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ORIGINAL ARTICLE

Role of Chicoric Acid and 13-*Cis* Retinoic Acid in *Mycobacterium tuberculosis* Infection Control by Human U937 Macrophage

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Abstract

Mycobacterium tuberculosis (Mtb) survives and proliferates within the main cells of the innate immune system, macrophages. The goal of our study was to investigate the immunostimulatory effects of 13-*cis* retinoic acid (RA) and chicoric acid (CA) in human U937 macrophages against H37Ra Mtb infection by evaluating its potential role in the cell surface expression of HLA-DR, CD14 molecules as well as nitric oxide (NO) production and prevention of the Mtb growth within macrophages. In this study, we investigated the effects of 13-*cis* RA and CA on Mtb-infected macrophages using flowcytometry and Griess methods, respectively. Moreover, inhibitory effect of 13-*cis* RA and CA on Mtb growth within macrophages were assessed using colony-forming unit. 13-*Cis* RA and CA enhanced the cell surface expression of HLA-DR and CD14 molecules on U937 macrophages and prevented the growth of Mtb within macrophages. In addition, 13-*cis* RA and CA, have increased NO generation compared to untreated control macrophages, significantly ($p < 0.001$). Both drugs have a significant inhibitory effect on Mtb growth but CA at the highest concentration was more potent than 13-*cis* RA ($p < 0.05$). The results of our study showed that infected U937 macrophages treated with 13-*cis* RA and CA represented significant increases in NO production, CD14 and HLA-DR expression and also prevents intracellular survival of Mtb. Therefore, 13-*cis* RA and CA may have a significant therapeutic approach in the control of Mtb infection.

Keywords *Mycobacterium tuberculosis* · 13-*Cis* retinoic acid · Chicoric acid · U937 macrophages

Introduction

Tuberculosis (TB) is an infectious chronic disease caused by *Mycobacterium tuberculosis* (Mtb). Mtb is a slow-growing bacterium with a doubling time of 18 h. Mtb grows and survives within macrophages and this chronic interaction lasting years. The ability of Mtb to grow and survive persistently inside macrophages before the primary acute phase can be ascribed to the activation of immune mechanism of macrophages. Nitric oxide contributes substantially to the control of chronic Mtb infection through stimulation of heat-shock protein (HSP) production. HSP starts stationary phase of mycobacteria growth (Cunningham-Bussel et al. 2013; Joseph et al. 2012; Ryndak et al. 2015). One-third of the world people are infected with Mtb and at risk of manifesting the clinical symptoms of TB disease (Estrella et al. 2011). Therefore, control and treatment of TB remains a very important health problem worldwide.

Regarding this issue, the implementation of medicinal plants in the therapy of various diseases has always been an interesting field of the investigations for developing new medications. Herbal drugs are capable to enhance host resistance to infections. There are many herbal medicine with immunostimulatory and antimicrobial properties for treatment of many infectious diseases (Enioutina et al. 2017).

For example, *Echinacea* is an herbal plant, including nine species. *Echinacea* has numerous pharmacological functions. *Echinacea angustifolia, Echinacea pallida*, and *Echinacea purpurea* have been used in the United States and Europe (Zhai et al. 2007). *Echinacea* is used in the treatment of the upper respiratory tract infections (Caruso and

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Gwaltney 2005; Islam and Carter 2005), wound damage (Speroni et al. 2002) inflammation (Raso et al. 2002; Speroni et al. 2002) and tumors (Currier and Miller 2002). The most consistent result of many studies indicate that *Echinacea* has immunomodulatory properties (Barrett 2003). For instance, *Echinacea* showed profound effects on immune cell number, (Cundell et al. 2003) granulocyte migration, cytotoxicity (Currier and Miller 2000, 2001), macrophage phagocytosis, and cytokine production (Cundell et al. 2003; Goel et al. 2002).

In the present study, we examined the immunostimulatory roles of cichoric acid [chicoric acid (CA), or dicaffeoyltartaric acid]. CA is derived from caffeic acid and tartaric acid (Kuban-Jankowska et al. 2016). CA is the major phenolic component of *E. purpurea* (Mølgaard et al. 2003). It was found in many plants, such as lemon balm, dandelion leaves, sea grass, algae, horsetail, fern, basil, chicory and lettuce (Lee and Scagel 2013). CA is one of the active constituents of *E. purpurea* and has been shown to have benefits for human health (Barnes et al. 2005; Lee and Scagel 2010).

Furthermore, several studies have been reported that CA stimulates phagocytosis in vitro and in vivo (Zhai et al. 2007). In addition, CA has antioxidant (Dalby-Brown et al. 2005; Grignon-Dubois and Rezzonico 2013), antiviral (Crosby et al. 2010; Pellati et al. 2004) and immunostimulatory properties. Moreover, the antiproliferative (Elansary and Mahmoud 2015) and anti-cancer effects of CA have been demonstrated in several studies (Tsai et al. 2012).

In addition to CA, we also evaluated the immunostimulatory properties of 13-*cis* retinoic acid (RA). Previous studies have shown that both 13-*cis* RA and vitamin D augment the anti-mycobacterial function of human macrophages by regulation of vitamin D receptor/retinoid X receptor (VDR/RXR) response sequence (Anand et al. 2008).

In the present study, we investigated the expression of CD14 and HLA-DR on Mtb H37Ra infected macrophages after treatment with 13-*cis* RA and CA. Macrophages express several types of receptors with different immunologic functions. Some mediate cross-talk with T cells (HLA-DR, CD1d, CD80, CD86), while other receptors-like CD14 bind mycobacterial cell wall components altering cytokine signaling, moreover, CD14 may make U937 more responsive to chemokines enabling homing, infiltration, and bacterial detection (Estrella et al. 2011). Since, CD14 functions as an outstanding main pattern recognition receptor of the innate immunity, mediating inflammation, signal transduction activation including NF-κB and MAPK pathways, synthesis of cytokines such as TNF-α, IL-1, IL-6, IL-8, IL-18, increase of phagocytic ability, enhancement of leukotriene synthesis thus plays an important role in immune response, inflammation and tissue homeostasis (Beschorner et al. 2002).

While, HLA-DR expression on monocytes has been detected to correlate highly with infection in many clinical conditions. HLA-DR have fundamental and remarkable role in the processing and presentation of antigen by macrophages to helper T cells. Several studies described that the HLA-DR expression on monocytes was decreased in patients with sepsis (Lekkou et al. 2004). Hence, CD14 and HLA-DR expression levels are point to Mtb H37Ra-infected macrophages activation after treatment with 13-*cis* RA and CA.

Previous studies have demonstrated that human monocytoid cell line, U937 cells could be infected with live Mtb in vitro, and the U937 cell line is an appropriate model for Mtb drug discovery and pathogenesis researches (Forero et al. 2005; Wang et al. 2015). The phorbol 12-myristate 13-acetate (PMA) induce differentiation of U937 macrophages and provide a model that resembles human alveolar macrophages (Passmore et al. 2001).

Due to the problems associated with the antibiotic therapy such as drug resistance and toxicity, the present study was conducted for finding alternate methods for treating TB. However, there are no studies that have examined the effects of 13-*cis* RA and CA on H37Ra Mtb-infected U937 macrophage. To date, the immunostimulatory effects of 13-*cis* RA and CA on H37Ra Mtb-infected U937 macrophage have not been investigated. Furthermore, the effects of 13-*cis* RA and CA on H37Ra Mtb-infected U937 macrophage are not fully understood. The goal of our study was to investigate the immunostimulatory effects of 13-*cis* RA (isotretinoin) and CA in PMA-induced human U937 macrophages against Mtb H37Ra infection by evaluating its potential role in the cell surface expression of HLA-DR and CD14 molecules as well as nitric oxide (NO) production and prevention of the Mtb growth within macrophages.

Materials and methods

Mycobacterium tuberculosis culture

Avirulent *M. tuberculosis* H37Ra was obtained from Iranian National Research center for Tuberculosis and Lung Diseases. Mtb H37Ra were grown to log phase at 37 °C in Middlebrook 7H9 Medium (Difco Laboratories, Detroit, MI, USA) enriched with 10% OADC Enrichment which consists 0.05% (w/v) oleic acid, 5% (w/v) bovine serum albumin, 2% (w/v) dextrose, 0.85% (w/v) NaCl, and 0.004% (w/v) catalase (all from Sigma, UK). Moreover, 0.2% (v/v) glycerol and 0.05% (v/v) Tween 80 were added to the medium. Mycobacteria were cultured under aeration condition and diluted to a concentration of McFarland's no. 1 standard (~ 10^8 bacilli/ml) and stored at − 80 °C in 7H9 broth containing 20% glycerol.

Differentiation of U937 Monocytic Cell Line to Macrophage

The U937 cell was grown in RPMI-1640 medium enriched with 10% heat-inactivated fetal bovine serum (FBS), 2.5 mM of L-glutamine, 100 U/ml of penicillin, and 100 U/ml of streptomycin (all from Gibco, UK) at 37 °C and 5% CO_2 in humidified incubator. U937 cells were stimulated with 100 nM PMA (Sigma, UK) for 3 days to permit differentiation into macrophages. The medium was replaced with fresh RPMI plus 10% FBS and cells were cultured for 48 h before beginning the experiments. Macrophage differentiation was checked by microscopic examination of cell morphology.

Infection of U937 Macrophages with Mtb H37Ra

Mycobacterial clumps were interrupted by passing the culture through a 27-gauge needle. Differentiated U937 human macrophages were infected with Mtb H37Ra at a multiplicity of infection of 5–10 bacteria per cell and incubated for 4 h at 37 °C in 5% CO_2. Then, extracellular Mtb H37Ra was removed by successive washes with RPMI medium (4–5 times).

Macrophage Surface Molecules Analysis

Differentiated macrophages were stimulated for three days using varying doses of 13-*cis* RA (Sigma, UK) and CA (Sigma, UK) (500 and 1000 μM) and were infected with Mtb H37Ra. The infected cells were washed once with phosphate-buffered saline (PBS) and stained, on ice for 30 min, with anti-human CD14 fluorescein isothiocyanate (FITC) and HLA-DR phycoerythrin (PE) antibodies (eBioscience, USA). The cells were post-fixed with 2% paraformaldehyde before analysis using BD Facscan.

Mycobacterial Survival Assay

Bacterial loads were determined 72 h after infection of U937 macrophages treated with or without 13-*cis* RA and CA (500, 1000, and 1500, 2000 μM). For colony-forming unit (CFU) assessment, macrophages were lysed with 0.25% sodium dodecyl sulfate in PBS, were plated on Middlebrook 7H10 medium supplemented with 10% OADC and incubated at 37 °C for 21 days.

NO Production Measurement

The NO production by infected macrophages treated with or without 13-*cis* RA and CA (500, 1000, and 1500 μM) was measured using the Griess reagent (Sigma, UK). The cell culture supernatants were collected and centrifuged at 4000 rpm for 10 min to eliminate debris. Thereafter, 100 μl of culture supernatants were used to determine nitrite concentration. The absorbance was measured at 540 nm, after 15-min incubation at room temperature, using a spectrophotometer. The concentration of nitrite in samples was computed using a standard reference curve of known nitrite concentrations prepared in RPMI medium.

Macrophage Viability

Macrophage viability was determined by the Trypan blue exclusion dye in all steps of the experiment. The cells were washed with sterile PBS. Then, 0.09 ml of 0.4% Trypan blue dye was added to 0.01 ml of the cell suspension (1:10) and live and dead cells were counted under an optical microscope using Neubauer chambers. The percentage viability was accounted as (number of live cells/total number of cells) × 100.

Statistical Analysis

The analysis was carried out using Prism 5.0, GraphPad software. Experiments were performed independently, and where appropriate, summary results are presented as means ± SEM. The group means were compared by one-way ANOVA and post hoc Tukey's multiple comparison test. Differences were considered significant for $p < 0.05$.

Results

13-*Cis* RA and CA Increase CD14 and HLA-DR Expression

In the present study, 13-*cis* RA and CA (500 and 1000 μM) had effects on HLA-DR and CD14 expression of U937 macrophages, and significantly were upregulated in U937 activated with 13-*cis* RA and CA compared to PMA and in general there was a dose–response to 13-*cis* RA and CA. The results are shown in Fig. 1. Moreover, the difference between the effects of 13-*cis* RA and CA on CD14 and HLA-DR was significant ($p < 0.05$). The viability of marophage cultures were not show significant differences and approximately were 95%.

13-*Cis* RA and CA Inhibit Growth of Mtb H37Ra In Vitro

In the present study, immunostimulatory effect of 13-*cis* RA and CA on U937 macrophages infected with Mtb H37Ra was assessed by determining CFU. 13-*Cis* RA and CA were used at final concentrations of 500, 1000, 1500, 2000 μM and growth inhibition of Mtb H37Ra in macrophages, was determined by CFU assay. 13-*Cis* RA and

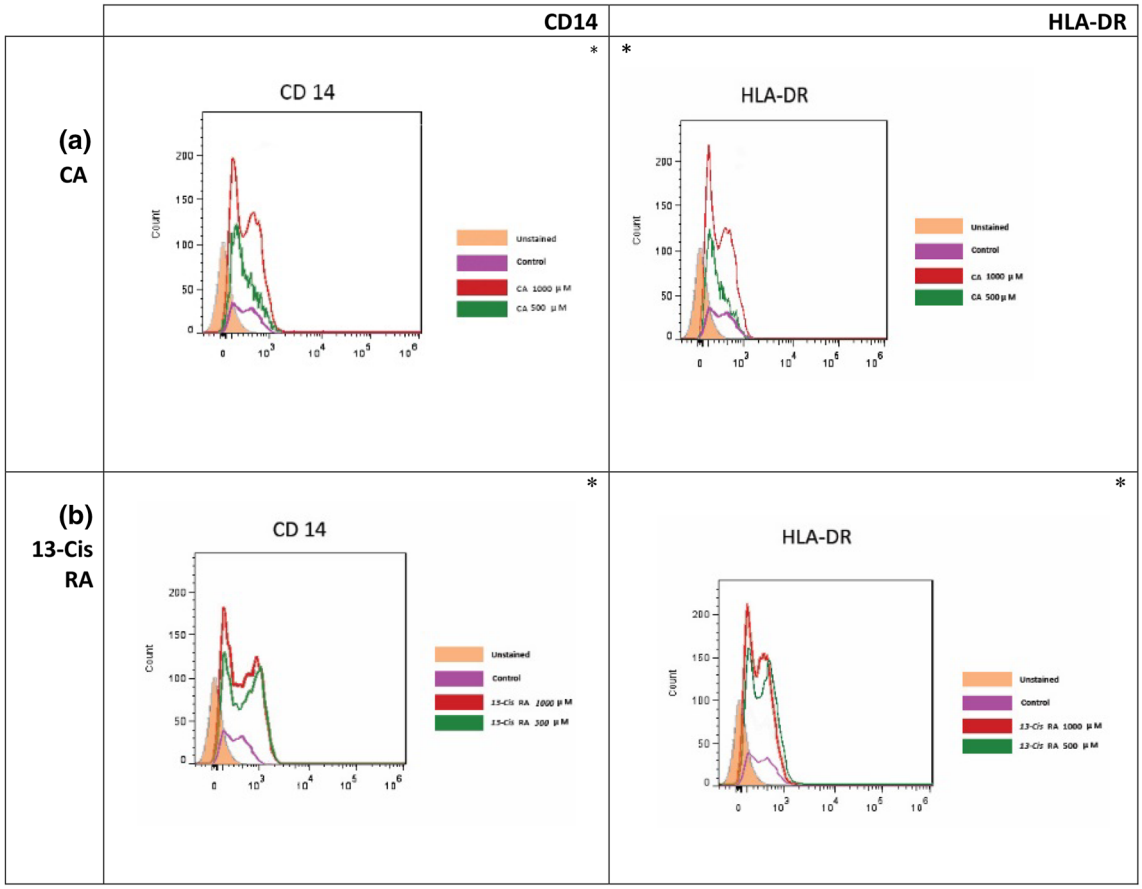

Fig. 1 13-*Cis* RA and CA increase the expression of surface receptors on human U937 macrophages. Mtb H37Ra-infected U937 macrophage were treated with 13-*cis* RA and CA (500 and 1000 µM); afterward were stained for surface receptors using either fluorescent antibodies and analyzed using Flowjo software. 13-*Cis* RA **(a)** and CA **(b)** significantly enhanced the expression of macrophage HLA-DR and CD14 compared to Mtb H37Ra-infected control U937. $*p \leq 0.05$

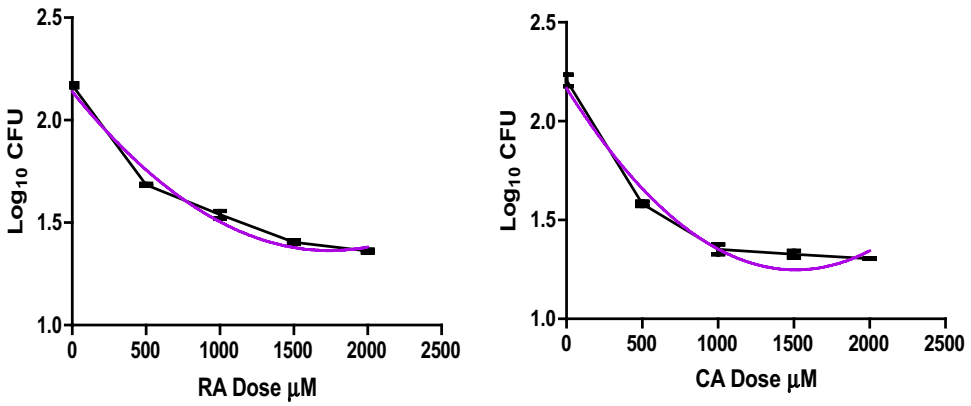

Fig. 2 13-*Cis* RA and CA decrease the H37Ra Mtb growth in U937 macrophages. Disturbance in growth kinetics of Mtb in the presence of different concentrations of 13-*cis* RA and CA was monitored by enumerating CFUs on day 21. Graphs were plotted on log scale. The purple curve is the trend line. Experiments were carried out three times with duplicate cultures and results were analyzed using one-way ANOVA test and reported as mean ± SEM. Both drugs have a significant inhibitory effect on Mtb growth but CA significantly was more potent at the highest concentration (> 1000 µM) than 13-*cis* RA

CA were able to inhibit the growth and survival of Mtb H37Ra (Fig. 2). Although both drugs have a significant inhibitory effect on Mtb growth but CA significantly was more potent at the highest concentration (> 1000 µM) than 13-*cis* RA in the inhibition of Mtb survival ($p < 0.05$).

13-*Cis* RA and CA Increase NO Production

A key mycobactericidal effector mechanism of phagocytic cells is production of NO by inducible NO (iNOS) synthetase. In fact, nitrite is the indirect marker of NO production, and was measured in U937 macrophages in response to Mtb H37Ra infection. Treatment of infected macrophages with 13-*cis* RA and CA (500, 1000 and 1000 µM) induced significant nitrite production compared control cells (Table 3). Figure 3 clearly shows that treatment of macrophages with 13-*cis* RA and CA, have significantly increased NO generation compared to untreated control macrophages.

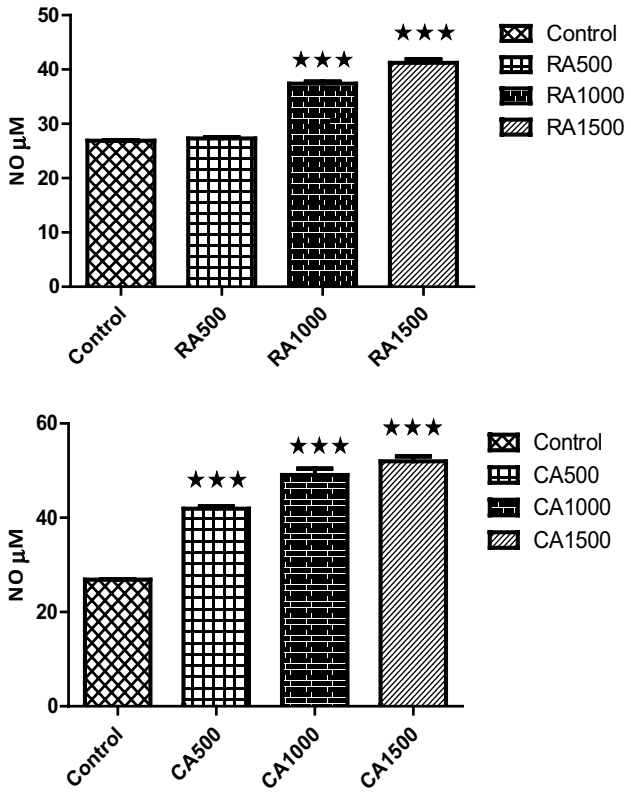

Fig. 3 13-*Cis* RA and CA increase the expression of NO by U937 macrophages. Mtb-infected U937 macrophages were treated with 13-*cis* RA and CA (500, 1000 and 1500 µM). NO production (µM) was measured using Griess reagent. The results demonstrates as means ± SEM (standard error of mean). ***$p \leq 0.001$

Discussion

In the present study, we observed that 13-*cis* RA and CA were able to activate the U937 macrophages against Mtb H37Ra. The results of our study indicates that surface molecules CD14 and HLA-DR expression increased on infected macrophages. In addition, 13-*cis* RA and CA enhanced production of nitric oxide which lead to restrain Mtb H37Ra growth compared to untreated macrophages.

The stimulatory effect of 13-*cis* RA on macrophage responses to Mtb might be including respiratory burst, autophagy, and antimicrobial protein production (Kramer et al. 1993). Previous studies have demonstrated the stimulatory effect of RA on dendritic cell differentiation and migration. RA also boosts the tumor immunity by increasing the macrophages within the tumor (Pino-Lagos et al. 2010). Previous investigation showed that vitamin A and its metabolites (all-trans retinoic acid, 9-*cis* RA, 13-*cis* RA) as well as nuclear RA receptors have important role in the regulation of innate and adaptive immunity (Kramer et al. 1993).Therefore, our finding of a select benefit of 13-*cis* RA on Mtb-infected U937 macrophages could confirm the immunostimulatory effects of 13-*cis* RA for enhancing the immune responses against Mtb infection.

In addition, CA has been shown to have anti-HIV activity (Crosby et al. 2010; Pellati et al. 2004), immunostimulatory properties, inducing phagocytosis (Goel et al. 2002), increase immune cell number (Cundell et al. 2003), granulocyte migration, natural killer cytotoxicity (Currier and Miller 2000) and cytokine production (Cundell et al. 2003; Goel et al. 2002). Moreover, CA is a high free-radical scavenger (Barnes et al. 2005; Pellati et al. 2004). It has been reported that antibacterial properties of CA against *Yersinia* sp. bacteria due to decreasing the activity of YopH virulence factor has been described (Kuban-Jankowska et al. 2016). Interestingly, our study supported the effects of CA on activation of infected macrophages.

As we show in Table 1, the expression of CD14 and HLA-DR on human U937 macrophages treated with 500 and 1000 µM concentration of 13-*cis* RA and CA increased significantly compared to untreated macrophages. Besides, 13-*cis* RA in comparison to CA was more potent in stimulation of CD14 and HLA-DR expression on infected macrophages. It seems that, 13-*cis* RA and CA-mediated activation of macrophage function in response to Mtb H37Ra was dependent on CD14 expression, which were similar to Anandaiah study that showed signaling of IB/NF-κB in infected macrophages was dependent on CD14 expression (Anandaiah et al. 2013). However, there was no significant difference between the percentages of HLA-DR compared to CD14 expression, this suggests that infected macrophages can express similarly these markers in response

Table 1 13-*Cis* RA and CA increase expression of HLA-DR and CD14 on human U937 macrophages

	HLA-DR expression Mean ± SEM	CD14 expression Mean ± SEM
13-*Cis* RA		
Control	37.2 ± 0.28	31.45 ± 0.55
500 µM	49.4 ± 0.28	48.95 ± 0.48
1000 µM	58.05 ± 0.23	55.185 ± 0.85
CA		
500 µM	45.4 ± 0.78	49.55 ± 0.8
1000 µM	49.95 ± 0.27	52 ± 0.64

Mtb H37Ra-infected U937 macrophage were treated with 13-*cis* RA and CA (500 and 1000 µM); afterward were stained for surface receptors using either fluorescent antibodies and analyzed using Flowjo software. 13-*Cis* RA and CA significantly enhanced the expression of macrophage HLA-DR and CD14 compared to control macrophages ($p < 0.05$)

to treatments. In fact, CD14 is one of the mediators in inflammation, signal transduction activation and cytokine production. In addition, HLA-DR have a central role in the antigen presentation by macrophages to helper T cells (Estrella et al. 2011). Thus, CD14 and HLA-DR trigger innate immunity subsequent to immune defense.

Prasad et al. (2012) reviewed that the deficiency of vitamin A in patients with TB might have participated to the progression of TB disease. A study by Estrella et al. (2011) showed that RA and vitamin D were able to activate macrophages due to the induction of NADPH oxidase. It has been reported that vitamins A and D cause dose-dependent inhibition of *Mycobacterium avium* and Mtb complex. The results of this study have shown that vitamin D, vitamin A and its metabolites prevent mycobacterial growth in vitro (Greenstein et al. 2012; Prasad et al. 2012). Our study supported this hypothesis as shown in Table 2, Mtb H37Ra infected-U937 macrophages were activated after treatment with 13-*cis* RA resulted in killing of most Mtb, significantly. This suggested that 13-*cis* RA mediated special antimicrobial activities of infected macrophages in a dose-dependent manner (Fig. 2).

It is postulated that mycobacterium recruits tryptophan aspartate-containing coat protein (TACO) into the phagosomes and prevents its fusion with the lysosome (Anand and Kaul 2005; Anand et al. 2008). It was ascertained that RA has anti-tuberculosis activity. RA and vitamin D augment the activity of human macrophages against Mtb in vitro through downregulating the TACO gene via regulation of VDR/RXR response sequence (Ferrari et al. 1999).

Several studies have reported that a low level of vitamin A by evaluating retinol in the blood of TB patients compared to healthy controls (Ramachandran et al. 2004). The ligand 3 for RXR receptor is 9-*cis* RA (Germain et al. 2006) and the isomers of RA (i.e., all-trans RA, 13-*cis* RA, and 9-*cis* RA) exist in equivalence (Marill et al. 2003). Srinivasan et al. (2013) reported that vitamin A deficiency among adults with tuberculosis, the concentrations of 13-*cis* RA in the plasma and pleural fluid of patients with TB were also less than that of the detected values in the healthy volunteers. Moreover, the results of Aibana's study in 2017 have explained that the association between vitamin A deficiency and TB development. Vitamin A supplementation among persons at high risk for TB may prepare a safe, and effective tool of impeding progression from latent TB infection to disease (Aibana et al. 2017).

Consequently, these evidences are consistent with our results to credit that 13-*cis* RA may prevent the survival of the mycobacteria in the macrophages. Thereby, it offers a promising therapeutic approach for control of Mtb infection.

Furthermore, we investigated the effects of CA on survival of Mtb H37Ra infected-U937 macrophages. Regarding CFU results, Mtb growth were inhibited in the presence of CA, while survived in untreated control. Moreover, CA significantly is more potent at the highest concentration (> 1000 µM) than 13-*cis* RA in the inhibition of Mtb survival (Table 2).

Previous studies have demonstrated that when the concentration of NO is greater than 1 µM, reactive nitrogen oxide species causes oxidative and nitrosative damage by changing DNA, inhibiting enzyme function, and inducing lipid peroxidation, which participate in the antimicrobial effects of NO (Schairer et al. 2012). In this study, we could confirm our assumption that treatment of Mtb-infected macrophages with these drugs increased NO production significantly (Table 3) that resulting in intracellular killing of Mtb. Our data indicate that various concentrations of

Table 2 13-*Cis* RA and CA decrease Mtb H37Ra growth in U937 macrophages

	Control	500 µM	1000 µM	1500 µM	2000 µM
Reduction in growth (Log10) of Mtb H37Ra					
13-*Cis* RA	2.17 ± 0.01	1.69 ± 0.005	1.54 ± 0.018	1.41 ± 0.009	1.36 ± 0.007
CA	2.17 ± 0.01	1.58 ± 0.011	1.35 ± 0.025	1.32 ± 0.018	1.30 ± 0.002

Table shows values of $\log_{10}$ scale of Mtb H37Ra growth. The growth of Mtb in treated macrophages decreased compared to control macrophages, significantly ($p < 0.0001$)

Table 3 13-*Cis* RA and CA increase NO production in U937 macrophages

	Control	500 µM	1000 µM	1500 µM
NO production (µM)				
13-*Cis* RA	26.88 ± 0.067	27.31 ± 0.16	37.41 ± 0.37	41.22 ± 0.64
p value		NS	< 0.001	< 0.001
CA	26.88 ± 0.067	41.90 ± 0.52	49.06 ± 1.36	51.96 ± 1.06
p value		< 0.001	< 0.001	< 0.001

Table demonstrates amounts of NO production by Mtb-infected U937 macrophages treated with various concentrations of 13-*cis* RA and CA compared to control macrophages. The results of these experiments were analyzed using one-way ANOVA test.

NS non-significant

CA in comparison to 13-*cis* RA increased NO production, significantly. Thus, CA is more effective than 13-*cis* RA in activation of iNOS to produce NO.

Although the effects of CA on increasing phagocytosis and natural killer cytotoxicity were observed, there have been few dedicated studies on the effects of CA on Mtb growth.

In conclusion, the results of our study showed that infected U937 macrophages treated with 13-*cis* RA and CA represented significant increase in NO production, CD14 and HLA-DR expression and also prevented intracellular survival of Mtb. The potential benefit of 13-*cis* RA and CA supplementation in the treatment of Mtb disease has not yet been established. The current finding that 13-*cis* RA and CA activate macrophage in vitro suggests a potential therapeutic role for partial supplementation for persons at risk for Mtb infection. However, the precise cellular mechanisms leading to these effects of 13-*cis* RA and CA on macrophages are required to more studies. Interestingly, Fratazzi et al. (1997) have shown that the U937 macrophages behave similarly to primary macrophages when infected with Mtb. Moreover, according to previous study about the features of avirulent and virulent strains the differences are not significant. Paul et al. (1996) observed that H37Ra and H37Rv are similar in their capacities to enter human macrophages, phagocytosis is mediated through complement receptors as well as growth rates in human macrophages. Thus, presumably the effects of 13-*cis* RA and CA on macrophage infected with virulent strain of Mtb are similar to avirulent strain.

This study provides the rationale to pursue additional in vitro investigations to allow the design of appropriate clinical trials to define the role of 13-*cis* RA and CA as a preventive or therapeutic adjuvant for Mtb infection.

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