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Short communication

Hydroxytyrosol modulates Par j 1-induced IL-10 production by PBMCs in healthy subjects

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ABSTRACT

Several papers have demonstrated the importance of substances from natural sources which can exert powerful anti-inflammatory effects. In this respect, hydroxytyrosol, one of the major elements of the phenolic components of olive oil, has been extensively studied for its anti-inflammatory activities and safety profile. In this report, we demonstrate that the co-stimulation of human PBMCs from healthy subjects with the Par j 1 allergen and hydroxytyrosol induced a statistically significant increase in the amount of Par j 1-induced IL-10, demonstrating that hydroxytyrosol can modulate an allergen-specific immune response potentiating a suppressive immune response towards an allergen. Our work opens the way to further studies to elaborate the possibility of using hydroxytyrosol as a nutrient for allergy prevention.

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1. Introduction

In the last few decades, the prevalence of allergic reactions has significantly increased in industrialized countries, creating an urgent need for improved prophylactic and therapeutic approaches (Holgate and Broide, 2003). Peripheral tolerance to allergens involves multiple suppressive factors, but the key role played by the cytokine interleukin (IL)-10 in the suppression of IgE production and the induction of protective allergen-specific IgG antibodies has been demonstrated during the course of successful allergen specific immunotherapy and in healthy subjects (Palomares et al., 2014; Shamji and Durham, 2011).

Parietaria pollen is one of the major sources of allergenic molecules in the Mediterranean area, and its allergenic composition has been studied by our group, showing the importance of the two major allergens Par j 1 and Par j 2 (Colombo et al., 2003). In particular, we recently reported the immunological response induced by Par j 1, showing that this allergen was capable of activating components of the innate and adaptive immune system leading to the induction of IFN- γ and IL-10 producing cells in healthy subjects (Bonura et al., 2013).

Polyphenols are a group of secondary metabolites commonly found in plant products such as tea, fruit, extra-virgin olive oil, and so forth, which are typical components of the Mediterranean diet (MD) (Hu et al., 2014). In recent years, an increasing body of evidence has demonstrated that these products contain molecules which have strong protective effects against oxidative stress and degenerative diseases (Killeen et al., 2014). The main source of fat in the MD is olive oil, whose principal phenolic compounds are secoiridoids that, in the intestinal tract, are hydrolyzed to hydroxytyrosol (HT) and tyrosol (Visser et al., 2002). Within this group of molecules, HT exerts notable anti-inflammatory effects, acting on the NF- κ B transcription factor to reduce its activation and translocation to the nucleus (Killeen et al., 2014). Furthermore, it has been demonstrated that, in macrophages stimulated by lipopolysaccharide, HT inhibited the secretion of several well-known inflammatory cytokines, such as IL-1 α , IL-1 β , IL-6, IL-12 and TNF- α (Richard et al., 2011). HT can be adsorbed in a dose-dependent manner from olive oil in humans, and its bio-availability has been demonstrated in several *in vivo* studies (Visioli et al., 2000). In terms of its safety profile, HT did not show untoward effects, even when administered at very high doses in formulations coming from olive mill waste water; however, less is known about the use of pure HT (Aunon-Calles et al., 2013a,b). Several other health-promoting properties have been attributed to HT, including: antioxidant, cardioprotective, antitumoral, antimicrobial, antidiabetic and neuroprotective activities (Visioli and Bernardini, 2011),

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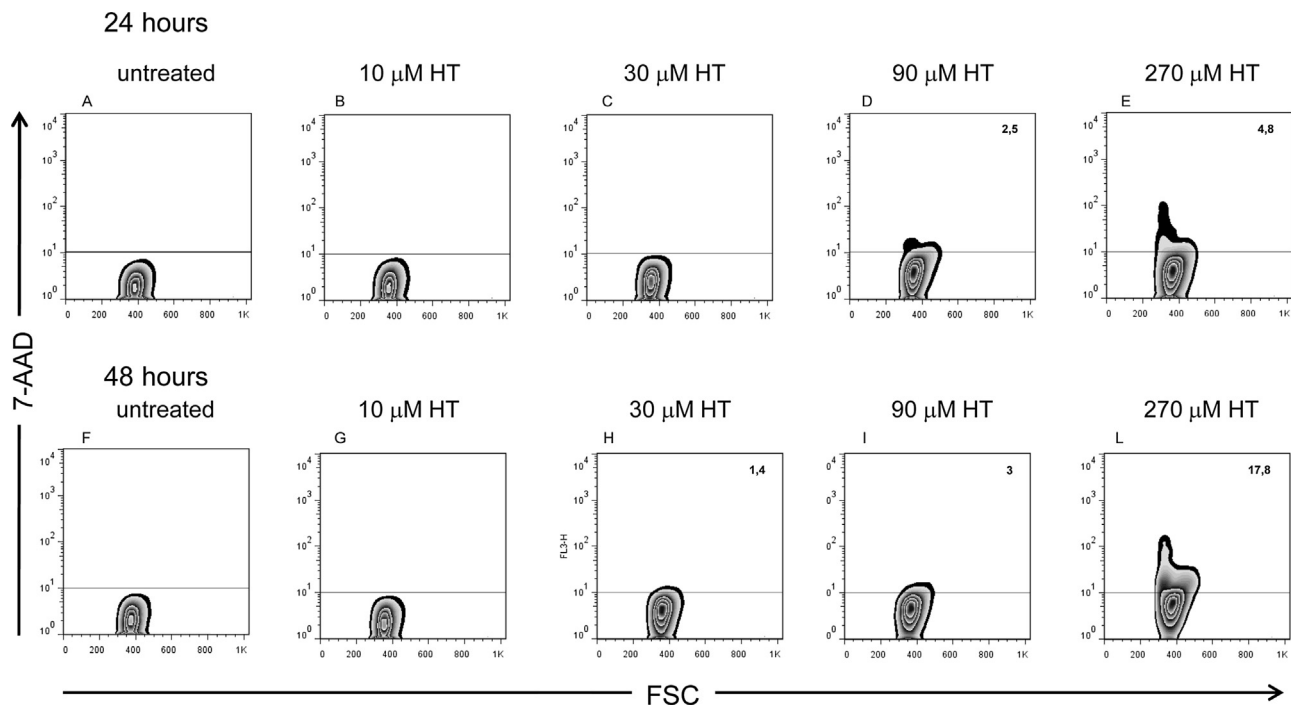


Fig. 1. 7-AAD incorporation. Panels A–E show a density plot analysis of PBMCs from one donor subject studied by means of 7-AAD incorporation after 24 h of increasing concentrations of HT. Panels F–L show cell viability after 48 h of treatment. Numbers indicate the percentage of dying cells after blank subtraction (sample with no antigen stimulation).

but very little is known about its potential beneficial effects in the field of allergies.

In this paper, we investigate the ability of HT to modulate Par j 1-induced IL-10 secretion ex vivo in PBMCs from healthy volunteers suggesting an anti-inflammatory effect induced by HT which is capable of potentiating a healthy immune response towards one of the major *Parietaria* allergens.

2. Material and methods

2.1. Sample preparation

3-Hydroxytyrosol purchased from Sigma-Aldrich (H4291) was resuspended in water for cell culture (Thermo Fisher, Milan, Italy). The allergen used in this study was the Par j 1 (EMBL accession number X85012), which was prepared as previously described (Bonura et al., 2012). The content of LPS in the recombinant allergen was assayed using the Multi-test Limulus Amebocyte Lysate (LAL) pyrogen plus test (Bio-Whittaker, USA) (endogenous endotoxin content ≤ 0.003 ng LPS/ μ g of recombinant protein).

2.2. 7-Aminoactinomycin D staining

Peripheral Blood Mononuclear Cells (PBMCs) were prepared from the heparinised blood of two donors by standard Ficoll density gradient separation and seeded at the concentration of 5×10^5 cells/well in a 48-well plate in RPMI 1640 complete medium with 25 mM HEPES, L-Glutamine and supplemented with Penicillin (100 U/ml) and Streptomycin sulfate (100 mg/ml) (Invitrogen, Milan, Italy), 10% FCS serum (Euroclone, Milan, Italy), 1% Non-Essential Amino Acid (AANE) (Euroclone, Milan, Italy) and 1 mM Sodium Pyruvate (Euroclone, Milan, Italy). Donors signed an informed consent before undergoing blood sampling. Cells were treated with 10 μ M, 30 μ M, 90 μ M, 270 μ M HT and incubated at 37 °C and 5% CO₂ for 24 and 48 h. Cells with no antigenic stimulation served as negative controls. PBMCs were then harvested and

stained with 7-Aminoactinomycin D (7-AAD) [50 μ g/ml] (Beckton Dickinson, San Jose, CA, USA). All measurements were made with a FACSCalibur Flow Cytometer (Beckton Dickinson, San Jose, CA, USA). At least 2×10^4 cells were analysed using FlowJo software (Tree Star, USA). A lymphocyte gate based on forward and side scatter properties was set. 7-AAD⁺ cells were selected on FSC vs 7-AAD.

2.3. Hydroxytyrosol modulation of Par j 1-induced IL-10 secretion

PBMCs were prepared from heparinised blood of healthy donors ($n = 5$) by standard Ficoll density gradient separation. Donors signed an informed consent before undergoing blood sampling. Healthy donors were defined as subjects with no allergic symptoms and a negative Skin Prick Test to *Parietaria* extract (Stallergènes, Milan, Italy). Cells were resuspended at a concentration of 5×10^6 /ml in RPMI 1640 complete medium as above reported in 10% AB serum (Euroclone, Milan, Italy). PBMCs were stimulated with 10 μ g/ml of the recombinant allergens Par j 1, 1 μ M of HT and a mixture of the rPar j 1 allergen plus HT at the above-reported concentrations. Untreated samples were defined as cells with no antigenic stimulation. Immediately before FACS analysis, Propidium Iodide (PI) (0.5 mg/ml, Sigma) was added to each sample to distinguish live and dead cells. Lymphocytes were gated on their FSC and SSC characteristics. Live cells and CD14⁺ cells were selected on an SSC vs CD14/PI. IL-10 positive expression was set from 10² fluorescence decade. According to the values of the total population frequency ($\leq 0.3\%$), the IL-10 positive cells were analysed by means of Mean Fluorescence Intensity (MFI), and significance was evaluated by the CV (Table E1 Supplementary material in the online version at DOI: <http://dx.doi.org/10.1016/j.imbio.2016.07.009>). A minimum of 50,000 events were collected and analysed on a FACSCalibur flow cytometer, and data were analysed using FlowJo software (Tree Star, USA). Isotype controls for each antibody were included in all experiments.

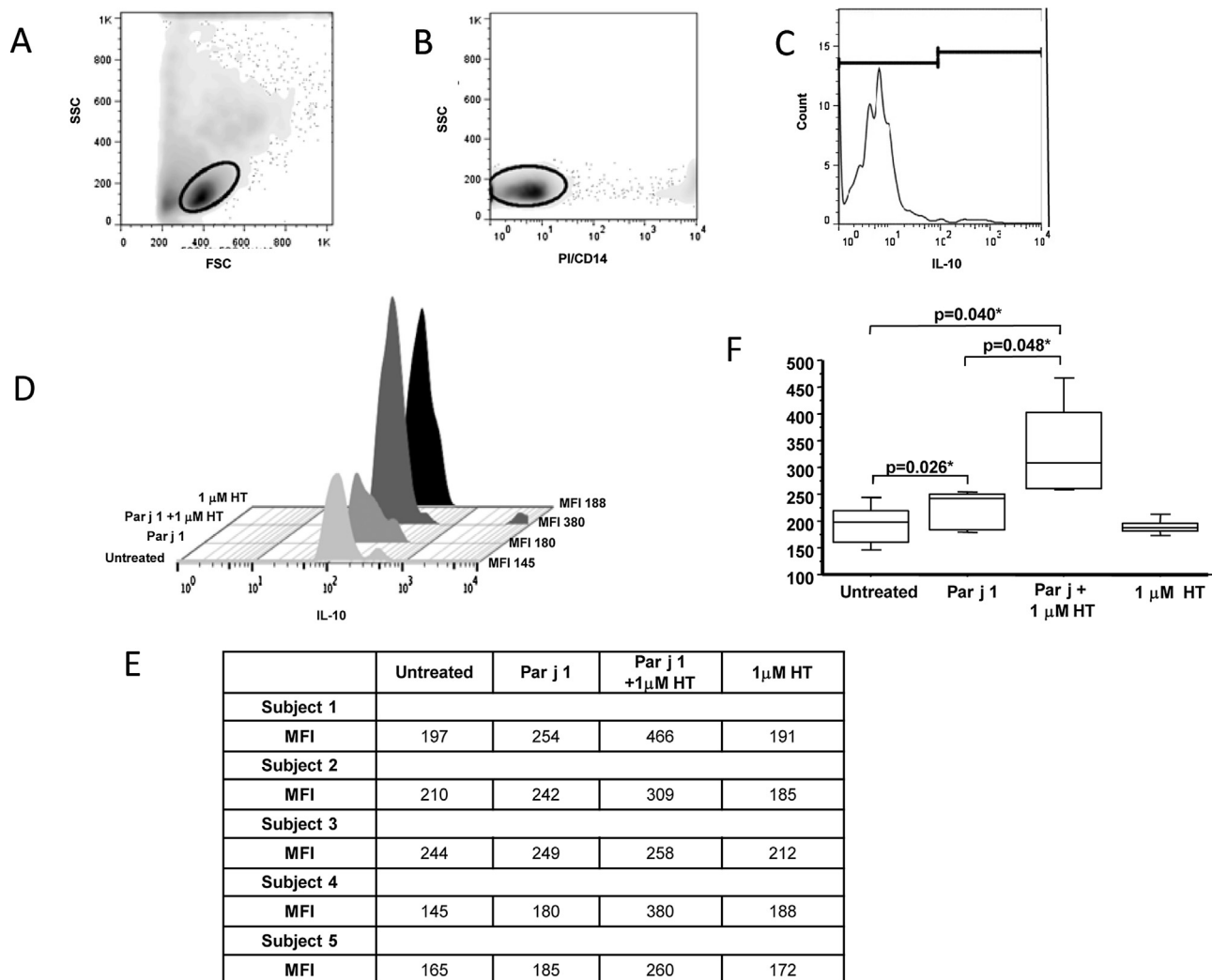


Fig. 2. PBMCs IL-10 secretion induced by the Par j 1, HT and the Par j 1/HT mixture. Panels A–C show the gate strategy used to study IL-10 secreting cells within the PBMCs population. Data reported within panel D show the analysis of one donor representative out of five. Numbers indicate the calculated MFI for each tested molecule. Panel E shows the calculated MFI for all the subjects. Panel F displays Analysis of Variance for Repeated Measures providing an overall p value of 0.002. Differences between experimental conditions were evaluated by means of Student's *t*-test for paired data (*). Bars indicate (from the bottom to the top) 10th, 25th, 50th (median), 75th, and 90th percentiles. Unless otherwise indicated, differences were not significant.

2.4. Statistical analysis

Analysis of Variance for Repeated Measures (RMANOVA) and Student's *t*-test for paired data were used for testing differences among different experimental conditions. Each experimental condition entered the RMANOVA model as within factor.

3. Results

3.1. 7-AAD staining

To test cell viability after HT treatment, PBMCs from healthy donors were studied by means of 7-AAD incorporation. As shown in Fig. 1, HT shows a dose-response increase in the percentage of 7-AAD-positive cells above the concentration of 90 μ M HT (Fig. 1 panel D) at 24 h and 30 μ M (Fig. 1 panel H) HT at 48 h. For this reason, in our further assay, we decided to test this molecule at a concentration of 1 μ M and for no longer than 16 h. It is noteworthy that the range of concentrations of HT that we used in our experimental set up resembled those detected in human plasma after the consumption of phenol-enriched olive oil and compatible with human dietary habits (Rubio et al., 2012).

3.2. Hydroxytyrosol modulates Par j 1 induced IL-10 production

Starting from our preliminary analysis of cell viability in *in vitro* cell culture experiments, we decided to investigate the pattern of IL-10 production in PBMCs from healthy volunteers ($n = 5$) stimulated with HT at a concentration far below the minimum level of toxicity previously identified in the cell culture assay (1 μ M HT), in presence of the recombinant Par j 1 [10 μ g/ml] and a mixture of both the rPar j 1 and HT.

Fig. 2 panels A to C show the gate strategy used for the selection of the IL-10-producing cells, while panel D shows the histograms of the MFI representative of one experiment out of five of the IL-10⁺ cells. Fig. 2 panel E shows the MFI calculated for all the samples. A statistical analysis performed by RMANOVA showed a statistically significant increase ($p = 0.002$) in IL-10 secretion in the Par j 1 treated cells, and a further increase was observed in the Par j 1/HT mix (Untreated vs Par j 1 $p = 0.026$; Untreated vs mix Par j 1/HT $p = 0.040$; Par j 1 vs Par j 1/HT $p = 0.048$) (Fig. 2 panel F). No significant increase was observed in negative control cultures.

4. Discussion

In a previously published pilot study, we described that during a natural immune response, the major allergen of the *Parietaria* pollen Par j 1 was able to stimulate both arms of the innate and adaptive human immune system via different cell types, leading to the production of IFN- γ and IL-10 cytokines (Bonura et al., 2013). For these reasons, we decided to employ the *Parietaria* pollen allergen as a model to study the effect of HT on the modulation of Par j 1-induced IL-10 secretion in PBMCs from healthy subjects. For this purpose, we first decided to perform toxicological studies to understand whether pure HT showed any toxic effects *in vitro* on human primary cell lines. In fact, previously published papers had reported that pure HT can have a cytotoxic effect *in vitro* due to its capability of inducing H₂O₂ production in commonly used cell culture media (i.e. RPMI 1640) (Odiatou et al., 2013). In Fig. 1, we reported the 7-AAD incorporation after a dose-response curve with pure HT in human PBMCs, showing that this substance did not affect cell viability below the concentration of 90 μ M after 24 h of stimulation. Starting from this observation we moved forward addressing the question whether HT can modulate Par j 1 immune response. For these reasons, PBMCs from healthy volunteers were incubated with the recombinant Par j 1, with rPar j 1 plus 1 μ M HT and the same concentrations of HT only, showing that this polyphenol can have a synergistic effect leading to a more robust IL-10 response during a naturally occurring tolerogenic response to the Par j 1 allergen (Fig. 2 Panel F).

A statistical analysis performed by RMANOVA and Student's *t*-test for paired data for testing differences among different experimental conditions was employed showing a statistically significant increase of the MFI in the samples treated with both the allergen and HT ($p=0.002$). These data are in agreement with our previously reported analysis (Bonura et al., 2013) showing that Par j 1 is able to induce IL-10 in human PBMCs (untreated vs Par j 1; $p=0.040$). In this report, we demonstrate that this response is potentiated by co-incubation with HT (Par j 1 vs Par j 1 + 1 μ M HT; $p=0.048$). It is noteworthy that the range of concentrations of HT that we used in our experimental set up resembled those detected in human plasma after the consumption of phenol-enriched olive oil and compatible with human dietary habits (Rubio et al., 2012). In our opinion, the data reported in this paper are of particular relevance since they may represent a further step in the comprehension of the mechanism by which HT anti-inflammatory activity could be applied through the induction of IL-10 production. Furthermore, our analysis demonstrated that the co-administration of HT and an allergenic molecule to PBMCs can potentiate a tolerogenic immune response via an increase of IL-10 secretion. To the best of our knowledge, this is the first report demonstrating that HT can modulate an allergen-specific immune response potentially able to strengthen an anti-inflammatory phenotype.

In conclusion, given the fact that HT displays an excellent safety profile (Hu et al., 2014), our pilot work opens the way to further studies to elaborate the pharmacological mechanisms behind the possibility of using HT as a nutrient for allergy prevention in addition to its already approved use for its preventive effect on LDL oxidation (EFSA, 2011).

Conflict of interest

The authors declare that they have no conflicts of interest.

Aknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.imbio.2016.07.009>.

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Table E1. Coefficient of Variation. CV of IL-10 producing cells were reported for each of the samples reported within Figure 2

	Untreated	Par j 1	Par j 1 +1«M HT	1«M HT
Subject 1				
CV	0,7	0,6	1,1	n/a
Subject 2				
CV	0,84	0,56	1,8	0,3
Subject 3				
CV	1,2	0,7	0,8	0,7
Subject 4				
CV	0,3	0,5	0,4	0,4
Subject 5				
CV	0,6	0,6	0,5	0,6