New insight into the immunomodulatory mechanisms of Tretinoin in NMRI mice

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ARTICLE INFO	ABSTRACT
	Objective(s): Recent evidence have proposed that Tretinoin produced in the gut preferentially promote differentiation of FoxP3+Treg cells but inhibits Th17 lymphocytes, and this may be the main immunomdulatory mechanism of Tretinoin *in vivo*. This study was done to investigate the effects of Tretinoin in outbred white mice after challenge with sheep red blood cells (SRBC). ***Materials and Methods:*** Twenty male NMRI-mice randomly allocated in two equal groups. Mice were treated with 1×10^9 SRBCs emulsified in CFA intraperitoneally twice with one weak interval. Animals were bled 5 days after last injection. Moreover, 48 hr before bleeding time, 1×10^9 SRBCs were injected into the left hind foot pad of mice. Tretinoin (25 mg/kg-every other day) were intraperitoneally injected into the treatment group from the beginning of the study and continued throughout the study. The levels of anti-SRBC antibody and the specific cellular immune responses were measured by microhemagglutination test and footpad thickness, respectively. Moreover, splenocytes were checked for proliferation rate, respiratory burst, cytokine production and FoxP3+Treg cells frequency. ***Results:*** Tretinoin markedly alleviated cellular immunity and concurrently potentiated humoral immunity after mice challenge with SRBCs. Furthermore, aside from reducing NBT reduction and lymphocyte proliferation, Tretinoin markedly suppressed the secretion of interleukin-17 and conversely, increased the production of interleukin-10. However, the level of IFN-γ and the frequency of FoxP3+Treg cells did not alter significantly. ***Conclusion:*** The *in vivo* immunomudlatoty effects of Tretinoin may be partly due to immune deviation from pro-inflammatory cytokine interleukin-17 to anti-inflammatory cytokine interleukin-10, but not absolutely depend on the expansion of FoxP3+Treg cells.

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Introduction

Retinoids are a class of natural and synthetic derivatives of vitamin A which play important functions in embryogenesis, vision, growth and reproduction (1, 2). Tretinoin (all-*trans* retinoic acid) is an active metabolite of retinoids and modulate a wide range of biologic processes such as cell proliferation, differentiation and survival via specific family of nuclear receptors, the retinoic acid receptors (RARs), which function as ligand-inducible transcription factors (2-5).

The concept that vitamin A derivatives are important for immunity goes back to the early 20th century when Edward Mellanby and his colleague Harry Green reported on vitamin A as anti-infective agents (6). In this regard, Tretinoin has been shown to exert anti-neoplastic and immunomodulatory properties (3, 4). It has been clinically administrated for the treatment of cancers such as acute promyelocytic leukemia and Kaposi sarcoma and inflammatory diseases including acne and psoriasis (3). Previous reports showed that Tretinoin alleviated inflammatory responses and tissue destruction and attenuated a variety of autoimmune diseases in animal models, such as rheumatoid arthritis, type I diabetes, inflammatory bowel disease and experimental autoimmune encephalomyelitis (7-11).

Recent evidence indicated that immune responses are finely regulated by the opposing effects of Th-17 and FoxP3+Treg lymphocytes (12, 13). On the other hand, some new documents indicated that Tretinoin produced by CD103+ dendritic cells in the gastrointestinal lumen preferentially promotes generation of FoxP3+ Treg cells but concurrently inhibits generation of Th17 cells in an antigen specific manner, and this may be the main immunomdulatory mechanism of Tretinoin *in vivo* (5, 14).

The present study was done to investigate the effect of Tretinoin on T-helper cells responses and its effects on humoral and cellular immunity in mice after challenge with sheep red blood cells (SRBC).

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Materials and Methods

Reagents

Tretinoin, Complete Freund's adjuvant (CFA), Nitro blue tetrazolium, dioxin, dimethyl sulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St.Louis, MO). RPMI 1640 and fetal calf serum were bought from GIBCO/Life Technologies Inc. (Gaithersburg, MD). The cytokine assay by enzyme-linked immunosorbent assay (ELISA) kits for interferon gamma (IFN-γ), interleukin (IL)-10 and IL-17 were procured from Bender MedSystems (Vienna, Austria). Mouse Regulatory T Cell Staining Kit was purchased from eBioscience (San Diego, CA).

Animals

Six to eight-week-old male NMRI mice were purchased from Pasteur Institute of Iran. The animals were housed for one week before the experiment, maintained under constant condition of temperature (22-24℃) and 12 hr light/dark cycle and received food and water ad libitum. Animal welfare was observed in compliance with the regulations of the Ministry of Health, IR Iran approved by the Medical Ethics Committee of the University for Animal Studies.

Experimental design, Immunological challenge and evaluation

Mice were randomly allocated into 2 groups: Control mice and Treatment group. Each group had 10 animals. Tretinoin (2.5 mg/ml) was suspended in 2% DMSO and stored at -80℃ as aliquots before use. Tretinoin (25 mg/kg body weight, every other day) were intraperitoneally injected into the treatment group from the beginning of the study (onset of immunization) and continued throughout the study when the mice were bled. This dosage was chosen in accordance with pervious works on murine autoimmune models (8, 15-18). Normal control mice received an equal volume of PBS containing 2% DMSO with the same schedule of treatment group.

When the experiment began, animals were intraperitoneally immunized twice with one weak interval by 1×10^9 sheep red blood cells (SRBC) emulsified in CFA. Mice were bled from their hearts 5 days after last injection and the levels of anti-SRBC antibody were measured by microhemagglutination test as described previously (19).

Moreover, 48 hr before bleeding time, 1×10^9 SRBCs in 50 μl of PBS were administered subcutaneously into the left hind foot pad of each mouse and simultaneously the same volume of PBS was injected into the right foot pad as a negative control.

Footpad thickness was measured before bleeding time with a dial caliper and the mean percentage increase in footpad thickness was measured according to the following formula: [(Thickness of left footpad] _ (Thickness of right footpad] * 100/ (Thickness of right footpad).

Cytokines production

Spleen cells were aseptically isolated from mice at bleeding time. In brief, single-cell suspensions of splenocytes were prepared in RPMI 1640 medium supplemented with 10% fetal calf serum and red blood cells (RBCs) were removed by RBC lysis buffer. Next, cell suspensions (2×10^6 cells/ml) were incubated in 24-well plates and pulsed with 50 μl PHA solution (1 mg/ml). The culture supernatants were collected after 72 hr. IFN-γ, IL-17 and IL-10 production were assumed by ELISA according to the manufacturer's instructions.

Splenocytes proliferation

Proliferation potential of lymphocytes in splenocyte population was evaluated by MTT assay. The splenocytes were plated in 96-well flat-bottomed plates in RPMI 1640 medium supplemented with 10% fetal calf serum (1×10^5 cells/100 μl/well) and stimulated with 50 μl PHA solution (1 mg/ml) or medium alone. After 72 hr incubation, cultures were pulsed with 20 μl of the MTT solution (5 mg/ml) for 4 hr at 37℃. Then 150 ml DMSO was added and shaken vigorously to dissolve formazan crystal. The optical density (OD) at 550 nm was measured using microplate reader (Dynatech, Denkendorf, Germany). The experiments were done in triplicate sets. The results were expressed as the proliferation index according to the ratio of OD_{550} of stimulated cells with MOG_{35-55} to OD_{550} of non-stimulated cells.

Respiratory burst in splenocyte population

Respiratory burst of phagocytic cells in splenocyte population was checked using NBT dye reduction as described previously with some modification (20-22). In brief, 100 μl of suspension of splenocytes with 0.1 ml of *Staphylococcus aureus* suspension (108 cell/ml) and 0.1 ml of 0.1% NBT in PBS (pH=7.4) were mixed. The mixture was incubated at room temperature for 15 min and subsequently kept at 37℃ for additional 15 min. The reduced dye was extracted in dioxan and quantitated at 520 nm.

Treg analysis by flow cytometry

Mouse Regulatory T Cell Staining Kit was used to check the $FoxP3^+CD4^+CD25^+$ regulatory T ($FoxP3^+Treg$) cells according to manufacturer's instructions. Briefly, 100 μl of freshly prepared splenocytes (1×10^6 cells) were stained with anti-mouse CD4 and anti-mouse CD25 for 30 min at 4℃. After staining surface markers, cells were incubated with fixation/permeabilization working solution for 30 min at 4℃. Then, the cells were washed with
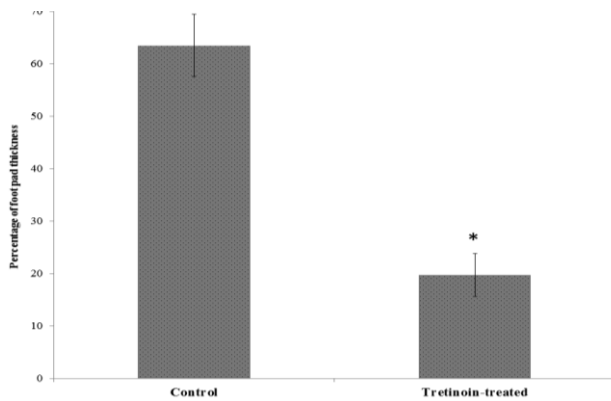
Figure 1. Effects of administration of Tretinoin on cellular immunity. Mice were intraperitoneally immunized twice with one weak interval by 1×10^9 sheep red blood cells (SRBC) emulsified in CFA. 3 days after last intraperitoneal immunization, 1×10^9 SRBCs in 50 μl of PBS were administered subcutaneously into the left hind foot pad of each mouse and the same volume of PBS was injected into the right foot pad as a negative control. Footpad thickness was measured before bleeding time with a dial caliper and the mean percentage increase in footpad thickness was measured. The values were presented as mean ± S.D. (*$P<0.001$ versus control mice)
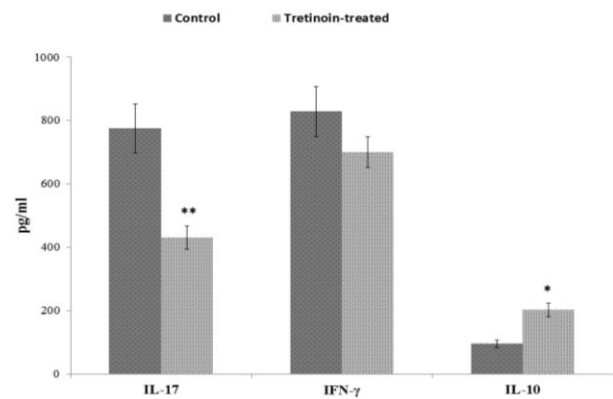

Figure 2. Cytokines production assay after treatment with Tretinoin. Spleen cells isolated from immunized mice with SRBC were cultured with 50 μl of PHA (1 mg/ml) for 72 hr. The levels of IFN-γ, IL-17 and IL-10 in culture supernatants were determined after 72 hr by ELISA. The results were shown as mean ± SD (*$P<0.01$, ** $P<0.001$ versus control mice)

permeablilization buffer and stained with anti-mouse Foxp3 antibody or isotype control at 4^0 for at least 30 min in dark. The cells were then washed with permeabilization buffer and suspended in flow cytometry staining buffer. All samples were analyzed on DAKO flow cytometer (Partec, Germany), using Cyflogic software (version: 1.2.1).

Statistical analysis

Data were analyzed by Student's_t-test and presented as means ± SD. *P*-values less than 0.05 were considered statistically significant.

Results

Footpad thickness after challenge with SRBC was performed as an indicator for evaluation of delayed type of hypersensitivity (DTH) reaction. As shown in Figure 1, Tretinoin-treated mice showed significantly lower DTH responses than the control mice. Conversely, mean antibody titer in treatment group (260±11.22) was significantly higher than the mean antibody titer in control mice (35.87±9.76) (*P*<0.001).

A significant decrease in IL-17 and a significant increase in IL-10 secretion in cells from Tretinoin-treated mice were found compared to cells from vehicle-treated group (Figure 2). However, the level of IFN-γ was diminished in treatment group but this reduction was not significant (Figure 2). Moreover, splenocyte proliferation and respiratory burst showed a significant reduction in Tretinoin-treated mice compared to the normal control animals (Figure 3).

Evaluation method of Foxp3⁺Treg cells is shown in Figure 4A, B, and C. No significant change in the expression of these cells was observed in the
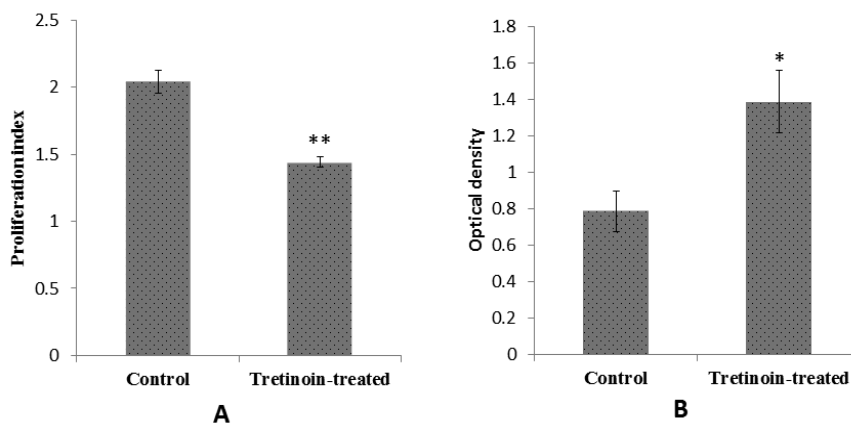

Figure 3. Effects of Tretinoin administration on lymphocytes proliferation and respiratory burst in phagocytic cells. Splenocytes were isolated from sensitized mice with SRBC. A) Splenocytes cultured with 50 μl PHA solution (1mg/ml) for 72 hr. Then, lymphocytes proliferation were evluated by MTT test. B) splenocytes with *Staphylococcus aureus* suspension and NBT were mixed and incubated for 30 min as detailed under materials and methods. The reduced dye was extracted in dioxan and quantitated at 520 nm. The values were presented as mean ± SD (*$P<0.01$, ** $P<0.001$ versus control mice)
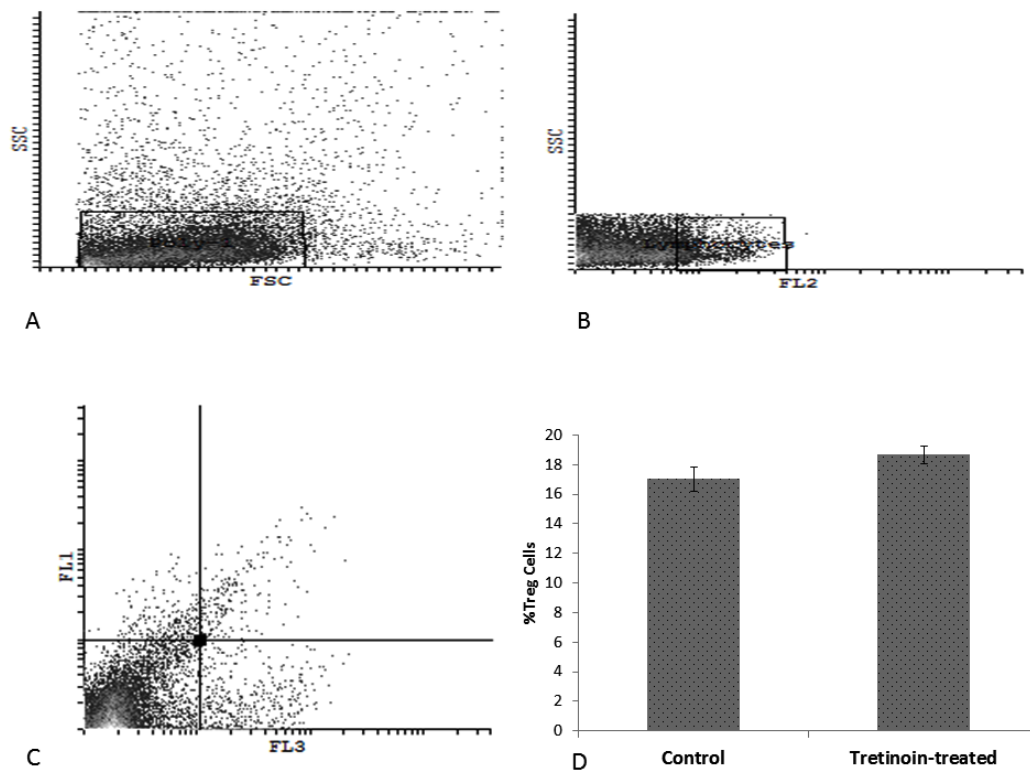
Figure 4. Evaluation of Tretinoin administration on FoxP3⁺Treg cells frequency. At the bleeding time, spleens were removed from immunized mice with SRBC. A single-cell suspension was prepared, and the cells were stained as detailed under materials and methods. Representative dot plots illustrated the regions and gates for immune phenotypic analysis. Lymphocytes were gated on a forward vs. side scatter dot plot (A) and CD25⁺ T cells were gated by CD25/side scatter futures (B). Then, we analyzed CD4⁺ FoxP3⁺on CD25⁺ gated cells (C). (FL1, CD4⁺; FL2, CD25⁺; FL3, FoxP3⁺). Flow cytometry analysis demonstrated that. No significant change in the expression of CD4⁺CD25⁺FoxP3⁺ was observed in the treatment group compared to the control mice (D). Data were shown as mean±SD

treatment group compared to the control mice (Figure 4D).

Discussion

Recently, several lines of evidence showed that retinoic acid derivatives play an essential role in the differentiation of T cell subsets and the migration of T cells into tissues (6). T cell-mediated immunity has a substantial role in determining the extent of organ-specific autoimmune diseases (23-25). DTH is one of the typical response patterns of T cell-mediated immunity (25). The first requirement for DTH reaction is the priming of a special effector class of antigen specific T cells (26). DTH response has long been believed to be mediated by Th1 cells (27, 28). However, this concept was doubted because mice deficient in components of the IL-12/Th1 axis including IL-12α (IL-12p35), IFN-γ or IFN-γ receptor were more susceptible to autoimmunity. This deficiency in understanding of immunopathology was resolved by discovery of IL-17-producing CD4⁺ T cells (Th17) (23, 27, 29). IL-17 (also called IL-17A) has a potent pro-inflammatory property (30) and is a crucial factor for promotion of DTH induction (26). It seems that Th17 cells imitate the inflammatory response, while Th1 cells determine the tissue damage (28). Overall, it seems that Th17 cells are more pathogenic than Th1 cells in immunopathologic conditions (30).

After immunizations with CFA, killed mycro-bacteria produce the microenvironment for the Th17 polarization of the T cells that are specific for the antigen mixed into the adjuvant (20). Interestingly, immunizations with complete Freund's adjuvant (CFA) initiate mixed Th1 and Th17 immunity in the absence of Th2 immunity, while injection with alum or incomplete Freund's adjuvant (IFA) promote highly polarized Th2 responses (31).

Our results showed that treatment with Tretinoin after challenge with SRBC significantly suppressed IL-17 production and consequently DTH reaction. However, the level of IFN-γ did not show any significant differences between groups. These findings offer new insight into the potential mechanisms underlying the immunomodulatory effects of Tretinoin.

FoxP3⁺Treg cells have an crucial role in maintaining of self-tolerance through their inhibitory properties on effector T lymphocytes especially during Th17 immune responses (32). *In vitro* studies have been demonstrated that Tretinoin can diminish Th17 differentiation and reciprocally promote differentiation of FoxP3⁺ Treg cells (3, 33, 34). Despite the *in vitro* studies, Tretinoin in our study did not have any significant effect on FoxP3⁺ Treg cell population. A

previous study showed a significant reduction in Th17 cells generation without any significant change in Treg cells development in mice orally infected with *Listeria monocytogenes* and treated with Retinoic acid. Authors reported that TGF-β might be a limiting factor in the decline of Treg cell expression induced by exogenous Retinoic acid *in vivo* (35). Also, it has been showed that the high level of IL-6 could suppress de novo generation of antigen specific Treg cells (36). Of note, TGF-β is an essential factor for development of FoxP3⁺Treg, while TGF-β plus IL-6 promote generation of Th17 cells (12). Therefore, we propose that minor expansion of Treg cells following Tretinoin administration may be due to the profound production of inflammatory cytokines (such as IL-6) or conversely low level production of TGF-β in cytokinic milieu. However, these remain to be clarified.

IL-10 is a cytokine with anti-inflammatory properties and plays an important role in suppressing and terminating macrophage activity in DTH reactions and subsequent preventing of tissue destruction (37, 38). This cytokine was profoundly increased in Tretinoin treatment group.

Significant reduction in IL-17 production and conversely increase in the level of IL-10 could explain the decrease of the respiratory burst in mononuclear phagocytic cells in mice treated with Tretinoin.

In general, cellular and humoral arms of immunity are reciprocally regulated (25). Therefore, increasing the humoral immune response following reducing DTH reaction in the Tretinoin treatment group is not far-fetched. In this regard, pervious evidence showed that retinoic acid promotes antibody responses to T cell–dependent antigens (6).

Altogether, immune deviation from cellular immunity to humoral responses concurrent with a significant decrease in lymphocyte proliferation may be explaining some beneficial effect that reported following Tretinoin therapy in some organ-specific autoimmune models.

Conclusion

The *in vivo* immunomudlatoty effects of Tretinoin may be partly due to immune deviation from pro-inflammatory cytokine IL-17 to anti-inflammatory cytokine IL-10, but not absolutely depend on the expansion of FoxP3⁺Treg lymphocytes. However, other mechanisms may also be involved, and these remain to be clarified.

Acknowledgment

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