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Supplemental information

Inhibition of pro-inflammatory signaling in human primary macrophages by enhancing arginase-2 via target site blockers

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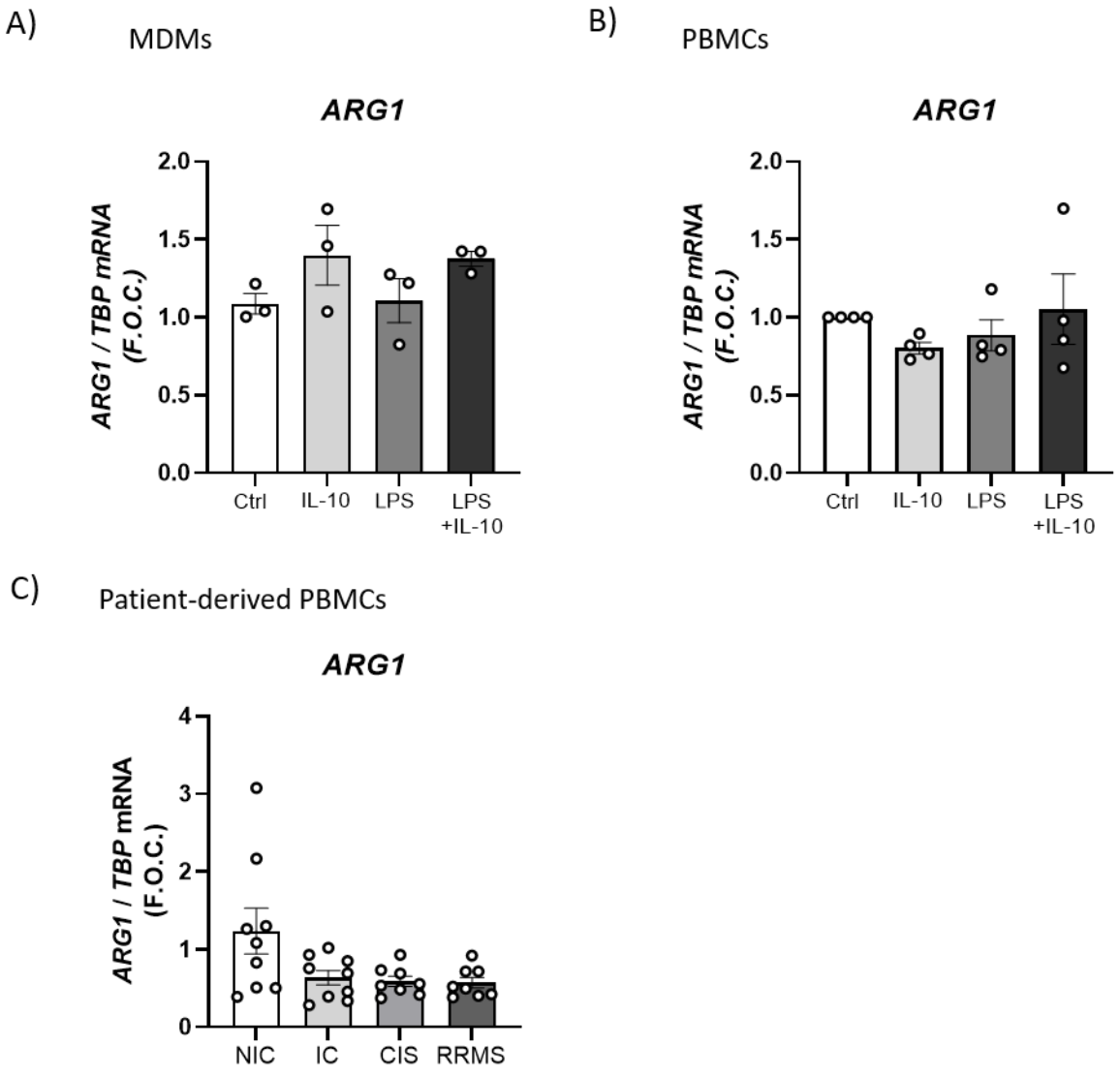

Figure S1: *Arg1* expression in stimulated human MDMs and PBMCs and in unstimulated patient derived PBMCs and miRNA expression in MDMs. A) *ARG1* expression was assessed using quantitative RT-PCR in stimulated MDMs (n=3). **B)** *ARG1* expression was assessed using quantitative RT-PCR in stimulated PBMCs (n=4). **C)** *ARG1* was analysed in human PBMCs from four different participant groups; NIC-non-inflammatory controls (n=9), IC-inflammatory controls (n=9), CIS-clinically isolated syndrome (n=9) and in patients with RRMS- Relapsing Remitting Multiple Sclerosis (n=9) where samples were taken during the remission phase. *TBP* was used as the endogenous control. Results were graphed as fold over control (F.O.C.). Statistical analysis: Graphs A and B were analysed using a one-way ANOVA and Tukey's multiple comparisons test and graph C was analysed using a Kruskal-Wallis Test and Dunn's multiple comparisons test.

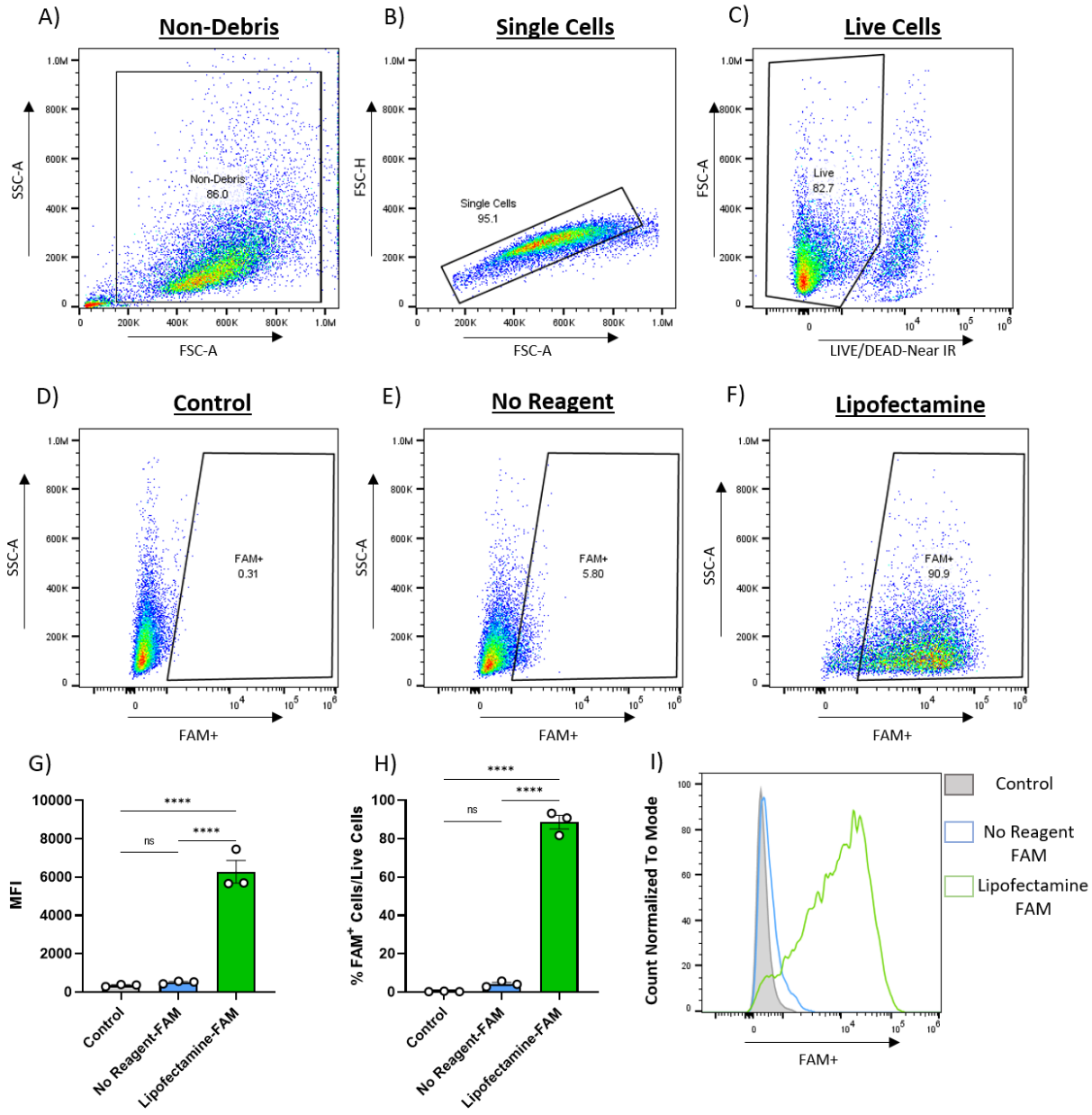

Figure S2: Target site blocker (oligonucleotide) uptake in MDMs. Negative Control A Sequence Target Site Blocker conjugated to the fluorescent dye, FAM, (NC-TSB) (50nM) was transfected into MDMs using Lipofectamine 3000 for 5hrs in serum free RPMI. NC-TSB (50nM) with no Lipofectamine was used as the 'No Reagent FAM' control. MDMs were also analysed without NC-TSB (Control). Cells were rested overnight and then prepared for flow cytometry analysis. **A)** Gates were established on the non-debris followed by **B)** Single cell gating. **C)** Gates were established on the live cell population. Live cells were analyzed for FAM+ MDMs in the following treatment conditions **D)** Control, **E)** No Reagent and **F)** Lipofectamine. **G)** Median fluorescent intensity (MFI) and **H)** the percentage of FAM+ cells relative to live cells were graphed (n=3 independent experiments). A representative histogram highlighting the changes in MFI of FAM under the 3 conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison test for graphs G-H. Not statistically significant (ns). ****=p<0.0001.

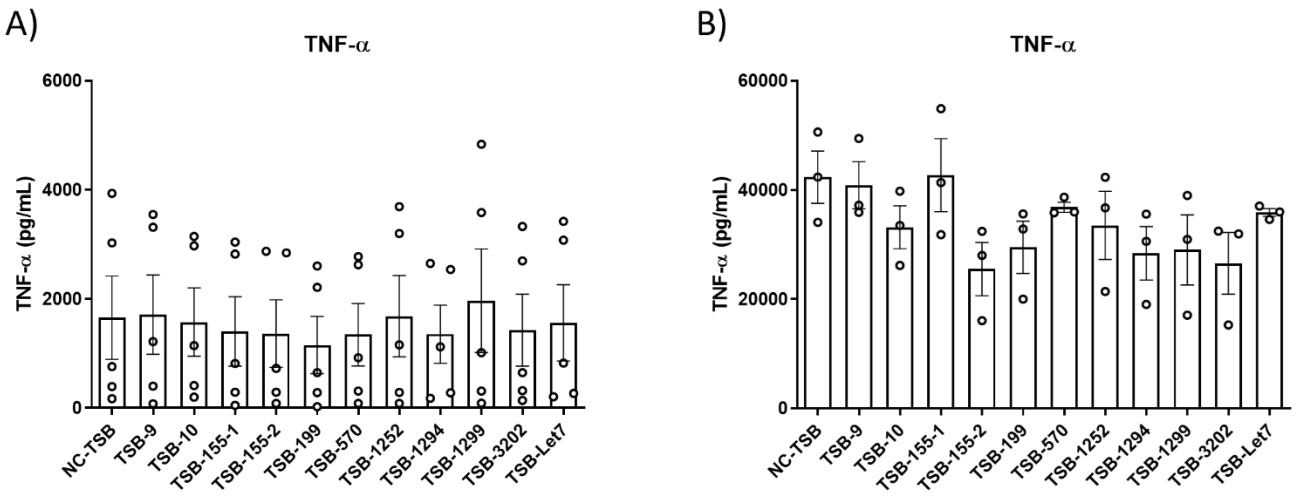

Figure S3: TNF-α secretion from MDMs treated with TSBs and LPS. 11 Target site blockers (100nM) were transfected into MDMs using Lipofectamine 3000. Cells were rested overnight and then stimulated with and without LPS (100ng/mL) for 24hr. Supernatants were collected from 8 independent experiments (n=8 donors) using experimental triplicates. Supernatants were analysed for TNF-α by ELISA and raw values were graphed with the **A)** lower concentrations and **B)** higher concentrations represented on independent graphs due to spread of the data. Statistical analysis was performed using multiple unpaired *t*-tests.

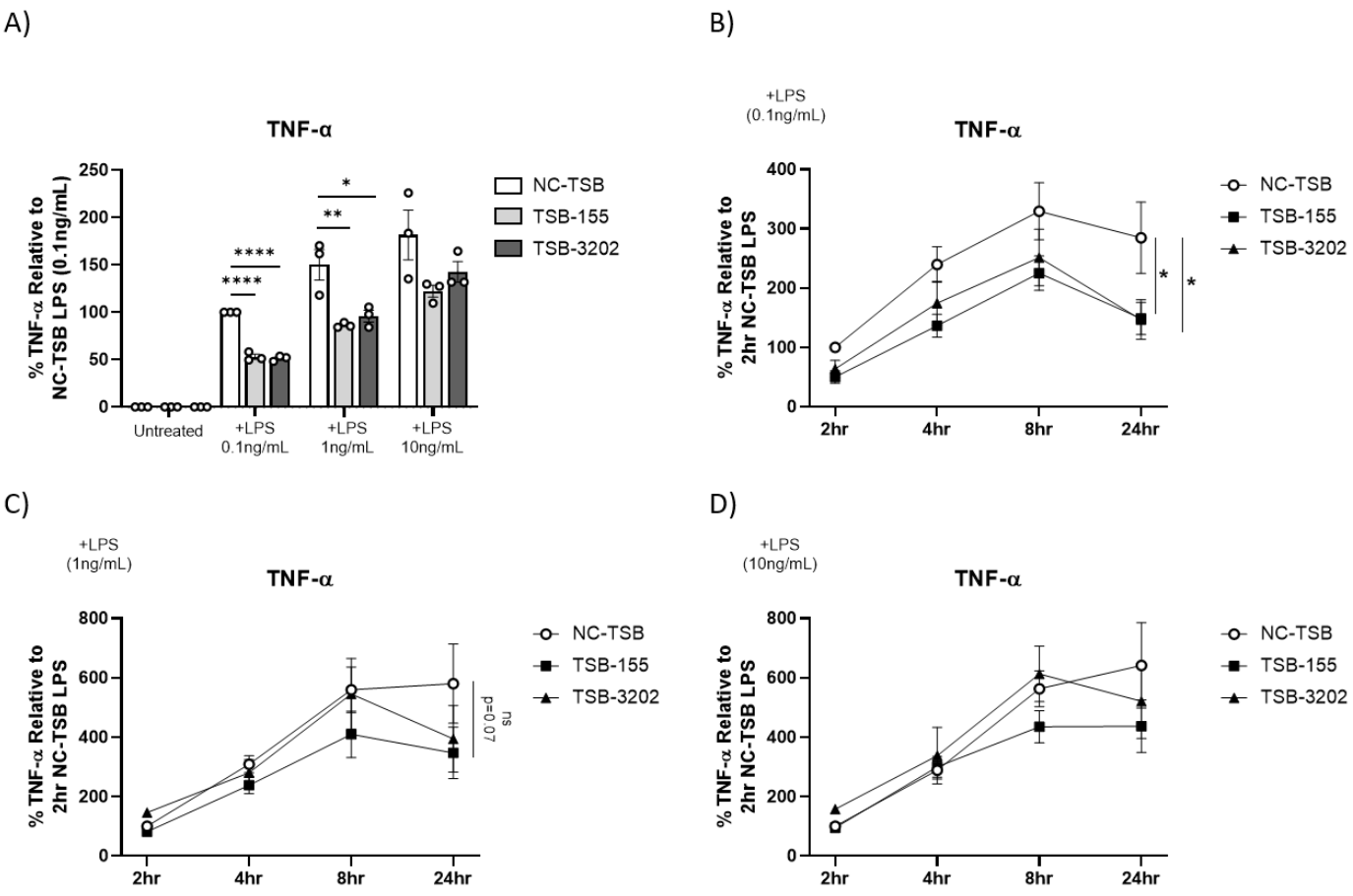

Figure S4: Effects of Arg2 target site blockers (TSBs) on TNF-α secretion from human MDMs following LPS stimulation. TSB-155 and TSB-3202 (100nM) were transfected into MDMs using Lipofectamine 3000 for 5hr and NC-TSB as the control, cells were rested overnight and then stimulated with and without LPS 0.1ng/mL, 1 ng/mL and 10 ng/mL for 24hr. **A)** Supernatants were analysed by ELISA for TNF-α (n=3). TNF-α (n=3) and graphed as a percentage relative to NC-TSB stimulated with LPS. TNF-α was analysed by ELISA in supernatants harvested at 2hr, 4hr, 8hr and 24hr post LPS stimulation at a concentration of **B)** 0.1ng/mL **C)** 1ng/mL and **D)** 10ng/mL (n=3). Statistical analysis was performed on graph A using multiple one-way ANOVAs with Dunnett's multiple comparison test. Graphs C and D were analyzed using a two-way ANOVA. ns= not statistically significant, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

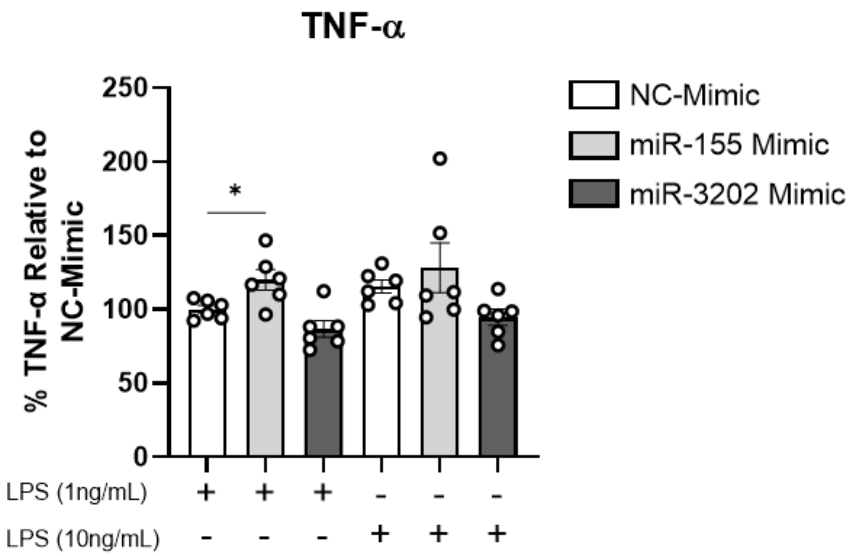

Figure S5: Effects of miR-155 and miR-3202 on TNF-α secretion from human MDMs stimulated with LPS. miR-155 mimic (40nM) and miR-3202 mimic (40nM) were transfected into human MDMs using Lipofectamine 3000 for 5hr using the negative control (NC) mimic as the control. The media was changed, and cells were rested overnight. Cells were stimulated with 1 ng/mL of LPS or 10ng/mL of LPS for 24hr. Supernatants were analysed for TNF-α by ELISA. 3 independent experiments were performed (n=3) and graphed using biological duplicates. Statistical analysis was performed using multiple unpaired t-tests.*=$p<0.05$.

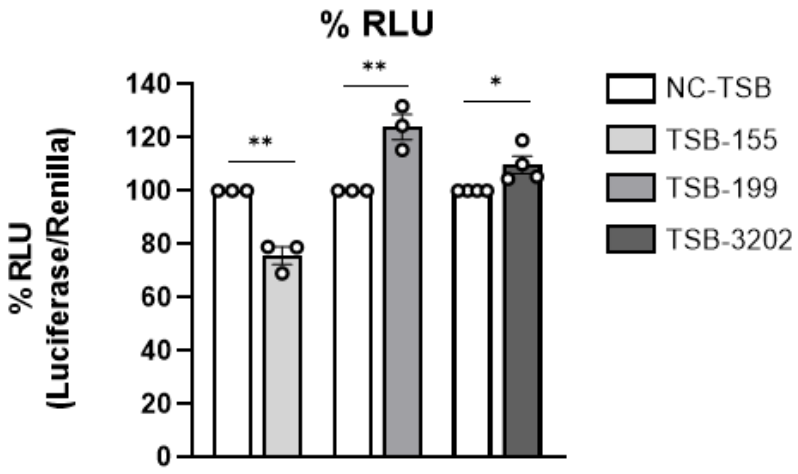

Figure S6: Effect of TSB-155, -199 and -3202 in *ARG2* luciferase assay. TSB-155, -199, -3202 were assessed using a luciferase reporter activity assay of the human *ARG2* 3′UTR and reported as percentage relative light units (RLU). 3-4 independent experiments were performed in triplicate and the averages were graphed. Statistical analysis was performed using multiple independent unpaired t-tests. *= $p<0.05$ and **=$p<0.01$.

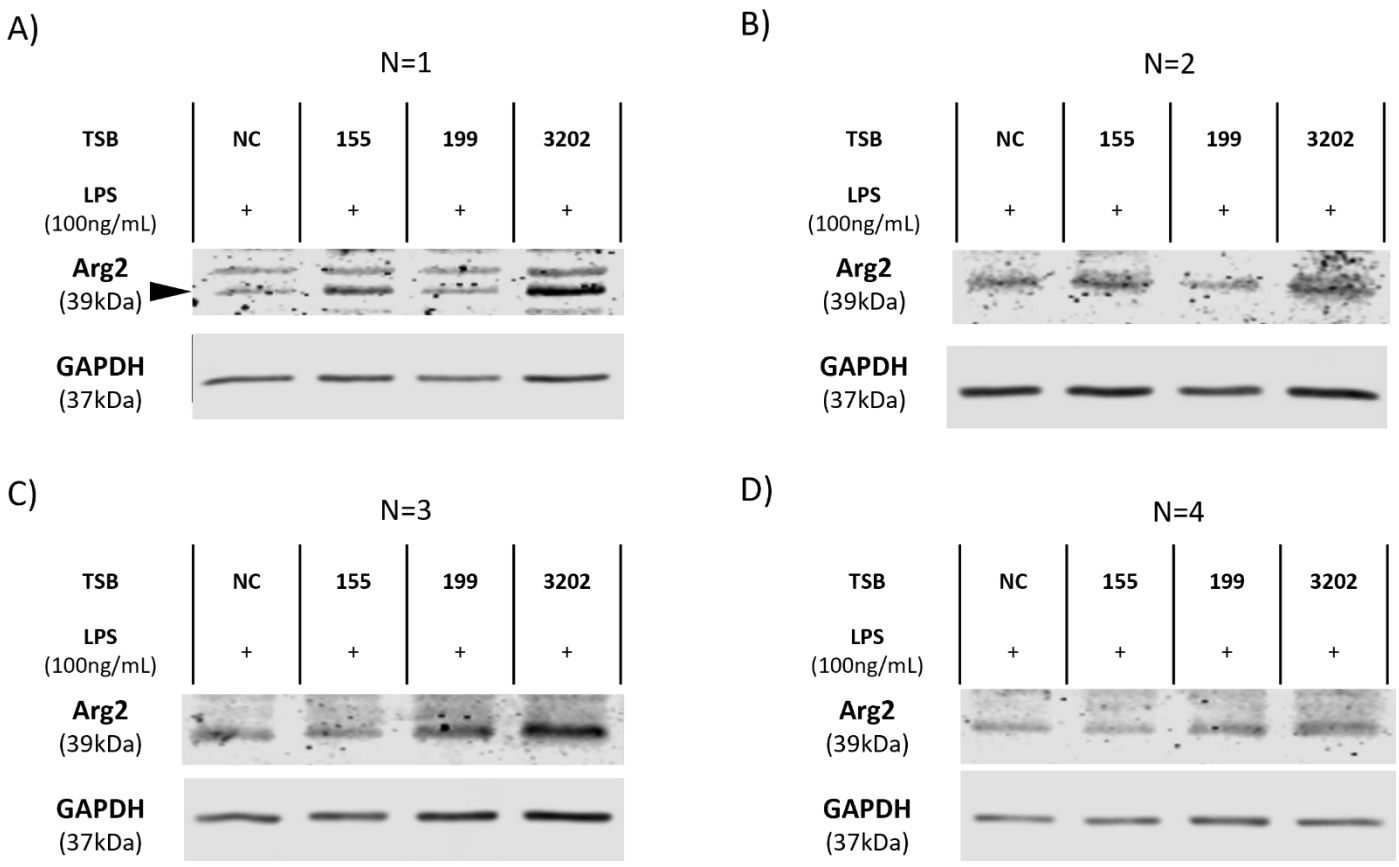

Figure S7: Arginase-2 protein expression in MDMs. Target site blockers (100nM) were transfected into MDMs using Lipofectamine 3000. Cells were rested overnight and then stimulated with LPS (100ng/mL) for 24hr. Western blotting was performed and membranes were imaged using the Odyessy® CLx imaging system. Arginase-2 and GAPDH were analysed in four independent experiments using blood from four different donors. **A)** N=1, **B)** N=2, **C)** N=3, **D)** N=4.

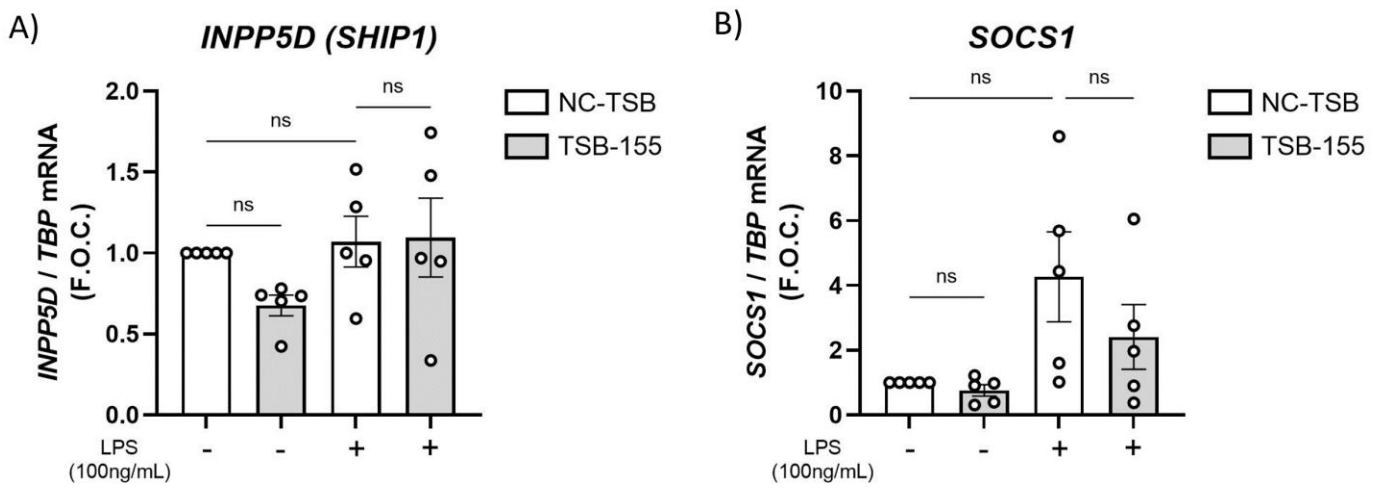

Figure S8: Effects of Arg-2 Target site blocker 155 (TSB-155) on miR-155 targets *INPP5D* (*SHIP1*) and *SOCS1*. Target site blocker 155 (TSB-155) (100nM) was transfected into MDMs using Lipofectamine 3000 using Negative control TSB (NC-TSB) as the control. Cells were rested overnight and then stimulated with LPS (100ng/mL) for 24hr. **A)** *INPP5D (SHIP1)* and **B)** *SOCS1* were analysed by RT-PCR using *TBP* as the endogenous control (n=5 independent experiments). Statistical analysis was performed using a one-way ANOVA (Kruskal-Wallis test) with Dunn's multiple comparison applied. ns = not statistically significant.

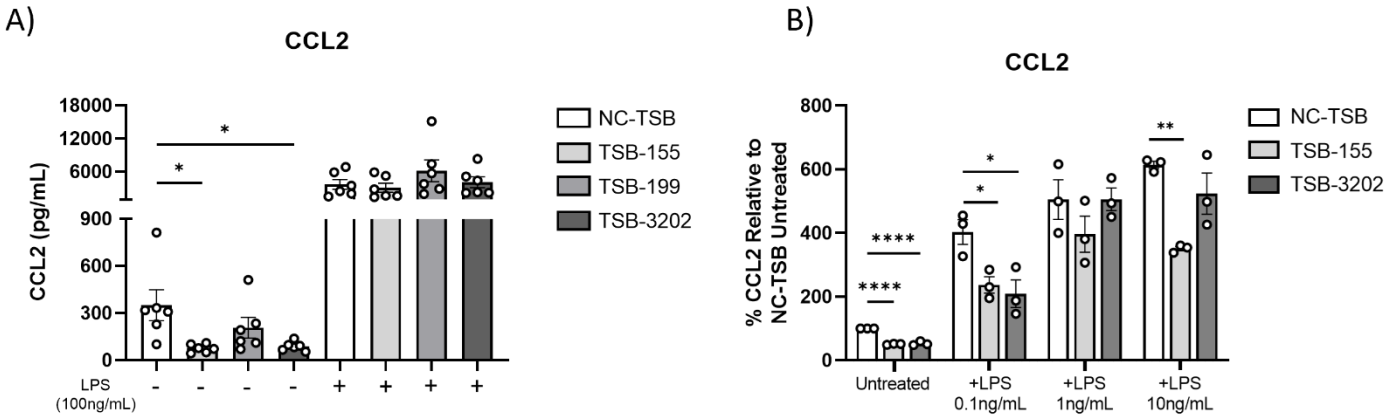

Figure S9: Effects of TSBs on CCL2 in human MDMs stimulated with LPS. A) TSB-155, -199 and -3202 (100nM) were transfected into MDMs using NC-TSB as the control. Cells were rested overnight and then stimulated with LPS (100ng/mL) for 24hrs. MDM supernatants were analysed by ELISA for CCL2 using biological duplicates (n=6 independent experiments) and graphed in pg/mL. **B)** TSB-155, -199 and -3202 (100nM) were transfected into MDMs using NC-TSB as the control. Cells were rested overnight and then stimulated with and without different doses of LPS; 0.1ng/mL, 1ng/mL and 10ng/mL for 24hrs (n=3 independent experiments). CCL2 was graphed as a percentage relative to the unstimulated NC-TSB. Statistical analysis was performed using a one-way ANOVA with Dunnett's multiple comparison test.*=$p<0.05$.

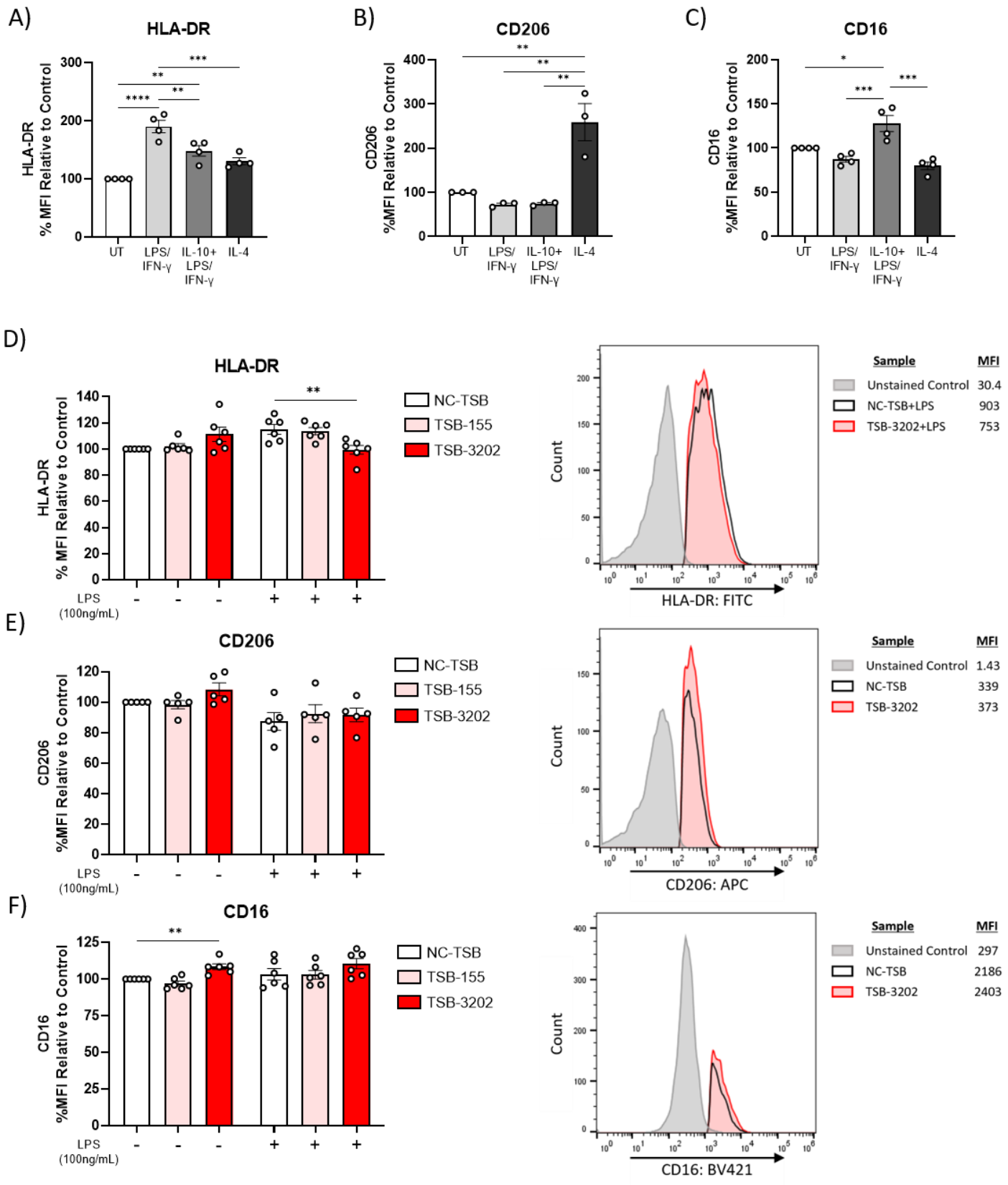

Figure S10: Flow cytometry analysis of the effects of Arg2 TSBs on human MDM phenotype. Human MDMs were treated for 48hr with the following stimuli to induce various phenotypes: LPS (100ng/mL) + IFN-γ (20ng/mL), IL-10 (100ng/mL) + LPS (100ng/mL) + IFN-γ (20ng/mL), or IL-4 (20ng/mL) using untreated MDMs

(M0) as the control. **A)** HLA-DR, **B)** CD206 and **C)** CD16 were analysed in polarised MDMs by flow cytometry and the percentage median fluorescent intensity (MFI) was calculated relative to the M0 control ($3 \leq n \leq 4$). In parallel, TSB-155 and TSB-3202 (100nM) were transfected into MDMs using NC-TSB as the control. Cells were rested overnight and then stimulated with LPS (100ng/mL) for 24hrs. **D)** HLA-DR, **E)** CD206 and **F)** CD16 were analysed in TSB-treated MDMs by flow cytometry and the percentage MFI was calculated relative to the NC-TSB ($5 \leq n \leq 6$). Representative histograms are shown highlighting the changes in MFI of each marker between the unstained control, NC-TSB and TSB-3202, with and without LPS ($4 \leq n \leq 5$). Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison test for graphs A-C or Dunnett's multiple comparison test for graphs D-E. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.

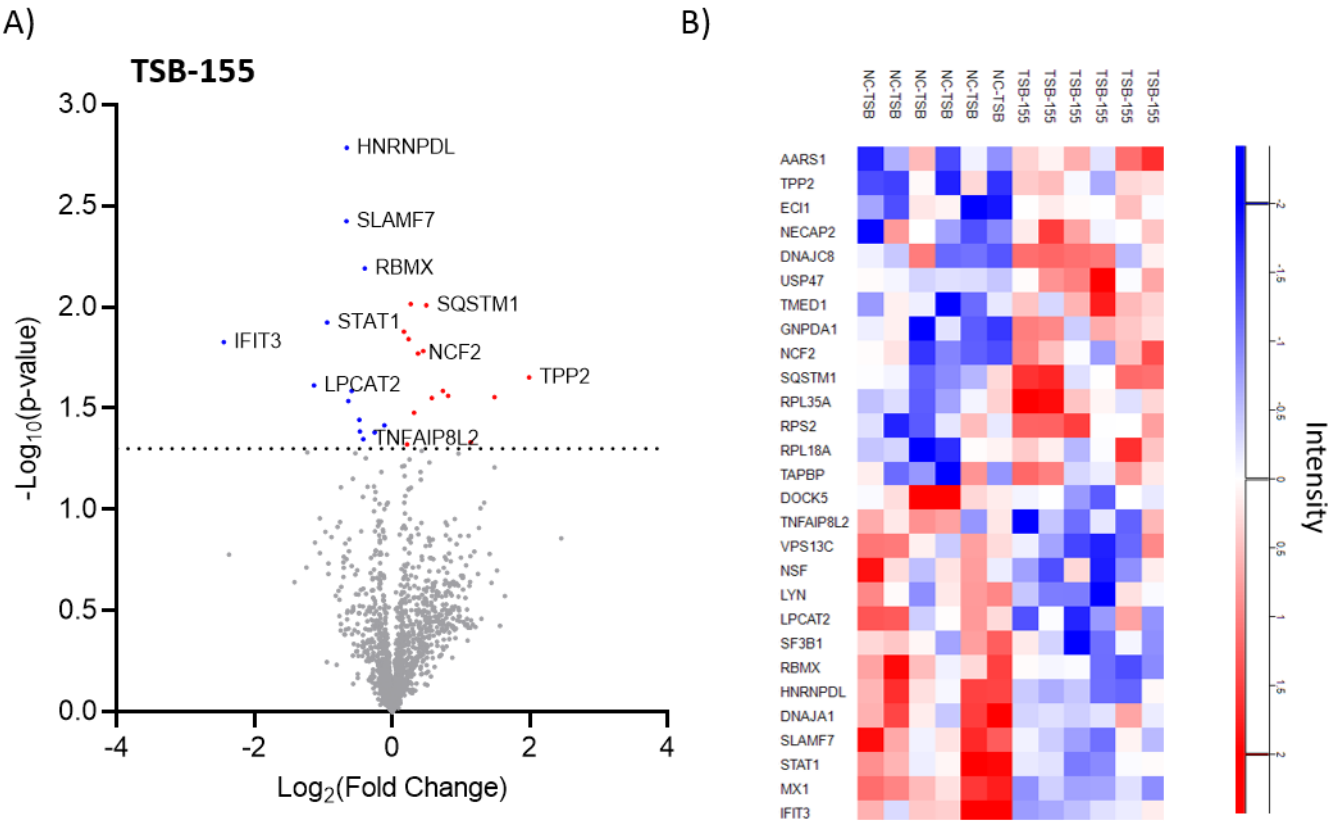

Figure S11: Mass spectrometry based proteomic analysis of the effects of Arg2 TSB-155 on human MDMs. TSB-155 (100nM) was transfected into MDMs using NC-TSB as the control. Cells were rested overnight and then stimulated with and without LPS (100ng/mL) for 24hrs. Cells were harvested in urea (6M) and peptides were isolated. Mass spectrometry analysis was performed on a Q-Exactive mass spectrometer. Treatments were compared to the NC-TSB. **A)** Volcano plot showing the effects of TSB-155 based on a Log_2 (fold change) and $-Log_{10}$ (p-value). Proteins with a significant p-value of <0.05 ($-Log_{10}$ p-value of >1.3) were highlighted in red (increased fold change) and blue (decreased fold change). **B)** Heat map representing the effects of TSB-155 compared to NC-TSB, where LFQ intensities of significantly changed proteins were represented as z-scores (n=3 independent experiments, performed in duplicate). Statistical analysis was performed on proteomic data using a student's T test to identify the significantly changed proteins.

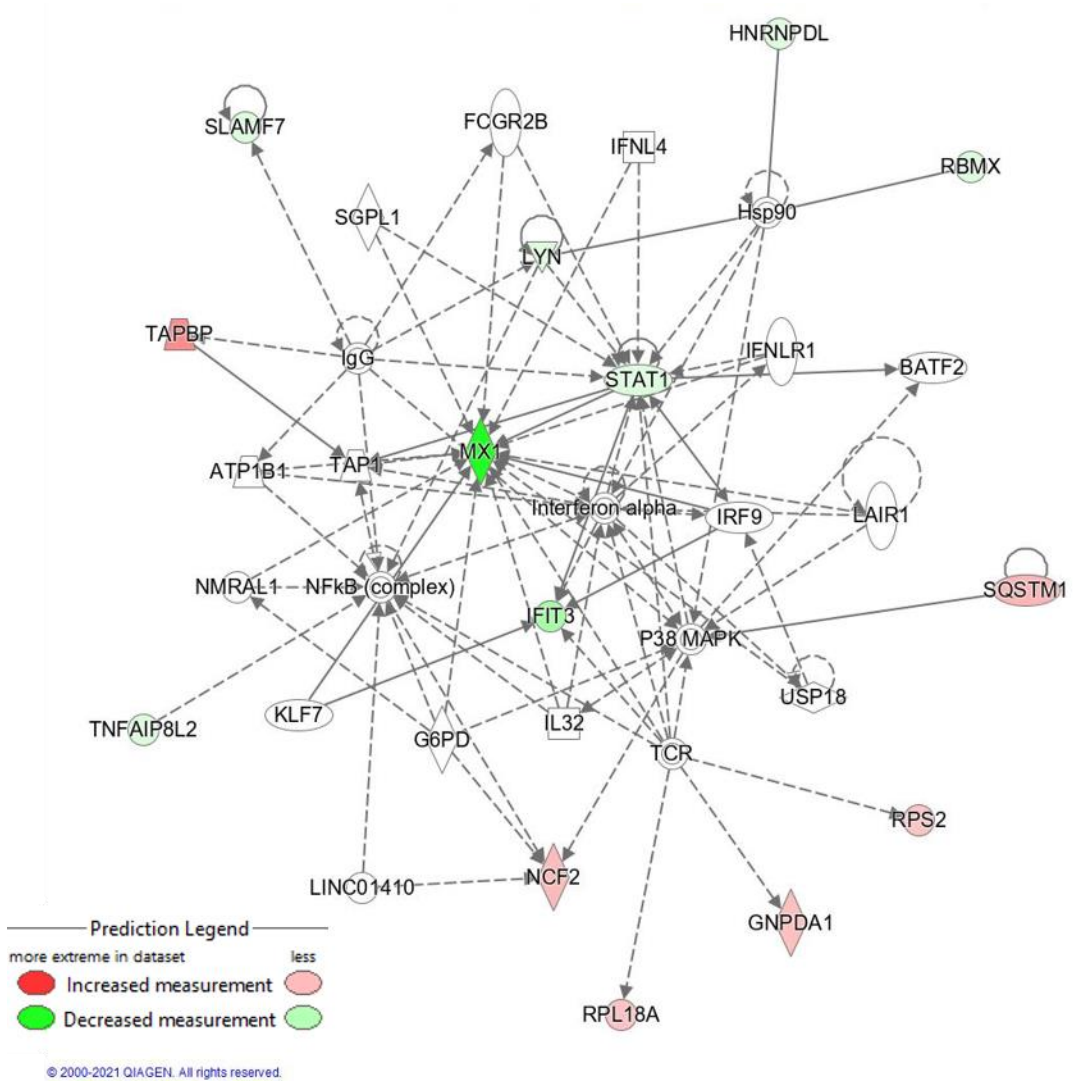

Figure S12: Network 1 generated by Ingenuity Pathway Analysis based on differentially regulated proteins altered by TSB-155. Mass spectrometry based proteomics was performed on MDMs treated with TSB-155. The significantly differentially regulated protein list was analysed using Ingenuity Pathway Analysis software from Qiagen. STAT-1, IFIT3, MX-1, NF-kB and Interferon alpha are key central nodes at the centre of this network.

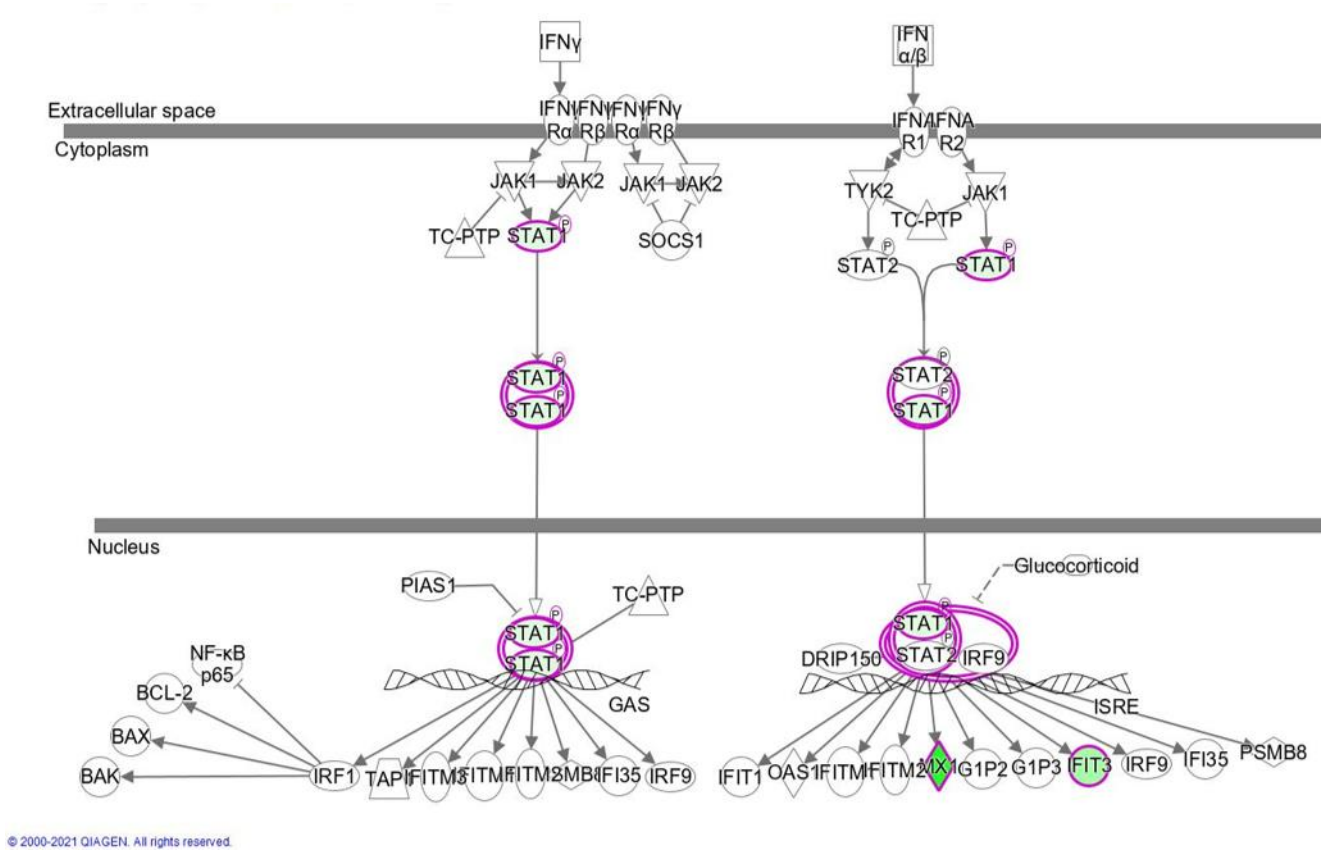

Figure S13: Components of the interferon signalling/STAT-1 pathway altered by TSB-155 and TSB-3202 generated by Qiagen Ingenuity Pathway Analysis based on differentially regulated proteins. Coloured proteins were decreased by TSBs. Mass spectrometry based proteomics was performed on MDMs treated with TSB-155 and TSB-3202. Interferon signalling was one such pathway predicted to be regulated by these TSBs. STAT-1, MX1 and IFIT3 were all significantly decreased in MDMs treated with TSB-155 and TSB-3202.

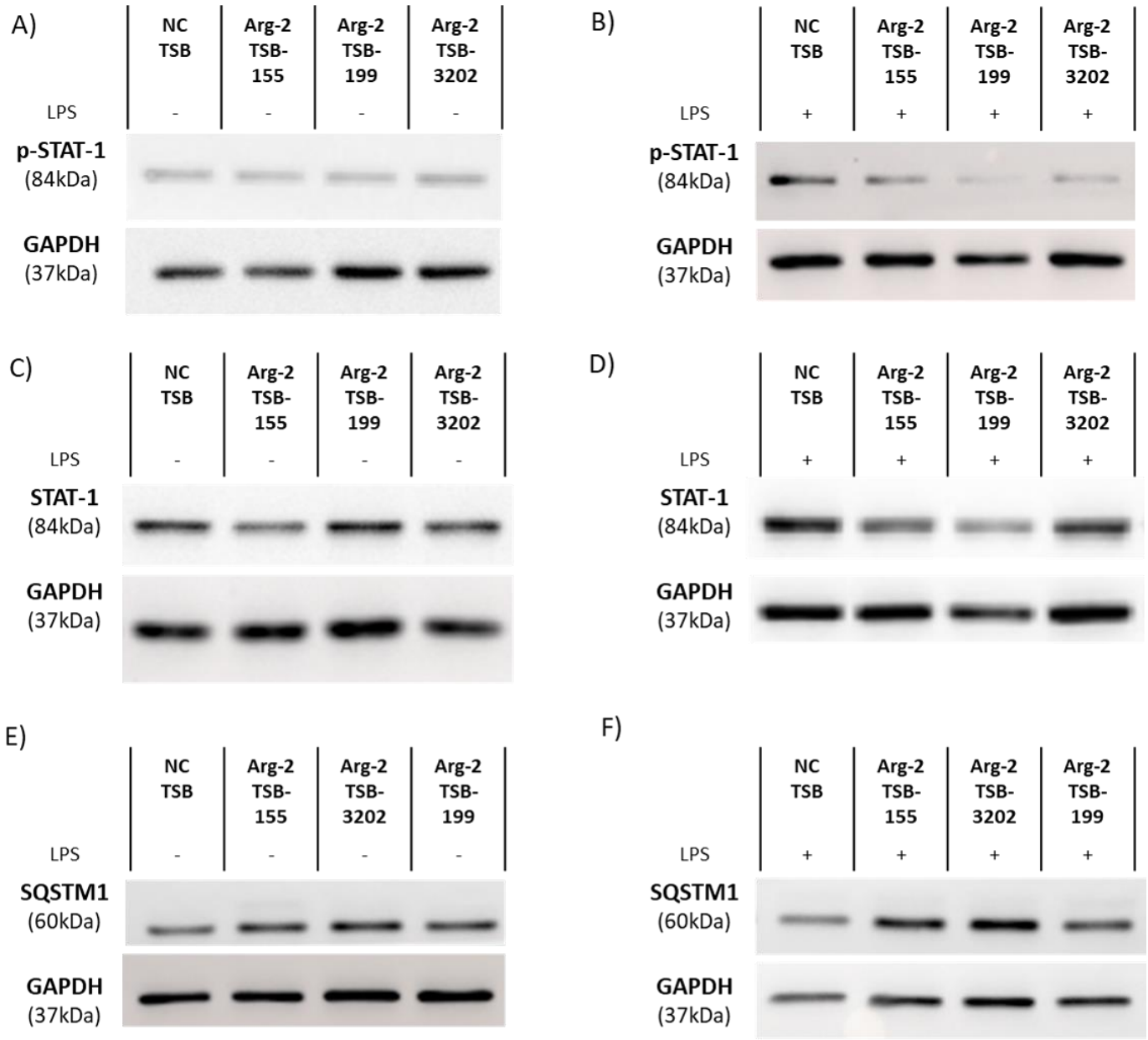

Figure S14: Validation of mass spectrometry data using western blot. Target site blockers (100nM) were transfected into human MDMs using Lipofectamine 3000. Cells were rested overnight and then stimulated with and without LPS (100ng/mL) for 24hr. Western blotting was performed and membranes were imaged using the Odyessy® CLx and the Vilber Fusion Fx (Vilber) imaging systems. **A-B)** Phosphorylated STAT-1 (p-STAT-1), **C-D)** STAT-1 and **E-F)** SQSTM1 representative blots are shown. Please note the intentional change in TSB ordering between blots A-D and E-F. GAPDH was used as the loading control.

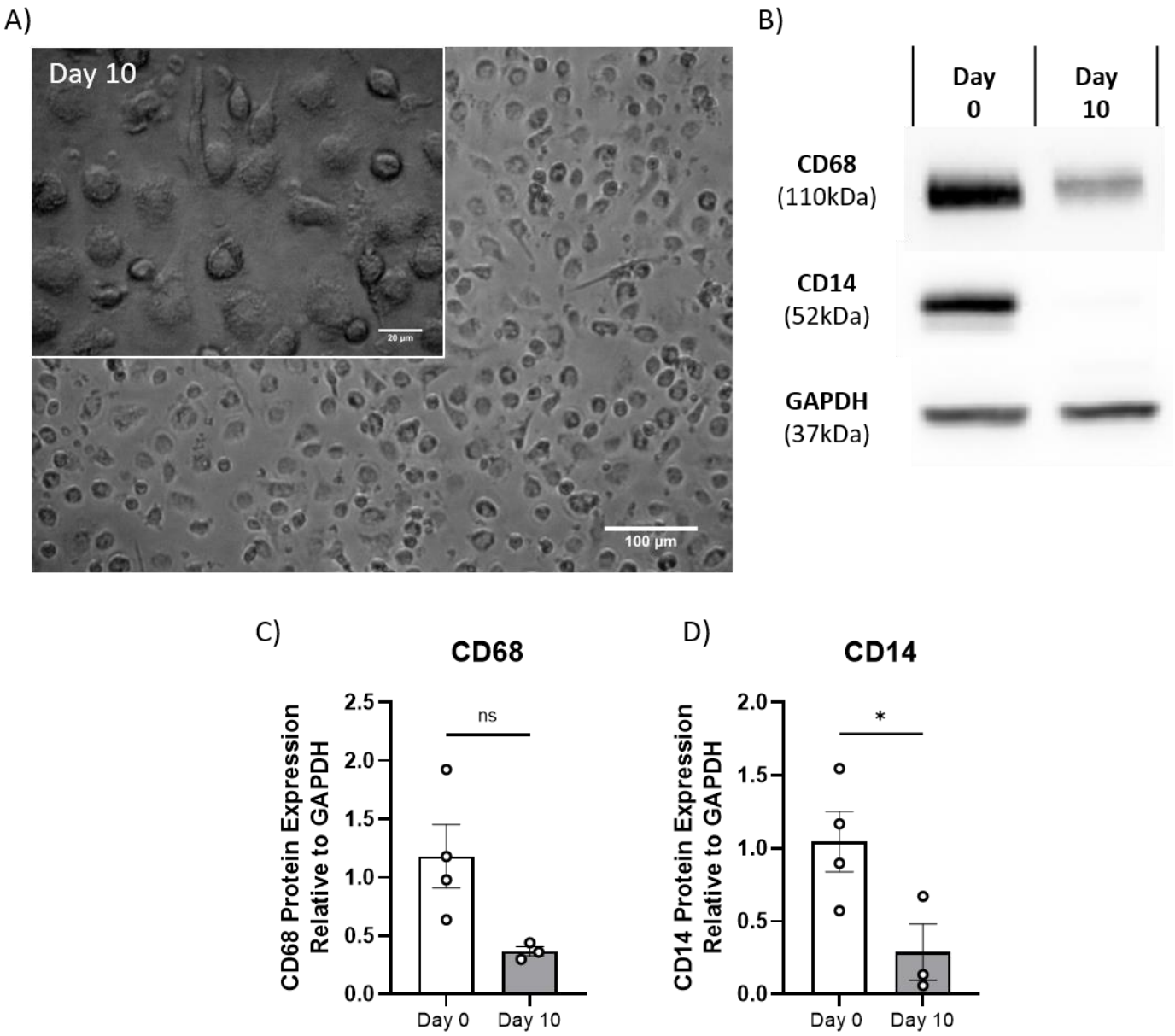

Figure S15: MDM characterisation. CD14+ isolated monocytes were cultured over 10 days into macrophages and characterised by Western blot. **A)** Light microscopy images of cells (MDMs) after 10 days in culture were taken using a 40X and 10X objective lens. **B)** Both monocytes (Day 0) and MDMs (Day 10) were analysed for the pan-macrophage marker, CD68, and the monocyte marker, CD14, using GAPDH as the endogenous control by Western blot. Densitometry analysis was performed on Western blots for **C)** CD68 and **D)** CD14. Statistical analysis was performed using an unpaired t test. *=$p<0.05$, **=$p<0.01$, ns=not statistically significant.

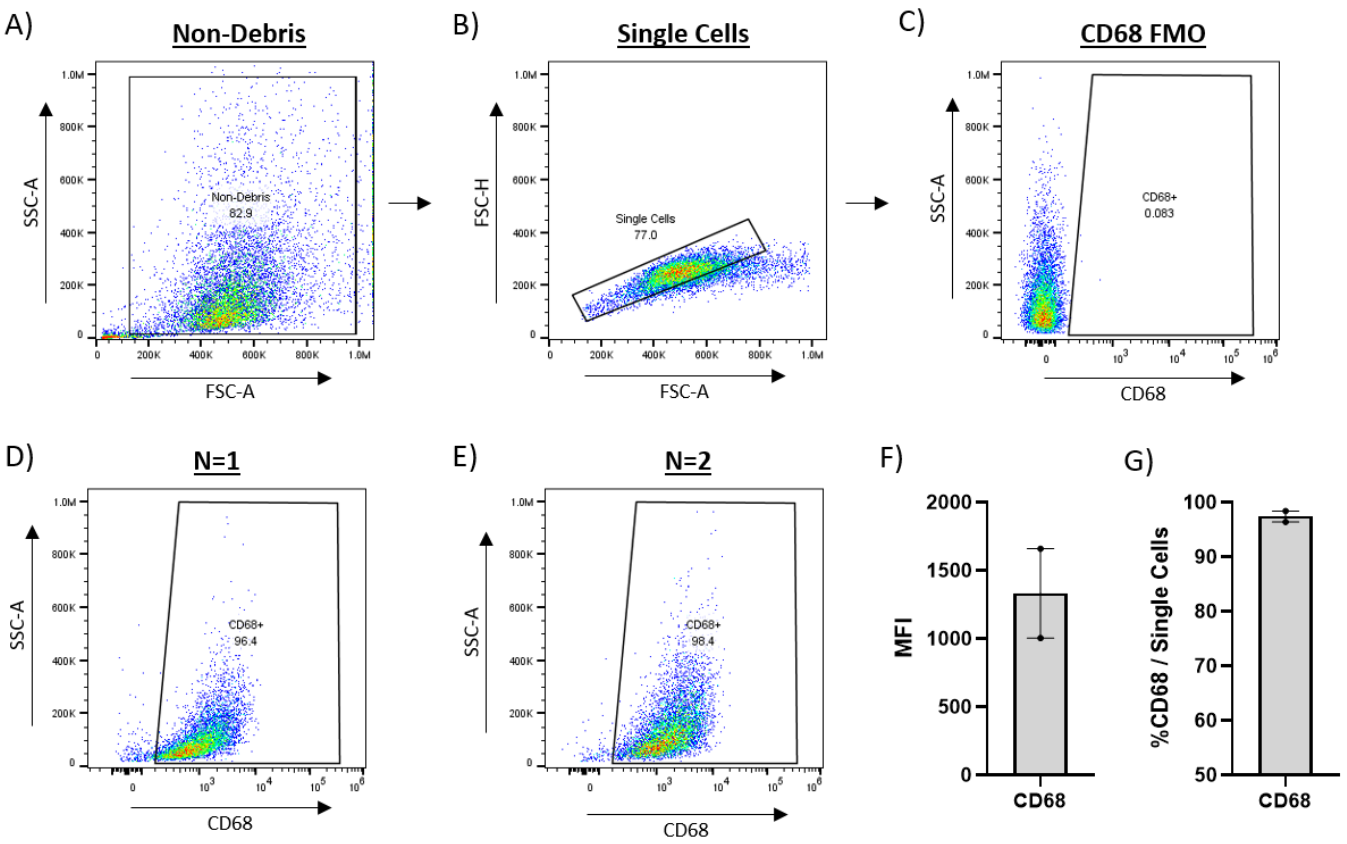

Figure S16: Flow cytometry on MDMs for the macrophage marker, CD68. A) All events were analysed using SSC-A and FSC-A and gates were established to remove debris. **B)** Gates were established for single cells using FSC-H and FCS-A. Cells were stained with and without CD68. **C)** Representative image of the fluorescent minus one (FMO) control for CD68. FMO was used to establish gates to ensure accurate gating on samples. **D)** Representative image of CD68+ cells analysed (n=1). **E)** Representative image of CD68+ cells analysed (n=2). **F)** Median Fluorescent Intensity (MFI) of CD68 and **G)** percentage of single cells positive for CD68 (n=2)**.**

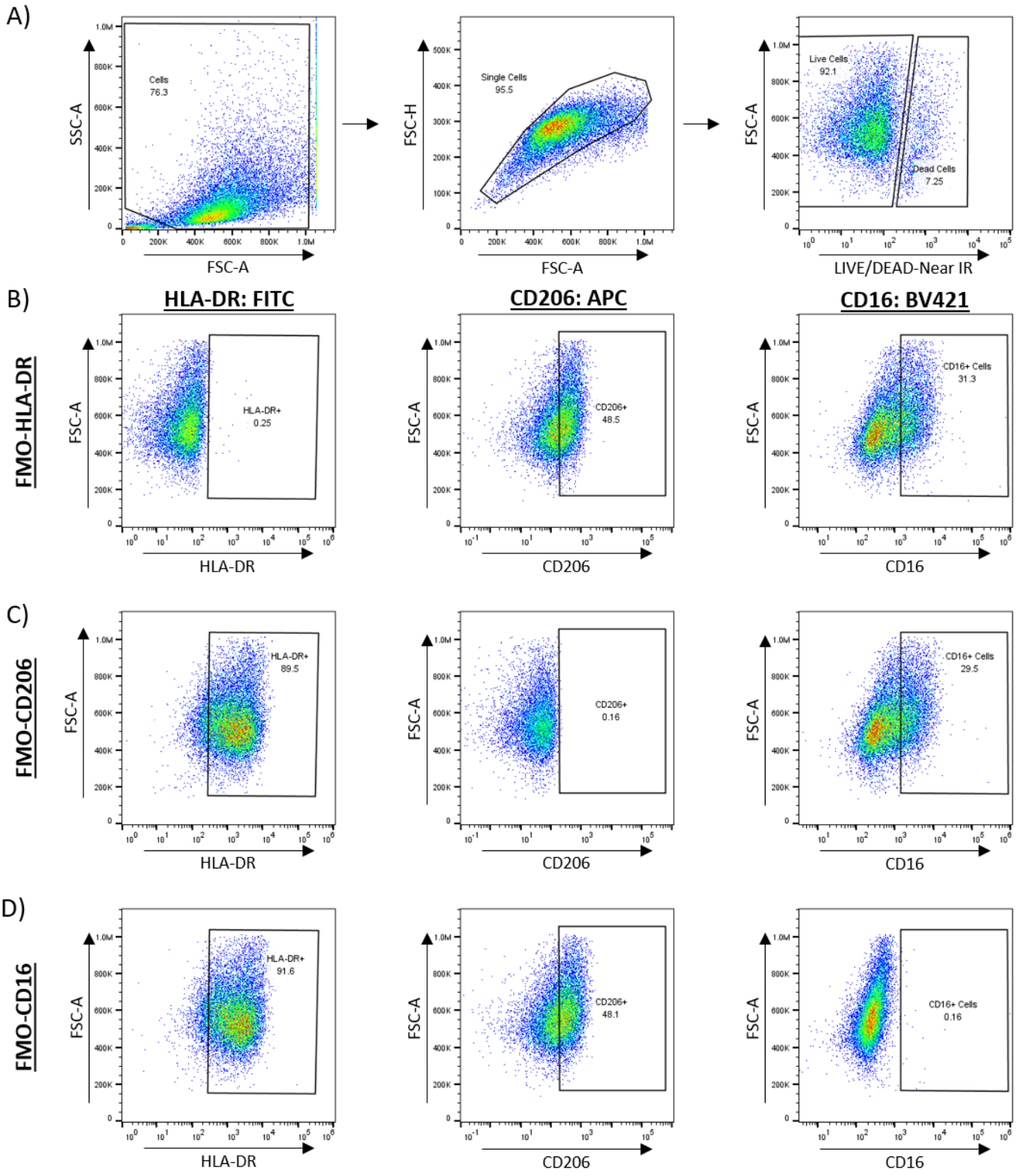

Figure S17: Flow cytometry gating strategy and fluorescent minus one controls. A) All events were analysed using SSC-A and FSC-A and gates were established to remove debris. Gates were established for single cells using FSC-H and FCS-A. Cells were incubated with Live/Dead Near IR to stain the dead cells. Gates were established on the live cell population which was used for analysis of the respective antibodies. Cells were triple stained with the antibodies HLA-DR, C206 and CD16. Displayed here are representative images of the FMO

controls for **B)** HLA-DR **C)** CD206 and **D)** CD16. FMOs were used to establish gates to ensure accurate gating on samples. Conjugates used include, Allophycocyanin (APC), Fluorescein Isothiocyanate (FITC) and Brilliant Violet (BV) 421.

Table S1: Details of the microRNA recognition elements (MREs) on the *ARG2* 3'UTR mRNA and the miRNA binding sites used for design of TSBs.

MicroRNA	**MRE Position on *ARG2* 3'UTR**	**miR Binding sites for TSB design**
hsa-miR-1299	36-43	gtttcacaacaggcattccagaattatgaggcattga (SEQ ID NO:2)
hsa-miR-199a, hsa-miR-199b	163-169	attttggtgaccaatactactgtaaatgtatttggtt (SEQ ID NO:3)
hsa-miR-10b, has-miR-10a	196-203	ggttttttgcagttcacagggtattaatatgctacag (SEQ ID NO:4)
hsa-miR-570	255-261	cataaacagcatttattaccttggtatatcatactgg (SEQ ID NO:6)
hsa-miR-1252	291-298	gtcttgttgctgttgttccttcacatttaagtggttt (SEQ ID NO:7)
hsa-miR-3202	448-454	gttctggtccacaaacccttcctatagaagttcaat (SEQ ID NO:8)
hsa-let-7a, -7b, -7c, -7e, -7f, -7g, -7i, hsa-miR-98	739-746	tagggataacactgtctacctcacagaaatgttaaac (SEQ ID NO:9)
hsa-miR-1294	741-748	gggataacactgtctacctcacagaaatgttaaactg (SEQ ID NO:10)
hsa-miR-9	774-780	actgagacaataaaaaccaaagcataa (SEQ ID NO:11)
hsa-miR-155 (1)	39-46	cacaacaggcattccagaattatgaggcattgagggg (SEQ ID NO:12)
hsa-miR-155	379-386	ctgtcagcccacagcagcaatatgcttattctatcca (SEQ ID NO:13)

Table S2: *In vivo* ready miRCURY LNA Power Target Site Blocker (Qiagen) sequences designed to inhibit the binding of specific miRNAs to the MRE of *ARG2* 3'UTR.

TSB	**MicroRNA**	**TSB Sequence (5'-3')**
TSB-1299	hsa-miR-1299	TTCTGGAATGCCTGTTGTGAA
TSB-199	hsa-miR-199a, hsa-miR-199b	TACAGTAGTATTGGTCA
TSB-10	hsa-miR-10b, has-miR-10a	ATACCCTGTGAACTGCA
TSB-570	hsa-miR-570	CAAGGTAATAAATGCTGTTT
TSB-1252	hsa-miR-1252	TGAAGGAACAACAGCAAC
TSB-3202	hsa-miR-3202	GGGAAGGGTTTGTGGACCA
TSB-Let7	hsa-let-7a, -7b, -7c, -7e, -7f, -7g, -7i, hsa-miR-98	GTGAGGTAGACAGTGTT
TSB-1294	hsa-miR-1294	TGTGAGGTAGACAGTGTT
TSB-9	hsa-miR-9	GCTTTGGTTTTTATTGT
TSB-155-1	hsa-miR-155 (1)	CATAATTCTGGAATGCCTGT
TSB-155	hsa-miR-155	ATATTGCTGCTGTGGGCT
NC-TSB	N/A	ACGTCTATACGCCCA

Table S3: Details of primary and secondary antibodies used for Western blot.

Primary Antibody	**Dilution**	**Catalogue Number**	**Company**
Arginase-2	1:500	ab137069	Abcam
β-Actin (C4)	1:3000	SC-47778	Santa Cruz
CD14 (D7A2T)	1:1000	56082S	Cell Signalling Tech
CD68	1:1000	SC-20060	Santa Cruz
CD206	1:500	12981	Cell Signalling Tech
GAPDH (D16H11)	1:3000	5174	Cell Signalling Tech
GPx1/2	1:500	SC-133160	Santa Cruz
iNOS	1:1000	14-5920-82	eBiosciences
SQSTM1/p62	1:1000	5114	Cell Signalling Tech
Phospho-STAT-1 (S727)	1:1000	9177	Cell Signalling Tech
STAT-1 (D1K9Y)	1:2000	14994	Cell Signalling Tech
Anti-mouse IgG, HRP-linked Antibody	X2 Primary Antibody	7076	Cell Signalling Tech
Anti-rabbit IgG, HRP-linked Antibody	X2 Primary Antibody	7074	Cell Signalling Tech

Table S4: Primer sequences for quantitative real time PCR. Primers used for cloning *ARG2* 3'UTR in the pmirGLO luciferase plasmid are also reported.

Gene	Primer	Sequence (5'-3')
ARG1	Forward	ACAAAACAGGGCTACTCTCAGG
	Reverse	CGAGCAAGTCCGAAACAAG
ARG2	Forward	TCAGTGCTGCGGATCATGT
	Reverse	CACTCCTTTTCTTTTCTGCCCTT
CD163	Forward	CGAGTTAACGCCAGTAAGG
	Reverse	GAACATGTCACGCCAGC
CCL2	Forward	CCCCAGTCACCTGCTGTTAT
	Reverse	TGGAATCCTGAACCCACTTC
CCL18	Forward	TCTATACCTCCTGGCAGATTC
	Reverse	TTTCTGGACCCACTTCTTATTG
IL1B	Forward	GCTGGAGAGTGTAGATCCCAAA
	Reverse	AGACGGGCATGTTTTCTGCT
IL10R	Forward	CCAGACATCAAGGCGCATGT
	Reverse	GATGCCTTTCTCTTGGAGCTTATT
IFIT3	Forward	AGGGCAGTCATGAGTGAGGTC
	Reverse	TGAATAAGTTCCAGGTGAAATGGCA
MRC1 (CD206)	Forward	GCTGCCAACAACAGAACGCT
	Reverse	TCAGCTGATGGACTTCCTGGT
SLAMF7	Forward	CTTTGGCAGCTCACAGGGTCA
	Reverse	TGGTGACAAGAGGGGTTGTGT
SQSTM1	Forward	CATTGCGGAGCCTCATCTCCT
	Reverse	CAAGTCCCCGTCCTCATCCTTTC
STAT1	Forward	ACCAGTGCACAGAATCCTCCA
	Reverse	TTCGTACCACTGAGACATCCACA
TBP	Forward	GCGGTTTGCTGCGGTAATC

	Reverse	TCTGGACTGTTCTTCACTCTTGG
TNFA	Forward	CTCGAACCCCGAGTGACAA
	Reverse	GCTGCCCCTCAGCTTGAG
SHIP1 (INPP5D)	Forward	GACACAGGAGTCAAGGCCC
	Reverse	AAACATCTCGGGCTTCGTCA
SOCS1	Forward	TTCGCCCCTTAGCGTGAAGATGG
	Reverse	TAGTGCTCCAGCAGTCGAAGA
Arg-2 3'UTR (with pmirGLO overlap and XhoI restriction site)	Forward	AACGAGCTCGCTAGCCTCGAGCACTGTGCACTGACATGT
	Reverse	CAGGTCGACTCTAGACTCGAGTCATCTTACTGGAGCTCGC

Table S5: Quantitative real time PCR Taqman Probes.

Taqman Probe	**Catalogue**	**Assay ID**	**Mature miRNA Sequence**
hsa-miR-155-5p	4427975	002623	UUAAUGCUAAUCGUGAUAGGGGU
U6 snRNA	4427975	001973	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACG ATACAGAGAAGATTAGCATGGCCCCTGCGCAAGGATGA CACGCAAATTCGTGAAGCGTTCCATATTTT
Taqman Advanced Probe	**Catalogue**	**Assay ID**	**Mature miRNA Sequence**
hsa-miR-155-5p	A25576	483064_miR	UUAAUGCUAAUCGUGAUAGGGGUU
hsa-miR-199a-3p	A25576	477961_miR	ACAGUAGUCUGCACAUUGGUUA
hsa-miR-3202	A25576	479675_mir	UGGAAGGGAGAAGAGCUUUAAU
hsa-miR-423-3p	4427975	002626	AGCUCGGUCUGAGGCCCCUCAGU

Table S6: Details of antibodies used for flow cytometry analysis.

Primary Antibody	**Dilution**	**Clone**	**Isotype**	**Fluorophore**	**Catalogue Number**	**Company**
CD16	1:50	3G8	Mouse $IgG1_K$	BV421	302038	Biolegend
MRC1	1:100	15-2	Mouse $IgG1_K$	APC	321110	Biolegend
HLA-DR	1:100	L243	Mouse $IgG2a,_K$	FITC	307604	Biolegend
CD68	1:100	eBioY1/82A	Mouse / $IgG2b,_K$	PE-eFluor 610	61-0689-42	eBioscience

Table S7: Significantly differentially expressed proteins from proteomic analysis. Please see Supplemental Excel File.

Table S8: Lists of significantly differentially expressed proteins uploaded for upstream regulator Qiagen IPA analysis. Please see Supplemental Excel File.