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T-cell activation induces dynamic changes in miRNA expression patterns in CD4 and CD8 T-cell subsets

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Abstract

T-cell activation affects microRNA (miRNA) expression in T-cell subsets. However, little is known about the kinetics of miRNA regulation and possible differences between CD4 and CD8 T cells. In this study we set out to analyze the kinetics of activation-induced expression regulation of twelve pre-selected miRNAs. The dynamics of the expression of these miRNAs was studied in sorted CD4 and CD8 CD45RO- T cells of healthy individuals stimulated with αCD3/αCD28 antibodies. Analysis of miRNA levels at day 3, 5, 7 and 10 showed significant activation-induced changes in expression levels of all twelve miRNAs. Expression levels of nine miRNAs, including miR-21, miR-146a and miR-155, were induced following activation, whereas expression of three miRNAs, including miR-31, were decreased following activation. The expression changes of miR-18a and miR-155 was relatively early, at day 3, whereas expression of miR-451, miR-21 and miR-146a was evident at day 5, 7 and 10, respectively. Four miRNAs showed a differential regulation between CD4 and CD8 T cells. Induction of miR-18a and miR-21 was more pronounced and occurred earlier in CD4 T cells compared to CD8 T cells. Downregulation of miR-223 and miR-451 was also more pronounced in CD4 T cells compared to CD8 T cells. In conclusion, we show a complex pattern of miRNA expression regulation upon T-cell activation with early and late as well as CD4 and CD8 T-cell specific changes. These differences might be the result of differences in kinetics and efficiency of CD4 and CD8 T cells in response to antigen priming.

Key words: microRNA, T cell, activation
**T-cell activation induced changes in miRNA expression in CD4 and CD8 T cells**

**Introduction**

Proper development, maturation and differentiation of T cells requires tight regulation of the underlying cellular processes by a complex network of transcription factors and epigenetic marks (1-4). In the past decade, it has become evident that microRNAs (miRNAs) play a significant role in regulating these processes in T cells (5-9). Distinct expression patterns of miRNAs have been described in specific T-cell subsets, including T helper and regulatory T cells, antigen-inexperienced naïve CD4 T cells as well as memory CD4 T cells (10,11).

Specific miRNA expression patterns most likely reflect the proliferative history and maturation stage of specific T-cell subsets (12). MiRNAs are dynamically regulated during T-cell maturation and play a role in differentiation of specialized T-cell subsets (10,13). Deletion of dicer, a critical enzyme in miRNA maturation, skews Th1 immune response via a compromised ability to repress production of interferon gamma (IFN-γ) (14). Several individual miRNAs including miR-155, and the miR-17–92 cluster are involved in the development of Th1 and Th17 immune responses (15,16).

Primary activation of naïve T cells has recently been shown to affect miRNA expression in T cells (17-19). Several miRNAs, such as miR-21, miR-31 and miR-155, are functionally involved in CD4 T-cell activation (15,20,21). MiR-9, miR-21 and miR-146a have been described to be altered upon T-cell receptor (TCR) stimulation and, in part, determine the strength of the T-cell response (17,22,23). Dynamic regulation of multiple miRNAs upon external stimuli has also been observed during T-cell activation and differentiation stages (10,24,25). MiR-155 was shown to be upregulated upon activation and its expression was found to be proportional to the strength of TCR signaling. MiR-146a controls the intensity and the duration of NF-κB signaling downstream of TCR activation (23,26,27).

TCR-mediated recognition of antigenic peptides presented by MHC class I and II molecules together with ligation of the co-stimulatory receptor CD28 ensures proper activation and effector cell differentiation of CD4 and CD8 T cells (28). TCR stimulation was suggested to trigger distinct responses in CD4 and CD8 T cells that are controlled by intrinsic regulatory mechanisms (29-31). Proliferation of CD4 T cells depends on prolonged presence of the antigenic stimulation, whereas CD8 T cells are able to divide and differentiate into effector cells also in the case of discontinued presence of the antigen (30). Undefined cell-intrinsic differences in CD4 and CD8 cell subsets have been proposed to underlie the differential kinetics in activation and proliferation. Given the marked differences in miRNA profiles and the proposed effects of T-cell activation on miRNA expression patterns of CD4 and CD8 T cells, we hypothesized that changes in miRNA levels define the kinetics of activation of CD4 and CD8 T cells. Although some previous studies report on changes in miRNA expression levels upon activation of either CD4 or CD8 T cells none of these studies directly compared CD4 and CD8 T cells. In this study, we analyzed T-cell activation induced changes in
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expression of thirteen selected miRNAs in sorted CD4 and CD8 CD45RO- T cells over a period of 10 days.

Results and Discussion

Peripheral blood mononuclear cells (PBMC) were isolated from 7 healthy donors (3 males and 4 females) with a median age of 27 (range 24 to 28 yrs of age). CD45RO- CD4 and CD8 T-cell subsets were stained with anti-CD3, anti-CD4 and anti-CD45RO antibodies and sorted following the strategy as shown in Supplementary Figure S1. Sorted CD4 and CD8 CD45RO- T-cells were activated with αCD3/αCD28 monoclonal antibodies for 10 days. To confirm effective activation we analyzed expression of the Interleukin (IL)-2 receptor α-subunit (CD25) on days 3, 5, 7 and 10. A marked increase in the percentage of CD25-positive cells was observed in both CD4 and CD8 T cells of all seven individuals at all-time points. In addition, we observed induction of CD45RO expression consistent with an acquired memory phenotype (Figure 1 and Supplementary Figure S1). Together, these observations confirmed that the majority of both CD4 and CD8 T-cell subsets had acquired an activation-induced phenotype (Figure 1).

We next assessed the expression kinetics of 13 selected miRNAs following stimulation of CD4 and CD8 T cells. 11 out of 13 miRNAs (miR-155, miR-21, miR-146a, miR-31, miR-9, miR-17, miR-18a, miR-19a, miR-19b, miR-20a, miR-92a) were selected based on previously published literature on T-cell activation and 2 out of 13 miRNAs (miR-223 and miR-451) were chosen based on our own previous observations (data not published). Expression levels of miR-9 were very low (Ct above 35) in both CD4 and CD8 T cells at all-time points. The other 12 miRNAs showed higher expression levels and underwent significant and dynamic changes in both CD4 and CD8 T-cell subsets (Supplemental Figure S2). The fold changes in expression levels in comparison to the median value of CD4 T cells on day 0 are shown in Figure 2. Nine out of 12 miRNAs, i.e. miR-155, miR-21, miR-146a and all six members of the miR-17~92 cluster were induced upon T-cell activation, while three miRNAs, miR-31, miR-223 and miR-451, were downregulated.

![Figure 1. Phenotype of activated CD4 and CD8 T cells.](image)

Expression analysis of A, cell surface activation marker CD25 and B, CD45RO analyzed after 3, 5, 7 and 10 days stimulation with anti-CD3/CD28. Shown is the percentage of positive cells for the indicated marker. Black bars indicate CD4 T-cells, open bars indicate CD8 T cells. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
T-cell activation induced changes in miRNA expression in CD4 and CD8 T cells

Interestingly, we identified several specific patterns of miRNA expression with early (day 3), intermediate (day 5) and late (day 7, 10) induction following T-cell stimulation (Table 1). MiR-155 levels reached maximum levels at day 5. Upregulation of miR-155 has been previously described upon Th1 differentiation in CD4 cells and in effector memory CD8 T cells upon viral exposure (15,32). Expression of miR-18a, a member of the miR-17~92 cluster, was strongly induced at day 3 upon T-cell activation and levels remained more or less stable until day 10 within CD4+ T cells. Induction of other members of the miR-17~92 cluster followed a similar pattern but was less prominent consistent with previously published literature (33). Enhanced expression of the members of the miR-17~92 cluster was shown to be required for the proper differentiation of memory cells (13,33). The functional impact of miR-17~92 induction in CD4 T cells has not been investigated yet. Induction of miR-21 was evident at day 5 (CD4) and day 10 (CD8), somewhat delayed in comparison to miR-155 and miR-18a in both CD4 and CD8 T cells. Induction of miR-21 has previously been shown upon TCR/CD28 stimulation in primary naïve T cells. Functional studies showed that miR-21 negatively regulates signal transduction of T cells via targeting the AP-1 transcription factor (17,34). Induction of miR-146a occurred even later, with maximal levels being reached at day 10. Upregulation of miR-146a upon TCR stimulation has been reported previously in both CD4 and CD8 T cells (23,35). MiR-146a modulates activation induced cell death via targeting Fas-associated death domain (FADD) in CD4 T cells (23).

Downregulation of miR-31, miR-223 and miR-451 was evident at day 3. Levels remained low in both CD4 and CD8 T cells, with the exception of miR-223 levels in CD8 T cells, which returned back to normal after 7 days. MiR-31 levels were decreased in both CD4 and CD8 T cells (Figure 2). However, miR-31 was reported to be induced upon TCR/CD28 engagement and to positively regulate T-cell activation via direct targeting of KSR2, a repression factor of RAS2 kinase (20). This potential discrepancy may be explained by differences in concentrations of αCD3/αCD28 used and duration of the stimulation. Expression of miR-31 was shown to be upregulated after 12 hours of stimulation, whereas the earliest time point we assessed miR-31 levels was after 3 days of stimulation. Expression of miR-223 is highly induced in inflamed T cells of rheumatoid arthritis patients (36), which leads to decreased production of interleukin (IL)-10, via suppressing insulin-like growth factor (IGF)-1 (37). Low levels of miR-223 might thus be required for proper production of IL-10 and protection against autoimmune diseases. However, changes of miR-223 levels with regard to T cell activation have not been studied so far. Similarly to miR-223, expression of miR-451 was positively correlated with rheumatoid arthritis activity and very little is known about the regulation of miR-451 in immune cells in general (11).
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Figure 2. T-cell activation-induced dynamic changes in miRNA expression. Expression pattern of A, miR-155; B, miR-18a; C, miR-21; D, miR-146a; E, miR-17; F, miR-20a; G, miR-19a; H, miR-19b; I, miR-92; J, miR-223; K, miR-31; L, miR-451 in CD4 and CD8 T cells at day 0, 3, 5, 7 and 10 following T-cell stimulation. MiRNA expression was normalized to expression of RNU44. Expression changes from day 3, 5, 7 and 10 were compared to day 0 (median value of CD4 t=0 set to 1) and fold change (FC) has been calculated respectively. Data are presented as median (solid line) with interquartile range (dashed lines). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Table 1. Up- and downregulation of miRNAs upon αCD3/αCD28 T cell activation

To gain insight in the relevance of these activation induced changes in miRNA levels, we summarized the currently known target genes validated in T cells (Supplementary Table S1). A large number of these validated targets are involved in T cell activation and co-stimulation pathways. AP-1 and KSR2 regulate the signaling cascade downstream of the TCR, whereas others such as ICOS, CREB1 and PHLPP2 are linked to the T cell co-stimulatory signaling pathway (16,17,20,38,39). In addition, some of the other validated targets such as STAT1 and SOCS1 are responsible for transcriptional regulation of genes relevant to T cells, such as the growth factor interleukin (IL)-2 (40,41).

Figure 3. Schematic overview of dynamic changes of miRNAs in CD4 and CD8 CD45RO T cells. Expression of miR-155, miR-21, miR-18a, miR-223 and miR-451 in CD4+ and CD8+ T cells following stimulation. Solid line indicates CD4 pattern and the dashed line indicates the pattern in CD8 T cells.

In a direct comparison of the activation-induced expression patterns of CD4 and CD8 T cells we noticed marked differences for 4 out of 12 miRNAs, i.e. miR-18a, miR-21, miR-223 and FC miRNA Day CD4 CD8

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**T-cell activation induced changes in miRNA expression in CD4 and CD8 T cells**

Table 1. Up- and downregulation of miRNAs upon αCD3/αCD28 T cell activation

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</table>

To gain insight in the relevance of these activation induced changes in miRNA levels, we summarized the currently known target genes validated in T cells (Supplementary Table S1). A large number of these validated targets are involved in T cell activation and co-stimulation pathways. AP-1 and KSR2 regulate the signaling cascade downstream of the TCR, whereas others such as ICOS, CREB1 and PHLPP2 are linked to the T cell co-stimulatory signaling pathway (16,17,20,38,39). In addition, some of the other validated targets such as STAT1 and SOCS1 are responsible for transcriptional regulation of genes relevant to T cells, such as the growth factor interleukin (IL)-2 (40,41).

**Figure 3. Schematic overview of dynamic changes of miRNAs in CD4 and CD8 CD45RO+ T cells.**

Expression of miR-155, miR-21, miR-18a, miR-223 and miR-451 in CD4+ and CD8+ T cells following stimulation. Solid line indicates CD4 pattern and the dashed line indicates the pattern in CD8 T cells.

In a direct comparison of the activation-induced expression patterns of CD4 and CD8 T cells we noticed marked differences for 4 out of 12 miRNAs, i.e. miR-18a, miR-21, miR-223 and
miR-451 (Figure 3). Expression of miR-18a was induced in both CD4 and CD8 T cells. In CD4 T cells, miR18a expression peaked at day 3 and gradually declined thereafter, while in CD8 T cells a less profound induction of miR-18a levels were observed but over a prolonged period. We observed delayed induction of miR-21 in CD8 versus CD4 T cells, which may in part be explained by a somewhat delayed response of CD8 T cells upon activation as judged by their CD45RA to RO progression (Figure 1). CD8 T cells are essential for protection against viruses, intracellular bacterial infection and tumor cells. CD4 T cells play an important role in the initiation and persistence of CD8 T-cell responses (42). Downregulation of miR-223 and miR-451 was more pronounced in CD4 when compared to CD8 T cells. These differences can at least in part be explained by differences in basal expression levels at day 0, which were 1.6 and 3.5 fold lower in CD8 T cells for miR-223 and miR-451 respectively (Supplementary Figure S2). In CD8 T cells levels of miR-223 were decreased at day 3, but returned back to basal levels after 7 days, while in CD4 T cells miR-223 remained low. MiR-451 inhibits proliferation of mesangial cells by targeting tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta (YWHAZ) (43), but its role in CD4 and CD8 T cells remains to be investigated. The observed differences in miRNA induction between CD4 and CD8 T cells may in part be explained by the differential kinetics of CD4 and CD8 T-cell responses upon antigenic challenge (30,31). The less pronounced changes of miRNA levels in CD8 T-cells, do not reflect the rapid differentiation of CD8 T-cell subsets into effector cells after primary stimulation. This might indicate that miRNAs are less important in regulating CD8 T cell activation as compared to CD4 T cell activation, although this needs to be studied in more detail.

Overall, we show dynamic changes in the levels of specific miRNAs during the process of T-cell activation with clear differences between CD4 and CD8 T cells. Also, we observed marked timing differences with early responses for miR-18a, miR-223, miR-31 and late responses for miR-21 and miR-146a upon activation.

Materials and Methods

Ethics statement

All participants provided written informed consent according to the Declaration of Helsinki to participate in this study that was approved by The Medical Ethical Committee (METC) (project number: 2009.118) of the University Medical Center Groningen UMCG.

Isolation of CD4 and CD8 T cells

Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by density gradient centrifugation using Lymphoprep (Axis-shield, Oslo, Norway) immediately after blood withdrawal into heparin vacutainer tubes (Becton Dickinson, Franklin Lakes, USA). CD4+, CD4- CD45RO- T cells were isolated from PBMCs by fluorescence-activated cell sorting (FACS) (MoFlo, Beckman Coulter, Brea, USA) using anti-human CD3 (OCT-3,
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- Anti-human-CD4 (OCT-4, eBioscience), and anti-human-CD45RO (UCHL1, BD Biosciences, San Diego, USA).

**T-cell activation with αCD3/CD28 mAbs**

T cell stimulation of isolated CD45RO- T cells was performed with plate-bound anti-human-CD3 and soluble anti-human-CD28 mAbs. Briefly, culture plates were incubated with goat-anti-mouse-IgG2a Ab (#1080-01, Southern Biotechnology) at 4°C overnight. After washing with PBS, cells were incubated with hybridoma-culture supernatant, containing anti-human-CD3 IgG2a mAb (clone WT32, approx. conc. 1μg IgG/mL) at RT for 1h. Unbound anti-CD3 antibody was removed by washing 4 times with an excess of PBS and T cells were seeded at a density of 0.5x10^6 cells/mL in medium supplemented with 5% V/V hybridoma-culture supernatant containing anti-human CD28 IgG1 mAb (clone 20-4669) at a final concentration of about 0.1μg IgG/mL. Cells were split every 2-3 days and placed in fresh medium supplemented with anti-CD3 and anti-CD28. At indicated time points, cells were harvested, stained for flow analysis or lysed with Qiazol reagent (Qiagen) and stored at -20°C until RNA extraction procedure.

**FACS analysis of cell surface markers**

Flow cytometry analysis was performed to assess changes in expression of CD45RO and CD25 upon stimulation by staining with monoclonal antibodies against human CD45RO (FITC labeled, UCHL1) and CD25 (APC labeled, BC96). Cells were analyzed on a FACS Calibur flow cytometer using Cell Quest software (BD Biosciences). Data were analyzed using the Kaluza Flow Analysis Software (Beckman Coulter).

**RNA Isolation and quantitative reverse-transcription-polymerase chain reaction (qRT-PCR)**

Total cellular RNA was extracted using the miRNasy Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer’s instructions. The RNA quantity was measured on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). MiRNA expression levels were analyzed by qRT-PCR. RNA was reverse transcribed using the Taqman MicroRNA Reverse Transcription kit in combination with multiplexed reverse transcription primers of TaqMan microRNA Assays (Life Technologies, Carlsbad, USA): for miR-21 (ID: 000397), miR-146a (ID: 000468), miR-155 (ID: 002623), miR-31 (ID: 002279), miR-17 (ID: 002308), miR-18a (ID: 002422), miR-19a (ID: 000395), miR-19b (ID: 000396), miR-20a (ID: 000580), miR-92a (ID: 000431), miR-223 (ID: 002295), miR-451 (ID: 00105) and RNU44 (ID: 001094)(44). The qPCR reaction was performed using qPCR MasterMix Plus (Eurogentec, Liege, Belgium), and Taqman gene expression assays. All reactions were run in triplicate. Mean cycle threshold (Ct) values for all genes were quantified with the Sequence Detection Software (SDS, version 2.3, Life Technologies), using ABI7900HT thermo cycler (Life Technologies). RNU44 served as an endogenous control. Relative expression levels were calculated using the 2^-ΔΔCt formula, where ΔCt = Ct gene – Ct ref gene.
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Statistical analysis

Results obtained from qRT-PCR are expressed as median and range. For comparisons of unstimulated and stimulated CD4 and CD8 T cells we applied the Kruskal-Wallis nonparametric test with Dunn’s posttest, data from day 0 was compared to other days of stimulation (day 3, 5, 7, 10). Statistical analyses were performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and SPSS Statistics version 22.0 (IBM Corp. Armonk, NY, USA). P < 0.05 was considered statistically significant.
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**Supporting information**

Supplementary Figure S1. T-cell sorting strategy and activation induced phenotype. A, Representative FACS plot depicting the gating strategy used to isolate CD4 and CD8 T CD45RO- cell populations. B and C, Representative FACS plot depicting T cell activation on day 7 as shown by expression of CD25 and CD45RO expression in activated CD4 and CD8 T cells. Isolated CD4 and CD8 T cells were stimulated with αCD3/αCD28 mAbs for 10 days and analyzed at day 0, 3, 5, 7 and 10.
Supplementary Figure S2. T-cell activation induced dynamic changes in miRNA expression.

Expression patterns of miRNAs in CD4 (left panel) and CD8 T cells (right panel). Expression pattern of A, miR-155; B, miR-18a; C, miR-21; D, miR-146a; E, miR-17; F, miR-20a; G, miR-19a; H, miR-19b; I, miR-92; J, miR-223; K, miR-31; L, miR-451 in CD4 and CD8 T cells at day 0, 3, 5, 7 and 10 following T-cell stimulation. MiRNA expression was normalized to expression of RNU44. Each dot in the graph indicates a single sample used for analysis. Data are presented as relative expression, for each time point the medians are indicated. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
**Supplementary Figure S2.** T-cell activation induced dynamic changes in miRNA expression in CD4 and CD8 T cells

Table S1. Validated target genes in T-cells for all miRNAs with significant changes upon T-cell activation

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* No distinction was made between miR-17 and miR-20a as they both have the same seed sequence and thus likely target a similar set of genes. The same accounts for miR-19a and miR-19b. For miR-451 no validated targets in T-cells have been published.
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References


References


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CHAPTER 4

Small RNA sequencing of T-cell clones revealed a microRNA signature associated with population doubling time.

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Work in progress