

Effect of All-Trans Retinoic Acid (ATRA) on Viability, Proliferation, Activation and Lineage-Specific Transcription Factors of CD4+ T Cells

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ABSTRACT

All-trans retinoic acid (ATRA), as an active metabolite of vitamin A, has been shown to affect immune cells. This study was performed to evaluate the effect of ATRA on viability, proliferation, activation and lineage-specific transcription factors of CD4+ T cells. CD4+ T cells were separated from heparinized blood of healthy donors and were cultured in conditions, some with, some without ATRA.

Viability was assessed by PI flowcytometry and proliferation was measured by MTT assay. CD69 expression was determined by flowcytometry as a measure of cell activation. Lineage-specific transcription factors (FOXP3, ROR γ t and T-bet) were examined by intracellular staining and flowcytometry. High doses of ATRA (0.1-1 mM) caused extensive cell death in both PBMCs and CD4+ T cells. Doses of ATRA equal to or lower than 10 μ M did not adversely affect cell viability and proliferation in comparison to culture medium without ATRA.

Doses of ATRA between 10 μ M and 1nM significantly increased cell activation when compared to culture medium without ATRA. ATRA could increase FOXP3+ and also FOXP3+ROR γ t+ T cells while it decreased ROR γ t+ and T-bet+ T cells. This study showed that doses of ATRA up to 10 μ M are safe when using with CD4+ T cells in terms of cell viability, proliferation and activation.

We could also show that ATRA diverts the human immune response in neutral conditions (without adding polarizing cytokines) by increasing FOXP3+ cells and decreasing ROR γ t+ cells. ATRA could be regarded as a potential therapy in inflammatory conditions and autoimmunities.

Keywords: ATRA; CD4+ T cells; FOXP3; ROR γ t; Transcription factors; T-bet

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INTRODUCTION

Vitamin A is present in blood and liver in the form of retinol and retinyl esters, respectively. Retinoid

dehydrogenases are then responsible to convert these metabolites into retinal and afterwards irreversibly into retinoic acid. ATRA is an active acid derivative of vitamin A which has an intense influence on cell proliferation and differentiation. ATRA binds to retinoic acid receptors (RARs) and forms holocomplexes which act as transcription factors that regulate target genes.¹ It also mediates non-genomic activities via signaling pathways through epigenetic mechanisms.²

Different doses of ATRA seem to act differently on cells. However, most groups have used ATRA at pharmacological concentrations (~1 μ M).³ High doses decrease viability of immature dendritic cells (DCs) and monocytes^{4,5} and facilitate myocardial proliferation.⁶

Vitamin A has long been believed as a dietary supplement to enhance resistance to infections.¹ It plays crucial roles in physiological functions of body such as vision, embryogenesis, metabolism and gene transcription.⁷ Vitamin A has been shown to affect diverse immune cells including B cells and their antibody responses.^{8,9} It also affects T cell numbers, their skewing to different subpopulations and their homing.¹

Recently there has been great interest on diverse subsets of T helper (Th) cells¹⁰ and the role of ATRA on the differentiation of Th1, Th17 and T regulatory (Treg) cells.

ATRA has been reported to be effective on FOXP3 induction in peripheral CD4+ T cells in numerous studies. In humans, some studies report that ATRA alone³ and some studies claim ATRA along with TGF- β ¹¹ can convert human naïve T cells into FOXP3+ Treg cells during activation. These induced Treg cells have been shown to be stable and potently suppressive.¹¹ It has been shown that ATRA, reciprocally, can suppress Th17 cells and regulates the balance between pro- and anti-inflammatory immunity.¹² ATRA has also been shown to suppress IFN- γ production and thus affect Th1 subset of Th cells.¹³ However, most studies have been conducted in polarizing conditions favoring different Th subsets.

In order to determine safe and effective doses of ATRA, in this study, we evaluated the effect of different doses of ATRA on viability, proliferation and activation of CD4+ T cells and peripheral blood mononuclear cells (PBMCs). We also aimed to assess the effect of ATRA on pro- and anti-inflammatory

transcription factors (ROR γ t, T-bet and FOXP3) of activated CD4+ T cells in neutral conditions (without adding polarizing cytokines) in order to evaluate the changes in the percentage of different T helper cell subsets in healthy donors.

MATERIALS AND METHODS

Cells

PBMCs were isolated from heparinized blood of 5 healthy donors by density-gradient centrifugation with Ficoll-Hypaque (Lymphoflot, Biotest, Germany). CD4+ T cells were purified by negative isolation kit (Dyna, Oslo, Norway), which contains a mixture of 8 monoclonal antibodies (monoclonal antibodies for CD8, CD14, CD16, CD19, CD36, CD56, CDw123 and CD235a) and Depletion Dynabeads. The purity of cells was over 95% and viability, determined by exclusion of trypan blue, was more than 98%.

PBMCs and CD4+ T cells (2×10^5 /well) in a total volume of 200 μ l of medium were activated with PHA (5 μ g/ml) or plate-bound anti-CD3 (2 μ g/ml) and soluble anti-CD28 (1 μ g/ml) in 96-well plates for 72 hours in the presence or absence of ATRA of various concentrations. The optimal concentration of PHA, anti-CD3 and anti-CD28 antibodies was determined by preliminary experiments.

ATRA

ATRA (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) under Argon inert gas at stock concentrations of 0.01 M and stored at -70 °C as aliquots in lightproof conditions before use. Different doses of ATRA were prepared with RPMI 1640 medium (supplemented with 10% FCS and 1% L-Glutamine) and were included in the cultures. Cultures with ATRA were protected from light all through culture period. For lineage-specific transcription factor evaluation, 0.1 μ M of ATRA was used.

Viability Determination by Propidium Iodide (PI)

PI binds with double-stranded DNA and rapidly enters into non-viable cells. It is excited in 488 nm and emits at 617nm. In order to evaluate viability of cells, 10 μ l of 0.5 mg/ml PI solution was added to cells and after 30 minutes, cells were washed once and analyzed by flowcytometry.

Proliferation Assay

The colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to measure cell proliferation. Cells were cultured in RPMI 1640 for 72 hours with different ATRA concentrations and MTT (0.5mg/ml) was added to cells after 20 minutes of incubation at 4-8 °C. Plates were incubated for 4 hours at 37 °C and 5% CO₂. Dimethyl sulfoxide (DMSO) was added as MTT solvent and absorbance was measured at 540 nm.

Flowcytometry for CD69 Surface Marker

Stimulated cells in the presence of different doses of ATRA were evaluated after 72 hours for CD69 surface marker expression according to the manufacturer’s instructions. CD69 (Early activation antigen) is specifically expressed on effector T cells and is a good marker to evaluate T cell activation.

Flowcytometry for Lineage-specific Transcription Factors

Activated cells (by anti-CD3 and anti-CD28 antibodies) which had been cultured for 72 hours in a cell incubator with 5% CO₂ at 37°C were fixed and permeabilized according to the manufacturer’s instructions. They were then stained by fluorescent-conjugated antibodies for FOXP3, RORγt and T-bet transcription factors and analyzed by BD flowcytometer and CellQuest Pro software. All buffers and antibodies were purchased from eBioscience, USA.

Ethics

The study was approved by the ethical committee of Tehran University of Medical Sciences. All study protocols were explained to all healthy donors and informed consent was obtained.

Statistics

One sample-t test and independent-samples t test were used to compare mean values with standards or between two groups, respectively. *P*-values less than 0.05 were considered significant.

RESULTS

Viability of Cells in the Presence of Different Doses of ATRA

PBMCs and CD4+ T cells were cultured in 96-well plates for 72 hours in the presence of different doses of ATRA (1mM-1nM) or culture medium without ATRA. High doses of ATRA caused significant increase in cell death in both PBMCs and CD4+ T cells compared to culture medium without ATRA (1mM ATRA: 100% cell death in both cell types, *P* =0.004; and 0.1 mM ATRA: 42% and 46% cell death in PBMCs and CD4+ T cells, respectively, *P* =0.04; compared to 15.6% cell death in cultures without ATRA).

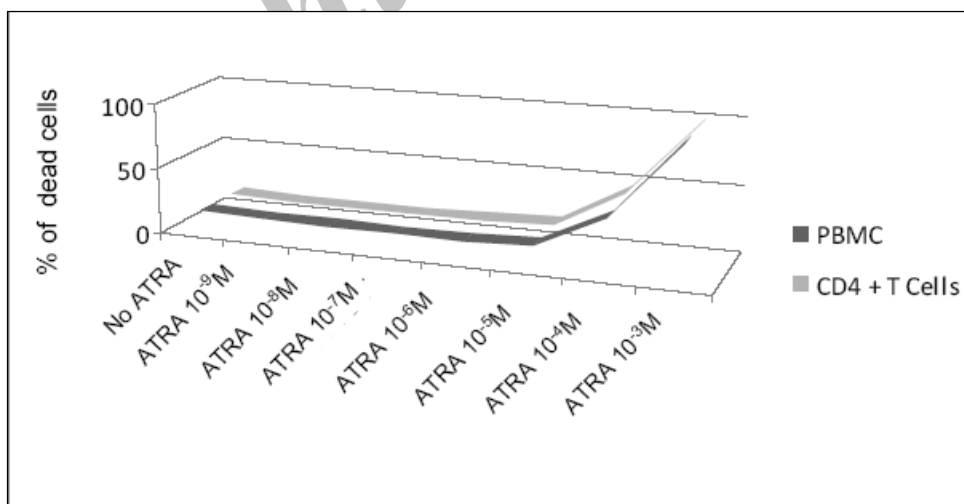


Figure 1. Effect of different doses of ATRA on PBMC and CD4+ T cell viability. Flowcytometric analysis of PI staining of PBMC and CD4+ T cells cultured in different ATRA concentrations showed the percent of dead cells in each condition.

-PHA	+ PHA	CD4+ T cells	-PHA	+ PHA	PBMCs
		No ATRA			No ATRA
		ATRA 10 ⁻⁵ M			ATRA 10 ⁻⁵ M
		ATRA 10 ⁻⁷ M			ATRA 10 ⁻⁷ M
		ATRA 10 ⁻⁹ M			ATRA 10 ⁻⁹ M

Figure 2. Proliferation of PBMCs and CD4+ T cells stimulated with PHA. The morphological appearance of PBMC and CD4+ T cells cultured with diverse doses of ATRA for 72 hours by light microscopy.

ATRA levels lower than 10µM did not significantly decrease PBMC and CD4+ T cell viability in comparison to culture medium alone. Figure 1 shows PBMC and CD4+ T cell viability in the presence of diverse doses of ATRA.

Proliferation of Cells in the Presence of Different Doses of ATRA

Cells cultured in 96-well plates, were evaluated by MTT proliferation assay after 72 hours. Cells were stimulated with PHA while ATRA was added to wells

in 10 µM to 1 nM concentrations. PHA-Stimulated cells showed a significant increase in optical density (OD) compared to non-stimulated cells. Doses of ATRA lower than 10 µM did not significantly affect OD and thus cell (PBMC and CD4+ T cell) proliferation in comparison to culture medium without ATRA. Figure 2 shows the morphology of CD4+ T cells in the presence and absence of PHA and presence of different doses of ATRA. PBMCs significantly showed higher OD results compared to CD4+ T cells ($P<0.001$).

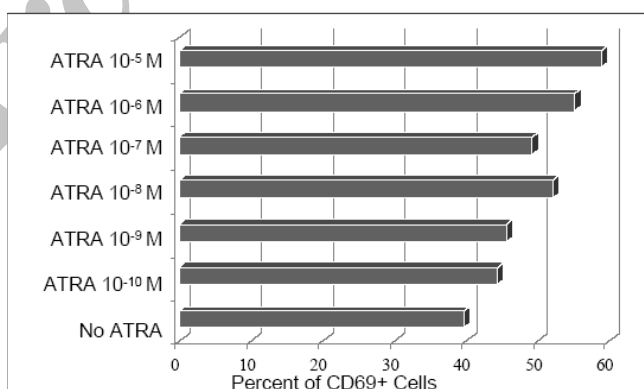


Figure 3. CD69 expression in CD4+ T cells stimulated with anti-CD3 and anti-CD28 antibodies in different cell culture conditions. Percent of CD69+ cells in CD4+ T cells stimulated with anti-CD3 and anti-CD28 antibodies in the presence of different doses of ATRA.

Effect of ATRA on CD4+ T Cells

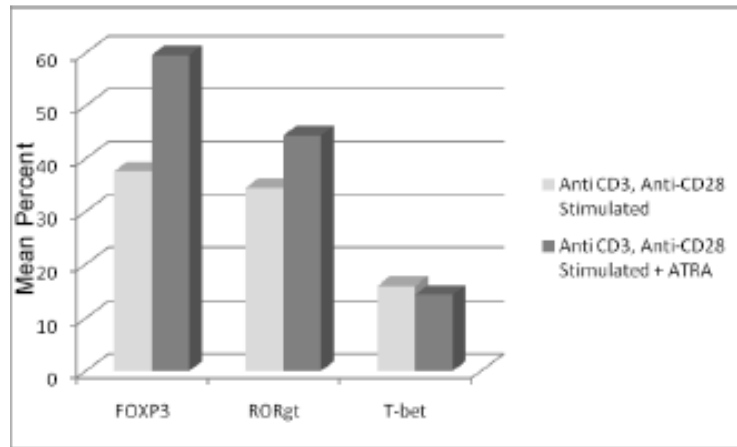


Figure 4. Mean percent of FOXP3, RORγt and T-bet expression in activated human CD4+ T cells with or without ATRA.

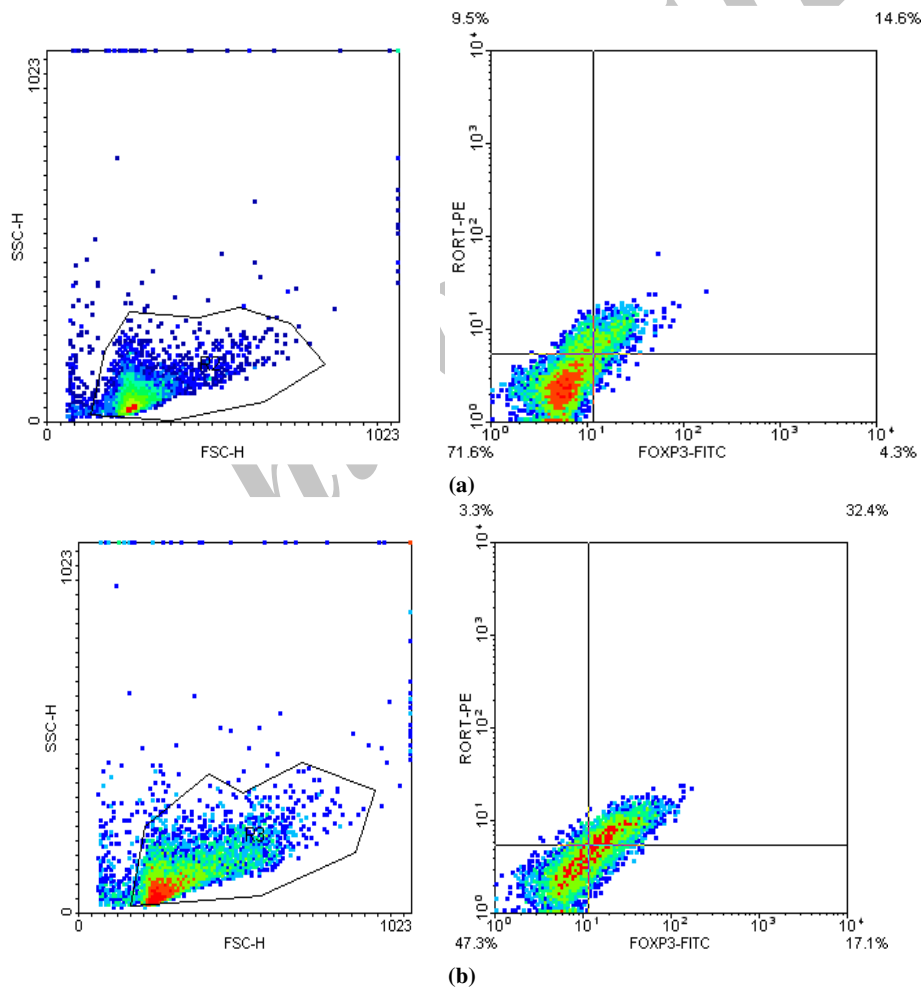


Figure 5. A sample of flowcytometric results for FOXP3 and RORγt in conditions of anti-CD3 and anti-CD28 stimulation alone (a) or in combination with ATRA (b).

CD4+ T Cell Activation in the Presence of Different Doses of ATRA

CD4+ T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 for 72 hours and CD69 marker was detected by flowcytometry. Doses of ATRA between 10 μ M and 1 nM significantly increased cell activation when compared to culture medium without ATRA ($P < 0.05$). Figure 3 shows percent of activated cells stimulated with anti-CD3 and anti-CD28 antibodies in the presence of different doses of ATRA compared to condition of no ATRA addition.

Lineage-specific Transcription Factors

FOXP3, ROR γ t and T-bet expressions were measured by intracellular flowcytometry after 72-hour-culture of CD4+ T cells in two conditions of anti-CD3 and anti-CD28 stimulation without ATRA or in combination with ATRA. The mean percent of cells expressing FOXP3 increased by 60% after ATRA addition, compared to culture conditions without ATRA. For ROR γ t, the mean increase, after addition of ATRA, was 28% and the mean T-bet expression decreased 10% after inclusion of ATRA in cultures (figure 4). When double positive cells were analyzed, it was revealed that the mean percent of ROR γ t+ FOXP3-cells decreased by 51% after addition of ATRA, while the mean percent of ROR γ t+FOXP3+ cells increased by 63% (figure 5). The mean percent of T-bet+FOXP3+ cells also increased, but not significantly.

DISCUSSION

ATRA has been used in numerous studies in recent years. It has been used as an anti-inflammatory agent which reduces Th1/Th17 responses in 1 μ M dose.¹⁴ In two separate studies, ATRA has been shown to increase T regulatory cells and inhibit Th17 development.^{15,16} It has also been shown that ATRA induces immune tolerance by generating highly suppressive T regulatory cells from PBMCs.¹¹

In order to evaluate the effect of ATRA on transcription factors, we activated CD4+ T cells in neutral conditions. Our study showed that ATRA alone can induce FOXP3+ T cells. Kim et al also revealed that ATRA in combination with low levels of TGF- β (produced by T cells themselves or present in culture media) can induce FOXP3+ T cells.³ In the present study, ATRA gave rise to ROR γ t increase but this increase was mostly in ROR γ t+ FOXP3+ cells which

have been suggested to be cells in transition during Th17 or Treg responses or a distinct subset of cells that stably co-express ROR γ t and FOXP3 with exclusive functional properties.¹⁷ Recent studies have mentioned the existence of double positive cells from FOXP3 and ROR γ t that produce IL-10 instead of IL-17.⁷ Like some other studies,¹⁴⁻¹⁶ we observed ROR γ t decrease after ATRA addition to the culture media, which implied a shift of immune responses from Th17 to Treg type. Our results also showed cells coexpressing T-bet and FOXP3 and these cells slightly increased after ATRA administration. These results demonstrated the need to assess several transcription factors at the same time in order to be able to evaluate cells positive for more than one transcription factor.

The optimal concentration of ATRA for in-vitro cell culture applications is 2-10 nM which is considered to be a physiological concentration range.^{18,19} However, a number of *in vitro* studies, higher than physiological levels of retinoic acids were used.²⁰ Our study showed that treatment of the cells *in vitro* with higher than physiological levels of ATRA (up to 1 μ M) do not affect lymphocyte viability. A study performed by Ueki et al on human eosinophils showed that retinoic acids did not show an adverse effect on eosinophils and ATRA, dose-dependently delayed eosinophil apoptosis in doses ranging from 1 μ M to 1 nM.¹⁹ Their study also confirmed that the effect of DMSO (used to make ATRA stock aliquots) was negligible at the concentrations between 1 μ M- 0.1 nM.¹⁹ A study performed on dendritic cells (DCs) revealed dose-dependent increased cell death during the generation of DCs particularly at the time of their differentiation.⁵ In another study by Liu PT et al, doses of ATRA higher than 0.1 μ M were reported to be toxic on monocytes.⁴ It can be concluded that ATRA affects different cells in different ways.

In accordance with the study by Nozaki et al,¹³ our results showed that PBMC and CD4+ T cell proliferations were not affected by doses of ATRA up to 1 μ M. We observed that the same number of PBMCs and CD4+ T cells with the same PHA stimulation, showed dissimilar ODs. It is supposed that PBMCs form numerous small colonies when stimulated with PHA, but CD4+ T cells generate big similar colonies and that's the reason for their different OD results.

When CD4+ T cells were stimulated with anti-CD3 and anti-CD28, we observed that 1 μ M up to 1 nM doses increased cell activation. Thus it can be summed

up that higher-than- physiological concentrations used in *in vitro* studies might cause CD4+ T cell activation and it is safe to use these doses in terms of cell viability and proliferation.

Furthermore, our results showed that ATRA in neutral conditions and without polarizing cytokines could affect human Th1, Th17 and Treg cells and change their balance towards anti-inflammatory state. Future studies with long-term cultures are needed to evaluate the stability of double positive cells and the application of ATRA in human inflammatory diseases.

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