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Date:
2018-01-01

Citation:
Do, T., Tan, R., Bennett, M., Medvedovic, M., Grom, A. A., Shen, N., Thornton, S. & Schulert, G. S. (2018). MicroRNA networks associated with active systemic juvenile idiopathic arthritis regulate CD163 expression and anti-inflammatory functions in macrophages through two distinct mechanisms. Journal of Leukocyte Biology, 103 (1), pp.71-85. https://doi.org/10.1002/JLB.2A0317-107R.

Persistent Link:
https://hdl.handle.net/11343/294097

MicroRNA networks associated with active systemic juvenile idiopathic arthritis regulate CD163 expression and anti-inflammatory functions in macrophages through two distinct mechanisms

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Summary sentence: MicroRNA utilize multiple mechanisms to regulate CD163 expression and integrate well characterized macrophage phenotypes and functional properties seen in active systemic juvenile idiopathic arthritis.

Character count: 27,433

Running title: MicroRNA regulation of CD163

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Funding: This work was supported by the Single Cell Phenotyping Core of the Cincinnati Rheumatic Diseases Resource Center P30-AR070549, PO1-AR048929 from the National Institutes of Health to

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/JLB.2A0317-107R.

AAG, and a Scientist Deveopment Award from the Rheumatology Research Foundation and Procter Scholar Award from the Cincinnati Children's Research Foundation to GSS.

Abbreviations:

DEGs: differentially expressed genes

Hgb: Hemoglobin

HMGB1: High-mobility group box 1

Hp: Haptoglobin

MAS: Macrophage activation syndrome

MDM: Monocyte-derived macrophage

SJIA: Systemic juvenile idiopathic arthritis

ABSTRACT:

Systemic juvenile idiopathic arthritis (SJIA) is a severe childhood arthropathy with features of autoinflammation. Monocytes and macrophages in SJIA have a complex phenotype with both pro- and anti-inflammatory properties that combine features of several well characterized *in vitro* conditions used to activate macrophages. An important anti-inflammatory phenotype is expression of CD163, a scavenger receptor that sequesters toxic pro-inflammatory complexes that is highly expressed in both active SJIA and macrophage activation syndrome (MAS). CD163 is most strongly upregulated by IL-10 (M(IL-10)), and not by other conditions that reflect features seen in SJIA monocytes such as M(LPS+IC). MicroRNA play key roles integrating cellular signals such as those in macrophage polarization, and as such we hypothesize microRNA regulate macrophage functional responses in SJIA including CD163 expression. We find that two microRNA previously found to be elevated in active SJIA, miR-125a-5p and miR-181c, significantly reduced macrophage CD163 expression through two distinct mechanisms. Neither microRNA was elevated in M(IL-10) with robust CD163 expression, but were instead induced in M(LPS+IC) where they restricted CD163 mRNA expression. Mir-181 species directly targeted CD163 mRNA for degradation. In contrast, miR-125a-5p functions indirectly, as transcriptome analysis of miR-125a-5p overexpression identified "cytokine-cytokine receptor interactions" as the most significantly repressed gene pathway, including decreased *IL10RA*, required for IL-10-mediated CD163 expression. Finally, overexpression of miR-181c inhibited CD163 anti-inflammatory responses to hemoglobin or high mobility group box 1 (HMGB1) complexes. Together, these data show that microRNA utilize multiple mechanisms to integrate well characterized polarization phenotypes and regulate macrophage functional properties seen in SJIA.

Introduction

Systemic juvenile idiopathic arthritis (SJIA) is a severe and distinctive subtype of JIA characterized by presence of daily fevers and other signs of systemic inflammation, along with chronic arthritis. Although the etiology of SJIA is unknown, it has many features consistent with an autoinflammatory disorder, with expansion and activation of innate immune cells and proinflammatory cytokines including IL-1 and IL-6 (1). As such, monocytes and macrophages are proposed to be key effector cells in SJIA. Children with active SJIA have increased circulating monocytes, and these cells display enhanced surface expression of numerous activation markers including the scavenger receptor CD163 (2). Children with SJIA are at high risk for macrophage activation syndrome (MAS), an episode of overwhelming inflammation characterized by proliferation of cytolytic T cells and hemophagocytic macrophages, and leading to a cytokine storm (3). Interestingly, bone marrow biopsies of patients with MAS show markedly increased macrophage markers including CD163 (4), and serum levels of soluble CD163 (sCD163) were elevated in SJIA patients with both overt and subclinical MAS (5,6).

CD163 is as an innate pattern recognition receptor exclusively expressed on myeloid cells (7). This receptor functions as a hemoglobin scavenger receptor, binding and engulfing circulating hemoglobin:haptoglobin complexes. CD163 directly mediates some anti-inflammatory functions of monocytes and macrophages by inducing IL-10 and heme oxygenase-1 production after ligand binding (8). Indeed, mice deficient in CD163 had enhanced mortality in mouse models of sepsis (9). Monocyte expressed CD163 likely plays key functional roles during active SJIA and MAS as well. These patients have a pronounced erythropoiesis gene expression signature, associated with high red blood cell turnover and markedly elevated serum ferritin levels that can mediate oxidative injury (10). There is also enhanced haptoglobin production in SJIA (10,11), which functions together with CD163 to clear free hemoglobin, limiting tissue damage and triggering release of anti-inflammatory compounds. Indeed, along with elevated levels of IL-10, serum HO-1 levels have been shown to be a

highly specific marker for active SJIA (12). CD163 also serves as a scavenger receptor for high mobility group box 1 (HMGB1), a damage-associated molecular pattern (DAMP) overexpressed in MAS which serves to amplify inflammatory responses (13). Notably, along with CD163, macrophages in SJIA and MAS produce high levels of proinflammatory mediators including TNFα, IL-6 and IL-18 (14,15), and these cells are believed to have key roles in SJIA pathogenesis (1). Taken together, CD163 expression on monocytes and macrophages mediates key anti-inflammatory responses and may serve to limit tissue damage and inflammation, particularly during MAS.

Activated monocytes and macrophages adopt distinct functional phenotypes based upon signals from their local environment, in a process known as polarization. These polarized populations elaborate effectors optimized for a variety of functions, including inflammation, tissue remodeling and repair, and immunoregulation. While previously considered as a dichotomy between classically activated "M1" and alternatively activated "M2" macrophages, recent work has shown that macrophages can be activated towards a diverse spectrum of distinct phenotypes (16). Expression of CD163 has largely been associated with several well characterized *in vitro* conditions, and particularly induced by the anti-inflammatory cytokine IL-10 (M(IL-10) or previously "M2c") (17–20). In contrast, other alternatively activated phenotypes such as those induced by LPS along with immune complexes (M(LPS+IC) or "M2b") produce both proinflammatory mediators including TNFα, IL-1 and IL-6 along with immune regulatory properties and IL-10 production, but have lower CD163 expression (21). How these activation states represent monocytes in inflammatory conditions such as SJIA is unclear. Importantly however, previous immunophenotyping and transcriptional profiling has demonstrated that monocytes in SJIA do not align with a single defined polarization state but rather exhibit features reflecting multiple activation phenotypes, including classical activation, M(IL-10) and M(LPS+IC) (2,10,22,23).

In agreement with this, effective macrophage polarization requires the integration of multiple cellular signaling pathways into coordinated changes in gene expression. As such, microRNA have emerged as important epigenetic contributors to this process. MicroRNA are small

non-coding RNA that serve as transcriptional negative regulators by directly binding to the 3' untranslated regions (UTR) of specific target transcripts to mediate mRNA degradation and/or inhibit translation (24). MicroRNA have been shown to play key roles in regulating gene expression programs involved in cell proliferation, differentiation, and inflammation, including monocyte and macrophage polarization (25,26). Our recent work found a broad spectrum of microRNA with increased expression in monocytes from children with active systemic JIA (27). We also found that one of these microRNA, miR-125a-5p, functioned as a key regulator of M(LPS+IC) phenotypes, which resembles some of the features seen in SJIA monocytes. Mir-125a has also recently been shown to stabilize Treg-medicated immune homeostasis in mouse models of autoimmunity, but its functions in myeloid cells are incompletely understood (28). Other microRNA increased in active SJIA also have key roles in inflammation, including the miR-181 family which can regulate macrophage cytokine gene expression (29,30)These findings suggest that microRNA may function to coordinate and integrate polarization-associated gene expression programs essential to monocytes in active systemic JIA.

Here, we examined microRNA regulation of CD163 expression in activated monocytes and macrophages. We hypothesized that microRNA function as key post-transcriptional regulators that integrate and balance polarized phenotypes in these cells during active SJIA, and in particular function as negative regulators of CD163. Indeed, we identify a network of microRNA that restrict expression of CD163 during M(LPS+IC) activation, despite robust autologous IL-10 production. This inhibition is modulated through two distinct mechanisms – both direct targeting of CD163 mRNA and indirectly through global effects on cytokine receptor gene expression including the IL-10 receptor. MicroRNA expression also significantly reduced CD163-mediated anti-inflammatory properties of macrophages, supporting key roles for these epigenetic factors in maintaining functional polarization phenotypes during hyperinflammation.

Materials and Methods

Cell isolation and culture: THP-1 monocytic cells were obtained from ATCC (Manassas, VA). Isolation of CD14+ peripheral blood monocytes was as described (27). Monocyte-derived macrophages (MDMs) were isolated from peripheral blood from healthy adult donors through the Cell Processing and Manipulation Core. Briefly, peripheral blood mononuclear cells were isolated by density gradient centrifugation through Ficoll-Paque Plus (GE Healthcare, Pittsburgh, PA), washed twice in RPMI and then placed in culture medium supplemented with 5 ng/ml monocyte colony stimulating factor (R&D Systems, Minneapolis, MN) at $2x10^6$ cells/ml. After 5-7 days of differentiation, cells were washed 3 times to remove non-adherent lymphocytes prior to cell stimulation.

Cell Stimulation/Polarization: All ligands were purchased from R&D Systems except for lipopolysaccharide (LPS) which was from Sigma Aldrich (St. Louis, MO). Prior to stimulation, THP-1 cells were differentiated overnight after addition of 50 ng/mL phorbol myristate acetate (PMA). The cells were left untreated or stimulated with IL-10 (1-50 ng/ml), IFNγ (20 ng/ml), LPS (10-100 ng/ml), IL-4 (20 ng/ml), or TGFβ (0.5 ng/ml). IgG-coated wells were prepared as described (26).

Flow Cytometry: CD163 mRNA transcripts were detected in single cells using the PrimeFlow™ kit according to manufacturer's directions (Affymetrix, eBioscience, Santa Clara, CA). Briefly, THP-1 cells were stained for surface CD163 using anti-human CD163 (Trilium, Brewer, ME). The cells were then fixed, washed and permeablized prior to labelling with CD163 mRNA Target Probes. The next day, PrimeFlow RNA PreAmp Mix was added directly to the cell suspension and incubated for 1.5 hours at 40^{o}C. The samples were then washed prior to adding diluted Label Probes to complete signal amplification prior to analysis. To visualize IL10RA or phosphor-STAT3 levels, THP-1 cells or MDMs were fixed with 0.4% paraformaldehyde and permeabilized with 90% methanol prior to staining with Alexa 647-conjugated anti-human IL10RA (Becton Dickinson, San Jose, CA) or

Pacific Blue-conjugated anti-human phosphor-STAT3 (Thermo Fisher). Samples were then filtered and transferred to 12 x 75 mm polystyrene tubes, and cells were acquired using a BD LSR Fortessa analytical cytometer. Data was analyzed by FACSDiva and FlowJo software.

Gene Expression: Cellular mRNA levels were quantified using realtime RT-PCR. Briefly, total RNA was isolated as described using Trizol and purity and concentration were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher, Waltham, MA). Approximately 1 μg of RNA was used to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was performed in 20 μl reactions using Viia7 Realtime PCR Instrument with gene-specific primers and SYBR Green Supermix (Life Technologies). Message copy numbers were normalized against the copy number of the housekeeping gene GAPDH as described (31). Primers specific for *GAPDH* and *CD163* were described previously (27). Additional primers are as follows: *TFGBR2* forward 5'-GTAGCTCTGATGAGTGCAATGAC-3', reverse 5'-CAGATATGGCAACTCCCAGTG-3' (32); *IL10RA* forward 5'-TTCTTTGCCTTTGTCCTGC-3'; reverse 5'-ACCTTCAAAAAGGCCTCCTC'3' (33). For quantification of microRNA levels, individual Taqman assays (Life Technologies, Carlsbad, CA) were performed using Bio-Rad iCycler thermocycler and a Viia7 Realtime PCR Instrument, calculated using the comparative C_T method and normalized to RNU48.

MicroRNA antagomir and mimic and RNA interference: To knock-down microRNA expression, THP-1 cells were differentiated overnight as described above and then treated with 300nM negative control or miR-125a-5p micrOFF antagoMIR (Ribobio, Guangzhou, China). To augment microRNA expression, THP-1 cells differentiated overnight with PMA were transfected with pre-miR microRNA precursors. Briefly, negative control pre-miR or miR-125a-5p (Dharmacon, Lafayette, CO) were incubated using RNAiMAX reagent using protocol described by Gantier and colleagues (34), with a final mimic concentration of 300nM. Efficiency of microRNA inhibition or overexpression was

determined using individual Taqman assays as described below. Similarly, IL10RA expression was inhibited using ON-TARGETplus siRNA SMARTpool (Dharmacon) transfected as above.

RNA immunoprecipitation assay: PMA differentiated THP-1 cells were treated with IL-10 and concurrently transfected with miR181c mimics or Negative Control mimics as described above. After 24 hours, cells were lysed and RNA-induced silencing complex (RISC) was immunoprecipitated using 5μg isotype control antibody or anti-Ago2 using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (EMD Millipore, Billerica, MA) according to manufacturer's directions. Following immunoprecipitation, RNA was extracted by phenol chloroform and precipitated via ethanol at -80°C overnight. The purified immunoprecipitated RNA was assessed by NanoDrop™ 2000 Spectrophotometers and analyzed using qRT-PCR as described above.

Luciferase reporter system: The 3' untranslated region of the human CD163 transcript (NM_004244) cloned downstream of the luciferase reporter gene in vector pMirTarget (WT-CD163) was obtained from OriGene (Rockville, MD). The putative miR-181 family binding site was altered using site-directed mutagenesis (Mut-CD163) with the GeneArt mutagenesis kit (Thermofisher, Waltham, MA) per manufacturer's directions, and sequence confirmed by Sanger sequencing. HEK293T cells were seeded into 12-well plates with complete DMEM containing 10% fetal bovine serum (FBS), 2mM of L-glutamine, and 100U/ml Pencillin/Streptomycin for 4-6 hours prior to transfection. A mixture of 4 uL Lipofectamine 2000 (Life Technologies Inc.), 2000 ng of WT- or Mut-CD163 vectors, 4.0 pmol of hsa-miRNA mimics (Dharmacon, Lafayette, CO), and 5ng of control vectors pRL-TK (Promega, Madison, WI) were prepared in Opti-MEM and incubated for 20 min at room temperature. Once transfection mixture was added to each well, cells were incubated at 37°C in 5% CO2 for 72 h. Dual-Luciferase Reporter Assay System (Promega) was used to evaluate the luciferase activity of each well according to the manufacturer's instruction using GloMax® Discover instrument (Promega).

Genome-wide microRNA target analysis: To identify potential microRNA target genes, miR-125a-5p mimic was overexpressed in THP-1 cells as described above. Extracted RNA was amplified using the NuGEN Ovation RNA-Seq System v2, after which the Nextera XT DNA Sample Preparation Kit was used to prepare DNA library templates. Libraries were then sequenced using the Illumina HiSeq2500, with approximately 35-40million reads per sample.

RNA-seq data analysis follows the protocol described in Anders et al. (35). Reads were aligned to the reference human genome (hg19) using the TopHat aligner (36). Counts of the reads aligned to the coding region of each gene were summarized using ShortRead (37) and associated Bioconductor packages for manipulation and analysis of next-generation sequencing data. Statistical analysis to identify differentially expressed genes was performed based on a negative-binomial distribution of read counts as implemented by the edgeR Bioconductor package (38) using FDR adjusted p-values (39). Cluster analysis of genes and samples were performed using Bayesian infinite mixture model based clustering (40,41) of the log-2 transformed read counts normalized for the length of different gene coding regions and the number of reads obtained for each sample (42). Significantly enriched gene pathways were determined using Database for Annotation, Visualization and Integrated Discovery (DAVID) (43).

CD163-mediated cytokine production: THP-1 cells were differentiated with PMA as described and stimulated with hemoglobin-haptoglobin (Hgb-Hp) or HMGB1-haptoglobin (HMGB1-Hp) complexes. Both Hgb-Hp and HMGB1-Hp complexes (Sigma) were prepared at a 1:1 molar ratio, and then added to cells at the final concentration of 20 μg/ml. Where indicated, THP-1 cells were transfected concurrently with microRNA mimics. After 48h incubation, the supernatant was collected and analyzed as described below. Anti-CD163 blocking antibodies RM3/1 were obtained from Santa Cruz Biotechnology (Dallas, TX) and added to macrophages at 20 μg/ml concurrently with complexes. Levels of secreted IL-1betta, IL-6, IL-8, IL-10, and IL-23 in culture supernatants were detected using specific ELISA (R&D Systems) or using the Milliplex™ Multiplex kits (MilliporeSigma, Darmstadt,

Germany) according to manufacturer's protocol.

RESULTS

Expression of CD163 mRNA is specifically induced by M(IL-10) polarization and not by other polarized phenotypes

While CD163 expression has been associated broadly with alternatively activated macrophages, the precise polarized phenotypes marked by CD163 are not fully understood. To define the *in vitro* conditions that induce CD163 expression in monocytes and macrophages, we used two distinct methods to quantify CD163 mRNA changes. First, CD163 mRNA levels were determined using quantitative RT-PCR in PMA-differentiated THP-1 macrophages. Multiple polarizing conditions, including classical M(LPS+IFNγ) activation, M(IL-4) or "M2a" alternative activation, M(LPS+IC), and M(TGFβ) failed to induce significant CD163 mRNA expression (Figure 1a). Indeed, other "alternative" polarizing conditions, and in particular M(LPS+IC), significantly reduced CD163 mRNA expression at 24 hours. Only when macrophages were polarized with M(IL-10), or "M2c", was CD163 expression significantly increased. Similarly, primary human CD14+ blood monocytes and MDM showed significant upregulation of CD163 mRNA only with M(IL-10) treatment and not with any other polarizing conditions, and significant repression of CD163 mRNA under M(LPS+IC) conditions (Figure 1a). As an alternative approach to quantify CD163 in polarized macrophages, we used RNA flow cytometry with PrimeFlow. This technique uses fluorescent *in situ* hybridization with oligonucleotide probe sets and signal amplification to obtain gene expression information (44). These probes can also be combined with fluorescently-labelled antibodies to allow simultaneous detection of mRNA and protein at a single-cell resolution in monocytes and macrophages (Thornton, Schulert et al, in preparation). As shown in Figure 1b, several polarizing conditions including M(LPS+IFNg), M(LPS+IC), and M(TGFβ) failed to induce changes in either CD163 mRNA levels or surface protein expression in THP-1 cells. Interestingly M(IL-4) treatment led to a small but consistent increase in CD163 surface protein expression with only a minor change in mRNA levels.

Only polarization towards M(IL-10) significantly increased macrophage CD163 mRNA and surface protein expression (Figure 1b). Recent studies have reported that more prolonged stimulation with M(LPS+IC) can induce CD163, possibly due to autocrine production of IL-10 in this condition (16). However in our hands, prolonged stimulation with M(LPS+IC) for 48 or 72 hours did not increase CD163 mRNA levels above that seen in untreated macrophages, despite continued robust IL-10 expression (Figure 1c). On the other hand, LPS has been shown to repress CD163 expression with as little as 6 hours treatment (45). Indeed, we found that polarization induced changes in CD163 mRNA levels occurred as early as 3 or 6 hours, with significant increases in M(IL-10) treated cells and decreases in M(LPS+IC) treated cells (Figure 1d). Taken together, these findings demonstrate that CD163 mRNA expression is specifically induced only upon M(IL-10) polarization, repressed by M(LPS+IC) treatment, and minimally altered by other well-characterized polarizing conditions.

Increased expression of multiple microRNA associated with SJIA can regulate CD163

MicroRNA play key roles in integrating signal transduction pathways and serving to "fine-tune" gene expression programs in monocyte and macrophage polarization. As such, we hypothesize that monocyte microRNA elevated in active SJIA can regulate polarization phenotypes including CD163 expression. Our previous work demonstrated elevated miR-125a-5p in monocytes from children with active SJIA, and that miR-125a-5p levels correlated with clinical and laboratory features of systemic inflammation (27). We also found that miR-125a-5p expression was specifically induced in conditions with limited CD163 expression, most notably M(LPS+IC) polarization. This previous work also showed that two members of the miR-181 family, miR-181a and miR-181c, were upregulated in active SJIA compared to patients in clinically inactive disease or healthy control children (27). Porcine miR-181 family members have been shown to target monocyte CD163 to suppress viral infection (46); however, whether these microRNA impact CD163 expression in humans is unknown. First, we found that miR-125a-5p, miR-181a, and miR-181c are expressed in THP-1 cells (Figure 2a), as well as primary CD14+ monocytes and MDM (Supplemental Figure 1). To determine

whether these microRNAs can inhibit CD163 expression in human macrophages, THP-1 cells were transfected with mature microRNA mimics. Overexpression of either miR-125a-5p or miR-181c, but not miR-181a, significantly reduced levels of IL-10 induced CD163 mRNA compared to cells transfected with control mimic (Figure 2b). Taken together, these findings suggest that miR-125a-5p and miR-181c, upregulated in monocytes from children with SJIA, inhibit CD163 mRNA expression.

miR-125a-5p and miR-181c limit upregulation of CD163 mRNA in M(LPS+IC) conditions

As shown above, CD163 expression in monocytes and macrophages is specifically repressed in M(LPS+IC) polarization, but not other polarizing conditions. To further define the role of microRNA in regulating CD163 in macrophage polarization, we determined polarization specific changes in microRNA expression. Interestingly, expression of miR-125a-5p, miR-181a and miR-181c was significantly increased only under M(LPS+IC) polarizing conditions, and not in other phenotypes including M(IL-10) (Figure 2c). Of note, M(LPS+IC) polarization significantly represses CD163 expression in monocytes and macrophages despite robust autocrine IL-10 production (Figure 1), which is the hallmark of this phenotype (47). Indeed, we found that M(LPS+IC) treatment induces miR-125a-5p and miR-181c expression in as little as 6 hours (Figure 2d), concomitant with the fall in CD163 mRNA expression under this same condition (Figure 1d). Given this, we hypothesized that miR-125a and miR-181 family members restrict CD163 expression under M(LPS+IC) conditions through a feedback loop. Specific microRNA antagomirs were used to diminish intracellular microRNA levels during macrophage polarization (Supplemental Figure 2). Of note, miR-181c antagomir also led to a slight but significant decrease in miR-181a expression (Supplemental Figure 2c). Treatment of THP-1 macrophages with miR-125a-5p antagomir markedly increased levels of CD163 mRNA after M(LPS+IC) polarization (Figure 2e). Inhibition of miR-181c also led to a smaller but significant increase in CD163 expression (Figure 2e). Taken together, these results show that upregulation of miR-125a-5p and miR-181 family members limits induction of CD163 during M(LPS+IC).

Direct targeting of CD163 mRNA by miR-181 family members but not by miR-125a-5p

To date, functional analysis of microRNA targeting human CD163 for degradation in human macrophages has not been performed. To explore this, the microRNA target prediction program TargetScan (48) was used to identify putative microRNA:mRNA interactions. We identified a conserved miR-181 family binding site in the 3'-UTR of CD163 (Figure 3a). Notably this site is among the best predicted by this algorithm, with a Context++ score of 91%. This is in agreement with previous findings that porcine miR-181 can target CD163, although the microRNA seed sequences are not fully conserved (46). In contrast, miR-125a-5p was not predicted to bind CD163 by TargetScan or by MirDB, which uses a distinct machine learning model to predict microRNA:mRNA binding (49).

To validate this putative miR-181 binding experimentally, we first utilized an RNA immunoprecipitation (RIP) assay, where total RNA delivered to the RNA-induced silencing complex (RISC) for degradation are co-precipitated along with protein components. This method allows for direct examination of microRNA-mediated targeting within the cell type of interest. Overexpression of miR-181c in THP-1 macrophages markedly enhanced the delivery of CD163 mRNA to the RISC, compared to overexpression of negative control RNA mimics (Figure 3b). As a complementary method to confirm the predicted CD163-miR-181 binding site, we utilized a luciferase reporter vector with the CD163 3'-UTR cloned downstream of the Firefly luciferase gene. Co-transfection of this construct in 293T cells along with specific miR-181a or miR-181c mimics led to significantly reduced luciferase activity, compared to negative control RNA mimic (Figure 3c). In contrast, miR-125a-5p mimic did not significantly alter luciferase activity, further supporting the lack of a specific binding site for CD163. Finally, site-directed mutagenesis was used to alter the predicted miR-181 family binding site in the CD163 3'-UTR to reduce specific binding (Figure 3a). Indeed, co-transfection of miR-181a or miR-181c did not reduce luciferase activity using this mutant construct

(Figure 3c). Together, these findings show that miR-181 family members directly bind to the CD163 3'-UTR to target this mRNA for microRNA-mediated degradation in macrophages.

Overexpression of miR-125a-5p induces global transcriptional changes impacting macrophage polarization

As shown above and in contrast to miR-181 family members, miR-125a-5p does not appear to directly interact with CD163 mRNA. To determine how miR-125a-5p overexpression may impact macrophage phenotypes and affect CD163 expression, a genome-wide target analysis was performed. Total RNA was extracted from THP-1 cells overexpressing either negative control or miR-125a-5p mimic, and differentially expressed genes (DEGs) were determined by RNA-Seq. Correcting for multiple comparisons, this identified 28 DEGs, 21 with significantly decreased expression and seven with increased expression upon miR-125a-5p mimic transfection (Table 1 and Figure 4a, FDR corrected $p<0.1$). Analysis of repressed genes upon miR-125a-5p overexpression showed that 38% (8/21) were predicted targets of this microRNA (Table 1). To identify broader gene networks targeted by miR-125a-5p, the wider samples of 291 DEG (Supplemental Table 1, uncorrected $p<0.01$) was utilized to perform pathway analysis using Ingenuity. The most significantly enriched KEGG pathway represented by miR-125a-5p-repressed genes was "cytokine-cytokine receptor interactions" (Table 2; FDR-corrected p=5.31E-16). Other highly enriched pathways included "Toll-like receptor signalling pathway", "Jak-STAT signalling pathway", and "chemokine signalling pathway". Indeed, among the most significantly downregulated genes identified were *IL10RA*, encoding IL-10 receptor subunit alpha, and *TGFBR2*, encoding TGF-beta receptor type-2 (Table 1). Repression of both genes upon miR-125a-5p overexpression was confirmed using specific qRT-PCR (Figure 4b). Using flow cytometry, we also showed that miR-125a-5p mimic overexpression decreased IL10RA protein levels compared to NC mimic (Figure 4c). Both gene products are receptors for key cytokines which mediate macrophage activation, and in the case of IL-10 is a potent inducer of CD163 mRNA expression. This supports a hypothesis that miR-125a-5p inhibits IL-

10 induced CD163 expression through reducing levels of the IL10 receptor. Indeed, we show that miR-125a-5p overexpression in macrophages was sufficient to markedly reduce signalling through the IL-10 receptor, as determined by IL-10 induced STAT3 phosphorylation (Figure 4d). To examine this further, macrophage *IL10RA* expression was suppressed using siRNA (Supplemental Figure 2). When these macrophages were then stimulated with IL-10, there was a significant reduction in CD163 mRNA upregulation compared to cells treated with scrambled siRNA (Figure 4e), confirming that *IL10RA* expression is essential for CD163 expression. Taken together, these findings suggest that miR-125a-5p is induced in M(LPS+IC) conditions to limit CD163 expression in response to autocrine IL-10 production, through inhibiting expression of key cytokine receptors.

MicroRNA expression alters CD163-mediated anti-inflammatory properties of macrophages

A major function of CD163 on monocytes and macrophages is clearance of potentially damaging endogenous products including free hemoglobin (Hgb) and HMGB1, which are elevated in active SJIA. CD163 binds to these DAMPs complexed with haptoglobin (Hp), leading to their internalization and production of anti-inflammatory mediators including IL-10. We hypothesized that microRNA inhibiting CD163 expression would similarly impair the ability of macrophages to respond to these complexes. First, we confirm that stimulation with either Hgb-Hp and HMGB1-Hp complexes, but not Hgb or HMGB1 alone, trigger increased IL-10 production from THP-1 cells (Figure 5a). This production is CD163-dependent, as co-treatment with a CD163 blocking antibody significantly reduces this IL-10 production (Figure 5a). We also found that HMGB1-Hp complexes fail to induce pro-inflammatory cytokines including IL-1β, IL-6, IL-8 and IL-23 (Supplemental Figure 4). THP-1 cells were then transfected with specific microRNA mimics to miR-181c. Compared to cells transfected with negative control mimics, miR-181c mimic-transfected THP-1 cells failed to induce significant IL-10 production in response to either Hgb-Hp or HMGB1-Hp complexes (Fig 5b). The effects of miR-125a-5p mimic could not be tested as our previous work has shown that this treatment itself enhances IL-10 production (27). These findings suggest that microRNA expression

can regulate key CD163-dependent functional properties involved in limiting tissue damage during hyperinflammation as seen in SJIA and MAS.

DISCUSSION

CD163-expressing monocytes and macrophages are increasingly recognized as important myeloid effector cells in multiple autoimmune and autoinflammatory rheumatic disorders. Macrophages with high CD163 levels can be detected in discoid lupus skin lesions (50) and in lupus nephritis (51). In addition, secreted markers of M(IL-10) or "M2c" macrophages, including sCD163, were increased in adult (52) and juvenile-onset (53) systemic lupus erythematosus, and correlated with disease activity (52). Patients with active SJIA demonstrate activation of blood monocytes with increased CD163 expression (1). In addition, emergence of MAS in patients with SJIA is associated with hemophagocytic macrophages in tissues demonstrating markedly increased in production of both pro-inflammatory mediators such as TNFα, IL-6 and IL-18, as well as sCD163 and surface CD163 expression (4,5,14,15). In turn, CD163-expressing monocytes and macrophages serve key roles during inflammation by clearing damaging substrates such as Hgb and HMGB1 and producing anti-inflammatory mediators. Indeed, CD163 expression reduces mortality in a mouse model of sepsis (9), and sCD163 has suppressive effects on lymphocytes *in vitro* (54). MicroRNA are increasingly recognized as having key roles in regulating functional polarization of monocytes and macrophages. Here, we identify a network of microRNA in monocytes that are increased in SJIA and integrate polarization phenotypes, regulating both CD163 expression and anti-inflammatory functions (Figure 6). Members of the miR-181 family directly target CD163 transcripts for degradation. In contrast, miR-125a-5p functions indirectly to mediate broad changes in macrophage phenotypes through effects of cytokine receptors and intracellular signalling pathways, and in particular responsiveness to IL-10. These microRNA regulatory networks have key implications for the control of macrophage functional polarization and the phenotype of myeloid cells in SJIA.

Epigenetic factors including microRNA are potent modifiers of gene expression programs, and as such there is burgeoning interest in defining their roles in inflammation and rheumatic diseases. Indeed, several reports have highlighted altered circulating or cellular microRNA levels in SJIA (55–57). Our previous monocyte microRNA expression profiling identified a broad spectrum of microRNA with altered expression in children with active SJIA (27). Here, we find several of those microRNA, miR-125a-5p and miR-181 family members, regulate both CD163 expression and key anti-inflammatory functions in monocytes and macrophages. Interestingly, these microRNA are also induced specifically under M(LPS+IC) polarizing conditions, and function as a feedback loop to limit CD163 expression in this phenotype despite autologous IL-10 production. We show here that miR-181 family members regulate CD163 through direct targeting of this mRNA in human macrophages. It is unclear why in our hands *in vitro*, the miR-181c mimic reduces CD163 expression but the miR-181a mimic does not (Figure 2b). This may be due to the higher baseline abundance of miR-181a in THP-1 cells, MDMs and primary monocytes (Supplemental figure 1). In porcine macrophages, several members of the miR-181 family similarly regulate CD163 expression (46). Previous work has suggested that miR-181 family members play key roles in monocytes and macrophages, by directly downregulating proinflammatory cytokine genes including IL-1α and IL-6, and promoting endotoxin tolerance (30,29). MiR-181a also affects key transcription factors including C/EBPα to modulate functional properties of polarized macrophages (58).

In marked contrast, we demonstrate here that miR-125a-5p regulates CD163 not through direct targeting but through induction of global changes in macrophage gene expression. We show that the most significantly enriched gene pathway upon miR-125a-5p overexpression was "cytokine-cytokine receptor interactions," specifically reducing expression of *IL10RA*, which is required to induce CD163 expression in response to IL-10. We also found significantly reduced expression of *TGFBR2*. Notably, TGFβ has been previously reported to induce macrophage CD163 expression (59), although we observed minimal induction in our hands (Figure 1). These findings are in agreement with emerging evidence that miR-125a has broad roles in promoting immunoregulatory phenotypes

in both myeloid and lymphoid cells. We and others have found that miR-125a inhibits antimicrobial functions and promotes alternative macrophage polarization in human and murine cells (27,60,61). Recent work has also found that miR-125a-5p has key roles in T regulatory cells by decreasing sensitivity to inflammatory cytokines such as IL-6 and stabilizing immune homeostasis (28,62). While the precise transcriptional targets for miR-125a in human macrophages remain unclear, our data support this regulator having genome-wide effects on the ability of macrophages to respond to cytokines and other polarizing conditions.

CD163 is both a marker of activated monocytes and macrophages and serves key functional roles during inflammation (9). It is an anti-inflammatory scavenger receptor, binding and engulfing mediators of tissue damage including free haemoglobin and HMGB1, and in turn releasing immune regulatory cytokines. CD163 expression in monocytes and macrophages is regulated by multiple activating stimuli and polarization conditions. Macrophage polarization induced by IL-10 has been long recognized as a potent stimulus for CD163 mRNA expression, and indeed CD163 is among the most robustly upregulated genes by this cytokine (18,63). Our findings using both qRT-PCR and Primeflow RNA flow cytometry are consistent with this, demonstrating significant CD163 gene expression only upon IL-10 stimulation compared to other polarizing conditions, including significant repression of CD163 mRNA by M(LPS+IC) treatment. We do find that while IL-4 leads to only minor increases in CD163 mRNA levels, it does enhance surface protein expression. This may explain in part the common classification of CD163 as a more universal “M2” marker (64) despite experimental evidence disputing a role for IL-4 in CD163 gene expression (19,65). Signal transduction events leading to CD163 gene expression are not fully elucidated. The robust induction of CD163 gene expression by both IL-10 and IL-6 (63) would support a role for STAT3 signalling. However, this is unlikely to occur through direct activation, as CD163 is not among the early STAT3-dependent genes induced by IL-10 (17), and the CD163 promoter was also not identified in a genome-wide analysis of STAT3 binding in macrophages (66). However, several studies show that CD163 expression in

monocytes and macrophages involves integration and cross-talk between multiple cytokine signalling pathways (17,65,67).

It is increasingly clear that monocyte and macrophage polarization is not a dichotomy between classical M1 and alternative M2 activation, but rather a spectrum of phenotypes optimized for particular environments and inflammatory states (16,45,68,69). While there is strong evidence that monocytes are key effector cells in SJIA (70–72), our studies begin to characterize how those states are regulated and integrated to lead to the phenotype seen in active disease. Both transcriptional profiling of PBMCs and direct monocyte immunophenotyping in SJIA patients demonstrates features of multiple well characterized *in vitro* conditions (2,10,22,23). This includes increased CD163 expression, further enhanced during MAS, with higher levels of sCD163 and strikingly high surface expression on macrophages in tissue (4–6). Here we find that miR-125a and miR-181 family members, which are elevated in active SJIA, are induced in M(LPS+IC) conditions and serve to limit CD163 expression in the setting of high autologous IL-10 production. Of note, M(LPS+IC) has key similarities to that seen in SJIA, including increased expression of TNFα, IL-1, IL-6 and IL-10 (27). In contrast, CD163 expression is associated with distinct polarizing conditions, most notably M(IL-10), which lacks expression of these microRNA. This supports a hypothesis where the monocyte phenotype in the hyperinflammatory environment of active SJIA represents a mixed phenotype including features resembling M(LPS+IC) and M(IL-10), with microRNA including miR-125a and miR-181 "fine-tuning" that mixture (Figure 6). Indeed, further increases in CD163 expression associated with MAS may signal that the mixed phenotype has been altered, and monocyte and macrophage functions have been drastically changed. This hypothesis is further supported by recent findings by us and others that emergence of MAS is associated with high circulating levels of interferon-gamma and interferon-induced chemokines (73,74). These mediators are associated with classical monocyte and macrophage activation and were significantly higher than those seen in patients with SJIA without MAS. Indeed, interferon-gamma has been shown to reduce the accumulation of numerous microRNA in macrophages including miR-125-5p (75). Further study

is needed to characterize the regulation of monocytes and macrophages in SJIA and MAS, and the role of key functions such as CD163 in disease pathogenesis.

Authorship:

N.S., S.T., A.A.G. and G.S.S. conceived the study, designed the experiments, and/or gave technical support and conceptual advice. T.D., R.T., and G.S.S. performed the experiments. M.B. and M.M. performed RNA-seq data analysis. S.T., A.A.G. and G.S.S. analysed the data. G.S.S. wrote the first draft of the manuscript. All authors commented on and contributed to the final draft of the manuscript.

Acknowledgements:

We thank the Cell Processing and Manipulation Core in the Translational Cores, and Physicians and Nurses at CCHMC for obtaining and processing these samples. We also thank the CCHMC Translational Research Trials Office for providing the regulatory and administrative support for this endeavor. The Translational Cores are supported in part by the Children's Hospital Research Foundation as well as the Center of Excellence in Molecular Hematology (P30 DK090971). The RNA sequencing datasets described in this paper are available in the Gene Expression Omnibus repository (GSE104853).

COI statement:

Dr. Grom has received consulting fees from Novartis and Baxalta, and has research collaborations with Novartis and NovImmune. Dr. Schulert has received consulting fees from Novartis. All other authors declare no conflicts of interest.

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Figure Legends:

Figure 1: CD163 expression is specifically induced by M(IL-10) and repressed by M(LPS+IC) conditions. A, CD163 mRNA expression in polarized THP-1, peripheral blood monocytes and monocyte-derived macrophages (MDMs) as determined by qRT-PCR after 24 h stimulation with the indicated conditions. B, CD163 protein and mRNA expression as determined by Primeflow RNA flow cytometry in THP-1 cells after 24h stimulation with the indicated ligands. C, Macrophages fail to express CD163 during M(LPS+IC) treatment despite autologous IL-10 production. PMA-differentiated THP-1 cells were left untreated or stimulated with M(LPS+IC), and CD163 (top panel) and IL-10 (bottom) expression determined using qRT-PCR. Data are representative of 2-3 independent experiments per condition. D, M(LPS+IC) treatment leads to rapid repression of CD163 mRNA despite IL-10 induction. PMA-differentiated THP-1 cells were left untreated or stimulated with M(IL-10) or M(LPS+IC), and CD163 (left) and IL-10 (right) expression determined using qRT-PCR. *$p<0.05$, **$p<0.01$, error bars represent mean ± SEM.

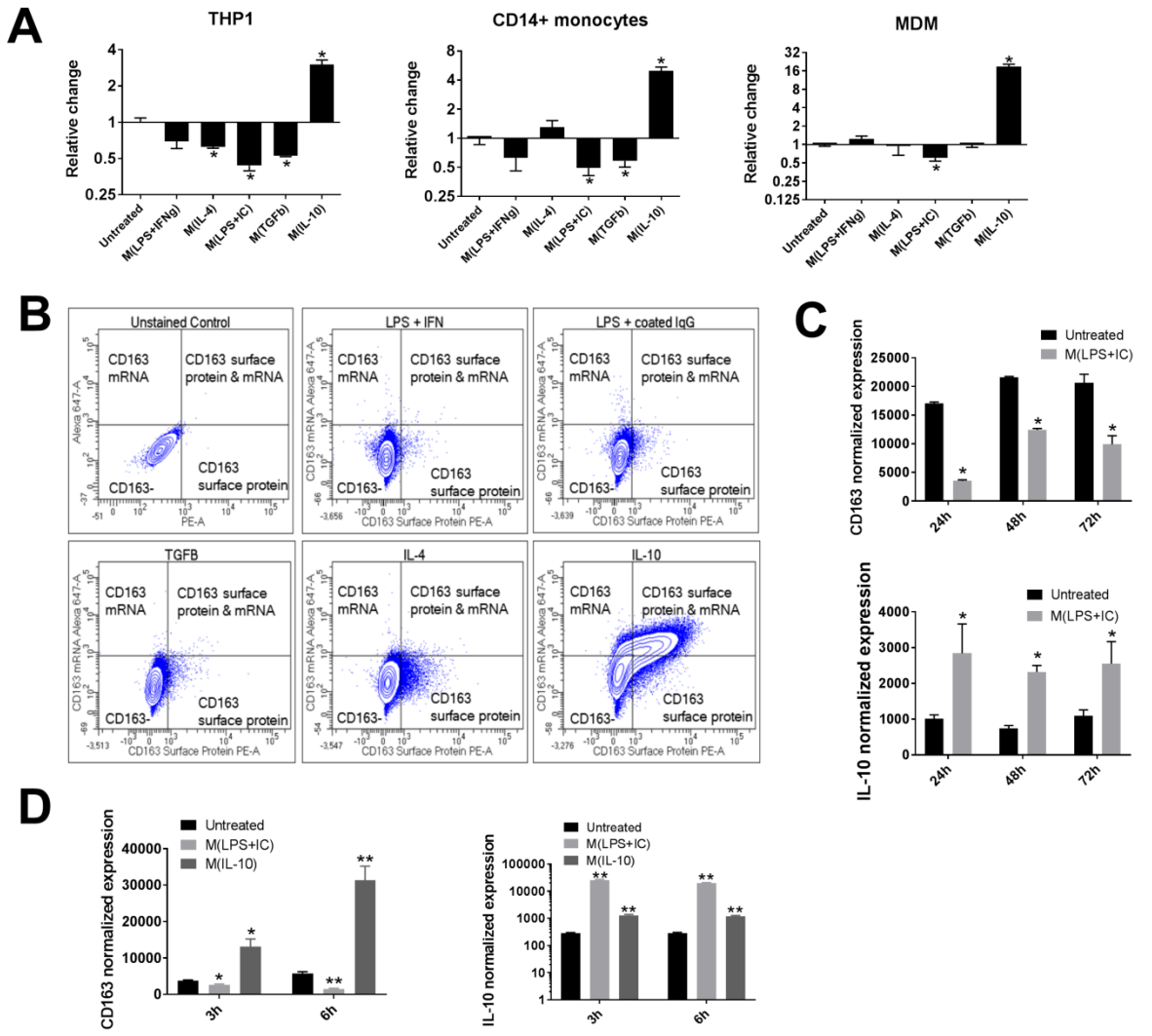

Figure 2: CD163 expression is regulated by multiple microRNA which restrict expression in M(LPS+IC) polarization. A, Levels of microRNA in THP-1 cells, as determined by Taqman qRT-PCR assays. B, CD163 expression is decreased by overexpression of miR-125a and miR-181c. PMA-differentiated THP-1 monocytic cells were transfected with negative control (NC) mimic or specific miR-125a or miR-181c mimics, and then left untreated or stimulated with IL-10 for 24 h. CD163 mRNA levels were determined by qRT-PCR. C, MicroRNA that regulate CD163 are induced by M(LPS+IC), which limit CD163 expression. PMA differentiated THP-1 cells were polarized for 24 h in the indicated conditions. MicroRNA levels were determined using Taqman qRT-PCR. D, M(LPS+IC) treatment leads to early induction of microRNA which restrict CD163 expression. MicroRNA levels were determined in PMA differentiated THP-1 cells as above. E, Inhibition of microRNA levels enhances CD163

expression in M(LPS+IC). PMA differentiated THP-1 cells were treated with NC or specific miR-125a or miR-181c antagomirs and polarized in M2b conditions for 24 h. *p<0.05.

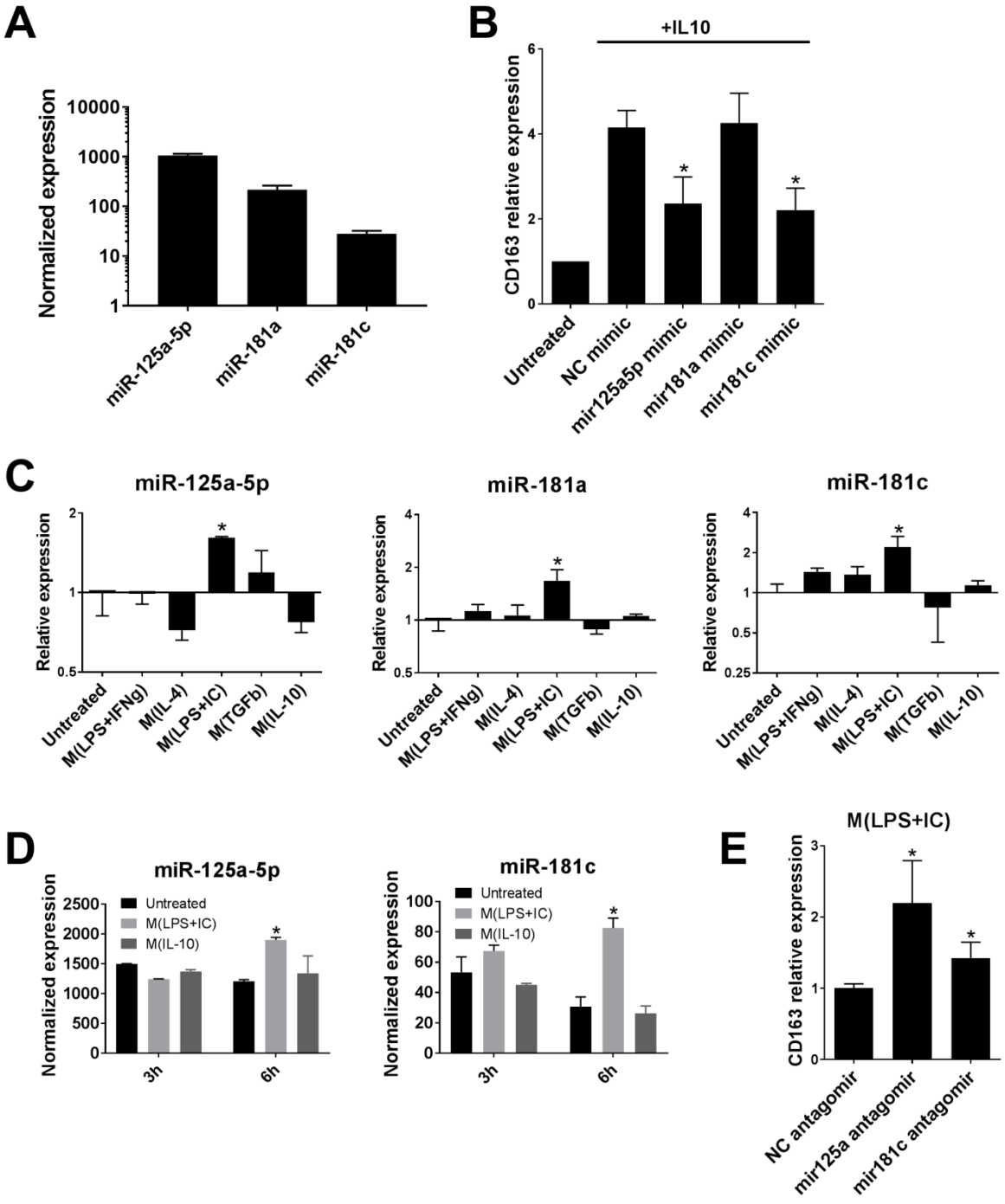

Figure 3: miR-181 family members directly regulate CD163 by binding to 3' UTR. A, Schematic representation of the predicted miR-181 family CD163 binding site and its mutagenesis. B, Overexpression of miR-181c enhances delivery of CD163 to the Ago2 RNA-induced silencing

complex. RNA immunoprecipitation was performed using isotype control or anti-Ago2 antibody in THP-1 cells transfected with negative control (cont) or miR-181c (181c) mimic. CD163 mRNA levels were determined using qRT-PCR. Data are representative of three independent experiments. C, miR-181 interact with CD163 3' UTR. 293T cells were co-transfected with luciferase reporter vectors containing wild-type (WT) or mutated (Mut) CD163 3'UTR, as well as negative control (cont), miR-181a, miR-181c or miR-125a mimics. Luciferase activity is relative to Renilla expression and normalized to expression with negative control mimic. Error bars represent mean ± SEM. *p<0.05.

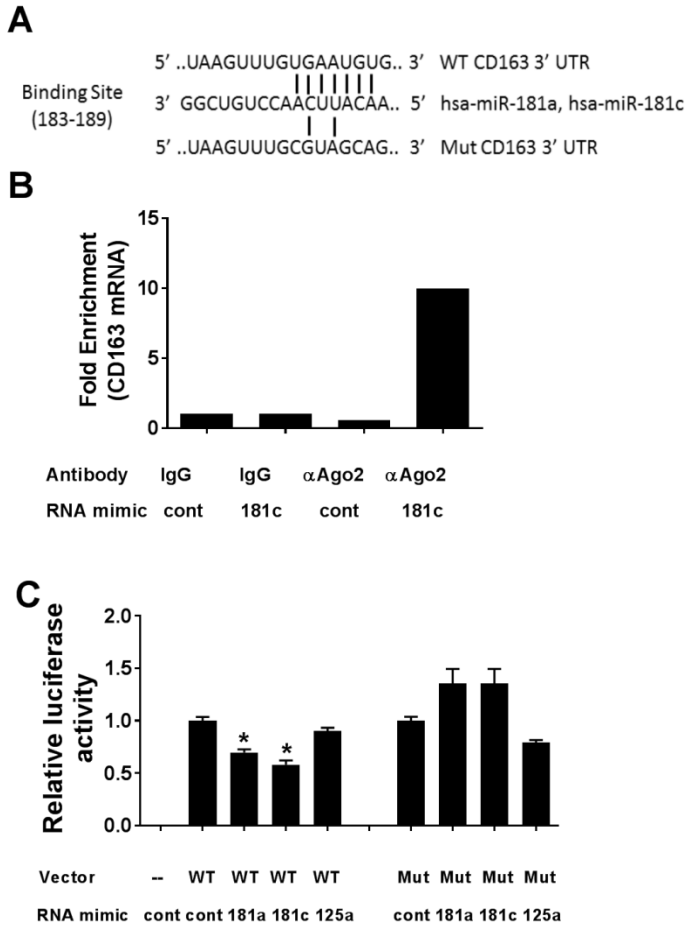

Figure 4: Genome-wide target analysis of miR-125a overexpression. A, RNA-Seq analysis was performed on PMA differentiated THP-1 monocytic cells overexpressing negative control (NC) or miR-125a mimics. Heatmap shows differentially expressed genes (p<0.05, FDR<0.1). B, qRT-PCR showing decreased expression of *IL10RA* and *TGFBR2* in THP-1 cells overexpressing miR-125a mimic. *p<0.05 vs NC mimic. C, miR-125a reduces IL10RA protein levels as determined by flow cytometry.

PMA differentiated THP-1 cells were transfected with NC or miR-125a mimics, and then stained with Alexa 647-conjugated anti-human IL10RA antibody. Representative experiment shown on left, pooled data from three independent experiments shown on right. *p<0.05 vs NC mimic. D, STAT3 phosphorylation in response to IL-10 stimulation. MDMs were transfected with NC or miR-125a mimics, and then stimulated with IL-10 for 30 minutes. Overlays show unstained cells (red), untreated (blue) and IL-10 treated (yellow). Data are representative of three independent experiments. E, IL10RA expression is required for IL-10 induced CD163 expression. PMA-differentiated THP-1 cells were transfected with NC or specific IL10RA siRNA SMARTpool and left untreated or stimulated with M(IL-10). CD163 expression was determined by qRT-PCR. Error bars represent mean±SEM. *p<0.05 vs NC siRNA.

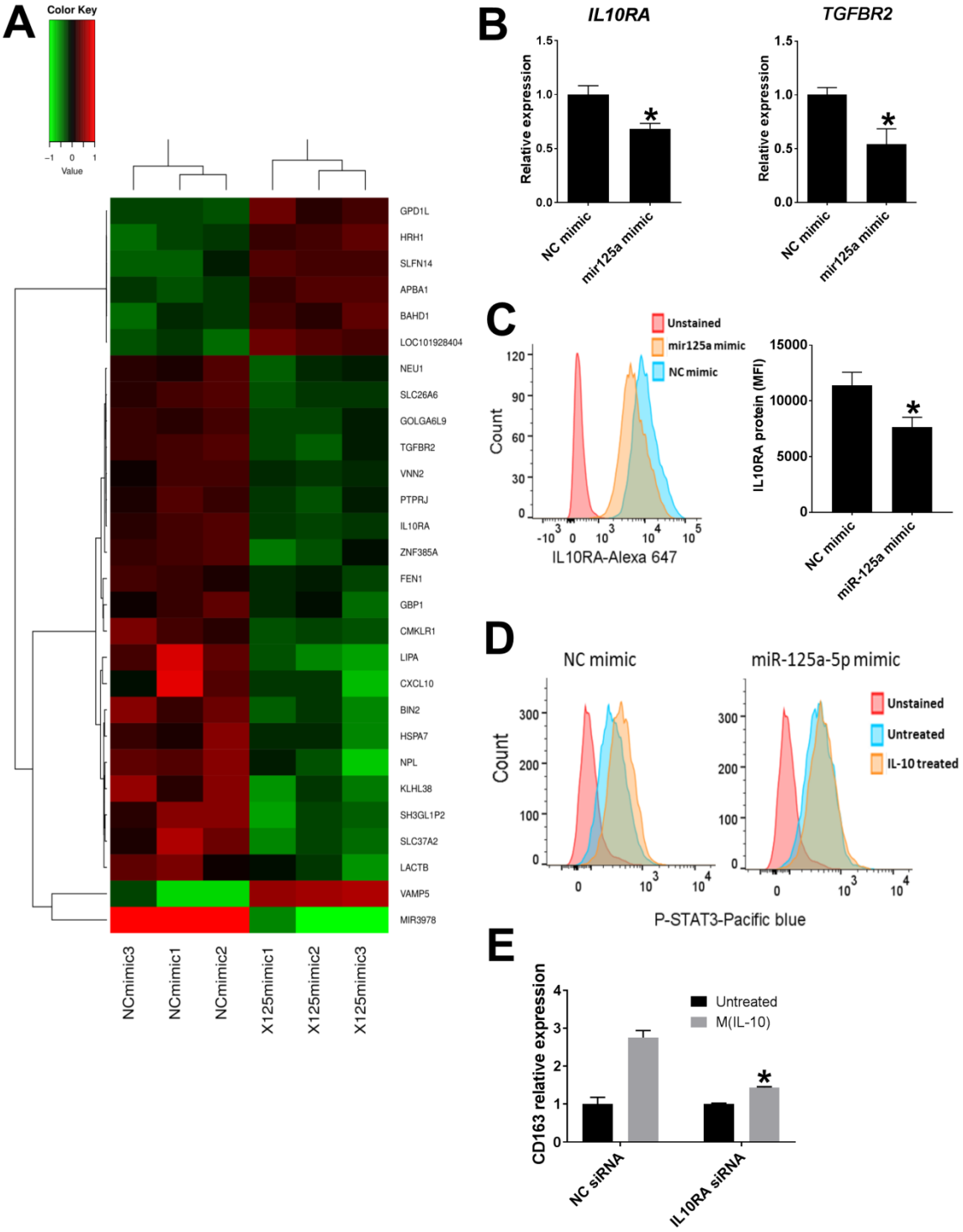

Figure 5: MicroRNA regulate CD163-mediated anti-inflammatory functions of macrophages. A, THP-1 cells left untreated or stimulated for 24 h with hemoglobin (Hgb), hemoglobin-haptoglobin (Hgb-Hp), HMGB1, or HMGB1-haptoglobin (HMGB1-Hp) complexes with or without anti-CD163 monoclonal antibody. IL-10 levels were measured in culture supernatant by ELISA. B, THP-1 cells

were treated with Hgb-Hp or HMGB1-Hp complexes and concurrently transfected with negative control or specific miR-181c mimics. After 48h, IL-10 levels were measured in culture supernatant. Error bars represent mean ± SEM. *p<0.01.

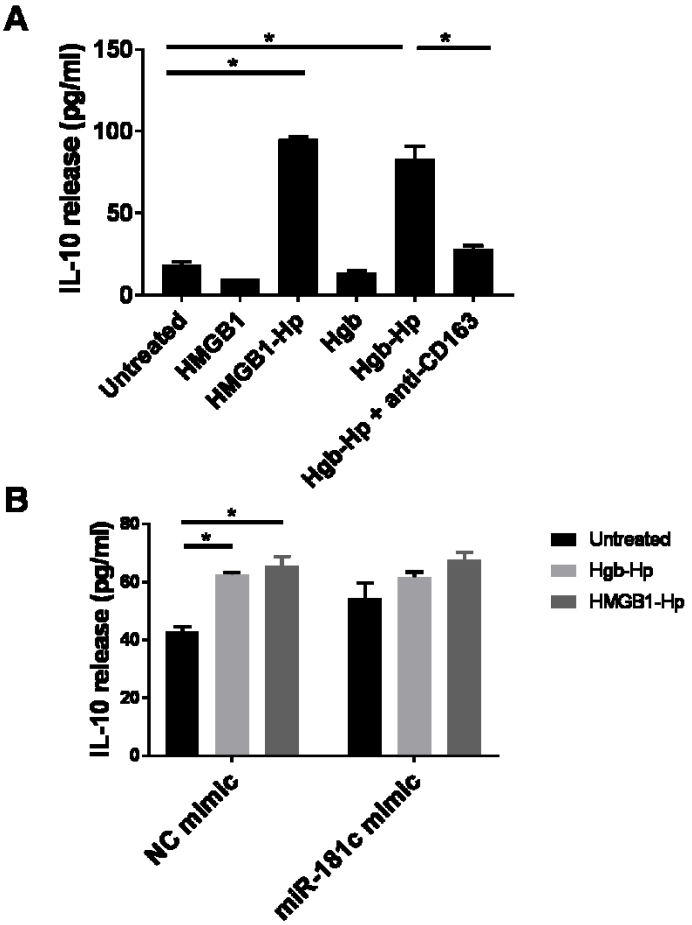

Figure 6: Model of microRNA-mediated regulation of CD163 expression in SJIA. 1) During active SJIA there are increased circulating levels of DAMPs including free hemoglobin, with corresponding increase in haptoglobin, as well as cytokines including IL-10. 2) Monocytes and macrophages are stimulated by IL-10, with increased transcription and protein expression of CD163. 3) CD163 mediates clearance of hemoglobin-haptoglobin complexes through their endocytosis and lysosomal degradation. 4) Complex clearance also leads to increased monocyte and macrophage IL-10 production, enhancing M(IL-10) phenotypes. 5) Monocytes in SJIA also have features of M(LPS+IC) polarization, including increased levels of miR-125a-5p and miR-181 family members. These microRNA inhibit IL-10 induced CD163 expression by reducing levels of IL10R (miR-125a-5p) and by

direct targeting of CD163 mRNA (miR-181 family), and thereby integrate these polarization phenotypes.

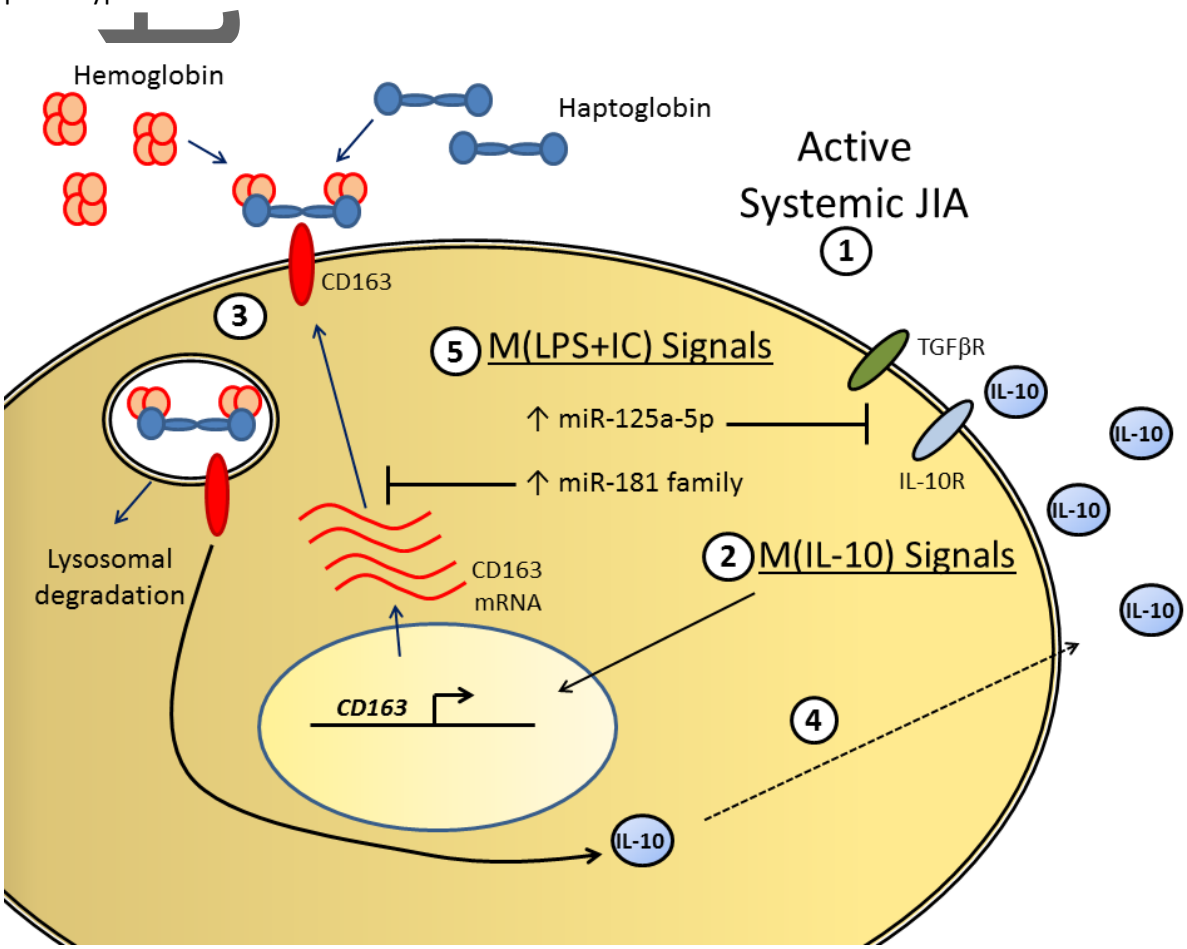

Table 1: Significantly downregulated genes with miR-125a overexpression, FDR <0.1

symbol	name	Mean RPM NC	Mean RPM miR-125mimic	Fold Change	P value	miR-125a target predication		
						Diana	miR-DB	Targetscan
VNN2	vanin 2	8.96092	0.70279	0.0887	0.00014			
MIR3978	microRNA 3978	16.721	2.42534	0.1493	8.57E-06			
KLHL38	kelch-like family member 38	18.585	2.99232	0.17002	3.25E-05			
SH3GL1P2	SH3-domain GRB2-like 1 pseudogene 2	50.2613	18.2999	0.36571	3.69E-05			
LIPA	lipase A, lysosomal acid, cholesterol esterase	2011.84	985.423	0.48831	1.11E-12	X	X	
GOLGA6L9	golgin A6 family-like 9	95.6906	49.2444	0.51399	3.31E-05			
CMKLR1	chemokine-like receptor 1	144.181	75.3111	0.52139	4.60E-06			
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	376.382	203.009	0.53884	5.04E-07	X	X	X
SLC37A2	solute carrier family 37 (glucose-6-phosphate transporter), member 2	1179.34	642.896	0.54369	5.12E-10		X	
BIN2	bridging integrator 2	730.31	416.675	0.56995	1.09E-10	X		X
CXCL10	chemokine (C-X-C motif) ligand 10	1147.94	662.303	0.57518	9.01E-05			
LACTB	lactamase, beta	349.954	217.054	0.61834	0.0001		X	
SLC26A6	solute carrier family 26 (anion exchanger), member 6	179.185	113.901	0.63449	0.00013	X	X	X
HSPA7	heat shock 70kDa protein 7 (HSP70B)	333.958	214.898	0.64333	0.00011			
ZNF385A	zinc finger protein 385A	426.298	278.541	0.6517	6.47E-06	X	X	
NEU1	sialidase 1 (lysosomal sialidase)	654.174	450.283	0.68737	2.15E-05	X		X
TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	1026.85	712.818	0.6923	3.62E-07			
IL10RA	interleukin 10 receptor, alpha	1083.78	765.624	0.70474	3.45E-07			
GBP1	guanylate binding protein 1, interferon-inducible	2045.46	1474.69	0.7194	4.74E-05			
PTPRJ	protein tyrosine phosphatase, receptor type, J	2533.17	1841.87	0.72511	2.95E-06			
FEN1	flap structure-specific endonuclease 1	976.636	740.504	0.75647	0.00014			

Table 2: Top downregulated KEGG pathways with miR-125a overexpression

DESCRIPTION_BRIEF	nGenes	zScore	pValue	FDR
Cytokine-cytokine receptor interaction	146	8.679057	2.00E-18	5.31E-16
Lysosome	116	5.232405	8.37E-08	4.55E-06
Toll-like receptor signaling pathway	83	4.502723	3.35E-06	0.00012
Jak-STAT signaling pathway	92	4.409067	5.19E-06	0.00018
Steroid biosynthesis	16	3.874876	5.33E-05	0.001379
RIG-I-like receptor signaling pathway	51	3.343673	0.000413	0.007815
MAPK signaling pathway	193	3.148086	0.000822	0.013547
Autoimmune thyroid disease	24	2.861423	0.002109	0.028726
Intestinal immune network for IgA production	31	2.834747	0.002293	0.030728
Cytosolic DNA-sensing pathway	40	2.828726	0.002337	0.0311
Natural killer cell mediated cytotoxicity	88	2.716573	0.003298	0.041066
TGF-beta signaling pathway	61	2.662627	0.003877	0.046449
Non-homologous end-joining	12	2.631005	0.004257	0.049909
Allograft rejection	23	2.566882	0.005131	0.058018
Chemokine signaling pathway	146	2.519142	0.005882	0.064369
Chronic myeloid leukemia	70	2.367486	0.008955	0.089356