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## Resveratrol decreases TNF $\alpha$ -induced ICAM-1 expression and release by Sirt-1-independent mechanism in intestinal myofibroblasts

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### ABSTRACT

Up-regulation of intercellular adhesion molecule-1 (ICAM-1) and its soluble form are involved in the chronic inflammation. For the first time, we demonstrated that resveratrol (RE), a natural polyphenol with antioxidant and anti-inflammatory properties, reduces the increase of expression and release of ICAM-1, due to TNF $\alpha$ -induced oxidative stress, in a myofibroblast cell line derived from human colonic (18Co cells). RE is scavenger of radical oxygen species (ROS) and modulates signaling pathways in which Sirt-1 and NF- $\kappa$ B are involved. Effectively, in TNF $\alpha$ -stimulated 18Co cells RE decreases ROS production and increases Sirt-1 expression and activity, but it reduces TNF $\alpha$ -induced ICAM-1 up-regulation by a Sirt-1-independent mechanism, as demonstrated by EX527 and Sirt-1 siRNA treatments. RE inhibits TNF $\alpha$ -induced activation of NF- $\kappa$ B by reducing both ROS and the degradation of I $\kappa$ B- $\alpha$ , an endogenous inhibitor of NF- $\kappa$ B, with consequent decrease of NF- $\kappa$ B nuclear translocation. This study also shows that NF- $\kappa$ B is not the only factor involved in the TNF $\alpha$ -induced ICAM-1 up-regulation and confirms our previous evidence according to which TNF $\alpha$  increases ICAM-1 levels by redox- and non-redox-regulated mechanisms. RE can represent good and useful support in therapies for intestinal inflammatory diseases in which TNF $\alpha$  plays a crucial role in the increase of adhesion molecule expression.

### 1. Introduction

Intercellular adhesion molecule-1 (ICAM-1)<sup>1</sup>, a glycoprotein expressed on the surface of various cell types, represents an important regulator of inflammation contributing to the leukocyte infiltration into inflammatory site by inducing adhesion and activation [1]. ICAM-1 expression is up-regulated by pro-inflammatory factors and/or oxidative stress [2–5] in various inflammatory and immunological disorders [6–9]. Moreover, ICAM-1 up-regulation is related to intestinal pathological conditions such as inflammatory bowel disease (IBD) [10,11]. In particular, IBD patients present an up-regulation of ICAM-1 expression on membranes of intestinal epithelial and fibroblast cells, and an enhancement of soluble ICAM-1 (sICAM-1) form, probably deriving from ICAM-1 shedding on the surface of intestinal cells, occurs in serum of Crohn’s disease (CD) patients [11–14]. The increase of ICAM-1 and sICAM-1 can be crucial in chronic inflammation and this contributes to

excessive infiltration of leukocytes to the inflammatory site [15] and stimulates cytokine production [16,17]. We have previously reported that tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), a crucial pro-inflammatory mediator involved in IBD inflammatory processes [18], up-regulates ICAM-1 expression and its release in a myofibroblast cell line derived from human colonic mucosa, CCD-18Co (18Co) cells [5]. In these cells, TNF $\alpha$  increases the production of radical oxygen species (ROS) that are in part responsible for TNF $\alpha$ -induced ICAM-1 expression and release. In fact, N-acetylcysteine (NAC), an antioxidant precursor of GSH synthesis, prevents the increase of expression and release of ICAM-1 due to TNF $\alpha$ -induced oxidative stress [5]. 18Co cells show many properties of intestinal subepithelial myofibroblasts (ISEMFs) that are involved in different functions, including the mucosal repair and inflammatory processes, both in healthy and ill intestine [19]. These cells play an important role in the physiopathology of IBD by secreting pro-inflammatory mediators such as cytokines, chemokines,

**Abbreviations:** CD, Crohn’s disease; H2DCF-DA, 2’-7’-dichlorodihydrofluorescein diacetate; ICAM-1, Intercellular adhesion molecule-1; ISEMFs, intestinal subepithelial myofibroblasts; sICAM-1, soluble ICAM-1; IBD, inflammatory bowel disease; NAC, N-acetylcysteine; NF- $\kappa$ B, nuclear factor-kappa B; ac-NF- $\kappa$ B, acetyl-NF- $\kappa$ Bp65; PDTC, pyrrolidine dithiocarbamate; RE, Resveratrol; ROS, radical oxygen species; Sirt-1, sirtuin-1; TNF $\alpha$ , tumor necrosis factor- $\alpha$

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metalloproteinases and adhesion molecules [12,20–22]. In particular, ISEMFs isolated from colonic mucosa of CD patients are characterized by oxidative stress that induces the up-regulation of pro-inflammatory mediators that in turn can cause an overproduction of ROS [23–27]. For this, the effect of natural phytochemicals with antioxidant properties, such as flavonoids and other polyphenols, in the modulation of intestinal inflammation has been investigated [28–30]. Resveratrol (RE) (trans-3,5,4'-trihydroxy-stilbene), a polyphenol present in red wine, some berries and peanuts, shows antioxidative and anti-inflammatory functions in IBD [29]. In experimental models for chronic inflammatory intestinal disease RE reduces neutrophil recruitment, TNF $\alpha$  production and the activity of adhesion molecules [31,32]. Studies performed in intestinal cells treated with lipopolysaccharide show that RE exerts its role by decreasing cyclooxygenase-2 expression and inhibiting nuclear factor-kappa B (NF- $\kappa$ B) activation [33,34]. Considering this and that a therapeutic role for RE has been suggested in IBD [29], in this study we investigated the effect of RE on TNF $\alpha$ -induced oxidative stress and on consequent up-regulation of ICAM-1 expression and release in 18Co cells. RE effect on NF- $\kappa$ B was also studied in TNF $\alpha$ -up-regulated expression of ICAM-1 by evaluating the involvement of sirtuin-1 (Sirt-1), a deacetylase that regulates various biological anti-inflammatory processes [35,36]. In fact, RE up-regulates activity and expression of Sirt-1 that is related also to down-regulation of NF- $\kappa$ B activity by deacetylating RelA/p65 subunit at Lysine 310 [37]. In fact, numerous properties of RE are due to Sirt-1-dependent mechanisms [38,39].

## 2. Materials and Methods

### 2.1. Cell culture and treatment

CCD-18Co (18Co) cells, obtained from American Type Culture Collection (Manassas, VA), were used in our experiments with population doubling level (PDL) = 27–36, given that the line begins to senescence at about PDL = 42. PDL was calculated using the equation  $PDL = \ln(CH/CS) \times 1/\ln 2$ , in which CH is viable cell harvested number and CS is cell seeded number. The substances used for cell culture and treatments were purchased from Sigma-Aldrich, (St. Louis, Missouri, USA). Cells were cultured at 37 °C in a 5% CO<sub>2</sub> atmosphere in minimum essential medium with 2 mM glutamine and 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum. Culture media were supplemented with 72 mg/l penicillin and 100 mg/ml streptomycin. Confluent and 24-hour serum-starved 18Co cells were pre-treated or not with various concentrations of RE (25–200  $\mu$ M) or pyrrolidine dithiocarbamate (PDT) (1–50  $\mu$ M) 1 h before and during the subsequent stimulation (2 or 24 h) or not with 10 ng/ml TNF $\alpha$ . RE concentration range includes those used in our previous study to detect the ability of RE to prevent oxidative stress in these cells [40]. In other experiments, 20 mM NAC was added or not to cells during the last 16 h of starvation and during the subsequent stimulation or not with 10 ng/ml TNF $\alpha$  as previously reported [5]. In experiments with EX527, 10 and 20  $\mu$ M of this compound were added to 24-hrs serum-starved 18Co cells 1 h before pre-treatments and subsequent TNF $\alpha$ -stimulations. 0.008 % DMSO (vehicle for RE and EX527) was present in the respective RE- and/or EX527-untreated cells. Cells were stimulated for 2 h with TNF $\alpha$  for detection of I $\kappa$ B- $\alpha$  levels and NF- $\kappa$ B nuclear translocation, and for 24 h for detection of ICAM-1, Sirt-1, NF- $\kappa$ Bp65 (NF- $\kappa$ B) expression. To relate acetylation NF- $\kappa$ B p65 to Sirt-1 expression and activation, acetylated NF- $\kappa$ B p65 (ac-NF- $\kappa$ B) levels were detected after 24 h of TNF $\alpha$ -stimulation. Some treatments to detect ICAM-1 expression were performed in cells transiently transfected with 75 nM human Sirt-1 siRNA corresponding to two DNA target sequences of human Sirt-1 (5'-GUGUCAUGGUCCUUUGCA[dT][dT]-3'; 5'-UGCAAAGGAACCAUGACAC[dT][dT]-3') (Sigma-Aldrich), or scrambled siRNA (Scr siRNA) (Universal Negative Control # 1, Sigma Aldrich), using lipofectamine RNAiMAX™ (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The ability of

siRNA to silence Sirt-1 expression levels in control cells was checked 24 h after transfection by Western blot analysis. The concentrations of all compounds used did not reduce the viability according to Trypan blue exclusion test.

### 2.2. Intracellular ROS production assay

The intracellular production of ROS was assayed, as previously described [41], in 18Co cells seeded in 12-well plates, and treated as described above. 30 min before the end of various treatments, 5 g/l 2'-7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) was added in culture medium. After PBS washing, 18Co cells were lysed in 50 mM Tris/HCl pH 7.5, 1% Triton X100, 150 mM NaCl, 100 mM NaF, 2 mM EGTA (RIPA buffer) and fluorescence intensity was detected using a Fluoroskan AscentFL microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 485 nm excitation and 518 nm emission wavelengths. Data have been normalized on total protein content and values were expressed as percentage of ROS production measured in untreated and unstimulated cells (control).

### 2.3. ICAM-1 and sICAM-1 assay

sICAM-1 release and intracellular ICAM-1 levels were measured in cell lysates and in the respective culture media, using Human ICAM-1 ELISA kit (Usen Life Sciences Inc., Wuhan, Hubei, PRC) according to the manufacturer's instructions. 18Co cells were seeded in 12-well plates and treated as described above. For cell lysates preparation, cells were detached with trypsin and collected by centrifugation at 130 x g for 10 min. Cells, washed three times in cold phosphate buffer saline (PBS), were suspended in PBS and ultrasonicated for 4 times. Subsequently, cell lysates were centrifuged at 1500 x g for 10 min at 4 °C to remove cellular debris and ICAM-1 assay was performed in the supernatants. Data have been normalized on total protein content and sICAM-1 and ICAM-1 levels were expressed as percent of the respective levels measured in control.

### 2.4. Sirt-1 expression assay

Sirt-1 expression was measured in 18Co cells, seeded in 12-well plates and treated as described above, by using Human SIRT1 ELISA kit (Abcam, Cambridge U.K.). Briefly, cell extracts were obtained in cells previously solubilized in Cell Extraction Buffer and centrifuged at 18,000 x g for 20 min at 4 °C according to the manufacturer's instructions. Data have been normalized on total protein content and Sirt-1 expression was expressed as percent of the respective levels measured in control.

### 2.5. Western blotting

Ac-NF- $\kappa$ B, NF- $\kappa$ B and I $\kappa$ B- $\alpha$  levels were analysed by Western blot in 18Co cells seeded in p-60 plates treated and stimulated or not as described above. Cells were lysed in ice-cold RIPA buffer containing phosphatase and protease inhibitor cocktails, purchased from Sigma (St. Louis, MO) and, after 15 min on ice, were centrifuged at 11,600 x g for 10 min and supernatants were subjected to Western blot analysis. Preparation of nuclear extracts for detection of NF- $\kappa$ B translocation was performed as described by Bai et al. [42]. Protein concentrations were determined by the bicinchoninic acid solution (BCA) protein reagent assay (Pierce) [43] using bovine serum albumin as standard (Sigma, St. Louis, MO). Equal amount of total proteins (30–35  $\mu$ g) were loaded in each line and were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE) on 10 % (w/v) gel and electrotransferred to PVDF membrane (GE Healthcare, Chicago, IL) that was probed with specific antibodies anti-acetyl-NF- $\kappa$ Bp65/Lys310 or anti-NF- $\kappa$ Bp65 or anti-I $\kappa$ B- $\alpha$  (Cell Signalling Technology Inc., Danvers, MA) or anti- $\beta$ -actin or anti-GAPDH or anti-Histone H3 (Santa Cruz

Biotechnology, Inc.). Secondary antibodies conjugated to horseradish peroxidase were used to detect antigen-antibody complexes with a chemiluminescence reagent kit (GE Healthcare, Chicago, IL). Amersham Imager A600 (GE Healthcare, Chicago, IL) was used for capture of high resolution digital images of the bands whereas densitometric analysis was performed using ImageJ Software (National Institutes of Health, USA).  $\beta$ -actin or GAPDH or Histone H3 bands were used for normalization in densitometric analysis and values were expressed as percentage variations relative to controls.

### 2.6. Statistical analysis

All experiments were carried out three or four times. Data are expressed as the mean  $\pm$  SEM and statistical significance of the differences was determined by using one-way ANOVA analysis with Bonferroni's multiple comparison test, using the GraphPad Prism Software.  $p \leq 0.05$  was considered statistically significant.

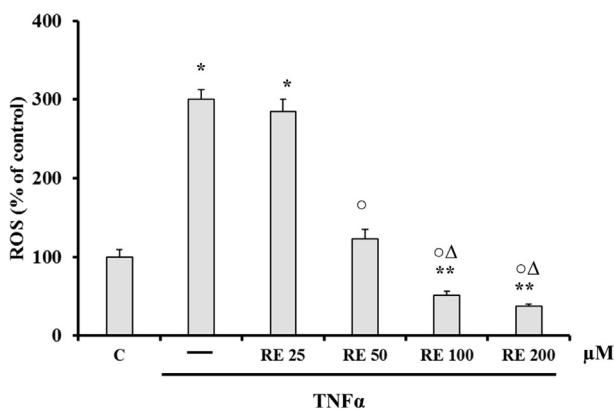
## 3. Results

### 3.1. Effect of RE on TNF $\alpha$ -induced intracellular ROS production.

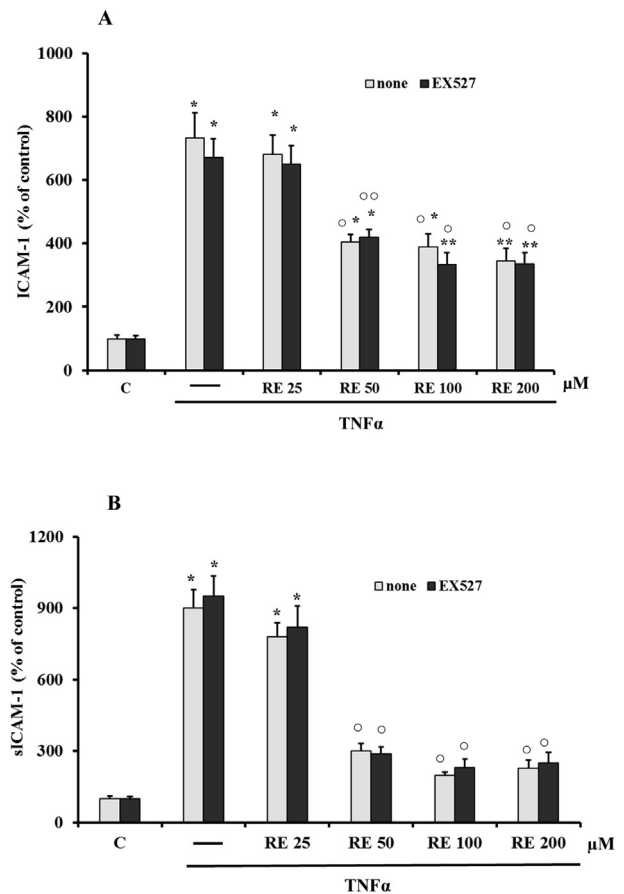
Initially, it has been investigated the effect of RE at various concentrations (25–200  $\mu$ M) on TNF $\alpha$ -induced ROS production in 18Co cells. Fig. 1 shows that the significant increase of ROS, detected in 10 ng/ml TNF $\alpha$ -stimulated cells, was not prevented by pre-treatment with 25  $\mu$ M RE. Differently, all other concentrations of RE down-regulated TNF $\alpha$ -induced ROS production. In fact, 50  $\mu$ M RE restored ROS to control values, whereas 100  $\mu$ M and 200  $\mu$ M RE decreased significantly ROS levels at values lower than those of control (Fig. 1).

### 3.2. Effect of RE on TNF $\alpha$ -induced ICAM-1 expression and sICAM-1 release.

Detection of ICAM-1 expression and sICAM-1 release were performed in 18Co cells by ELISA kit in cell lysates and in culture media, respectively. Fig. 2 A, B shows that ICAM-1 and sICAM-1 were up-regulated by 10 ng/ml TNF $\alpha$  and that the pre-treatment with 25  $\mu$ M RE did not affect this according to ROS levels. On the contrary, pre-



**Fig. 1.** Intracellular ROS production in 18Co cells pre-treated or not with RE and stimulated with TNF $\alpha$ . Starved 18Co cells, pre-treated or not with various concentrations of RE (25–200  $\mu$ M), were stimulated or not for 24 h with 10 ng/ml TNF $\alpha$ , as reported in Materials and Methods. The intracellular ROS production was detected by measuring the fluorescence intensity of the intracellular oxidation-sensitive probe H<sub>2</sub>DCFDA. The values, normalized on total protein content and expressed as percentage of untreated and unstimulated cells (control, C), are the mean  $\pm$  SEM of four experiments repeated in triplicate. \* $p \leq 0.001$ ; \*\* $p \leq 0.01$  compared to C cells;  $\circ$  $p \leq 0.001$  compared to RE untreated and TNF $\alpha$  stimulated cells;  $\Delta$  $p \leq 0.01$  compared to 50  $\mu$ M RE pre-treated and TNF $\alpha$  stimulated cells.

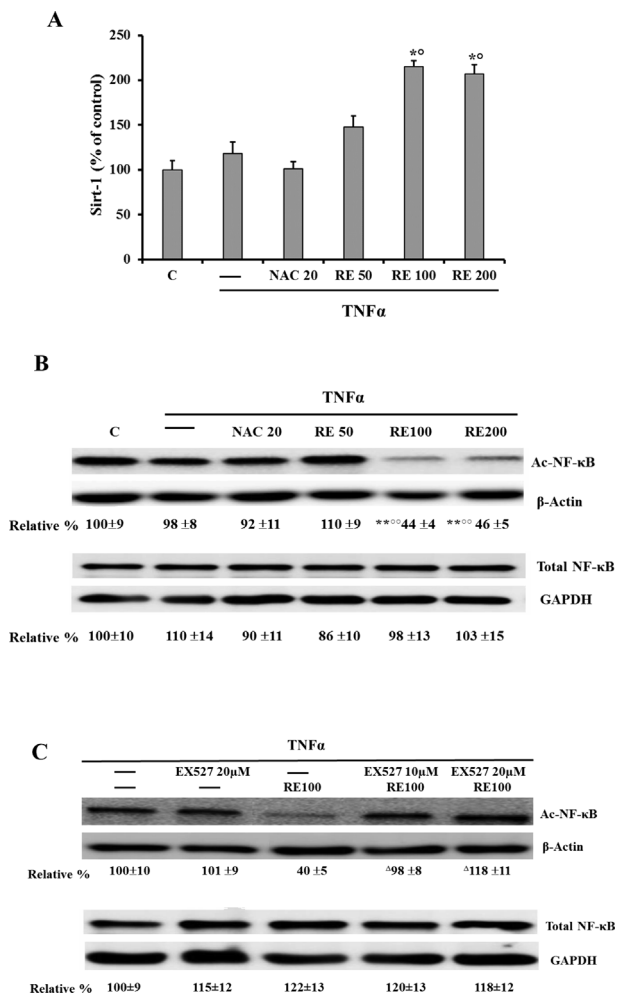


**Fig. 2.** ICAM-1 expression and sICAM-1 release with or without EX527 in 18Co cells pre-treated or not with RE and stimulated with TNF $\alpha$ . Starved 18Co cells, pre-treated or not with various concentrations of RE (25–200  $\mu$ M), were stimulated or not for 24 h with 10 ng/ml TNF $\alpha$  in the presence or the absence of 10  $\mu$ M EX527, as reported in Materials and Methods. Expression of ICAM-1 levels (A) and sICAM-1 levels (B) were assayed by ELISA kit in cell lysates and in culture media, respectively. The values, expressed as percentage of untreated and unstimulated cells (control, C), are the mean  $\pm$  SEM of four experiments repeated in triplicate. \* $p \leq 0.001$ ; \*\* $p \leq 0.05$  compared to C cells;  $\circ$  $p \leq 0.001$ ;  $\Delta$  $p \leq 0.05$  compared to RE untreated and TNF $\alpha$  stimulated cells.

treatment with 50–200  $\mu$ M RE decreased ICAM1-expression and release in the same way and significantly as compared to TNF $\alpha$ -treated cells, indicating the lack of a dose-response relationship. However, at these concentrations of RE ICAM-1 expression levels were not similar or lower than control values, differently to that occurred for ROS production and sICAM-1 release. In fact, 50–200  $\mu$ M RE restored TNF $\alpha$ -induced sICAM-1 levels to control values (Fig. 2 B). Considering that RE is a strong activator of Sirt-1 [44], we evaluated the effect of RE on ICAM-1 expression and release in the presence of 10  $\mu$ M EX527, a Sirt-1 inhibitor [45]. This inhibitor did not affect ICAM-1 expression and sICAM-1 release in all conditions used (Fig. 2A and B).

### 3.3. Effect of RE on Sirt-1 expression and activity in TNF $\alpha$ -stimulated cells.

Subsequently, expression and activity of Sirt-1 in 18Co cells stimulated with TNF $\alpha$  and pre-treated or not with various concentrations of RE, were detected. Sirt-1 activity was measured by evaluating the levels of acetylated-NF- $\kappa$ B subunit Rel/p65 at lysine 310 (ac-NF- $\kappa$ B). Fig. 3A and B shows that TNF $\alpha$  did not induce an increase of the expression and activity of Sirt-1 in non-pre-treated or in 50  $\mu$ M RE pre-treated cells, as compared to control cells. Differently, pre-treatment with 100 and 200  $\mu$ M RE induced a similar and significant increase in Sirt-1 expression with consequent remarkable decrease in ac-NF- $\kappa$ B



**Fig. 3.** Ac-NF-κB, total NF-κB levels and Sirt-1 expression in 18Co cells pre-treated or not with RE and stimulated with TNFα. Starved 18Co cells, pre-treated or not with various concentrations of RE (25–200 μM), were stimulated or not for 24 h with 10 ng/ml TNFα, as reported in Materials and Methods. Sirt-1 expression was assayed by ELISA kit in cell lysates (A). The values, expressed as percentage of untreated and unstimulated cells (control, C), are the mean ± SEM of four experiments repeated in triplicate. Ac-NF-κB and NF-κB levels in the absence (B) or in the presence of 10 or 20 μM EX527 (C) were detected in cells lysates by Western blot analysis using anti-acetyl-NF-κBp65/Lys310 or anti-NF-κBp65. Blots are representative of three experiments and the normalized values with β-actin or GAPDH bands are reported in the bottom. The values are expressed as percentage ± SEM relative to those obtained in untreated and unstimulated cells (control, C) for B, or to those obtained in EX527-untreated and TNFα-stimulated cells, for C. \*p ≤ 0.001; \*\*p ≤ 0.01 compared to C cells; °p ≤ 0.001; °°p ≤ 0.01 compared to untreated and TNFα stimulated cells; ^p ≤ 0.01 compared to 100 μM RE pre-treated and TNFα stimulated cells.

levels, in TNFα-stimulated cells. On the contrary, NAC, an antioxidant that reduces TNFα-up-regulated ICAM-1 expression similarly to RE [5], did not affect expression and activity of Sirt-1 in 18Co cells stimulated with TNFα (Fig. 3A and B). To verify that EX527 at concentration used for the detection of ICAM-1 (Fig. 2) was effectively able to inhibit Sirt-1, we measured ac-NF-κB levels in the absence or in the presence of this inhibitor. Fig. 3C shows that the highest concentration used of EX527 did not affect ac-NF-κB levels in TNFα-stimulated cells. On the contrary, 10 and 20 μM EX527 significantly increased levels of ac-NF-κB in 100 μM RE pre-treated and TNFα-stimulated cells, as compared to RE-pre-treated cells without inhibitor. Moreover, EX527 restored ac-NF-κB levels to those detected in TNFα-stimulated cells. The levels of NF-κB expression did not change and were similar in all experimental

conditions (Fig. 3B and C). Therefore, even if RE is able to induce in these cells expression and activity of Sirt-1, this deacetylase does not seem to be involved in the effect of RE on TNFα-induced ICAM-1 expression and release.

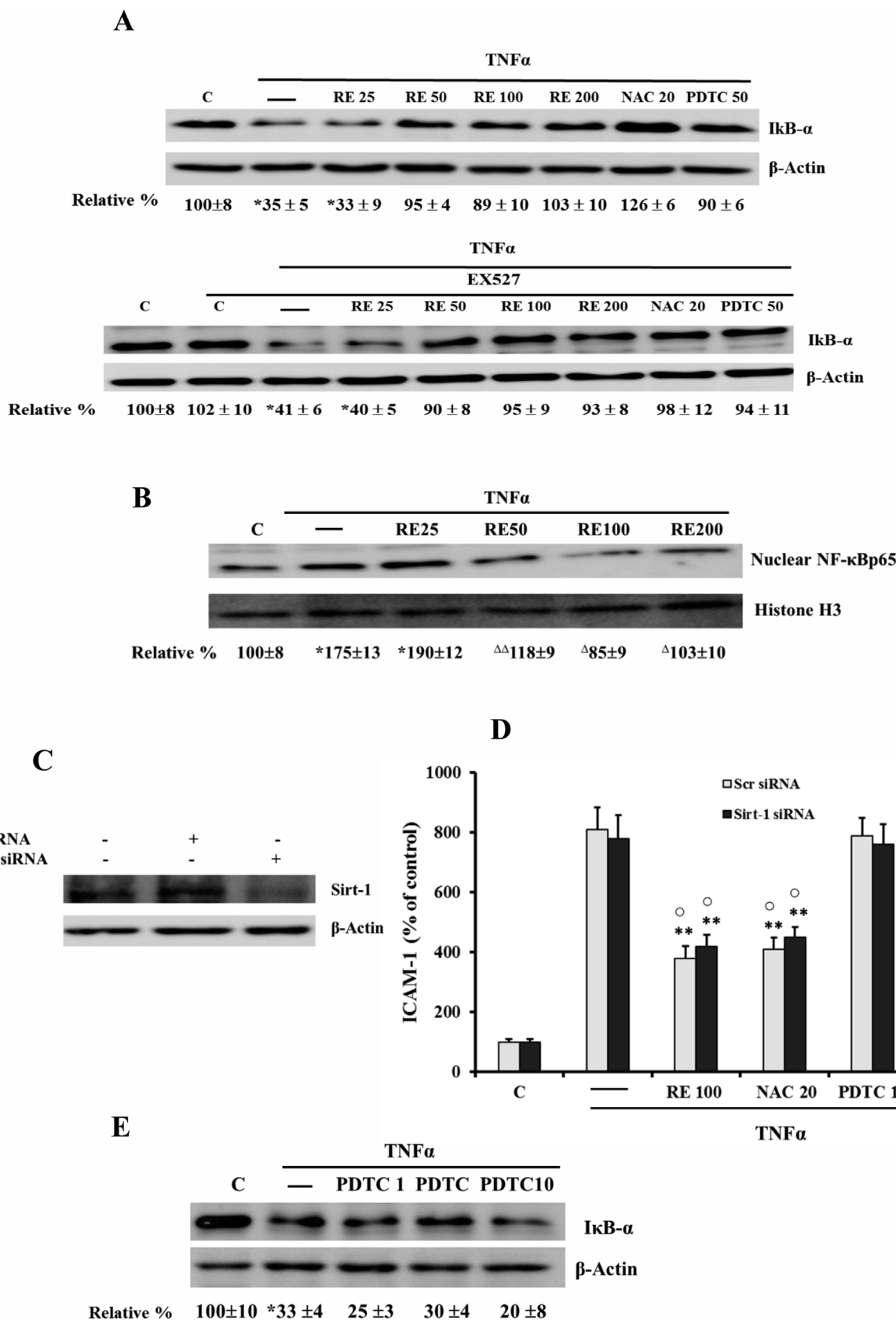
### 3.4. Effect of RE on IκB-α degradation in TNFα-stimulated cells.

Under physiological conditions NF-κB is inactivated by interaction with its endogenous inhibitor, IκB-α protein, that when degraded influences subsequent nuclear translocation of NF-κB subunits [46]. For this, the role of RE in TNFα-treated 18Co cells on IκB-α degradation and nuclear translocation of NF-κB were studied. Fig. 4A shows that the IκB-α protein was significantly degraded in 18Co cells stimulated with 10 ng/ml TNFα, as compared to control cells. IκB-α degradation was not affected by pre-treatment with RE 25 μM in agreement with its effect on ROS production and ICAM-1 levels. All other concentrations used of RE were able to inhibit TNFα-induced IκB-α degradation by restoring the levels of this protein to those of control, similarly to 20 mM NAC and 50 μM PDTC, a specific inhibitor of NF-κB [47]. IκB-α levels were not reversed by EX527 treatment (Fig. 4A) and were related to nuclear NF-κB levels in 18Co cells pretreated or not with RE and stimulated with TNFα (Fig. 4B). In fact, Fig. 4B shows that TNFα induced an increase of nuclear NF-κB levels that were restored to control levels in the presence of 50–200 μM RE but not with 25 μM RE. The influence of NAC and PDTC on ICAM-1 expression levels was compared to 100 μM RE in TNFα-stimulated cells transfected or not with specific Sirt-1 siRNA that induced a decrease in Sirt-1 expression in control cells after 24 h of transfection (Fig. 4C). Fig. 4D shows that NAC and 50 μM PDTC had a similar effect to 100 μM RE and did not restore ICAM-1 levels to those of control. siRNA of Sirt-1 did not reverse neither the effect of RE nor that of NAC or PDTC (Fig. 4C), confirming the non-involvement of Sirt-1 in the role of RE on TNFα-induced ICAM-1 expression. 10 μM PDTC was not able to reduce ICAM-1 expression (Fig. 4D) and concentrations of this compound lower than 50 μM did not affect IκB-α degradation detected in TNFα-stimulated 18 Co cells (Fig. 4E).

## 4. Discussion

RE, a natural polyphenolic compound involved in the treatment of various pathologies, possesses many pharmacological activities, including antitumor, anti-inflammatory, anti-aging, antioxidant and anti-diabetic effects [48]. The beneficial properties of RE are due to its ability to neutralize ROS or to regulate signaling pathways in which Sirt-1 and NF-κB are involved [49]. For the first time, this study shows that RE is able to reduce the oxidative stress and to down-regulate ICAM-1 expression and release by a Sirt-1 independent mechanism in TNFα-stimulated 18Co cells. In particular, at the lowest concentration (25 μM) RE does not influence ROS production and ICAM-1 expression and release, differently to that occurs with the other concentrations of RE used. In fact, at these concentrations, RE is not able to restore ICAM-1 expression to control values differently to that occurs for sICAM-1, even if RE totally prevents TNFα-induced ROS levels. It is possible that RE effect on sICAM-1 values can be both due to diminished expression of ICAM-1 and to the consequent inhibition of ROS-activated proteases involved in ICAM-1 shedding. The protective role of RE on TNFα-induced oxidative stress is in accordance with its preventive effect on oxidative stress induced by buthionine sulfoximine or starvation in 18Co or in MLO-Y4 cells, respectively [40]. Moreover, RE prevents oxidative stress caused by high levels of glucose in smooth cells [50] and TNFα-induced ROS production in HUVEC cells [51]. Sirt-1, belonging to NAD<sup>+</sup>-dependent deacetylase family, plays a crucial role in the regulation of various biological and metabolic activities by modulating transcriptional factors such as p53, NF-κB and forkhead box O, with consequent change of gene expression responsible for the protection against apoptosis, oxidative stress and aging [52]. RE is an





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activator of Sirt-1 and often exhibits its anti-aging, anti-inflammatory and antioxidant properties through the up-regulation of this deacetylase [53-55], as it occurs in the mitigation of TNF $\alpha$ -induced inflammation in

fibroblasts and in the attenuation of triglyceride in adipocytes [56,57]. The up-regulation of adhesion molecules that occurs in pathological conditions can be inhibited by RE. In fact, it is able to prevent the

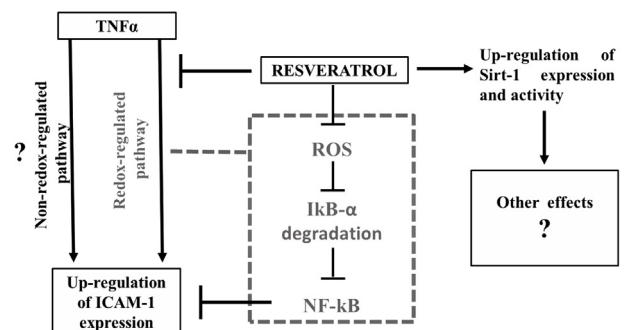
**Fig. 4.** I $\kappa$ B- $\alpha$  levels, nuclear NF- $\kappa$ B and ICAM-1 expression in 18Co cells pre-treated or not with RE, NAC or PDTC and stimulated with TNF $\alpha$ . Starved 18Co cells, pre-treated or not with various concentrations of RE (25–200  $\mu$ M) or PDTC (1–50  $\mu$ M) or 20 mM NAC, were stimulated or not for 2 h with 10 ng/ml TNF $\alpha$ , as reported in Materials and Methods. I $\kappa$ B- $\alpha$  (A) and nuclear NF- $\kappa$ Bp65 (B) levels were detected in cell lysates and in nuclear extracts, respectively, by Western blot analysis using anti-I $\kappa$ B- $\alpha$  or anti-NF- $\kappa$ Bp65. Blots are representative of three experiments and the normalized values with  $\beta$ -actin or Histone H3 bands are reported in the bottom. The values are the mean percentage  $\pm$  SEM relative to those obtained in the untreated and unstimulated cells (control, C). Expression of ICAM-1 levels (D) by ELISA kit were assayed in lysates of cells transfected with Sirt-1 siRNA or Scr siRNA, pre-treated as reported above and stimulated for 24 h with 10 ng/ml TNF $\alpha$ . The values, expressed as percentage of respective untreated and unstimulated cells (control, C), are the mean  $\pm$  SEM of four experiments repeated in triplicate. Sirt-1 levels (C) were detected in lysates of cells transfected or not with Sirt-1 siRNA or Scr siRNA by Western blot analysis using anti-Sirt-1. \* $p \leq 0.01$ ; \*\* $p \leq 0.05$  compared to C cells;  $^{\circ}p \leq 0.05$  compared to untreated and TNF $\alpha$  stimulated cells;  $^{\wedge}p \leq 0.01$ ;  $^{\Delta\Delta}p \leq 0.05$  compared to TNF $\alpha$  stimulated cells or 25  $\mu$ M RE pre-treated and TNF $\alpha$  stimulated cells.

expression of adhesion molecules induced by pro-inflammatory mediators in endothelial cells [48,55,58-60], and to exert its anti-inflammatory effect by suppressing ICAM-1 and VCAM-1 levels in colon and serum in a rat model of ulcerative colitis [32]. The mechanism by which RE inhibits adhesion molecules expression can be mediated by Sirt-1 as it happens in lipopolisaccharide-stimulated HUVEC cells and in obese septic mice [55]. Other data show that RE reduces ICAM-1 expression and leukocyte adhesion in the retina of mice with ocular inflammation by dual mechanisms involving the antioxidant properties of RE and its ability to activate Sirt-1 [61]. In the present study, high RE concentrations in TNF $\alpha$ -stimulated 18Co cells up-regulate the expression of Sirt-1 and induce a significant decrease in the deacetylation of NF- $\kappa$ B, reverted by Sirt-1 inhibitor, EX527, without interfering on total NF- $\kappa$ B expression levels. However, Sirt-1 is not involved in the capacity of RE to decrease ICAM-1 levels as demonstrated by experiments performed in the presence of EX527 and in cells transfected with specific Sirt-1 siRNA. Indeed, this is evident also considering that 50  $\mu$ M RE treatment reduces TNF $\alpha$ -induced ICAM-1 expression and ROS levels but does not increase Sirt-1 expression and activation. It seems that, in these experimental conditions, RE affects ICAM-1 levels only by its antioxidant role similarly to that previously obtained with NAC [5] that, on the contrary, does not affect expression and activity of Sirt-1. Then, we suggest that RE reduces ICAM-1 expression and release by decreasing TNF $\alpha$ -induced oxidative stress in 18Co. Effectively, TNF $\alpha$  does not affect Sirt-1 expression and does not enhance NF- $\kappa$ B activity through its acetylation in our experimental conditions. This can explain why RE can act through a Sirt-1 independent manner, even if it reduces the levels of ac-NF- $\kappa$ B below those of control. In 18Co cells, TNF $\alpha$  activates NF- $\kappa$ B by inducing ROS and decreasing I $\kappa$ B- $\alpha$  levels, an endogenous inhibitor of NF- $\kappa$ B. TNF $\alpha$  stimulates NF- $\kappa$ B activity by a signaling pathway that involves proteins of complex I [62], and oxidative stress can affect NF- $\kappa$ B activity [63]; in both cases, an increased degradation of I $\kappa$ B- $\alpha$  occurs. We demonstrated that in 18Co stimulated with TNF $\alpha$ , RE inhibits ROS production and I $\kappa$ B- $\alpha$  degradation with consequent decrease of NF- $\kappa$ B nuclear translocation and ICAM-1 expression. Indeed, RE blocks NF- $\kappa$ B activation in various cell types and this can occur also by inhibiting mechanisms involved in I $\kappa$ B- $\alpha$  degradation [64]. In 18Co cells, we suggest that TNF $\alpha$ -induced oxidative stress activates NF- $\kappa$ B considering that all RE concentrations, except for 25  $\mu$ M, are able to restore I $\kappa$ B- $\alpha$  to control levels also in the presence of EX527. These data confirm that, in our experimental conditions, RE does not involve Sirt-1 in the inhibition of NF- $\kappa$ B, considering also that 50  $\mu$ M RE inhibits NF- $\kappa$ B and this is in agreement with ROS levels and ICAM-1 expression but not with Sirt-1 expression level. Indeed, in murine macrophages, RE also regulates the inflammatory responses by decreasing and increasing ac-NF- $\kappa$ B and I $\kappa$ B- $\alpha$  levels, respectively, without affecting total NF- $\kappa$ B levels [65]. However, these effects in macrophages are reversed by EX527 treatment [65] differently to that occurs in 18Co cells, in which EX527 reverses the effect of RE on NF- $\kappa$ B acetylation but not on I $\kappa$ B- $\alpha$  and ICAM-1 levels. 50  $\mu$ M PDTC, with antioxidant properties often related to its inhibitory role on NF- $\kappa$ B [66,67], and NAC restore I $\kappa$ B- $\alpha$  values, but not ICAM-1 levels to control values in TNF $\alpha$ -stimulated 18Co. EX527 treatment or Sirt-1 siRNA transfection did not reverse these effects, similarly to that occurs with RE. Indeed, to investigate and clarify RE role and/or Sirt-1 involvement

on NF- $\kappa$ B, PDTC is frequently used [66,68-70]. In our study, the behavior of RE is similar to that of PDTC and NAC, clarifying that effectively RE suppresses TNF $\alpha$ -induced NF- $\kappa$ B activation and ICAM-1 expression without Sirt-1 involvement. Moreover, PDTC at concentration lower than 50  $\mu$ M did not affect I $\kappa$ B- $\alpha$  and ICAM-1 levels similarly to that occurs with 25  $\mu$ M RE. These data altogether indicate that the activation of NF- $\kappa$ B is involved effectively in TNF $\alpha$ -up-regulated ICAM-1 expression but the activation of this transcriptional factor is not the only mechanism implicated. Moreover, they confirm that RE regulates TNF $\alpha$ -induced NF- $\kappa$ B activation and ICAM-1 expression by attenuating ROS production in a Sirt-1-independent manner. RE reduces totally TNF $\alpha$ -induced ROS production but not ICAM-1 expression, confirming our previous data that show the partial involvement of TNF $\alpha$ -induced oxidative stress in the increase of ICAM-1 expression [5]. Then, it is strengthened the hypothesis that the enhancement of ICAM-1 expression in TNF $\alpha$ -stimulated myofibroblasts is due to redox-dependent and -independent mechanisms, and RE, acting as antioxidant, is able to reduce the increase of ICAM-1 expression due to redox-dependent mechanism of TNF $\alpha$ . The anti-inflammatory role of RE in intestinal cells was studied in CaCo-2 cells, in SW480 human colon adenocarcinoma cells [33,34], and in animals. RE attenuates inflammatory mediators secretion and molecule adhesion levels in colon and serum [31,32] and, by reducing the expression of adhesion molecules in intestinal myofibroblasts, RE also decreases sICAM-1 levels. This effect can down-regulate development of cancer that can be consequent to the chronic inflammation [71]. In fact, an increase of ICAM-1 and its soluble form can be involved in the tumor differentiation and in the development of metastasis, respectively [72,73].

## 5. Conclusions

For the first time, this study shows that, RE reduces TNF $\alpha$  up-regulated ICAM-1 expression and release through a Sirt-1 independent and redox-regulated mechanism in 18Co cells (Fig. 5). In fact, RE increases Sirt-1 expression and activity in 18Co, but these effects are not involved in RE regulation of TNF $\alpha$ -induced NF- $\kappa$ B activation partially involved in the increase of ICAM-1. RE inhibits I $\kappa$ B- $\alpha$  degradation and



**Fig. 5.** Schematic representation of mechanism by which RE down-regulates TNF $\alpha$ -induced oxidative stress and ICAM-1 expression in 18Co cells. TNF $\alpha$  induces ROS production that activates I $\kappa$ B- $\alpha$  degradation and NF- $\kappa$ B pathway signalling. This mechanism, in part involved in the increase of ICAM-1 expression, is inhibited by RE in a Sirt-1-independent manner.

NF- $\kappa$ B nuclear translocation by reducing ROS production. The results suggest also that NF- $\kappa$ B pathway is not the only mechanism involved in TNF $\alpha$ -induced ICAM-1 up-regulation, and they supports our previous hypothesis according to which TNF $\alpha$  increases ICAM-1 expression by redox and other non-redox regulated mechanisms. Moreover, RE is efficient in ICAM-1 down-regulation in 18Co stimulated with TNF $\alpha$  at lower concentrations than those used for other antioxidants, such as NAC. For this, we speculate that RE, being present in food and natural supplements, may be useful in supporting the anti-inflammatory and anti-TNF $\alpha$  therapies used in intestinal inflammatory diseases, such as IBD, in which an increased expression of adhesion molecules due to TNF $\alpha$  occurs.

#### Declaration of interest

None.

#### Conflicts of interest

The authors declare that they have no competing interests.

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#### Author contributions

TI and MTV conceived and designed the study. VD performed the experiments and prepared figures. TI, MS, AGB and MTV analyzed and interpreted the data. TI and MTV wrote the manuscript. VD and AGB edited and revised the manuscript. All authors read and approved the final manuscript

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