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## Red wine extract disrupts lymphocyte Th17 cells differentiation in a cancerous context.

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**Red wine extract disrupts lymphocyte Th17 cells differentiation in a cancerous context**

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**Keywords:** Red wine extract; Polyphenols; lymphocytes Th17; Interleukins

## Abstract

### Scope

It is well established that immune response and inflammation promotes tumoral progression.

Immune cells communicate through direct contact or through cytokine secretion and it is the expression of those mediators and of the pro-inflammatory status that will tip the balance towards tumor progression or anti-tumor immunity. We have recently demonstrated that a red wine extract (RWE) was able to decrease inflammation through an action on the NLRP3 inflammasome complex. This study determined whether a RWE impacts other key actors of inflammation through an action on immune Th17 cells.

### Methods and Results

Using a RWE containing 4.16g of polyphenols per liter of wine, we showed *in vitro* and *in vivo* that RWE exerts a reduction of tumor growth associated with a decrease of the tumor-infiltrating lymphocytes. More specifically, the process of lymphocyte T differentiation into protumoral Th17 cells was altered by RWE *in vitro* and *in vivo* as revealed by the decrease in key actors' expression controlling this process such as STAT3 and ROR $\gamma$ t. Subsequently, this disruption was associated with an inhibition of inflammatory IL-17 secretion.

### Conclusion

The result highlighted the main involvement of Th17 immune cells in the biological effect of a red wine extract.

## 1. Introduction

For several years, numerous epidemiological studies have shown that there is a strong link between inflammation and the immune system. Indeed, immune cells play a role in first-line defense following various traumas caused by infectious agents, chemical substances, physical agents or even post-traumatic tissue lesions. In contrast, inflammation has been described as an initiator event of major diseases with a significant impact in terms of public health such as cardiovascular diseases, autoimmune diseases, eye diseases, age-related diseases, and more particularly neurodegenerative diseases and the occurrence and development of cancers. In this last context, tumoral cells can produce various chemokines through different process. Among the most well-known processes, the release of interleukin-1 beta (IL-1 $\beta$ ) following the activation of a multiprotein complex, the inflammasome, is responsible for inflammation that can be acute but also chronic or low-noise, causing many disorders including the occurrence of cancers. A second well-known process is the secretion of pro-inflammatory interleukins by immune cells that are able to overproduce various factors promoting proliferation, inflammation, neoangiogenesis. Among these proteins, we found IFN- $\gamma$ , TNF $\alpha$ , IL-6, IL-17A, and IL-25 that strongly contribute to tumoral growth.

Therefore, one of the major current issues would be to find compounds capable of modulating these production processes of pro-inflammatory substances and thus help reduce the occurrence of pathologies or reduce their progression. In this way, various studies have pointed the interest of polyphenolic compounds to prevent various diseases without toxicities. For example, numerous studies reveal that phytoconstituents or micronutrients can protect against cancers [1]. Especially, Levi et al. show an inverse relation between resveratrol (a non-flavonoid polyphenol) and breast cancer risk [2]. Another cohort study in Finland [3] shows a link between flavonol consumption and reduced risk of lung cancer. These findings lead to the suggestion that polyphenols and flavonoids have beneficial health effects. Very

1  
2  
3 recently, we have highlighted the potential anti-inflammatory effect of an extract of red wine  
4 enriched in polyphenol compounds (RWE) on macrophages [4]. This extract was able to  
5  
6 strongly decrease IL-1 $\beta$  secretion through a modulation of the expression of key proteins  
7  
8 involved in the inflammasome complex, i.e., NLRP3 and apoptosis-associated speck-like  
9  
10 protein containing a CARD (ASC). Moreover, we have demonstrated that RWE disrupted the  
11  
12 priming signal and the activating signal leading to inflammasome activation in macrophages  
13  
14 [4]. This important way could then prevent inflammation in various contexts, but at present  
15  
16 there is no data on the effect of a complex mixture such as a polyphenol-rich red wine extract  
17  
18 on the immune response and its involvement in tumor progression.  
19  
20  
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22

23  
24 Considering the fundamental role playing by the lymphocytes T in cell-mediated  
25  
26 immunity, and more particularly by the lymphocytes T helper (Th cells) are also known as  
27  
28 CD4<sup>+</sup> T cells, we investigated the potential effect of a red wine extract (RWE) on Th17  
29  
30 immune cells that have inflammatory properties derived from their ability to produce various  
31  
32 interleukins (i.e. IL-17A, IL-17F, IL-6, IL-21, IL-22 and TNF $\alpha$ ). We demonstrated that RWE-  
33  
34 enriched in polyphenolic compounds reduced colorectal tumoral growth in mice which was  
35  
36 associated with a decrease of the percentage tumor-infiltrating lymphocytes. These effects  
37  
38 involve an alteration of the lymphocyte T differentiation into pro-inflammatory Th17 cells  
39  
40 where the key regulators of this process, the proteins ROR $\gamma$ t (Retinoid-related Orphan  
41  
42 Receptor gamma t) and STAT3 (Signal Transducer and Activator of Transcription 3), were  
43  
44 affected by RWE treatment. Subsequently, this disruption of Th17 polarization leads a  
45  
46 strongly decrease of pro-inflammatory IL-17 secretion and underlines the potential anti-  
47  
48 inflammatory properties of complex mixture of polyphenol compounds.  
49  
50  
51  
52  
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54  
55

## 56 **2. Materials and Methods**

### 57 **2.1. Cell lines**

1  
2  
3 Human colorectal cancer cell lines SW620, HCT116 and murine colorectal cancers CT26 and  
4  
5 MC38 were obtained from the American Type Culture Collection. Cells were maintained in a  
6  
7 5% CO<sub>2</sub> humidified atmosphere at 37°C and cultured in DMEM supplemented with 10%  
8  
9 (v/v) fetal calf serum (Dutscher). Cells were routinely tested for mycoplasma contamination  
10  
11 using the Mycoalert Mycoplasma Detection Kit (Lonza).  
12  
13

## 14 **2.2. Preparation of red wine extract**

15  
16 Red wine extract (RWE) was obtained from French red wine, Santenay 1er cru Les Gravières  
17  
18 2012 (Côte d'Or, France) selected by the BIVB (Bureau Interprofessionnel des Vins de  
19  
20 Bourgogne) and provided by CTIVV (Centre Technique Interprofessionnel de la Vigne et du  
21  
22 Vin). Red wine extract dry powder was prepared and analyzed as previously described [5].  
23  
24 Briefly, phenolic compounds were adsorbed on a preparative column, then alcohol desorbed.  
25  
26 The alcoholic eluent was gently evaporated, and the concentrated residue was lyophilized and  
27  
28 finely sprayed to obtain the phenolic extract dry powder. Briefly, the alcoholic eluent and  
29  
30 water were gently evaporated using a rotary evaporator set, and the concentrated residue was  
31  
32 deposited on the column (Diainon® HP-20). The reservoir was filled with distilled water and  
33  
34 the flow was adjusted to about 20 drops/min. The polyphenol fraction was eluted with an  
35  
36 ethanol-0.1% glacial acetic acid solution (flow adjusted to 40 drops/min). The individual  
37  
38 eluent fractions were collected and concentrated to dryness using a rotary evaporator set. One  
39  
40 liter of red wine produced 104 g of phenolic extract, which contained 5.04 mg/g of total  
41  
42 phenolic compounds expressed as gallic acid equivalent.  
43  
44  
45  
46  
47  
48

## 49 **2.3. HPLC analysis**

50  
51 The phenolic composition of the wine samples was determined by HPLC analysis according  
52  
53 to the method Guererro *et al.* [6]. Briefly, HPLC analysis was performed on a HPLC system  
54  
55 equipped with a diode array detector, a fluorescence detector and a C18 column (5 µm, 250  
56  
57 mm × 4.6 mm) for compound separation. For anthocyanins and flavonols, the mobile phase  
58  
59  
60

1  
2  
3 consisted of water containing 5% formic acid (v/v) and methanol in various proportions at a  
4  
5 flow rate of 1 mL/min. Anthocyanins were quantified at 520 nm as malvidin-3-glucoside and  
6  
7 flavonols at 360 nm as quercetin-3-rutinoside. Flavan-3-ols and phenolic acids were analyzed  
8  
9 using a gradient containing water with 2% acetic acid (v/v) and methanol–water–acetic acid  
10  
11 (90:8:2, v/v/v). Phenolic acids were quantified at 320 nm as caffeic acid, flavan-3-ols as  
12  
13 catechin using the fluorescence signal (excitation wavelength, 290 nm; emission wavelength,  
14  
15 320 nm) and stilbenes as resveratrol (excitation wavelength, 330 nm; emission wavelength,  
16  
17 320 nm).

#### 21 **2.4. Cell proliferation and viability measurements**

22  
23 Human and murine colorectal cancer cells were seeded in sextuplicate into 96 well-plates  
24  
25 ( $1.5 \times 10^4$  cells per well) and treated for 48 and 72h at 37°C with increasing concentrations of  
26  
27 RWE (from 1.9 to 250  $\mu\text{g/mL}$ ). At the end of the treatments, cells were washed with PBS,  
28  
29 stained for 5 min with a crystal violet solution (0.5% (w/v) crystal violet in 25% (v/v)  
30  
31 methanol) and then gently rinsed with water. Absorbances were read at 540 nm with a  
32  
33 Biochrom Asys UVM 340 spectrophotometer, after the extraction of the dye by 0.1 M sodium  
34  
35 citrate in 50% ethanol. The inhibitory concentrations 50 % ( $\text{IC}_{50}$ ) were calculated by a 4-  
36  
37 parameter nonlinear regression with SigmaPlot version 6 software (Systat software, Inc).  
38  
39  
40

#### 42 **2.5. Apoptosis identification**

43  
44 Apoptosis was determined using the annexin V-FITC and 7-aminoactinomycin D (7AAD)  
45  
46 staining from BD Biosciences according to the manufacturer's instructions. Briefly, cells were  
47  
48 seeded in 12-well plates ( $5 \cdot 10^4$  cells/well) 24 h before treatments. The following day, cells  
49  
50 were treated with the indicated concentrations of RWE for 48 and 72 h. At the end of the  
51  
52 treatments, cells were harvested, washed two times with cold PBS and then incubated for 15  
53  
54 min at room temperature with a mixture of annexin V-FITC/7AAD (5  $\mu\text{L}$  each per sample) in  
55  
56 50  $\mu\text{L}$  of binding buffer. At the end of the incubation, 100  $\mu\text{L}$  of binding buffer was added  
57  
58  
59  
60



1  
2  
3 and cells were kept on ice until analysis. Annexin V binding and 7-AAD incorporation were  
4  
5 detected by an LSRII flow cytometer (BD Biosciences) and analyzed using Flowjo software  
6  
7 (Tristar, Ashland, OR, USA).  
8

## 9 10 **2.6. Mouse strains**

11  
12 Six-week-old BALB/c and C57BL/6Rj female mice were purchased from Janvier Labs (Saint-  
13  
14 Berthevin, France). All mice were housed and maintained 22-24°C on a 12-h light-dark cycle  
15  
16 in a designated pathogen-free area accredited by the Federation of Laboratory Animal Science  
17  
18 Associations (FELASA). All experiments involving mice were performed in accordance with  
19  
20 the institutional guidelines and were approved by the University of Burgundy's Ethics  
21  
22 Committee on the Use of Laboratory Animals. Animals were provided *ad libitum* access to a  
23  
24 chow diet.  
25  
26

## 27 28 **2.7. Tumor growth experiments**

29  
30 Eight-week-old BALB/c mice were randomly divided into two experimental groups (14 mice  
31  
32 per group). Mice were individually maintained on normal chow (control mice group) or fed  
33  
34 *per os* with 100 mg/kg/day of RWE (RWE mice group), for three weeks prior to the induction  
35  
36 of tumor formation and throughout tumors growth. To induce tumor formation,  $4 \times 10^5$   
37  
38 mycoplasma free CT26 cancer cells were injected subcutaneously into the left flank of balb/c  
39  
40 mice. Tumor size was monitored three times a week by caliper measurements of the widest  
41  
42 diameter and the narrowest diameter. Time of death was considered when mice reached  
43  
44 experimental endpoints related to graft size, animal weight and general behavior in  
45  
46 accordance with the guidelines prescribed by the Ethics Committee at the University of  
47  
48 Burgundy.  
49  
50  
51

## 52 53 **2.8. Isolation and analysis of tumor-infiltrating lymphocytes (TILs)**

54  
55 Xenograft tumors from mice groups (4 per group) described in the previous section were  
56  
57 harvested 12 days after tumor formation induction and then dissociated in RPMI with the  
58  
59  
60

gentleMACS™ dissociator (Miltenyi Biotec) according to the manufacturer's protocol. Lysates were centrifuged and cells were stained with an antibody cocktail containing anti-CXCR3-BV421 (CXCR3-173, BD Biosciences), anti-CD4-VioBright FITC (REA604, Miltenyi Biotec), anti-CD45RA-APC-Vio770 (30F11, Miltenyi Biotec) and anti-CCR6-PE (REA277, Miltenyi Biotec). Tumor infiltrating Th17 cells were identified as CD4<sup>+</sup>, CD45RA<sup>-</sup>, CCR6<sup>+</sup>, CXCR3<sup>-</sup>. After surface staining, 2 ml of red blood cells lysis solution (BD Biosciences) was added for 10 minutes, centrifuged (400 x g, 5 minutes) and then resuspended in flow cytometry buffer (eBioscience). All events were acquired by a BD LSR-II cytometer equipped with BD FACSDiva software (BD Biosciences), and data were analyzed using FlowJo software (Tree Star). The gating strategy is illustrated in Figure 5A.

## 2.9. In vitro Th17 cells differentiation

Naïve CD4<sup>+</sup> T cells (CD4<sup>+</sup> CD62L<sup>hi</sup>) were obtained from spleens and lymph nodes of C57BL/6Rj mice. CD4<sup>+</sup> T cells were purified from spleen and lymph nodes with anti-CD4 microbeads (Miltenyi Biotec), then were further sorted as naïve CD4<sup>+</sup>CD62L<sup>hi</sup> T cells. Isolated naïve T cells were routinely 98% pure. Isolated naïve CD4<sup>+</sup> T cells were stimulated with plate-bound antibodies against CD3 (145-2C11, 2 µg/mL; BioXCell) and CD28 (PV-1, 2 µg/mL; BioXCell) and polarized into effector CD4<sup>+</sup> T lymphocyte subsets with cytokines. Mouse, IL-6 (25 ng/mL), and TGF-β (2 ng/mL) were all purchased from MiltenyiBiotec. Anti-IL-4 (clone 11B11; 10 µg/mL) and anti-IFNγ (clone XMG1.2; 10 µg/mL) antibodies were obtained from Bio-XCell. In experiments, RWE have been added at the final concentrations of 0.2, 2, 20 and 100 µg/ml. As previously described, cells were classically harvested on day 3 (unless otherwise specified) for detection of cytokines by ELISA and for quantitative real-time PCR (qRT-PCR) analysis as previously described [7].

## 2.10. Measurement of IL-17A production

1  
2  
3 After a 72-hour polarization, cell culture supernatants were assayed by ELISA for mouse IL-  
4 17A (Biolegend), according to the manufacturer's protocol.

### 7 **2.11. Western Blotting analysis**

9  
10 Purified naïve T cells were differentiated for 6 or 24 hours into Th17 cells with or without  
11 RWE, then collected and pelleted by centrifugation (5 minutes, 450g, 4°C). Cells were lysed  
12 in boiling buffer [1% SDS, 1 mM sodium orthovanadate, and 10 mM Tris (pH 7.4)]  
13 containing protease inhibitor cocktail for 20 minutes at 4°C. Cell lysates were subjected to  
14 sonication (10 seconds at 10%) and protein concentration was assessed using the Bio-Rad DC  
15 Protein Assay Kit. Proteins were then denatured, loaded, and separated on SDS-PAGE and  
16 transferred on nitrocellulose membranes. After blocking with 5% nonfat milk in Phosphate-  
17 Buffered Saline containing 0.1% Tween 20 (PBST), membranes were incubated overnight  
18 with primary antibody diluted in PBST containing 5% BSA: rabbit monoclonal antibodies  
19 anti-STAT3 and anti-phospho-STAT3-Tyr705 (Cell Signaling Technology), rat monoclonal  
20 antibody was used: anti-ROR $\gamma$ t (eBiosciences), or mouse monoclonal antibody anti- $\beta$ -actin  
21 (Sigma-Aldrich). Afterwards, the membranes were incubated with antibodies HRP-  
22 conjugated polyclonal goat anti-mouse (Jackson ImmunoResearch), polyclonal goat anti-  
23 rabbit (Cell Signaling Technology) and polyclonal goat anti-rat (Santa Cruz Biotechnology) at  
24 room temperature for 1 h and developed using the ECL reagents (Supersignal West Femto  
25 maximum sensitivity substrate, ThermoFisher). Digital chemiluminescence images were  
26 captured and analyzed using the ChemiDoc<sup>TM</sup> XRS+ imaging system (BioRad). Image  
27 processing and analyses were carried out using Image Lab 5.2.1 build 11 Bio-Rad software.

### 51 **2.12. Quantitative real-time PCR**

52  
53 Total RNA from T cells was extracted with TriReagent (Ambion), reverse transcribed using  
54 M-MLV Reverse Transcriptase (Invitrogen), and was analyzed by RT-qPCR with the SYBR  
55 Green method according to the manufacturer's instructions using the 7500 Fast Real-Time  
56  
57  
58  
59  
60

1  
2  
3 PCR System (Applied Biosystems). Expression was normalized to the expression of mouse or  
4 human *Actb*. Primers designed to assess gene expression are as follows: *Actb mus musculus*  
5 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17a mus musculus* 5'-  
6 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17a mus musculus* 5'-  
7 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
8 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
9 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
10 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
11 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
12 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
13 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
14 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
15 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
16 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
17 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
18 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
19 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
20 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
21 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
22 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
23 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
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30 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
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47 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
48 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
49 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
50 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
51 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
52 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
53 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
54 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
55 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
56 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
57 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
58 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
59 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-  
60 5'-atggaggggaatacagccc-3' and 5'-ttctttgcagctcctctggtt-3'; *il17f mus musculus* 5'-

## 2.14. Statistical analysis

Results are shown as means  $\pm$  SD for triplicate assay samples (otherwise mentioned), reproduced independently at least three times. Statistical analysis of data was carried out with Prism GraphPad6.0 Prism Software. The significance of the differences between mean values was determined by a one-way ANOVA with Holm-Sidak correction. *P*-values  $<0.05$  were considered significant ( $*p < 0.05$ ,  $**p < 0.01$  and  $***p < 0.001$ ). For *in vivo* studies, mice survivals were estimated by the Kaplan-Meier method and were compared by the log-rank test.

## 3. Results

### 3.1. RWE decreases colorectal cancer cells proliferation

We have previously shown that red wine extract (RWE) can decrease the number of colonic aberrant crypt foci (AFC) in the CF-1 mouse model for AOM induced preneoplastic colonic AFC [8] suggesting the potential interest of polyphenol extract from red wine in a precancerous context. In order to better understand the potential effects of RWE, we investigated its effects on immune cells in colorectal cancer context. Indeed, it is now clearly established that a modulation of specific immune cells can influence the development or regression of cancers. To determine the potential effect of RWE on immune cells, we have collected a French red wine, Santenay 1er cru Les Gravières 2012 (Côte-d'Or, France) from which we made a dry extract enriched in polyphenols that we so-called Red Wine Extract

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3 (RWE). In order to appreciate its polyphenolic content, we performed a qualitative and  
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5 quantitative analysis by HPLC of RWE. As shown in figure 1, RWE showed high levels of  
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7 phenolic acids (caftaric acid, caffeic acid), anthocyanidins (malvidin), catechins (catechin and  
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9 procyanidin dimers), stilbenes (resveratrol and its glycosylated form piceid) and flavonols  
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11 (quercetin). These contents are important to consider since we have previously shown that the  
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13 qualitative and quantitative composition of bioactive compounds of the wine could influence  
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15 the biological effects of this one. In particular, we showed that there was a synergistic effect  
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17 against the proliferation of colon cancer cells resulting from an association between  
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19 resveratrol and quercetin [8]. This effect is no longer additive for a combination of resveratrol  
20  
21 / catechin or resveratrol / catechin / quercetin. Histogram of figure 1 reveals that a high level  
22  
23 of total resveratrol (1.92 mg/g of RWE) and quercetin (0.96 mg/g of RWE) as compared to  
24  
25 our previous studies [8], suggesting a potential benefit effect to induce the death of colon  
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27 cancer cells. In this way, we first evaluated the RWE toxicity in our models of colon cancer  
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29 cell lines (Fig. 2). Human colorectal metastatic cancer cells SW620, colorectal carcinoma  
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31 cells HCT116 and murine colorectal cancer cells MC38 and CT26 were exposed to various  
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33 concentrations of RWE (0.1 to 250  $\mu\text{g}/\text{mL}$ ) during 48 and 72 h (Fig. 2A, B). As revealed by  
34  
35 the cytotoxic curves, RWE presented a cytotoxic activity in all colorectal cancer cell lines  
36  
37 tested in time and concentration-dependent manner. Indeed, the inhibitory concentration of 50  
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39 % of tumor cells ( $\text{IC}_{50}$ ) is more lower in human cancer cell lines after 48h of treatment than  
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41 murine colorectal cancer cells (Table I), but this difference fades to 48 hours of treatment.  
42  
43 Secondly to determine whether these antiproliferative effects of RWE are associated with an  
44  
45 induction of cell death, we analyzed the RWE impact on apoptosis process. To highlight this,  
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47 cancer cell lines were double staining with Annexin V / 7 AAD after treatment with 100 and  
48  
49 200  $\mu\text{g}/\text{mL}$  of RWE. Consistent with cytotoxicity curves, results show that polyphenol extract  
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51 induced at early time of 48h treatment a more apoptosis in human colorectal cancer cells  
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3 SW620 and HCT116 as compared to murine colorectal cancer cells MC38 and CT26 for  
4  
5 which there are no significant differences from the control (Fig. 3A). However, at 72h we  
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7 observe a strong induction of death in all colon cancer cell lines with a sharp increase in the  
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9 rate of late apoptosis (Fig. 3B).  
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### 14 **3.2. RWE decreases colorectal tumor progression in xenografted mice model**

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16 In a second step, we sought to correlate the inhibitory effects of proliferation and induction of  
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18 apoptosis *in vitro* with the ability of RWE to prevent tumor progression. In order to  
19  
20 demonstrate this, C57 BL6 mice received a fixed amount of RWE (100 mg/kg/days) for 3  
21  
22 weeks before receiving subcutaneous murine tumoral colon CT26 cells. Our results showed  
23  
24 that RWE delays the growth of tumors as early as five days after tumor implantation as  
25  
26 compared to the tumoral growth of mice controls (Fig. 4A). This difference is sharply  
27  
28 accentuated 15 days after the injection of CT26 cells (Fig. 4A). This regression of tumor  
29  
30 growth induced by RWE is associated with a significantly increase of survival rate in mice  
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32 receiving RWE complementation (Fig. 4B).  
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### 40 **3.3. RWE disturbs tumor-infiltrating lymphocytes**

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42 To precise the relationships between antitumoral effect of RWE and the immune cells, we  
43  
44 evaluated the percentage of tumor-infiltrating lymphocytes. Among T lymphocytes coexist  
45  
46 many T cell populations that are not homogenous where each subgroups present specific  
47  
48 function and structure. We have recently shown that resveratrol was able to affect lymphocyte  
49  
50 Th17 differentiation and subsequently disrupts interleukin-17 (IL-17) production [7]. These  
51  
52 resveratrol effects were associated with a reduction in various *in vivo* models of tumor growth  
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54 and metastasis [7]. Due to this importance of Th17 in the biological response of some  
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56 polyphenols, we have investigated whether RWE could affect more specifically the CD4<sup>+</sup> Th  
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3 cells. To further analyze these immune cells, we use polychromatic flow cytometry to sort  
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5 CD4<sup>+</sup> T cells subsets with differential expression of CD45, CXCR3 and CCR6. We observed  
6  
7 that after 3 weeks of RWE treatment, a decreases of the percentage of tumor-infiltrating  
8  
9 lymphocytes (CD45<sup>+</sup>CD4<sup>+</sup>CXCR3<sup>-</sup>CCR6<sup>+</sup>) in tumor at 12 days after inoculation of tumor  
10  
11 (Fig. 5). This significant reduction of RWE-induced TIL reduction is associated with a  
12  
13 decrease of IL-17 as measured by ELISA method in plasma level of mice treated by RWE  
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17 (not shown).  
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### 21 **3.4. RWE counteracts IL-17 production and genes expression**

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23 Among CD4 activated cells, specific markers CXCR3-CCR6<sup>+</sup> characterized Th17 that  
24  
25 specific immune cells critical for progression of cancer through the production of various pro-  
26  
27 inflammatory cytokines we identified the Th17 such as IL-6, IL1, IL-8 and IL-17. This last  
28  
29 interleukin is responsible firstly for chronic and acute inflammation contributing to tumoral  
30  
31 growth but secondly this interleukin promotes tumor angiogenesis through the stimulation of  
32  
33 vascular endothelial growth factor (VEGF) by both tumoral cells and fibroblasts. To corraled  
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35 precise the RWE effect on the specific imune T cells Th17 and its main itnerluekin 17  
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37 secretion, we have isolated naive T cells from lymph nodes and mice spleen, then they were  
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39 diffrentiated *invitro* into pro-inflammatory Th17 with a cocktail of specific csytokines with or  
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41 without various concentration of RWE during 3 days. Using ELISA method, we measured IL-  
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43 17 secretion which is strongly decreases with RWE treatment in a dose dependent manner as  
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45 compared to the control (Fig. 6A). This inhibition of IL-17 secretion is associated with a  
46  
47 decrease of genes encoding this interleukin, RWE being also able to decrease mRNA levels of  
48  
49 *il-17A* and *iL-17F* genes (Fig. 6B).  
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### 58 **3.5. Alteration of lymphocytes T differentiation by RWE *in vitro* and *in vivo***

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3 Differentiation of lymphocytes T is mainly under the control of signaling pathway involving  
4  
5 key proteins such as the homodimer STAT3 and the nuclear transcriptional factor ROR $\gamma$ t. In  
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7 fact, the phosphorylation of STAT3 stabilizes the protein. Once phosphorylated, STAT3 is  
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9 translocated into the nucleus to active the transcription of gene encoding the protein ROR $\gamma$ t  
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11 which in turn will activate the transcription of genes encoding interleukins, particularly IL-17.  
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13 As previously, immunoblotting of Th17 from *in vitro* differentiation of naive T cells reveals  
14  
15 that a RWE treatment during three days decreases phosphorylation of STAT3 and  
16  
17 subsequently strongly decreases ROR $\gamma$ t protein expression in concentration-dependent  
18  
19 manner (Fig. 7A & B). In order to evaluated whether RWE could affect the lymphocyte T  
20  
21 cells differentiation in animal models, we have pretreated mice with (100 mg/kg/days) for 3  
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23 weeks, and we have isolated as previously naive T cells from lymph nodes and mouse spleen  
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25 before their *in vitro* differentiation into Th17. Interestingly, immunoblotting highlight that  
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27 RWE treatment during 3 weeks significantly decreases ROR $\gamma$ t protein expression ( $p < 0.05$ )  
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29 and therefore the ability of naïve T cells to differentiate into pro-inflammatory Th17 (Fig. 7C  
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31 & D).  
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#### 41 **4. Discussion**

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43 In the present report, we studied the effects of a red wine extract (RWE) rich in  
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45 polyphenolic compounds on the immune lymphocyte T cells *in vitro* and *in vivo* and the link  
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47 with the antitumoral properties of polyphenol extract. We found that antitumoral effects of  
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49 RWE *in vivo* are associated with significant reduction of the percentage of tumor-infiltrating  
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51 lymphocytes (TILs). These lymphocytes play often a dual role in the balance between pro-  
52  
53 and anti tumoral effects. Thus, we highlight that RWE affects lymphocytes T into specific  
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55 lymphocytes T helper called Th17 which are able to overproduce a pro-inflammatory  
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57 interleukin, IL-17. We subsequently showed that RWE reduced IL-17 secretion-associated  
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3 pro-inflammatory Th17. The molecular mechanism seems to involve a disruption of a specific  
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5 signaling cascade where the nuclear transcriptional factor, protein ROR $\gamma$ t, which play an  
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7 essential role in Th17 differentiation is strongly decrease by RWE.  
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10 The immune system can recognize many cancers (*i.e.* breast, ovarian, colon cancers).  
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12 Natural antitumor immunosurveillance fights against the appearance and development of  
13  
14 cancers. Thus, it has been shown that local immunological factors may therefore have a  
15  
16 positive effect on tumor progression or on the contrary play a deleterious role on tumor  
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18 progression. Indeed, we have been able to show that a lymphocyte population called  
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20 regulatory T lymphocytes could inhibit the antitumor immune response in humans as well as  
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22 in animals [9]. These regulatory T cells are able to inhibit the T dependent immune response  
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24 and the increase in their number is a factor of poor prognosis in patients with ovarian cancer  
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26 [10]. Another team has shown that probably only patients with a high infiltration of CD8  
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28 effector cells into ovarian tumor and few regulatory T cells would have a good prognosis [11].  
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31 Conversely, a deleterious role of inflammation and the immune system is seen added to all  
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33 these mechanisms that can pervert the immune system including *via* mechanisms involving  
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35 the production of pro-inflammatory cytokines such as TNF $\alpha$ , IL-6 and IL-1 $\beta$ . These cytokines  
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37 induce a modification of the T cell polarization towards a Th17 polarization (IL-17 producing  
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39 cells). This population exerts a protumoral role by increasing tumor angiogenesis and making  
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41 tumor cells resistant to cell death. Therefore, by decreasing the production of pro-  
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43 inflammatory substances such as IL 17, RWE is able to reduce tumor growth but also  
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45 angiogenesis. These results allow us to provide a possible explanation for the observation of  
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47 many teams including Schini-Kerth's team who showed in a model of colon cancer that red  
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49 wine extract reduced neoangiogenesis [5]. In fact, the modulation of immune cells could  
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51 appear as the upstream element of the antitumor effects observed for the RWE but also for  
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53 many polyphenols. Indeed, we have recently shown that resveratrol was able to modulate  
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3 lymphocytes T differentiation into Th17 in similar manner as metformin [7]. The antitumoral  
4 effect of resveratrol was dependent of Th17-IL-17 system. Indeed, using IL-17 knock down  
5 mice where the antitumoral resveratrol activity observed in normal mice was abolished in IL-  
6 17 KO mice [7]. Furthermore, resveratrol effect on the IL-17 production contributes to reduce  
7 VEGF levels and subsequently contributes to strongly decrease the number of pulmonary  
8 metastasis in tumorigenesis-induced mice [7].  
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17 The molecular mechanism of antitumoral effect and more specifically the RWE-induced  
18 immunomodulation seems more complicated, because of the relatively complex composition  
19 of the mixture (Fig. 1) that contains a high number of polyphenolic compounds that may  
20 interact with each other or target various signaling pathway actors. We previously  
21 demonstrated that the qualitative and quantitative composition of a wine in bioactive  
22 molecules such as polyphenolic compounds is crucial in the biological effects that can be  
23 observed and that they are antagonistic or synergistic [8]. We previously showed that  
24 resveratrol and quercetin combined exhibited a synergistic effect inducing apoptosis,  
25 antiproliferative effects, and cell cycle disruption compared to the compounds alone in colon  
26 cancer cells. Interestingly, in this same study, the combination of resveratrol+catechin or  
27 resveratrol+catechin+quercetin produced only an additive effect on the inhibition of tumor  
28 cell proliferation or on cell cycle arrest, and catechin+quercetin did not induce a synergistic  
29 effect on apoptosis [8]. In this way some reports have shown a potential effect of quercetin on  
30 Th17 in some models of inflammation. For example, quercetin was able to reduces Th17 cell  
31 polarization through an inhibition of a mitogen-activated protein kinase Toll-like receptor 4  
32 (MAPK-TLR4) signaling pathway and subsequently decrease IL-17 production in a model of  
33 dental pulpitis [12]. Interestingly, in a model of rheumatoid arthritis using osteoclasts,  
34 quercetin reduces Th17differentiation and IL-17-stimulated RANKL (Receptor Activator of  
35 Nuclear factor kappa-B Ligand) production in RA-FLS (Rheumatoid Arthritis-Fibroblasts-  
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3 Like Synoviocytes) and IL-17-stimulated osteoclast formation [13]. In similar manner,  
4 catechin, and more particularly epigallocatechin-3-gallate (EGCG) suppressed expansion and  
5 cell cycle progression of naïve CD4<sup>+</sup> T by modulating cell cycle-related proteins. EGCG also  
6 inhibited naïve CD4<sup>+</sup> T-cell differentiation into Th1 and Th17 effector subsets by impacting  
7 their respective signaling transducers and transcription factors in some models of autoimmune  
8 diseases [14-17].  
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17 But in the concept of immunosurveillance in a cancerous context, various other immune  
18 cells play key roles such as cytotoxic T cells (CTL or CD8<sup>+</sup> T cells) that are able to induce the  
19 death of infected somatic cells or tumor cells. The activation and differentiation of CD8<sup>+</sup> cells  
20 is an essential step for the establishment of a response against intracellular pathogens and  
21 against tumor cells. For example, in a tumor context, the polyphenol resveratrol led to an  
22 increase in the number of cytotoxic T-lymphocyte associated protein 4 (CTLA-4; also known  
23 as CD152)-positive cells and in the gene expression levels of CTLA-4, FoxP3, IL-10 and  
24 TGF- $\beta$  these two last TGF- $\beta$  and IL-10 being anti-inflammatory cytokines and controlling  
25 pathogenic, as well as aging-associated inflammatory conditions [18]. In a same manner,  
26 other immune cells such as regulatory T cells (Treg cells) appear essential in cancer  
27 progression, since the presence of Treg cells is likely to correlate with cancer progression  
28 [19], and specific depletion of Treg cells markedly inhibits tumor growth and maintains a  
29 strong and persistent antitumor immune response [20]. Again many polyphenols can act on  
30 these immune cells [21, 22]. Additional studies are now in progress to determine the impact of  
31 a polyphenol-enriched extract on the different subsets of immune cells and more particularly  
32 on T cells that play a central role in cell-mediated immunity.  
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56 **Author contributions :** P.C. performed the experiments and analyzed the data; F.C.  
57 performed immunological detection; E.L., F.C. performed Th17 isolation; E.C.V. and T.R.  
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3 performed HPLC analysis; C.A, P.C. and V.S.K. performed RWE production and analyzed  
4 the data; V.A. and F.G. helped in setting up the project and analyzed the data; V.A. and D.D.  
5 revised the manuscript; D.D. supervised the overall project.  
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18 **Conflicts of Interest:** the authors declare no conflict of interest.  
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For Peer Review

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32 anti-inflammatory effect. *Int Immunopharmacol* 2018, 54, 153-162.  
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## Legends of figures

**Figure 1. HPLC quantification of red wine extract (RWE) obtained from the Santeney 1er cru Les Gravières 2012.** The content of compounds was expressed of mg of compound per g of RWE.

**Figure 2. Concentration-dependent inhibition of colon cancer cells proliferation by RWE.** After 24h of culture, cells were incubated with or without RWE during 48h (A) or 72h (B) at the indicated concentration. The percentage of viable cells was determined by crystal violet assay. Results were expressed as percentage of control (A and B : mean  $\pm$ SD of three independent experiments with n=6).

**Figure 3. RWE-induced apoptosis in colon cancer cell lines.** After 24h of culture, SW620, HCT116, MC38, CT26 cell lines were incubated with vehicle (Co) or with RWE (100 or 200  $\mu$ g/ml) and then were stained with Annexin V/7AAD after 48h (A) or 72h (B). The data are mean  $\pm$ SD of three independent experiments. *P*-values were determined by the multiple Student's *t*-test. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

**Figure 4. RWE reduced colorectal tumor growth and increased survival level of mice.** Eight-week-old C57BL/6Rj female mice daily received *per os* RWE of RWE (100 mg/kg/day; RWE mice group) or the vehicle (control mice group) during 3 weeks before bearing CT26 colorectal tumors (n=10 per group) and for 19 days after initiation of tumor formation. Tumor size in mm<sup>2</sup> over time is represented as median  $\pm$  s.e.m. B) Kaplan-Meier cumulative survival plots for mice groups described in A), with *p*-values assessed by the log-rank test. \*  $p < 0.05$ .



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3 **Figure 5. RWE decreases Tumor-Infiltrating Lymphocytes (TILs).** Eight-week-old  
4 C57BL/6Rj female mice daily received *per os* RWE of RWE (100 mg/kg/day; RWE mice  
5 group) or the vehicle (control mice group) during 3 weeks before bearing CT26 colorectal  
6 tumors (n=10 per group) and for 12 days after initiation of tumor formation. Xenograft tumors  
7 from mice groups (4 per group) were harvested at day 12 and dissociated prior to cell surface  
8 staining with specific antibodies and analysis by flow cytometry. The results are expressed in  
9 percentage  $\pm$  s.e.m of infiltrated Th17 cells (CD4<sup>+</sup>, CD45RA<sup>-</sup>, CCR6<sup>+</sup>, CXCR3<sup>-</sup>) in xenografts  
10 tumors from mice groups is shown. *P*-values were determined by the Mann-Whitney U-test. \*  
11 *p*<0.05.  
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26 **Figure 6. RWE-induced interleukin 17 inhibition in lymphocytes Th17.** CD4<sup>+</sup> T cells  
27 isolated from draining lymph nodes or mouse spleen from C57bl/6 mice and then they were  
28 differentiated into Th17 cells in the presence of anti-CD3 and anti-CD28 for 24h in presence  
29 or not of 0.2, 2, 20, 50, 100 and 150  $\mu$ g/ml of RWE during 3 days. IL-17 A protein expression  
30 was determined by ELISA (A) and expression of il17A and il17F were determined using  
31 qRT-PCR analysis.  
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42 **Figure 7. RWE affects key regulator protein of Th17 differentiation.** A) CD4<sup>+</sup> T cells  
43 isolated from draining lymph nodes or mouse spleen from C57bl/6 mice and then they were  
44 differentiated into Th17 cells in the presence of anti-CD3 and anti-CD28 for 24h in presence  
45 or not of 0.2, 2, 20 and 100  $\mu$ g/ml of RWE. Expression of p-STAT3, STAT3 and ROR $\gamma$ t in  
46 naïve T cell and differentiated Th17 cells were assessed by western blotting. B-actin was used  
47 as loading control. One representative of three independent experiments. B) Densitometry  
48 quantification of western blotting obtained in A). Data are expressed as fold induction of one  
49 representative experiment. C) Eight-week old female balb/c mice received of daily oral  
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3 administrations of 100 mg/kg/day of RWE or the vehicle during 3 weeks before CD4<sup>+</sup> T cells  
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5 isolated from draining lymph nodes or mouse spleen from C57bl/6 mice and then they were  
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7 differentiated into Th17 cells in the presence of anti-CD3 and anti-CD28 for 24h. As in A)  
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9 Expression of ROR $\gamma$ t in naïve T cell and differentiated Th17 cells were assessed by western  
10  
11 blotting. B-actin was used as loading control. D) Densitometry quantification of western  
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13 blotting obtained in C). Data are expressed as fold induction of the average of the 5 mice used  
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15 in control and treated. Statistical analyses were performed by the Mann-Whitney's test and  
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17 are shown a significant with  $p < 0.05$  (\*).  
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**Table I. Determination of IC<sub>50</sub> for RWE on the four colorectal cell lines tested.**

Cell lines	IC <sub>50</sub> of RWE (µg/ml)	
	48h	72h
SW620	123.7 ± 11.9	60 ± 15.7
HCT116	200.0 ± 4.3	74.5 ± 11 .6
MC38	213.5 ± 30.4	200 ± 6.6
CT26	121.0 ± 9.8	150 ±30.0

Figure 1

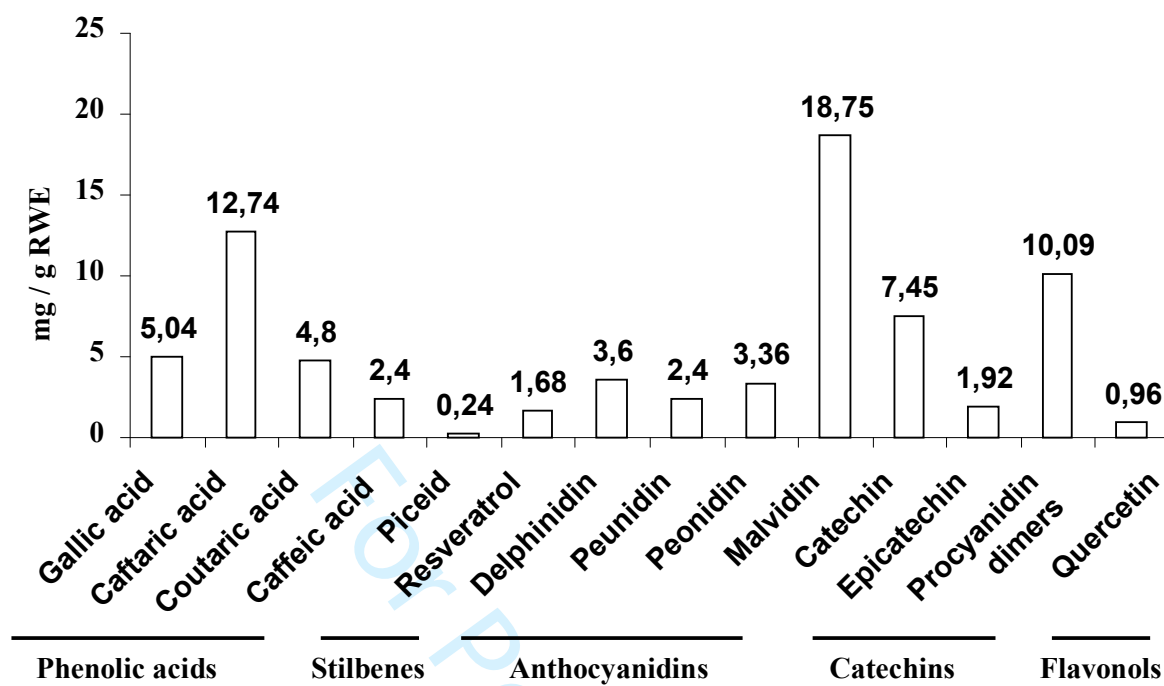


Figure 2

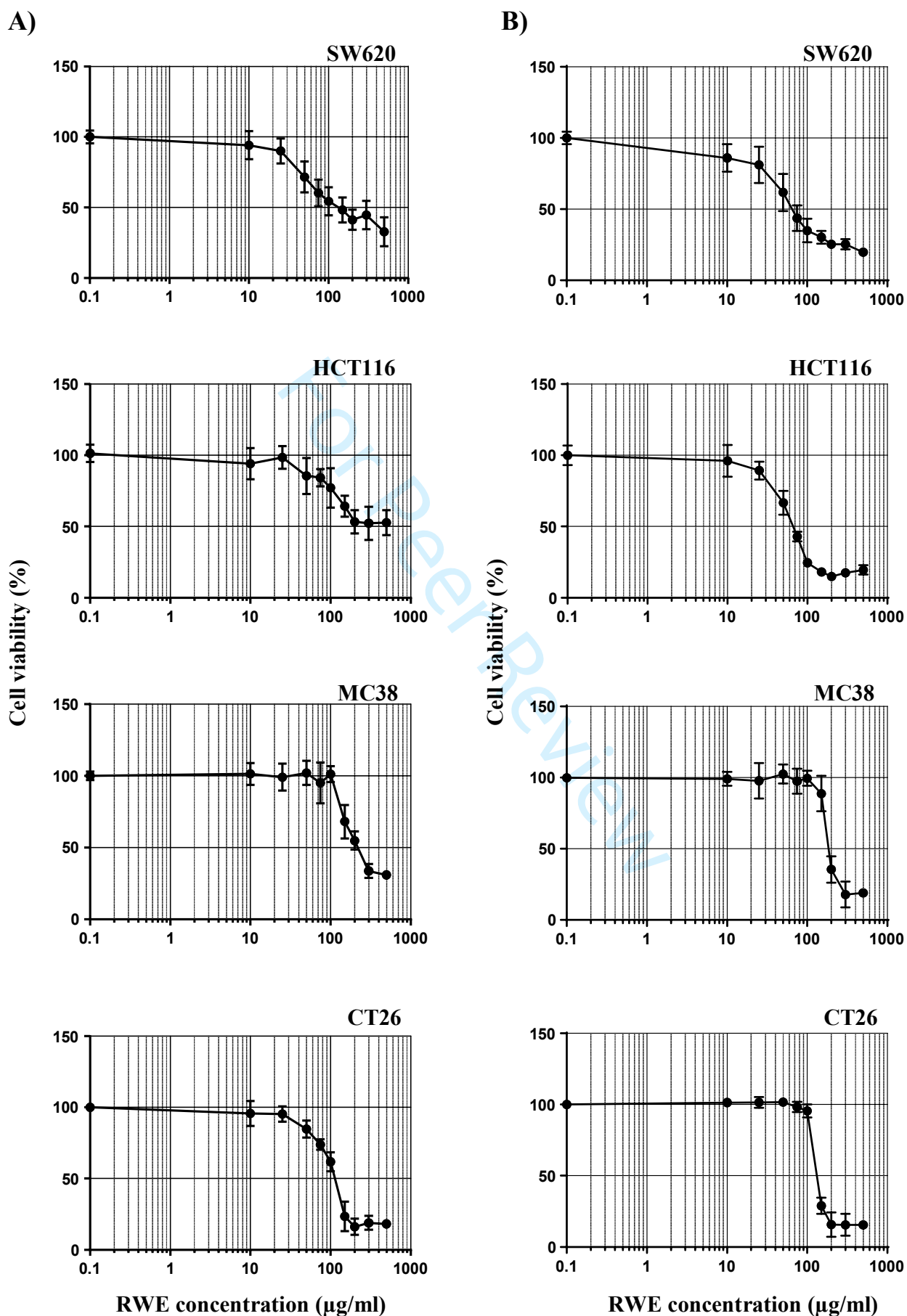


Figure 3

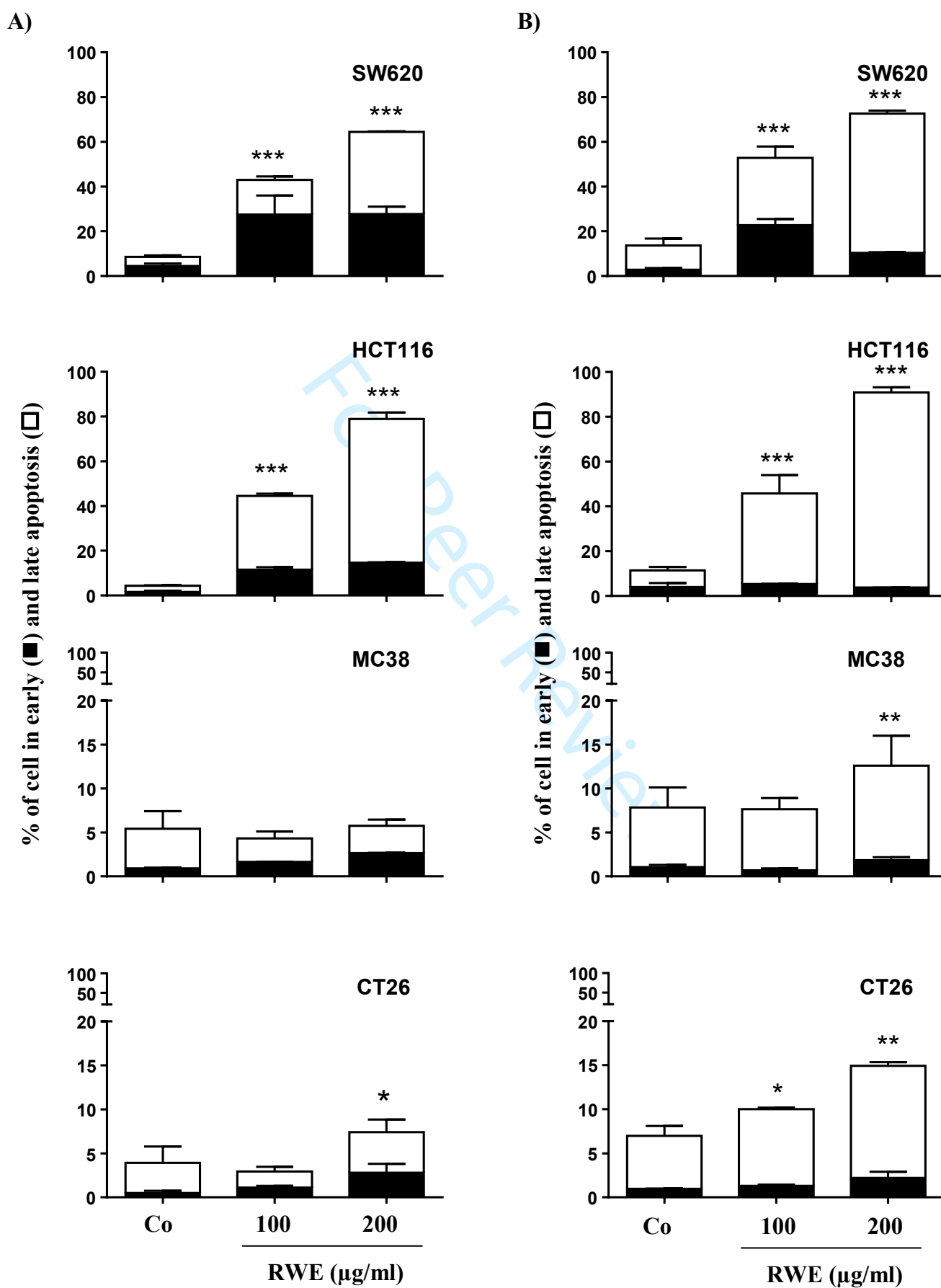


Figure 4

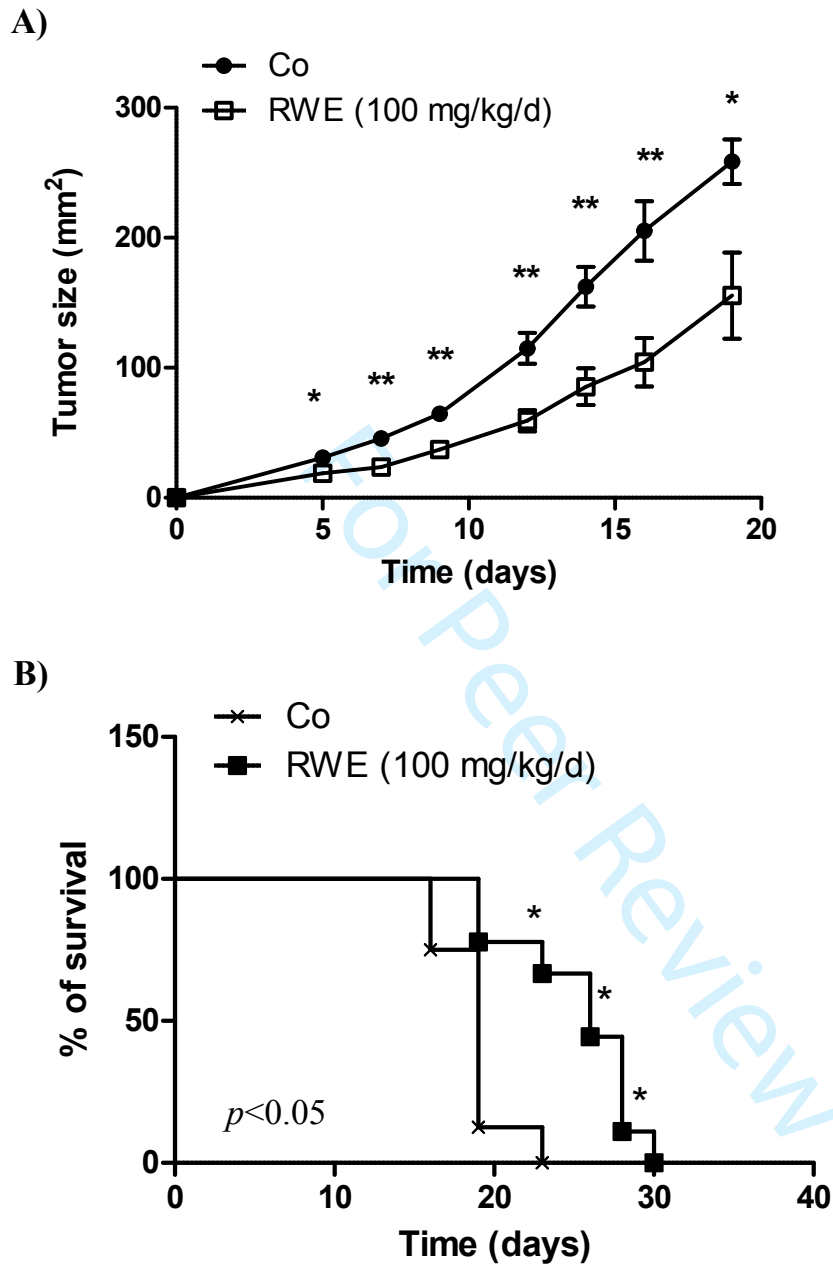


Figure 5

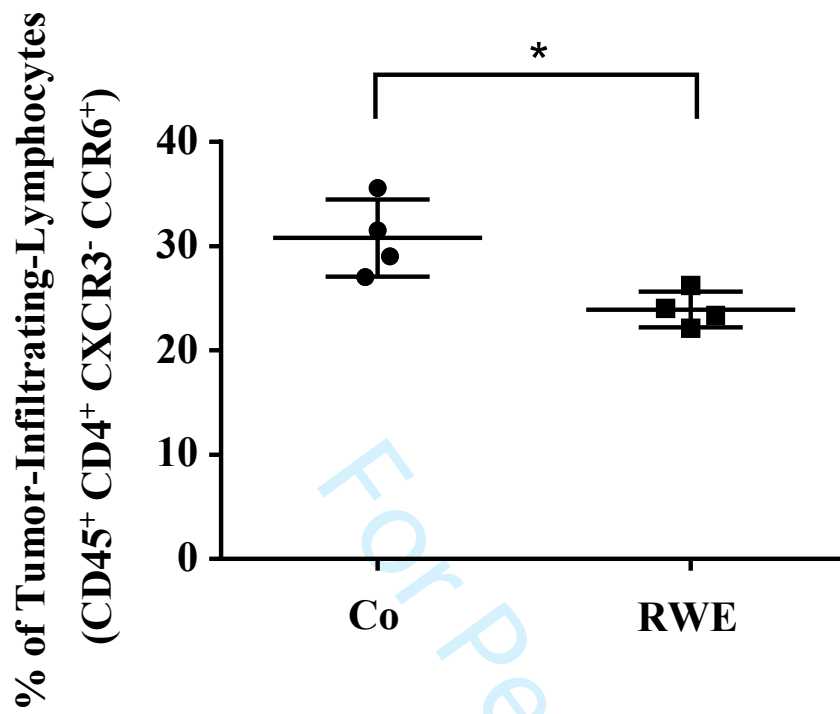




Figure 6

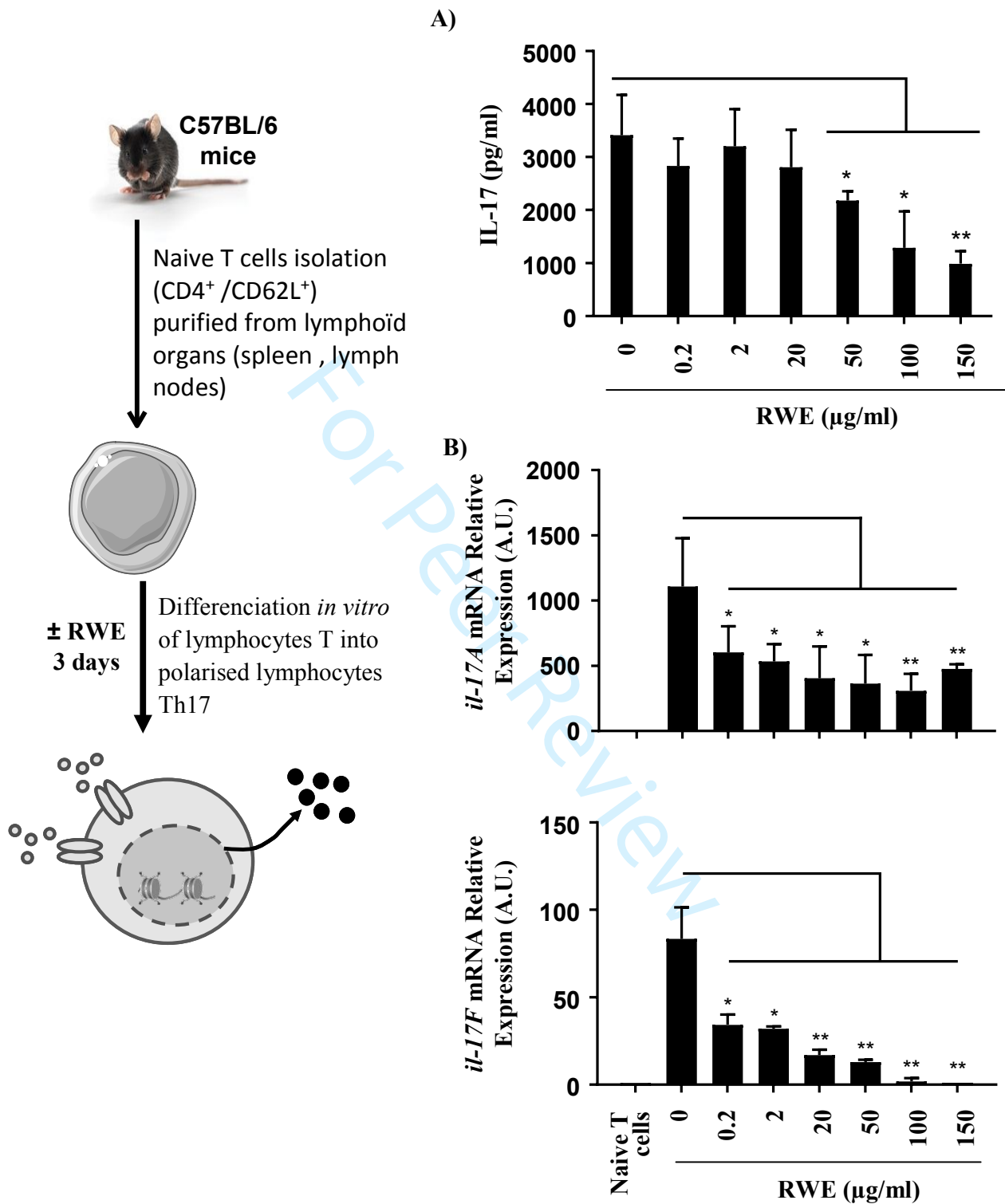
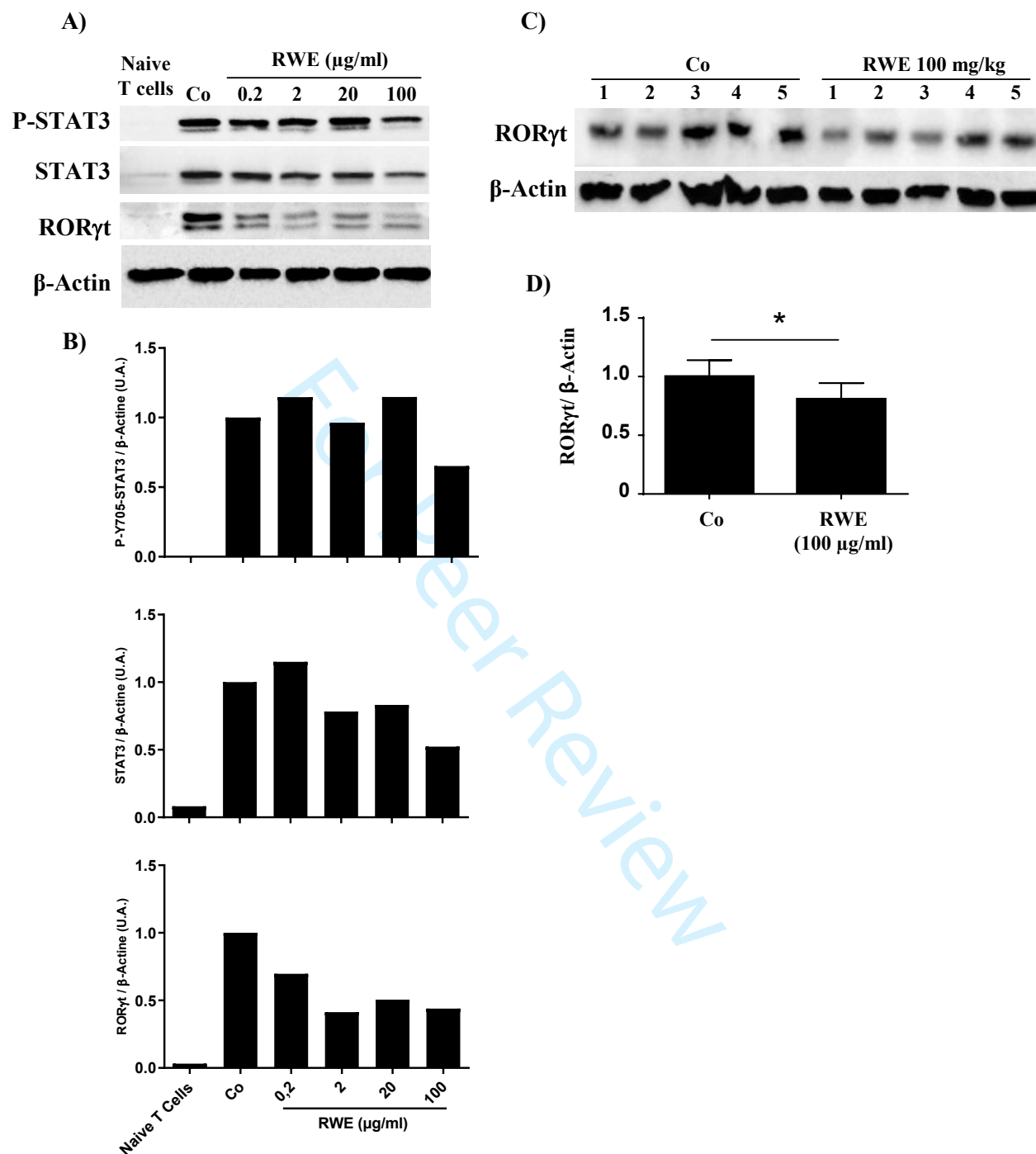
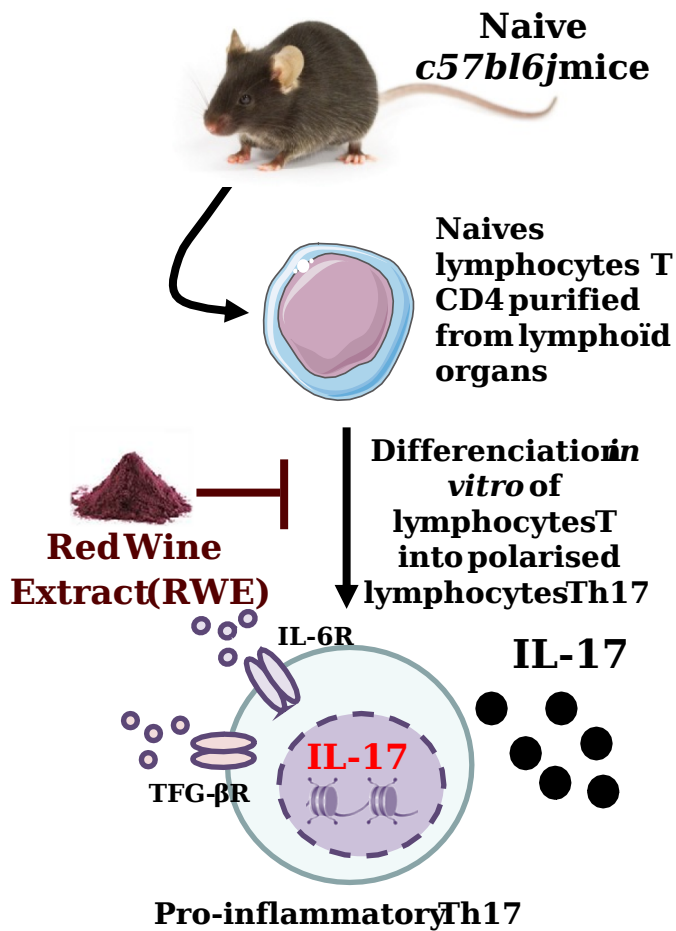


Figure 7



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3 Red Wine extract was able to alter differentiation of lymphocyte T cells into pro-  
4 inflammatory Th17 through an alteration of nuclear transcriptional factor, the protein ROR $\gamma$ t.  
5 Subsequently, Red Wine Extract disrupts pro-inflammatory interleukin IL-17. These events  
6 are associated in vivo with a decrease of tumor-infiltrating lymphocytes and with a reduction  
7 of colorectal tumor growth.  
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