR-155 and Arg2 3’ UTR through the MRE at position 30–37.

Next, we wanted to evaluate whether Arg2 TSB was able to interfere with the miR-155-dependent regulation of endogenous Arg2 levels in an inflammatory context. MiR-155 up-regulation was achieved in vitro by stimulating primary bone marrow-derived macrophages (BMDM) (Figure 1C) and RAW 264.7 murine macrophage cell line (Figure S1A) with lipopolysaccharide (LPS), a Toll-like receptor 4 agonist with a well established ability to potentely boost miR-155 expression downstream of NFκB activation in macrophages. No significant difference in miR-155 expression was observed between negative control (NC) and Arg2 TSB-transfected cells, neither when unstimulated nor when treated with LPS. We then measured the endogenous levels of Arg2 mRNA and protein in BMDM and RAW 264.7 cells after TSB transfection, either in the presence or absence of LPS. Our results show that Arg2 TSB could not boost Arg2 endogenous levels in the absence of LPS. However, in the presence of LPS, a 1.37-fold (p < 0.001) (Figure 1D) and 2.55-fold (p < 0.0001) (Figure S1B) increase in Arg2 mRNA was observed in Arg2 TSB-transfected BMDM and RAW 264.7 cells, respectively, when compared with NC TSB-transfected cells. This was mirrored at the protein level, where we found Arg2 increases of 1.33-fold (p = 0.029) (Figure 1E) and 1.74-fold (p = 0.015) (Figure S1C) in the same cells.

We next sought to investigate arginase catalytic activity (conversion of L-arginine to L-ornithine) by measuring the by-product urea in TSB-transfected cells. A 1.53-fold (p = 0.0018) and 1.45-fold (p = 0.0046) increase in arginase activity was measured in Arg2 TSB-transfected BMDM (Figure 1F) and RAW 264.7 cells (Figure S1D), respectively, in presence of LPS when compared with NC TSB-transfected cells.

Overall, these results demonstrate that Arg2 TSB can effectively block miR-155-mediated repression of Arg2 by preventing its access to the binding site located at position 30–37 of Arg2 3’ UTR, ultimately leading to increased levels of endogenous Arg2 mRNA, protein, and enzymatic activity in LPS-stimulated cells.

**Arg2 TSB modulates surface markers and metabolic parameters in macrophages**

We have recently shown that Arg2 plays a pivotal role in resolving a pro-inflammatory phenotype in macrophages. After confirming that Arg2 TSB was indeed able to boost Arg2 expression in macrophages, we sought to assess whether this increase had an effect on macrophage phenotype in a variety of biological in vitro assays.

First, we assessed the impact of Arg2 TSB transfection (in absence and presence of LPS) on macrophage polarization by assessing the expression of a panel of pro-inflammatory (CD80 and CD86) and anti-inflammatory (CD71 and CD206) macrophage surface markers by flow cytometry. In the absence of LPS, we did not observe any significant differences in any marker expression. However, in LPS-treated BMDM, Arg2 TSB-transfected cells showed a significant decrease of the pro-inflammatory marker CD80 (Figure 2A) and a significant increase of the anti-inflammatory marker CD71 (Figure 2C) when compared with NC-transfected macrophages. Levels of CD86 were also decreased (but not significantly) upon Arg2 TSB transfection (Figure 2B), while CD206 levels were not changed (Figure 2D).

Next, we assessed the impact of Arg2 TSB transfection on macrophage metabolism. OxPhos is a key metabolic process at the mitochondria that produces energy in aerobic conditions. When macrophages are in a pro-inflammatory stage (e.g., when stimulated with LPS), they decrease their commitment to OxPhos to favor glycolysis by reducing their basal oxygen consumption rates (OCR), maximal respiratory capacity (MRC), and OxPhos-linked ATP production, while an intact OxPhos metabolism is observed in anti-inflammatory macrophages. We have recently shown OxPhos is enhanced by the presence of Arg2 at the mitochondria. Therefore, here we investigated whether Arg2 TSB transfection in macrophages would increase OxPhos parameters (OCR, MRC, and ATP production) using metabolic flux analysis in both the absence and presence of LPS. In
unstimulated BMDM, we observed that OCR, MRC, and ATP production were significantly increased in Arg2 TSB-transfected cells (Figures 2E and 2F, green vs purple line/bar). In cells stimulated with 10 ng/mL LPS for 24 h (Figures 2E and 2F, blue vs red line/bar), we observed a significant increase in MRC and an increased trend in basal OCR and ATP production, albeit not statistically significant. We observed very similar results in RAW 264.7 cells upon transfection with TSBs in presence and absence of LPS (Figures S1E and S1F). Overall, this suggests that Arg2 TSB-transfected macrophages boost their oxidative metabolism, even in the presence of a powerful agonist such as LPS, although to a lesser extent than when unstimulated.

Figure 1. Arg2 TSB effectively blocks miR-155-mediated repression of Arg2
Data are presented as mean ± SEM and were compared by two-way ANOVA (using multiple comparisons test, *p ≤ 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). (A) Visual map of the in silico predictions of miR-155 binding to its MRE within Arg2 3' UTR. (B) Arg2 3' UTR luciferase activity reported as percent change in relative light units (RLU) in RAW 264.7 cells. Site-directed mutagenesis was applied to pmir_Arg2_wt vector to disrupt the miR-155 MRE. RAW 264.7 cells were co-transfected with wt (first to fourth bars) or miR_155 mutant (fifth to eighth bars) plasmid and TSB and miR mimic (100 ng plasmid, 100 nM TSB, 25 nM mimic, n = 3, in triplicate). Samples co-transfected with WT plasmid, non-targeting control (NC) TSB and NC miR mimic were used as reference and set at 100%. For both WT and miR_155 mutant plasmids, only the comparison between NC and Arg2 TSB (co-transfected with NC or miR-155 mimics) are highlighted in the graph. (C) miR-155 expression upon stimulation with LPS (100 ng/mL, 24 h) and TSB transfection in primary BMDM (n = 4, in triplicate). LPS-stimulated samples transfected with NC TSB are set as 1. (D) Arg2 TSB effect on endogenous levels of Arg2 mRNA in primary BMDM (n = 4, in triplicate). Unstimulated samples transfected with NC TSB are set as 1. (E) Arg2 TSB effect on endogenous levels of Arg2 protein in primary BMDM (n = 3, in single). LPS-stimulated samples transfected with NC TSB are set as 1. (F) Arginase activity assay in primary BMDM upon transfection of Arg2 TSB (n = 3, in triplicate). Urea was measured as a byproduct of arginase activity and fold arginase activity was measured by setting LPS-stimulated samples transfected with NC TSB as 1.

Arg2 TSB encapsulated in biocompatible poly lactic-co-glycolic acid nanoparticles decreases pro-inflammatory cytokines secretion in macrophages
We have previously shown that Arg2 can limit IL-1β production in LPS-stimulated macrophages, suggesting this enzyme could play a role in modulating secretion of pro-inflammatory cytokines in macrophages. We, therefore, investigated the secretion of pro-inflammatory mediators including IL-6, tumor necrosis factor (TNF), and, most important, IL-1β upon TSBs transfection. While we have observed functional effects of Arg2 TSB in BMDM when transfected with classical transfection reagents (i.e.,
lipofectamine 3000) in flow cytometry and metabolic analyses (Figures 2A–2F), we did not measure any significant changes in cytokine levels when BMDM were transfected with Arg2 vs NC TSBs using lipofectamine 3000 (Figure 3A). Therefore, we encapsulated TSBs in polyactic-co-glycolic acid (PLGA) nanoparticles (NPs) as a proof-of-principle delivery strategy to macrophages where nucleic acid cargo could exert its therapeutic action more efficiently owing to protection from degradation by using a biocompatible polymeric carrier (PLGA). NPs were prepared using PLGA loaded with NC or Arg2 TSB (and a blank empty PLGA). Their diameter was 231.3 ± 6.9, 229.7 ± 5.3, and 244.8 ± 8.3 nm for the empty, NC TSB-, and Arg2 TSB-PLGA NPs, respectively and the polydispersity index value was of approximately 0.1 for all the systems (Figure 3B). All NPs are negatively charged with zeta-potential ranging from −11 mV to −15 mV on average (Figure 3C). Transmission electron microscopy confirmed the size range of approximately 200 nm and showed a round morphology, as expected (Figure 3D). Importantly, unlike transfection with lipofectamine, PLGA NPs did not decrease cell viability or increase cytotoxicity when compared with untransfected macrophages (Figures 3E, 3F for BMDM, S2A and S2B for RAW 264.7 cells).

Our results show that the encapsulation of Arg2 TSB, and hence possibly its greater stability and lower toxicity, is required to obtain a significant decrease in pro-inflammatory mediators in transfected cells in vitro. Importantly, we showed that IL-1β secretion is significantly decreased to approximately 32% (p = 0.0009) in BMDM (Figure 3G), when transfected with Arg2 TSB-PLGA compared to NC TSB-PLGA. IL-6 and TNF levels significantly decreased to approximately 56% (p < 0.0001, Figure 3H) and approximately 80% (p = 0.02, Figure 3I) also. Similar effects were also observed in the RAW 264.7 macrophage cell line, where Arg2 TSB transfection, when encapsulated into PLGA NPs, led to significant decrease in IL-1β, IL-6, and TNF (Figure S2C, fifth to tenth bars in each graph). Overall, these data demonstrate that biocompatible PLGA NPs could represent a valuable delivery strategy for modulating secretion of pro-inflammatory cytokines by macrophages.

**Arg2 TSB delivery decreases cytokine secretion in an in vivo model of acute inflammation**

Given these in vitro observations, we next sought to test the efficacy of Arg2 TSB in an in vivo model of acute inflammation where mice were injected with 200 µg TSB (10 mg/kg dose) and subsequently challenged with an intraperitoneal injection (i.p.) of 5 mg/kg LPS for 8 h. We first checked Arg2 TSB ability of enhancing Arg2 expression in the LPS in vivo model. Arg2 mRNA (Figure 4A) and protein (Figure 4B) levels were increased in peritoneal exudate cells (PECs) and spleen of mice injected with Arg2 TSB when compared with NC TSB. We next measured the pro-inflammatory mediators IL-6, TNF, and IL-1β to assess whether increasing Arg2 expression through Arg2 TSB could lead to the decrease of these cytokines, similar to what we observed in vitro. In serum, Arg2 TSB-injected mice had significantly lower levels of IL-1β (p = 0.01, a decrease from 313.8 ± 110.6 to 42.02 ± 12.97 pg/mL) (Figure 4C) and IL-6 (p = 0.04, a decrease from 18,889 ± 2174 to 13,925 ± 1619 pg/mL) (Figure 4D) when compared with NC TSB-injected mice in the presence of LPS challenge. TNF levels were also decreased, albeit not statistically significantly (Figure 4E). Overall, these in vivo data support the hypothesis that a therapeutic strategy aimed to specifically increase Arg2 expression could lead to lower systemic levels of pro-inflammatory mediators that could potentially dampen the overall inflammatory status in a context of acute inflammation.

**DISCUSSION**

This work shows that a TSB specifically designed to block the interaction between miR-155 and Arg2 mRNA can enhance Arg2 expression and modulate its function as a regulator of the inflammatory status of macrophages. Arg2 is a mitochondrial protein involved in arginine metabolism; however, we and other investigators have shown that its role in macrophages goes beyond its enzymatic activity and places Arg2 as a key player in the resolution of the inflammatory response and mediators IL-6, TNF, and IL-1β. Interestingly, we observed that Arg2, thus avoiding widespread off-targets effect (typical of anti-miR strategies owing to the intrinsic ability of miRNAs to regulate multiple mRNAs) and the over-expression of Arg2 when it is not required. Moreover, the TSB will only work in situations when miR-155 expression levels are high, i.e., in cases of inflammation.
Once the TSB was designed, we first verified that Arg2 TSB was specific for miR-155 MREs on the Arg2 3’ UTR via luciferase assay and effective at increasing Arg2 expression and enzymatic activity in murine primary macrophages and macrophage cell line. Arg2 TSB transfection and the subsequent increase of Arg2 mRNA did not significantly affect miR-155 expression levels. This was expected, as TSBs do not interact with miRNAs but with their target mRNAs; however, it was important to show given the mounting evidence about a regulatory mechanism called target RNA-directed microRNA degradation (TDMD), whereby high complementarity between miRNA and target mRNAs can actually trigger the degradation of the bound miRNA.28-30 Our data did not show TDMD happening upon Arg2 TSB transfection, and this is compatible with the literature around TDMD showing that a more extensive pairing through the 3’ region of the mRNA, not present in the interaction between miR-155 and Arg2 3’ UTR, seems to be required for the interaction.

Once we had verified the ability of Arg2 TSB to boost Arg2, we assessed whether it was able to recapitulate some of the features we identified as being modulated by Arg2 in murine macrophages. Previously,7 we have shown in detail that Arg2 can skew macrophages and secretion of the pro-inflammatory cytokines in murine macrophages and macrophage cell line. Arg2 TSB transfection and we showed TDMD happening upon Arg2 TSB transfection, and this is compatible with the literature around TDMD showing that a more extensive pairing through the 3’ region of the mRNA, not present in the interaction between miR-155 and Arg2 3’ UTR, seems to be required for the interaction.

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Figure 4. Arg2 TSB delivery decreases pro-inflammatory cytokines secretion in an LPS in vivo model

WT C57Bl/6J mice were given i.p. injection of Arg2 vs NC TSB for 24 h followed by LPS at 5 mg/kg (or PBS control) for 8 h. (A) Arg2 mRNA expression in PECs (left) and spleen (right). Data are presented as mean ± SEM and were compared by one-way ANOVA where mice injected with NC TSB and LPS were set at 1. (B) Arg2 and β-actin in spleen. Blot representative of seven mice from each TSB injection (three of which were then injected with PBS and four with LPS for 8 h). (C) Pro-inflammatory cytokines levels in serum in the LPS model of acute inflammation. Data are presented as mean ± SEM and were compared by one-way ANOVA (Sidak’s multiple comparison test, *p < 0.05).
their polarization toward an anti-inflammatory state. To the best of our knowledge, this is the first report of an effect of Arg2 modulation on surface markers, and although the mechanisms governing this are not understood yet these data further corroborate the involvement of Arg2 in determining the polarization status of macrophages.

There are limitations to this study. While we observed functional effects of Arg2 TSB in multiple assays (flow cytometry, metabolic analysis, cytokine secretion) and could suggest a rationale behind some given our previous investigation, it was not possible to explore all mechanisms underpinning these functional changes as they were beyond the scope of this work. Ideally, a proteomic study upon transfection of Arg2 TSB could have informed more accurately about the selection of pro- and anti-inflammatory markers to be measured in vitro and in vivo. We did not test Arg2 TSB when encapsulated in the PLGA NPs in the LPS injury model because the TSBs were in vivo ready and the amount of NPs for in vivo injection would have required a scale-up of NP preparation which was not feasible. Finally, while this work represents a proof of concept of therapeutic strategy aimed to reprogram macrophages in murine in vitro and in vivo models, further investigation on the role of Arg2 in human macrophages and its therapeutic potential needs to be carried out.

In conclusion, in this work we have shown that, by blocking the specific interaction between miR-155 and its key targets Arg2 by using a chemically stable TSB, macrophages are able to shift their physiological state away from a pro-inflammatory phenotype toward an anti-inflammatory one. This strategy could be beneficial in the context of diseases mediated by macrophages such as inflammatory, autoimmune or neurological diseases, where it is desirable to reprogram macrophages from a pro-inflammatory toward an anti-inflammatory state as a therapeutic avenue to control inflammation and promote repair.

MATERIALS AND METHODS

Cell culture and treatments

All cells were incubated in a humidified incubator at 37°C with 5% CO2 levels.

RAW 264.7 murine macrophage cell line was obtained from ATCC and cultured in Dulbecco’s Modified Eagle’s Medium (Sigma, Cat# D5796) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, Cat# F9665) and 1% penicillin-streptomycin (pen/strep, 100 U/mL, Sigma, Cat# P4333). Cells were routinely tested to be Mycoplasma negative. Cells were passaged twice a week (1:10) in complete RPMI-1640 supplemented with 10% heat-inactivated FBS and 1% pen/strep. We also added 20% L929-conditioned media to the culture to induce BMDM differentiation, after which cells were incubated for 6 days. In experiments, BMDM were seeded and stimulated in complete DMEM supplemented with 10% L929-conditioned media.

L929 murine fibroblast cell line was obtained by ATCC and maintained in RPMI medium supplemented with 10% FBS and 1% pen/strep. L929 conditioned media was generated from 20 × 10⁶ L929 cells plated in 40 mL complete RPMI-1640 in T175 flasks for 10 days after which the media was filtered and frozen at −20°C until use.

Fresh media was added to the cells before stimulation experiments. LPS (Sigma Escherichia coli O111:B4) was diluted from stock concentration of 1 mg/mL in complete DMEM, and used at a final concentration of 100 ng/mL (unless specified otherwise). Cells were typically stimulated for 24 h before conducting further assays.

Target site-blocker and dual-luciferase assay

TSBs are locked-nucleic acid antisense oligonucleotides that specifically compete with miRNAs for the binding to individual MREs of a target mRNA, hence preventing them from gaining access to those sites. One TSB was designed to compete with miR-155-5p (miR-155) binding to its specific site within the Arginase-2 (Arg2) 3’ UTR (MRE at position 30–37, as predicted by TargetScan v7.1 http://www.targetscan.org/mmu_71/) and its sequence is GTAATGCTGTTGTGAA (Qiagen, Cat # 339199, ID YT0070992-FDA, MW 5416.27 Da). A scrambled TSB (i.e., not targeting anywhere in the genome, sequence AGCTCTATACGCCCA, Qiagen, Cat# 339199, ID YT0070993-FDA, MW 5016.05 Da) was used as a NC in all experiments.

The full-length (270 bp) murine Arg2 3’ UTR was amplified using Q5 High-Fidelity DNA Polymerase (NEB) and inserted into XhoI-digested pmirGLO vector (Promega) downstream to the firefly luciferase (luc2) reporter gene using the GenBuilder Cloning Kit (Gen-script). Plasmids were isolated from bacterial cultures with the Plasmid Midi Kit (Qiagen, Cat # 12143). To prove the specificity of Arg2-TSB for that particular binding site, a mutagenesis reaction was performed to disrupt its MRE at position 30–37 within the 3’ UTR region using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) using the wt plasmid (i.e., pmir_Arg2_wt) as a template. Presence of the mutation in the mutant plasmid (i.e., pmir_Arg2_mut) was subsequently checked by screening with allele-specific oligonucleotide PCR and Sanger sequencing. The sequence of cloning, mutagenesis, and sequencing primers is reported in Table S1.

In luciferase assay experiments, RAW 264.7 cells were seeded in a 96-well plate at a final density of 80,000 cells/well and incubated for 24 h. Cells were then co-transfected with 100 ng pmir_Arg2_wt or pmir_Arg2_mut and 100 nM of Arg2-TSB or NC TSB and 25 nM suspension was prepared by passing the cells through a 70-μm cell strainer (Corning). They were then plated in 10-cm petri dishes in complete DMEM supplemented with 10% heat-inactivated FBS and 1% pen/strep. We also added 20% L929-conditioned media to the culture to induce BMDM differentiation, after which cells were incubated for 6 days. In experiments, BMDM were seeded and stimulated in complete DMEM supplemented with 10% L929-conditioned media.
analyses. The experiments were performed in triplicate and were used for statistical analyses.

**Gene and protein expression analysis**

For gene expression (miR-155 and Arg2 mRNA), BMDM and RAW 264.7 cells were seeded in a 24-well plate at a final density of 2.5-3.75 x 10^5 cells/well and after 24 h they were transfected with 100 nM Arg2 TSB or NC TSB in DMEM serum-free medium and lipofectamine 3000 transfection reagent (ThermoFisher Scientific). At 24 h after transfection, cells were stimulated with 100 ng/mL LPS as previously stated for a further 24 h. Total RNA was then extracted using Tri-Reagent (Sigma, Cat# T9424). For miR-155 expression, TaqMan MicroRNA Reverse Transcription kit (ThermoFisher Scientific, Cat# 4366596) and predesigned TaqMan MicroRNA Assays (ThermoFisher Scientific, Cat# 4427975, Assay ID 002571 for mmu-miR-155 and 001973 for U6 snRNA) were used for individual miRNA quantification. For Arg2 mRNA expression, equal quantities were reverse transcribed into cDNA using High Capacity cDNA reverse transcription kit (ThermoFisher Scientific, Cat# 4368814) following the manufacturer’s protocol. Quantitative RT-PCR was performed on the 7900 HT and 7500 Real-Time PCR System. Primers for Rplp0, forward 5’-GGATCCAGAAGGTGATGGAA-3’; reverse 5’-AGAGCTGACAGGACACCTG-3’ and three housekeeping genes were used: unstained cells, single stained live/dead cells, and full minus one controls or full minus two controls. Single stain compensation controls were generated using Ecomp beads (Invitrogen, Cat# L3000008) and three housekeeping genes respectively was determined using the C_{(ΔΔCt)} method in at least two independent experiments (in triplicate). Arg2 mRNA levels were also measured in PECs and spleen in the in vivo study using the same primers.

Arginase assay

Arginase activity was determined in BMDM and RAW 264.7 cells at 48 h after transfection (24 h after LPS stimulation) using the Arginase Activity Assay Kit (Sigma, Cat# MAK112-1KT) following the manufacturer’s protocols in three independent experiments (in triplicate). Briefly, cells were lysed in low-stringency lysis buffer followed by the addition of arginine substrate buffer (provided in the kit) for 2 h at 37°C. Reaction was stopped by addition of urea reagent, and incubated for a further 1 h at RT, after which the absorbance was read at 430 nm to quantify the urea produced against a standard curve.

Flow cytometry

BMDM were plated in 12-well plates at a final density of 5 x 10^5 cells/well, transfected with 100 nM Arg2 TSB or NC and stimulated with LPS (10ng/mL) as described above. After 24 h of LPS stimulation, the medium was removed and cells were gently scraped in PBS. Cells were collected by centrifugation at 600 x g, followed by staining using LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (1:1000, Invitrogen, Cat #L10119). Cells were washed in fluorescence-activated cell sorting (FACS) buffer (dPBS, 2% FBS, 1 mM EDTA) and incubated with Fc Block for 10 min on ice (1:100, Biolegend, Cat # 101301). Cells were then incubated with antibodies against extracellular antigens F4/80, CD80, CD86, and CD71 for 30 min on ice, followed by two wash steps in FACS buffer. Cells were fixed and permeabilized using Cyto-Fast Fix/Perm Buffer Set (Biolegend, Cat# 426803) followed by intracellular staining for CD206. All data were acquired on the Attune NxT Flow Cytometer (ThermoScientific). The following controls were used: unstained cells, single stained live/dead cells, and full minus one controls or full minus two controls. Single stain compensation controls were generated using Ecomp beads (Invitrogen, Cat# 01-2222) stained with 0.5 x antibody concentration used for cell staining. Cells were gated on forward scatter side and side scatter, single cells, and live cells. There were 10,000 live events acquired per
sample. RAW FCS files were analyzed using FlowJo Software (FlowJo). Analysis of all markers was performed on the F4/80 positive population. Antibodies listed as follows (fluorochrome, dilution, clone, company, cat number): CD80 (BV421, 1:100, 16–10A1, Biolegend, 104725) CD86 (SuperBright660, 1:160, GL1, Invitrogen, 63-0862-80), F4/80 (Alexa Fluor 488, 1:800, BM8, Biolegend, 123119), CD206 (PercP/Cyanine5.5, 1:80, C068C2, Biolegend, 141715), CD71 (APC, 1:160, RI7217, Biolegend, 113819).

**Metabolic flux analysis**

Cells were plated in 6-well tissue culture plate at a final density of 10^6 cells/well and after 24 h they were transfected with 100 nM Arg2 TSB or NC TSB in DMEM serum-free medium and lipofectamine 3000 transfection reagent (ThermoFisher Scientific). At 6 (BMDMs)-24 (RAW 264.7) hours after transfection, cells were gently scraped, counted, and re-seeded on an XF 96-well plate (Agilent) in complete DMEM (+10% L929 supernatant in media for BMDM) at a final density of 5 × 10^4 cells/well. Cells were then stimulated with 10 ng/mL LPS for further 24 h followed by MitoStress test as described in.7 Five independent experiments (with multiple wells/treatment) were performed for this assay in BMDM, while preliminary data for one experiments was obtained for RAW 264.7 cells.

**Cytokine measurements**

For cytokine measurements, cells were seeded in 96-well plates at a final density of 50–80,000 cells/well. After 24 h, cells were either transfected with Arg2/NC TSB (100 nM) and lipofectamine 3000 as previously stated or with empty PLGA/NC TSB PLGA/Arg2 TSB PLGA NPs resuspended in serum-free DMEM at a NPs concentration of 3.33 mg/mL (typically for each experiment 200 μg of NPs in 600 μL of serum-free DMEM, 100 μL/well) in at least three independent experiments (in triplicate). After 24 h, cells were stimulated with LPS at 100 ng/mL and supernatants removed and analyzed for mouse LPS using the RNeasy Mini kit (Qiagen, Cat# 74106) and stored at –80°C. Spleens were excised, cut in half, snap frozen in liquid nitrogen, and stored at –80°C until time of assay. RNA was extracted from one-half of the spleen by Tri-Reagent extraction and the second half homogenized, assayed for protein quantification by Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Cat # 23227) and western blotting as described above.

**PLGA NPs preparation**

Arg2 TSB and NC TSB (Qiagen) were encapsulated in DOTAP/PLGA NPs using the double emulsion solvent evaporation method as previously described.32,46 Briefly, TSBs were condensed with a cationic lipid DOTAP at an N/P (defined as the molar ratio of amine to phosphate groups) ratio of 4:1. Briefly, for each preparation of 50 mg of PLGA NPs, Arg2 (8.1921 μg) and NC (8.09239 μg) TSB were diluted in 200 μL of RNAse-free water and DOTAP (67.62 μg) was dissolved in 200 μL Terr-butanol. The TSB solution was added dropwise to the lipid mixture, mixed, and lyophilized overnight. For empty PLGA NPs, 200 μL RNAse-free water were added to the lipid mixture, mixed, and lyophilized overnight. We dissolved 50 mg PLGA Resomer RG 502 H (Sigma Cat# 719897) in 2.5 mL chloroform and briefly sonicated. Lyophilized TSB/DOTAP was resuspended in RNAse-free water, added to the PLGA solution dropwise with a glass Pasteur pipette and sonicated for a total of three bursts of 5 s in continuous pulses mode at 70% amplitude to form the primary water-in-oil emulsion. The primary emulsion was added dropwise to a 2% poly(vinyl alcohol) (PVA) solution and sonicated on ice for 10 min in continuous pulses mode at 70% amplitude to form a secondary water-in-oil-in-water emulsion and then added to 2% PVA. The emulsion was mechanically stirred in the fume hood overnight to allow the solvent to evaporate and allow NPs formation. NPs were then collected by centrifugation at 20,000 × g for 15 min at 4°C and washed three times with NaCl 1.13% in deionized water to remove residual PVA. After this, TSB-PLGA NPs were resuspended in RNAse-free water and freeze-dried for 24 h.

**PLGA NPs characterization and morphology**

Size and zeta-potential of the TSB-PLGA NPs were measured by dynamic light scattering and by laser Doppler electrophoresis, respectively, on a Zetasizer Nano Series (Malvern Instruments). Measurements were made at RT. PLGA NPs were prepared at a concentration of 0.5 mg/mL in deionized water and 1 mL was used for measurement in the instrument. At least three independent batches of NPs, each prepared in triplicate, were used to determine the size distributions and the surface charge of the PLGA-LSB NPs. NPs were visualized by transmission electron microscopy to further confirm size and determine the morphology. Briefly, PLGA-LSB NPs were prepared at a concentration of 1 mg/mL in deionized water. We placed 5 μL NPs suspension on a Silicon Monoxide/Formvar coated grid (Mason Technologies). Samples were allowed to air dry for approximately 10–15 min before being negative stained with 2% uranyl acetate alternative solution. Excess stain was removed using...
TBS-PLGA NPs effect on macrophage viability

RAW 264.7 cells and BMDM were seeded in 96-well plates at a final density of 40–80,000 cells/well. After 24 h, cells were either left untransfected (control) or transfected with Arg2/NC TSB (100 nM) using lipofectamine 3000 as previously stated or with empty/NC TSB/Arg2 TSB PLGA NPs resuspended in serum-free DMEM at a NPs concentration of 3.33 mg/mL (typically for each experiment 200 μg of NPs in 600 μL serum-free DMEM, 100 μL/well) in three independent experiments (in triplicate). The impact of PLGA NPs on cell viability was assessed using CellTititer 96 Aqueous One Solution Cell Proliferation MTS Assay (Promega, Cat# G3582). To check the cytotoxicity of TSB-PLGA NPs, supernatants were used to measure lactate dehydrogenase release from dying cells using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Cat# G1780).

Statistical analyses

Analyses were performed using GraphPad PRISM 8.0. Results are expressed as mean ± SEM and compared as indicated using one-way or two-way ANOVA, as appropriate, followed by Tukey’s, Dunnett’s, or Sidák’s multiple comparisons tests. Differences were considered statistically significant when p ≤ 0.05.

Data availability statement

All data generated or analyzed during this study are included in this published article (and its supplementary information files).
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