Identifying Functional MicroRNAs in Macrophages with Polarized Phenotypes

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Macrophages respond to external stimuli with rapid changes in expression of many genes. Different combinations of external stimuli lead to distinct polarized activation patterns, resulting in a spectrum of possible macrophage activation phenotypes. MicroRNAs (miRNAs) are small, noncoding RNAs that can repress the expression of many target genes. We hypothesized that miRNAs play a role in macrophage polarization. miRNA expression profiles were determined in macrophage-derived macrophages (MDMs) incubated in conditions causing activation toward M1, M2a, M2b, or M2c phenotypes. One miRNA guide strand and seven miRNA passenger strands were significantly altered. Changes were confirmed in MDMs from six separate donors. The amplitude of miRNA expression changes in MDMs was smaller than described studies of monocytes responding to inflammatory stimuli. Further investigation revealed this correlated with higher basal miRNA expression in MDMs compared with monocytes. The regulation of M1- and M2b-responsive miRNAs (miR-27a, miR-29b, miR-125a, miR-146a, miR-155, and miR-222) was similar in differentiated THP-1 cells and primary MDMs. Studies in this model revealed cross-talk between IFNγ- and LPS-associated pathways regulating miRNA expression. Furthermore, expression of M1-associated transcripts was increased in THP-1 cells transfected with mimics of miR-29b, miR-125a-5p, or miR-155. The apparent inflammatory property of miR-29b and miR-125a-5p can be at least partially explained by repression of TNFαIP3, a negative regulator of NF-κB signaling. Overall, these data suggest miRNAs can contribute to changes in macrophage gene expression that occur in different exogenous activating conditions.

Macrophages are abundant in diverse tissues and organs where they can function as immune effectors, immune regulators, tissue remodelers, or quiescent scavengers. Some external stimuli can cause macrophages to undergo a dramatic and coordinated change in expression of multiple gene products, changing the functional capacity of the cell. The diversity of environments surrounding macrophages in different tissues corresponds to an equally diverse constellation of macrophage phenotypes in the host (1). Experimentally, there are some well-defined stimuli that cause macrophages to adopt distinct polarized “activation” phenotypes (1, 2). The longest recognized is classical macrophage activation (now called M1 activation), which is induced by IFNγ plus either TNFα or molecules that induce TNFα (e.g. LPS), and is characterized by enhanced microbicidal effector activity (3). “Alternative activation,” now renamed M2a activation, results from IL-4 exposure and has been associated with tissue remodeling and inhibition of inflammation (2, 4, 5). The combination of Fcγ receptor stimulation plus LPS induces “type 2” or M2b macrophage activation, a name that reflects a role in antigen presentation leading toward Th2 differentiation (6). Several anti-inflammatory stimuli, including IL-10 or TGFβ, have been used to generate macrophages that lack microbicidal responses (2). The latter have been called M2c or “deactivated” macrophages, although they retain functional capacities such as phagocytosis and HLA class II molecule expression (2, 7). The regulatory mechanisms controlling the expression of the constellation of genes in macrophages responding to activating conditions are not fully defined.

MicroRNAs (miRNAs) are small, non-coding RNA molecules that suppress gene expression post-transcriptionally. This
occurs after loading of the functional “guide” strand of the miRNA into the miRNA-induced silencing complex, whereas the non-functional “passenger” strand is degraded. When loaded into this effector complex, a single miRNA type can potentially hybridize to and suppress many mRNA transcripts. Indeed, there is evidence that some evolutionarily conserved miRNAs target hundreds of transcripts (8, 9). The capacity to simultaneously modify the expression of multiple genes led us to hypothesize that miRNAs are partially responsible for the coordinated changes in gene expression occurring during macrophage polarization. This hypothesis is supported by a number of published studies implicating different miRNAs in the human monocyte/macrophage response to inflammatory stimuli (10–14). To address our hypotheses, we employed a quantitative RT-PCR (RT-qPCR)-based profiling assay to document the changes in abundance of miRNAs induced by activation of primary human macrophages with four distinct polarizing conditions to span the spectrum of described activation patterns (M1, M2a, M2b, and M2c). Our data revealed that a number of miRNAs were consistently altered under distinct polarizing conditions. Interestingly, despite confirming robust macrophage polarization in our experimental system through measurements of numerous mRNA and protein activation biomarkers, the amplitude of miRNA expression changes detected here were smaller than those reported for monocytes or monocytic cell lines responding to inflammatory conditions. Study of basal expression abundance showed basal levels of all miRNAs investigated were 20 to >700-fold higher in MDMs compared with monocytes, consistent with the global increase in miRNA abundance that has been reported during differentiation of monocytes to macrophages and monocytic cell lines to macrophage-like cells (12, 14–16). This creates an environment in MDMs where “fold changes” in expression levels are smaller in amplitude than in monocytes, in which basal miRNA expression is low. Due to the elevated global miRNA levels in MDMs, we found that monitoring miRNA passenger strand expression was useful for detecting small increases in corresponding miRNA guide strand expression. The biological relevance of some of the regulated miRNAs was evident from the increased expression of several activation-induced inflammatory genes following transfection of a monocytic cell line with mimics of these miRNAs. Both mir-29b and mir-125a-5p repressed the expression of TNFAIP3, a negative regulator of NF-κB signaling, at least partially explaining the inflammatory property of these miRNAs. These data suggest that miRNAs are modifiers of macrophage gene expression that contribute to regulating macrophage gene expression responses to polarizing environmental conditions.

**EXPERIMENTAL PROCEDURES**

**Human Subjects**—Human study protocols were approved by the University of Iowa Institutional Review Board.

**Cell Purification and Culture**—Leukocyte fractions from peripheral blood of healthy human donors were obtained from the University of Iowa DeGowin Blood Center. Peripheral blood mononuclear cells were isolated by density sedimentation using Ficoll-Paque PLUS (GE Healthcare) and maintained in Petri dishes in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen)). After 4 days, non-adherent cells were removed by rinsing and the adherent MDMs were dislodged with 0.25% trypsin/1 mM EDTA (Invitrogen) and cell scraping, and incubated in RP-10 overnight in a Teflon container. In separate experiments, monocytes were isolated by positive selection from peripheral blood mononuclear cells using CD14 magnetic beads (Miltenyi Biotec). RNA from freshly isolated monocytes was purified using TRIzol reagent (Invitrogen).

The THP-1 monocytic leukemia cell line was maintained in RPMI-10 and allowed to differentiate by incubation overnight in RP-10 with 5 ng/ml PMA (Sigma-Aldrich) (17) in a Teflon container. All cells were maintained at 37 °C with 5% CO2.

**Macrophage-polarizing Conditions**—MDMs or PMA-differentiated THP-1 cells were left untreated or were activated toward the following phenotypes: M1 (20 ng/ml IFNγ + 10 ng/ml LPS), M2a (20 ng/ml IL-4), M2b (IgG-coated wells + 100 ng/ml LPS), or M2c (0.5 ng/ml activated TGF-β1). For M2b polarization, wells of tissue culture plates were coated with IgG by incubating plates in HBSS supplemented with human γ-globulin (50 μg/ml) for 1 h at room temperature. Unbound γ-globulin was removed by washing with RP-10. Cells were exposed to each of the polarizing conditions or buffer control when cells were transferred to tissue culture plates. The duration of the treatments is indicated for each experiment. Sources of reagents were as follows: IFNγ (PeproTech), phenol-extracted *Escherichia coli* 055:B5 LPS (Sigma-Aldrich), IL-4 (PeproTech), human γ-globulin (Jackson ImmunoResearch Laboratories), and TGF-β1 (R&D Systems).

**Cytokine Analysis**—Supernatants were collected from MDMs incubated in M1, M2a, M2b, or M2c polarizing conditions for 72 h. Cytokines were quantified using Bio-Plex cytokine assays (Bio-Rad). Data were collected with a Luminex 200 flow cytometer (Bio-Rad) and processed using Bio-Plex Manager software (Bio-Rad).

**RT-qPCR**—Total macrophage RNA was purified using TRIzol reagent (Invitrogen), and the RNA concentration was determined with an ND-1000 spectrophotometer (NanoDrop).

**TaQMan low density array (TLDA)** human miRNA assays (version 2.0; Applied Biosystems) were used to calculate miRNA expression changes between control and polarized MDMs collected from three independent experiments. Briefly, cDNA was generated using the TaqMan reverse transcription kit and Megaplex primer pools A and B (version 2.0). PCR amplification was first performed using TaqMan PreAmp Master Mix and Megaplex PreAmp Primers (version 2.0). Finally, TLDA cards A and B were loaded with pre-amplified reactions, and PCR was performed on a 7900HT fast real time PCR system (Applied Biosystems). Fold changes in the expression of each miRNA were calculated (ΔΔ*Ct* method) using SDS (version 2.3) and SDS RQ Manager software (version 1.2) in tandem. Follow-up analysis of specific miRNAs was performed using TaqMan microRNA assays. RNU48 was used as an endogenous control.

**TaqMan gene expression assays** (Applied Biosystems) were used to quantify the expression of mRNA transcripts. Random hexamer-primed cDNAs were generated using SuperScript III.
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reverse transcriptase (Invitrogen). β-Actin was used as an endogenous control for ΔΔCt calculations.

Transfection of miRNA Mimics—THP-1 cells were incubated in PMMA (5 ng/ml) for 6 h to induce differentiation toward a macrophage phenotype (17). Cells were transfected with the indicated pre-miR miRNA precursor molecules (Applied Biosystems) using RNAiMAX (Invitrogen). Cell lysates were collected 48 h post-transfection for RT-qPCR analysis.

Plasmids—Expression vectors for miR-29b-1 and miR-125a were generated by cloning human genomic sequence into the Xhol/NotI sites of a CMV promoter-based expression plasmid (18). Sequences described in the GRCh37 human genome release of chromosome 7 nucleotides 68595231–68594974 (− strand) and chromosome 19 nucleotides 24464650–24464885 (+ strand) were cloned for the miR-29b-1 and miR-125a expression vectors, respectively. The miRNA expression cassette was then subcloned into the EcoRI site of pAAV-hrGFP (19) (plasmids were kindly provided by Ryan Boudreau and Beverley Davidson, University of Iowa). Luciferase reporter constructs were generated using the psiCHECK2 vector (Promega). Positive control oligonucleotide duplexes were ligated into the Xhol/NotI sites. Each control 3′-UTR contained two miRNA binding sites separated by an SpeI restriction site. Binding sites were composed of sequences with inverse complementarity to the miRNA sequence. The majority of the TNAFAP3 3′-UTR (initial 1400 of 1993 nucleotides) was cloned into the Pmel/NotI sites of psiCHECK2. Mutation of the miR-29b-1 or miR-125a-5p TargetScan-predicted binding sites (supplemental Fig. S1) was performed using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies). Oligonucleotides (Integrated DNA Technologies) used in PCR reactions and in positive control oligonucleotide duplexes are listed in supplemental Table S1. Sequencing was performed to confirm that all constructs contained the expected DNA sequence.

Dual-Luciferase Assays—HEK293 cells were cultured in DM-10 (DMEM (Invitrogen) with 10% fetal bovine serum) in 96-well plates. At ~50% confluence, cells were co-transfected with 50 ng pAAV-hrGFP- and 50 ng psiCHECK2-based plasmids using TransIT293 transfection reagent (Mirus). At 48 h post-transfection, cell lysates were used in a Dual-Luciferase reporter assay system (Promega). Ratios of Renilla and firefly luciferase (Rluc:Fluc) were determined for each sample. The Rluc:Fluc ratio for each psicHECK2-based reporter in miRNA overexpression samples was divided by the Rluc:Fluc ratio for that psiCHECK2-based plasmid in samples transfected with the pAAV-hrGFP vector (no miRNA overexpression). These values were then normalized to the samples transfected with psiCHECK2 vector lacking an insert in the 3′-UTR (arbitrarily set to 1).

Statistical Analyses—Unless otherwise indicated, the linear mixed model analysis for a randomized block design was used to compare mean Ct values or luciferase ratios between treatments. The mixed model included treatment as the fixed effect and the replicate experiment as the random effect. By including the experiment as the random effect, we are able to account for between experiment variability, with treatments compared within experiments. Post hoc test for pairwise comparison with the reference treatment was done using Tukey’s test or Dunnett’s test as indicated.

RESULTS

Biomarkers of Polarized MDM Activation Phenotypes—Our approach to characterizing miRNAs involved in different macrophage activation phenotypes was to screen for miRNAs whose abundance was altered significantly after incubating human MDMs in four distinct polarizing conditions. It was first necessary to verify that the chosen conditions led to the expected activation patterns. MDMs were incubated in conditions chosen to induce their differentiation toward four polarized activation phenotypes as follows: M1 (IFNγ and LPS), M2a (IL-4), M2b (IgG and LPS), or M2c (TGF-β1) (20–22). Polarization toward the anticipated phenotypes was verified by measuring selected cytokines as biomarkers (supplemental Table S2). Seventy-two hours after the addition of polarizing stimuli, IL-12 (p70) was elevated in supernatants of M1-polarized MDMs compared with control MDMs. The proinflammatory molecules IL-1β, IL-6, and TNFα were increased in supernatants from MDMs incubated in either M1- or M2b-polarizing conditions, consistent with other similarities between these phenotypes (20). Although the basal levels of several cytokines that we measured were low in the supernatants of untreated MDMs, the abundance of all five showed a trend toward lower concentrations under the M2c-polarizing condition (supplemental Table S2). This is consistent with observation that TGF-β1 suppresses many biological macrophage activities (2).

IL-10 was increased in supernatants of MDMs incubated in M2b conditions as reported (23). However, a similar increase in IL-10 in supernatants of MDMs polarized toward a M1 phenotype was not anticipated. To determine whether this might reflect a primary response to polarizing stimuli or a secondary response to mediators of inflammation, we investigated the kinetics of IL-10 expression under both conditions. Indeed, IL-10 transcripts were induced in M2b-, but not M1-, polarized MDMs 2 h post-treatment (Fig. 1B), IL-10 mRNA was increased in both conditions (Fig. 1F).

We investigated other biomarkers of the different activation states that could be assessed by RT-qPCR. We chose markers to investigate based upon published microarrays comparing M1- and M2a-polarized MDMs (22). Selected MDM transcripts were measured 8 h after incubation in polarizing treatments (Fig. 1, C–F). Transcripts encoding CCL13 and CCL17 were increased by incubation in M2a-polarizing conditions. M1 conditions consistently induced CXCL9 mRNA expression, and the induction was even greater at the earlier (2 h) time point (Fig. 1A). These data confirmed that the polarization conditions used in this study resulted in distinct macrophage phenotypes.

Expression Profiles of miRNAs in Polarized MDMs—Prior studies of monocytic cell miRNAs responding to inflammatory stimuli document a progressive increase over 8 h and plateau thereafter (10, 13). Therefore, we analyzed miRNA expression in RNA samples extracted from MDMs 8 h after exposure to polarizing conditions. The same RNA samples analyzed for cytokine/chemokine expression mRNA 8 h post-treatment in
The abundance of several miRNAs was regulated differentially in MDMs incubated in distinct polarizing conditions. Polarization-specific biomarkers were analyzed by RT-qPCR assays using RNA collected from MDMs at (A and B) 2 h post-treatment and (C–F) 8 h post-treatment. Data indicate mean fold change with S.E. (n = 3). Horizontal bars indicate significant differences in ΔCt values between the underlying treatments (p < 0.05, Tukey post test). NT, no treatment. G, mean fold changes in the expression of 249 miRNAs determined by TLDA RT-qPCR assays in polarized MDMs at 8 h post-treatment compared with untreated MDMs (n = 3). Labels highlight miRNAs in which changes were significant (p < 0.05, Tukey post test).

Fig. 1 were used as templates for TLDA (version 2.0). These RT-qPCR arrays evaluated the expression of 667 human miRNAs and six putative endogenous controls in treated or untreated MDMs. Four of the controls (RNU48, RNU6A, RNU24, and RNU44) showed no significant changes in abundance between all polarizing conditions, indicating that any of these small RNAs would be reliable endogenous controls. The abundance of the remaining “controls” (RNU43 and RNU6B) fluctuated (supplemental Fig. S1). Of the four reliably stable endogenous controls, RNU48 was utilized to calculate changes in abundance of miRNAs in each of the four polarized MDM conditions relative to the untreated MDMs (supplemental Table S3) and in all subsequent miRNA RT-qPCRs.

Among the miRNAs characterized in the TLDA assays, 249 distinct miRNAs were expressed in MDMs as determined by cycle threshold (Ct) values of <32 in the untreated MDM samples. This total exceeds the reported number of miRNAs expressed in a variety of tissues by ~2.5-fold, according to deep-sequencing efforts using an expression threshold of 100 sequence tags per million (24), suggesting miRNAs of low abundance were included in our analysis. The abundance of 13 of the 249 expressed miRNAs changed >2-fold in MDMs exposed to one or more of the polarizing conditions (Fig. 1G). The eight miRNAs with statistically significant two-or-more-fold changes in expression are labeled in Fig. 1G.

Several general observations can be drawn from the data. First, the relatively small changes in miRNA induction corresponded to vigorous responses to our experimental polarizing conditions. In support of this, we note that response to the M1- and M2b-polarizing conditions was very robust as the concentrations of secreted IL-6 and TNFα could not be accurately calculated without extrapolation as they exceeded the upper limit of the standard curve (Table S2). Second, there were significant changes in expression of some miRNAs in each polarized MDM phenotype except M2c. Third, more miRNAs were significantly induced rather than repressed during macrophage activation. Finally, most of the statistically significant changes in miRNA abundance occurred in miRNA passenger strands (marked with an asterisk) rather than the active guide strand (no asterisk). Passenger strands are usually excluded from miRNA-induced silencing complex and thus are thought to lack biological functions (25, 26), although exceptions have been noted (27).

Changes in expression of several miRNAs were confirmed using RT-qPCR with TaqMan miRNA assays. Replicate assays using MDMs from six independent blood donors, including the original TLDA assays, showed that the abundance of miR-125a-3p, miR-193b, or miR-27a* was significantly increased in MDMs exposed to M1, M2a, or M2b polarizing conditions, respectively (Fig. 2). The BIC (B-cell integration cluster) transcript, which encodes miR-155 and miR-155*, has been shown previously to be induced by inflammatory stimuli such as LPS (28). Thus, it was not surprising that miR-155* was induced by both M1 and M2b conditions, which both include LPS, miR-26a-2* and miR-29b-1* were also co-regulated in both M1 and M2b conditions, suggesting these may also be LPS-responsive. Interestingly, miR-222* had reduced expression under M1-polarizing conditions, yet it had increased expression in both M2a and M2b polarized MDMs.

Increases in miRNA Passenger Strand Abundance Correlate with Lower Magnitude Increases in Corresponding miRNA Guide Strand Abundance—It was surprising that most miRNAs whose expression significantly changed by >2-fold were miRNA passenger strands. An analysis of the corresponding miRNA guide strands revealed parallel trends in expression profiles that only reached statistical significance in the case of miR-27a and miR-155. (Compare passenger strand expression in Fig. 2A–F with corresponding guide strand expression in Fig. 3, A–F.)
The suppressed expression of the miR-222 passenger strand, miR-222*, did not correlate with decreased miR-222 guide strand abundance in M1-polarized MDMs (Figs. 2F and 3F). Similarly, the decreased abundance of miR-26a-2* (Fig. 2A) was not associated with a corresponding decrease in miR-26a (Fig. 3A). Therefore, the parallel trends in regulation of miRNA passenger and guide strands seemed to hold true only in conditions where the passenger strand was increased, but not reduced, in abundance.

Transient Accumulation of miRNA Passenger Strands Precedes Accumulation of miRNA Guide Strands when Basal Expression of miRNA Guide Strands Is High—Despite a robust response of MDMs to polarizing conditions (Fig. 1 and supplemental Table S1), the fold-changes in expression of most miRNA guide strands were small, even when changes in passenger strand expression indicated significant alterations in expression of the miRNA. Based on previous reports (12, 14–16), we hypothesized that miRNA expression levels may globally increase during monocyte-to-macrophage differentiation, accounting for the decreased ratios. To address this, we compared expression of the same miRNAs using RT-qPCR analysis of RNA from monocytes purified directly from human peripheral blood mononuclear cells. ΔCt values of the freshly isolated monocytes were compared with ΔCt values derived from MDMs. For all miRNAs analyzed, the basal expression levels were substantially higher in MDMs than in monocytes with increases ranging from ~20-fold increase in miR-193b to ~700-fold higher expression of miR-155 compared with monocytes (Fig. 4).

Although miRNA guide strands clearly accumulated during the monocyte differentiation to macrophages, the same was unlikely to be true for miRNA passenger strands due to the relatively short half-life of these molecules. We hypothesized that the consistently larger fold changes in the abundance of miRNA passenger strands compared with guide strands resulted from different basal steady-state concentrations. We tested this hypothesis by examining changes in miRNA abundance over time in PMA-differentiated THP-1 cells treated with LPS (Fig. 4, B and C). Indeed, the abundance of the passenger strands miR-155* or miR-125a-3p peaked after 3 or 8 h of LPS treatment, respectively, whereas the guide strands miR-155 and miR-125a-5p continually accumulated following LPS treatment. These observations suggest that there is a window of time following an acute stimulus in which relatively large fold
changes in miRNA passenger strand expression may be considered markers indicating smaller but functionally important changes in the corresponding guide strand.

miRNA and mRNA Expression in Differentiated THP-1 Cells—Anticipating that it may be technically simpler to manipulate miRNA function in a monocytic cell line than in primary macrophages, we analyzed biomarkers and miRNAs in PMA-differentiated THP-1 cells (supplemental Fig. S2, A–D). Similar to MDMs, CXCL9 or IL-10 transcripts were predominantly induced in M1- or M2b-polarizing conditions, respectively, and induced to a lower level in the alternate condition. However, unlike observations using MDM samples, transcripts for the M2a activation biomarkers CCL13 and CCL17 were not preferentially induced by IL-4 treatment of THP-1 cells. As such, we used this cell line as a model to test the function of miRNAs up-regulated in M1 or M2b polarizing conditions.

Five miRNA strand pairs (miR-27a, miR-29b, miR-125a, miR-155, and miR-222) were regulated similarly in THP-1 cells (Fig. 5) and in MDMs exposed to M1 or M2b conditions (Figs. 2 and 3). Also, similar to MDMs, passenger strands in THP-1 cells were induced to a greater fold change than the corresponding guide strands.

IFNγ Inhibits Some LPS-responsive miRNAs—In vivo, macrophages are simultaneously exposed to multiple external stimuli. As such, cross-talk between signaling pathways is important for fine-tuning the cellular responses to a specific external environment (1). Because M1- and M2b-polarizing conditions consist of LPS plus a second stimulus, we examined whether these stimuli were additive or antagonistic for miRNA expression.

miRNA guide and passenger strand pairs (Fig. 6, A–E, and A’–E’) and one additional guide strand, miR-146a (Fig. 6F), were analyzed in THP-1 cells treated with IFNγ and/or LPS, the components of M1 polarization. IFNγ alone accounted for the increase in miR-29b expression, whereas LPS alone accounted for increases in miR-125a-5p, miR-146a, and miR-155 expression. Interestingly, IFNγ antagonized the LPS-induced increase in miR-125a-5p and miR-146a expression. Considering both the guide and passenger strand, IFNγ antagonized LPS-induced expression of miR-222/miR-222′ as well.

The miRNAs analyzed in Fig. 6 were also analyzed in THP-1 cells exposed to components of M2b-polarizing conditions, IgG-coated plates and/or LPS (Fig. 7). Similar to Fig. 6, LPS accounted for most of the increased expression of miR-125a-5p, miR-146a, and miR-155 in THP-1 cells. The remaining miRNAs (miR-27a, miR-29b, and miR-222) were increased after combined treatment with IgG and LPS rather than each component alone in guide and/or passenger strands, suggesting an additive effect of Fcγ receptor- or LPS-induced signaling pathways on expression of these miRNAs.

Cytokine Expression Is Altered in THP-1 Cells in Response to Ectopic miRNA Expression—To examine the functional relevance of the miRNAs that were regulated in response to polarizing conditions, we queried whether transfecting PMA-differentiated THP-1 cells with mimics of these miRNAs could alter expression of cytokines and chemokines characteristic of the M1 and M2b activation phenotypes. CXCL9, IL-6, and TNFα mRNA expression were analyzed following transfection of THP-1 cells with miRNA mimics (Fig. 8, A, C, and E). Experimental augmentation of miR-29b or miR-155 resulted in dose-dependent increases in the M1 marker, CXCL9, as well as the M1/M2b markers IL-6 and TNFα (Fig. 8, B, D, and F). Furthermore, the miR-125–5p mimic, but not the miR-125a-3p mimic, led to increased levels CXCL9 that reached statistical significance. These results indicate that at least some of the miRNAs whose expression in macrophages is increased in response to M1 and M2b polarizing conditions can independently lead to biological changes characteristic of these macrophage activation phenotypes.

miR-29b and miR-125a-5p Repress Expression of Negative Regulator of NF-κB Signaling—The inflammatory property of miR-29b has not been described previously. A negative regulator of NF-κB signaling, A20 (encoded by TNFAIP3), is predicted by TargetScan to be a target of not only miR-29b but also miR-125a-5p (supplemental Fig. S3), whose miRNA mimic induced the expression of CXCL9. A luciferase reporter containing the 3′-UTR of TNFAIP3 was repressed in cells expressing transcripts containing the miR-29b-1 and miR-125a hairpin structures (Fig. 9A). The repression of the reporter was likely mediated by the predicted guide strands for these miRNAs because the positive controls containing targets for the guide strands but not the passenger strands were repressed when co-transfected with the corresponding miRNA expression vectors. Mutation of three nucleotides within the seed region of the predicted miRNA binding sites (supplemental Fig. S3) did not rescue the expression of the TNFAIP3 luciferase reporter (Fig. 9B). This suggests either that the miR-29b- and miR-125a-5p
mediated repression of the TNFAIP3 luciferase reporter was indirect or that the miRNAs recognized the mutated forms of the binding sites.

**DISCUSSION**

Mammalian macrophages are induced to adopt a spectrum of widely divergent phenotypes in response to diverse external stimuli. The current study was based upon the hypothesis that miRNAs are regulators that coordinate, in part, the global changes in expression of many genes that occur after macrophage exposure to different activating conditions.

Expression profiling experiments have documented changes in miRNA expression in human and murine monocytic cells responding to selected inflammatory conditions (10–14, 2012).
concentrations (2, 10, and 50 nM) of either miR-29b or miR-155 was used. Data
significant differences in
polarization (13).
already described as LPS-responsive, in both M1- and M2b-
typotypes. The increased abundance of passenger strand miR-
was still larger than those of the corresponding guide miRNA
strands.
We used MDMs and PMA-differentiated THP-1 cells as models of differentiated, tissue-derived macrophages of the
host. The literature documents several miRNAs, including
miR-29b, miR-146a, miR-155, miR-193b, and miR-222, to be
elevated during monocytic cell differentiation toward macro-
phages (12, 14–16). These miRNAs appeared to be regulated in
MDMs. However, with the exception of miR-193b, the ampli-
tude was too small to reach statistical significance, and the
apparent regulation was only detected when measurements of
the corresponding passenger miRNA strands also were taken
into account. The amplitude of changes was larger and reached
statistical significance when PMA-differentiated THP-1 cells
were analyzed. Yet, despite the increased amplitude in guide
miRNA expression changes, the amplitude of passenger strands
was still larger than those of the corresponding guide miRNA
strands.

FIGURE 8. Introduction of mimics of miRNAs can alter macrophage phenotypes. A, C, and E, RNA was collected from THP-1 cells 48 h post-transfection with miRNA mimics (50 nm). This was used as a template in real time RT-qPCR analysis of IL-6, TNFα, and CXCL9 transcripts. Asterisks indicate significant differences in ΔCt values (p < 0.05, Dunnett post test). B, D, and F, RNA was collected and analyzed similar to A–C, except a range of miRNA mimic concentrations (2, 10, and 50 nm) of either miR-29b or miR-155 was used. Data represent the mean with S.E. fold changes in mRNA abundance between cells transfected with the indicated mimic and the negative control mimic (n = 3).

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miRNA expression changes, the amplitude of passenger strands
was still larger than those of the corresponding guide miRNA
strands.

Compared with our study, relatively large changes in miRNA
expression have been reported in monocytes (10) or undiffer-
entiated THP-1 cells exposed to activating stimuli (13). Inter-
estingly, we found that elevated basal miRNA abundance in
MDMs relative to monocytes correlated with a small amplitude
fold-change in miRNA expression in MDMs exposed to polar-
izing conditions than in activated monocytes. Coley et al. (16)
reported an increase in Dicer and other proteins involved in
miRNA biogenesis during monocyte-to-macrophage differen-
tiation, providing a mechanistic explanation for the elevated
basal miRNA expression levels that we observed.

Transfection with miRNA mimics confirmed the biological
relevance of the guide strands of miR-155, miR-29b, and miR-
125a-5p. The former two each induced macrophage expression
of IL-6, TNFα, and CXCL9, characteristically expressed during
M1 (CXCL9) or during both M1 and M2b (IL-6, TNFα) polar-
ization. The miR-125a-5p mimic induced the expression of
CXCL9, an M1 marker. These observations confirm our con-
tention that significantly elevated passenger strands are indica-

FIGURE 9. miR-29b and miR-125a-5p repress expression of a luciferase reporter under control of the 3′-UTR of TNFAIP3, a negative regulator of NF-κB signaling. A, Dual-Luciferase assays were performed using lysates of Hek293 cells 48 h after transfection with psicHECK2-based plasmids with the TNFAIP3 3′-UTR, or a perfect match target of the indicated miRNA, downstream of the Renilla luciferase reporter. The pAAV-hrGFP-based plasmids, pGFP-29b, or pGFP-125a, were co-transfected with the reporter. Renilla luciferase activity (RLuc) was normalized to activity of firefly luciferase (FLuc), also encoded on the psicHECK-2 vector. B, Independent experiments were performed as described in A using psicHECK2-based plasmids containing mutations within the predicted miR-29b and miR-125a-5p binding sites of the TNFAIP3 3′-UTR, TNFAIP3Δ29b, and TNFAIP3Δ125a, respectively. Normalized RLuc/Fluc ratios are shown as mean with S.E. (n = 3). Asterisks indicate significant differences in luciferase ratios (p < 0.01, Dunnett post test).

28–34). In these experiments, a subset of miRNAs has been
repeatedly documented to be induced following inflammation-
inducing stimuli, which induce differentiation toward the M1
phenotype. We reasoned that miRNAs may be involved not
only in macrophage responses to inflammatory conditions but
also in the modifications of gene expression required to gener-
ate a spectrum of macrophage activation patterns. To better
represent the entire spectrum, therefore, our study was
designed to identify miRNAs that respond to stimuli inducing
four different patterns of macrophage activation (M1, M2a,
M2b, and M2c). The experiments illuminated miRNAs that
were uniquely regulated in human macrophages polarized
toward M1 (miR-125a-3p and miR-26a-2*), M2a (miR-193b),
or M2b (miR-27a*, miR-29b-1*, miR-132*, and miR-222*)
phenotypes. The increased abundance of passenger strand miR-
155* signified involvement of miR-155, an miRNA that was
already described as LPS-responsive, in both M1- and M2b-
polarization (13).
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tors of functionally important changes in the corresponding guide strands.

Further investigation suggested that the inflammatory properties of miR-29b and miR-125a-5p may at least partially be explained by these miRNAs enhancing NF-κB signaling by repressing the expression of a negative regulator of NF-κB signaling A20, encoded by TNFAIP3. Because TNFAIP3 is induced by NF-κB signaling, it is tempting to speculate that miR-29b and miR-125a-5p may act to limit the accumulation, rather than reduce basal levels, of this transcript.

During miRNA biogenesis, primary miRNA transcripts are processed sequentially by the endonucleases Drosha and Dicer to generate a ~22-bp double-stranded miRNA duplex. One strand, the guide strand, is loaded into miRNA-induced silencing complex where it influences gene expression, whereas the other strand is excluded and degraded quickly (35). For most miRNA duplexes, there is a preferred guide strand (25, 26). The identity of the preferred guide strand for each miRNA duplex, based on its classification by miRBase (36), was confirmed by estimating the relative basal abundance of each strand in unstimulated MDMs based on Ct values from quantitative RT-PCRs (data not shown). A surprising result of the current study was the observation that seven of the eight miRNAs that had statistically significant changes in the TLDA screen above a 2-fold cutoff were miRNA passenger strands. Alterations in passenger strand abundance were mirrored by smaller, statistically insignificant changes in abundance of the corresponding miRNA guide strands in MDMs. We hypothesized that, due to their lower basal expression, changes in the abundance of miRNA passenger strands were more easily detected than changes in the corresponding guide strands in response to acute macrophage stimulation. This hypothesis was supported by kinetic analyses of both strands of LPS-responsive miR-125a or miR-155, showing that fold-increases in passenger strands were larger and peaked earlier in response to LPS than fold-increases in the corresponding guide strands. The transient nature of the increase in miRNA passenger strand abundance may be a result of the short half-life of these molecules. Given the observed expression kinetics of guide versus passenger strands, we conclude that analysis of miRNA passenger strand expression provides a transient marker for delayed increases in the functional guide strand miRNA expression following acute stimulation of macrophages. These observations, therefore, should be applicable to miRNA expression studies in other cell types responding to acute stimulation.

Several miRNAs that initially seemed to be M1- or M2b-specific based on TLD assay results were later determined to be LPS-responsive and were regulated in similar directions in M1- and M2b-polarized macrophages. An exception to this trend was miR-222, which in both MDMs and differentiated THP-1 cells had reduced abundance in M1, but increased abundance in M2b, treatment conditions. This observation led us to investigate whether miRNA expression is affected by cross-talk between signaling pathways that were simultaneously activated during M1 or M2b polarization. Several miRNAs (miR-125a-5p, miR-146a, and miR-222) showed reduced response to LPS in the presence of IFNγ, whereas IgG treatment enhanced LPS-mediated induction of a subset of miRNAs (miR-27a, miR-29b, and miR-222). Thus, variations in miRNA expression can result from cross-talk between signaling cascades initiated by different external stimuli. miRNAs whose abundance is altered in response to a given stimulus often act in a feedback loop, regulating the expression of proteins that transduce the signal (37). Consistent with the fact that miRNAs often target the pathways that regulate their expression, miR-146a targets STAT1 (38), an important component of IFNγ signaling. It will be of interest to determine whether either miR-125a or miR-222 also modulate IFNγ signaling.

Given the complex variability of macrophage activation, the involvement of miRNAs gives the cell a logical means of translating a myriad of external stimuli into a spectrum of responses at the level of gene expression. Adding to this complexity, multiple miRNAs can contribute to the expression of one transcript and one miRNA can simultaneously modify the expression of many genes. Bioinformatic target prediction algorithms estimate up to two-thirds of the human transcriptome may be regulated by miRNAs (39). Thus, the modulation of miRNA expression and the subsequent effect on the expression of their target genes could provide the macrophage a mechanism through which it can diversify and specifically respond to environmental stimuli with different patterns of activation.

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