HIV-1 infection in macrophages and genes involved throughout: Big eaters versus small invaders

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chapter TWO

NEXT-GENERATION SEQUENCING OF microRNAS UNCOVERS EXPRESSION SIGNATURES IN POLARIZED MACROPHAGES

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ABSTRACT

microRNAs are small non-coding RNAs that regulate gene expression at a post-transcriptional level, and play a crucial role in the development of cells of the immune system. Macrophages are essential for generating inflammatory reactions upon tissue damage and encountering of invading pathogens, yet modulation of their immune responses is critical for maintaining tissue homeostasis. Macrophages can present different phenotypes, depending on the cytokine environment they encounter in the affected tissues. In this study, we have identified expression signatures of miRNAs that are differentially regulated during maturation of monocytes and polarization of macrophages by cytokines. We present a comprehensive characterization of miRNA expression in human monocytes and M1, M2a and M2c polarized macrophages, using next-generation sequencing. Furthermore, we showed that miRNA expression signatures are closely related to the various immune functions of polarized macrophages and therefore are involved in shaping the diverse phenotypes of these cells. The miRNAs identified here serve as markers for identification of inflammatory macrophages involved in the development of immune responses. Our findings contribute to understanding the role of miRNAs in determining the macrophage function in healthy and diseased tissues.

INTRODUCTION

microRNAs (miRNAs) are important post-transcriptional regulatory elements of gene expression, and play a central role in differentiation and maturation of cells of the immune system[1,2] . miRNAs are non-coding RNA molecules of about 15-25nt in length, transcribed by the RNA polymerase II, and encoded by introns of protein-coding genes, exonic 3' untranslated regions (UTRs) or independent regions in the genome. miRNAs that bind to mRNA targets with nearly perfect sequence complementarity, induce mRNA degradation. However, in most cases mismatches in the miRNA-target interaction will end in repression of translation of the mRNA[3].

miRNAs play a central role in balancing the immune response established upon tissue damage or pathogen recognition, and failure of regulation by miRNAs is related to the development of inflammatory diseases[4,5]. Since miRNAs are evolutionary conserved sequences, invading pathogens have developed mechanisms to utilize host miRNAs to their advantage, as it is the case of Hepatitis C virus (HCV) and miR-122[6]. miR-122, which is specifically expressed in the liver, binds to the 5' non-coding region of the HCV genome and protects the viral RNA from degradation by the endonuclease Xrn1[7]. Likewise, pathogens such as viruses are capable of encoding their own miRNAs and therefore can modulate cellular function towards a beneficial outcome of infection of their host[8]. For instance, human cytomegalovirus (hCMV) and simian virus 40 (SV40) encode viral miRNAs that mediate evasion of cytotoxic T lymphocyte responses by targeting host or viral proteins[9,10]. To fully comprehend the role of miRNAs in pathogen interactions, it is essential to understand how changes in miRNA expression shape the function of mature immune cells.

Macrophages are versatile immune cells that engage in various processes for maintaining tissue homeostasis and mounting immune responses against invading pathogens[11-13]. Macrophages are found in many tissues, where they are surrounded by different cytokine microenvironments that can modify their phenotype and function. IFNγ and TNFα or LPS-stimulated macrophages (M1) are associated with pro-inflammatory responses, while macrophages stimulated with Interleukin 4 (IL-4) (M2a) or Interleukin 10 (IL-10) (M2c) mediate anti-inflammatory responses and are associated with tissue remodeling[14,15]. Numerous studies have described the gene expression landscape in these polarized cells, yet few reports have focused on analyzing expression of some miRNAs in polarized macrophages[16].

This study describes a comprehensive characterization of miRNA expression in human monocytes and polarized macrophages using next-generation Sequencing by Oligonucleotide Ligation and Detection (SOLiD™). Our results were able to provide information on the expression of more than 1900 known miRNAs reported in miRBase. In this report we have identified a repertoire of miRNAs that have a specific expression signature in polarized macrophages and that are important for macrophage phenotype and function.

RESULTS

To better understand miRNA expression during maturation and polarization of macrophages, we sequenced small RNAs of human primary monocytes and cytokine-polarized macrophages and analyzed their expression dynamics compared with unstimulated cells. We obtained small RNA sequences from monocytes, 5-day cultured macrophages (MΦ) with or without stimulation with IFNγ+TNFα (M1), IL-4 (M2a) or IL-10 (M2c). We used the next-generation SOLiD™ platform to obtain sequences from all small RNA species present in our samples, without bias due to specific primers. This platform is also not limited to a specific subset of small RNAs since it sequences all RNA species. This is in contrast to expression arrays that measure only miRNAs with pre-defined probes. The total number of reads obtained from these samples ranged from 2 to 14 million reads per sample. From these sequences, 10-26% mapped uniquely to miRNA sequences in the miRBase.

We identified 779 known miRNA sequences in our samples, out of the 1919 human miRNAs reported in miRBase version 18 and compared with 365 miRNAs analyzed in similar studies, by using miRNA qPCR arrays[21]. We selected miRNAs that were differentially expressed between MΦ and monocytes, M1, M2a or M2c polarized cells (figure 1a). All these miRNAs had substantial read counts, fold changes and absolute changes in expression levels. In total, 303 miRNAs fulfilled the selection criteria. From these, 45 miRNAs showed low or no expression in monocytes, but were considerably up or down-regulated in M1, M2a or M2c macrophages, compared with MΦ. The remaining 258 miRNAs showed considerable up or down-regulation in MΦ, M1, M2a, or M2c macrophages when compared with monocytes. Hierarchical clustering analysis of all these miRNAs, across monocytes, MΦ and M1, M2a or M2c polarized macrophages (figures 1b-c), did not exhibit exact expression patterns, or clusters of miRNAs regulated under specific polarization conditions. These diverse expression patterns indicate that expression of miRNAs is a very dynamic process, and that many miRNAs could be involved in shaping the phenotype of these cytokine polarized macrophages. The 45 miRNAs which lacked sufficient expression in monocytes, but were differentially expressed during macrophage polarization, are shown in figure 1b. The 258 miRNAs that were expressed in monocytes and were regulated in at least one of the polarization conditions are shown in figure 1c.

To validate our results, we selected 50 candidates from the 303 miRNAs described above (indicated in red, figure 1b-c), for confirmation by qPCR in samples isolated from additional donors. Some of the selected miRNAs have been associated with modulation of the immune response: miR-155-5p, miR-21-5p, miR-132-3p, miR-146a-5p and miR-146-b-5p among others (the involvement of these miRNAs in regulation of immune responses is described in the discussion). Other miRNAs were selected for confirmation if they exhibited large differences in expression among monocytes and

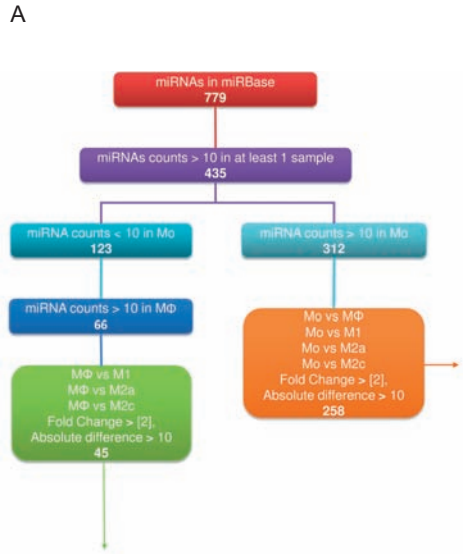
macrophages, or unpolarized and polarized cells. Expression of a large number of candidate miRNAs was differentially regulated during macrophage differentiation and polarization. These results matched the sequencing outcome for 60% of the miRNAs analyzed, either for all cell types or for at least one of them. This indicated that at least 30 miRNAs are consistently expressed in polarized macrophages among the samples from 1 donor, used for RNA-sequencing and additional samples from the multiple donors utilized for confirmation of miRNA expression. 40% of the confirmed miRNAs showed expression pattern different from what we found with next-generation SOLiD™ sequencing, which is probably due to biological variation between different donors. With this analysis we identified a number of miRNAs that are expressed in monocytes and that are differentially regulated during maturation of phenotypically different macrophages. These miRNAs were divided in 4 groups according to their expression profile among polarized macrophages and are described below.

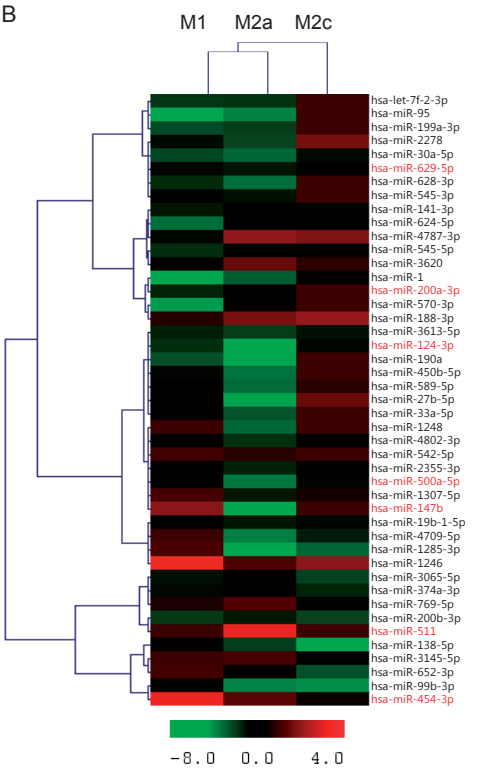
Figure 2 shows the confirmation of expression of miRNAs that were only regulated during maturation of monocytes into macrophages. Expression of miR-223-5p, miR-454-3p and miR-93-5p was down-regulated in MΦ, compared with monocytes (Figure 2a-c) and expression of miR-106-3p, miR-132-3p, miR-335-5p, miR-34a-5p, miR-362-3p, miR-424-5p was up-regulated in macrophages (Figure 2d-i), when compared with monocytes.

Expression of 7 miRNAs was regulated during polarization of macrophages, but did not change during maturation of monocytes into MΦ (figure 3a-g). miR-125b-5p, miR-181a-5p, miR-193b-3p, miR-125a-5p and were significantly up-regulated in M1 macrophages but also regulated in M2 polarized cells (figure 3a-d). miR-145-5p was uniquely up-regulated in M1 (figure 3e), whereas miR-181b-5p was uniquely down-regulated in M2a cells (figure 3f), and miR-200a-3p was exclusively down-regulated in M2c macrophages (figure 3g).

Several miRNAs were differentially expressed during maturation of monocytes, but also under one macrophage polarization condition (figure 4). miR-150-5p and miR-27a-5p were down-regulated in MΦ, compared with monocytes, but also regulated under different polarization conditions (Figure 4a-b). Other miRNAs were up-regulated in MΦ compared with monocytes and also regulated during polarization, when compared with MΦ: miR-146a-5p, miR-193a-5p and miR-29b-3p (Figure 4c-e) were up-regulated and miR-629-5p was down-regulated in M1 cells (Figure 4f); miR-500a-5p and miR-502-3p were up-regulated in M2a macrophages (Figure 4g-h); miR-21-5p, miR-22-3p, miR146b-5p were up-regulated, and miR-339-3p were down-regulated in M2c cells (Figure 4i-l).

Other miRNAs were differentially regulated during maturation of monocytes and also in two or three of the polarizing conditions (figure 5). In monocytes, miR-147b was highly expressed (figure 5a), while the rest of miRNAs in this group had low expression levels in these cells (figure 5b-h). Additionally, miR-221-3p, miR-222-3p and miR-511 displayed low levels in monocytes, M1 and M2c, and


A
miRNAs in miRBase
779
miRNAs counts > 10 in at least 1 sample
435
miRNA counts < 10 in Mo
123
miRNA counts > 10 in Mo
312
miRNA counts > 10 in MΦ
66
Mo vs MΦ
Mo vs M1
Mo vs M2a
Mo vs M2c
Fold Change > [2],
Absolute difference > 10
258
MΦ vs M1
MΦ vs M2a
MΦ vs M2c
Fold Change > [2],
Absolute difference > 10
45


B
M1
M2a
M2c
hsa-let-7f-2-3p
hsa-miR-95
hsa-miR-199a-3p
hsa-miR-2278
hsa-miR-30a-5p
hsa-miR-629-5p
hsa-miR-628-3p
hsa-miR-545-3p
hsa-miR-141-3p
hsa-miR-624-5p
hsa-miR-4787-3p
hsa-miR-545-5p
hsa-miR-3620
hsa-miR-1
hsa-miR-200a-3p
hsa-miR-570-3p
hsa-miR-188-3p
hsa-miR-3613-5p
hsa-miR-124-3p
hsa-miR-190a
hsa-miR-450b-5p
hsa-miR-589-5p
hsa-miR-27b-5p
hsa-miR-33a-5p
hsa-miR-1248
hsa-miR-4802-3p
hsa-miR-542-5p
hsa-miR-2355-3p
hsa-miR-500a-5p
hsa-miR-1307-5p
hsa-miR-147b
hsa-miR-19b-1-5p
hsa-miR-4709-5p
hsa-miR-1285-3p
hsa-miR-1246
hsa-miR-3065-5p
hsa-miR-374a-3p
hsa-miR-769-5p
hsa-miR-200b-3p
hsa-miR-511
hsa-miR-138-5p
hsa-miR-3145-5p
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hsa-miR-99b-3p
hsa-miR-454-3p
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0.0
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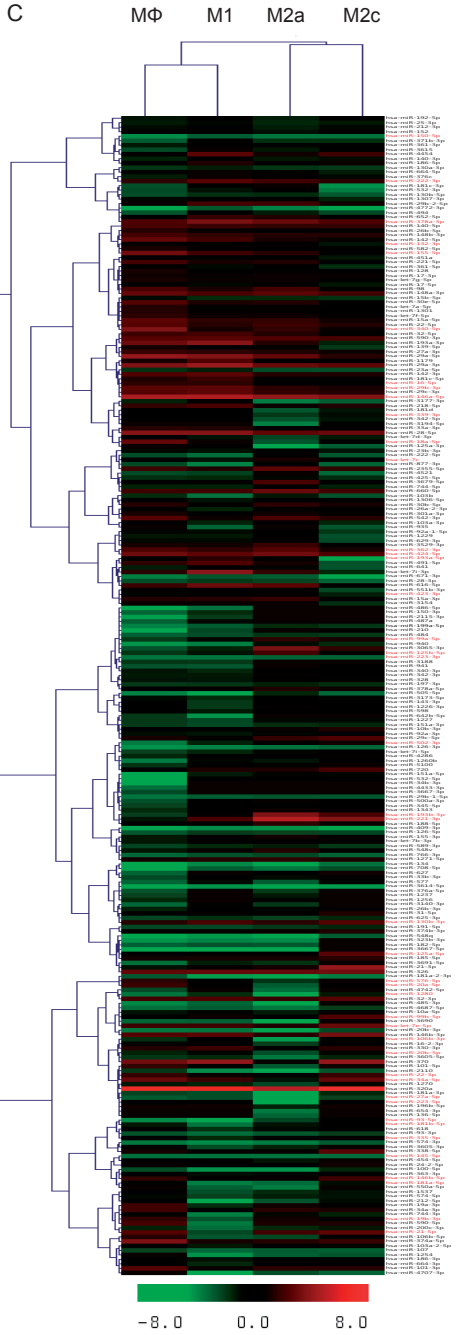

C
MΦ
M1
M2a
M2c
-8.0
0.0
8.0

◄ **Figure 1. Selection and characterization of miRNAs differentially expressed in polarized macrophages. A)** Selection of differentially expressed miRNAs: 779 miRNA from the miRBase 18 database were identified in sequences obtained with next-generation SOLiD sequencing method from samples obtained from 1 healthy donor: monocytes (Mo), unstimulated macrophages (MΦ) or macrophages stimulated with IFNγ+TNFα (M1), IL-4 (M2a) or IL-10 (M2c) (red). From these, 435 miRNAs had normalized counts higher than 10 in at least 1 sample (purple). These miRNAs were divided in two categories. In the first category, 123 miRNAs had low or undetectable expression in monocytes (below 10 counts) (dark turquoise). From these, 66 miRNAs, with counts higher than 10 in unstimulated macrophages (MΦ), were selected (blue). 45 miRNAs which had fold expression values higher than 2 with an absolute difference in counts of more than 10, when comparing MΦ to M1, M2a or M2c polarized cells were selected (green). The second category was composed of 312 miRNAs that were expressed in monocytes with counts higher than 10 (light turquoise). From these, 258 miRNAs had a fold expression higher than 2 in MΦ, M1, M2a or M2c cells, when compared with monocytes, with an absolute difference in counts of more than 10 (orange). **B-C)** Hierarchical clustering analysis of miRNAs differentially expressed in polarized macrophages, as selected above. The values represent fold change in expression (log2 transformed), compared with unstimulated macrophages. miRNAs selected for further confirmation are indicated in red. **B)** Fold expression of miRNAs regulated in M1, M2a or M2c macrophages, compared with MΦ. **C)** Fold expression of miRNAs regulated in MΦ or any other polarization condition, compared with monocytes (Mo).

were highly expressed in M2a macrophages (Figure 5f-h). Furthermore, we observed that the expression levels of 14 remaining miRNAs did not change significantly in monocytes or macrophages (data not shown).

The miRNA expression data are summarized in figure 6 and table 1, highlighting only the significant changes found in miRNA expression levels in monocytes and polarized macrophages, when compared with MΦ. The results are divided in 4 groups, based on miRNA expression signatures: miRNAs that are only regulated during maturation of monocytes into MΦ (figure 6a); miRNAs whose expression is not regulated during maturation of monocytes into MΦ but are differentially expressed under polarizing conditions (figure 6b); miRNAs that are differentially expressed during maturation of monocytes, and also in one (figure 6c) or more polarization conditions (figure 6d). With these result we have generated a catalogue of 50 miRNAs that are characteristically expressed in monocytes and macrophages. Expression signatures of 36 of these miRNAs revealed differential regulation during maturation of monocytes and/or polarization of macrophages.

To comprehend the cellular processes that these miRNAs control during macrophage maturation and polarization, we identified possible targets for the 50 miRNAs analyzed by qPCR, using the Ingenuity® Knowledge Base and Ingenuity Pathway Analysis software. Certain target genes can be regulated by several miRNAs, and also inhibition of gene expression does not always end in degradation of the target mRNA. However, in this study we decided to focus on the miRNAs that do down-regulate mRNA levels of the target gene. We selected possible target genes that are also differentially expressed in monocytes

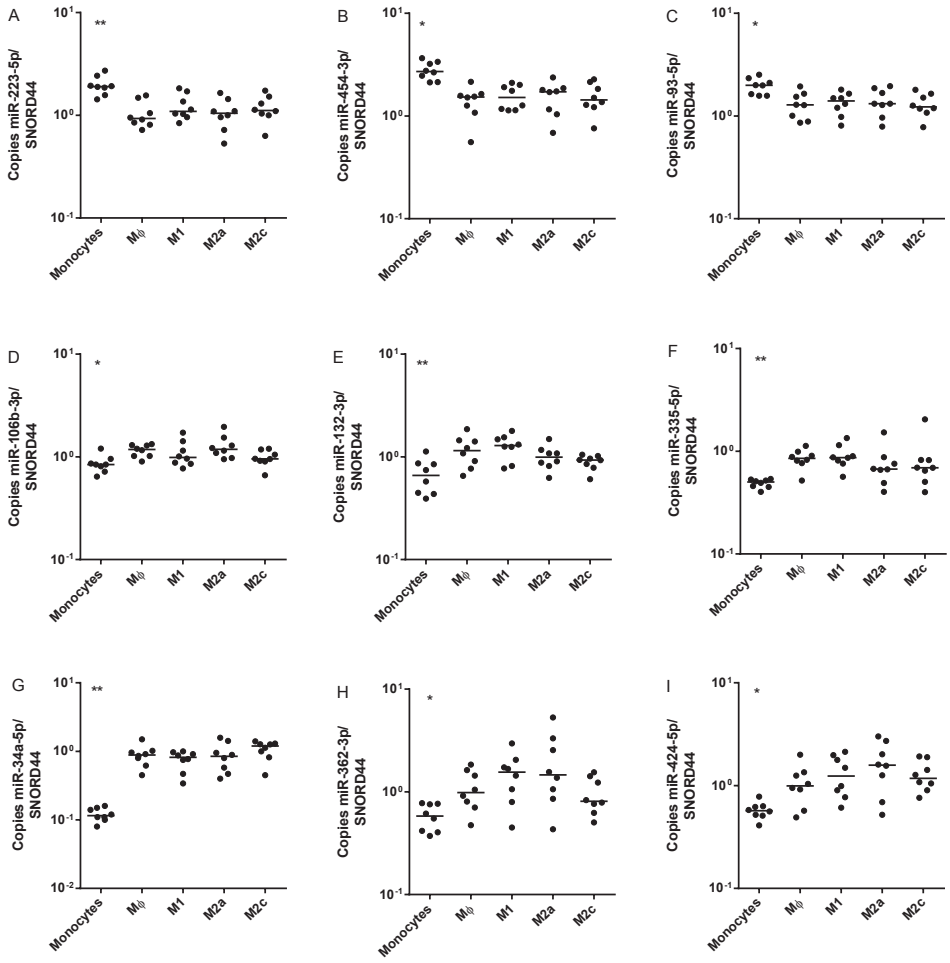


Figure 2. Expression of selected miRNAs in monocytes and polarized macrophages. Expression of A) miR-223-5p, B) miR-454-3p, C) miR-93-5p, D) miR-106b-3p, E) miR-132-3p, F) miR-335-5p, G) miR-34a-5p, H) miR-362-3p, I) miR-424-5p was analyzed by qPCR in monocytes, 5 day unstimulated monocyte-derived macrophages (MΦ) or stimulated with IFNγ+TNFα (M1), IL-4 (M2a) or IL-10 (M2c). Significant changes in expression of these miRNAs were only observed in monocytes compared MΦ. Expression values from samples from different donors are represented by the dots and the horizontal bar represents the median of all samples. Expression values were normalized to SNORD44 for input. Significant differences in expression, when comparing monocytes, M1, M2a, or M2c to MΦ, are indicated with asterisks (Wilcoxon signed ranked test,*p<0.05, **p<0.01, ***p<0.001).

and polarized macrophages (GSE49240[20]). From the 50 miRNAs included in this analysis, 15-17 (depending on the cell type) were connected to genes whose expression was also differentially regulated in each cell type (Supplementary

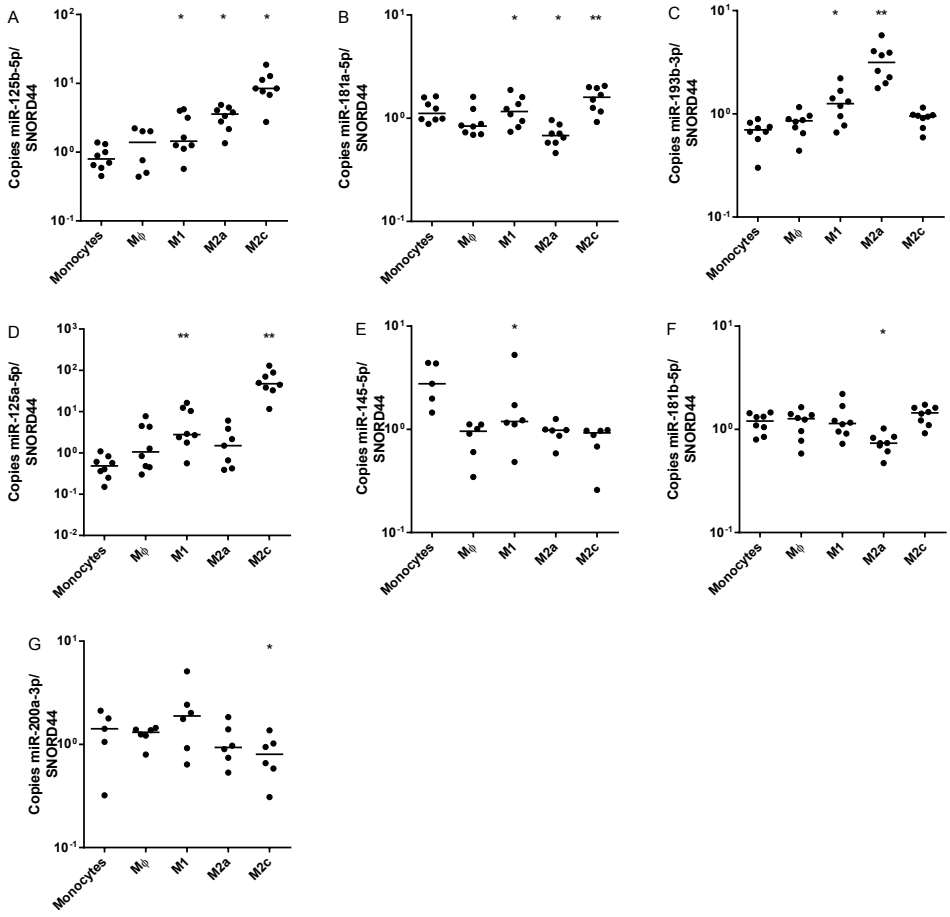


Figure 3. Expression of selected miRNAs in monocytes and polarized macrophages. Expression of A) miR-125b-5p, B) miR-181a-5p, C) miR-193b-3p, D) miR-125a-5p, E) miR-145-5p, F) miR-181b-5p, G) miR-200a-3p, was analyzed by qPCR in monocytes, 5 day unstimulated monocyte-derived macrophages (MΦ) or stimulated with IFNγ+TNFα (M1), IL-4 (M2a) or IL-10 (M2c). Significant changes in expression of these miRNAs were observed in M1, M2a or M2c cells compared with MΦ. Expression values from samples from different donors are represented by the dots and the horizontal bar represents the median of all samples. Expression values were normalized to SNORD44 for input. Significant differences in expression, when comparing monocytes, M1, M2a, or M2c to MΦ, are indicated with asterisks (Wilcoxon signed ranked test,*p<0.05, **p<0.01, ***p<0.001).

Table S2 a-d). Next, we analyzed biological functions that are associated with these miRNAs and their targets (Supplementary Table S3). Here we focused only in the 10 functions that showed the highest association (Figure 7). In monocytes, we observed an increase in biological functions in the categories of "Cellular Development", "Cellular Growth and Proliferation" and "Cellular Movement"

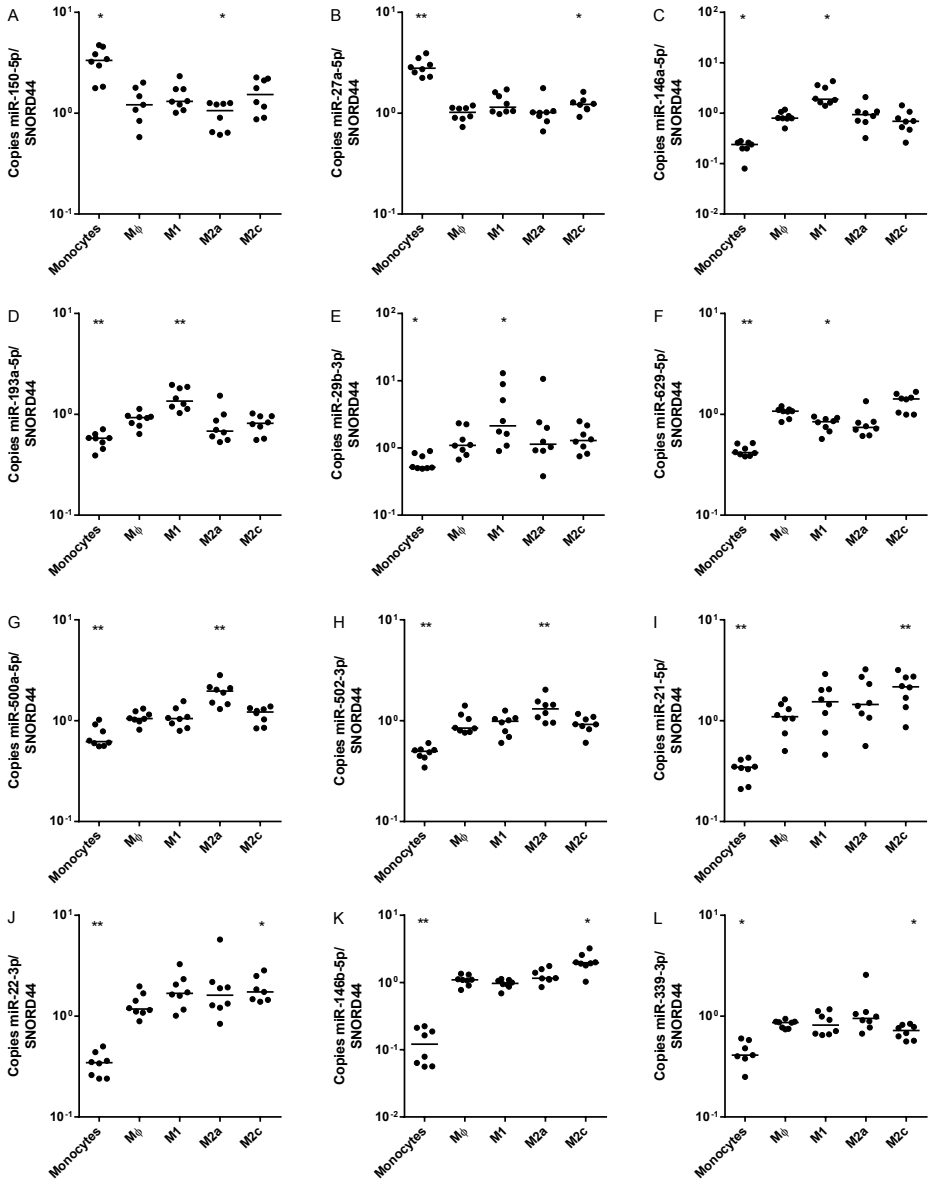


Figure 4. Expression of selected miRNAs in monocytes and polarized macrophages. Expression of A) miR-150-5p, B) miR-27a-5p, C) miR-146a-5p, D) miR-193a-5p, E) miR-29b-3p, F) miR-629-5p G) miR-500a-5p, H) miR-502-3p, I) miR-21-5p, J) miR-22-3p, K) miR-146b-5p and L) miR-339-3p was analyzed by qPCR in monocytes, 5 day unstimulated monocyte-derived macrophages (MΦ) or stimulated with IFNγ+TNFα (M1), IL-4 (M2a) or IL-10 (M2c). Significant changes in expression of these miRNAs were observed in Monocytes and one of the polarized macrophages, compared with MΦ. Expression values from samples from different donors are represented by the dots and the horizontal bar represents ►

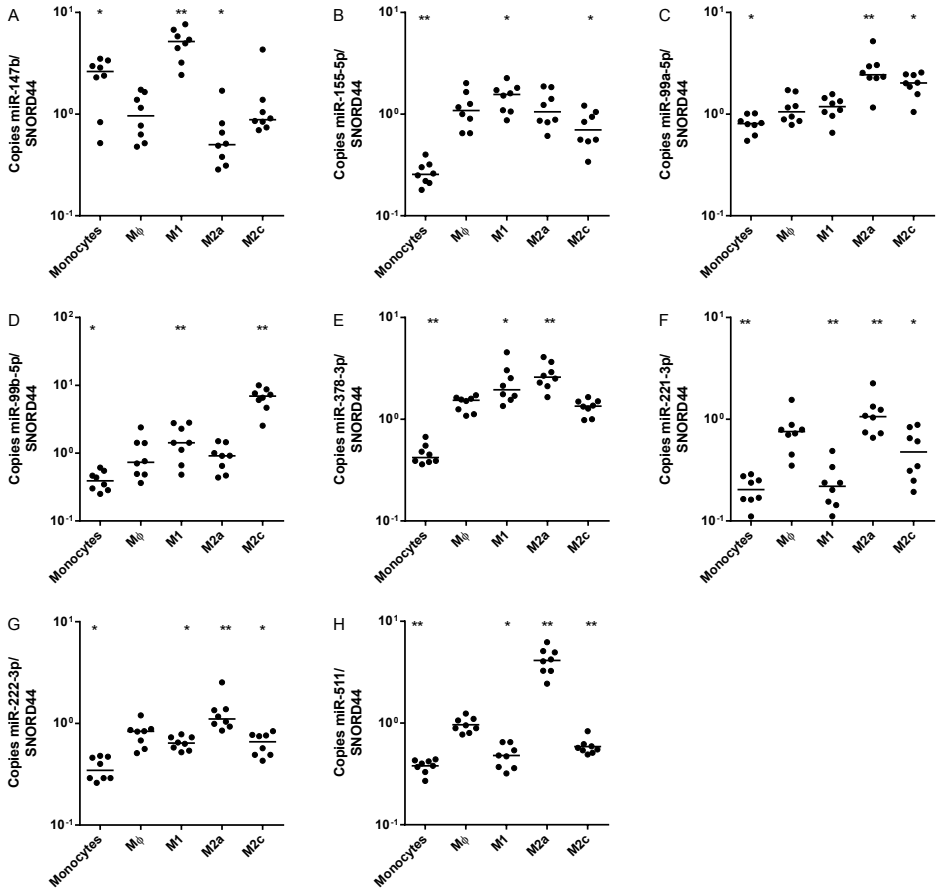


Figure 5. Expression of selected miRNAs in monocytes and polarized macrophages. Expression of A) miR-147b, B) miR-155-5p, C) miR-99a-5p, D) miR-99b-5p , E) miR-378a-3p, F) miR-221-3p, G) miR-222-3p, H) miR-511 was analyzed by qPCR in monocytes, 5 day unstimulated monocyte-derived macrophages (MΦ) or stimulated with IFNγ+TNFα (M1), IL-4 (M2a) or IL-10 (M2c). Significant changes in expression of these miRNAs were observed in Monocytes and polarized macrophages, compared with MΦ. Expression values from samples from different donors are represented by the dots and the horizontal bar represents the median of all samples. Expression values were normalized to SNORD44 for input. Significant differences in expression, when comparing monocytes, M1, M2a, or M2c to MΦ, are indicated with asterisks (Wilcoxon signed ranked test,*p<0.05, **p<0.01, ***p<0.001).

► the median of all samples. Expression values were normalized to SNORD44 for input. Significant differences in expression, when comparing monocytes, M1, M2a, or M2c to MΦ, are indicated with asterisks (Wilcoxon signed ranked test,*p<0.05, **p<0.01, ***p<0.001).

that were associated with the miRNA-target genes (Figure 7a). On the other hand, M1 macrophages showed an increase in functions in the categories of "Hematological System Development and Function", "Inflammatory Response", "Hematopoiesis" and "Organismal Injury and Abnormalities" (Figure 7b), which corresponds to the function of these macrophages in mounting pro-inflammatory responses. In M2a macrophages, functions related to "Cellular Movement" and "Inflammatory Response" were decreased (Figure 7c), which also points towards the characteristic anti-inflammatory phenotype of these cells. In M2c macrophages we saw that most of the depicted functions were decreased, which suggests that these cells have a resting and inactivated phenotype (Figure 7d). These results indicate that some of these miRNAs are likely to be involved in controlling some

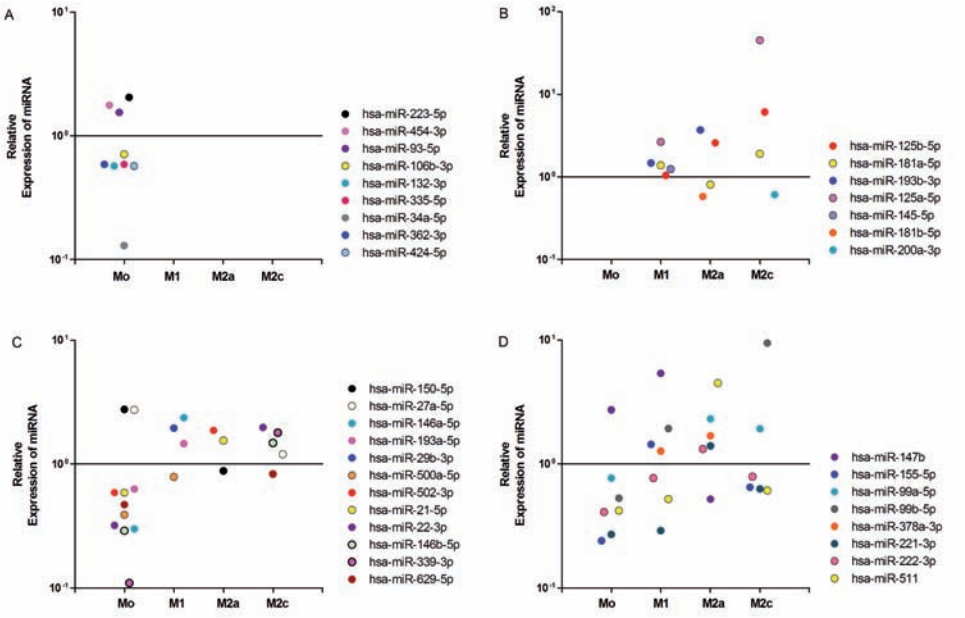


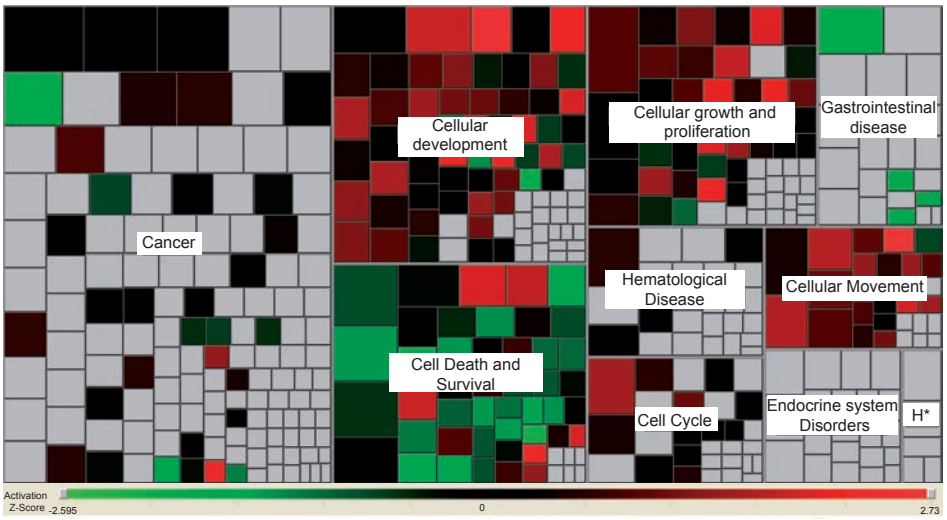
Figure 6. Expression signatures of miRNAs in monocytes and polarized macrophages. miRNA expression was analyzed by qPCR in monocytes, 5 day unstimulated monocyte-derived macrophages (MΦ) or stimulated with IFNγ+TNFα (M1), IL-4 (M2a) or IL-10 (M2c) cells. The median of the miRNA expression in Monocytes, M1, M2a or M2c polarized macrophages relative to the median of the miRNA expression in MΦ, is depicted by the colored dots. miRNAs were grouped according to the expression signature they display in monocytes and polarized macrophages when compared with MΦ: **A)** miRNAs that are only significantly regulated during maturation of monocytes into macrophages; **B)** miRNAs that are not differentially expressed in monocytes, compared with macrophages, but are differentially expressed under polarizing conditions; **C)** miRNAs that are differentially expressed in monocytes, compared with macrophages and also in one polarization condition; **D)** miRNAs that are differentially expressed in monocytes, compared with macrophages, and in various polarization conditions. The horizontal line is set at 1 to represent expression in MΦ.

Table 1. Differentially expressed miRNAs in monocytes and polarized macrophages compared with MΦ.

Monocyte	M1	M2a	M2c	Previous miRNA Ids [a]	Figure [b]
miR-223-5p [c]				*miR-223**	2a
miR-454-3p				*miR-454*	2b
miR-93-5p				*miR-93*	2c
miR-106b-3p				*miR-106b*	2d
miR-132-3p				*miR-132*	2e
miR-335-5p				*miR-335*	2f
miR-34a-5p				*miR-34a*	2g
miR-362-3p					2h
miR-424-5p				*miR-424*	2i
	miR-125b-5p	**miR-125b-5p**	**miR-125b-5p**	*miR-125b*	3a
	miR-181a-5p	*miR-181a-5p*	**miR-181a-5p**	*miR-181a*	3b
	miR-193b-3p	**miR-193b-3p**		*miR-193b*	3c
	miR-125a-5p		**miR-125a-5p**	*miR-125a*	3d
	miR-145-5p			*miR-145*	3e
		miR-181b-5p		*miR-181b*	3f
			miR-200a-3p	*miR-200a*	3g
miR-150-5p		*miR-150-5p*		*miR-150*	4a
miR-27a-5p			**miR-27a-5p**	*miR-27a**	4b
miR-146a-5p	**miR-146a-5p**			*miR-146; miR-146a*	4c
miR-193a-5p	**miR-193a-5p**				4d
miR-29b-3p	**miR-29b-3p**			*miR-29b*	4e
miR-629-5p	*miR-629-5p*			*miR-629*	4f
miR-500a-5p		**miR-500a-5p**		*miR-500; miR-500a*	4g
miR-502-3p		**miR-502-3p**			4h
miR-21-5p			**miR-21-5p**	*miR-21*	4i
miR-22-3p			**miR-22-3p**	*miR-22*	4j
miR-146b-5p			**miR-146b-5p**	*miR-146b*	4k
miR-339-3p			*miR-339-3p*		4l
miR-147b	**miR-147b**	*miR-147b*			5a
miR-155-5p	**miR-155-5p**		*miR-155-5p*	*miR-155*	5b
miR-99a-5p		**miR-99a-5p**	**miR-99a-5p**	*miR-99a*	5c
miR-99b-5p	**miR-99b-5p**		**miR-99b-5p**	*miR-99b*	5d
miR-378a-3p	**miR-378a-3p**	**miR-378a-3p**		*miR-422b; miR-378*	5e
miR-221-3p	*miR-221-3p*	**miR-221-3p**	*miR-221-3p*	*miR-221*	5f
miR-222-3p	*miR-222-3p*	**miR-222-3p**	*miR-222-3p*	*miR-222*	5g
miR-511	*miR-511*	**miR-511**	*miR-511*	*miR-511-5p*	5h

[a] miRNA names previously used to identify the indicated miRNA, as found in miRBase.
[b] Corresponding Figure where expression of the indicated miRNA is depicted.
[c] In **Bold**, miRNAs that are significantly up-regulated; in *italic*, miRNAs that are significantly down-regulated.

A



B

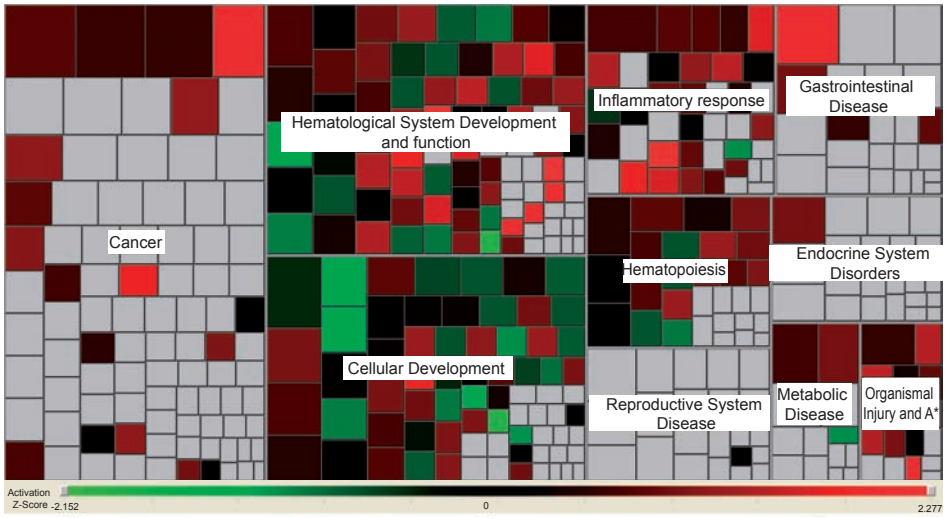
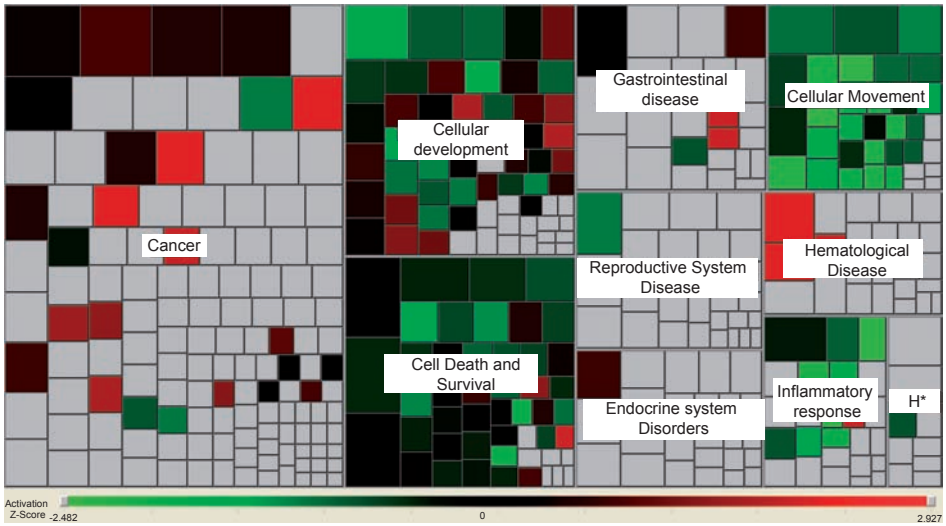


Figure 7. Biological functions associated with miRNAs and their possible target genes, in monocytes and polarized macrophages. Tree map representation of the biological functions associated with miRNAs and their possible target genes. Biological function analysis of the target genes and miRNAs selected was performed in IPA, using fold expression values of genes and miRNAs in monocytes, 5 day macrophages stimulated with IFNγ+TNFα (M1), IL-4 (M2a) or IL-10 (M2c) cells when compared with unstimulated macrophages (MΦ). The 10 categories of functions most associated with genes and miRNAs differentially expressed in **A)** Monocytes, **B)** M1 macrophages, **C)** M2a macrophages and **D)** M2c macrophages are depicted here. The association of each category and the genes in

C



D

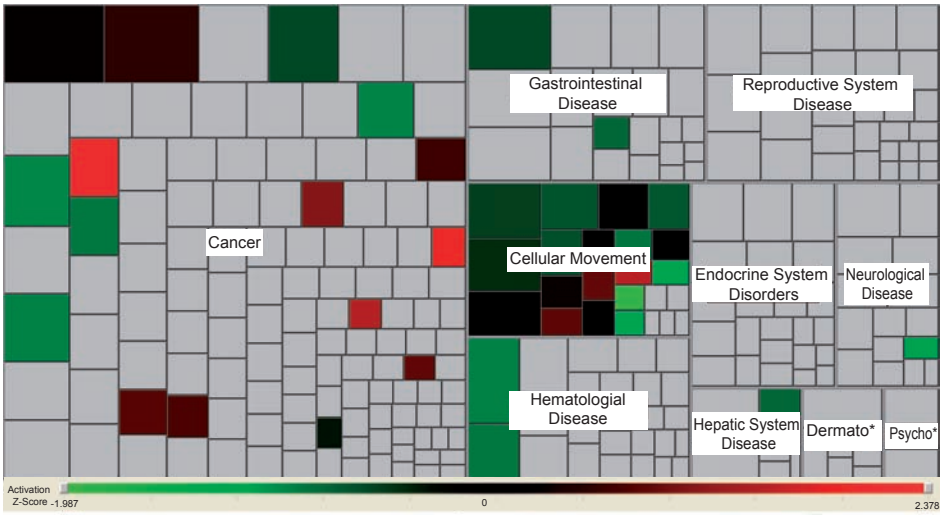


Figure 7. *(continued)* each dataset is determined by the p-value calculated with a built-in Fisher's exact test. The p-values are inversely represented by the size of each category. The amount of squares in each category represents the different associated functions. The size of each square represents the amount of molecules that were found to be significantly associated with such function. Activation Z-score was determined with the built-in algorithm and is represented by color, according to the color bar at the bottom of each graph. Gray represents an incalculable Z-score. In **A**, H*: Hepatic System Disease. In **B**, A*: Abnormalities. In **C**, H*: Hepatic System Disease. In **D**, Dermato*: Dermatological Disease and Conditions; Psycho*: Psychological Disorders.

of the most important processes in macrophage maturation and defining the phenotype and functions of polarized macrophages.

DISCUSSION

In this study, we have analyzed the expression of miRNAs that are differentially regulated during monocyte differentiation and cytokine polarization of human macrophages using next-generation sequencing. We observed that although miRNA expression is highly variable, specific signatures in miRNA and mRNA expression relate to the macrophage role in the immune systems. We believe that the data presented here, contributes to the understanding of global gene expression in human macrophages and confirms that miRNA expression is closely related to cellular function, thus helping to further characterize these cells and to comprehend how they fulfill their crucial role in the immune system.

miRNA expression patterns were highly variable in macrophages, contrary to the global changes in gene expression observed during polarization[22], where groups of genes specific to a cell type are evident and their identification is less complicated. Here we observed that there is no clear distinction in the expression patterns of miRNAs in differentiated and polarized macrophages (Figure 1). This may be due to the synergistic function of miRNAs, in which one miRNA can have various mRNA targets, but also one mRNA can be targeted by multiple miRNAs. Relatively minor changes in the expression of one miRNA may not have a large effect in the expression of multiple targets. However, if a miRNA strongly targets many genes, even slight changes in its expression may affect mRNA levels of all its targets, and this may have great consequences for the phenotype of a cell. miRNA expression, as well as gene expression, can significantly fluctuate among different individuals, due to genetic polymorphisms for instance, and this could explain why we observe high variation in expression of some miRNAs among different donors. Also, miRNA expression is a very dynamic process and it is dependent on cellular factors that regulate transcription. At the same time, these same miRNAs also regulate expression of those transcription factors, therefore forming self-regulatory loops, as it is the case with NF-κB-driven miRNAs[23]. Consequently, understanding expression levels of numerous miRNAs in macrophages is the first step to reveal miRNA mode of action and finally comprehend how they are able to fine tune gene expression and modulate immune responses.

miRNAs are involved in differentiation of hematopoietic precursors as well as differentiation of these cells into myeloid progenitors and subsequently monocytes[24]. In accordance, we found that miR-146a-5p, miR29b-3p, miR-150-5p, and miR-424-5p were differentially regulated during maturation of monocytes into macrophages.

Several miRNAs are known to participate in modulation of immune responses in macrophages. miR-155-5p, miR-21-5p and miR-132-3p are mediators of

inflammation, and miR-146a-5p and miR-146-b-5p are negative regulators of these responses[2,25-27]. Also miR-511 and miR-147b have been associated with modulation of Toll-Like Receptor 4 (TLR)-mediated signaling and NF-κB activation[28,29]. miR-125b-5p is involved in controlling expression of TNFα[30] and has been associated with polarization of macrophages through down-regulation of Interferon Regulatory Factor 4 (IRF4)[31]. Our results showed differential regulation of all these miRNAs during polarization of macrophages, which not only confirms the validity of our study, but they provide a detailed characterization of miRNA expression across phenotypically different macrophages.

Another study also described expression of various miRNAs in polarized macrophages[16]. Although our results were in part similar to those published by Graff *et. al.*, discrepancies between our and their findings were also observed for some miRNAs. This might in part be explained by differences in the techniques used: Graff *et. al.* used miRNA expression arrays, whereas we used the next-generation SOLiD™ platform. However, the major differences in miRNA expression are probably due to differences in the culture procedures to obtain polarized macrophages, since we did not use LPS to stimulate M1 macrophages and used IL-10 instead of TGF-β to obtained M2c macrophages. LPS is a strong inducer of TLR4 signaling, which leads to expression of specific genes and miRNAs in macrophages. Additionally, our study also described expression of numerous miRNAs in monocytes, and how this changes during maturation of these cells into macrophages.

In M2a polarized macrophages, we observed high up-regulation of miR-511, which has been associated with alternative activation of macrophages[32]. miRNAs involved in differentiation and maturation of myeloid cells, such as miR-424-5p[33], miR-181a-5p[34], miR-223-3p[35] and members of the miR-17-92 cluster (miR-18a-5p, miR-19b-3p, miR-20a-5p)[36], were also characterized on our study. M2c polarization induced up-regulation of miR-146b-5p and down-regulation of miR-155-5p, which has also been reported in other studies using Taq-man based miRNA arrays on IL-10 stimulated monocytes[37].

The involvement of other miRNAs in macrophage maturation and polarization also in relation to cancer has been reviewed by Squadrito and collaborators[24]. Several miRNAs identified here, have been implicated in cancer and tumorogenesis processes. We observed down-regulation of miR-222-3p, miR-339 and miR-200a-3p in M2c macrophages. miR-222-3p and miR-339 are expressed in glioblastoma tumor cells[38] and miR-200a-3p expression has been linked to metastasis and tumorogenesis in various types of cancer[39]. Expression of these miRNAs may be related to the function of M2c macrophages as tumor-associated macrophages (TAMs)[40].

From our results, we have recognized a few miRNAs that showed very high or low expression in polarized macrophages, compared with unstimulated macrophages; the expression profile of these miRNA may help identify specific macrophage populations in healthy or diseased tissues. M1 macrophages present

high expression of miR-146a-5p, miR-29b-3p, miR-147b, and low expression of miR-221-3p. M2a macrophages display high levels of miR-193b-3p and miR-511 and low expression of miR-181a-5p and miR-181b-5p. M2c macrophages express high levels of miR-125b-5p and miR-125a-5p, miR-99b and uniquely low levels of miR-200a-3p (figure 6a-d).

A large number of miRNAs are associated with development of inflammatory diseases. miR-629-5p and miR-362-3p have been associated with Inflammatory Bowel Disease, Crohn's Disease and Ulcerative Colitis[41,42]. The expression of miR-145-5p, miR-22-3p, miR-221-3p and miR-454-5p has been associated with disease progression in Multiple Sclerosis[43] and high plasma levels of miR-150-5p are associated with Atherosclerosis[44]. Macrophages are likely to be involved in these pathologies, since they are major players in regulation of inflammation. Proper regulation of gene expression by miRNAs in these cells is crucial for maintaining tissue homeostasis and preventing disease development. Here we described how these miRNAs are expressed in phenotypically different macrophages derived from healthy individuals. Our study may serve as an independent reference for other studies that aim to study miRNAs in relation to several diseases. miRNAs can be secreted by cells of the immune system through exosomes, and mediate cell-to-cell communication[45]. These miRNA-carrying exosomes can be secreted into the plasma of other body fluids[46], and could serve as disease biomarkers[47,48].

On the other hand, macrophages play a main role in the first line of defense against bacterial and viral infections. In previous studies, it has been reported that Human Immunodeficiency Virus type 1 (HIV-1) infection is inhibited in polarized macrophages[49-54]. We have previously observed that miRNAs that directly target the viral RNA[55,56] are not responsible for inhibition of HIV-1 replication in polarized cells[57]. Moreover, there have been reports that show differences in miRNA expression profiles between infected, exposed and uninfected HIV-1 patients[58]. However, it is yet to be described whether miRNAs that target cellular factors which are important for HIV-1 infection, may be differentially regulated in polarized macrophages. Certainly this also needs to be elucidated for other pathogens that interact with tissue macrophages.

In this study, we have identified miRNA expression profiles, during differentiation and polarization of macrophages, using next-generation sequencing. The sequences that mapped to miRBase described here corresponded only to the 10-26% of all sequences obtained in our experiments. Simultaneously, sequences corresponding to novel miRNAs or even other small RNA species were also obtained. Although, contamination by fragmented RNA of tRNAs is possible, there are numerous possibilities for discovering new small RNAs that could be involved in regulation of gene expression and therefore cellular function of macrophages[59].

Analysis of the biological processes associated with some of our candidate miRNAs and their possible target genes, revealed the functional relevance of

miRNAs and the importance of the regulation of gene expression that they achieve. We observed that relationships between gene and miRNA expression correlated with the macrophage phenotype upon maturation from monocytes or cytokine stimulation. This is an indication that these miRNAs and their targets may be crucial in determining macrophage function in relation to their role in the immune system. M1 cells were found to have an increase in functions associated with inflammatory responses, whereas M2a and M2c showed a decrease in such functions, among others. Our findings evidence the impact of gene expression regulation by miRNAs and their importance in the role of macrophages in the immune system.

Our study offers a catalogue of differentially expressed miRNAs that are specifically expressed during macrophage differentiation and polarization, and that are essential for cellular function. Modulation of gene expression by miRNAs is crucial for maintaining proper performance in macrophages, and alterations in this process lead to failure of macrophage innate immune functions. Therefore, comprehensive characterization of miRNAs expression immune cells is an essential step towards understanding their role in the tissues. The information presented here will open numerous possibilities to explore functional and mechanistic aspects of miRNAs involved regulation of immune responses and infectious diseases.

MATERIALS AND METHODS

Isolation of monocytes and cell culture

Monocytes were obtained from buffy coats from healthy blood donors. Written informed consents were obtained from all donors in accordance with the ethical principles set out in the declaration of Helsinki. This study was approved by the Medical Ethics Committee of the Academic Medical Center and the Ethics Advisory Body of the Sanquin Blood Supply Foundation in Amsterdam, The Netherlands. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation. Monocytes were isolated by adherence to plastic and cultured in Iscove's modified Dulbecco's medium (IMDM; Lonza, Basel, Switzerland) supplemented with 10% [v/v] heat-inactivated human pooled serum (HPS), penicillin (100 U/ml; Invitrogen, Carlsbad, CA), streptomycin (100 µg/ml; Invitrogen) and ciproxin (5 µg/ml; Bayer, Leverkusen, Germany) for 5 days in the presence of different cytokines: IFN-γ at 50 U/ml (Sigma-Aldrich), in combination with TNF-α at 12.5 ng/ml (Peprotech, Rocky Hill, NJ, USA), IL-4 at 50 ng/ml (Peprotech), IL-10 at 50 ng/ml (Peprotech) or medium alone at 37°C in a humidified atmosphere supplemented with 5% CO_2.

Library Preparation and SOLiD Sequencing

Total RNA was isolated from monocytes and 5-day cultured macrophages (unstimulated or cytokine-polarized), using TriPure Isolation Reagent (Roche)

according to the manufacturer's instructions. RNA quality was determined using NanoDrop® Spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, USA) and the Agilent 2100 Bioanalyzer with the RNA6000 Nano kit and the Small RNA Chip Kit (Agilent, Santa Clara, CA, USA). All samples used subsequently for sequencing had an A280/260 value higher than 1.8 and a RNA integrity number (RIN) higher than 6. Library preparation was carried out following the SOLiD™ Total RNA-Seq Kit Protocol (PN 4445374, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol for Small RNA library preparation. Briefly, RNA samples were first enriched for their small RNA fraction, by size selection between 15nt and 40nt on 15% TBE-Urea acrylamide gels. The small RNA-enriched samples were hybridized and ligated for cDNA synthesis. cDNA libraries were further purified with the MinElute® PCR Purification Kit (Qiagen, Venlo, The Netherlands). After a size-selection step on 10% TBE-Urea gels, cDNA samples were amplified on gel using 19 cycles instead of 15 recommended by the manufacturer. For multiplexing purposes, the cDNA samples were amplified using the SOLiD™ 3' Primers from the SOLiD RNA Barcoding Kit. cDNA libraries were purified using the PureLink™ PCR Micro Kit (Invitrogen, Carlsbad, CA, USA) and subjected to a second round of size selection on gel and purification according to the manufacturer's instructions. The quality of the cDNA library was assessed with a smear analysis using the Bioanalyzer 2100 software (Agilent).

cDNA libraries were pooled together at an equimolar ratio and used for the emulsion PCR reaction. Workflow Analysis Runs (WFA) showed that the bead preparation had titration metrics above 74% and Noise-to-Signal ratio bellow 5%. The libraries were sequenced to 35bp read length in a 1-well deposition chamber using the Multiplex Fragment Sequencing reagents and the SOLiD™ 4 Analyzer.

Analysis of Sequencing Data

Quality of raw reads was checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). After removing sequencing adapters (FAR http://sourceforge.net/projects/theflexibleadap, Pyrodigm trimming http://dawhois.com/www/pyrodigm.com.html), trimmed reads were aligned against miRBase 18 (http://www.mirbase.org) using BWA[17]. Unique mapping reads were filtered based on mapping quality and counted using R/Bioconductor (http://www.bioconductor.org, Rsamtools, IRanges, ShortRead). Statistical analysis was performed in R/Bioconductor with the use of DESeq[18]. Hierarchical clustering analyses of selected miRNAs were performed in Multi Experiment Viewer from TM4, version 4.8.1[19], after a Log2 transformation of the count values and calculating median centered values for each miRNA. The clustering was done with average linkage method using a Pearson correlation. The data has been made available in the EMBL–EBI ArrayExpress public database (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-MTAB-1969.

qPCR

Expression levels of miRNAs were confirmed using RT-qPCR. RNA was isolated from unstimulated or cytokine-polarized MDM at day 5 after isolation, using TriPure Isolation Reagent (Roche). cDNA was generated with the qScript microRNA cDNA synthesis kit (Quanta BioScineces Inc., Gaithersburg, MD, USA) following the manufacturer's protocol. For quantification of miRNAs, specifically designed primers (Supplementary Table S1) were used in combination with the PerfeCTa Universal PCR Primer (Quanta BioSciences Inc.). The qPCR was performed in a Lightcycler 480 II using SYBR Green I Master (Roche). The following cycling conditions were used: denaturation: 95°C for 10 min; amplification: 40 cycles of 95°C for 10 sec, 58°C for 20 sec and 72°C for 30 sec. Purity of the PCR products was confirmed by melting curve analysis. SNORD44 expression levels were used to correct for cDNA input, with the Human Positive Control Primer (Quanta BioSciences Inc.). A serial dilution of the pooled cDNA was used as a relative standard curve. Differences in the expression levels of miRNAs were analyzed with a Wilcoxon signed ranked test using GraphPad Prism 5 (GraphPad Software, La Jolla, California, USA).

Transcriptional profiles study and data mining

Total RNA was isolated from monocytes and 5-day cultured macrophages (unstimulated or cytokine-polarized), using TriPure Isolation Reagent (Roche) according to the manufacturer's instructions. RNA samples were cleaned with RNeasy Mini Spin columns (Qiagen RNeasy Mini Kit, Qiagen, Valencia, CA, USA) according to the cleanup protocol from the manufacturer. The concentration was analyzed by the Nanodrop ND-1000 spectrophotometer and A260/280 values were above 2.0 for all samples. Microarrays were performed in the Illumina Hg12v4 platform. Background-subtracted data were normalised by Quantiles in R Bioconductor. All datasets have been deposited in Geoprofiles: SubSeries GSE49240 (samples GSM1195728-39), part of the SuperSeries GSE35495[20]. Statistical differences and data processing was performed in TMev v4.8.1. Genes with fold change ≥ 2 were considered differentially expressed.

microRNA Target analysis

Possible targets for selected microRNA were identified with the microRNA Target Filter tool from IPA (Ingenuity Systems Inc., Redwood City, CA, USA). Targets were selected from miRNA-mRNA interactions that had been experimentally validated and reported in the Ingenuity® Knowledge Base, TarBase and miRecords. Targets were selected by comparison of the expression of the miRNA to their target genes in expression data obtained from gene expression experiments described above. We selected miRNA targets only if the miRNA was up-regulated and the target gene was down-regulated, or vice versa. Biological function analysis of the target genes and miRNAs selected in monocytes, M1, M2a and M2c macrophages,

when compared with unstimulated cells, was performed in IPA and the statistical significance was determined with the built-in Fisher's exact test and Z-score algorithm. The results for the top 10 Categories for biological functions associated with each cell type were used in HeatMap Explorer 2.0 (www.labescape.com) to generate TreeMap views of the results.

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