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MiR-146a and ADAPTIVE IMMUNITY:

A Novel Player in the Regulation of Normal Immune function and Inflammation

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Contents

Abstract

MicroRNAs (miRNAs) have been shown to affect haematopoietic differentiation, acting as important determinants of cellular fate specification.
The aim of this work is to investigate the role of miRNAs in the activation of T lymphocytes. By microarray analysis, we observed differential expression of miRNAs in distinct subclasses of T lymphocytes: $CD8^+$ naïve, $CD8^+$ memory effector, $CD8^+$central memory; $CD4^+$ naïve, $CD4^+$ memory effector and $CD4^+$ central memory. We found that miR-146a is expressed in separated primary T cells, in both $CD4^+$ and $CD8^+$ cells, but not in naïve T cells.
Furthermore, a significative up-regulation of miR-146a expression levels was also obtained in the Jurkat T cell line, stimulated with αCD3-αCD28 antibodies, or with PMA and ionomycin, which are stimuli that mimic antigen recognition by TCR.
Therefore, we attempted to discover the mechanisms of miR-146a transcriptional regulation. Some indications about the pathways possibly involved were obtained by treatment of induced Jurkat cells with specific pharmacological inhibitors of pathways triggered by TCR signalling.. We found that Cyclosporine (CsA), a Calcineurin inhibitor, significantly impaired miR-146a up-regulation, thus suggesting that the pathways affected by CsA play a key role in the induction of miR-146a upon TCR engagement. This observation prompted us to further investigate with molecular tools the transcription factors which are responsible for miR-146a expression in T lymphocytes. A deeper investigation to carefully characterize the *cis* regulatory elements involved in miR-146a regulation was performed through a bioinformatic analysis of miR-146a promoter and a luciferase assay. These approaches allowed us to identify conserved consensus sites for T lymphocytes-specific transcription factors, which are involved in pathways triggered by TCR signalling and are required for miR-146a induction. The results obtained indicate that NF-κB and c-ETS are involved in miR-146a induction in T cell activation pathway.
Moreover, we also demonstrated that miR-146a is also induced in Jurkat cells by an inflammatory stimulus, TNF-α treatment, and NF-κB is the main transcription factor responsible of miR-146a induction.
Another intriguing aspect of miRNAs study is the identification of miRNAs biological role in a cell. We identified miR-146a targets

taking advantage of a 3'-untranslated region (UTR) luciferase reporter assay. We tested a number of predicted miR-146a targets, relevant for T cell physiology. In particular, we report here that miR-146a down-modulates the apoptosis process via translational inhibition of FADD and it reduces AP-1 transcriptional activity, through the down-regulation of c-FOS

Altogether, our findings reveal miR-146a involvement in T cell activation process, significantly affecting key properties of lymphocytes.

INTRODUCTION

MICRORNAs: NEW KEY MODULATORS OF CELLULAR PROCESSES

Gene regulation mediated by the action of small RNAs is a widely spread mechanism, evolutionarily conserved across diverse *phyla*, from prokaryotes to the more complex eukaryotes.This kind of regulation is present at many levels; several classes of small silencing RNAs have been identified thus far including small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNas) and microRNAs (miRNAs).

MiRNAs are an abundant class of small silencing RNAs, whose functional role in many biological processes have been emerged in the last decade. It was long known in the *Caenorhabditis elegans* community that a decrease in the expression of the heterochronic gene *lin-14*, regulating developmental timing, was critical for early larval stage transition and that the *lin-4* gene was required to reduced *lin-14* levels and allow the larval transition to occur[1]. In 1993 a groundbreaking work by two groups showed that repression occurred through complementary binding of small RNA *lin-4* to the 3'-UTR (untranslated region) of the *lin-14* mRNA [2,3]. In working to clone the *line-4* gene, Lee, Feinbaum, Ambros rigorously narrowed down the genomic interval of interest and systematically mutated all ORFs there to demonstrate that the *line-4* gene product is not a protein. Instead, they found the *line-4* gene product is a small ncRNA that shows antisense complementarily of the *line-14* 3'-UTR [1]. A later study found and cloned a gene, *let-7*, that encodes a similar small ncRNA which acts in a similar manner [4]. Numerous miRNAs have been cloned in the following years and they have shown to negatively regulate protein translation.

MiRNAs are small endogenous non coding RNAs of about 22 nucleotides in length involved in the post-transcriptional regulation. They derive from long primary transcripts (pri-miRNA), which can exceed 1 kb and typically are transcribed by polymerase II [5]. These pri-miRNA are processed into hairpins of 60-70 nucleotides in length, called pre-miRNA by a complex containing the nuclear RNAse III

enzyme Drosha and DGCR (Di Gorge Sindrome Critical Region 8 homologue); they are then exported to the cytoplasm and further processed by an enzymatic complex containing the RNAse III enzyme Dicer and TRBP (human immunodeficiency virus 1-transactivating response RNA-binding protein) yielding the mature, double stranded, 22 nucleotides long miRNAs [6,7] (Fig.A).

Using one strand of the miRNA, called the guide strand, the miRISC is believed to either block mRNA translation, reduce mRNA stability or induce mRNA cleavage, after imperfect binding between the 5'-end of the miRNA and complementary sequence, in the 3'-UTR of the target mRNA, (Fig.A) [8,9]. Interaction specificity relies on the nucleotides occupying positions 2 to 7 or 2 to 8 within the miRNA. This region is called "seed" [8,9]. Significantly, this redundancy within the miRNA:mRNA binding region means that individual miRNAs are able to target multiple mRNA and has led to the speculation the miRNAs might have a similar role to transcription factors [10,11].

It is generally assumed that members of the same family of miRNA (i.e. sharing the same seed sequence) will affect the same targets.

In the last few years 678 mature human miRNA sequences have been listed in the miRNA registry (http://www.microrna.sanger.ca.uk /sequences) with approximately 1000 predicted miRNAs, each potentially targeting about 200 genes [12]. Therefore, it is estimated that the expression of >30% of the human genome may be regulated by miRNAs.

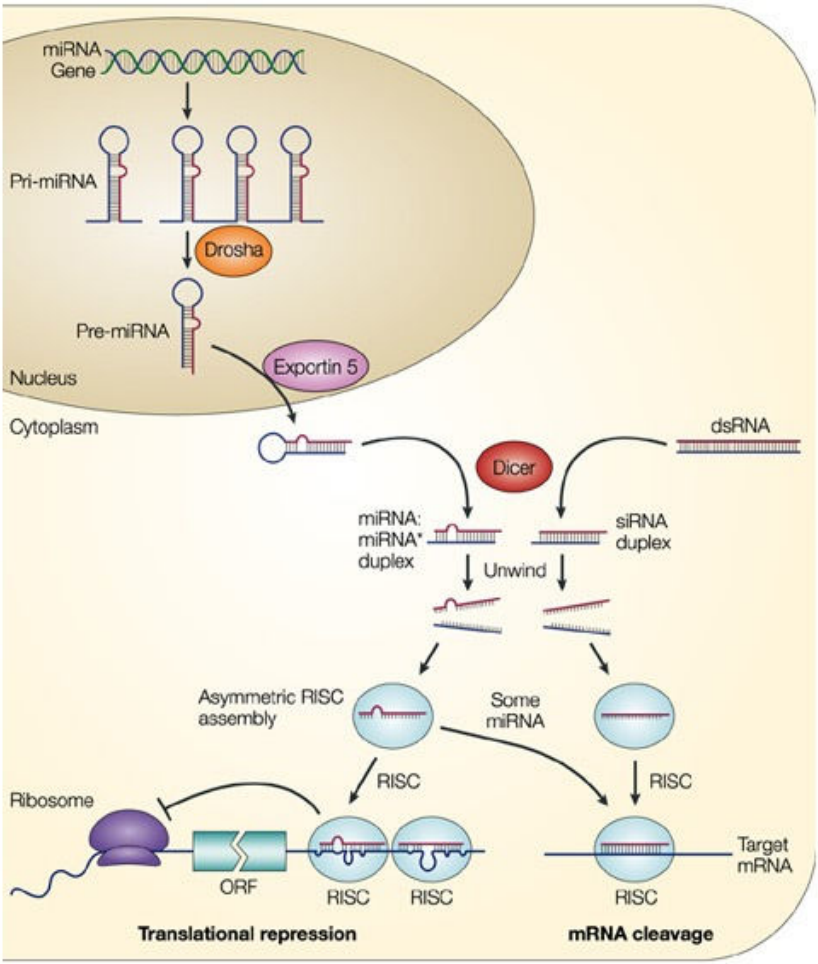


Fig. A: Biogenesis of MiRNAs *Nat. Rev. Genet., 5, 522-531*

MicroRNAs in haematopoiesis and in the immune response

Study of miRNAs flourished during the last decade and it is now apparent that they can potentially modulate every aspect of cellular activity, targeting genes involved in a wide range of physiological processes, from apoptosis [13] to proliferation[14] and differentiation [15].

Many vertebrates miRNAs are expressed in a tissue- or cell type-specific manner [7]; for example miR-142 is primarily expressed in lymphoid cells [16], miR-223 is preferentially expressed in myeloid cells[17] and miR-1 is expressed in the muscle [15]. These findings are consistent with a role of microRNAs as tissue identity specificators.

MiRNAs also participate to the regulation of haematopoietic differentiation, acting in a lineage and developmental stage-specific manner[18,19]. This has emerged from studies that revealed selective expression of miR-181 in the thymus and miR-223 in the bone marrow and indicated their involvement in the differentiation of pluripotent hematopoietic stem cells (HSCs) into the various blood cells lineages, including B and T cells [19,21] (Fig. B).

Some microRNAs are important to fine tune lymphoid-specific pathways; for example it has been shown that the over-expression of miR-181 in haematopoietic precursor cells can bias lymphoid differentiation toward the B cell lineage at the expense of T cells [19,22].A significant role of microRNAs in the immune response has also been shown for miR-146a and miR-155; both of them are involved in the control of innate immunity, in response to pro-inflammatory stimuli[23,24](Fig. B).

Regulatory T (Treg) cells were also shown to express a distinct miRNA subset, which resembles a profile of miRNAs expressed in activated but not naive T cells [19,20].

The examples mentioned above suggest that, in addition to the effects of lineage specific transcription factors and cytokines, microRNAs affect lymphoid differentiation, acting as important determinants of cellular fate specification [19,21].

Nature Immunology 9, 839-845 (2008)

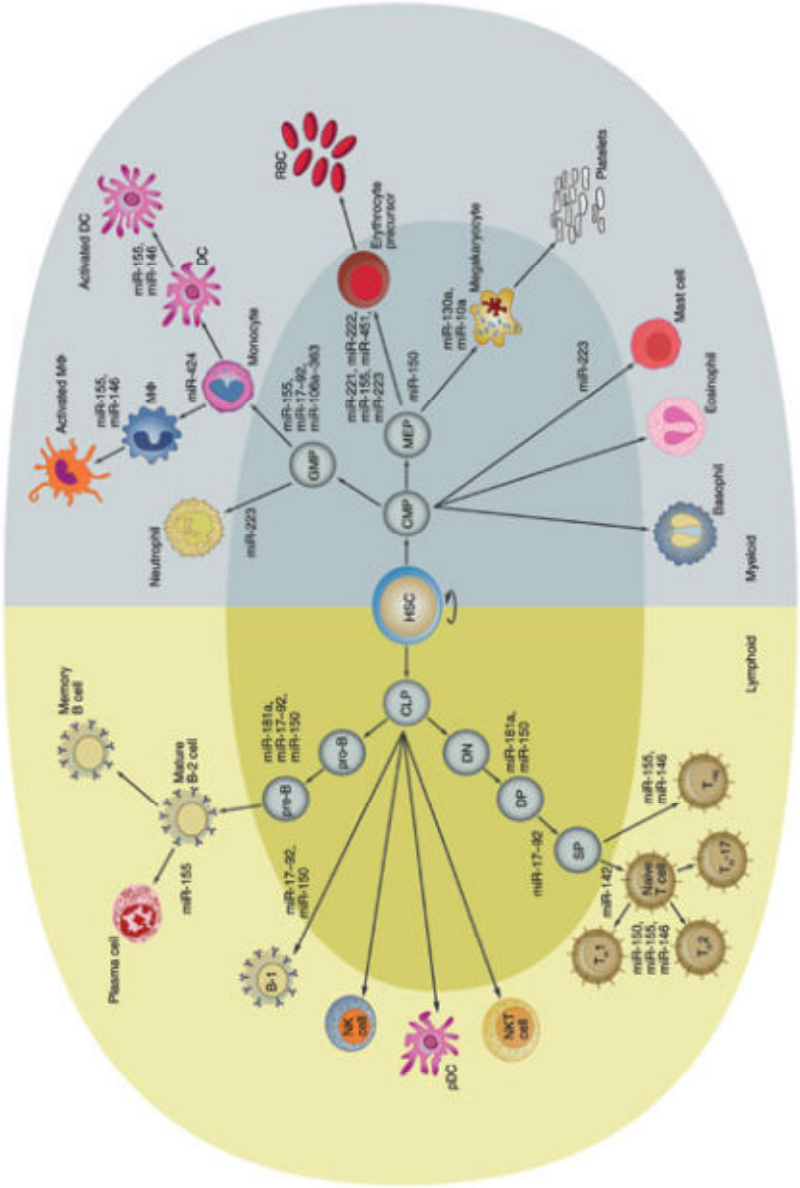


Fig. B: Overview of the involvement of miRNAs in hematopoiesis and immune system function

T LYMPHOCYTE ACTIVATION

T lymphocyte activation is induced *via* the interaction of antigenic peptides, associated with major histocompatibility complex molecules (MHC) on the surface of antigen-presenting cells (APC), with the T cell receptor (TCR)/CD3 complex on T cells [25]. A co-stimulatory signal, mediated by the interaction of the auxiliary receptor expressed on T lymphocyte (CD28) with co-stimulatory molecules expressed by APC cells (B7.1 and B7.2), is also needed to achieve proper activation [26].

TCR engagement by the antigen triggers a signal cascade which ultimately leads to the activation of transcription of several genes and induces the T lymphocytes to entry the cell cycle.

TCR elicits its function through the regulation of phospholipase Cγ (PLCγ) an enzyme involved in the metabolism of inositol phospholipids[27]. The phosphorylation and following activation of this enzyme, occurring within minutes after antigen recognition, simultaneously induces the phosphatidyl-inositol and the DAG (diacylglycerol) signal transduction pathways.

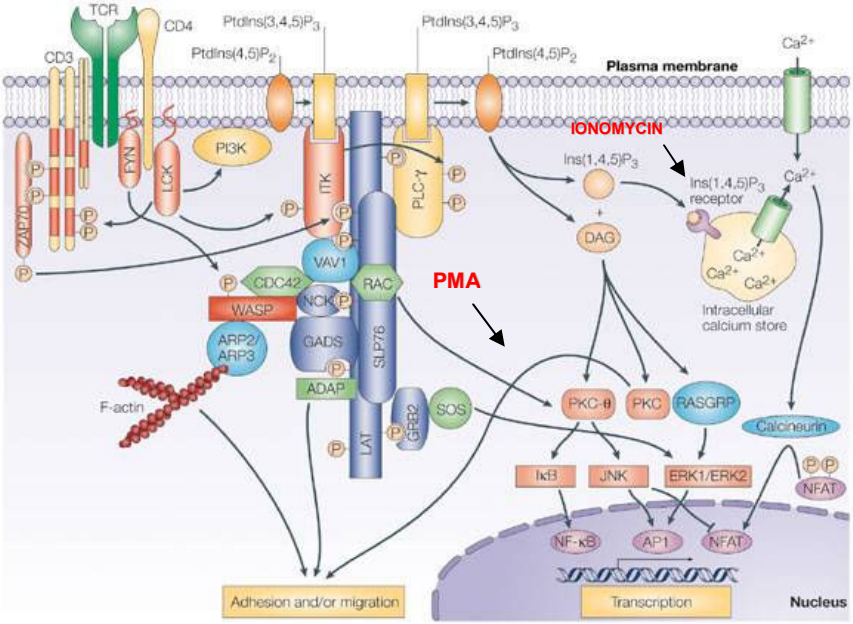
The first pathway triggers the activation of protein kinase Cθ which, in turn, leads the phosphorylation of several cytoplasmic proteins and induces the transcription of downstream genes, through the activation of two main transcription factors: AP-1 (an heterodimeric transcriptional activator, composed of members of the Jun and Fos families) and NF-κB [27,28] (Fig. C).

The DAG signal, generated from membrane phospholipids, elicits a sustained entry of Ca^{2+} from the external milieu. The increase of the cytoplasmic Ca^{2+} concentration leads to gene transcription mediated by the nuclear factor of activated T cells (NFAT)[29].

Finally, the synergic effects of these two signal transduction pathways induce transcription of interleukins, in particular IL-2, which is an important growth factor, fundamental to induce proliferation of activated T lymphocytes [30].

Following activation by antigen presentation, T cells go through several phases:

1) an interleukin-2 (IL2)- dependent proliferation of the cells ("**clonal expansion**") and differentiation of the progeny in effector cells,



Adapted from *Nature Reviews Immunology 3, 939-951 (2003)*

Fig. C: TCR signalling pathway

2) an "**effector phase"**, in which T lymphocytes either provide help or kill infected target cells;
3) a final "**deletion phase**", in which most of the T cell clone eventually undergoes apoptosis, while few T cells survive and enter the memory T-cell pool.
The proliferation of T lymphocytes in response to the antigenic stimulus is mediated by an autocrine loop: activated T cells produce cytokines (e.g. IL-2) and at the same time they express on their membrane the receptors for those cytokines [30].
During the first phase T cells are resistant to apoptosis; entering the effector phase, T cells become progressively more sensitive to cell death in the presence of IL-2 and are finally deleted in the deletion phase. Therefore, IL-2 plays a dual role: it is firstly mandatory for clonal expansion and later for sensitizing T cells towards apoptosis [31].
The isolation of cell lines suitable for the study of T-cell activation and maturation, together with the development of protocols for the

activation of T-lymphocytes *ex vivo*, has allowed molecular characterization of the pathways involved [32].

The leukemic Jurkat T-cell line has been central to most of the important discoveries in T-cell biology. Jurkat cells have been demonstrated to be very useful for the biochemical characterization of Antigen-TCR interaction and to investigate the related signal transduction pathways [32]. Moreover, genetic studies in mice and the characterization of human immunodeficiencies reveal that the core aspects of TCR signalling which have been defined in Jurkat cell line are also relevant in a physiological setting [2]

In vitro study of T lymphocyte activation makes use of polyclonal activators (Fig. C), which bind to several TCR complexes, not depending on their specificity, mimicking the effect of the interaction with the complex MHC-peptide. For example, activation of T cells can be induced by signals generated by phorbol esters, such as PMA (activation of PKC) and ionomycin (induction of Ca^{2+} influx). The synergistic effect of these two signals leads to the activation of distinct signalling pathways which ultimately converge to induce IL-2 transcription within 2-5 h from the beginning of the stimulation[33]. Therefore, these molecules bypass the interaction between antigen and lymphocyte, inducing the activation of signalling down-stream of both TCR and co-stimulatory pathway; for this reason they are widely used for *in vitro* studies.

REGULATION OF CYTOKINES SIGNALING AND INFLAMMATION

In response to cell injury elicited by trauma or infection the inflammatory response sets in, constituting a complex network of molecular and cellular interactions detected to facilitate a return to physiological homeostasis and tissue repair. The response is composed of both local events and a systematic activation mediated by cytokines. If tissue health is not restored or in response to a stable low grad irritation, inflammation becomes a chronic condition, that continuously damages the surrounding tissues [34].

The inflammatory response involves the sequential release of mediators and the recruitment of circulating leukocytes, which become activated at the inflammatory site and thus release further mediators. This response is self-limiting and in most cases it is resolved through the release of endogenous anti-inflammatory mediators and the clearance of inflammatory cells [35]. The persistent accumulation and activation of leukocytes is a hallmark of chronic inflammation [34, 36].

The cellular components of the inflammatory response include leukocytes (innate immune system) as well as lymphocytes T, B, NK (adaptive immune system). The first step in the initiation of the inflammatory response is the activation of the innate immune system. This non specific response serves to locate the injurious agent, restrict the tissue damage and eliminate the harmful agent, initiate the adaptive immune system and determine the path that will be followed (cellular / humoral response). As lymphocytes arrive to the inflammatory area, antigen-presenting cells (APCs) present infectious agent antigens of macrophages to T cells. This is the ignition signal for the activation of the adaptive immunity, consisting of cellular and humoral immunity. These cells co-operate using molecular signals, including cytokines, chemokines, vasoactive amines (histamine, serotonine), plasma proteases (kinine system, complement system), arachidonic acid metabolites (prostaglandins, leukotrienes, lipoxins), platelet-activating factor (PAF), nitric oxide (NO).

Cytokines play important roles in the different phases of the immune response. In fact, they are responsible for the induction and outcome of the inflammatory process, regulation of the acute phase response, initiation of lymphocyte activation, control of antibody production and/or generation of cytotoxic lymphocytes.

Cytokines are soluble factors which act as intercellular messenger between cells of the immune system by binding to specific receptors which are poorly expressed on resting cells and are up-regulated following cell activation.
Each cell types is able to produce a specific set of cytokines and receptors in response to different stimuli; several receptors and cytokines can be expressed by different cell types, thus creating a network in which each cell produces, for a short period of time, a large variety of cytokines able to bind and stimulate different cells [35]
Cytokine receptors, consisting of two or more membrane protein subunits, can be separated in different classes including the hematopoietin receptor superfamily (e.g. receptor for IL-2, -4, -10), the Tumor-Necrosis-Factor receptor (TNF-R) superfamily, the Interferon-receptor (IFN-R) superfamily [37]. Dimerization receptor subunits upon binding of the proper cytokine leads to the activation of the receptor associated cytoplasmic protein tyrosine kinases (JAKs) which either recruit members of the STAT family or activate the Ras-mitogen pathway [37].
Tumor-Necrosis-Factor-*a* (TNF-α), interleukin-1b (IL-1*b*) and IL-6 are the classical pro-inflammatory cytokines. Their ability to activate both local and systemic effects is well established [37]. Locally, they contribute to the activation of the inflammatory cells and together with chemokines, which induce the expression of adhesion molecules, cause their local recruitment. When the causes of the inflammatory reaction are of a high intensity, the production of cytokines is increased and they are released in the circulation provoking the "acute phase response". On the other hand, "inhibitory" cytokines such as IL-10 damp down the activation of some effector functions of T lymphocytes and mononuclear phagocytes, by inhibiting the release of pro-inflammatory cytokines and therefore turning off the inflammatory process [35] .
In fact, it was recently shown that IL-10, produced by T lymphocytes and macrophages can inhibit cytokine production in monocytes by blocking endotoxin-induced NF-κB activation, although the mechanism for this effect is unknown [38].
NF-κB is thought to play a pivotal role in immune and inflammatory responses through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines and growth factors [39]. NF-κB is usually kept inactive in the cytoplasm through association with endogenous inhibitor proteins of the IκB (inhibitor of NF-κB) family. In

response to pro-inflammatory stimuli, such as TNF-α, the signalling pathway converges on the activation of NF-κB [40,41]. The NF-κB induction regulates both pro- and anti-apoptotic pathways.
It was shown that NF-κB not only plays an essential role in the initiation of inflammation, allowing the production of inflammatory cytokines [37], but it is also involved in the resolution of inflammation, during which it induces the expression of anti-inflammatory genes and also induces apoptosis, thus turning off the inflammatory response [42].

MiR-146a AND INFLAMMATION

MiR-146 was first identified as an immune system modulator in a systematic effort to find miRNAs which influence the mammalian response to microbial infection [23]. Exposure of human monocytic THP-1 cells to various bacterial components, such as lipopolysaccharide (LPS) and peptidoglycan, or to pro-inflammatory cytokines results in rapid induction of the expression of mature miR-146 [23].
Moreover, in B cells with latent infection with Epstein-Barr virus, miR-146a expression increases as a result of signalling of the virus-encoded latent membrane protein 1 (LMP-1), which acts as a constitutively active homolog of the TNF-receptor [43].
MiR-146 family is composed by two members: miR-146a and miR-146b, located on separate chromosomes in the context of unrelated genes. These miRNAs differ in their mature sequence by just two nucleotides at the 3' end, suggesting that they could probably regulate the same target mRNAs. Transcription of both pri-miR-146a and pri-miR-146b is up-regulated upon LPS stimulation [23], but only mature miR-146a is detected, suggesting that this miRNA family has a rather sophisticated mode of regulation of expression, involving more than one point of control. Promoter analyses have shown that transcriptional up-regulation of miR-146a, induced by Toll-like receptor-4 (TLR-4) and LMP-1, is NF-κB dependent [23,43]. Although the physiological functions of the miR-146 family are not yet fully defined, some interesting targets have been validated and support a role for miR-146a in inflammation. In particular it was demonstrated that miR-146a is able to down-regulate the expression of IRAK1 and TRAF6 adaptor

molecules, whose involvement in TLR signalling in monocytes is well established [23].
Since both miR-146 targets (IRAK1 and TRAF6) act in the same linear signalling cascade, the cumulative effect of a drop in their protein abundance would probably have a considerable effect on TLR signalling and might be involved in regulation of the innate immune response. In addition, a microarray survey of miR-146a targets after its enforced over-expression in Akata B cell line has demonstrated miR-146a-mediated inhibition of a group of interferon - responsive genes [43]. Whether their regulation by miR-146a is direct or indirect remains to be determined.

Activation-Induced Cell Death (AICD) in T cells

T cell receptor is able to deliver multiple signals which lead to the induction of selective T cell functions by activating specific intracellular signaling pathways. In particular, TCR engagement in mature T lymphocytes triggers both proliferation and cell death. These two opposite outcomes are differentially regulated and both of them are fundamental for the maintenance of the homeostasis of the immune system.
Thymocytes which fail to rearrange their TCR gene die by neglect [43] while those that recognize self-antigens are eliminated by apoptosis in a process called "negative selection" [44].
In peripheral T cells, instead, the deletion of autoreactive T lymphocytes occurs by a form of apoptosis induced by repeated TCR stimulation [45]. Furthermore, during an immune response, the majority of T lymphocytes, which undergo clonal expansion and proliferation in response to antigens *in vivo,* subsequently die by apoptosis, in order to maintain T cell homeostasis and self-tolerance [45].
The physiological process of efficient removal of these activated lymphocytes is known as Activation-Induced Cell Death (AICD) [46]. It is now well established that AICD predominantly occurs by apoptosis, induced by repeated TCR stimulation[45,46].
Unless survival signals are received, AICD may be a default pathway in all activated T cells [45,47]. Indeed, T cell activation leads to the increased expression of Fas antigen (CD95) and Fas ligand (FasL)[48].

The Fas antigen is a death receptor and it belongs to the family of Tumor Necrosis Factor (TNF) receptor [49]. Activated T cells expressing both Fas and FasL are killed either by themselves ("suicide") or by interacting with each other ("fratricide") [50]. Although the Fas/FasL system is primarly used for AICD of $CD4^+$ cells, the TNF/TNF-R2 system seems to substantially contribute to the elimination of $CD8^+$ cells [51]. Moreover, AICD also involves perforin/granzyme B and it can occur in a caspase-independent manner [52].

The functional relevance of Fas and FasL factors in AICD is underlined by the evidence that mice having mutations in either Fas or FasL show an abnormal accumulation of T cells in the periphery at about 8 weeks of age [51]. Moreover, autoimmune diseases develop in mice and humans with inherited defects in Fas or FasL [49,53].

Engagement of Fas by FasL results in aggregation of the receptor and recruitment of the cytoplasmic adaptor protein FADD (Fas- associated death domain) [54].

The amino-terminal death effector domain (DED) of FADD interacts with homologous DED within the prodomain of the initiator caspase 8, acting as a platform for its recruitment [55].

Upon activation of the Fas receptor by FasL, FADD also recruits the initiator caspase-8 (also called FADD-like IL-1β- converting enzyme, FLICE)[55,56]. The Fas receptor, FADD and pro-caspase 8 form a functional Death-Inducing Signaling Complex (DISC).

The activated caspase 8 is then released into the cytosol where it triggers the activation of a caspase cascade which initiates apoptosis [55] (Fig. D).

In addition to TCR ligation, pharmacological drugs, such as phorbol esters (PMA) and Ca^{2+} ionophores (ionomycin), which mimic the two physiological signals (inositol 1,4,5-triphosphate and DAG, respectively) required for T cell activation, can also induce FasL expression and then AICD. For these reasons the use of these drugs represents a useful experimental approach to study the apoptotic process [57].

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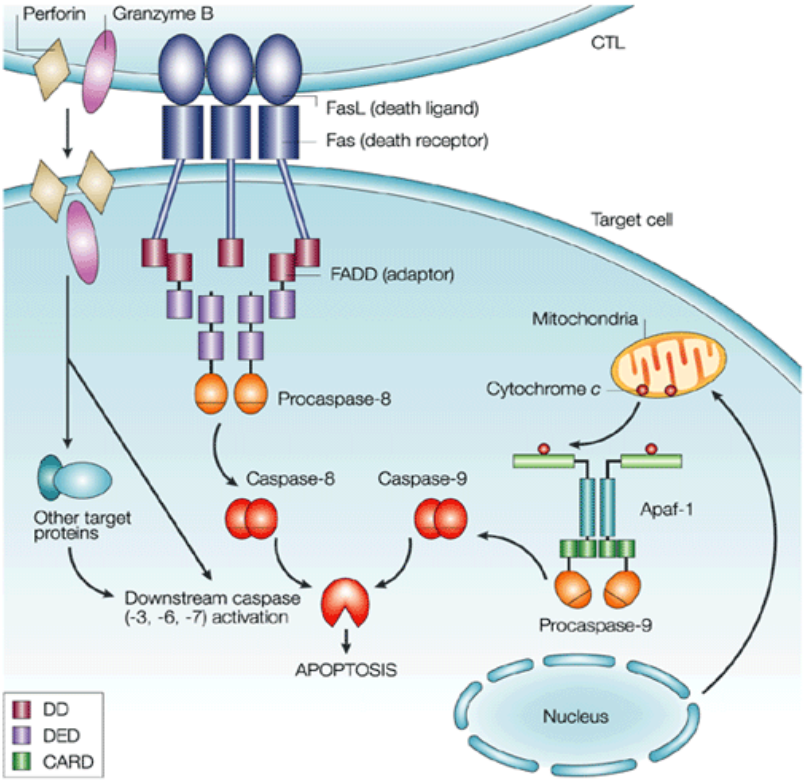


Nature 1:50-58 (2001)

Fig.D: Apoptosis cascade

FADD adaptor protein in Apoptosis and beyond

FADD (Fas Associated Death protein with Death Domain) is a key protein adaptor, transmitting the death signal mediated by death receptors. All the main death receptors, like TNF-R, Fas, TRAIL, require FADD adaptor for transmitting their apoptotic signal [57]. Consequently, FADD is a crucial protein which controls multiple essential processes, including cellular homeostasis and elimination of

pathological cells, particularly during the course of an immune response, both in mice and humans [57].

Human and mouse FADD proteins are very similar; they consist of 208 and 205 amino acids respectively and share 80% similarity and 68% identity [58].

The pivotal role, that FADD plays in the apoptotic signalling of death receptor, was highlighted by generating mice with T cells that have germ line deficiency in FADD or those carrying a dominant negative form of FADD (FADDdd): they are resistant to apoptosis [58].

Biological functions of FADD are regulated via cellular sub-localization, protein phosphorylation [59,60] and inhibitory molecules [56].

Control of FADD recruitment to the DISC can occur following several mechanisms, depending on the cell type and the death receptor [56]. The best characterized death receptor signaling inhibitor affecting FADD functions is the FLICE-inhibitory protein (c-FLIP) [56].

Inhibition of death receptors- induced apoptosis by endogenous c-FLIP results from binding of c-FLIP to the death-effector-domain (DED) of FADD, thus hindering pro-caspase 8 activation.

Therefore, the equilibrium between FADD and the expression of its inhibitors determines the outcome of the death receptor-stimulated cells: apoptosis or survival [61] (Fig.D).

Besides its role in transmitting the apoptotic signal into the cell, FADD have been shown to be a multifunctional protein, implicated in survival/ proliferation and cell cycle progression [62]

FADD-deficient mice are embryonic lethal with heart developmental problem and die around day 10 of gestation. Chimeric mice generated by injecting *FADD-/-* embryonic stem cells into the *RAG-1-/-* blastocysts do not develop any lymphoproliferative diseases. Analysis of these mice showed T cell development abnormalities and mature T lymphocytes lack the full potential to proliferate in response to TCR signals [63] . More recently, analysis of T-cell specific FADD deficient mice showed an inhibition of T cell development at the proliferative step between DN and DP transition [64]. These observations suggest that FADD protein plays a role in mediating not only cell death signalling but T cell proliferation as well.

It was demonstrated that FADD $^{-/-}$ peripheral T cells are cycling but are defective in their co-stimulatory response when stimulated. Analysis of several cell cycle proteins showed normal down-regulation of p27 inhibitor, but increased level of p21, decreased levels of cyclin D2 and

constitutive activation of several cyclin-dependent kinases in activated T cells [62]. However, further studies are necessary to elucidate the molecular mechanisms by which FADD targets the cell machinery
It was supposed that the reduced proliferation resulted from the lack of IL-2 production or unresponsiveness to IL-2, but enzyme-linked immuno-absorbent assay (ELISA) did not show any alteration in IL-2 levels[58]. Several characteristics of FADD-deficient chimeras are strikingly similar to those observed in IL-2Rβ-deficient mice, including the defect of T cell proliferation in response to IL-2, and a higher percentage of CD 69^+ T cells [58,62].
All these observations suggest a role for FADD in cytokine signaling and an unexpected link between cell proliferation and apoptosis.

APOPTOSIS - REGULATING miRNAs: THE APOPTOMIRs

Since the current knowledge on miRNAs world suggests that these small RNAs may modulate several biological processes, from differentiation to proliferation, it is not surprising that they also play an important role in regulating key steps of the apoptotic pathways by affecting the expression of apoptosis-regulating genes. Thanks to the growing knowledge on this field, it is now quite clear that an increase number of miRNAs play a role in apoptosis.
We refer to this class of Apoptosis-regulating miRNAs as "**apoptomiRs**".
The first miRNA described as an apoptotic regulator was the *Drosophila* gene *bantam* [65]. It directly down-regulates the pro-apoptotic factor *hid*, suppressing the apoptotic activity. Furthermore, another miRNA, miR-14 was found to act as an anti-apoptotic factor by suppressing the death activator *reaper* during the eye formation[66].
Many efforts have been directed to identify miRNAs relevant for proliferation or cell death; for this purpose *Cheng et al.* individually knocked down 90 human miRs in HeLa and A459 cells [67]. Among those miRNAs identified as regulators of apoptosis there was miR-21. This miRNA has been shown to down-regulate PTEN as well as PDCD4 and the pro-apoptotic tumor-suppressor p53 in cancer cells[68].

In a groundbreaking work it was shown an inverse correlation between miR-15a/miR16-1 expression and BCL-2 levels in CLL cells, providing the first evidence of direct miRNA modulation of apoptotic machinery in human tissue [12]. Another microRNA which regulates BCL2 family members is miR-29a,b and c, which target MCL-1[69]. These few examples suggest that tight regulation of anti-apoptotic function through miRNA is critical in development and other cellular processes and the breaking of such equilibrium is a key event for the development of malignancies.

Therefore, a single miRNA is able to provide a significant survival advantage to cancer cells upon its deregulation, and this may be a fundamental mechanism, adopted by cancer cells to elude apoptotic cell death. On the other side, the alterations in the apoptotic pathways are detrimental also in non cancerous cells, because of the disrupted homeostasis inside a particular cellular compartment; for example in immune cells the altered equilibrium between life and death may lead to the development of autoimmune disorders.

MATERIALS AND METHODS

Reagents. PD98059, SP600125 and Cyclosporine inhibitors were purchased from Sigma Aldrich. For the isolation of CD4+ and CD8+ primary T lymphocytes: CD45RO, CD45RA or CD27 MicroBeads, and MACS® Columns from Miltenyi Biotech (Bergisch Gladbach, Germany) Miltenyi MACS Purification kit was used. PE-conjugated α-CD4, PE-cyanine (Cy) 7-conjugated anti-CCR7, FITC-conjugated α-CD27, PE-conjugated α-CD127, allophycocyanin (APC)-conjugated α-CD45RA, FITC-conjugated α-CD69, and control isotype-matched antibodies were purchased from from BD Pharmigen (San Diego, CA). FITC-conjugated anti-CD45RO from Caltag Laboratories (Burlingame, CA). Dynabeads CD3/CD28 T Cell Expander (αCD3/CD28) from Dynal Biotech (ASA, Oslo, Norway). PMA and ionomycin from Sigma-Aldrich (Milan, Italy). TNF-α and IFN-α from Sigma-Aldrich (Milan, Italy). Recombinant IL-2 from Chiron (Amsterdam, Netherlands). MiR-146a over-expression was obtained by using Mimic-146a (Dharmacon) and Mimic scramble was used as control (Dharmacon).
Ann-V was purchased from BD Biosciences and PI from Sigma Aldrich.

Cell cultures. The human leukemic T cell line, Jurkat (DSMZ) was used. Cells were grown in RPMI 1640 (Gibco) supplemented with 10% heat inactivated Foetal Bovine Serum (Sigma-Aldrich), Penicillin/ Streptomycin (Gibco) and L-glut (Gibco) at 37°C in 5% CO_2.
293T cells used for miR-146a targets identification were grown in D-MEM (Gibco) supplemented with 10% Foetal Bovine Serum, Penicillin / Streptomycin, and L-glut.

Reverse transcription of mature miRNAs. qRT-PCR amplification of miRNAs was performed as described [70] with minor modifications: we used a different adapter sequence for each miRNA instead of the same sequence for all miRNAs. This modification allowed us to perform reverse transcription of all miRNAs in a given sample at the same time. Primers used are listed in Supplementary Table 1. 400ng of total RNA were denaturated for 3 minutes at 95º C and reverse transcribed with

the Superscript III RNAse H- Reverse Transcriptase (Invitrogen) for 30 minutes at 50º C. 10µl reaction contained each RT primer at a final concentration of 0.1µM. Superscript enzyme was inactivated by incubation at 95º C for 5 minutes. Each reverse transcription primer consists of two different regions: the 3'region is 7 to 12nt long and is complementary to the 5'end of miRNA; the 5'region (herein referred to as adapter) is a 20 to 22nt sequence not represented in the mammalian genomes.

qRT-PCR of mature miRNAs. RT reaction was brought to 800µl and 10µl were used for each qPCR reaction. qPCR was performed with iCycler (BioRad) using iQ-SYBR-green supermix (BioRad) according to the manufacturers' instructions.
Forward primers for PCR amplification are 14 to 17nt long LNA modified DNA oligonucleotides [70]. Three to five non consecutive nucleotides were LNA modified in the 5'end of each oligonucleotide. Melting temperature of LNA primers was checked at http://lna-tm.com/. Reverse primers for PCR amplification are the reverse complementary sequence of adapters. PCR amplifications were performed in triplicates with a relative error of about 30%.
Mature miRNAs were quantified with the standard curve method. Standard curves were obtained by qPCR amplification of serial dilutions of synthetic DNA templates. U6 small nuclear RNA was measured with the same method and used for normalization. Blank and RT minus controls were included. All oligonucleotides were purchased from Sigma-Aldrich (St. Louis, MO).

Lentiviral transduction. Viral particles were obtained by co-transfection of 293T cells with the pRRL-miR-146a construct (see DNA plasmid constructs) and the PLP-1, PLP-2 and PLP-VSVG plasmids. Medium was replaced 6 hrs after transfection, and viral particles were harvested 48 hrs after transfection. Jurkat cells were transduced overnight at a MOI of 15. Thereafter, cells were selected with 2µg /ml puromycin.

RNA isolation. RNA used for mRNA and miRNA quantification analyses were obtained by a modified TRIzol method. Briefly, cell were suspended in TRIzol reagent (Invitrogen) and processed

according to manufacturer's protocol up through the addition of isopropanol. Samples were allowed to precipitate in isopropanol overnight at -20°C and then centrifuged at 12000x g for 15 min at 4°C. Isopropanol was decanted, samples were washed with ethanol 70% and then resuspended in nuclease- free water.

Northern Blot. 30μg of total RNA were separated by electrophoresis in a 15% agarose gel and transferred to Gene Screen Plus nylon membrane, After 3 min of UV- cross linking, membranes were prehybridized for 2h at 50°C in the hybridization buffer (50% formamide, 6X SSC, 10X Denhart's solution, 10mM EDTA, 0,1% SDS and 100 μg/ml single-stranded salmon sperm DNA). Hybridization was carried out overnight at 50°C with miR-146a probe (3x 10^6 cpm/ml).The probe was labeled with [γ-^{32}P] deoxyoligonucleotide antisense to miR-146a, using T4 PNK system (NEB Biolabs). Then the membranes were washed 3 times with 2X SSC and 0,1X SDS for 20 min and analyzed using IMAGEJ software for quantitative analyses and auto-radiographed on X-ray films (Kodak) using an intensifying screen at -80°C. After autoradiography the blot was reprobed with a probe for U6 as a loading control.

Treatment with pharmacological inhibitors. Jurkat cells were pre-incubated with CsA or PD98059 or SP600125 for 30 min before stimulation with αCD3-αCD28 antibodies. Various concentrations of inhibitors were used, as indicated in Fig.4a,b,c. Cells were harvested for RNA extraction 24h after stimulation.

DNA plasmid constructs. miR-146a lentiviral construct was obtained as follows: a region of about 300 nt containing miR-146a was amplified from Jurkat cells genomic DNA and cloned into the BamHI Xho sites of a pcDNA 4 TO vector (Invitrogen). The MluI-XhoI fragment was PCR amplified and cloned into pSuperior-Puro (Oligoengine, Seattle) into the BstBI XhoI sites. The XmaI-XhoI fragment was finally cloned into the AgeI-XhoI sites in pRRLSIN.cPPT.PGK-GFP.WPRE (Addgene, plasmid #12252, Cambridge MA, USA) thus obtaining pRRLSIN.cPPT.PURO.CMV-TO-miR-146a.WPRE, herein referred to as pRRL-146a.

Cloning of miR-146a promoter reporter constructs. The pmLuc-1 AccepTor plasmid (Novagen) was used for generation of promoter reporter constructs. Briefly, the relevant regions were amplified by PCR from genomic DNA, using primers listed in supplementary table 1. PCR products were ligated into the pMLuc-1 AccepTor plasmid according to manufacturer instructions. Deletions were introduced using the site QuickChange II Site Directed Mutagenesis kit (Stratagene). Primers used for mutagenesis are listed in supplementary table1. The introduction of mutations was confirmed by DNA sequencing.

Cloning of putative mir-146a targets UTR. A region encompassing about 300nt upstream and downstream of putative micro-RNA seed was amplified by PCR on genomic DNA with the following PCR protocol: 5x GoTaq Flexi buffer (Promega) 10μl MgCl (Promega) 3μl, 10mM dNTPs (Promega) 1μl GoTaq (Promega) 0,25μl 10 μM Reverse and Forward oligos 1,25μl, genomic DNA 0,2μg and H_2O to 50μl. The PCR program was as follows: 30 cycles (95°C for 30 sec, 58°C for 45sec, 72°C for 60sec). PCR products were run on a 1% agarose gel to confirm amplified fragment size and then were purified on a Nucleospin column (Macheray-Nagel), digested with Xba I (New England Biolabs), purified on a G50 sephadex column (GE Healthcare, Amersham) and cloned into the Xba I site of pGL-3 plasmid (Promega). Positive clones were screened by PCR (10μl, same protocol as above) using the seqluc primer on the plasmid) and the UTR specific reverse primer. Positive clones were further confirmed by enzymatic digestion. Midi-preps of the plasmids were prepared with the Endofree Midi kit from Qiagen.

Transient transfection and Dual-luciferase reporter assay

a) 146a promoter constructs. Lipid-mediated transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. Briefly, Jurkat cells (5 x10^5 cells/ ml) were plated in 6 well culture plates and transfected with 2μg pmLuc-1 AccepTor plasmid (Novagen) containing the promoter fragment and 2μg of pGL3TK for normalization of transfection efficiency. After 24h recovery period, transfected cells were either left untreated or induced with TNF-α (40ng/ml; Roche Diagnostics) or αCD3 (5μg/ml; clone

OKT-3) and αCD28 (5μg/ml) or PMA (10ng/ml) and ionomycin (1μg/ml). The luciferase activity was assayed using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer instructions.

b) 3'UTR of Target genes. 24 h before transfection 293T (1 x 10^5 cells/ 500μl) were plated in 24-well culture plates. The cells were then transfected with a pGL-3 vector containing the 3' UTR of interest (0,26μg), a miR-146a mimic or a mimic scramble as a control and with a pRLTK vector (1,3ng) for normalization of transfection efficiency (Promega). The constructs were diluted in 50μl of OPTIMEM (Invitrogen) and added to 50μl of OPTI-MEM containing 2μl of Lipofectamine 2000 (Invitrogen) incubated 5 min at room temperature (RT). The mix was incubated at RT for 20 min and then added to the cells. Cells were collected and lysed with 100μl of Passive Lysis Buffer (Promega). The luciferase activity was assayed as described above

Jurkat cells kinetics of Activation. 12 well-culture plate were coated over night at 4°C with a polyclonal rabbit anti-mouse immunoglobulins (Dako), 300μl in each well at the concentration of 10μg/ml. Wells were washed three times with cooled PBS and an equal concentration of αCD3 (5μg/ml) and αCD28 (5μg/ml) were added. The plates were incubated for 2-4h at 37°C or overnight at 4°C; afterward the wells were washed three times with the medium (RPMI1640, Gibco-Invitrogen). Jurkats (5 x 10^5 cells) were added to the wells and incubated for the indicated times.

Bioinformatic analysis of hsa pri-miRNA-146a promoter
Bioinformatic analysis of the miR-146a 1100bp promoter region was performed using the MULAN software, available on line (www.mulan.dcode.org)

CD69 staining. Jurkat and Jurkat pRRL-146a cells (5x 10^5 cells/ml) were washed in PBS, centrifuged at 1100 rpm for 10 min and resuspended in 50μl of PBS with 1μl of αCD69 FITC. The samples were incubated for 15 min at 4°C in the darkness and then were washed

twice with PBS. Cells were resuspended in 500µl of PBS and analyzed by FACS-Calibur (Becton Dickinson)

T cell stimulation and cell death analysis. For the induction of AICD, Jurkat T cells ($5x10^5$ cells/ml), transfected with mimic-146a or mimic scramble, were stimulated with PMA (100ng/ml) and ionomycin (1µg/ml) for 72h. Cells activation was measured by CD69 staining 4h after PMA and ionomycin induction. Apoptotic cell fractions were determined by annexin V-FITC and PI double staining according to the manufacturer's instruction and analyzed with FACS-Calibur (Becton Dickinson). Unstained cells served as negative control. Data were analyzed using CellQuest software (Becton Dickinson)

NF-κB reporter assay. Jurkat cells (4×10^5 cells/ ml) were co-transfected by Lipofectamine 2000 according to manufacturer's protocol, with 3µg of NF-κB reporter construct (Promega) and with 3µg of the pGL3TK plasmid. 24h later cells were split in two wells of a 24-wells plate; one well was induced with αCD3-αCD28 antibodies. After 24h from activation they were analyzed by luciferase assay.

AP-1 reporter luciferase assay. Jurkat pRRL-empty and Jurkat pRRL-146a cells (4×10^5 cells/ ml) were co-transfected with 3µg of AP-1 reporter construct (a nicely gift from Luca Simeoni) and with 1µg of the pRLTK plasmid, using Lipofectamine 2000, according to manufacturer's instructions. 6h after transfection, cells were stimulated with αCD3-αCD28 antibodies. After 24h activation cells were analyzed by luciferase assay.

RESULTS

MiR-146a is up-regulated in activated primary T cells

In order to identify those miRNAs whose expression is triggered by T cell activation, we studied the expression profile of miRNAs in primary T cells. Using a DNA microarray service provided by LC sciences, we analyzed distinct subclasses of sorted human primary T cell: naive T cells, $CD4^+$ memory effectors and $CD4^+$ central memory, $CD8^+$ effectors and $CD8^+$ memory effectors (data not shown).
Array analysis allowed us to focus our attention on a small subset of miRNAs which were differentially expressed in the cellular subtypes examined. Among the miRNAs which were up-regulated by different activation stimuli in primary T cells, there was miR-146a and miR-146b. The data obtained were validated by Real-Time RT-PCR (qRT-PCR), using primers which recognized the mature forms of selected miRNAs (Fig.1).
MiR-146 is a family of miRNAs composed by miR-146a and miR-146b, which differ from each other in their mature sequence only by two nucleotides at the 3'end; they are also encoded by two different genes on distinct chromosomes.

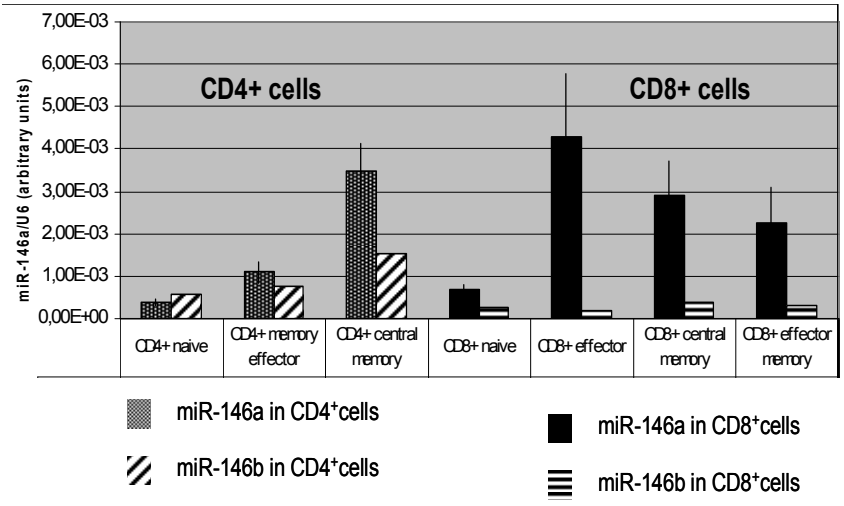


Fig.1. MiR-146a is up-regulated in differentiated primary T cells: miR-146a and miR-146b were measured in primary CD4+ and CD8+ cells by Real-Time qRT-PCR

We found that in $CD4^+$ memory effectors and $CD4^+$ central memory miR-146a was up-regulated ofabout 3-9 fold compared to $CD4^+$ naive cells (Fig.1). Similar higher levels of miR-146a were observed in $CD8^+$ memory effectors, $CD8^+$ central memory and $CD8^+$ effectors (Fig.1) compared to CD8+ naïve cells.
On the contrary, no differences of expression were observed for miR-146b neither in $CD8^+$ cells, nor in $CD4^+$ memory effector compared with naive cells; the up-regulation of miR-146b was found only in $CD4^+$ central memory cells (Fig.1).

MiR-146a is differentially up-regulated in various stimulation conditions in Jurkat T cells

We next moved towards an *in vitro* model to focus our attention on T lymphocytes activation and for this purpose we chose Jurkat T cells as experimental system.
In order to verify whether activation of T-lymphocytes results into induction of miR-146a, we measured miRNAs expression by microarray, validating it by Real-Time qRT-PCR.
As expected, miR-146a and miR-146b levels were up-regulated in Jurkat cells by either PMA+ ionomycin or αCD3- αCD28 antibodies.

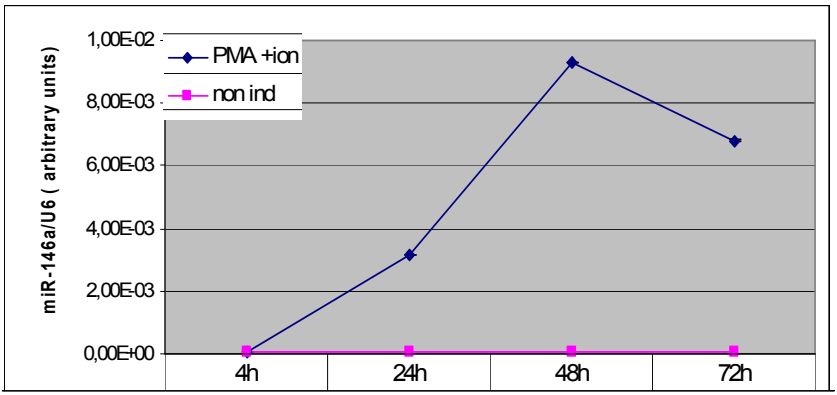


Fig.2a. **Mir-146a expression markedly increases after TCR stimulation:** Jurkat cells were stimulated with PMA (10ng/ml) and ionomycin (1μg/ml) and miR-146a levels were measured at different times by Real-Time qRT-PCR).

The fold increase of this up-regulation was quite different between the two microRNAs; in fact, miR-146a up-regulation was higher, reaching 300 fold increase upon PMA and ionomycin stimulation (Fig.2a), whereas miR-146b was up-regulated of about 3 fold (data not shown).
The increase of miR-146a expression was confirmed by Northern blot analysis (Supplementary Fig.1).
The kinetic of miR-146a expression showed that miR-146a levels increase 24h after PMA and ionomycin treatment in Jurkat T cells reaching a plateau 48h after stimulation (Fig.2a.).
A similar induction was highlighted by experiments where αCD3 and αCD28 antibodies were used to resemble a more physiological stimulation of TCR (Fig.2b); in this case however, miR-146a induction was less pronounced at 24h, while it was comparable to that observed after PMA and ionomycin stimulation at 72h.
Since miR-146b expression was not induced in Jurkat cells stimulated with αCD3 and αCD28 antibodies (data not shown) we focused our analysis on miR-146a.
In contrast to what observed in Jurkat cells, miR-146a levels in 293T cells did not significantly change after stimulation with phorbol esters (PMA) (Fig.2c), thus suggesting that the need of an increase in miR-146a levels following this treatment should require transcription factors typically expressed in T lymphocytes.

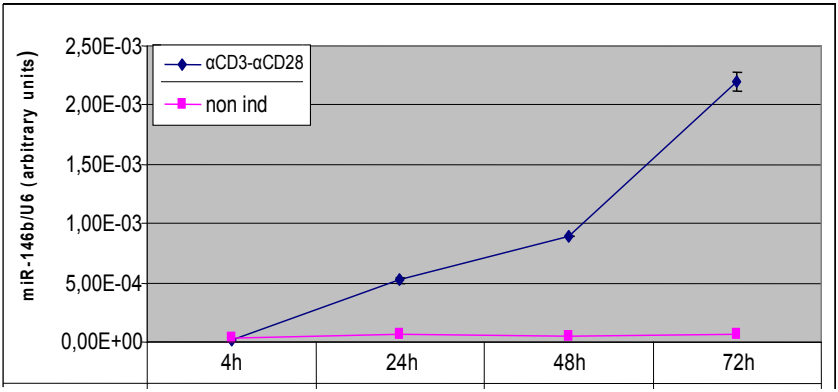


Fig.2b. MiR-146a expression markedly increases after TCR activating stimuli: Jurkat cells were stimulated with αCD3 (5μg/ml) and αCD28 (5μg/ml) and miR-146a levels were measured at different times by Real-Time qRT-PCR.

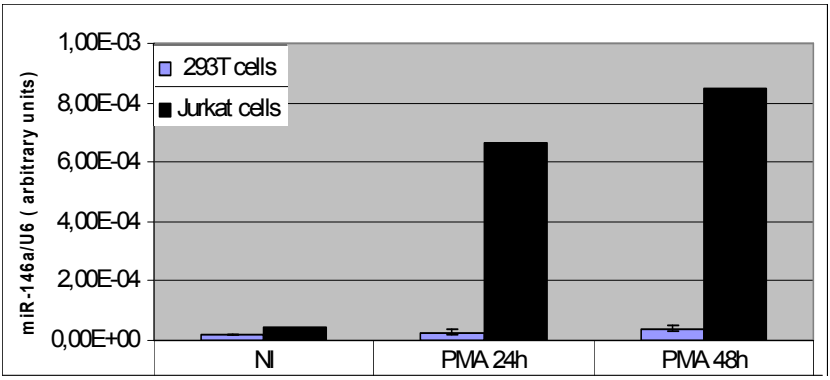


Fig.2c. PMA- mediated induction of miR-146a is specific for T cells: Jurkat cells and 293T cells were stimulated with PMA (10ng/ml) and miR-146a levels were measured at different times by Real-Time qRT-PCR

MiR-146a is expressed in response to TNF-α inflammatory stimulus, but not upon IFN-α stimulation

We tested miR-146a responsiveness to other stimuli, different from those which induce T cell activation.

MiR-146a responsiveness to various pro-inflammatory stimuli has been demonstrated in monocytes [23]. Prompted by these findings, we checked miR-146a expression in Jurkat cells stimulated with TNF-α, one of the early major cellular mediators in inflammatory response [71,72].

Upon binding of TNF-α to TNFR-1 expressed on T cells several pathways are triggered; among them apoptosis and NF-κB- mediated inflammation.

TNF-α induced a significant increase of miR-146a levels (Fig.3a), with a kinetic of activation in which an up-regulation of about 3 fold was observed 24h after induction. MiR-146a level of expression further increased 48h and 72h after induction, reaching ten fold higher levels compared to non-treated cells (Fig.3a).

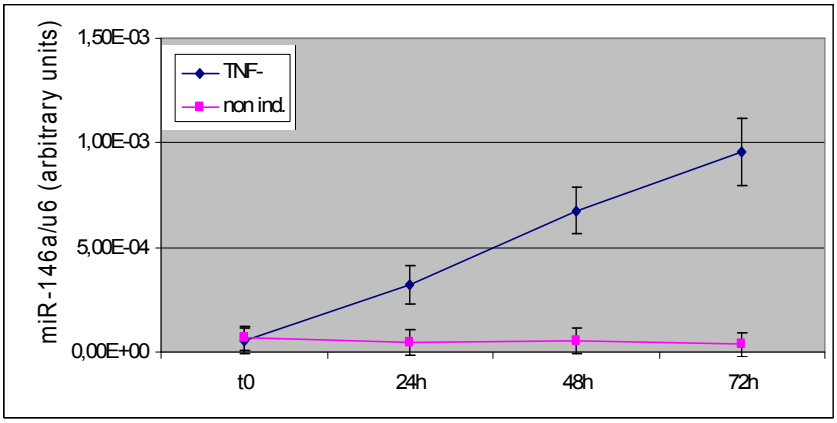


Fig.3a. MiR-146a expression in TNF-α induced Jurkat T cells Jurkat cells ($5x10^5$ cells/ml) were stimulated with TNF-α (40ng/ml) and miR-146a levels were measured at different times by Real-Time qRT-PCR

This experimental evidence suggested that miR-146a could be involved in the inflammatory response in T lymphocytes as was already shown in monocytes and B lymphocytes [23,43].

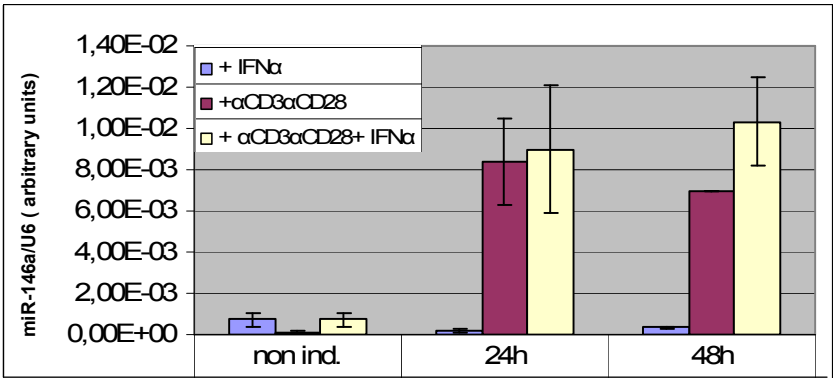


Fig.3b. MiR-146a is not induced by IFN-α: Jurkat cells ($5x10^5$ cells/ml) were stimulated with IFN-α alone, with αCD3 (5μg/ml) and αCD28 antibodies (5μg/ml) and with a double stimulus IFN-α (20ng/ml) + αCD3 (5μg/ml) and αCD28 antibodies (5μg/ml). MiR-146a levels were measured at different times by Real-Time qRT-PCR

Another stimulation condition tested in our experiments was IFN-α treatment with and without TCR concomitant stimulation, in order to assess if miR-146a in T cells was also induced by IFN-α downstream effectors, namely STAT proteins.
Our results show that, in Jurkat cells IFN-α does not induce miR-146a expression and has no effect on miR-146a up-regulation mediated by TCR (Fig. 3b). Altogether, our observations suggested that NF-κB and AP-1, transcription factors activated by TNF-α signalling, could be modulators of miR-146a expression in T lymphocytes in response to inflammation.

Pharmacological approaches indicate TCR signalling pathways affecting miR-146a expression

We have shown that miR-146a is up-regulated in Jurkat cells in various stimulation condition involving TCR signalling and TNF-α mediated induction.
It has been reported that multiple signalling pathways downstream of TCR activation contribute to the induction of early activation genes (e.g. IL-2) and therefore we employed various pharmacological inhibitors to investigate the contribution of some of these pathways to miR146a expression following TCR activation.
For this purpose, we used three different inhibitors, known to affect partially overlapping pathways triggered upon T cell activation. In particular we impaired NFAT, NF-κB, JNK and ERK pathways using different drugs.
To ensure that in Jurkat T cells, as in primary T lymphocytes, SP600125 behaved as a JNK inhibitor [73], we examined its effect on IL-2 transcription; in fact it has been demonstrated that this drug is able to strongly reduce the levels of its transcript [73].
As expected, , IL-2 levels dampened after treatment with SP600125 (data not shown), while miR-146a levels of expression showed only a subtle reduction 24h after TCR stimulation, using various concentration of inhibitor (Fig.4a). So this drug did not have a significative effect on αCD3-αCD28-mediated stimulation of miR-146a expression, thus demonstrating that AP-1 is not involved in miR-146a modulation.

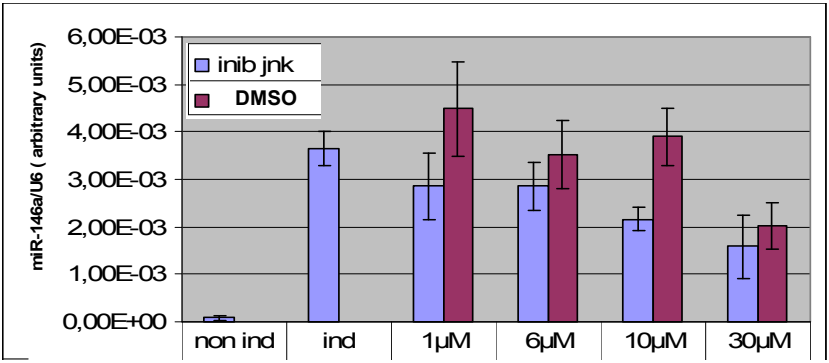


Fig.4a. MiR-146a expression is partially affected by JNK inhibitor: Jurkat cells treated with JNK inhibitor for 30 min were induced for 24h with αCD3 (5µg/ml) and αCD28 (5µg/ml) antibodies. MiR-146a levels were measured by Real-Time qRT-PCR

A similar approach was also used to test PD98059, which specifically inhibits MEK1 [73] and thus the ERK pathway (Fig.4b).
As reported in other papers [73] this drug only attenuates IL-2 expression resulting in a 40% inhibition of its mRNA levels (data not shown).
A similar effect was also observed on miR-146a levels in the presence of different concentrations of inhibitor, showing that PD98059 partially affected miR-146a expression in the presence of CsA 20µM (Fig. 4b).

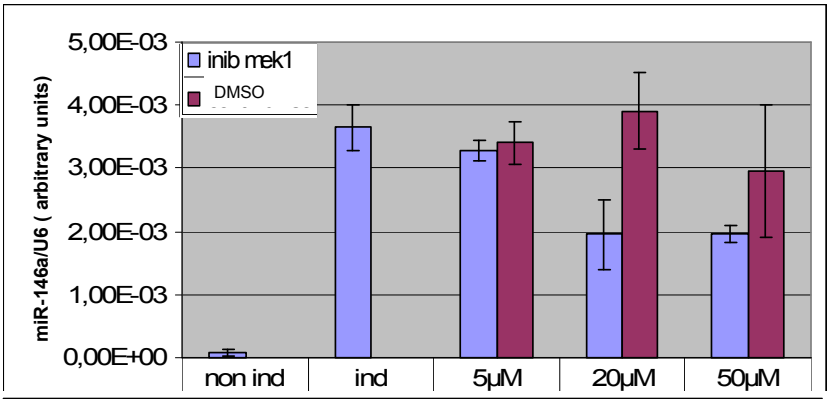


Fig.4b. MiR-146a expression is partially affected by MEK1 inhibitor: Jurkat cells treated with MEK1 inhibitor for 30 min were induced for 24h with αCD3 (5µg/ml) and αCD28 (5µg/ml) antibodies. MiR-146a levels were measured by Real-Time qRT-PCR

Finally, a third pharmacological inhibitor used in our experiments was cyclosporine (CsA), a specific Calcineurin inhibitor, able to suppress the production of cytokines during T cell activation. In particular CsA directly inhibits Calcineurin-mediated activation of NFAT and appears to have some effects on AP-1 and NF-kB activity [73] (supplementary Fig.2).
As expected treatment with CsA caused a strong reduction of IL-2 levels (data not shown); we observed also a substantial reduction of miR-146a expression (Fig 4c). This finding suggests that at least one of the three transcription factors, downstream of Calcineurin (NFAT, AP-1 and NF- kB) could be involved in modulating miR-146a expression.

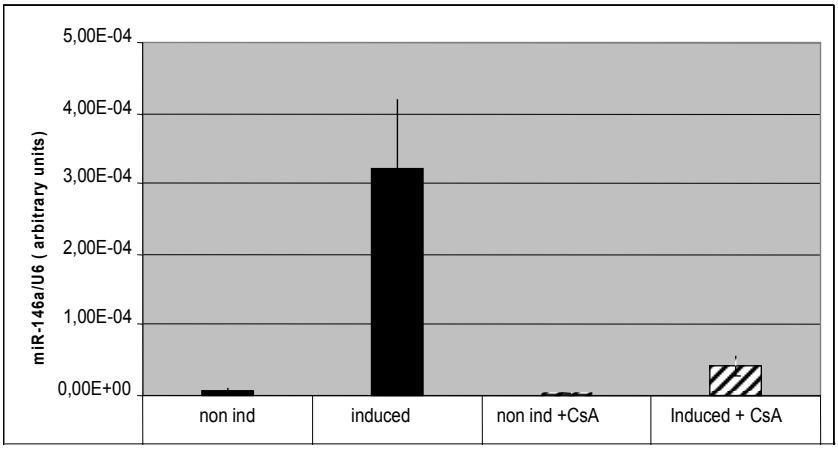


Fig.4c. Cyclosporine inhibits miR-146a expression:: Jurkat cells ($5x10^5$ cells/ml) treated with CsA inhibitor (0,5μg/ml) for 30 min were induced for 24h with αCD3 (5μg/ml) and αCD28 (5μg/ml) antibodies. MiR-146a levels were measured by Real-Time qRT-PCR

Analysis of miR-146a promoter region upon TCR stimulation and TNF-α treatment by a luciferase assay

The experiments illustrated above implicated a transcriptional regulation of miR-146a expression following an activating stimulus mimicking the TCR-engagement by antigen or in response to TNF-α inflammatory stimulus.
To further address this issue, we extended our study to identify the promoter elements which mediated the different responsiveness to these two stimuli. We cloned a 1100bp promoter region (Fig 5), mapping upstream of the pri-miRNA coding region. This 1100bp construct was tested in transient transfection assays in Jurkat cells stimulated either with αCD3-αCD28 antibodies (or PMA and ionomycin) or with TNF-α. As illustrated in Fig. 5, the region of 1100bp up-stream of miR-146a is able to drive expression of a reporter gene, in response to the same stimuli leading to miR-146a expression (Fig.2a, 2b and Fig.3a)

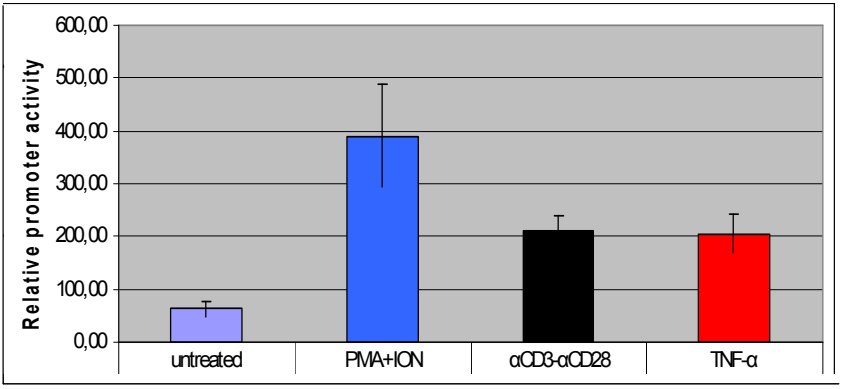


Fig.5. Effects of PMA+ionomycin, αCD3-αCD28 antibodies and TNF-α on miR-146a promoter reporter: Luciferase assay on miR-146a 1100bp promoter fragment in transfected Jurkat cells, stimulated for 24h with PMA (10ng/ml) and ionomycin (1μg/ml), αCD3 (5μg/ml) and αCD28 (5μg/ml) or TNF-α (40ng/ml). Relative promoter activity was measured by renilla/firefly Dual Luciferase assay.

We then performed a bioinformatic analysis of the promoter sequence to identify candidates for the transcriptional regulation of miR-146a.

The results of these analyses revealed the existence of putative conserved binding sites of interest; among them there were the two NF-κB sites previously described by Taganov et al. in monocytes [23].
Because of this finding we asked whether the down-modulation of miR-146a observed in the presence of CsA (Fig.4c) could depend on impairment of NF-κB activity, subsequent to Calcineurin inhibition. (supplementary Fig.2). Therefore, we directly tested CsA inhibition on the activity of miR-146a promoter fragment.
As shown in Fig. 6 CsA affected luciferase expression of miR-146a promoter construct determining a two fold decrease of luciferase levels.

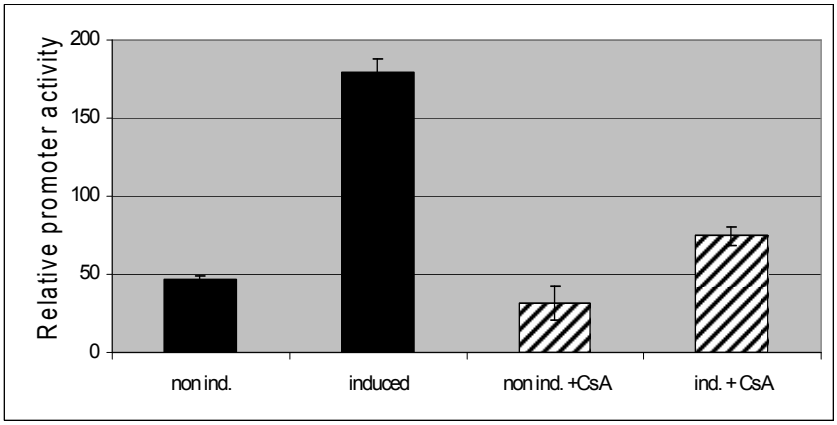


Fig.6. **Cyclosporine down-regulates the 1100bp promoter activity**: luciferase assay in Jurkat cells ($5x10^5$ cells/ml) treated with 0,5μg/μl CsA inhibitor; 24h after stimulation (fold induction of αCD3-αCD28 stimulation compared with non induced constructs). Relative promoter activity was measured by renilla/firefly Dual Luciferase assay

Site-directed mutagenesis demonstrates that TCR and TNFα stimulation modulate miR-146a expression through partially overlapping mechanisms

To further confirm the data obtained with the pharmacological approach and to finely map the *cis* elements required to drive the expression of miR-146a, we performed a site directed mutation of the relevant consensus sequences within the miR-146a promoter reporter construct. In addition to NF-κB, we identified conserved putative binding sites, within miR-146a promoter, for some interesting transcriptional factors which are known to play a role in T cell activation. Among them c-ETS and USF2 binding sites were studied. To investigate the contribution of these different binding sites to miR-146a promoter activity a mutagenesis analysis was performed (Fig.7a).

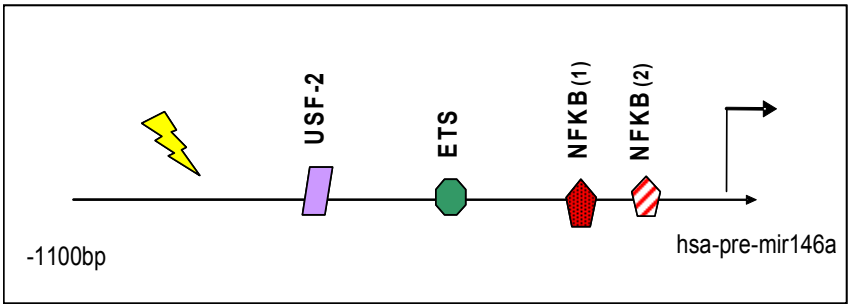


Fig 7a. Promoter region of 1100bp isolated upstream pre-miRNA coding sequence: Binding sites mutagenized are here illustrated

Mutagenized constructs were transiently transfected into Jurkat T cells, together with a control vector and the results of the luciferase activity was analyzed. As shown in Fig.7b, the mutation of either NF-$\kappa B_{(1)}$ and ETS sites almost completely abolished miR-146a promoter activity upon TCR stimulation.

By contrast, the mutation of NF-κB$_{(2)}$ site had a lower effect on miR-146a promoter activity. Finally, USF2 site mutation did not affect miR-146a promoter activity.

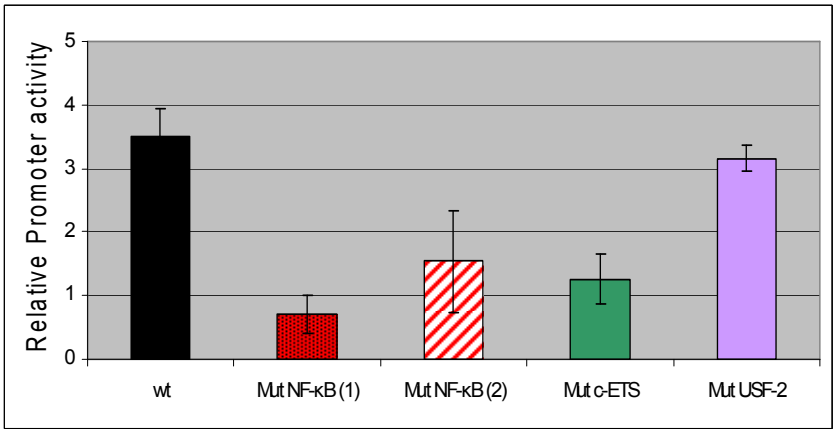


Fig. 7b. ETS and NF-κB modulate miR-146a expression: Jurkat cells were transfected for 48h with the wildtype (wt) or mutagenized (mut) promoter construct and with pGL3TK used for normalization of transfection. Cells were stimulated with αCD3 (5μg/ml) and αCD28 (5μg/ml) antibodies for 24h. Fold induction of αCD3 and αCD28 stimulation were here illustrated compared with non induced reporter constructs. Relative promoter activity was measured by renilla/firefly Dual Luciferase assay. (p-value<0,05).

Since TNF-α induced miR-146a expression in Jurkat cells (Fig.3a) we used miR-146a promoter construct to test its activity in the presence of an inflammatory stimulus. We found that, according to the results described above, TNF-α induced miR-146a promoter activity (Fig.7c).
Mutagenesis analysis showed that the disruption of NF-κB$_{(1)}$ site was *per se* sufficient to determine a complete loss of responsiveness to TNF-α stimulation. NF-κB$_{(2)}$ site, instead, had a subsidiary role, inducing only a partial reduction of luciferase activity (Fig.7c).
In contrast to the effects of mutation at the c-ETS site in Jurkat cells stimulated with αCD3-αCD28 antibodies (Fig. 7b) or with PMA and ionomycin (data not shown), TNF-α stimulation of the promoter

reporter was not affected by the ETS mutation (Fig.7c), indicating that the ETS site did not play a role in TNF-α response.

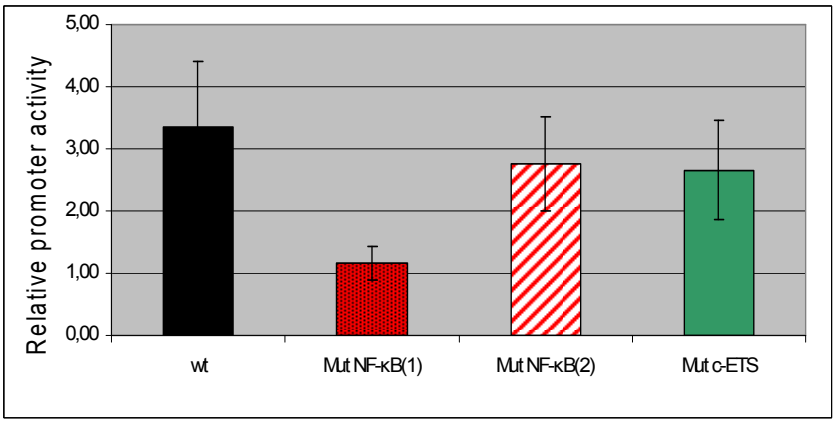


Fig. 7c. NF-κB is the main transcriptional factor modulating miR-146a expression in an inflammatory condition: Jurkat cells were transfected with the wildtype (wt) or mutagenized promoter construct (Mut) and with pGL3TK used for normalization of transfection. Cells were stimulated with αCD3(5μg/ml) and αCD28 (5μg/ml) antibodies for 24h. Fold induction of αCD3-αCD28 stimulation were here illustrated compared with non induced reporter constructs. Relative promoter activity was assayed using a renilla/firefly Dual-Luciferase Assay System. (p value< 0,05).

MAD-4, FADD and c-FOS are directly targeted by miR-146a

To examine the functional relevance of miR-146a up-regulation in T cells as part of the TCR-mediated activation process, we set out to identify miR-146a-regulated genes. We chose a bioinformatics approach, using prediction algorithms for the identification of microRNA targets. We referred to prediction methods such as PicTar[74,75] and TargetScan[76,77], both relying heavily on evolutionary conservation of the target seed site in mammals. Although conservation is a powerful way to improve the sensitivity over background, it is clearly not useful for the potentially large class of species-specific miRNAs-mRNA interactions which have recently been found [77].

In order to overcome this drawback, we also referred to the miRanda algorithm to identify potential binding sites for a given miRNA in genomic sequences [77,78]. Because of the lower level of stringency required by miRanda, it gives a larger number of putative targets.

Among the more than 900 computationally predicted targets for miR-146a we focussed on genes relevant for T cell biology, predicted by at least one of these algorithms.

We decided to validate the putative targets selected taking advantage of a 3'-untranslated region (3'-UTR) luciferase reporter assay.

We performed luciferase reporter assays on constructs containing the firefly luciferase gene, fused to 500bp of the 3'UTR of 22 genes. Among them there were: MAD4, BCL11A, LCK, FADD and c-FOS (see Supplementary Table 2 for the complete list of putative targets analyzed). In particular, we noticed that MAD4 was an interesting candidate, because it contained two miR-146a complementary seed site in its 3'untraslated region (3'UTR). Moreover, it has been reported that MAD4 is down-regulated in T lymphocytes upon TCR stimulation [79].

Another important candidate as miR-146a target was FADD, which has only a non conserved seed site and thus it is predicted only by miRanda algorithm. Nevertheless, it is noteworthy that the level of complementarity between FADD 3'UTR and miR-146a seed neighbouring nucleotides was greater than that of other putative targets.

Finally, c-FOS acts as an important transcription factor, forming a heterodimer with c-Jun, commonly known as AP-1 [80].

The reporter constructs were transiently transfected together with a mimic-146a or with a scramble mimic (see material and methods).

The data shown in Fig.8a,b,c demonstrate a miR-146a specific regulation of MAD4, FADD and c-FOS luciferase reporters.

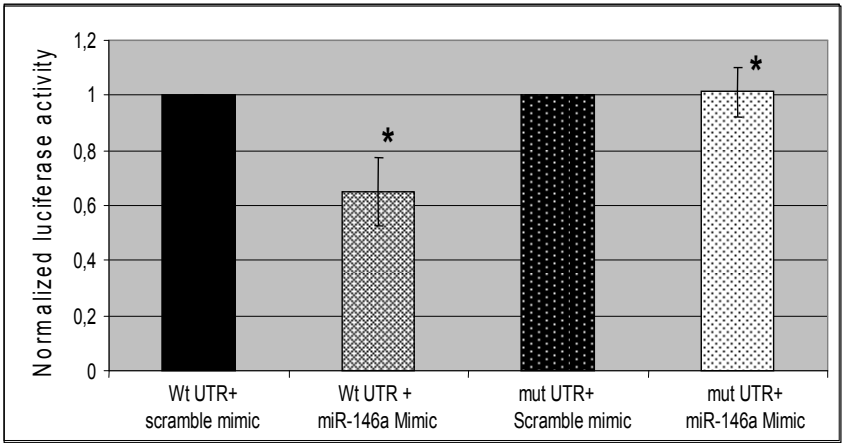


Fig.8a. MAD4 is targeted by miR-146a: luciferase assay of MAD4 3'UTR in 293T cells 24h after transfection The effects of miR-146a up-regulation were here illustrated on wildtype 3'UTR (wt UTR) and on mutated 3'UTR seed match (mutUTR). Luciferase activity was assayed using a Dual-Luciferase Reporter System.
(p value< 0,05).

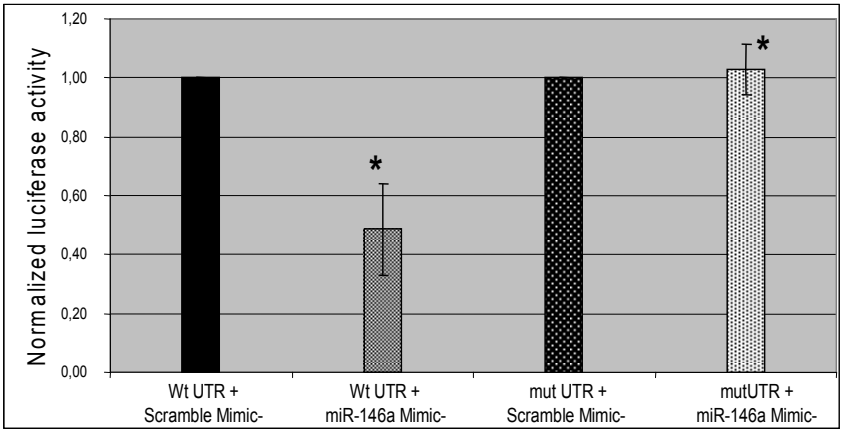


Fig. 8b. FADD is down-regulated by miR-146a: luciferase assay of FADD 3'UTR in 293T cells 24h after transfection The effects of miR-146a up-regulation were here illustrated on wildtype 3'UTR (wt UTR) and on mutated 3'UTR seed match (mutUTR) (*p value <0,01).

The down-regulation observed was specific to the predicted miR-146a target sites, as mutation of the 3'UTR seed match sequences relieved the inhibitory activity of miR-146a (Fig. 8a,b,c).

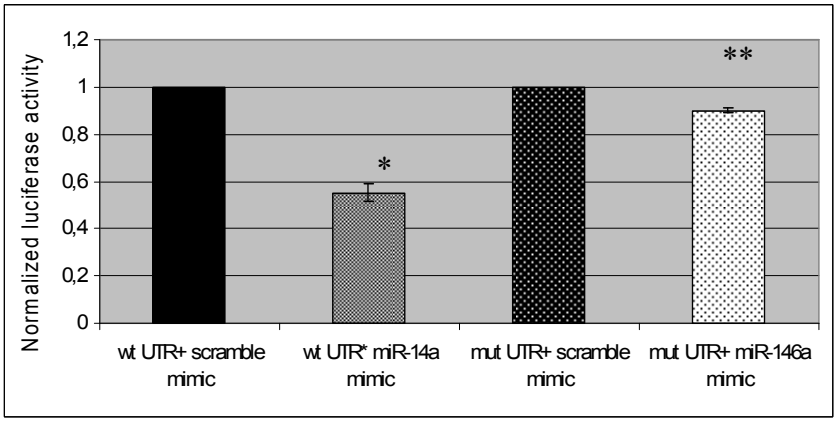


Fig.8c. c-FOS is targeted by miR-146a: luciferase assay of c-FOS 3'UTR in 293T cells 24h after transfection; data were normalized to the value obtained for mimic scramble transfected cells The effects of miR-146a up-regulation were here illustrated on wildtype 3'UTR (wt UTR) and on mutated 3'UTR seed match (mutUTR)
Luciferase activity was assayed using a Dual-Luciferase Reporter Assay System
(*p value <0,01; **p value< 0,05).

Given that microRNAs can also promote target mRNA degradation, we also tested whether FADD, c-FOS and MAD4 mRNA levels were affected by miR-146a over-expression. To do so we measured mRNA levels by Real-Time RT-qPCR. MiR-146a over-expressing and control Jurkat cells had essentially the same mRNA levels, thus suggesting that miR-146a did not promote FADD, MAD4 or c-FOS mRNA decay (Fig. 8d).

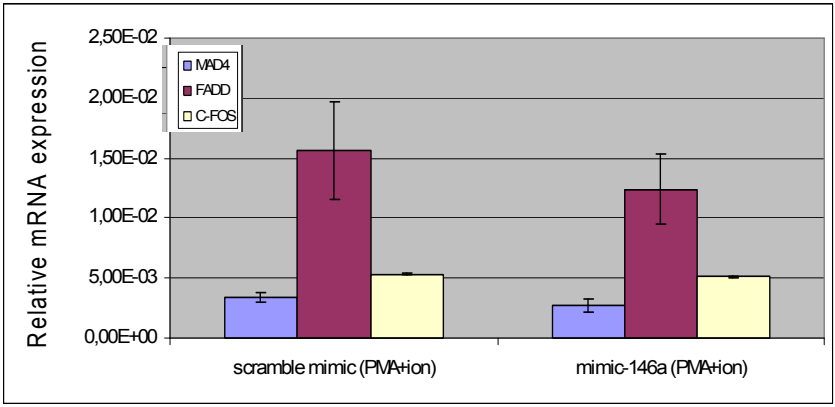


Fig.8d. MiR-146a over-expression does not promote mRNAs decay of target genes: Jurkat cells ($5x10^5$ cells/ml) stimulated for 24h with PMA and ionomycin, were transfected with mimic-146a or with a scramble mimic; cells were collected 48h after transfection. GAPDH mRNA was used to normalize data (Real-Time qRT-PCR).

These results gave a good indication that miR-146a was implicated in the post-transcriptional down-modulation of MAD4, FADD and c-FOS levels; hence the need of further analyses to elucidate the molecular phenotypes associated to miR-146a up-regulation.

MiR-146a over-expression attenuates AICD in Jurkat cells

To explore the phenotypes associated to miR-146a-mediated FADD down-modulation after TCR activation, we looked at possible effect on apoptosis. As mentioned above, FADD is an adaptor protein mainly involved in the apoptotic process and in particular in AICD in T cells. The Jurkat cell line has been widely used as a model for T cell AICD studies, since apoptosis can be easily induced by various activating signals. The AICD process, indeed, can be mimicked by cross linking the TCR complex with αCD3 antibodies or by activating downstream signalling molecules (PKC and Calcineurin) with PMA and ionomycin.

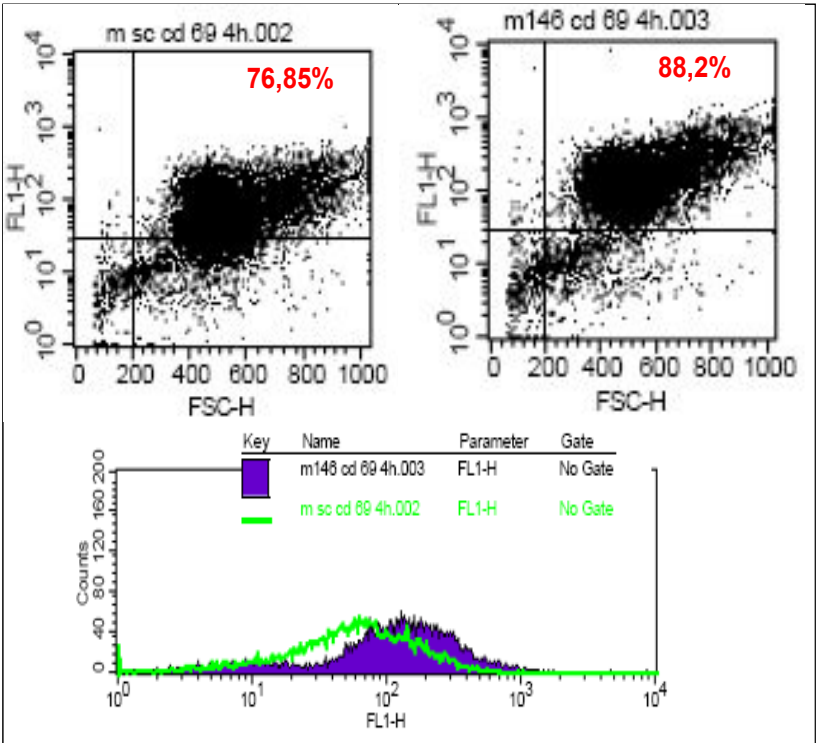


Fig. 9a. Mimic-146a expressing Jurkat cells are more activated than control Jurkat cells: Jurkat cells (5 x10^5 cells/ml) were stimulated with PMA (10ng/ml) and ionomycin (1μg/ml) and 2 x10^5 cells were stained with CD69-FITC 4h after stimulation. Data analysis was performed with FACS- Calibur

We transfected Jurkat cells with either mimic-146a or with scramble mimic and induced AICD by stimulating Jurkat cells with PMA and ionomycin for 72h.
We performed a CD69 staining to monitor the activation status of cells early after TCR stimulation. As expected, in non induced cells (both in the control cells and in mimic-146a Jurkat cells) CD69 is not expressed. As shown in Fig.9a, miR-146a over-expression caused a higher expression of CD69, thus evidencing a more activated status of these lymphocytes. These findings are in agreement with published studies that correlate FADD down-modulation with a higher percentage of CD69 positive cells [62,63].
Moreover, when we examined the apoptotic rate of Jurkat cells transfected with mimic-146a, 72h after PMA and ionomycin stimulation, we found that there was a smaller fraction of apoptotic cells compared with the control (Fig. 9b).
Taken together, these findings pointed out an anti-apoptotic role of miR-146a, here demonstrated in T lymphocytes, possibly due to FADD down-modulation, as suggested by 3'UTR-FADD luciferase assay (Fig. 8b).

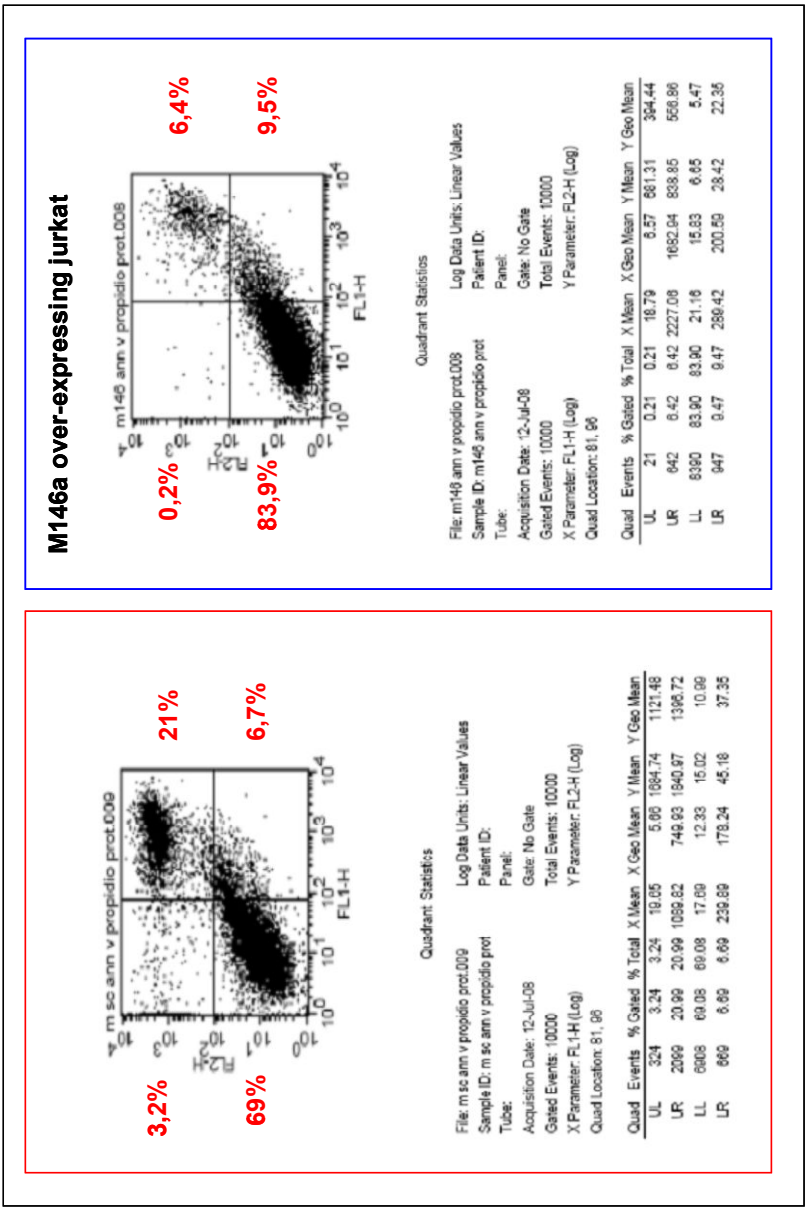


Fig.8b. MiR-146a acts as an anti-apoptotic factor: AnnV^{+}-PI^{+} double staining in Mimic146a cells compared with scramble mimic control cells (FACS analysis).

MiR-146a affects AP-1 activity as revealed by a luciferase reporter assay

To further investigate the effect of mir-146a on c-FOS expression we performed a luciferase assay using an AP-1 reporter plasmid, transiently transfected in pRRL-146a Jurkat cells.
pRRL-146a Jurkat cells are transduced with a lentiviral construct, which allows a stable expression of miR-146a of about 3 fold compared with Jurkat cells expressing the pRRL(-) empty lentiviral construct.
pRRL-146a Jurkat cells were stimulated with αCD3-αCD28 antibodies for 24 hours. As illustrated on Fig.10, the over-expression of miR-146a impairs AP-1 transcriptional activity.
This result is consistent with the observation that c-FOS is down-modulated by miR-146a (Fig. 8c) and indicates the involvement of miR-146a in the modulation of AP-1 activity after T cell activation

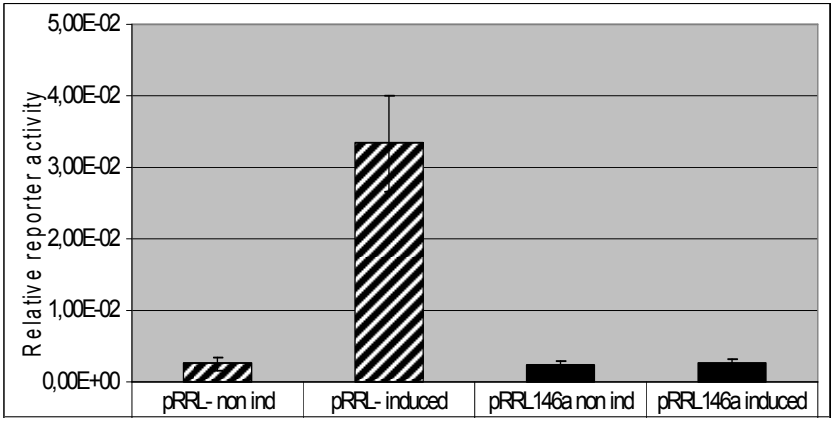


Fig10. AP-1 activity is down-modulated by miR-146a: luciferase reporter assay on lentiviral transduced Jurkat cells, over-expressing miR-146a (pRRL-146a) compared with control Jurkat cells (pRRL-); cells were stimulated with αCD3-αCD28 antibodies for 24h. Luciferase activity was assayed using a renilla/firefly Dual-Luciferase Reporter Assay System. (p value< 0,05).

DISCUSSION

The critical role played by miRNAs in immunity has emerged in the last years [14,18,21].

In the present study we found that miR-146a is expressed in primary $CD4^+$ and $CD8^+$ T lymphocytes, but not in naive T cells (Fig.1). This first experimental evidence, suggesting the induction of miR-146a expression upon TCR stimulation, prompted us to further investigate a possible role of miR-146a in T cell activation.

We detected up-regulation of MiR-146a levels in Jurkat T cells, stimulated with PMA and ionomycin or with αCD3-αCD28 antibodies, which mimic antigen recognition by TCR (Fig.2a,2b).

Therefore, we attempted to elucidate the pathways involved in miR-146a transcriptional regulation using Jurkat cells as an experimental model. A first approach consisted in the treatment of Jurkat cells with specific pharmacological inhibitors, interfering with the main pathways triggered by TCR signalling: NFAT, AP-1, ERK and NF-κB pathways.

We found that cyclosporine, a Calcineurin inhibitor, significantly impaired miR-146a up-regulation (Fig.4c), thus suggesting that the pathways affected by CsA play a key role in the induction of miR-146a upon TCR engagement. This observation prompted us to further investigate with molecular tools the transcription factors which are responsible for miR-146a expression in T lymphocytes.

A deeper investigation of miR-146a promoter region, through a bioinformatic analysis and a luciferase assay, allowed us to carefully characterize the *cis* regulatory elements involved in miR-146a transcriptional regulation. First we identified conserved consensus sites for T lymphocytes specific transcription factors, which are involved in pathways triggered by TCR signalling; in particular two sites for NF-κB, one site for c-ETS and one site for USF2 (Fig.7a). Subsequently, mutational analysis of the promoter region indicated that NF-κB and c-ETS binding sites are relevant for the modulation of miR-146a expression upon TCR stimulation (Fig.7b.) .

Both NF-κB and c-ETS are transcription factors fundamental for transmitting TCR activation signals in the T lymphocytes [81,82] and play a key role in modulating the expression of several cytokines (e.g. IL-2 and IL-12). Therefore, our finding that these two transcriptional factors strongly affect the expression of miR-146a provide a further link between miR-146a and T lymphocytes activation.

Since miR-146a has been shown to be induced in monocytes by inflammatory stimuli [23], we analyzed miR-146a expression in Jurkat cells treated with TNF-α, one of the early major cellular mediators in inflammatory and immuno-modulatory responses. Our observation that miR-146a is up-regulated following TNF-α stimulation (Fig.3a) and that, in this condition, NF-κB is the only main activator responsible of miR-146a promoter activation (Fig.7c), indicate a role of miR-146a in the modulation of the inflammatory response also in T lymphocytes.

Therefore, we have shown that at least two different signals drive miR-146a expression in Jurkat cells: TCR stimulation and inflammation (Fig.5). We demonstrated that these two signals use partially overlapping transcriptional mechanisms to induce miR-146a expression. In particular, NF-κB, the main transcription factor activated by TNF-α during the inflammatory response, is the activator of miR-146a expression in the inflammatory context, while during TCR activation the contribution of both NF-κB and c-ETS is indispensable.

The study of miR-146a molecular functions also represents a stimulating challenge ad so we aimed to elucidate the fine-tune regulation exerted by miR-146a in some important biological aspects of T cell immune response.

MiR-146a down-regulate FADD expression at posttranscriptional level as demonstrated by luciferase reporter assays performed using the 3'-UTR of FADD (Fig.8b). Moreover, miR-146a over expression in Jurkat cells determined a more activated status of cells, as revealed by a higher expression of $CD69^+$ marker (Fig.9a), and a decrease of the apoptotic rate (Fig.9b). Notably, both of these phenotypes are consistent with a down-regulation of FADD.

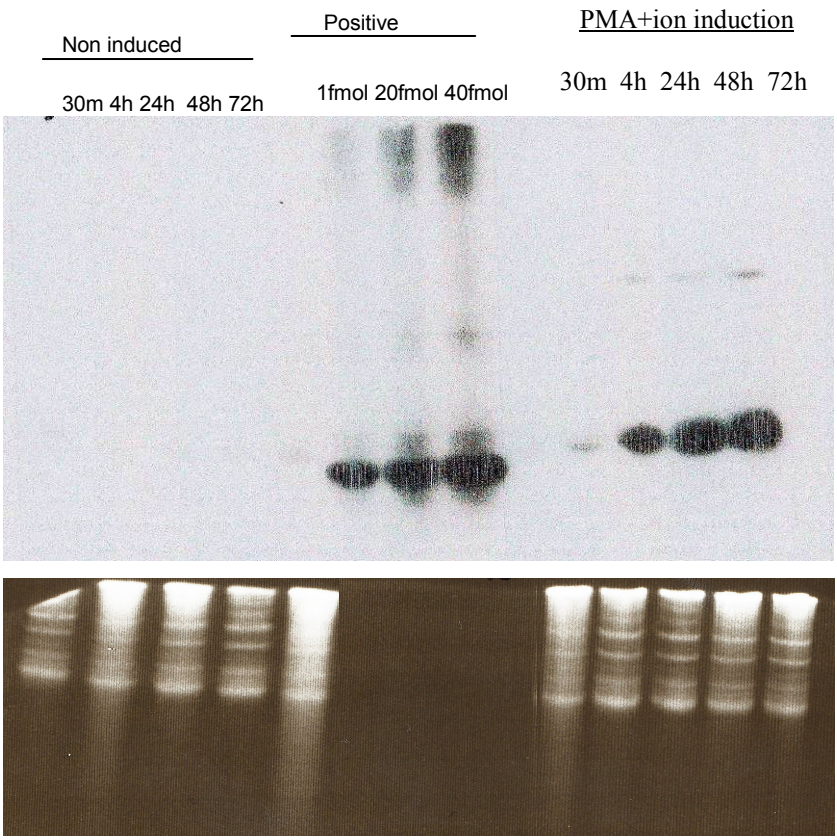
For the first time, we have shown that other signaling pathways, apart from inflammation, are influenced by miR-146a modulation and function. Indeed, we provided experimental evidences that support a role for miR-146a in modulating AICD, through the down-regulation of FADD.

We also showed miR-146a involvement in other pathways related to T cell activation, in particular we demonstrated through luciferase assays that MAD4 and c-FOS are down-modulated by miR-146a (Fig.8a,8c).

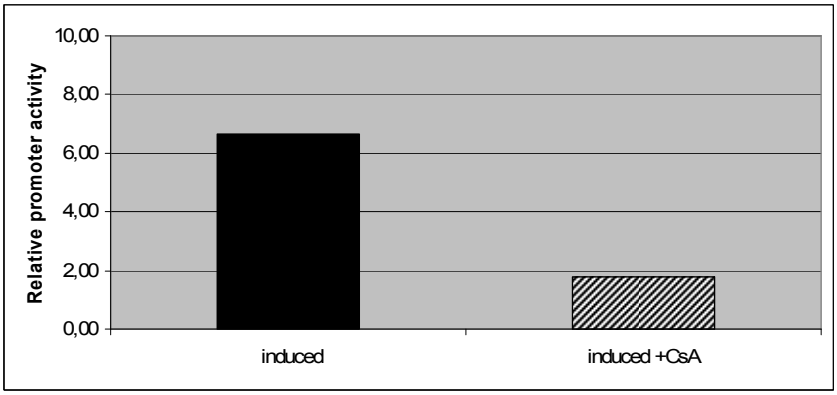
MAD4 is a member of the MADs family, a negative regulator of MYC function [83,84] and it promotes differentiation and suppresses the development of cancer *in vivo* [81]. Moreover, it was reported that in T lymphocytes MAD4 is down-regulated upon TCR stimulation[79]. It

would be intriguing to unravel which is the contribution of MAD4 down-regulation to the phenotype of miR-146a over-expressing cells; this aspect will be further investigated in following studies.
We focused on c-FOS, being an interesting transcriptional factor, which modulate the expression of several cytokines, important for T cell differentiation. The luciferase assay validating it as a target of miR-146a (Fig.8c) is consistent with the observation that miR-146a over-expression impairs AP-1 activity after T cell activation (Fig.10)
This result was obtained using a luciferase reporter vector for AP-1 transfected in the lentiviral pRRL-146a Jurkat cells (see material and methods). An advantage of the experimental approach here adopted is that in pRRL-146a Jurkat cell line, the expression levels of miR-146a are not as elevated as in the case of transient transfection of the mimic-146a (about 700 fold; Supplementary. Fig.3), thus demonstrating that even a modest up-regulation of miR-146a (3 fold) is sufficient to exert its modulatory effect on AP-1 activity.
This work illustrated a framework of an intriguing scenario, in which miR-146a contributes to the modulation of T cell function by fine tuning complex signaling networks, delimiting the intensity and duration of such signaling.
A focus for future studies will be the elucidation of the molecular mechanism of miR-146a action, thereby demonstrating a direct correlation between FADD repression and AICD impairment, both mediated by miR-146a.
Moreover, it would be very interesting to analyze interleukins levels in stimulated Jurkat cells over-expressing miR-146a, according to the molecular phenotype associated with c-FOS down-regulation, being AP-1 heterodimer an important modulator of IL-2 expression [80].
Therefore, many other questions regarding miR-146a still await answering. Understanding its functions in the complex molecular networks which orchestrate T cell immune response will contribute not only to a better comprehension of immune homeostasis but will identify novel targets for modulating immune response and inflammation in humans.

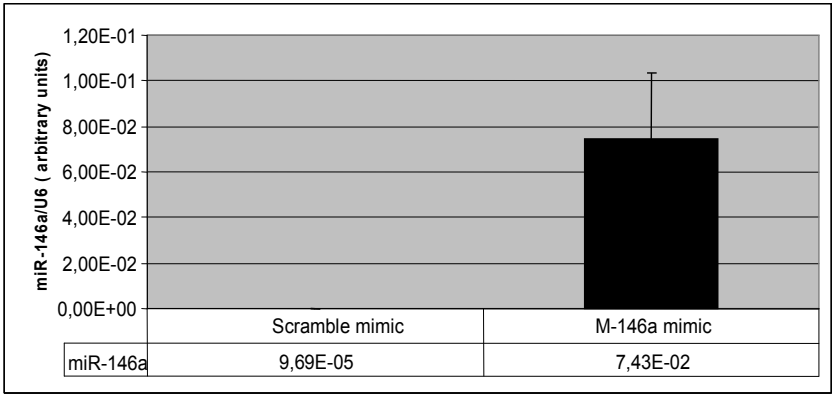
Supplemental Data



Suppl. Fig 1: Northern Blot: miR-146a expression in Jurkat cells stimulated with PMA (10ng/ml) and ionomycin (1µg/ml) for different times.



Suppl. Fig2. NF-κB reporter activity is inhibited by CsA: Jurkat cells were transfected with a NF-κB reporter plasmid and the day after transfection they were treated for 30 minutes with CsA (0,5µg/µl) and then stimulated with αCD3 (5µg/ml) and αCD28 (5µg/ml) for 24h. The luciferase assay was performed 48h after transfection. Fold induction of stimulation were here illustrated compared with non induced reporter construct. Relative NF-κB reporter activity was assayed using a renilla/firefly-Luciferase Reporter Assay System. (p value< 0,05).



Suppl. Fig3. Mimic-146a transfected Jurkat cells over-expresses miR-146a Jurkat cells were transiently transfected with miR-146a mimic or with a scramble mimic. iR-146a levels were measured 48h after transfection by RT-qReal Time-PCR

Supplementary Table 1. List of oligonucleotides (*LNA bases are preceded by a "+" symbol)

RT-qPCR analysis

***PCR for LNA U6**
5'-GCTTCGGCAGCACATATACT-3'
U6 template PCR
5'-GCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAG
AAGATTAGCATGGCCC-3'
PCR rev miR-146b
5'-CAGAGGTACGATAGTCAAGG
mir-146b template PCR
5'-CAGAGGTACGATAGTCAAGGAGCCTATGGTTCAGTTCTCA-3'
***PCR for LNA mir-146b**
5'-T+GA+GAA+CTGAATTCCATA-3'
RT miR-146b
5'- CAGAGGTACGATAGTCAAGGAGCCTATGG-3'
PCR rev/ RT U6
5'-GAATTTGCGTGTCATCCTTG-3'
PCR revmiR-146a
AACAACGAGTGCTATGGAAT
***PCR for LNA mir-146a**
5'-T+GA+GA+ACTGAATTCCATG-3'
PCR rev miR-146a
5'-TGAGAACTGAATTCCATGGGTT-3'
miR-146a template PCR
5'AACAACGAGTGCTATGGAATAACCCATGGAATTCAGTTCTCA-3'
MAD4-pcrFOR
5'-ACAACAGGTCTTCACACAAC-3'
MAD4_pcrREV
CTCCTCCAGTTTCTTGATG-3'
FADD pcr FOR
5'-TGCGGGAGTCACTGAGAATC-3'
FADD pcr REV
5'-CAGCCACCAGGTTCATCTG-3'
FOS pcrFOR
5'-AGCGGAGACAGACCAACTAG-3'
FOS pcr REV
5'-GCTGCCAGGATGAACTCTAG-3'

3'UTR luciferase assays

FADDutrFOR
5'- GCGCGCTCTAGAGGACCACAGGCATCTACACA-3'
FADDutrREV
5'-GCGCGCGAGAGGAGACTCAACTCACA-3'
FADD mut FOR
5'-CTAGAGGACCACAGGCATCTACACAAGATATCCTTTGGTTCTCTCAGGAAG GTACC-3'
FADD mut REV
5'-GCTACCTTCCTGGAGAGAACCAAAGGATATCTTGTGTAGATCCTGTGGTCCT CTAG-3'
MAD4utrFOR
5'-GCGCGCTCTAGATGCCTGCCCGCCAGCCAC-3'
MAD4utrREV
5'-GCGCGCCTCACCCAGTTCCCGCCCTT-3'
MAD4 mut FOR
5'-CAGCCACGCGTGTCAGCCCTCCGCCATGGCTTCAGTTGACGCCA-3'
MAD4 mut REV
5'-GAGAGGCTGGCGTCAACTGAAGCCATGGCGGAGGGCTGACACGCGTGGCTG-3'
IRAK1utrFOR
5'-GCGCGCTCTAGAGTTCACCTGGGCAGATCC-3'
IRAK1utrREV
5'-GCGCGCGATGATGCCAGCCTTCCT-3'
FOS mut FOR
5'-GAGGTGGTCTGAATGTTCTGACATTAATCCATGAAAACGTT-3'
FOS mut REV
5'-AACGTTTTCATGGATTAATGTCAGAACATTCAGACCACCTC-3'
GAPDH FOR
5'-GAAATCCCATCACCATCTTCCAGG-3'
GAPDH REV
5'-GAGGCCCAGCCTTCTCCATG-3'

Northern oligonucleotides

northern marker miR-146a
5'-TGAGAACTGAATTCCATGGGTT-3'
northern probe miR-146a
5'-AATTCAGTTCTCA-3'
northern probe U6
5'-AGTATATGTGCTGCCGAAGC-3'

MiR-146a promoter luciferase assays

NFKB1 mut rev
5’-CCCTCTCTGGAAAGTGGTTTCTAGATCCCTCCTCGGC-3’
NFKB1 mut for
5’-GCCGAGGAGGGATCTAGAAACCACTTTCCAGAGAGGG-3’
ETS mut for
5’-GCGGAGAGTACAGACGCCTGGGGACCCAGC-3’
ETS mut rev
5’-GCTGGGTCCCCAGGCGTCTGTACTCTCCGC-3’
NFKB mut for
5’-AGCCGATAAAGCTCTCACCATTTCCCCGCGGGGC-3’

NFKB2 mut rev
5’-GCCCCGCGGGGAAATGGTGAGAGCTTTATCGGCT-3’
USF mut for
5’-AGGAAGATTTCTCAGTGTTCCAAGTGCCAGGTCAGTTTACAGTTC-3’
USF mut rev
5’-GAACTGTAAACTGACCTGGCACTTGGAACACTGAGAAATCTTCCT-3’

Seq luc for
5’-GTGGATTACGTCGCCAGTC-3’

Supplementary Table2a. predicted targets cloned for 3'UTR luciferase assays

part1					validated target
miRanda (miRbase) TargetScan	**IRAK1**	*Interleukin-1 receptor-associated kinase 1 (IRAK-1).*	interleukin-1 receptor complex, transmembrane receptor protein serine/threonine kinase signaling pathway, signal transduction, transcriptional activator activity, protein amino acid autophosphorylation, positive regulation of transcription, **NF-kappaB-inducing kinase activity**, protein serine/threonine kinase activity, magnesium ion binding, trnsferase activity, activation of NF-kappaB-inducing kinase.		
PicTar, TargetScan	**MXD4**		**negative regulation of cell proliferation**, protein binding, **negative regulation of transcription from RNA polymerase II promoter** , nucleus, DNA binding		
PicTar, TargetScan	**SMAD4**	*Mothers against decapentaplegic homolog 4 (SMAD 4)*	negative regulation of cell proliferation, SMAD protein heteromerization, transcription cofactor activity, DNA-dependent, transcription, cytoplasm, protein binding, positive regulation of transcription from RNA polymerase II promoter		
miRanda (miRbase), PicTar, TargetScan	**KLF7**	*Krueppel-like factor 7 (Ubiquitous krueppel-like factor).*	transcription coactivator activity,**regulation of transcription from RNA polymerase II promoter**, zinc ion binding, metal ion binding		
TargetScan, PicTar	**BCL11A**	*B-cell lymphoma/leukemia 11A (B-cell CLL/lymphoma 11A)*	transcription corepressor activity,**T cell differentiation**, cytoplasm, B cell differentiation, hemopoiesis, zinc ion binding, nucleic acid binding, metal ion binding		
miRanda (miRbase), Pictar, Targetscan	**SPIB**	*Transcription factor Spi-B.*	transcription factor complex, RNA polymerase II transcription factor activity, macrophage differentiation, sequence-specific DNA binding,		

Supplementary Table2b .predicted targets cloned for 3'UTR luciferase assays

part2					
miRanda (miRbase), PicTar	**MAX isoform c**	*Protein max (Myc-associated factor X).*	protein binding, regulation of transcription.		
miRanda (miRBase)	**BCL6**	*B-cell lymphoma 6 protein (BCL-6) (Zinc finger protein 51) (LAZ-3 protein) (BCL-5)*	transcriptional repressor activity, inflammatory response, mediator complex, spermatogenesis, **negative regulation of apoptosis,** protein binding, **positive regulation of cell proliferation**, **inhibition th2 cells differentiation and gata3 expression**		
PicTar, TargetScan	**ZDHHC 17**	*Hypermethylated in cancer 2 protein (Hic-2) (Hic-3) (Zinc finger and BTB domain-containing protein 30)*	lipoprotein transport, protein-cysteine S-palmitoleyltransferase activity, transferase activity, acyltransferase activity, signal transducer activity, **positive regulation of I-kappaB kinase/NF-kappaB cascade,** protein binding, integral to membrane, protein palmitoylation		
PicTar	**OTUD7B (CEZANNE)**	*Zinc finger A20 domain-containing protein 1 (Cellular zinc finger anti-NF-kappa B protein) (Zinc finger protein Cezanne).*	cysteine-type peptidase activity, DNA binding, **negative regulation of I-kappaB kinase/NF-kappaB cascade**, cytoplasm, ubiquitin cycle, protein binding, nucleus, zinc ion binding, metal ion binding		
PicTar, TargetScan	**IRF9 , RNF31**	*Transcriptional regulator ISGF3 subunit gamma (Interferon regulatory factor 9) (IRF-9) (IFN-alpha-responsive transcription factor subunit)*	cytosol, protein binding, internal side of plasma membrane, intracellular, zinc ion binding, metal ion binding		

Supplementary Table2c. predicted targets cloned for 3'UTR luciferase assays

part3					
TargetScan	**RNF4**	*RING FINGER PROTIEN 4*	The protein encoded by this gene contains a RING finger motif and acts as a transcription regulator.		
PicTar, TargetScan	**EGR3**	*Early growth response protein 3 (EGR-3)*	intracellular, **regulation of transcription**, peripheral nervous system development, neuromuscular synaptic transmission, circadian rhythm, transcription factor activity, zinc ion binding, metal ion binding, muscle development		
miRanda (miRBase)	**ETV5 (ERM)**	*ETS translocation variant 5 (Ets-related protein ERM).*			
PicTar, miRanda (miRBase), TargetScan	**RAB10**	*PHD finger protein 20-like 1 (PHF20L1), transcript variant 1, mRNA*	intracellular protein transport, protein binding, Golgi apparatus, protein transport, small GTPase mediated signal transduction, GTP binding, membrane, nucleotide binding		
PicTar,TargetScan	**PIP5K2B**	*Phosphatidylinositol-4-phosphate 5-kinase type-2 beta*	internal protein amino acid acetylation, ribosome binding, intracellular, N-terminal protein amino acid acetylation, transferase activity, N-acetyltransferase activity, DNA packaging, acyltransferase activity, cytoplasm, peptide alpha-N-acetyltransferase activity, protein binding, nucleus, chromosome organization and biogenesis (sensu Eukaryota)		
miRanda (miRbase)	**LCK**	*Proto-oncogene tyrosine-protein kinase LCK (p56-LCK) (Lymphocyte cell-specific protein-tyrosine kinase) (T cell- specific protein-tyrosine kinase).*	protein binding, release of sequestered calcium ion into cytosol, protein serine/threonine kinase activity,**TCR signaling**		

Supplementary Table2d. predicted targets cloned for 3'UTR luciferase assays

part4						validated target
miRanda (miRbase)	**TNFAIP 8**	*tumor necrosis factor, alpha-induced protein 8*	**anti-apoptosis**			
miRanda (miRbase)	**FADD**	*FADD protein (FAS-associating death domain-containing protein) (Mediator of receptor induced toxicity)*	**immune response, cell-cell signaling, induction of apoptosis**, tumor necrosis factor receptor binding, **positive regulation of I-kappaB kinase/NF-kappaB cascade**, signal transduction, extracellular space, soluble fraction, integral to plasma membrane, membrane, zinc ion binding, metal ion binding			
PicTar, TargetScan	**EFNB2**	*Ephrin-B2 precursor (EPH-related receptor tyrosine kinase ligand 5) (LERK-5)*	cell differentiation, **cell-cell signaling**, ephrin receptor binding, protein binding, integral to membrane, lymph vessel development, plasma membrane, organ morphogenesis, integral to plasma membrane, nervous system development, morphogenesis			
miRanda (miRbase)	**CXCR4**	*C-X-C chemokine receptor type 4 (CXC-R4) (CXCR-4) (Stromal cell-derived factor 1 receptor) (SDF-1 receptor) (CD184 antigen).*	G-protein coupled receptor protein signaling pathway, germ cell development, neuron migration, patterning of blood vessels, **T cell proliferation**, germ cell migration, purinergic nucleotide receptor activity, G-protein coupled, integral to membrane, ameboidal cell migration, receptor activity, C-C chemokine receptor activity, regulation of cell migration, **C-X-C chemokine receptor activity**, growth cone, signal transduction, brain development, rhodopsin-like receptor activity, motor axon guidance			
miRanda (miRbase)	**CD180**	*CD180 antigen precursor (Lymphocyte antigen 64) (Radioprotective 105 kDa protein)*	protein-lysine 6-oxidase activity, copper ion binding, protein binding, signal transduction, extracellular space, cellular defense response, extracellular matrix (sensu Metazoa), scavenger receptor activity, cell adhesion, membrane			

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