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Anti-inflammatory Effect of Resveratrol and Polydatin by *In Vitro* IL-17 Modulation

Giulia Lanzilli,[1] Andrea Cottarelli,[2] Giuseppe Nicotera,[1] Serena Guida,[1] Giampiero Ravagnan,[2] and Maria Pia Fuggetta[1,3]

Abstract—Interleukin-17 (IL-17) is a proinflammatory cytokine produced, although not exclusively, by T helper 17 recently identified as a distinct T helper lineage mediating tissue inflammation. IL-17 is known to be involved in a number of chronic disorders although the mechanisms regulating its production in inflammatory disease are still unclear. The beneficial properties of the polyphenolic compound resveratrol including its anti-inflammatory, antioxidant, and antitumor effects, its role in the aging process and in the prevention of heart and neurodegenerative diseases are well-known. In addition, derivatives of resveratrol, including glucosylated molecules as polydatin have been linked to similar beneficial effects. We have investigated the effects of resveratrol and polydatin on the *in vitro* production of IL-17 in a model of inflammation *in vitro*. The results obtained by activated human peripheral blood mononuclear cells, stimulated with anti-CD3/anti-CD28 monoclonal antibodies and treated with these polyphenolic compounds at different concentrations show that both decrease IL-17 production in a concentration-dependent manner. This study confirms the anti-inflammatory activity of resveratrol and its derivatives and suggests a potential clinical relevance in the therapy of inflammatory diseases.

KEY WORDS: resveratrol; polydatin; interleukin 17; inflammation.

INTRODUCTION

Basic and clinical research provides evidence that inflammatory mechanisms play a central role in the pathogenesis and progression of many pathological conditions.

In particular, chronic inflammatory diseases are characterized by chronic or relapsing immune activation, and there is growing evidence that cytokine deregulation is responsible for the occurrence of these inflammatory and autoimmune pathologies [1–3]. Moreover, inflammation is an important environmental factor that promotes tumorigenesis and progression of established cancerous lesions, and recent studies have started to analyze the mechanisms linking the two pathologies [4]. It is therefore evident that modulation of cytokine production by T cells can play a fundamental role in the control of various pathologies, and substances able to modulate this production could have important immunopharmacological and therapeutic effects.

The family of CD4+ T cells includes different subtypes of T-helper (Th) lymphocytes characterized by specific cytokine profiles: Th1 cells produce interleukin 2 (IL-2), interferon-γ, and tumor necrosis factor (TNF) and are mainly responsible for cell-mediated immunity, such as retarded hypersensitivity; Th2 cells produce other types of interleukins such as IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 and are mainly responsible for the regulation of tumor immunity; T helper 17 (Th17) cells, produce IL-17 family, IL-22, IL-21, and CCL20 and have a relevant role in immune response to fungi and extracellular pathogens and inflammatory disorders [5, 6]. Moreover, Th17 cells have been characterized as potent inducers of tissue inflammation in several autoimmune diseases, such as inflammatory bowel disease, psoriasis, multiple sclerosis, systemic lupus erythematosus, rheu-

Giulia Lanzilli and Andrea Cottarelli contributed equally to this work.

[1] Institute of Translational Pharmacology (IFT), National Council of Research, Rome, Via Fosso del Cavaliere 100, 00173 Rome, Italy
[2] GLURES, Academic SPINOFF, Ca Foscari University of Venice, Venice, Italy
[3] To whom correspondence should be addressed at Institute of Translational Pharmacology (IFT), National Council of Research, Rome, Via Fosso del Cavaliere 100, 00173 Rome, Italy. E-mail: mariapia.fuggetta@ift.cnr.it

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matoid arthritis, and Bechet's disease through the activation of a wide range of inflammatory mediators. The production of IL-17 indeed induces the secretion of chemokines and other cytokines, such as IL-6, IL-8, PGE2, MCP-1, or G-CSF from many kinds of adherent cells like fibroblasts, keratinocytes, epithelial, and endothelial cells [7, 8]. Moreover, the Th17 response has been recently implicated in several models of infection [9].

Evidence from human diseases suggests that, like Th1 and Th2 cells, the *in vivo* development of Th17 cells from naïve T cells is dependent on antigen presentation by professional antigen presenting cells, co-stimulatory stimulation, and a specific cytokine milieu [10]. In addition, the differentiation factors responsible for their generation have revealed an interesting reciprocal relationship with regulatory T (T_{reg}) cells, which prevent tissue inflammation and mediate self-tolerance. Furthermore, cytokines that promote Th17 differentiation are distinct from those that promote Th1 and Th2 differentiation. Usually, when the activity of the effector cells is excessive and not balanced by regulatory cells of the immune system, there is the increased risk for development of autoimmune diseases. Therefore, in many autoimmune diseases, the activity of Th17 exceeds that of T_{regs} [11–13].

Interleukin-17 is the founding member of a group of cytokines called the IL-17 family. The IL-17 family is comprised of at least six members, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. Prototype member of the family has been designated IL-17A.

Currently, in addition to Th17 cell subset, a recent literature describes other cells producing IL-17 in response to stress, injury, or pathogens. These innate immune cells have been identified as: γδ T cell subset that has an important role at the level of mucosal barrier; iNKT cells, which are activated in response to self and non-self glycolipid antigens presented by the non-polymorphic MHC class I-like CD1d molecule; and *Paneth* cells that are highly specialized epithelial cells involved in mucosal homeostasis. They reside in diverse anatomical locations—mainly in the lungs, liver, skin, gut, and secondary lymphoid organs where they have a broad range of immune regulatory functions that has yet to be fully understood [14].

Moreover, IL-17 R mRNA exhibits a broad tissue distribution and has been detected in all cells and tissues tested [15]. Functional studies have provided evidence that IL-17 acts on epithelial cells, endothelial cells, fibroblasts, synoviocytes, and myeloid cells to induce secretion of a variety of mediators including IL-8, CXC ligand (CXCL) 1, CXCL6, IL-6, granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor, TGF-β, TNF-α, and IL-1β [16, 17].

Polyphenols, including anthocyanins, flavonoids, and stilbenes, constitute one of the most abundant and ubiquitous group of plant metabolites and are an integral part of the human diet. They are recognized for their beneficial implications in human health such as in the treatment and prevention of cancer and cardiovascular and neurodegenerative diseases. The wide range of biological effects exhibited is generally believed to be the outcome of their powerful antioxidant properties *in vitro*, which are described in numerous publications [18].

Resveratrol (3,4′,5-trihydroxy-trans-stilbene, RES) is a non-flavonoid polyphenol found in several dietary sources, such as grape seeds, berries, peanuts, and red wine.

Resveratrol exists as both the *trans*- and *cis*-isomer with numerous reports suggesting *trans*-resveratrol to be the more stable form in nature and the most bioactive form of this molecule. *Trans*-resveratrol can be readily converted to *cis*-resveratrol when exposed to UV light and is unstable when exposed to high pH. In addition to resveratrol isomers, derivatives of resveratrol that include its glucosylated, prenylated, methylated, and hydroxylated modifications have been linked with beneficial activities [19, 20].

In particular, polydatin, also known as piceid (resveratrol-3-*O*-β-mono-D-glucoside, POLY) is a glucoside of resveratrol in which the glucoside group bonded in position C-3 substitutes a hydroxyl group. This substitution gives rise to conformational changes of the molecule, resulting in changes in the biological properties. Piceid is more resistant to enzymatic oxidation than resveratrol, is soluble in water and unlike resveratrol which penetrates the cell passively it enters the cell via an active mechanism using glucose carriers [21]. This property makes polydatin a molecule endowed with greater bioavailability respect to resveratrol.

Resveratrol is known to have various beneficial effects such as anti-cancer, antioxidative, anti-inflammatory, and cholesterol-lowering activities besides prophylactic effects on cardiovascular diseases. Resveratrol has been shown to suppress proliferation of a wide variety of human tumor cells *in vitro* which has led to numerous preclinical animal studies to evaluate its cancer preventive and therapeutic potential. Moreover, it has been reported that resveratrol exhibits immunomodulatory properties showing antioxidant and anti-inflammatory activities [19, 22, 23]. In particular, Petro TM in its review [24] point out

the potential therapeutic use of resveratrol, for autoimmune disease through the modulation of Th17 and T_{reg} cells.

Similarly to resveratrol, polydatin is able to scavenge free radicals, inhibit platelet aggregation, and oxidize LDL [25, 26].

In this work, we evaluated the effects of different concentrations of RES or POLY on normal human lymphocytes activated *in vitro* with an inflammatory stimuli. The anti-inflammation outcome of RES and POLY was investigated and compared by evaluating the releasing of proinflammatory IL-17 cytokine in activated T cells.

The evaluation of the immunomodulating effects of RES or POLY was performed using PBLs stimulated via CD3/CD28 molecule activation. This method is currently used to analyze the production of interleukin Il-17 in patients with inflammatory diseases as polymyositis and dermatomyositis, or Crohn's disease and ulcerative colitis [27, 28]. Moreover, this technique, simply and reproducible, permits easy assess to the individual responses in the clinic.

MATERIALS AND METHODS

Chemicals

Natural RES and POLY were extracted and kindly supplied by Dr. Fulvio Mattivi, (Fondazione Edmund Mach, Istituto Agrario di San Michele all'Adige (IASMA) Italy). The purity of both compounds tested by HPLC-MS, UV, and NMR was higher than 99% (method patented and described in) [29, 30].

RES was dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, USA) at 100 mM stock solution, whereas POLY was dissolved in Dulbecco's modified Eagle's medium (DMEM,GIBCO Laboratories, Grand Island, NY, USA) at 100 mM. All stock solutions were stored at −80°C and diluted in culture medium just prior to use.

Isolation and Culture of Human Mononuclear Cells

Buffy coats were obtained from peripheral blood of healthy donors. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-hypaque gradient centrifugation (Sigma-Aldrich Corp.). The cells at the interface were removed, washed three times by centrifugation at 400×*g* for 10 min and finally resuspended at the appropriate concentration in RPMI-1640 GLUTAMAX (GIBCO) medium supplemented with 1% glutamine, 1% penicillin/streptomycin, and 10% heat-inactivated (56°C, 30 min) fetal calf serum (Hyclone Laboratories, Logan, UK), hereafter referred to as complete medium (CM).

Treatment of PBMC with RES or POLY and Measurement of Il-17 in Culture Supernatants

Isolated PBMC, suspended at the concentration of 10^6cells/ml in CM, were treated with 1 μg/ml of anti-CD3 mAb (Clone HIT3a, PharMingen International) and incubated at 37°C in 5%CO2 for 3 days. At the end of incubation, the anti-CD3-stimulated lymphocytes were washed, resuspended in CM containing 1 μg/ml of anti-CD28 mAb (clone CD28.2, PharMingen International), and incubated at 37°C in 5% CO_2 for additional 3 days.

Immediately before antibody stimulation PBMC were treated with graded concentration (0.01, 0.1, 1, 2.5, 5, 10, and 20 μg/ml) of RES or POLY. As control, the cells were treated with vehicle: DMSO and DMEM, respectively, for RES and POLY treatments.

Cells were analyzed for their cytokine-producing capability on day 6 at the end of treatments. Cell proliferation and viability were determined by the Trypan blue dye exclusion method by manually counting the cells with a hemocytometer. All experiments were carried out in triplicate.

Culture supernatants of cells stimulated with anti-CD3/anti-CD28 monoclonal antibodies and treated with different concentrations of RES or POLY were collected for IL-17 detection. The test was carried out by ELISA quantitative sandwich enzyme immunoassay technique (ELISA kit Quantikine, h-IL-17 immunoassay, R&D Systems, Minneapolis, USA). The detection limit of the assays was approximately 15.0 pg/ml and is specific for natural and recombinant human IL-17.

Flow Cytometric Analysis

Cytotoxic effects of RES and POLY treatment were evaluated by flow cytometric analysis. Stimulated and unstimulated lymphocytes, treated with RES and POLY at different concentrations (2,5, 5, 10, and 20 μg/ml) and control cells grown only in culture medium, were fixed in 70% ethanol, washed in PBS, stained with propidium iodide (PI) 50 μg/ml and incubated in presence of 100 KU/ml of RNAse in the dark at room temperature for 30 min. Cellular fluorescence was measured with a FACscan flow cytometer (Becton Dickinson, Mountain View, CA, USA) using an argon ion laser emitting at 488 nm. Ten thousand events were counted for each sample, and data collection was gated utilizing forward light scatter and side light

scatter to exclude cell debris and aggregates. Apoptotic cells are represented by a broad hypodiploid peak below the G1 peak (cells with a fractional DNA content) in the red fluorescence channel. This fraction is representative of cells with decreased staining for PI as an indicator for DNA fragmentation associated with apoptotic cell death. All data were recorded using the CellQuest software (Becton & Dickinson).

IL-17 mRNA Expression-RT-PCR

Total RNA was extracted from human mononuclear cells using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. The RNA was extracted from: (1) non-activated cells, (2) cells stimulated with anti-CD3/anti-CD28, and (3) cells stimulated with anti-CD3/anti-CD28 and treated with 20 μg/ml of POLY. Reverse transcription-PCR was carried out with the PCR CORE kit (Applied Biosystem, Roche) using random hexamers and 1 μg total RNA for first-strand synthesis. The cDNA encoding IL-17 was amplified using the oligonucleotides IL-17 forward (5′-ATGACTCCTGG-GAAGACCTCATTG-3′) and IL-17 reverse (5′-ATGACTCCTGGGAAGACCTCA TTG-3′) by 40 PCR cycles of 95° for 30 s, 70° for 30 s, and 72° for 30 s. As control, the constitutive human β_2 microglobulin cDNA was PCR amplified using the oligonucleotides β_2 forward (5′- GAA TTG CTA TGT GTC TGG GT- 3′) and β_2 reverse (5′- CAT CTT CAA ACC TCC ATG ATG- 3′). The amplified products were electrophoresed on 2% agarose gel in 1× TAE (Tris acetate EDTA).

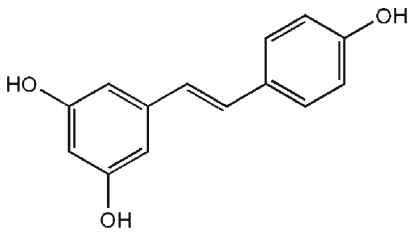
Statistical Analysis

Results are expressed in terms of means (M)± standard error (SE) of the mean. Statistical significance was determined by Student's test analysis.

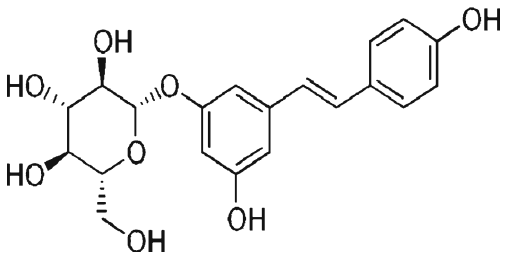
RESULTS

Effect of RES and POLY Treatment on IL-17 Production in Activated PBMC *In Vitro*

Mononuclear cells isolated from peripheral blood of human healthy donors were treated with RES or POLY at different concentrations (0.01, 0.1, 1, 2.5, 5, 10, and 20 μg/ml) and activated with monoclonal anti-CD3/anti-CD28 antibodies. After 6 days, cell samples were evaluated for IL-17 production in culture supernatants by ELISA immunoassay (Fig. 1).



Resveratrol



Polydatin

Fig. 1. Chemical structure of Resveratrol and Polydatin.

The results illustrated in Fig. 2 show that, at higher concentrations, RES and POLY treatments result in a dose-dependent inhibition of IL-17 production by stimulated PBMC. The results show that POLY treatment of activated PBMC induce a marked inhibition IL-17 statistically stronger of the inhibition obtained with

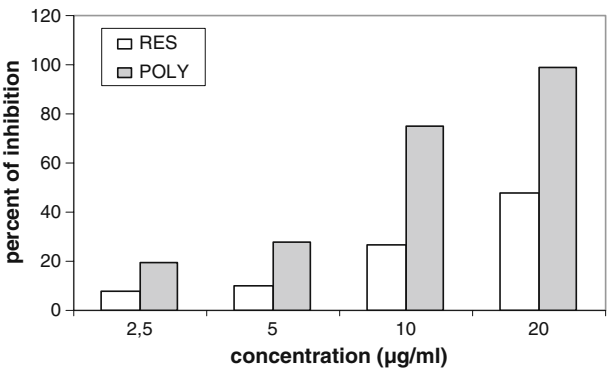


Fig. 2. Effect of RES and POLY on IL-17 production by PBMC activated with anti-CD3/ANTI-CD28 monoclonal antibodies. The results are expressed as percent inhibition of IL-17 production in samples (-means of triplicate) treated with different concentrations of RES or POLY respect to untreated stimulated PBMC. The cytokine production was detected by ELISA immunoassay.

RES at the same concentration. In particular, POLY treatment at 20 μg/ml induces an almost complete inhibition (98%) whereas RES treatment at 20 μg/ml shows no more than a 50% of inhibition.

On the other hand, at low concentration (0.01 μg/ml), only RES induces a significant increase of IL-17 produced by stimulated PBMC (Fig. 3a), whereas, POLY treatment at the same concentrations did not show any significant change, although a slight decrease of cytokine production can be observed early, starting from 1 μg/ml (Fig. 3b).

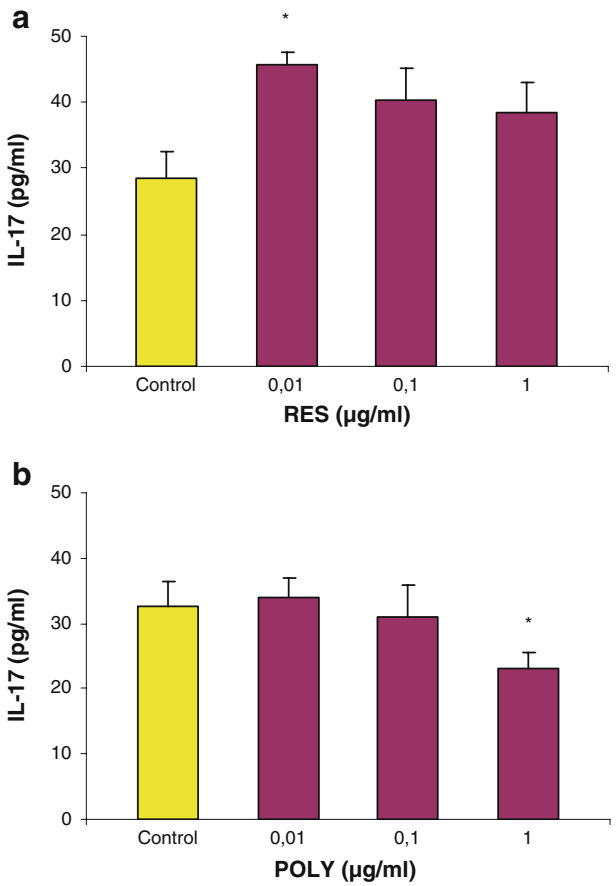


Fig. 3. Effect of low concentrations of RES or POLY on activated lymphocytes. The results are expressed in terms of IL-17 production (picograms per milliliter) in PBMC treated with different low concentrations of RES or POLY respect to untreated control. The Il-17 production was detected by ELISA immunoassay. In panel **a**, are showed the effects of treatment of PBMC with RES at 0.01, 0.1, and 1 μg/ml concentrations respect to untreated control (only vehicle, DMSO). *Bars* represent the SE of the mean.*$P<0.01$ (Student's*t* test). In panel **b**, are showed the effects of POLY at 0,01 0,1 1 μg/ml concentrations respect to untreated control (only vehicle, DMEM) *Bars* represent the SE of the mean.*$P<0.05$ (Student's*t* test).

Effect of RES and POLY Treatment on Cytotoxicity of Activated PBMC

To exclude that, downregulation of IL-17 in culture supernatants was due to cell cytotoxicity by RES or POLY treatment, cultured cells were manually counted to evaluate dead cells by TB exclusion. Data show that cell counts after stimulation and treatment remain substantially unaffected respect to control cells (data not shown).

To evaluate whether RES or POLY treatment induced apoptosis, we tested cultured lymphocytes by flow cytometry analysis. Briefly, unstimulated or stimulated lymphocytes, treated with 2.5, 5, 10 e 20 μg/ml of RES or POLY were fixed in acetone/methanol 1:4 in 50% PBS and labeled with PI 1 μg/ml. The results in Fig. 4 show that RES or POLY treatment does not induce a proapoptotic effect both in unstimulated or stimulated cells. Only at 20 μg/ml of POLY treatment a slight significative increase in the number of apoptosis can be observed, both in activated (5.2%) or unstimulated (5.9%) samples.

Effect of RES or POLY on the Expression of IL-17 mRNA in Anti-CD3/ANTI-CD28 Activated Lymphocytes

Accordingly with data from inhibition of IL-17 activity, IL-17 expression seems to be inhibited by POLY at RNA level as well. IL-17 cDNAs were transcribed and amplified from differently treated human mononuclear cells and results from a representative experiment are shown in Fig. 5. The IL-17 mRNA was clearly down-regulated in cells treated with POLY *vs* cell-activated and not activated, suggesting its possible regulatory role at transcriptional or post transcriptional level.

DISCUSSION

IL-17 is a cytokine involved in inflammatory conditions such as autoimmune diseases, psoriasis, arthritis, and inflammatory bowel diseases [10, 11, 13].

The data obtained in this study demonstrate the immunomodulating effects of RES and POLY in an *in vitro* model of inflammation. The *in vitro* production of IL-17 by activated PBMC treated with RES or POLY at different concentrations has been evaluated. The results showed that both RES and POLY decrease IL-17 production in a concentration-dependent manner. A strong inhibitory activity (approximately100%)

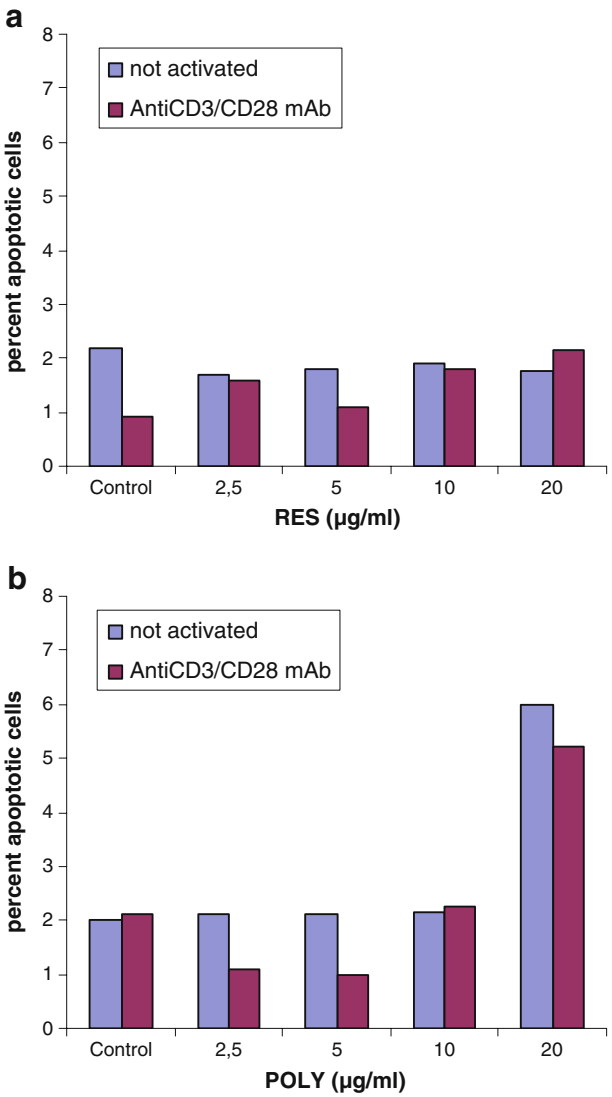


Fig. 4. Effect of RES and POLY on apoptosis induction in PBMC *in vitro*. Lymphocytes stimulated with antiCD3/antiCD28 mAbs, treated with RES (**a**) and POLY (**b**) at 2.5, 5, 10, or 20 μg/ml for 6 days, fixed and labeled with propidium iodide. The percentage of DNA loss was measured by flow cytometry and indicated as the percent of hypodiploid nuclei.

was obtained by POLY treatment at the concentration of 20 μg/ml. RES, at the same concentration, also induces a significant inhibition of IL-17 production, although the level of inhibition was lower (50%). These results suggest a more effectiveness of POLY in inhibiting IL-17 production. It is well known that in POLY, the hydroxyl group in position C-3 is replaced by a glucoside group that induce a conformational changes of the molecule and resulting in changed biological properties. POLY is more resistant to enzymatic oxidation than RES, it is soluble in water and, unlike RES which penetrates the cell passively, it enters the cell through glucose carriers [21, 25], thus, allowing maximum absorption and a better bioavailability. To establish whether RES and POLY could be active at low concentrations as well, we treated stimulated PBMC with either RES or POLY at 0.01, 0.1, and 1 μg/ml. The results showed that only RES at 0.01 μg/ml increases the IL-17 production. This finding is in agreement with our previous studies on immunomodulating activity of RES describing the effects on cell function and cytokine production in three models of *in vitro* immune response [31]. We previously demonstrated that *in vitro* treatment of immune cells with RES induces a biphasic effect: immunostimulation at low concentrations and strong inhibition at higher concentrations, for natural (NK cell) activity, antigen-specific immune responses and cytokine production. Son TG *et al.* successively confirmed this biphasic dose–response effect on different cells and pointed out the hormetic effect by several phytochemicals compounds [32].

RES is reported as a potent inducer of apoptosis [33, 34]. To exclude the possibility that a diminished amount of IL-17 after RES or POLY treatment was

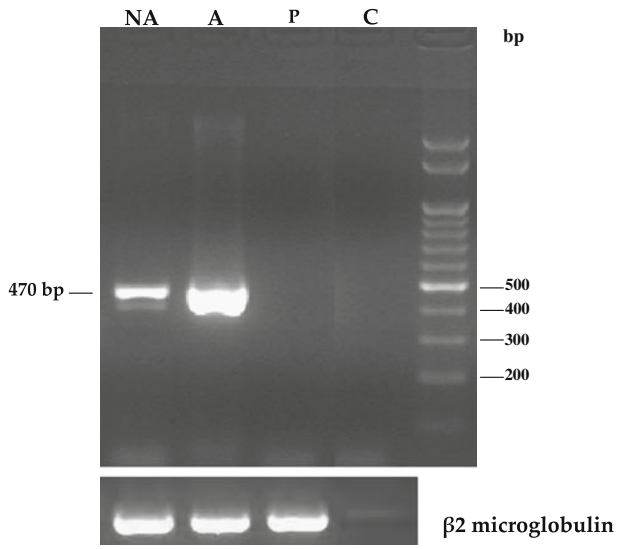


Fig. 5. Effect of poly on IL-17 transcription on treated and not treated PBMC. RT-PCR from untreated PBMC (lane *NA*), antiCD3/antiCD28 mAbs stimulated PBMC (lane *A*), and PBMC stimulated and treated with POLY at 20 μg/ml (lane *P*). *C* negative control.

related to a reduction of number of viable cells, we tested the effect of RES or POLY treatment on apoptosis induction. Flow cytometry analysis evidenced that only highest concentration of POLY induced a modest proapoptotic effect. These results are also in agreement with data from viable cell enumeration by TB exclusion (data not shown) and confirm that activated lymphocytes are more resistant to apoptotic cell death [35].

To further investigate the effect of POLY at the highest concentration (20 μg/ml), we studied the IL-17 gene expression at the molecular level in stimulated or unstimulated lymphocytes *in vitro*.

Accordingly, with data obtained about the IL-17 inhibition in supernatants, IL-17 expression seems to be inhibited in POLY-treated PBMC at RNA level. From preliminary data (Fig. 5), the IL-17 mRNA was clearly down-regulated in activated cells treated with POLY, suggesting its possible regulatory role at transcriptional or post-transcriptional level.

As previously described, uncommitted T helper cells can be induced to differentiate to specific lineages according to the local cytokine milieu, towards Th1, Th2, Th17, and T_{reg} phenotypes in a mutually exclusive manner [5–7]. Each phenotype is characterized by unique signaling pathway and expression of specific transcription factors, notably T-bet for Th1, GATA-3 for Th2, forkhead box P3 (FoxP3) for T_{regs} and receptor-related orphan receptor (ROR)alpha and RORgamma for Th17 cells. [11, 13, 36].

Overexpression of RORγt promotes Th17 differentiation; conversely, RORγt deficiency results in profound Th17 deficiency. However, RORγt defect did not completely abolish Th17 differentiation. Th17 cells also express another orphan nuclear receptor RORα, which is induced by TGF-β and IL-6 in a STAT3-dependent manner. Overexpression of RORα promotes Th17 differentiation and significantly upregulates IL-17 expression. RORα deficiency results in reduced IL-17 expression *in vitro* and *in vivo*. Furthermore, RORα and RORγt co-expression synergistically drive greater Th17 differentiation especially under non-favorable conditions [13, 37, 38].

Signal transducer and activator of transcription 3 (STAT3) acts as an important mediator in multiple biological processes induced by different cytokines [39]. RORγt the first transcription factor to be selectively expressed in Th17 cells is regulated by STAT3 [40]. Overexpression of a hyperactive STAT3 enhanced Th17 differentiation while STAT3 deficiency impairs Th17 differentiation *in vitro* [38, 39] and *in vivo* [41]. STAT3 has been shown to bind to IL-17 gene promoter [42] and appears to control IL-17 gene expression [43]. The precise biochemical function of STAT3 is unclear, but it could be supposed that it affects the regulation of lineage-specific master transcription factors expression. Moreover, recently, IL-21 was reported to regulate Th17 differentiation, as an autocrine factor induced by IL-6 [44]. STAT3 is also needed for IL-6 induction and is required for IL-21-mediated Th17 differentiation [41].

Resveratrol attenuates the activation of immune cells and modulates the subsequent synthesis and release of inflammatory mediators through the inhibition of transcriptional factors such as nuclear factor-kappaB [45]. Resveratrol has also been shown to control other various transcription factors as STAT3, HIF-1alpha, beta-catenin, and PPAR-gamma [46]. Resveratrol could significantly inhibit the JAK1/STAT3 signal transduction pathway, through down-regulation of pJAK1 and pSTAT3 expression and reducing the phosphorylation of JAK1 and STAT3 in a dose- and time-dependent manner [47].

It is reasonable to hypothesize that both RES and POLY, reducing the phosphorylation of STAT3, regulate RORγt, the first transcription factor selectively expressed in Th17 cells, and consequentially induce a reduction of IL-17 production. Further research is in progress in our laboratory to investigate this molecular pathway.

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