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ORIGINAL ARTICLE

MicroRNA profiling reveals opposing expression patterns for miR-511 in alternatively and classically activated macrophagesDanielle Karo-Atar, MSc¹, Michal Itan, PhD¹, Metsada Pasmanik-Chor, PhD², and Ariel Munitz, PhD¹¹Sackler School of Medicine and ²Bioinformatics Unit, Tel Aviv University, Tel Aviv, Israel**Abstract**

Background: Macrophages are heterogeneous cells, which possess pleotropic effector and immunoregulatory functions. The phenotypic diversity of macrophages is best exemplified by the ability of IL-4 or IL-13, two key cytokines in asthma to promote macrophages into a suppressive/anti-inflammatory phenotype (e.g. alternatively activated or M2) whereas exposure to IFN- γ followed by microbial trigger renders macrophages pro-inflammatory (e.g. classically activated or M1). Intriguingly, only limited data exists regarding the expression of miRNA in M2 macrophages. **Objective:** To define the miRNA profile of M2 and M1 macrophages. **Methods:** Bone marrow-derived macrophages were activated to classically and alternatively activated states using IL-4, IL-13 or IFN- γ followed by *Escherichia coli* stimulation. Thereafter, an unbiased miRNA “mining” approach was utilized and the expression of several miRNAs was validated following *in-vitro* and *in-vivo* macrophage activation (qPCR). miR-511 over-expression was performed followed by global transcriptional and bioinformatic analyses. **Results:** We report unique miRNA expression profiles in M2 and M1 macrophages involving multiple miRNAs. Among these miRNAs, we established that miR-511 is increased in macrophages following IL-4- and IL-13-stimulation and decreased in M1 macrophages both *in-vitro* and *in-vivo*. Increased miR-511 expression was sufficient to induce marked transcriptional changes in macrophages. Interestingly, bioinformatics analyses revealed that miR-511 altered the expression of gene products that are associated with hallmark alternatively activated macrophage functions, such as cellular proliferation, wound healing responses and inflammation. **Conclusions:** Our data establish miR-511 as a bona fide M2-associated miRNA. These data may have significant implications in asthma where the expression of IL-4 and IL-13 are highly increased.

Keywords

Alternatively activated macrophages, IL-4, macrophages, micro RNA, miR-511

History

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Introduction

Macrophages are heterogeneous immune cells, which possess key effector and immunoregulatory functions ranging from tissue damage to repair [1]. Therefore, molecular pathways regulating the heterogeneity and plasticity of these cells have been an area of intensive research. Several studies have raised the notion that the phenotypic diversity of macrophages is likely a continuum spectrum of various distinct cellular activation states [1,2]. Comparing the functional activities and genetic signatures of “classically-activated” macrophages (e.g. cells activated with IFN- γ along with exposure to microbes/microbial products, also termed M1) with those of “alternatively-activated” macrophages (i.e. cells activated with IL-4 or IL-13, also termed M2) nicely represent the extremes of this scale [3].

M2 macrophages are traditionally considered anti-inflammatory and immunosuppressive cells. These cells have fundamental roles in Th2 immunity, obesity, metabolism

and cancer [4–6]. M2 macrophages display a distinct gene signature profile, which includes the up-regulation of hallmark mediators and enzymes including Resistin-like molecule α (Relm- α), arginase 1, matrix metalloproteinases (e.g. MMP-12) and Th2-associated chemokines (e.g. CCL17) [5,7–9]. In sharp contrast, M1 macrophages are characterized by increased production of pro-inflammatory cytokines (e.g. IL-6 and TNF- α), elevated levels of nitric oxide and reactive oxygen species production, which ultimately lead to increased microbicidal activity and are associated with tissue damage [1,8].

IL-4 and IL-13 induce many overlapping effector functions in macrophages since both cytokines signal via a shared network of receptors (e.g. the type 1 IL-4R) and signaling cascades (e.g. STAT-6) [10,11]. Indeed, activation of macrophages with IL-4 or IL-13 induces the expression of similar M2-associated markers, such as Relm- α , arginase, YM1 and renders the anti-inflammatory function of these cells [12]. Thus, the downstream effects of IL-4- and/or IL-13-activated macrophages (mutually termed M2) received much attention over the past few years. Despite this growing interest, only limited data exists regarding the mutual roles of these

cytokines in inducing intrinsic regulatory mechanisms, such as miRNAs. Notably, identification of IL-4/IL-13-induced miRNAs may provide key insight into the downstream molecular checkpoints governing IL-4/IL-13-induced macrophage activities.

miRNAs are evolutionary conserved, short, single-stranded, non-coding RNAs that regulate specific gene expression via direct interactions between their 5' region (termed "seed sequence") and the 3' untranslated region (UTR) of target mRNAs. Consequently, direct binding of miRNAs to a target mRNA mainly results in mRNA degradation. Nevertheless, miRNAs can also inhibit protein translation [13]. Studies focusing on miRNA expression and function in macrophages have predominantly focused on M1 macrophages. These studies revealed the involvement of various miRNAs including miR-155, let-7e, mir-21, miR-146a-5p and miR-125b-5p in inflammatory activities of M1 macrophages [14–16]. To date only a limited number of miRNAs (e.g. miR-378, miR-511 and Let-7c) has been identified in M2 or tumor-associated macrophages, respectively [17–21]. While the latter studies begun to unravel the involvement of miRNAs in M2 macrophage polarization, a substantial paucity of data still exists in respect to the global expression pattern of miRNAs in polarized macrophages. Furthermore, whether IL-4 and IL-13 induce similar miRNA expression in macrophages is currently unknown.

In this study, we aimed to profile the expression of miRNAs, which are differentially expressed in M1 and M2 macrophages. To this end, we now show that miR-511 is induced in macrophages following IL-4- and IL-13-stimulation both *in-vitro* and *in-vivo* and is suppressed in M1 macrophage activating conditions. Unbiased transcriptional profiling following miR-511 over-expression suggested key roles for miR-511 in multiple M2-associated macrophage functions, such as cellular proliferation, cellular metabolism and healing responses. Collectively, these data highlight miR-511 as a hallmark M2-expressed miRNA and suggest miR-511 as a possible regulator of the M1/M2 macrophage activation checkpoint.

Materials and methods

Mice

Male and female, 6- to 8-week-old C57BL/6 wild-type (WT) mice were obtained from Harlan Laboratories (Rehovot, Israel) and grown in-house. In all experiments, age-, weight- and gender-matched mice were housed under specific pathogen-free conditions according to institutionally approved protocols of the Tel Aviv University Animal Care Committee.

Bone-marrow-derived macrophage activation

Bone-marrow-derived macrophages were obtained by flushing the femur and tibia of the mouse with 20 ml of Dulbecco's Modified Eagle's medium (DMEM). Thereafter, the media was supplemented with 20 ng/ml of recombinant mouse M-CSF (Peprotech, Rehovot, IL) and the cell suspension was cultured for 6–8 days. BM-derived macrophages were treated with recombinant IL-4 (10 ng/ml, Peprotech, 48 h), IL-13 (10 ng/ml, Peprotech, 48 h), or IFN- γ (10 ng/ml, 24 h)

followed by *Escherichia coli* (24 h, American Type Culture Collection #10799). Peritoneal macrophages were obtained by washing the peritoneal cavity using 10 ml of DMEM. Thereafter, the cells were left to adhere for 3–4 h. Purity of the adherent macrophages was consistently over 97% as assessed by CD11b and F4/80 expression [22]. Macrophage polarization was determined by assessing specific cytokine production and measuring the ratio between arginase 1 and iNOS [23] using the following primers: Arginase 1 (Fwd GAATCTGC ATGGGCAACC; Rev-GAATCCTGGTACATCTGGGAA C), iNOS (Fwd-CTTTGCCACGGACGAGAC; Rev-TCATTGTA CTCTGAGGGCTGAC).

Enzyme-linked immunosorbent assay

IL-6, CCL17 and TNF- α were measured by ELISA (Duo-Set, R&D Systems, Minneapolis, MN). Relm- α was determined as previously described [22,24]. The lower detection limits for the aforementioned mediators were 31.25, 15.6, 31.25 and 31.25, respectively.

TaqMan real-time PCR microRNA array

Total RNA was isolated using Trizol (Invitrogen, Grand Island, NY). TaqMan Rodent MicroRNA Arrays (Applied Biosystems, Foster City, CA), representing 585 mature miRNAs, were used according to the manufacturer's instructions. Cycle threshold (*Ct*) values were calculated using the SDS software v.2.3 (Applied Biosystems, Grand Island, NY) using automatic baseline settings and a threshold of 0.2. *Ct* values > 37 were considered to be below the detection level of the assay. Five endogenous controls were considered. Eventually, data were analyzed in comparison to snoRNA202, which served as the most reliable endogenous control in our settings (*Ct* differences between samples/treatment of <0.5). Data analysis was performed using GeneSpring (Agilent Technologies, Wilmington, DE) setting a 2-fold increase threshold.

IL-4 and IL-13-induced peritonitis

IL-4-induced peritonitis was performed using anti-IL-4:IL-4 complexes, which increase the *in vivo* half life of IL-4 [25]. IL-13 was administered via intraperitoneal delivery (10 μ g/mouse) every other day for 5 days. Forty-eight hours after the final IL-4 or IL-13 injection peritoneal lavage was performed. Peritoneal cells were left to adhere over night and thereafter; macrophages were collected for RNA extraction and miRNA expression assessment. Macrophage purity was assessed by anti-F4/80, anti-CD11b staining and was consistently >97%.

Allergic airway inflammation

Antigen-associated asthma was induced by challenging mice intranasally three times a week for 3 weeks with an extract of *Aspergillus fumigatus* (Asp) as previously described [26]. In brief, mice were lightly anesthetized with isoflurane inhalation, and 10 μ g of total protein (and not dry weight) in 50 μ l saline of Asp (Bayer Pharmaceuticals, Spokane, Washington, DC) or 50 μ l of saline solution alone was applied to the nasal cavity by using a micropipette with the mouse held in the supine position. After instillation, mice were held upright

until alert. Mice were euthanized 24–48 h after the last challenge and bronchoalveolar lavage was performed. Bronchoalveolar cells were left to adhere over night and thereafter; macrophages were collected for RNA extraction and miRNA expression assessment. Macrophage purity was assessed by anti-F4/80, anti-CD11b staining and was >97%.

Affymetrix cDNA microarray

Mouse Affymetrix (Cleveland, OH) microarrays (1.1 ST GeneChip[®], Affymetrix, Cleveland, OH) were performed and analyzed using established protocols of the Tel-Aviv University Bioinformatics Unit.

Statistical analysis

Data were analyzed by ANOVA followed by Tukey's *post hoc* test or Student's *t*-test using GraphPad Prism 4 (GraphPad Software, San Diego, CA). Data were presented as mean \pm SEM, and values of $p < 0.05$ were considered statistically significant.

Results

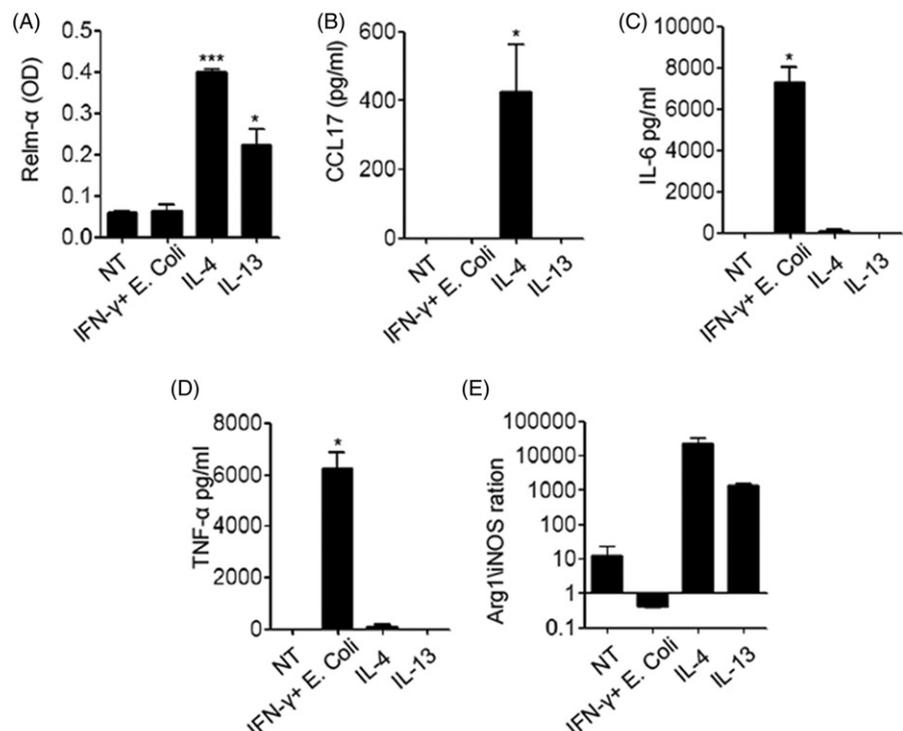
miRNA expression profiling in M1 and IL-4-activated macrophages

Bone marrow-derived macrophages were activated with IL-4, IL-13 or IFN- γ followed by heat inactivated *E. coli*. The polarized phenotype of the macrophages was validated by the expression of Relm- α and CCL17 for IL-4- and IL-13-activated cells, IL-6 and TNF- α for M1 polarized cells as well as the arginase 1:iNOS ratio (Figure 1A–E) [22,23,27]. IL-4 and IL-13 increased the expression of Relm- α (Figure 1A), while only IL-4 was capable of increasing CCL17 secretion (Figure 1B). Furthermore, Arg1/iNOS ratio was elevated in IL-4 and IL-13 polarized cells (Figure 1E). Notably, IL-4 or

IL-13 did not increase the secretion of IL-6 and TNF- α (Figure 1C–D). In contrast, activation of macrophages with IFN- γ followed by *E. coli*, which induces an M1-polarization phenotype, induced a marked increase in IL-6 and TNF- α secretion (Figure 1C–D), resulted in reduced Arg1/iNOS ratio (Figure 1E) but had no effect on Relm- α and CCL17 secretion (Figure 1A–B).

To identify the effects of IL-4 and M1 activating conditions in the regulation of downstream miRNA expression, we undertook an unbiased empiric miRNA profiling approach. This analysis revealed that out of the 585 miRNAs that were assessed, 196 miRNAs were differentially expressed in M1 and/or IL-4 polarized macrophages, which possess $\sim 30\%$ of the miRNAs, which were analyzed. Hierarchical clustering of differentially expressed miRNAs revealed unique miRNA expression profiles in IL-4 and M1 macrophages (Figure 2A). Specifically, skewing macrophages into an M1 state was accompanied with differential expression of 143 miRNAs, whereas IL-4 induced the differential expression of 117 miRNAs. Among these miRNAs, M1 polarizing conditions induced specific changes in 79 miRNA transcripts (Figure 2B, Table S1). In contrast, IL-4-induced macrophage activation resulted in specific decreased/increased expression of 53 miRNAs (Figure 2B, Table S2). Skewing macrophages into an M1- or M2-associated phenotype resulted in altered expression of 64 miRNAs (Figure 2B and Table S3). To better distinguish between the different clusters, selected differentially expressed miRNAs were grouped and further classified into four miRNA groups representing: (1) ‘‘generally activated macrophage miRNAs’’ – representing miRNAs that are increased/decreased in both M1 and IL-4-activated macrophages (representative miRNAs in this group can be found in Supplemental Figure 1); (2) ‘‘M1 specific miRNAs’’ – representing miRNAs which are specifically increased/

Figure 1. M1 and M2 polarization of macrophages. Bone marrow-derived macrophages were activated with IL-4 or IL-13 for M2-polarization or IFN- γ followed by *E. coli* for M1-polarization. The levels of secreted Relm- α (A), CCL17 (B) (as M2 markers), IL-6 (C) and TNF- α (D) (as M1 markers) were determined by ELISA. The ratio between Arginase 1 (Arg1) and iNOS (E) was determined using qPCR. Data represent $n = 3$; * $p < 0.05$, *** $p < 0.001$, NT – no treatment.



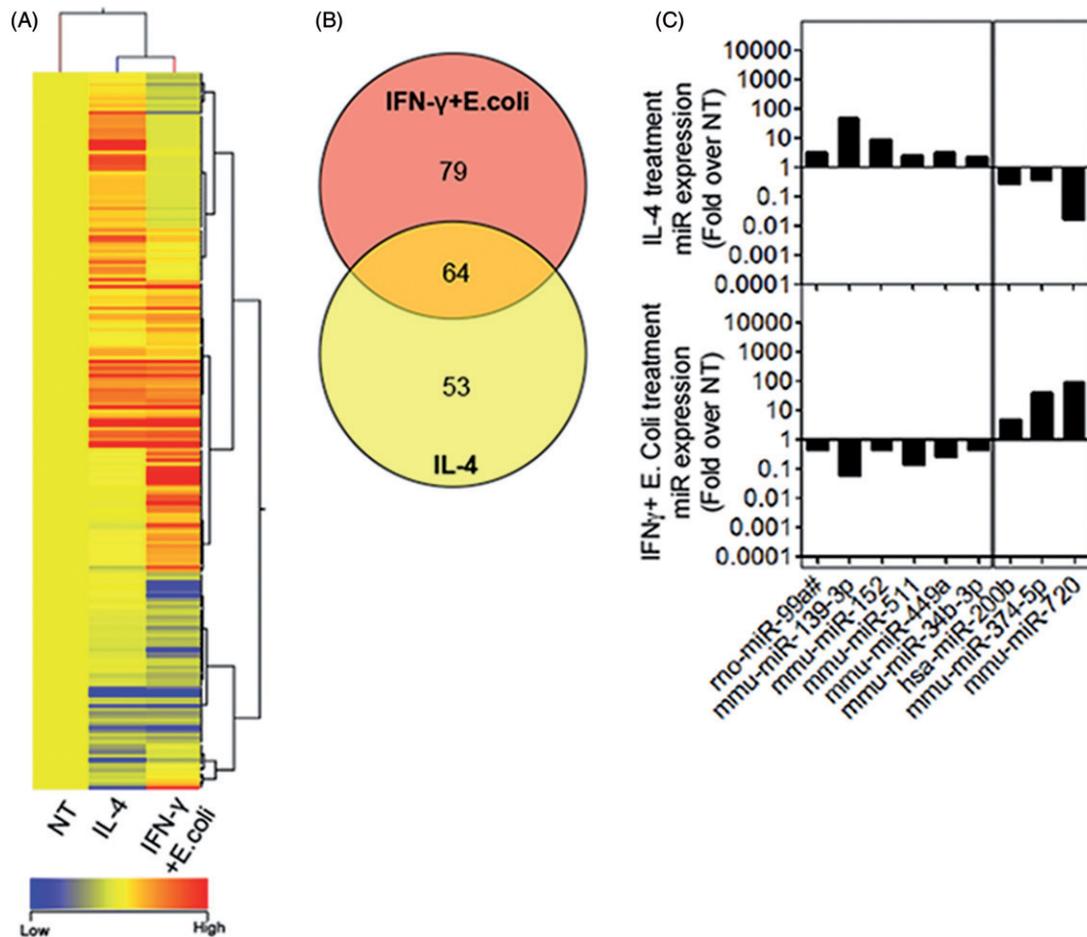


Figure 2. M1 and M2-polarized macrophages display distinct miRNA expression profiles. miRNA expression profiling of IFN γ + *E. coli*- and IL-4-activated macrophages (IL-4) was performed. Differential miRNA expression following the different treatments is demonstrated by heat map (A) and Venn plot (B) analyses. Cluster analysis of selected differentially expressed miRNAs (fold change >2) was performed. A cluster representing miRNAs that are oppositely regulated by IL-4 and IFN γ + *E. coli* induced macrophage activation is shown (C). Data represent $n = 2$; NT - no treatment.

decreased following M1 polarization and unchanged in IL-4 polarized cells (representative miRNAs in this group can be found in Supplemental Figures 1, 2, left panel); (3) “IL-4 specific miRNAs” – representing miRNAs that are specifically increased/decreased following IL-4 activation and unchanged in M1-polarizing conditions (representative miRNAs in this group can be found in Supplemental Figures 1, 2, right panel); and finally, (4) “M1/IL-4 oppositely-regulated miRNAs” – representing miRNAs which display contrasting expression following M1 and IL-4-activation (Figure 2C).

Validation of miRNA profiling data by qPCR analysis

To validate the array results, an independent cohort of M1-polarized and IL-4-activated BM-derived macrophages was subjected to qPCR analysis of selected miRNAs. Specifically, we chose previously established M1- or M2-associated miRNAs (e.g. miR-155 and miR-378, respectively) [15,18], which were also detected in our experimental settings (Supplemental Figures 1 and 3 and Tables S1–S2) and miRNAs that displayed opposing expression patterns following M1/IL-4-polarization (e.g. “oppositely-regulated miRNAs”, Figure 2C and Table S3). Certainly, miR-155, a

“hallmark” M1-associated miRNA, was also validated as an M1-specific miRNA in our data set (Figure 3A). In addition and in agreement with previous findings, miR-378 was up-regulated following IL-4-induced macrophage activation and to a lesser extent in M1 macrophages (Figure 3B). Finally, miR-449a and miR-511, which were identified in our miRNA array as “oppositely regulated miRNAs” (Figure 2C), were indeed confirmed as miRNAs that are induced by IL-4 and decreased in M1-polarizing settings (Figure 3C–D). Although miR-139-3P and miR-720 were the most differentially expressed miRs among the “oppositely regulated miRNAs”, we were unable to confirm this observation in independent experiments.

miR-511 and miR-449a are up-regulated *in-vivo* in IL-4C elicited macrophages

Intrigued by the differential regulation of miR-511 and miR-449a in IL-4 and M1 macrophages we were next interested to determine whether IL-4 could induce the expression of these miRNAs *in vivo* as well. To this end, a long acting form of IL-4 (IL-4C) was administered to the peritoneal cavity of wild type mice and IL-4C-elicited peritoneal macrophages were harvested. Consistent with our *in-vitro* data, intraperitoneal

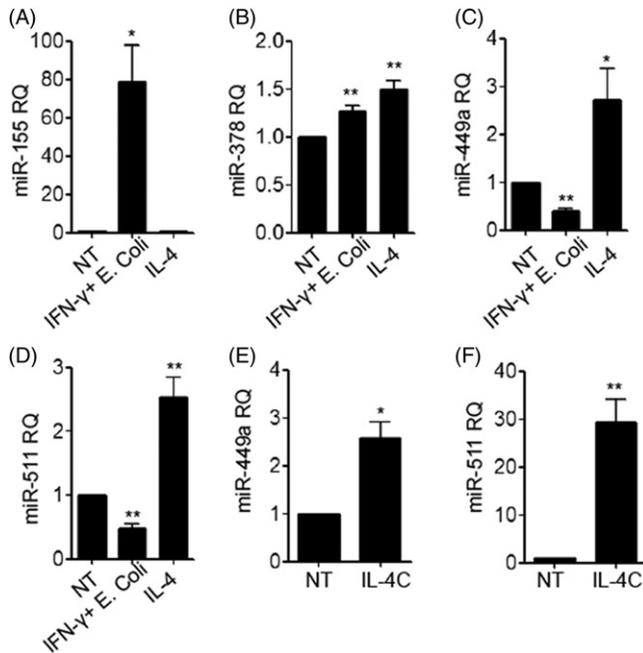


Figure 3. Validation of miRNA array reveals miR-511 as an IL-4-induced M1-repressed miRNA. qPCR analysis of miR-155 (A), miR-378 (B), miR-449a (C) and miR-511 (D) in IL-4 (IL-4) and IFN- γ followed by *E. coli* stimulation (M1) is shown. The expression of miR-449a (E) and miR-511 (F) in IL-4-elicited peritoneal cavity macrophages (IL-4C) is shown; $n=3$ experiments. * $p<0.05$, ** $p<0.01$; NT – no treatment.

injection of IL-4 was sufficient to induce the expression of miR-449a and miR-511 *in-vivo* (Figure 3E–F). Collectively, these results identify unique miRNA profiles in M1 versus IL-4-polarized macrophages.

IL-13 induces the expression of miR-511 but not miR-449a

IL-4 and IL-13 share the requirement for IL-4R α and the phosphorylation and translocation of STAT-6 for their optimal signaling [10]. Thus, IL-4 and IL-13 are likely to co-regulate downstream effector functions of macrophages in a similar fashion. Hence, we were interested to determine whether IL-13 regulated the expression of miR-511 and miR-449a in a similar fashion to IL-4. Indeed, IL-13 was capable of inducing similar miR-511 expression to that of IL-4 (Figures 3D and 4A). Surprisingly, however, in contrast to IL-4, IL-13 suppressed the expression of miR-449a (Figure 4B). Notably, the expression of previously known M1- or M2-associated miRNAs (e.g. miR-155 and miR-378) [15,18] was not regulated by IL-13 (Figure 4C–D). To assess whether IL-13 can induce the expression of miR-511 *in-vivo* as well, the expression of miR-511 was determined in IL-13-elicited peritoneal macrophages. Certainly, intraperitoneal injections of IL-13 were capable of inducing miR-511 in peritoneal macrophages as well (Figure 4E). Furthermore, the expression of miR-511 and miR-449a were increased and decreased, respectively, in bronchoalveolar macrophages that were obtained following induction of allergic airway inflammation by Asp (Figure 4F–G). These *in vivo* data are consistent with our previous findings that Asp-induced allergic airway inflammation yields higher levels of IL-13 in comparison

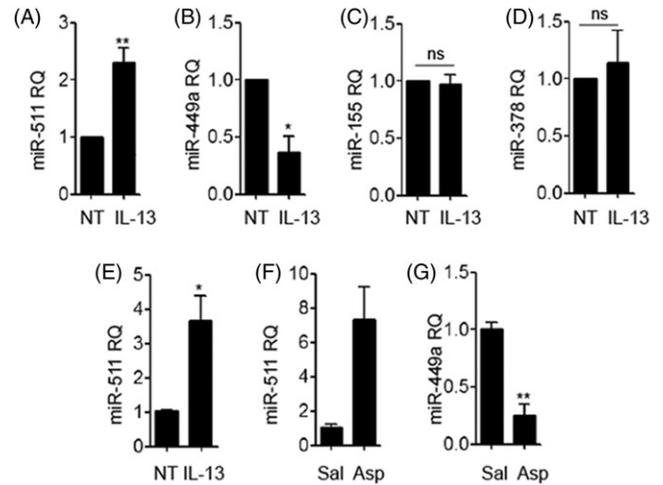


Figure 4. miR-511 is induced by IL-13 *in vitro* and *in vivo*. qPCR analysis of miR-511 (A), miR-449a (B), miR-155 (C) and miR-378 (D) in IL-13-activated BM-derived macrophages. The expression of miR-511 in peritoneal cavity macrophages following intraperitoneal injection of IL-13 (E) is shown. The expression of miR-511 and miR-449a in bronchoalveolar macrophages following allergen challenge (F–G) is shown; $n=4$ experiments. * $p<0.05$, ** $p<0.01$; NT – no treatment, Sal – Saline.

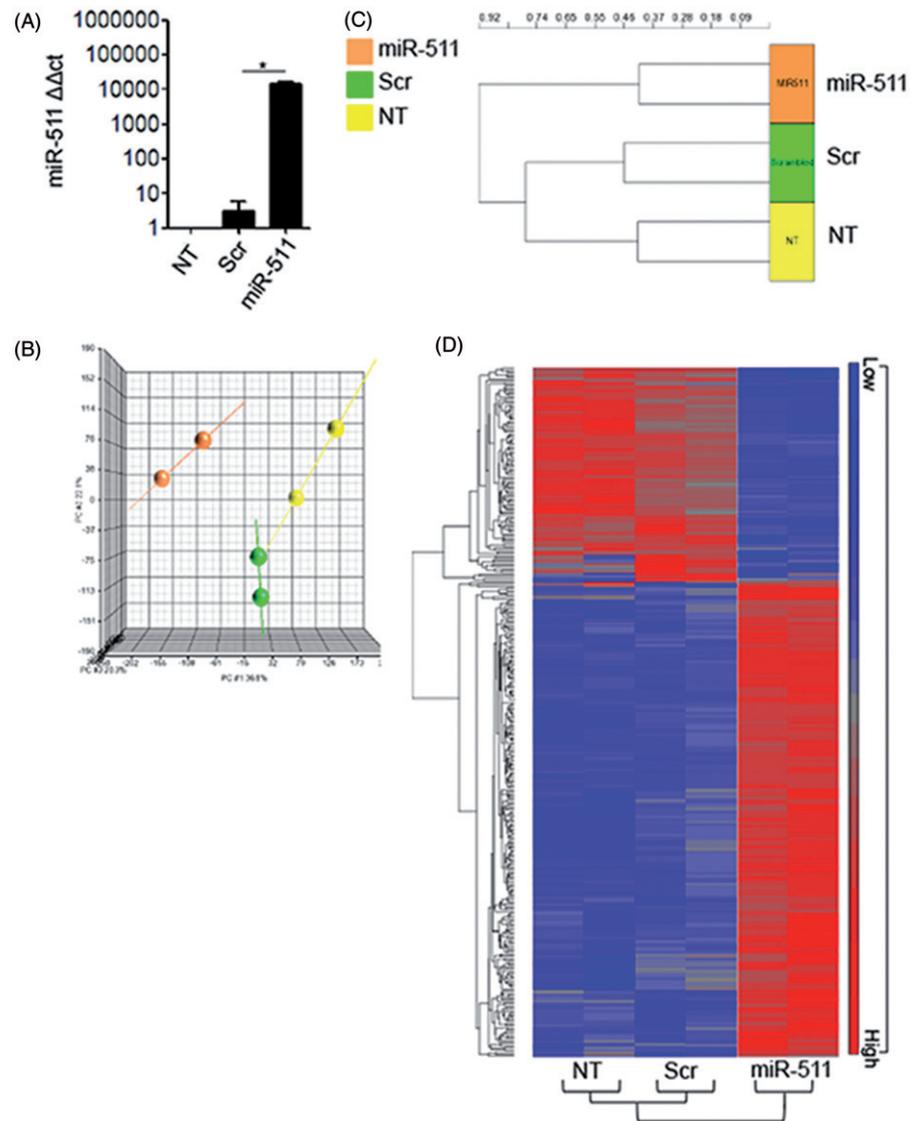
with IL-4 [26]. Taken together, our results establish miR-511 as a bona-fide M2-associated miRNA, which is suppressed in M1-polarizing conditions.

Over-expression of miR-511 in macrophages reveals a unique genetic signature

In attempt to define the roles of miR-511 in macrophages, global transcriptome analysis of macrophage mRNA expression was determined following miR-511 induction using microarray analysis. To this end, miR-511 (or scrambled control) was over expressed in BM-derived macrophages (Figure 5A) and mRNA microarray analysis was performed. Principal component analysis (PCA) demonstrated that over-expression of miR-511 induced alterations in the genetic signature of BM-derived macrophages in comparison with non-treated and scrambled control-treated samples, which were overall similar (Figure 5B–C). In fact, forced expression of miR-511 induced the differential expression of 340 genes (Figure 5D, Table S4, >1.5-fold change, $p<0.05$), among those, the expression of 106 genes was down-regulated and the expression of 234 genes was up-regulated. For assessment of miR-511 predicted targets, we used the MicroRNA.org [28] and TargetScan algorithms [29]. Intersecting both algorithms identified 271 transcripts that are predicted as miR-511 targets (Table S5). Next, in attempt to define whether any of the genes which were miR-511-predicted targets (Table S5), were also down-regulated by forced expression of miR-511 (Figure 5, Table S4), we intersected the 271 predicted miR-511 targets with the 340 genes that were found regulated by miR-511 in our microarray analysis. This bioinformatic analysis identified two candidate genes, *Npnt* and *Cdk7*, which were down-regulated (0.630 and 0.629, respectively) in response to miR-511 over-expression and also predicted miR-511 target genes.

Subsequent STRING analysis, which identifies known and predicted protein interactions revealed that miR-511 induced

Figure 5. Over-expression of miR-511 in macrophages induces a unique transcriptional signature. Bone marrow-derived macrophages were transfected with miR-511 mimic (miR-511), scrambled control (Scr) or left untreated (NT) for 48 h. Thereafter, global transcriptome analysis was performed using Affymetrix gene chips[®]. miR-511 over-expression following mimic transfection was validated using TaqMan qPCR analysis (A). Principal component (PCA) (B) and cluster analyses (C) of the microarray data are shown. Heat map (D) analysis of the differentially expressed mRNA transcripts (fold change > 1.5, $p < 0.05$) is shown. Data represent $n = 2$ experiments. * $p < 0.05$.



a transcriptome signature that consists of three major hubs (Supplemental Figure 4 and Table S4). Gene ontology analysis of these hubs revealed that miR-511 induction was associated with various cell proliferation and metabolic pathways, such as guanyl nucleotide binding, purine ribonucleotide binding (see Hub 1 in Table S6), cell division, cell cycle and mitosis (see Hub 3 in Table S6). In addition, miR-511 induced various genes associated with macrophage immune response, such as cytokine and chemokine activities including response to wounding, defense response and inflammatory response (see Hub 2 in Table S6).

Taken together, these results suggest miR-511 as an important regulator of M2 macrophage responses possibly by governing molecular checkpoints associated with IL-4/IL-13-induced macrophage activities.

miR-511 over-expression does not effect the M2 activation of macrophages

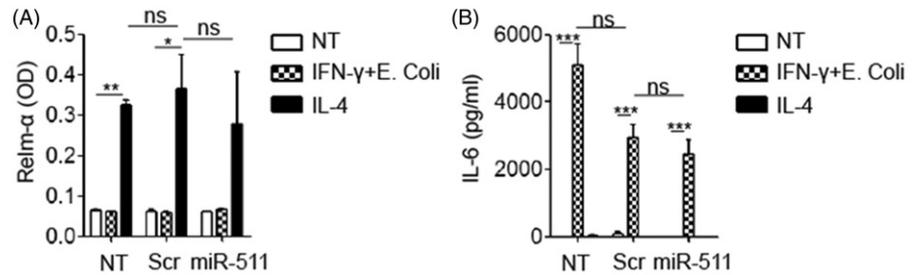
Next, we opted to assess the functional significance of miR-511 in the regulation of the M1/M2 polarization in macrophages. To this end, miR-511 was over expressed in

BM-derived macrophages. Thereafter, macrophages were activated with IL-4 or IFN- γ followed by heat inactivated *E. coli* and the polarized phenotype of the macrophages was assessed. Over-expression of miR-511 did not affect baseline Relm- α or IL-6 secretion in macrophages (Figure 6) suggesting that over-expression of miR-511 alone is not sufficient to skew the phenotype of macrophage towards an M2 phenotype. Furthermore, over-expression of miR-511 following M2 or M1-activation was not sufficient to affect the polarized phenotype of macrophages (Figure 6A–B).

Discussion

Macrophages are central cells of the immune system, which display substantial effector function diversity and mediator repertoire [1,8]. These functional properties of macrophages vary depending on their anatomical location and physiological context [12]. Specifically, activation of macrophages by the Th2 cytokines IL-4 or IL-13, leads to induction of a specific molecular signature that represents one extreme of macrophage heterogeneity collectively termed alternatively (or M2) macrophages. M2 macrophages have distinct roles in

Figure 6. Over-expression of miR-511 in macrophages does not induce a phenotypic change. Bone marrow-derived macrophages were transfected with miR-511 mimic (miR-511), scrambled control (Scr) or left untreated (NT) for 48 h. Thereafter, macrophages were activated with IL-4 for M2-polarization or IFN- γ followed by *E. coli* for M1-polarization. The levels of secreted Relm- α (A) (as M2 marker) and IL-6 (B) (as M1 marker) were determined. Data represent $n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NT – no treatment.



allergic settings as well as in tissue repair, wound healing and cancer but also in homeostatic activities (e.g. glucose metabolism and thermogenesis) [4,12]. Although emerging studies have begun to analyze the expression and function of miRNAs in M2 macrophages [13,17,19], there is still an unmet need to comprehensively define miRNA profiles in these cells following IL-4 and/or IL-13 stimulation. Using an unbiased miRNA “mining” approach we have now characterized the miRNA expression profile of M1 and M2 macrophages. We establish differential regulation of miR-511 following M1 and M2-polarizing conditions. Importantly, we further establish that miR-511 expression is increased by IL-4 and IL-13 *in vitro*, *in vivo* and in a disease model of allergic airway disease that is associated with increased IL-13 and IL-4 production. Finally, global transcriptome analyses following miR-511 over-expression revealed a substantial role for miR-511 in macrophages and suggest that miR-511 regulates diverse activities of M2 macrophages including cellular proliferation, metabolism, healing responses and inflammation. Collectively, our data suggest miR-511 as a hallmark bona fide M2-associated miRNA.

To date, the study of miRNAs in macrophages has been largely focused on the expression and function of miRNA's in settings of M1 activating conditions and their expression in settings associated with M2 macrophage polarization received considerably less attention. The Th2 cytokines, IL-4 and IL-13, exert their function via binding to the type 1 and/or type 2 IL-4Rs. IL-4 binds IL-4R α and can signal through the type 1 and type 2 IL-4Rs whereas IL-13 binds IL-13R α 1 and can therefore signal only via the type 2 IL-4R [10,30]. Thus, IL-4 and IL-13 are generally considered to exert similar functions. Despite this, recent data suggest that the downstream signaling events following IL-4 or IL-13 stimulation are not entirely identical. For example, while IL-4 and IL-13 can induce the tyrosine phosphorylation of IRS-2 and Jak1, IL-4 induces the tyrosine phosphorylation of Jak3 and IL-13 induces Jak2 phosphorylation [31–33]. Therefore, the ability of IL-4 and IL-13 to induce the expression of similar miRNA targets is not obvious. Indeed, while our analyses revealed that miR-511 is similarly induced by IL-4 and IL-13, the expression of miR-449a, was increased by IL-4 and decreased by IL-13. IL-4 and IL-13 are often used interchangeably as M2-polarizing cytokines. In order for a miRNA to be termed as a “bona-fide” M2-associated miRNA, it is expected that IL-4 and IL-13 will regulate it in a similar fashion. While our data highlight miR-511 as a potential genuine M2-associated miRNA we also demonstrate that these two cytokines can induce differential miRNA expression. Future experiments

comparing IL-4 and IL-13-induced miRNA transcriptional changes in macrophages will likely shed more light on the differential functions of these two cytokines.

Several examples exist regarding the ability of miRNAs to shape the balance of M1 and M2 macrophage polarization. For example, recent studies assessing the role of miR-155 in macrophages, demonstrated that IL-13R α 1 is a direct target of miR-155 [34]. Hence, induction of miR-155 in M1-polarized macrophages abolished IL-13-induced STAT6 activation leading to decreased IL-13-induced gene expression [34]. In addition, induction of miR-21 in alveolar myeloid cells by allergen challenge (via IL-13) was capable of decreasing IL-12p35 expression and secretion in these cells. The latter finding is critically important, as IL-12p35 expression is central to development of Th1 responses [35]. Thus, miRNA expression in polarized macrophages can globally skew immune responses. Importantly, the induction of miR-511 in M2-polarizing conditions was not restricted to *ex-vivo* generated macrophages (i.e. BM-derived cells) since primary (IL-4- or IL-13-elicited) peritoneal macrophages and macrophages that were obtained from the airways of allergic mice displayed increased miR-511 expression as well. Although both IL-4 and IL-13 were capable of inducing miR-511 expression, the relative expression of miR-511 varied dramatically between these two activation states, at least *in-vivo*. IL-4 induced the expression of miR-511 by nearly 30-fold whereas, IL-13 increased miR-511 levels by \sim 3-fold. These effects are likely due to the relatively higher expression of the type 1 IL-4R in macrophages in comparison with expression of the type 2 IL-4R [36]. Similarly, the expression of miR-511 in macrophages that were obtained from asthmatic mice was increased by \sim 8-fold and therefore resembled the expression, which was obtained by IL-13. This is likely due to the relatively higher expression of IL-13 in this specific experimental asthma model, as we have previously shown [26].

Interestingly and further supporting an important role for miR-511 in M2 macrophage polarization, IFN- γ and *E. coli* stimulation repressed miR-511 expression. This finding is of specific interest since miR-511 is one of few miRNAs that has been previously described as a miRNA that is induced in tumor-associated macrophage [17], which resemble M2 macrophages. Using a genetic-analysis approach, Squadrito et al. [17] demonstrated that miR-511 is located within the *Mrc1* gene, which is expressed and up-regulated by M2 macrophages and tumor-associated macrophages. Certainly, miR-511 was co-expressed with MRC1⁺ tissue macrophages and directly suppressed ROCK2 expression. Subsequently, miR-511 over-expression in BM-derived tumor-associated

macrophages resulted in suppression of tumor growth and blood vessel formation [17].

Given that the majority of miRNAs lead to RNA degradation, it is expected that over-expression of a specific miRNA will lead to down-regulation of specific mRNA targets. Our global microarray approach revealed that several predicted gene products including *Npnt* (a TGF- β responsive gene) [37] and *Cdk7* (a signaling molecule involved in cell replication) [38] were down-regulated by miR-511 over-expression and are hence likely direct miR-511 targets. Importantly, over-expression of miR-511 in macrophages and subsequent microarray analysis revealed that miR-511 potently induced (rather than repressed) numerous genes. This observation can be due to indirect effects that follow the interaction of miR-511 with its downstream targets or possibly positive regulation by miR-511. In support of the latter hypothesis, studies in human monocyte-derived dendritic cells demonstrated miR-511 as miRNA with capacity for positive regulation (rather than as a suppressor) [39].

Previous approaches have revealed the involvement of several additional miRNAs in IL-4-induced macrophage activation. Importantly, our screening approach has identified these miRNAs as well. For example, miR-324-5p, which was upregulated in our experimental model specifically following IL-4 stimulation, was recently shown to regulate the expression of *CUEDC2* and subsequently tune tumor-associated macrophage function (as assessed in IL-4-activated macrophages) in colon carcinogenesis [40]. Furthermore, miR-378 was recently described as an M2-associated miRNA. Following nematode infection, miR-378 expression was increased in wild type macrophages, whereas *Il4ra*^{-/-} macrophages displayed decreased miR-378 expression [18]. Mechanistically, miR-378 was a key regulatory checkpoint in IL-4-induced local macrophage proliferation by targeting *AKT1* expression resulting in decreased macrophage proliferative capacity [18]. Although the previous study did not dissect whether miR-378 was induced by IL-4 or IL-13 (which both require the IL-4R α chain for their function), our data suggests that miR-378 is specifically induced by IL-4 but not IL-13. This observation might be explained by the relative higher expression of the type 1 IL-4R in comparison with the type 2 IL-4 receptor in BM-derived cells, such as macrophages, which renders the cells more responsive to IL-4.

Our results fail to establish a functional role for miR-511 alone in macrophage polarization. This finding may suggest that multiple miRNAs regulate M1/M2 polarization and therefore over-expression of a single miRNA, such as miR-511 will not be sufficient to induce any phenotypic changes in macrophages. Despite this, our combined *in vivo* and *in vitro* data highlight that miR-511 could be used as a useful marker for M2 macrophage polarization in disease settings, such as asthma (especially allergic asthma[41]) where the levels of IL-4 and/or IL-13 are highly increased.

Key findings

In this study, we identified distinct miRNA expression profiles in M1 and M2 polarized macrophages. We show that the Th2 cytokines IL-4 and IL-13 induce specific miRNA signatures in macrophages and identify miR-511 as

a bona-fide M2 miRNA. These findings shed light into the molecular mechanisms underlying macrophage polarization and define functional differences between IL-4 and IL-13-polarized macrophages. These data might have significant implications in disease settings involving M2-polarized macrophages including asthma, cancer and metabolism.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary material available online

Supplementary Tables S1, S2, S3, S4, S5 and S6; Figures 1, 2, 3 and 4.