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Resveratrol enhances TNF-α production in human monocytes upon bacterial stimulation

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ABSTRACT

Background: Resveratrol is a key component of red wine that has been reported to have anti-carcinogenic and anti-aging properties. Additional studies conducted in vitro and in animal models suggested anti-inflammatory properties. However, data from primary human immune cells and in vivo studies are limited.
Methods: A pilot study was performed including 10 healthy volunteers. Plasma cytokine levels were measured over 48 h after oral application of 5 g resveratrol.
To verify the in vivo findings, cytokine release and gene expression in human peripheral blood mononuclear cells (PBMC) and/or monocytes was assessed after treatment with resveratrol or its metabolites and stimulation with several toll-like receptor (TLR)-agonists. Additionally, the impact on intracellular signaling pathways was analyzed using a reporter cell line and Western blotting.
Results: Resveratrol treated individuals showed a significant increase in tumor necrosis factor-α (TNF-α) levels 24 h after treatment compared to baseline. Studies using human PBMC or isolated monocytes confirmed potentiation of TNF-α production with different TLR agonists, while interleukin (IL)-10 was inhibited. Moreover, we observed significantly enhanced nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) activation using a reporter cell line and found increased phosphorylation of p105, which is indicative of alternative NF-κB pathway activation.
General significance: By administering resveratrol to healthy humans and utilizing primary immune cells we were able to detect TNF-α enhancing properties of the agent. In parallel, we found enhanced alternative NF-κB activation. We report on a novel pro-inflammatory property of resveratrol which has to be considered in concepts of its biologic activity.

1. Introduction

Resveratrol is a small polyphenol compound which is found in many nutrients such as pines, peanuts and mulberries [1]. Furthermore, it is contained in the skin of red grapes and thus also in red wine, which emphasizes its relevance as a dietary constituent. Since its first description as a protective agent in a murine cancer model in 1997 [2], many studies have suggested a variety of biological effects for this compound. The anti-carcinogenic effect was reproduced in numerous rodent studies [3]. A new field of resveratrol research arose when the substance was shown to be capable of inhibiting aging of metazoans in 2004 [4] and many of its beneficial effects were attributed to its ability to scavenge free radicals [5]. Promising results were obtained also regarding its neuroprotective action, which may be explained in part, by its ability to prevent toxic effects of several agents [6,7]. Interestingly, its ability to induce autophagy appears to be beneficial in models of Alzheimer's [8] and prion disease [9]. Furthermore, several anti-inflammatory effects of resveratrol have been described recently, e.g., its favorable effects in murine autoimmunity [10], experimental inflammatory bowel disease models [11,12] and chemoprevention of inflammation-induced hepatocarcinogenesis [13,14]. The differential biological effects of the substance which were shown in experimental animal

Abbreviations: FLA, flagellin; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; IL, interleukin; LPS, lipolysaccharide; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; SAC, *Staphylococcus aureus* cells; TNF-α, tumor necrosis factor-α

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models and in vitro resulted in the launch of several clinical studies to investigate the impact of this compound also in humans; so far there have been partially promising results [15].

However, as pointed out in the summary of the Resveratrol Conference in 2010 [16], there is yet insufficient evidence to recommend the intake of the substance beyond regular dietary doses. In particular, to date there is no clinical study which has investigated the impact of a defined amount of resveratrol alone on human immune parameters in vivo. Furthermore, although there is an abundance of data on the effect of resveratrol in cell lines [17–19], and various animal models [10–12,20], information concerning resveratrol's impact on immune function of primary human leukocytes is limited.

Therefore, the purpose of this study was to assess the inflammation modulating effects of this substance in humans and to delineate its impact on primary human leukocytes. As cytokines are well-established conductors of immune cell function, we focused on evaluating alterations of these mediators in immune cells by resveratrol. To this end, we launched an interventional pilot study including 10 healthy individuals and measured cytokine levels over the first 48 h after administration of resveratrol. Additionally, mononuclear cells from healthy donors were tested for the impact of resveratrol on cytokine release induced by different stimulants. In order to further dissect resveratrol's cytokine-modulatory properties we compared the modulating effects of the main metabolites found in our in vivo study to the effects of the unmodified substance. Using primary human immune cells we detected a hitherto undescribed cytokine-potentiating effect of resveratrol. Moreover, signaling pathways known to be involved in cytokine production like the NF-κB and the mitogen activated protein kinase (MAPK) pathways were analyzed to decipher the underlying molecular mechanisms.

2. Materials and methods

2.1. Interventional pilot study in humans

2.1.1. Study population

Healthy males aged 18–45 years with a body mass index of 18–28 kg/m^2 were eligible. Exclusion criteria were any abnormal laboratory or physical finding as well as a history of significant clinical disease and the use of concurrent medication during the study or of an investigational drug three weeks prior to the study. Additional exclusion criteria were known hypersensitivity to resveratrol or regular intake of the substance as well as a history of drug abuse, alcoholism and smoking.

The investigated individuals were aged 21–28 years, mean age was 24 years; the mean body weight was 77 kg, and the mean body mass index was 22.6 kg/m^2.

The study was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. It was approved by the Ethical Committee of the Medical University of Vienna (Vote Nr. 785/2010) and all subjects agreed to study participation by providing written informed consent.

2.1.2. Study design

This was a randomized, open-label, parallel-group, single-dose pilot study. 10 healthy male subjects were studied in two groups (resveratrol intervention, n = 8; placebo, n = 2). Blinding of the clinical study was not deemed necessary since primary endpoints are laboratory parameters and independent of knowledge of the administered treatment. In the intervention group 5 g resveratrol (Terraternal, Santa Clara, CA) was administered orally. The resveratrol dose was selected based on safety observations of previous clinical trials conducted in humans [21]. Since this was a pilot study, we used resveratrol only in a single dose without dose-escalation. All subjects received a standardized diet not containing polyphenols during the study period.

2.1.3. Study endpoints

A primary endpoint was to measure the inflammation modulating effect of the substance with a focus on plasma cytokine levels. Since cytokine levels in plasma are very volatile, this study was conducted only over a limited time period after treatment. Cytokines known to be involved in innate immune responses were analyzed such as IL12p40, IL-10, TNF-α, IL-8 and IL-6.

2.1.4. Blood sampling and bioanalytical methods

Blood samples for cytokine analysis were collected from venepunctures at pre-dose, 10 h, 24 h and 48 h after intervention.

The samples were centrifuged at 1500 *g* for 10 min at 4 °C and the plasma was separated and stored at −80 °C until analysis of cytokine levels by Luminex® (see below). The detection limit for TNF-α in human plasma was 1 pg/ml.

2.1.5. Measurement of resveratrol and its metabolites in the plasma

Resveratrol and its metabolites (trans-resveratrol-4′-glucoronide, trans-resveratrol-disulfate, trans-resveratrol-3-glucoronide, trans-resveratrol-4-sulfate, trans-resveratrol-3-sulfate) were analyzed with a high-performance liquid chromatography diode array detector (HPLC–DAD) using an Dionex UltiMate®3000 System (Thermo Fisher Scientific, Waltham, MA; column: Phenomenex Luna 5u C18(2) 100A) with a liquid gradient. The detection limit for resveratrol was 5 ng/ml and for resveratrol metabolites 6 ng/ml.

2.2. Cell culture

Buffycoats from healthy individuals were obtained from the Red Cross Austria. Peripheral blood mononuclear cells (PBMC) were isolated over density-gradient separation using endotoxin-free Lymphoprep (Nycomed Pharma AS, Oslo, Norway). For experiments utilizing PBMC, the cells were washed once after density-gradient separation in phosphate buffered saline (PBS; PAA, Pasching, Austria) and resuspended in RPMI 1640 (PAA) containing 10% fetal calf serum (PAA) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin; Sigma-Aldrich Corporation, St. Louis, MO). In order to purify CD14+ monocytes, PBMC were washed once in PBS and underwent MACS® separation according to the manufacturer's protocol (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, PBMC were washed in buffer (PBS + 0.5% human serum albumin, CSL Behring, Vienna, Austria; 5 mM EDTA, LifeTec Inc., Vienna, Austria), re-suspended in buffer (approx. 80 μl for 10^7 cells) and 20 μl CD14 antibody per 10^7 cells was added. After incubation for 15 min at 4 °C, cells were washed in buffer and separated over a MACS® column (Miltenyi Biotec GmbH). Afterwards, cells were washed and cultured in RPMI 1640 medium as described above.

2.3. Cytokine release assay

Cytokine release assays were performed as described before [22]. Briefly, PBMC were plated to a density of 1×10^6/ml and monocytes to a density of 5×10^5/ml. Cells were preincubated with resveratrol (Sigma-Aldrich Corporation) or resveratrol metabolites (resveratrol-3-O-sulfate and resveratrol-3-O-D-glucoronide; Bertin Pharma, Montigny le Bretonneux, France) in the indicated amounts for 60 min before stimulation with 100 ng/ml lipolysaccharide (LPS) 0111:B4 (Sigma-Aldrich Corporation), 1 μg/ml *Staphylococcus aureus* cells (SAC) (InvivoGen, San Diego, CA) or 100 ng/ml flagellin (FLA) (InvivoGen). After 18–20 h incubation the supernatants were collected and centrifuged for 5 min at 400 *g* and used for further analysis. Cytokine measurements were performed by Luminex® testing (Luminex, Austin, TX) for the presence of TNF-α, IL-10, IL-12p40, IL-1β, IL-6 and IL-8 using specific matched-pair antibodies and recombinant cytokines as standards (eBioscience, San Diego, CA) [22].

2.4. Western blotting

Monocytes (1×10^6/ml) were preincubated for 60 min with resveratrol (30 and 50 μM) and then exposed to LPS (10 ng/ml). Incubation was stopped after different time periods by centrifuging the samples for 5 min at 4 °C and 400 *g* and subsequent washing in PBS. The cells were then resuspended in 50 μl PBS and 50 μl Tris-Glycine SDS sample buffer (Laemmli Buffer, LifeTec Inc.) was added followed by 6 min heating at 99 °C. The probes were loaded on precast SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA) and transferred onto immunoblot nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (blocking buffer: Bio-Rad Laboratories; Tris, NaCl: Sigma-Aldrich Corporation) for 1 h at 4 °C. Membranes were washed 3 times for 5 min with Tris-buffered saline between each incubation step. After blocking, membranes were incubated with p-IκB, IκB, phospho-p105, phospho-ERK 1/2 MAPK, phospho-JNK MAPK, phospho-p38 MAPK and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) antibodies (1:1000 dilution, Cell Signalling Technology, Danvers, MA), respectively, overnight at 4 °C. After washing, membranes were incubated with horseradish-peroxidase-coupled secondary antibodies (Dako, Glostrup, Denmark) for 1 h at 4 °C and detection was performed with Pierce® ECL Western blotting substrate (Thermo Fisher Scientific, Waltham, MA).

2.5. Cell viability assay

Monocytes were treated as described for the cytokine release assay. After 20 h co-incubation with LPS and resveratrol cells were separated by washing with PBS/4 mM EDTA, washed once in PBS and resuspended in 25 μl PBS/EDTA. Immediately before performing flow cytometric analysis, propidium iodide (Sigma-Aldrich Corporation) was added to a final concentration of 50 ng/ml.

2.6. NF-κB-GFP reporter cell assay

The U937T NF-κB-GFP reporter cell line was generated as described [23]. U937T were cultured in RPMI 1640 as described above. 2×10^5 cells were pre-incubated with the indicated amounts of resveratrol for 30 min prior to stimulation with 100 ng/ml LPS. 10 and 20 h after stimulation, respectively, cells were washed in ice-cold PBS and subjected to flow cytometric analysis.

2.7. qPCR analysis of cytokine induction

5×10^6 monocytes were seeded on to 6-well-plates and preincubated for 60 min with the indicated amounts of resveratrol before stimulation with 100 ng/ml LPS. After 1.5 h and 3 h of stimulation cells were scraped off and the cell suspension was centrifuged for 5 min at 300 *g* at 4 °C. Subsequently, RNA was obtained utilizing an RNeasy Mini KitTM (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The purified RNA was then converted to 1st strand cDNA utilizing the First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada) according to the manufacturer's protocol. qPCR analysis was performed as described [24]. For normalization of gene expression, GAPDH was utilized as an endogenous control. The Livak method [25] was applied for determination of expression levels of the target gene compared to the endogenous control. The list of primer sequences is provided in the Supplementary methods (Table S1).

2.8. Statistics

The cytokine data of the interventional pilot study were analyzed by Wilcoxon signed rank test to compare measurements from the different time points after resveratrol treatment to baseline levels. The in vitro experiments were analyzed using paired, two sided *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. TNF-α levels are elevated after application of resveratrol in vivo

10 healthy male subjects were included and all of them completed the study. Oral resveratrol was well tolerated by the subjects under study and no adverse reaction was reported. Plasma cytokine levels were measured at pre-dose, 10 h, 24 h and 48 h after oral administration of 5 g resveratrol. Intriguingly, subjects in the resveratrol treated group were noted to have elevated levels of TNF-α at 10 h, 24 h and 48 h as compared to the pretreatment levels whereas no such effect was seen in the control group. The cytokine levels of the intervention group had average baseline TNF-α levels of 3.5 pg/ml, which rose 1.42 fold at 10 h ($p = 0.43$, compared to baseline), 1.57 fold at 24 h ($p = 0.03$) and 1.45 fold at 48 h ($p = 0.15$) (Fig. 1). We also measured levels of IL-10/6/8/12p40; these cytokines were mostly non-detectable and showed no significant alterations after drug administration (data not shown).

3.2. Resveratrol treatment of human PBMC skews cytokine release towards a pro-inflammatory state upon TLR-4 targeting

As cytokine levels from the in vivo pilot study suggested a hitherto undescribed pro-inflammatory effect of resveratrol, we performed additional experiments using human PBMC in order to analyze its effects on cytokine production in vitro. For this purpose, we pre-incubated the cells with the indicated amounts of resveratrol prior to stimulation with LPS, a well-established TLR-4 agonist. Interestingly, we observed a strong up-regulation of TNF-α secretion, whereas the release of the anti-inflammatory cytokine IL-10 was down-regulated compared to stimulation with LPS only (Fig. 2A and B). However, other cytokines were not significantly altered by resveratrol treatment (Fig. 2C–F). To exclude the possibility of endotoxin contamination, controls treated with resveratrol alone were included in the experimental setting. Such treatment did not cause cytokine induction, thus excluding this possibility (data not shown). Additionally, we evaluated solvent controls of ethanol (0.15%; the concentration equivalent to the highest resveratrol dose used) when stimulating with LPS (Fig. 2A–F). No significant alterations of cytokine production, however, were observed under these conditions. Thus, with these experiments we were able to show up-regulation of TNF-α production while demonstrating concomitant dampening of IL-10 release by human PBMC.

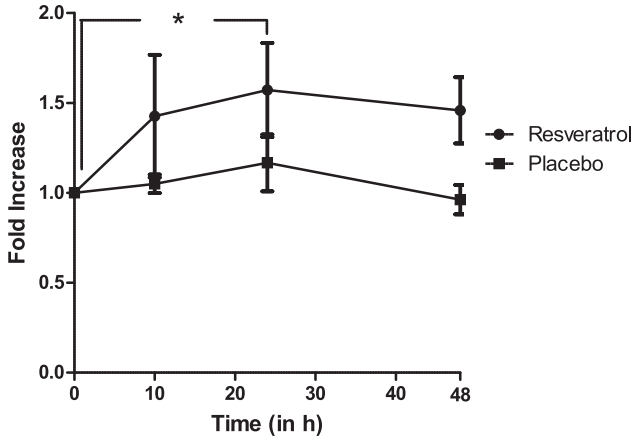

Fig. 1. TNF-α plasma levels are elevated after administration of resveratrol. 5 g of resveratrol was administered orally to 8 healthy male individuals, 2 individuals received placebo. Blood samples were obtained at 0, 10, 24 and 48 h and plasma cytokine levels were measured by Luminex®. *Significant compared to baseline TNF-α levels, $p < 0.05$ calculated by Wilcoxon signed rank test.

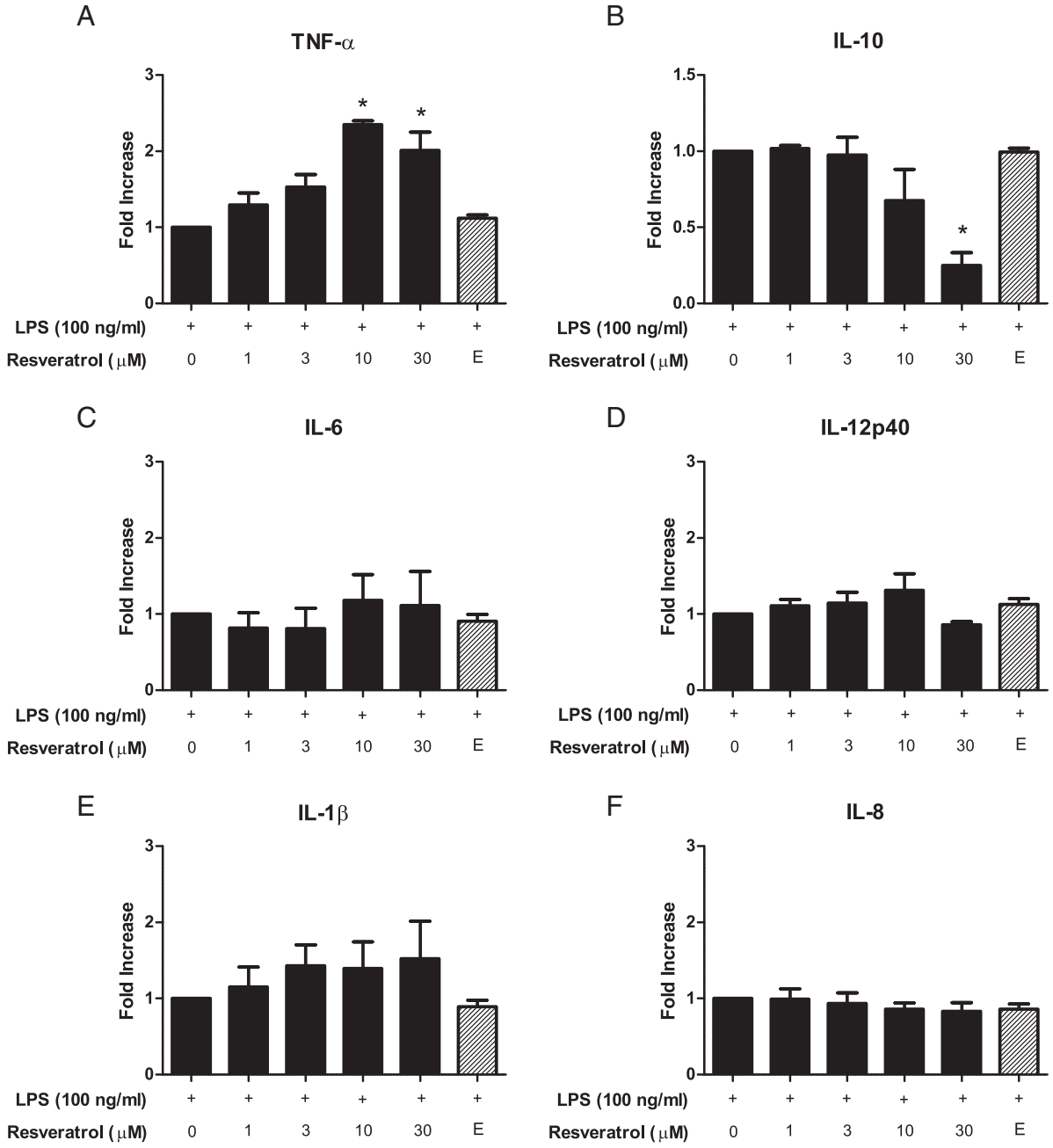

Fig. 2. Resveratrol enhances TNF-α production and dampens IL-10 release upon LPS stimulation in human PBMC. Panels A–F show the impact of the indicated concentrations of resveratrol and a solvent control (E, ethanol 0.15%) on the cytokine release in LPS stimulated human PBMC. Cells were treated with resveratrol for 1 h prior to stimulation with LPS and cytokine levels in supernatants obtained after 18–20 h of co-incubation were measured by Luminex®. Values are expressed as mean ± SEM from three independent experiments. *Significant compared to LPS alone, $p < 0.05$ calculated by paired *t*-test.

3.3. Enhanced TNF-α secretion upon TLR-4 stimulation is pronounced in human monocytes

PBMC are a heterogeneous group of leukocytes, containing monocytes and lymphocytes. To further analyze the observed alteration in cytokines, we set out to identify the source of enhanced TNF-α production. As monocytes are known to be potent TNF-α producers, we assessed the effect of resveratrol on purified CD14+ cells. In these experiments, we observed an even more pronounced up-regulation of TNF-α secretion, showing a 4-fold increase compared to LPS alone (Fig. 3A). Interestingly, IL-10 secretion of monocytes also seemed to be dampened, but did not reach significance in this setting (Fig. 3B). Again, the other cytokines investigated did not show significant alterations after treatment with resveratrol (Fig. 3C–F).

3.4. Resveratrol induces TNF-α expression in human monocytes at the transcriptional level

To further explore resveratrol's mode of action we next evaluated the cytokine-modulatory effect on the level of gene expression. Thus, we performed qPCR analysis of the investigated cytokines. Indeed, we were able to show significantly enhanced TNF-α transcription in human monocytes (Fig. 4A), whereas no significant alterations were detected in the other cytokine transcripts (Fig. 4B–F). These

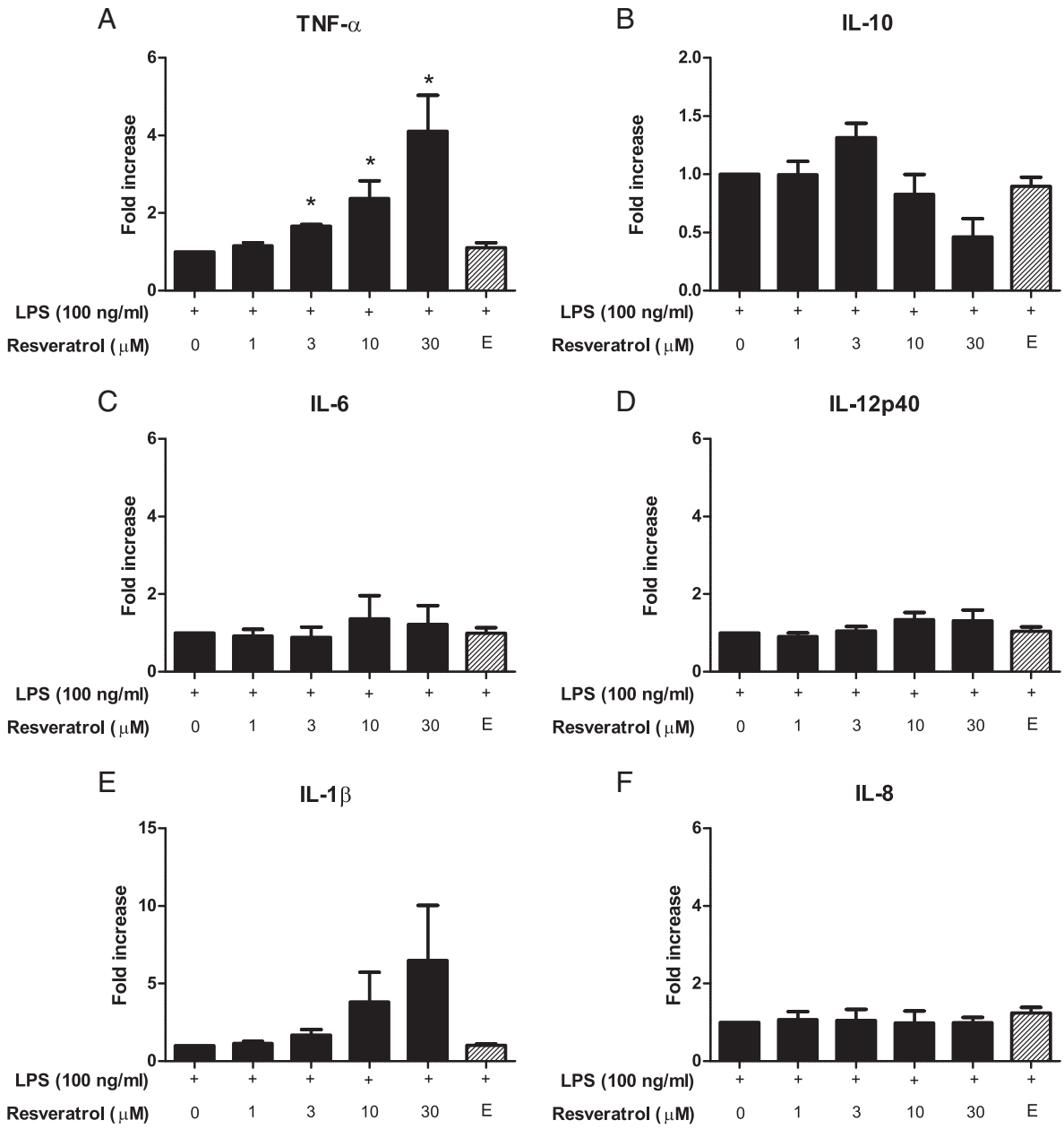

Fig. 3. Resveratrol enhances TNF-α release in human monocytes upon LPS stimulation. Panels A–F show the impact of the indicated concentrations of resveratrol and a solvent control (E, ethanol 0.15%) on cytokine release in LPS stimulated human monocytes. Cells were treated with resveratrol for 1 h prior to stimulation with LPS and the cytokine levels in supernatants obtained after 18–20 h of co-incubation were measured by Luminex®. Values are expressed as mean ± SEM from four independent experiments. *Significant compared to LPS alone, $p < 0.05$ calculated by paired *t*-test.

experiments indicate that resveratrol enhances TNF-α production of human monocytes by augmenting gene expression.

3.5. The main metabolites of resveratrol do not alter cytokine production in human monocytes

Resveratrol is known to be metabolized very rapidly thus leading to very low concentrations of the unmodified substance in the plasma [26]. Consistent with these previous findings we observed high levels of sulfo- and glucuronide-conjugated resveratrol compounds but barely measurable unmodified substance in the plasma of probands (Fig. 5A, Fig. S1). In order to potentially differentiate between a systemic effect of resveratrol's metabolites from a local effect of unmodified resveratrol in the gut, we investigated the cytokine-modulating properties of the two most prominent early metabolites found in our in vivo study, resveratrol-3-O-sulfate and resveratrol-3-O-D-glucuronide. In contrast to resveratrol's strong TNF-α potentiating effect, the two tested metabolites failed to induce significant changes in TNF-α secretion upon LPS-stimulation in monocytes (Fig. 5B). Similarly, expression of other cytokines was also not affected (Fig. S2).

3.6. Upregulation of TNF-α expression in human monocytes by resveratrol is not restricted to TLR-4 ligation

In order to find out whether the potent enhancement of TNF-α production was specific for the engagement of the TLR-4 pathway we investigated the impact of resveratrol on cytokine release induced by additional TLR agonists. For this purpose, we stimulated monocytes with 1 μg/ml SAC, a potent activator of TLR-2, and the TLR-5 agonist FLA (100 ng/ml). Consistent with our previous findings using LPS,

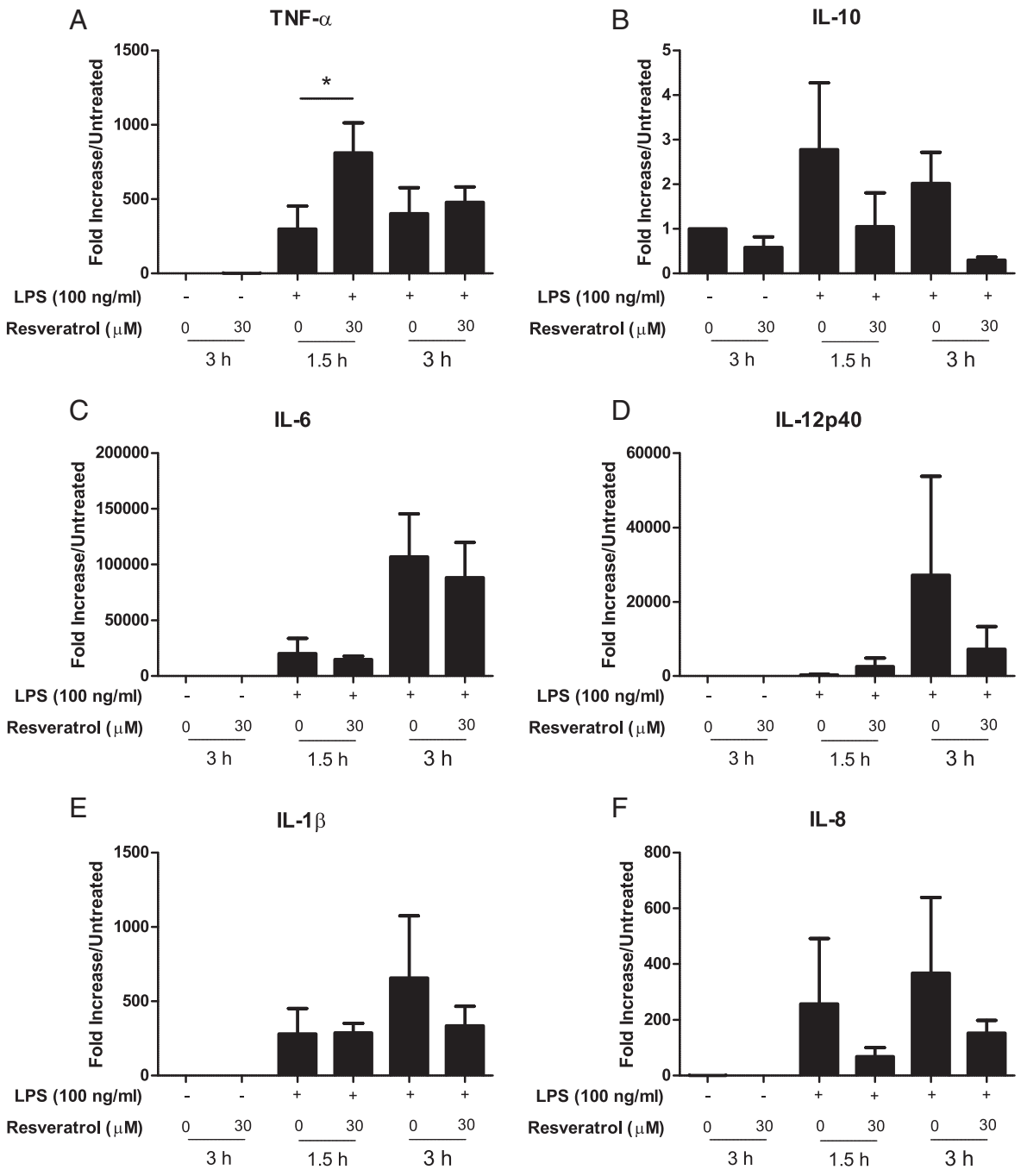

Fig. 4. Resveratrol augments TNF-α gene expression in LPS-stimulated monocytes. Panels A–F show the cytokine mRNA levels induced by addition of 30 μM resveratrol to LPS (100 ng/ml) stimulated human monocytes. Cells were treated with resveratrol for 1 h prior to stimulation with indicated amounts of LPS and RNA samples obtained after 1.5 h and 3 h. RNA samples were directly subjected to cDNA synthesis and analyzed by qPCR. Values are expressed as mean ± SEM from three independent experiments. *Significant compared to LPS alone, $p < 0.05$ calculated by paired *t*-test.

we observed a dose-dependent up-regulation of the TNF-α release by resveratrol for both TLR agonists (Figs. 6A, 7A). When stimulating monocytes with SAC, an IL-10 dampening effect of resveratrol was also observed (Fig. 6B). Moreover, resveratrol also significantly enhanced IL-6 production after SAC stimulation (Fig. 6C), whereas both IL-6 and IL-1β secretion were significantly enhanced when FLA was used for stimulation (Fig. 7C + E). These findings are in line with the observation of an overall enhanced pro-inflammatory state through TLR-stimulation by resveratrol co-treatment. Thus, we were able to prove that resveratrol's TNF-α enhancing effect is not specific for the TLR-4 pathway.

3.7. Resveratrol induces potent activation of NF-κB dependent-transcription enhancing alternative NF-κB signaling

Having shown that resveratrol is a potent enhancer of TNF-α secretion in human monocytes following stimulation with different TLR agonists, we wanted to clarify the signaling pathways involved in the enhanced release of this cytokine. NF-κB signaling is described as one of the major transcriptional regulators of TNF-α expression [27]. In order to test the impact of resveratrol on transcriptional regulation by NF-κB transactivation, a well-characterized monocytic NF-κB-GFP U937T reporter cell line [23] was utilized. Indeed, we observed a

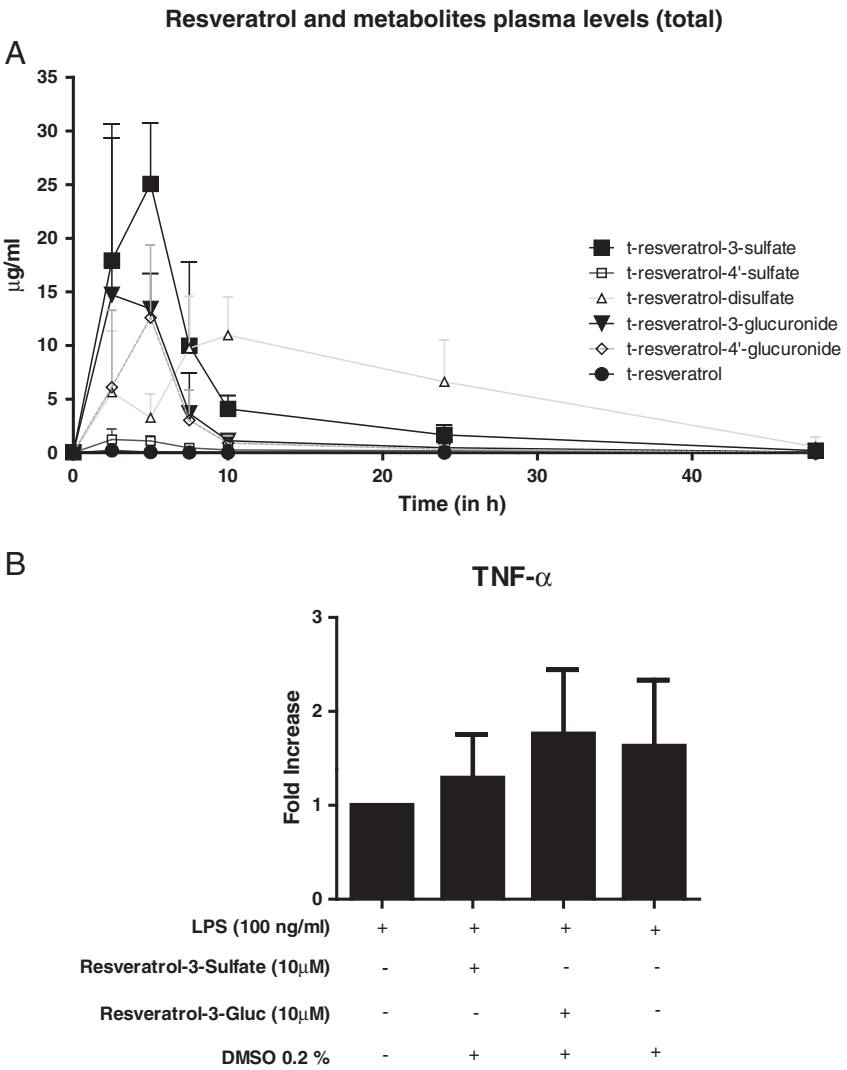

Fig. 5. Main resveratrol metabolites do not enhance TNF-α production of monocytes upon LPS stimulation. Panel A: 5 g of resveratrol was given orally to 8 healthy male individuals, 2 individuals received placebo. Blood samples were obtained at 0, 2.5, 5, 7.5, 10, 24 and 48 h and plasma levels of resveratrol and the indicated metabolites were analyzed using a high-performance liquid chromatography diode array detector as described in the Materials and methods section. Panel B shows the impact of 10 μM of trans-resveratrol-3-O-sulfate, trans-resveratrol-3-O-D-glucuronide and a solvent control (DMSO 0.2%) on the TNF-α release in LPS stimulated human monocytes. Cells were treated with resveratrol metabolites for 1 h prior to stimulation with LPS and the TNF-α levels in supernatants obtained after 18–20 h of co-incubation were measured by Luminex®. Values are expressed as mean ± SEM from three independent experiments.

profound upregulation of NF-κB dependent-transcription after 20 h of co-treatment with resveratrol and LPS (Fig. 8A–B), indicating that enhanced TNF-α production in myeloid human cells was caused by triggering NF-κB signaling. Similar results were obtained already after 10 h co-incubation suggesting enhancement in early signal transduction (Fig. S3). Canonical activation via IκB degradation and alternative activation via p105 cleavage represent different forms of NF-κB signaling in human monocytes. In order to investigate the impact of resveratrol upon these pathways we performed Western blotting experiments after treatment with the indicated amounts of resveratrol and stimulation with LPS. We did not observe substantial alterations in the degradation of IκB after resveratrol treatment, thus indicating no significant impact on the canonical NF-κB pathway. In contrast, we found a profound increase in p105 phosphorylation upon treatment with resveratrol (Fig. 8C), demonstrating enhanced alternative NF-κB signaling.

3.8. p38 phosphorylation is inhibited by resveratrol treatment, whereas other MAPK are not significantly affected

To further analyze the impact of resveratrol on intracellular signaling in human monocytes we evaluated phosphorylation of the MAPKs p38, ERK1/2 and JNK by Western blotting. Here, we found substantial inhibition of p38 formation in human monocytes by resveratrol treatment, whereas no significant alterations in ERK1/2 phosphorylation were observed (Fig. 8D). The impact of resveratrol treatment on JNK phosphorylation did not yield a clear picture due to donor variability (data not shown). Thus, we have demonstrated a differential impact of resveratrol on MAPK signaling leading to selective inhibition of p38 signaling.

4. Discussion

There is growing evidence over the past decade that resveratrol might have a strong impact on multiple biological systems. Particularly its impact on the immune system of humans, however, is insufficiently characterized and requires further research prior to clinical utilization.

In vivo immunomodulatory properties of the substance have so far only been described when administered in combination with other potentially active compounds [28,29]. Resveratrol was given as part of a plant extract containing 30 mg of the substance over a long period and cytokine levels were measured in a time-frame of several weeks. Earlier studies in humans have described the safe use of the substance

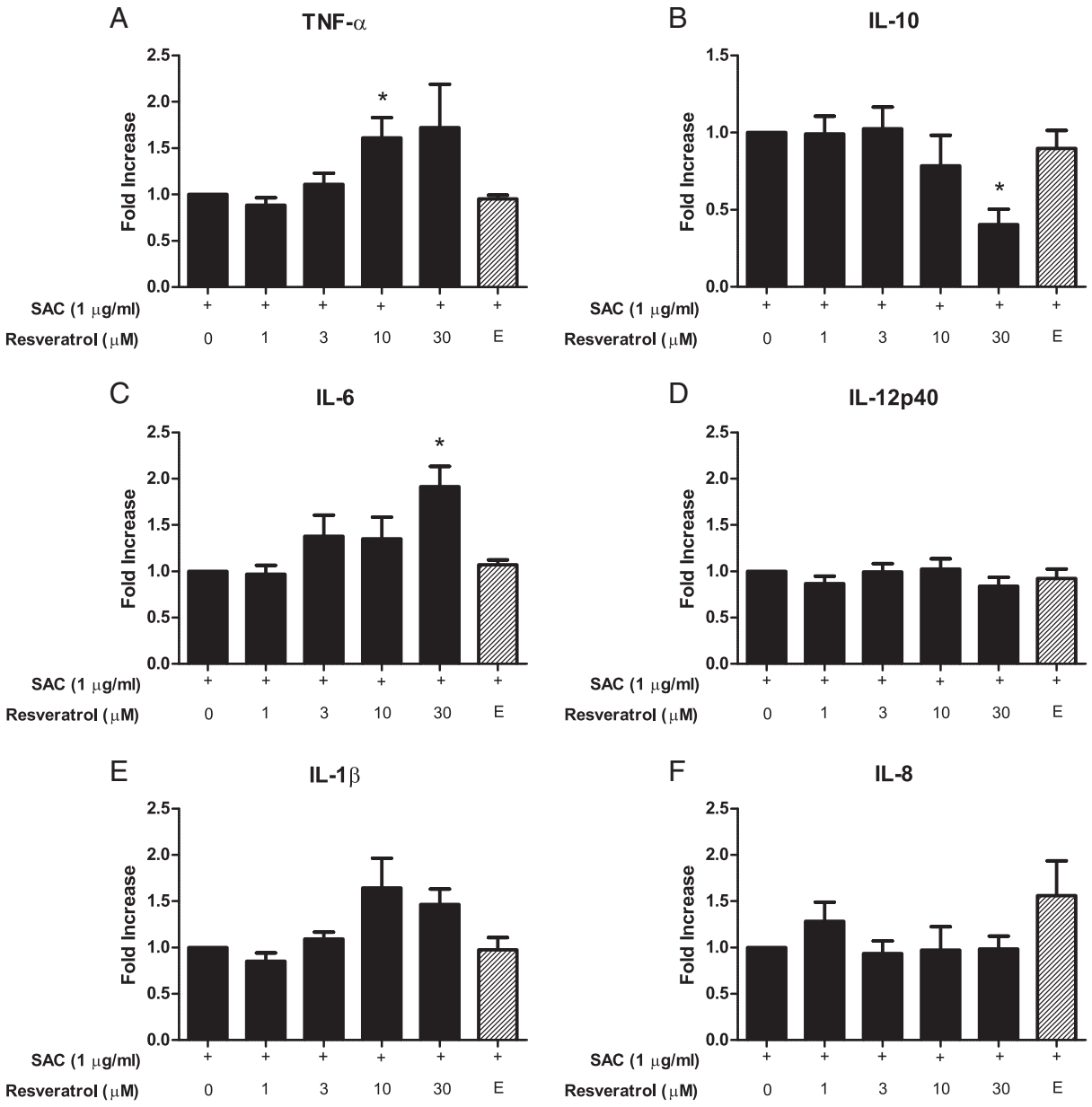

Fig. 6. Resveratrol enhances TNF-α release in human monocytes upon SAC stimulation. Panels A–F show the impact of the indicated concentrations of resveratrol and a solvent control (E, ethanol 0.15%) on cytokine release in SAC stimulated human monocytes. Cells were treated with resveratrol for 1 h prior to stimulation with SAC and the cytokine levels in supernatants obtained after 18–20 h of co-incubation were measured by Luminex®. Values are expressed as mean ± SEM from four independent experiments. *Significant compared to SAC alone, $p < 0.05$ calculated by paired *t*-test.

up to amounts of 5 g [21]. For the purpose of analyzing the cytokine-modulatory effect of the agent itself, we administered the pure compound at the highest safe dose. Due to the dynamics of cytokine release and the volatility of these mediators in human plasma, we chose to measure cytokine levels shortly after the application of resveratrol. Thereby, we found elevated plasma levels of TNF-α soon after the application of the agent compared to baseline levels. Interestingly, in the study by Ghanim et al. [29], TNF-α levels were also reported to increase shortly after resveratrol administration and to drop later in the course of the study.

As shown in the present study, resveratrol itself did not induce cytokine production in vitro, but was able to modulate cytokine expression induced by stimulation with different TLR-agonists. In contrast, its main metabolites that can be found in systemic circulation did not induce significant alterations in the cytokine profile of monocytes. These findings might indicate that the increase in TNF-α levels observed in our in vivo study could be due to potentiation of local innate immune cells exposed to TLR-stimulating agents from the gut's microenvironment. Hence, the presence of resveratrol in a particular diet might modulate cytokine-mediated leukocyte action and immune-surveillance in the gut.

Due to the pilot character of this study there are limitations, which have to be considered. Comparison of different routes of administration might help to further distinguish between local and systemic immune-modulation. A small sample size and the single dose administration might be issues which need to be addressed before making a definite statement about the cytokine-modulatory effects of resveratrol in vivo.

In contrast to several reports from other groups we found a pro-inflammatory action of resveratrol when addressing the cytokine-modulatory properties of this compound in vitro. Importantly, most of these observations describing an anti-inflammatory effect of resveratrol were made in animal models or in vitro studies utilizing cell lines, which were not of hematopoietic origin. Down-regulation of IL-1β, TNF-α and COX-2 was recently shown in a microglial cell line [17], and inhibition of iNOS production was reported in a colon cancer cell line [18]. A study

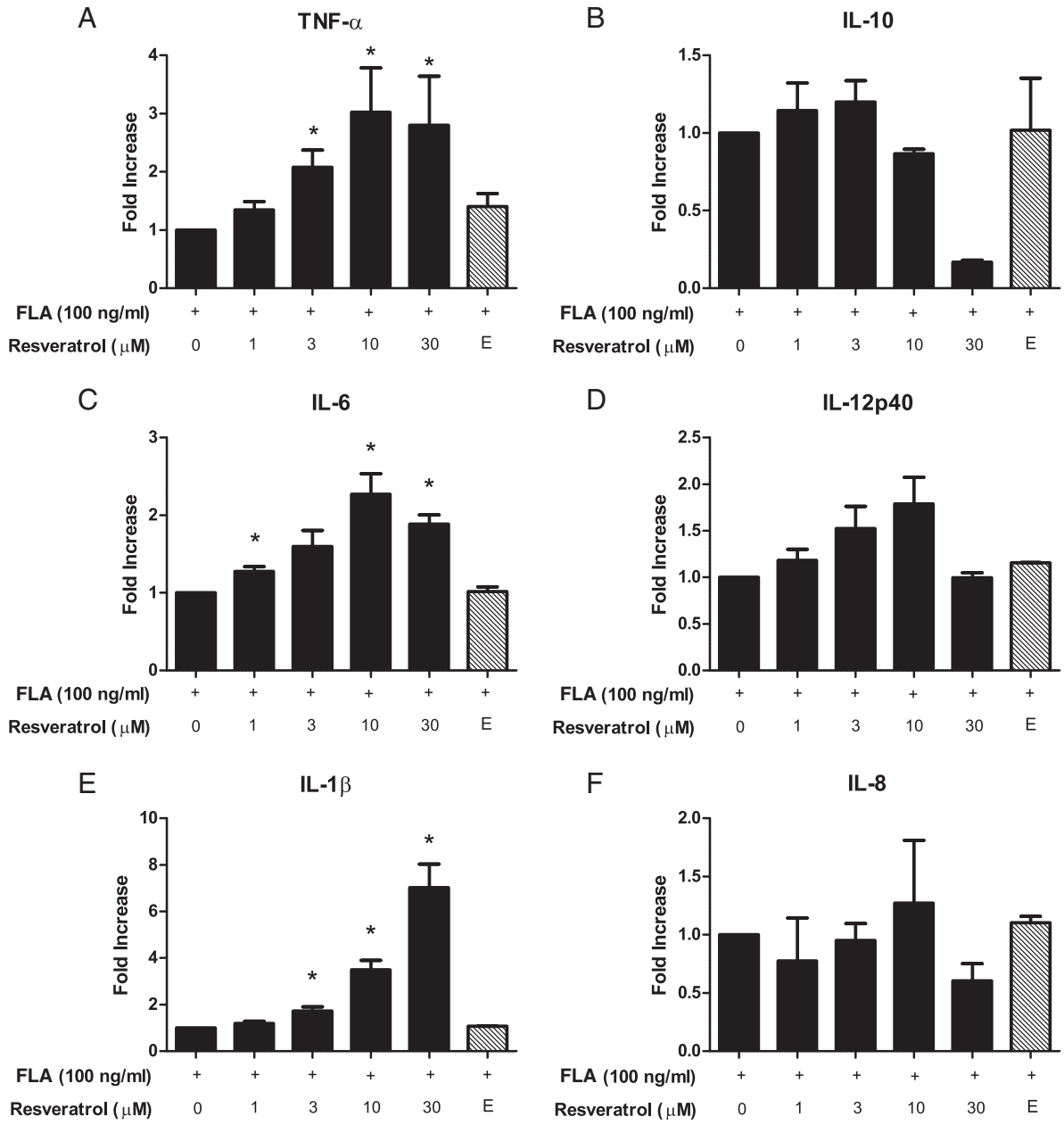

Fig. 7. Resveratrol enhances TNF-α release in human monocytes upon flagellin stimulation. Panels A–F show the impact of the indicated concentrations of resveratrol and a solvent control (E, ethanol 0.15%) on cytokine release in FLA stimulated human monocytes. Cells were treated with resveratrol for 1 h prior to stimulation with FLA and the cytokine levels in supernatants obtained after 18–20 h of co-incubation were measured by Luminex®. Values are expressed as mean ± SEM from four independent experiments. *Significant compared to FLA alone, $p < 0.05$ calculated by paired *t*-test.

using the human monocytic cell line THP-1 reported on dampened IL-8 production after incubation with resveratrol [30]. Cell lines usually require rather high amounts of the respective activating agonists in order to achieve measurable cytokine responses (the mentioned studies have used up to 1–2 μg/ml LPS). Thus, it is likely to be difficult to further up-regulate inducible cytokines in cell lines and even more problematical to translate such experimental conditions to pathophysiologically relevant situations in humans.

To date, only a few studies have been performed using primary human immune cells such as PBMC or monocytes [31–33]. The only study measuring TNF-α alterations upon TLR targeting was performed by Shah et al. [31] who reported no substantial changes in cytokine levels by resveratrol treatment. Divergent results might be explained by different LPS doses used in these studies (Shah et al. applied 10 μg/ml whereas we used 100 ng/ml) but may also be due to the evaluation of cytokine release at different time points (Shah et al. measured cytokine levels 4 h after LPS stimulation, whereas levels were quantified after 18 h of stimulation in our study).

Interestingly, resveratrol was recently shown to enhance pro-inflammatory cytokine production and thus to prevent the progression of chemically-induced induced hepatic cancer in a rat model of hepatocellular carcinoma [20]. Mbimba et al. found that the cancerogenic chemical agent diethylnitrosamine down-regulated the levels of TNF-α, IL-6 and IL-1β in the liver of rats which could be reversed by treatment with resveratrol. As resveratrol simultaneously prevents carcinogenesis in this model [34], it is possible that enhanced immune surveillance is responsible for resveratrol's anti-carcinogenic effects. In view of the results reported here, the

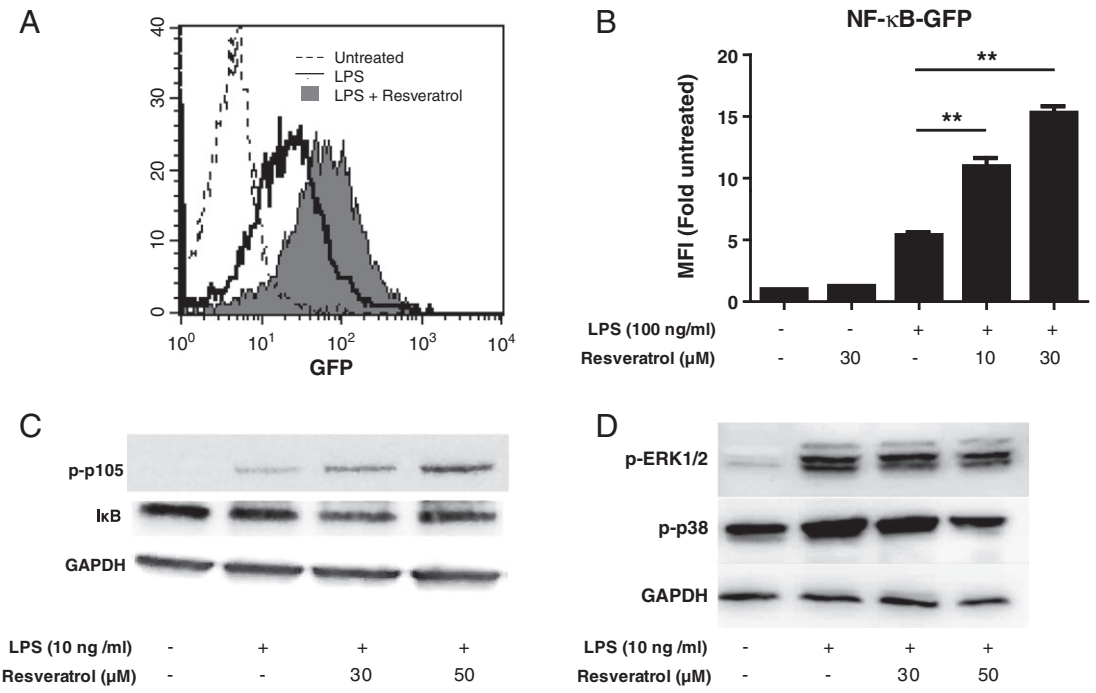

Fig. 8. Resveratrol enhances alternative NF-κB activation. Panel A depicts a representative result of NF-κB-GFP U937T pre-treated 30 min with 30 μM resveratrol and stimulated for 20 h with 100 ng/ml LPS. Panel B shows the summary of 3 independent experiments as performed in A. Results are expressed as mean ± SEM. *p < 0.05; **p < 0.01: Significant compared to LPS alone calculated by paired *t*-test. Panel C shows the enhanced p105 phosphorylation after treatment with resveratrol while IκB levels remain unaffected. Panel D shows the differential effects of resveratrol upon LPS-induced p38 and ERK1/2 activation. Panels C, D: human monocytes were treated with the indicated concentrations of resveratrol for 1 h prior to stimulation with LPS for 10 min. Cell lysates were prepared and subjected to Western blot analysis using antibodies as described in Materials and methods section. Representative blots of three independent experiments are shown.

source of the elevated TNF-α levels is likely to be innate immune cells of the gut and the liver which might contribute to enhanced immune-surveillance. Further research needs to be done to clarify the role of resveratrol's TNF-α enhancing effect in liver carcinogenesis.

To determine the molecular mechanisms underlying our findings we further focused on the signaling events leading to TNF-α production. Binding sites for AP-1, NF-κB and C/EBPβ are present in the promoter region of TNF-α in myeloid cells and have been shown to be highly responsive to LPS stimulation [27]. Our results with the NF-κB reporter cell line show a potently enhanced NF-κB-dependent signaling in myeloid cells when co-treated with resveratrol thus indicating that this pathway may be crucial for the resveratrol effect. Elevation of p50 levels in myeloid cells was shown to potently enhance NF-κB-dependent transcription of TNF-α even when p65 levels remained unaltered [27]. Phosphorylation events of p105 were shown to evoke 20S proteasomal cleavage which results in the release of active p50 units [35]. Thus, the enhanced phosphorylation of p105 by resveratrol co-treatment is likely to be involved in the enhanced TNF-α production described in this report. A large number of studies have addressed molecular targets of resveratrol, demonstrating a broad impact on cellular transcription machinery [36]. Particularly, inhibition of the NF-κB pathway was shown in different cell lines [17,18,37]. However, none of these studies has focused in detail on alternative NF-κB activation. Here we report a hitherto undescribed modulation of alternative NF-κB signaling in human monocytes by resveratrol.

In conclusion, our data reveal a novel aspect of resveratrol's biologic activity, indicating differential immune modulatory properties of the substance rather than exclusive anti-inflammatory effects. Thus, enhanced clearance of potential invaders might ultimately result in less inflammatory stimulation. Enhanced immune-surveillance might play a role in the reported anti-cancerogenic properties of the substance. Moreover, regarding the molecular mode of action we report a hitherto undescribed impact on the alternative NF-κB pathway in human monocytes. The differential cytokine modulatory effect resulting in altered immune effectiveness might not only have an implication in the field of immunology, but also for other areas like cancer research or vascular biology.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.09.009.

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