AKT family and miRNAs expression in IL-2 induced CD4+T cells

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ARTICLE INFO	ABSTRACT
	Objective(s): Study of non-coding RNAs is considerable to elucidate principal biological questions or design new therapeutic strategies. miRNAs are a group of non-coding RNAs that their functions in PI3K/AKT signaling and apoptosis pathways after T cell activation is not entirely clear. Herein, miRNAs expression and their putative targets in the mentioned pathways were studied in the activated CD4+T cells. ***Materials and Methods:*** Herein, proliferation rate and IL-2 secretion were measured in treated and untreated cells by IL-2. Putative targets of up-regulated miRNAs were predicted by bioinformatics approaches in the apoptotic and PI3K/AKT signaling pathways. Then the expression of two putative targets was evaluated by quantitative RT-PCR. ***Results:*** Proliferation rate of treated cells by IL-2 increased in a dose- and time- dependent manner. Naive and activated CD4+T cells induced by different dose of IL-2 secreted abundant amounts of IL-2. Also, in IL-2 un-induced cells (IL-2 depleted cells) after 3 days, decrease of proliferation has been shown. *In silico* analysis predicted putative targets of up-regulated miRNAs such as AKT1, AKT3 and apoptotic genes in the activated cells induced or un-induced by IL-2. Decrease of AKT3 was shown by Q-RT-PCR as a potential target of miRNAs overexpressed in IL-2 depleted cells. But there was no significant difference in AKT1 expression in two cell groups. ***Conclusion:*** Our analysis suggests that decrease of AKT3 was likely controlled via up-regulation of specific miRNAs in IL-2 depleted cells. Also it seems that miRNAs play role in induction of different apoptosis pathways in IL-2 induced and un-induced cells.

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Introduction

Protein kinase B (AKT/PKB) is a family including three kinases (AKT1/PKBα, AKT2/PKBβ, AKT3/PKBγ) which play role in cellular functions such as cell survival, metabolism, differentiation and proliferation (1). These isoforms have similar domains in protein structure and are phosphorylated by PI3K (2). In respect to important role of PI3K/AKT pathway in cell survival, these genes are considerable targets for cancer therapy and inflammatory suppression (3). It has been shown that PI3K/AKT pathway is necessary for T cell proliferation (4). IL-2/IL-2R binding activates PI3K/AKT pathway and phosphorylates AKT/PKB (5, 6). Akt activation leads to up-regulation of Bcl-2 and c-myc which inhibit apoptosis and increase cell target genes through mRNA degradation or translational repression (12). Dysregulation of miRNAs have been found in various cancers (13-17), neurological

disorders, metabolic (18) and immune proliferation (6). Also, AKT/PKB phosphorylates GSK3, which in turn leads to export NFAT into T cell nucleus. NFAT and AP-1(Fos/Jun) proteins in the nucleus bind to promoter of target genes such as IL-2 and induce cell proliferation (7). However, regulation of Akt family and its anti-apoptotic properties in T cell after TCR-engagement and IL-2 induction has remained unknown.

MicroRNAs (miRNAs) are small non-coding RNAs by ~22 nucleotide length (8) that play critical roles in biological and physiological processes (9). More than 700 miRNAs have been determined in the mammalian cells (10) that potentially regulate expression of about one-third of mRNAs (11). miRNAs bind to target mRNAs with perfect or imperfect complementarity and then suppress target genes through mRNA degradation or translational repression (12). Dysregulation of miRNAs have been

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found in various cancers (13-17), neurological disorders, metabolic (18) and immunesystem diseases (19, 20). miRNAs are important negative regulators in the different cells which can change expression of target genes promptly. In this respect, it appears that they can be promising therapeutic candidates for disorders in immune system, that requires precise and prompt modulation through complex signaling networks.

In our previous study, miRNA profiling was performed by a reproducible and high sensitive method (8) using miRNA Q-PCR array. Herein, bioinformatics prediction revealed that deregulated miRNAs in activated T cells after IL-2 induction or depletion target different genes involved in PI3K/AKT signaling as well as apoptotic pathways. Also, AKT1 and AKT3 expression were investigated as two putative targets of modulated miRNAs in the cell groups.

Materials and Methods
Cell culture
Human naïve $CD4^+T$ cells isolated from PBMC were cultured in DMEM supplemented with 10% FBS and antibiotics. Naïve $CD4^+T$ cells (1×10^5 cells/well) were seeded in 96-well plates and activated with/ without anti-CD3, CD2, CD28 microbeads (bead-to-cell ratio 1:2). After 3 days, different doses of IL-2 (0.375, 0.75 and 1.5 ng/ml, R&D Systems, Minneapolis, MN) were added for 24, 48 and 72 hr. Cell numbers were determined by trypan blue exclusion assay. Cells were grown at $37^\circ C$ and 10% CO_2 in humidified air. Percentage of $CD4^+$ $CD45R^+$ T cells after culture was identified by flow cytometry using anti-human CD4-FITC (RPA-T4; eBiosciences) and anti-human CD45RA-PE (JS-83). Mouse IgG1κ-FITC and mouse IgG1κ-PE were served as isotype controls. All mAb were purchased from eBiosciences (San Diego, CA, USA). Anti-human-CD2, CD3, CD28 microbeads (human T Cell Activation/Expansion Kit, Miltenyi Biotec GmbH) were a gift from Dr Kambiz Arasteh (asthma and allergy center, Imam Khomeini Hospital, Tehran, Iran).

BrdU assay
The BrdU procedure was carried out according to the manufacturer's instructions (Roche applied biosciences). Briefly, 10 µM BrdU labeling solution was added to each well for 18 hr. The microplate contents were centrifuged (1000 rpm, 10 min) and cells were dried using a hair dryer for 20 min. Cell fixation and DNA denaturing were performed with FixDenat solution for 30 min. After removing the solution, cells were incubated with anti-BrdU mAbs conjugated to peroxidase for 3 hr at room temperature. After washing, the reaction was started by adding substrate solution and then stopped after

3 min by adding 1 M H_2SO_4 solution. The optical density was measured at 450 nm with a reference wavelength at 630 nm using an ELISA plate reader. Cell proliferation ELISA, and BrdU (colorimetric) kit (Roche Applied Science, Mennheim, Germany) was a gift from Dr Mehdi Mahdavi (Virology department, Pasteur Institute, Iran).

IL-2 secretion assay
After treatment with IL-2 for 24, 48 and 72 hr , cell-free supernatants were harvested and kept frozen ($-80^\circ C$) until assayed. IL-2 concentrations were determined by commercially available ELISA kit (Human IL-2 ELISA Ready-SET-Go Kit, eBiosciences) following the manufacturer's protocol. The test sensitivity was 4 pg/ml. The absorbance was measured at 450 nm test wavelength and 545 nm reference wavelengths using an ELISA plate reader. Cytokine quantities in the samples were calculated from standard curves of recombinant cytokines using a regression linear method.

Investigation of potential targets of deregulated miRNAs
In the present study, we carried out miRNA profiling in IL-2 induced and depleted $CD4^+T$ cells (data not shown). Then, targets of up-regulated miRNAs were predicted by bioinformatics tools in PI3K/AKT and apoptotic signaling pathways. To predict potential targets of most modulated miRNAs, Targets can (http://www.targetscan.org/), miRanda (http://www. microrna.org/), Diana mT (http://diana.cslab.ece. ntua.gr/), miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), and miRDB (http://mirdb.org/cgi-bin/search.cgi) algorithms were used.

AKT1 and AKT3 expression analysis by Q-RT-PCR
To evaluate our predictions, we investigated AKT1 and AKT3 expression as potential targets of some modulated miRNAs by Q-RT-PCR. cDNA synthesis was carried out using total RNA with Prime Script Reverse Transcriptase (Takara bio inc. Japan) according to the manufacturer's instructions. Quantitative PCR (Q-PCR) was performed with Power SYBR® Green PCR Master Mix (ABI applied biosystems) using Applied Biosystems Step One™ instrument. PCR programs were as follows: $95^\circ C$ for 10 min, 40 cycles at $95^\circ C$ for 15 sec and $60^\circ C$ for 1 min. GAPDH gene was used as an endogenous internal control. Primer sequences were shown as follow: AKT1 (160 bp) Forward: 5'-GTGGCCAAGG ACGAGGTG-3', Reverse: 5'-ACAGGT GGAAGAACAGCT CGC-3', AKT3 (118bp) Forward: 5'-TGTGTACCGTGAT CTCAAGTTGG-3', Reverse: 5'-GAATGTCTTCATGGT GGCTGC-3' GAPDH (226 bp) Forward: 5'-GAAGG TG AAGGTCGGAGTC-3' and Reverse: 5'-GAAGATGGTG
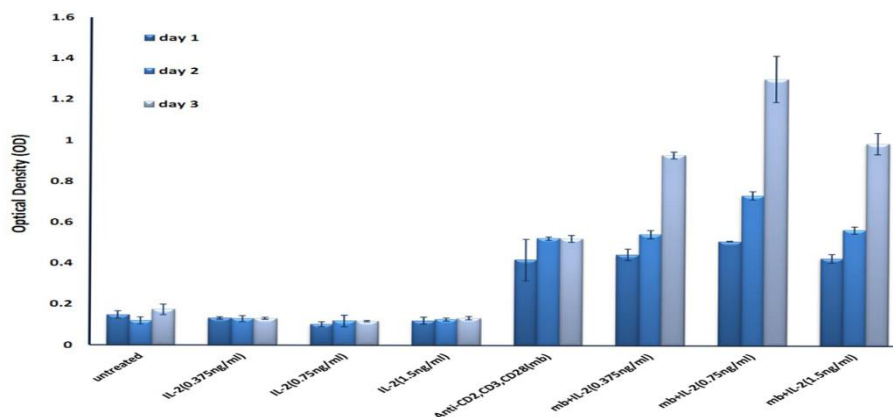
Figure 1. Proliferation analysis by BrdU incorporation (ELISA) assay. Optical density (OD) of treated and un-treated cells by anti-CD2, CD3, CD28 and/or IL-2 revealed the most proliferative rate in activated cells after IL-2 induction during 3 days. Significant difference ($P < 0.0001$ by one-way ANOVA) in the proliferation rate between IL-2 induced cells and activated ones with un-treated cells was statically measured. Representative data from at least three experiments are shown. The results are presented as mean ± SD

ATGGGATTTC-3'. All reactions were run in triplicate and the results were analyzed with $\Delta\Delta C_T$ method. Relative expression of gene was calculated by the equation $2^{-\Delta\Delta CT}$.

Statistical analysis

Results are presented as mean ±SD. Student's t-test or One-Way ANOVA test was used to compare data between two cell groups. Individual experiments were performed in triplicate.

Results
Cell proliferation and IL-2 secretion

Following exposure to anti-CD2/CD3/CD28 for 72 hr, the CD4+CD45RA+ T cells formed large cell colonies which revealed T cell activation (data not shown). The activated and un-activated CD4+T cells were induced by different doses of exogenous IL-2 during other 3 days. IL-2 could not induce cell proliferation in un-activated cells; but, in the activated cells, the increase of optical density (OD) after IL-2 treatment has been shown (Figure 1). Also, flow cytometric analysis showed that the percentage of CD4+CD45RA+ T cells declined after stimulation with both microbeads and IL-2 (Figure 2) compared to stimulation with microbeads alone, which is likely result of the differentiation of some of the activated CD4+T cells after IL-2 induction. In addition, the amount of secreted IL-2 in supernatant was determined between ~1000 to1200 pg/ml in activated and un-activated cells after stimulation with exogenous IL-2. But in cells treated by microbeads alone (activated cells) and in un-treated cells (naïve CD4+T cells), secretion of IL-2 was not determined by ELISA during 3 days (Figure 3). According to these results, IL-2 depletion occurred in CD4+ T cells after full-activation during next 3 days. This cell group herein was used as control and it is called "IL-2 depleted cells".
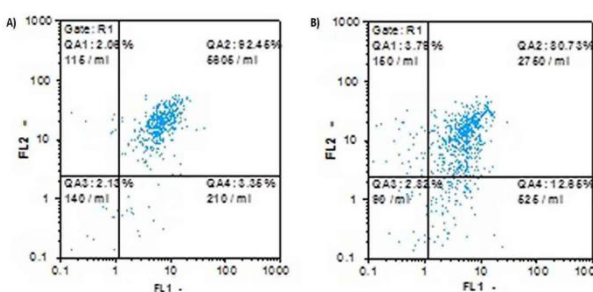

Figure 2. CD45RA+CD4+T cells count after activation with anti-CD2,CD3,CD28 microbeads alone (A) and along with IL-2(B). Flow cytometry analysis showed decrease of CD4+CD45RA+T cells in IL-2 induced cells compared to IL-2 depleted cells

Quantitative analysis of miRNAs potential targets

To explain biologic roles of deregulated miRNAs (data not shown) after T cell activation and IL-2 induction, their potential targets were identified by *in silico* analysis. Diana miRpath analysis revealed that modulated miRNAs potentially target several cell signaling pathways such as TCR-, IL-2-, PI3K/AKT-, and MAPK/ERK- signaling and apoptotic pathways. In addition, putative targets of up-regulated miRNAs were predicted by other algorithms (Table. 1). For example, AKT1 was a potential target of modulated miRNAs in both IL-2 induced and depleted cells. Whereas AKT3 was target of greater number of miRNAs in IL-2 depleted cells than IL-2 induced cells. Also, anti-apoptotic and pro-apoptotic genes such as BCL2 and BCL2L11 (Bim) were target of up-regulated miRNAs. Of these targets, we investigated expression levels of AKT1 and AKT3 by Q-RT-PCR. As expected, down-regulation of AKT3 (Figure 4), has been shown in IL-2 depleted cells. While, there was no significant difference in AKT1 expression levels between two cell groups.

Table 1. Potential targets of the most modulated miRNAs in two cell groups predicted by algorithms such as TargetScan, miRanda, Diana microT and miRWalk

Upregulated miRNAs in IL-2 depleted cells	Target genes	Upregulated miRNAs in IL-2 induced cells	Target genes
hsa-miR-342-3p	AKT2, BCL2L1, CREB1, GSK3, BCL2L11(Bim), CYCS	hsa-miR-634	AKT2, CYCS, BCL2L1, PUMA, BCL2L11
hsa-miR-942	AKT1, AKT3, APAF1, FAS , GSK3, CYCS, BCL2,FASL	hsa-miR-192*	PIM1,CYCS
hsa-miR-92a-2*	AKT1, AKT2, AKT3, APAF1, CYCS	hsa-miR-595	APAF1,CYCS, BCL2L1, GSK3, BCL2L11, BCL2
hsa-miR-328	AKT1, AKT3, PIM1, APAF1, CYCS, AKT2, BCL2L1 (BCL-XL), GSK3, NOXA, PUMA, BCL2L11	hsa-miR-580	PIM1, CYCS, CREB1, NFKB2, BCL2L11
hsa-miR-766	MDM2, PIM1,APAF1, CYCS, AKT2, BCL2L1, DR5, FAS, GSK3, PUMA, BCL2L11	hsa-miR-941	AKT1, GSK3
hsa-miR-938	APAF1, BCL2L1, GSK3, CDKN1A (p21), BCL2L11	hsa-miR-586	AKT1, APAF1, CREB1,BCL2L11, CYCS, BCL2,FASL
hsa-miR-106a	AKT3, MDM2, APAF1, CYCS, BCL2L1, CREB1, DR5, GSK3, CDKN1A, BCL2L11, BCL2, FASL	hsa-miR-558	AKT2, AKT3, PIM1, APAF1, BCL2L1,CREB1, GSK3, CDKN1A, BCL2L11, BCL2
hsa-miR-501-3p	AKT1,AKT3, APAF1, AKT2, NOXA, FASL	hsa-miR-487a	AKT2, CYCS, DR5,FAS , NOXA, AKT3,IL-2,FASL
hsa-miR-30d*	AKT2, AKT3, PIM1, APAF1, CREB, CYCS, FASL	hsa-miR-520f	AKT1, AKT2, AKT3, MDM2, CREB1, DR5, GSK3, CDKN1A, BCL2L11 ,FASL
hsa-miR-590-5p	APAF1, CREB1, DR5, BCL2 ,FASL	hsa-miR-181c*	AKT2, AKT3, PIM1,CREB1
hsa-miR-20a	MDM2, CREB1, DR5, FAS, GSK3, NOXA, CDKN1A, BCL2L11, AKT3, CYCS, FASL	hsa-miR-548c-5p	AKT3, NFKB1,MDM2, APAF1, BAX, DR5,GSK3,NOXA,BCL2L11
hsa-miR-192	PIM1, CYCS, CREB1, GSK3, BCL2L11,FASL	hsa-miR-886-3p	AKT2, DR5,GSK3,PUMA
hsa-miR-16-2*	AKT3, CYCS, FASL	hsa-miR-665	AKT1, AKT2, AKT3, MDM2, DR5 ,GSK3, CDKN1A, PUMA, BCL2L11
hsa-miR-425*	PIM1, CYCS	hsa-miR-512-3p	AKT1
hsa-miR-338-5p	AKT3, NFKB1, APAF1, CREB1,DR5, GSK3 , BCL2L11, BCL2,IL-2 ,FASL		
hsa-miR-23a	AKT2, APAF1, CYCS, DR5, FAS, NOXA, BCL2L11, BCL2		
hsa-miR-222	AKT2, MDM2, APAF1, CYCS, PUMA, BCL2L11, BCL2, FASL		
hsa-miR-20a*	AKT2, AKT3, BCL2L11, CYCS, FASL		
hsa-miR-93	AKT3, APAF1, CYCS, CREB1, FAS, GSK3, NOXA, CDKN1A, BCL2L11, BCL2, FASL		
hsa-miR-155	APAF1, CREB1,DR5, BCL2		
hsa-miR-218	AKT3, CYCS, CREB1,DR5 , GSK3		
hsa-miR-374a	AKT1, AKT3, MDM2, APAF1, CYCS, FAS, GSK3, NOXA		
hsa-miR-532-5p	AKT3, MDM2, CYCS, DR5		
hsa-miR-130b	AKT2, APAF1, CREB1, DR5, GSK3, CDKN1A, PUMA, BCL2L11		
hsa-miR-215	PIM1,CREB1, GSK3, BCL2L11		
hsa-miR-888	AKT2, AKT3, APAF1, CREB1,FAS, NOXA, BCL2L11		
hsa-miR-330-3p	AKT2, AKT3, APAF1, DR5,FAS , GSK3,NFKB1, BCL2L11		
hsa-miR-185*	AKT1, AKT2, PIM1, CDKN1A		
hsa-miR-371-3p	AKT2, GSK3, CDKN1A		
hsa-miR-32	DR5, GSK3, BCL2L11, FASL, FASL		
hsa-miR-105	AKT1, AKT2, AKT3, CREB1, GSK3, CDKN1A, BCL2L11		
hsa-miR-195	AKT2, AKT3, PIM1, DR5, FAS, CDKN1A, BCL2L11, BCL2		
hsa-miR-323-5p	FAS , GSK3, CDKN1A, PUMA		
hsa-miR-513a-3p	MDM2, PIM1,CREB1, FAS, GSK3		
hsa-miR-520a-3p	AKT1, AKT2, AKT3, CREB1,DR5,GSK3, CDKN1A, BCL2L11, BCL2 , FASL		
hsa-miR-130b*	PIM1,CREB1, FASL		
hsa-miR-548h	AKT3, MDM2, APAF1, DR5, GSK3, NFKB1, NOXA		

Continue from previous page

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hsa-miR-561	AKT2, AKT3, CREB1, DR5, FAS, GSK3, NOXA, CDKN1A, BCL2L11
hsa-miR-589*	AKT3, PIM1, FAS, GSK3
hsa-miR-34b*	AKT3, PIM1,CREB1
hsa-miR-424*	AKT1, AKT3, PIM1
hsa-miR-517c	FASL
hsa-miR-607	AKT2, AKT3, PIM1, CREB1, BCL2L1,DR5,FAS, GSK3,NOXA
hsa-miR-378	APAF1, BCL2L1, GSK3
hsa-miR-1236	AKT3, PIM1, APAF1, CREB1, GSK3,PUMA, DR5
hsa-miR-486-5p	PIM1, BCL2L1, GSK3
hsa-miR-148b	AKT3, CREB1, CDKN1A, PUMA, PUMA, BCL2L11,
hsa-miR-181a	AKT2, AKT3,CREB1,FAS, GSK3, NOXA, BCL2L11, BCL2, IL-2, FASL
hsa-miR-211	AKT2,APAF1, CREB1, BCL2L1, GSK3
hsa-miR-99b	GSK3
hsa-miR-24	AKT2, AKT3, MDM2, Pim1, APAF1, CREB1, GSK3, PUMA,BCL2L11, BCL2, FASL
hsa-miR-200c	AKT2, AKT3,CREB1,NOXA
hsa-miR-548b-3p	AKT1, MDM2, CREB1, FASL
hsa-miR-150	AKT2, AKT3, PIM1, APAF1, Bax,CREB1, PUMA
hsa-let-7i*	AKT1, AKT2, PIM1, FASL
hsa-miR-23b	APAF1, AKT2,DR5,FAS, GSK3, NOXA, BCL2
hsa-miR-21	DR5, APAF1, BCL2, FASL
hsa-miR-1260	AKT2, DR5, GSK3, PUMA
hsa-miR-31	Pim1, GSK3
hsa-miR-92a	MDM2, CREB1, DR5, GSK3, BCL2L11, FASL
hsa-miR-200b*	DR5, AKT2, AKT3
hsa-miR-744*	AKT1, AKT2, AKT3
hsa-miR-324-5p	GSK3, PUMA
hsa-miR-151-5p	AKT2, MDM2, GSK3
hsa-miR-25	AKT2, MDM2, APAF1, DR5,FAS,GSK3, BCL2L11, FASL
hsa-miR-940	AKT2, MDM2, APAF1, Bax,DR5,GSK3,CDKN1A, PUMA
hsa-miR-197	AKT2, NOXA, BCL2
hsa-miR-501-5p	AKT3, NFKB2
hsa-miR-181b	AKT2,AKT3, DR5, FAS,GSK3,NOXA, BCL2L11 BCL2, IL-2, FASL
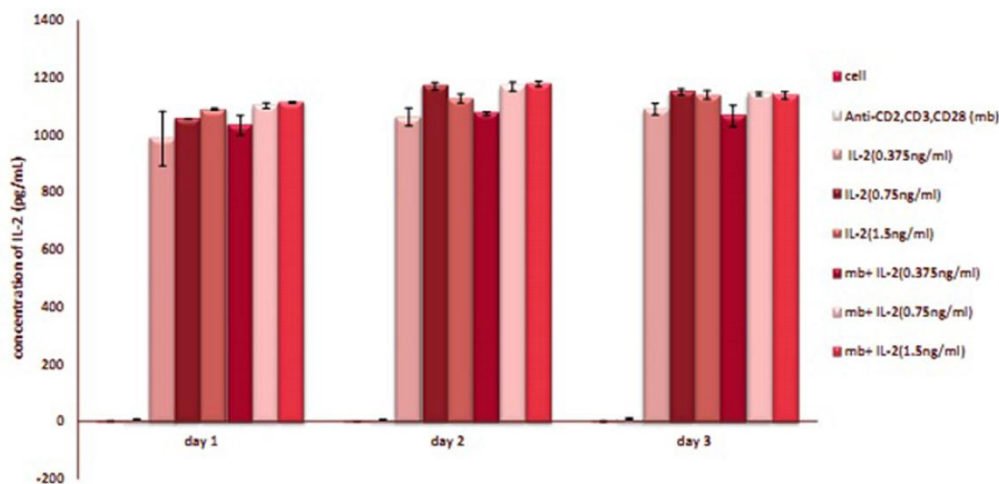
Figure 3. IL-2 production was measured in the supernatants of cultured cells during 3 days after IL-2 induction by ELISA. Cytokine production was significantly higher in the cultures from IL-2 treated cells compared to activated or un-treated cells ($P<0.0001$ by t-test). It is noticeable that cytokine secretion by un-treated CD4+CD45RA+ T cells and activated cells (by microbeads) was not detectable. Representative data from at least three experiments are shown. The results are represented as mean ±SD
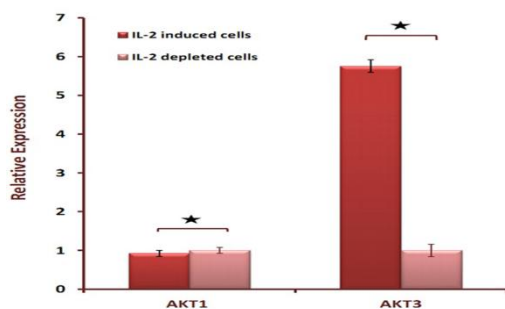
Figure 4. Quantitative expression of AKT1 and AKT3 in IL-2 induced cells compared to IL-2 depleted ones. Relative expression of AKT1 and AKT3 was normalized to GAPDH. Representative data from at least three experiments are shown. Asterisks indicate significant differences the groups (* P <0.05). The results are represented as mean ±SD

Discussion

miRNA profiling is an important approach to identify expression of miRNAs in the biological and physiological processes. The expression pattern of miRNAs in different cells allow to determine relation between transcriptome and microRNome. To investigate miRNAs involved in proliferation or death in IL-2 induced CD4+T cell after miRNA profiling, potential targets of up-regulated miRNAs were analyzed in PI3K/AKT and apoptotic pathways. To investigate the effects of IL-2 on T cell proliferation, the first naïve CD4+T cells were induced by different doses of IL-2. Exogenous IL-2 stimulated IL-2 secretion in naïve CD4+T cells, while cell proliferation was not induced in the cells. Therefore, naïve CD4+T cells were activated by anti-CD2, CD3, CD28 microbeads to express IL-2-Rα chain. TCR stimulation by Ag (21), Anti-CD3, or CD28 induces IL-2-Rα expression on T cell surface. Subsequently, IL-2 binds to IL-2 receptor (α, β and γ chains) and induces cell proliferation by activating downstream signaling pathways (21). The activated cells then were cultured with/without exogenous IL-2 for 3 days and the expression pattern of miRNAs deregulated after IL-2 induction was determined by Q-RT-PCR. IL-2 is a crucial growth factor for Ag-primed T cells that regulates cell survival and death via several downstream pathways including JAK/STAT and PI3K/AKT signaling (5). Previous studies have determined anti-apoptotic role of Akt /PKB family in IL-2 induced T cells; but, the expression pattern of AKT family and miRNAs and their relation to cell proliferation or death in these cells has not been sought, herein we investigated expression of two members of Akt family (AKT1 and AKT3) which potentially targeted by up-regulated miRNAs in IL-2 induced and depleted cells.

In IL-2 un-induced activated cells, depletion of IL-2 during 3 days indicated that these cells have a similar process to end immune response. In murine activated cells, decline of IL-2 during 48 hr after activation has been shown (22). In this study, IL-2 depletion led to decrease of proliferation on day 3 compared to day 2 in the activated CD4+T cells. With IL-2 withdrawal, the full-activated T cells slowly undergo apoptosis (26). While, IL-2 induction led to secretion of IL-2 in activated cells and increase of proliferation and survival during 3 days, that is similar to progression of immune response. PI (propidium iodide) staining in our previous study has revealed to increase apoptosis after 3 days in IL-2 depleted cells relative to IL-2 induced ones (23). In other studies, it has been shown that to maintain lymphocyte homeostasis (24), apoptosis happens after activation of regulatory T cells (Treg cells) (25) or after IL-2 depletion (*in vitro*) (22, 26). This result indicates that the absence or presence of a specific cytokine is determinant for immune cells destination. The IL-2 depleted cells herein suffered greater apoptosis than IL-2 induced cells. Thus, it is noticeable that over-expression of pro-apoptotic genes and/or under-expression of anti-apoptotic genes occur in the IL-2 depleted cells.

miRNAs regulate some aspects of immune responses, and their expression changes during T cell activation and differentiation (10, 27, 28). The role of some miRNAs that were up-regulated in IL-2 depleted cells such as miR-181a and miR-21 has been previously determined in T cell response or development. For example, miR-181a regulates T cell sensitivity to antigens by down-modulation of multiple phosphatases. Thus, miR-181a down-modulation in IL-2 induced cells likely increases the activation of these phosphatases to repress TCR/Ag engagement. Naïve T cells derived from miR-155 knockout mice were revealed greatly trend to Th2 rather Th1 differentiation (10). miR-21 is induced after TCR stimulation and likely is a negative regulator of TCR signaling, and ectopic expression of miR-21 decreases T cell activation (29). Increase of apoptosis in IL-2 depleted cells is associated with proliferation related pathways such as TCR- and IL-2 signaling. Thus, miR-21 over-expression in these cells likely led to suppress T cell activation by inhibition of TCR signaling. It appears that mentioned miRNAs after IL-2 depletion inhibit clonal expansion through regulation of TCR and IL-2 signaling. Also they may drive T cell to differentiation or apoptosis. In this study, bioinformatics tools predicted that greater number of up-regulated miRNAs such as hsa-miR-942, hsa-miR-92a-2*, hsa-miR-328, hsa-miR-330-3p and hsa-miR-374a potentially targeted AKT3 gene in IL-2 depleted cells. While, AKT1 was potentially targeted by up-regulated miRNAs in both IL-2 induced and depleted cells. AKT family members are differentially expressed during T cell development (2). Previously, it is defined that in the absence of Akt1, immature T cell suffers increased apoptosis (2). Herein, AKT1

expression was approximately at the same levels in two cell groups; so, it is suggested that AKT1 is important for survival of activated cell both in the presence and absence of IL-2. Also, it is reasonable that AKT1 expression was required for cell proliferation in activated cells, but it seems that Akt1 could not efficiently repress cell death due to IL-2 depletion; so that three days after T cell full-activation apoptosis overcame proliferation. Also, it is possible that anti-apoptotic effects of Akt1 were reduced by over-expression of apoptotic genes in the IL-2 depleted cells. On the other hand, we revealed that induction of IL-2 in the activated cells increased AKT3 expression and cell proliferation. Akt3 role has been determined in tumorigenesis and inhibition of apoptosis (30). Akt3 activates many oncogenic pathways including CREB, IKK, Cyclin D1 and PRAS40 (31), also it inhibits FoxO3a protein as an apoptotic inducer (32). Regarding AKT3 was a putative target of most up-modulated miRNAs in IL-2 depleted cells; it appears that down-regulation of AKT3 after IL-2 depletion may be regulated by miRNAs, partly. Also, increase of AKT3 after IL-2 induction likely plays role in the cell survival and decrease of apoptosis. In a study on MEF-Akt1, and 2-DKO cells, it has been determined that apoptosis significantly induced only after depletion of $\geq 80\%$ Akt3 protein in these cells. It was considerable that reintroducing Akt3 inhibited apoptosis in these cells (33). However, herein in the IL-2 induced cells AKT1 was down-modulated, but it seems that because of AKT3 up-modulation IL-2 induced cells suffered apoptosis less than IL-2 depleted cells. While, decrease of AKT3 and AKT1 together induced greater apoptosis in IL-2 depleted cells. Nevertheless, it is unclear yet why AKT1 or AKT3 were expressed differentially after T cell activation.

Regarding AKT signaling pathway is one of the main pathways implicated in cell survival (34), but no evidence has yet been reported that Akt inhibits pro-apoptotic protein such Bad or FasL in T cells. However, it seems that Akt plays role in the up-regulation of anti-apoptotic proteins including Bcl-2 and Bcl-xL (35).T cell apoptosis is induced either through intrinsic mitochondrial (result of antigen clearance and IL-2 withdrawal) or extrinsic (FAS mediated) pathway (36, 37). In this study, under-expression of Bcl-2, as a potential target of up-regulated miRNAs, was determined after IL-2 withdrawal (depletion) (data not shown). Over-expression of Bcl-2 inhibits intrinsic pathway (38). Previously, it has been shown that Bcl-2 expression decreased after IL-2 depletion in CD4+T cells blasts (39). Up-regulated miRNAs in IL-2 depleted cells may regulate intrinsic pathway by Bcl-2 targeting. Also, cytokine withdrawal apoptosis (CWA) is regulated by the pro-apoptotic members of Bcl-2 family such as Bim and Puma. These members

activate pro-apoptotic proteins Bak and Bax (37). Herein, *in silico* analysis predicted that Bim (BCL2L11) was potentially targeted by up-regulated miRNAs both in IL-2 depleted and induced cells. It is suggested that Bim may be controlled at post-transcriptional level partly after T cell activation or IL-2 induction.

It has been reported that Akt attenuates activation of intrinsic pathway through inhibition of Bak and Bax (40). Thus in IL-2 induced cells, apoptosis probably was inhibited by Akt activity, but in IL-2 depleted cells, decrease of AKT3 expression may have influence on increase of pro-apoptotic proteins. Other studies have revealed that activated Akt inhibits the intrinsic pathway of apoptosis (40). Akt3 likely plays role as an inhibitor in mitochondrial apoptosis pathway in IL-2 depleted T cells (26); in addition, decrease of AKT3 along with up-regulation of miRNAs likely led to activation of apoptosis intrinsic pathway. While in IL-2 induced cells it has been demonstrated that FASL signaling plays role in extrinsic apoptosis induction (26).

Conclusion

According to our results, it seems that IL-2 depletion leads to over-expression of miRNAs that play role in induction of apoptosis and inhibition of proliferation. However, IL-2 induction likely increases expression of miRNAs involved in cell death inhibition. On the other hand, AKT3 may be important for survival of IL-2 induced cells that its expression is regulated by miRNAs in part. Our findings suggested that up-regulated miRNAs likely regulate activation of apoptosis pathway after IL-2 depletion by suppression of anti-apoptotic genes such as Akt family and Bcl-2.

Conflict of interests

The authors declare no conflicts of interest.

Acknowledgment

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