



## Stimulus-specific regulation of CD63 and CD203c membrane expression in human basophils by the flavonoid quercetin

S. Chirumbolo<sup>a,\*</sup>, A. Conforti<sup>c</sup>, R. Ortolani<sup>b</sup>, A. Vella<sup>b</sup>, M. Marzotto<sup>a</sup>, P. Bellavite<sup>a</sup>

<sup>a</sup> Department of Morphological and Biomedical Sciences-Sect. Clinical Chemistry, University of Verona–University Hospital GB Rossi piazzale AL Scuro 10 37134 Verona, Italy

<sup>b</sup> Department of Pathology-Sect. Immunology, University of Verona–University Hospital GB Rossi piazzale AL Scuro 10 37134 Verona, Italy

<sup>c</sup> Department of Public Health-Sect. Pharmacology, University of Verona–University Hospital GB Rossi piazzale AL Scuro 10 37134 Verona, Italy

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### ABSTRACT

**Background:** Flavonoids, such as quercetin, were reported to inhibit histamine release and cytokine production by basophils, but there is no evidence describing their action on membrane markers and intracellular biochemical pathways.

**Objective:** The aim of the study was to examine the effect of several quercetin doses on an *in vitro* human basophil activation system that evaluates up-regulation of membrane markers in response to agonists.

**Methods:** Leukocyte buffy coats from K<sub>2</sub>-EDTA anti-coagulated blood were treated with different concentrations of quercetin and triggered with anti-IgE (“allergy model”) and with N-formyl-Met-Leu-Phe (fMLP) (“inflammation model”). Basophils were captured as CD123<sup>bright</sup>/HLA-DR<sup>non-expressing</sup> cells in a flow cytometry analysis and fluorescence values of CD63-FITC, CD203c-PE and CD123-PECy5 were used to produce dose response curves.

**Results:** Quercetin at a dose of 10 µg/ml strongly inhibited CD63 and CD203c membrane up-regulation triggered by both agonists, but it neither affected cell viability nor changed the expression of the phenotypic marker CD123. The anti-IgE model appeared highly sensitive to the effect of quercetin: a dose as low as 0.01 µg/ml was able to significantly decrease CD63 and CD203c membrane expression. In the fMLP model the dose response was different: quercetin doses from 0.01 to 0.1 µg/ml significantly increased up-regulation of membrane markers, achieving the highest effect with CD63.

**Conclusion:** Very low doses of quercetin, within the pharmacological range, inhibit IgE-mediated membrane marker's up-regulation but prime the response to the chemotactic peptide fMLP; this stimulus specificity may have implications on the possible therapeutic action of the flavonoid in different pathologies.

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### 1. Introduction

Flavonoids are a large group of polyphenolic secondary plant metabolites with low molecular weight that can be found in various fruits and vegetables and are thus common substances in human daily diet [1]. These natural products were known for their beneficial effects on health long before flavonoids were isolated as the effective compounds. Flavonoids can be divided into various classes on the basis of their molecular structure: flavones, flavonols, isoflavones, anthocyanins, flavanols, and flavanones. More than 4000 varieties of flavonoids have been identified, many of which are responsible for the attractive colors of flowers, fruit, and leaves. Besides being involved as protective substances in cardiovascular diseases [2], they have been recognized as having anti-oxidant [3], anti-inflammatory [4], anti-allergic [5], anti-infectious [6], anti-carcinogenic [7], pro-apoptotic

[8,9] and anti-hypertensive [10] properties. Quercetin, a member of flavonols, is the most abundant of the naturally occurring flavonoids – as it is widely present in the plant kingdom such as onions, apple, broccoli and berries [3,5] – and may have great potential for the prevention and treatment of the diseases. The nutraceutical property of this flavonol has been extensively evaluated [3], although many effects still need confirmation by human intervention trials [11].

The anti-allergic property of this flavonol has been explored mainly by investigating the secretory response of activated mast cells in both human and animal models [12–16], and by evaluating histamine release from human basophils [17,18]. However, little is known about the cellular and molecular target(s) of the action of quercetin on basophil, even though several reports have focused on the mechanism by which flavonoids exert their function [4,15,19]. Human basophils are involved in many inflammatory reactions and express several cell membrane antigens, which can be related to their immunological responsiveness. Among these markers two main molecules, the tetraspan CD63 and the ectoenzyme CD203c are used to assess basophil behavior to external triggering [20]. Challenging basophils with allergens, soluble compounds or agonists may result in

\* Corresponding author. Dipartimento di Scienze Morfologico-Biomediche, Università degli Studi di Verona, Laboratorio Analisi-Ospedale Policlinico “G.B. Rossi”, Piazzale L.A. Scuro 10, 37134 Verona, Italy. Tel.: +39 4582027485.

E-mail address: [salvatore.chirumbolo@univr.it](mailto:salvatore.chirumbolo@univr.it) (S. Chirumbolo).

a modified expression of these molecules on cell membrane, dynamics that can be easily evaluated by flow cytometry.

In this study we used a new flow cytometry approach [21] to investigate the effect of increasing doses of quercetin on the expression of CD63 and of CD203c in human basophils triggered by different agonists. To the best of our knowledge no evidence was reported concerning the effect of quercetin on these basophil activation markers.

Our first approach was to analyze the effect of quercetin in normal, non-allergic subjects (screened healthy blood donors), since this is the first investigation in humans about the effect of quercetin on the indicated basophil markers. The use of normal donors was preferred since quercetin also exhibited anti-infectious and anti-inflammatory properties [11]; thus, in order to appraise the potential mechanisms of this type of effect, the use of basophils from allergic subjects would be less indicated.

Recent evidence shows a strategic role of basophil in immunology that goes beyond IgE-mediated allergy [22] and different models have been proposed to study *in vitro* IgE-mediated and non-IgE-mediated pattern of basophil activation [20,23,24]. The simplest model, with anti-IgE and with the bacterial peptide fMLP, allowed for the identification of two kinds of basophil activation and degranulation by cytology study [25]. The differential pathways can be assumed as general paradigms of allergic and inflammatory (infectious) reactions. In this context, quercetin may act in different ways on basophils by modulating different functions according to the tissue environment *in vivo* or to the experimental conditions *in vitro*. In particular, since these cells are involved either in allergy or in acute or chronic inflammatory processes of various nature, it was of interest to test the drug effect on two major models of activation. Therefore, increasing doses of quercetin were tested on resting cells and on cells activated by anti-IgE monoclonal antibodies, a model that mimics the main pathway of allergy (IgE-mediated response), and by fMLP, a chemotactic agent taken as a simple paradigm for leukocyte inflammatory response to an infectious agent.

## 2. Methods

### 2.1. Reagents

N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), HEPES (4-2-hydroxyethylpiperazine-1-ethanesulfonic acid), quercetin dihydrate (minimum 98% HPLC), phorbol-12-myristate-13-acetate (PMA), Na<sub>3</sub>-EDTA, sodium heparin, saponin, trypan blue, propidium iodide, and distilled water (HPLC grade, Chromasolv® Plus) were purchased from Sigma (Sigma-Aldrich GmbH, Germany). Goat monoclonal anti-human IgE was purchased from Caltag Laboratories, (UK). Mouse anti-human monoclonal antibodies for flow cytometry evaluation CD123-PECy5 (isotype IgG<sub>1</sub>, clone 6H6), CD45-APCCy7 (isotype IgG<sub>1</sub> clone HI30), CD203c-PE (isotype IgG<sub>1</sub> clone NP4D6), CD63-FITC (isotype IgG<sub>1</sub> clone MEM-259) were purchased from Biolegend, San Diego CA, USA. HLA-DR-PECy7 (isotype IgG<sub>2a</sub> clone L243) was purchased from Becton Dickinson, Pharmingen CA, USA. Pure quercetin was dissolved in DMSO at a stock solution of 1 mg/ml and stored at -20 °C for a maximum of 1 week; working solutions were made into HEPES modified buffer (HEPES 20 mmol/L; NaCl 127 mmol/L; KCl 5 mmol/L; sodium-heparin 5 U/ml, pH 7.4) (HBE). fMLP was dissolved in dimethyl sulfoxide (DMSO) as a 2 × 10<sup>-2</sup> M stock solution, stored at -20 °C and thawed before use. Working solutions of fMLP and anti-IgE were freshly prepared in HBE supplemented with 5 mM CaCl<sub>2</sub> and with 2 mM MgCl<sub>2</sub> (HBC buffer). All reagents were pure and quality checked; whenever necessary disposable plastic ware and sterile apyrogenic solutions were used.

### 2.2. Subjects and sampling

A total of 48 blood donors volunteers (45% male, 55% female) were enrolled in this study. Recruitment was randomized and encompassed

an age range from 26 to 65 years (mean 46.34 ± 5.67 SD) in order to have a wide experimental population and to prevent age influence on cell releasability [26]. All subjects were non-allergic, non-atopic and had never reported any previous history or genetic diathesis of chronic allergy; moreover, none underwent neither drug therapy nor anti-histamine therapy in the 48 h before the peripheral venous blood withdrawal. All participants completed and signed a specific consenting form for providing samples and for data processing.

### 2.3. Cell recovery and preparation

In each experiment basophils were collected as leukocyte-enriched buffy coats from venous K<sub>2</sub>-ethylenediaminetetraacetic acid (EDTA) anti-coagulated peripheral blood from four screened healthy donors, according to previously described methods [21]. The buffy coat was used because basophils account for <1% of total peripheral blood leukocytes and because collecting leukocyte-enriched buffy coats revealed as a good approach to obtain basophils without using time-consuming and hardly feasible approaches such as Ficoll or immunomagnetic beads, which could result in relative low yields and spontaneous activation of these cells [21,27]. Buffy coats were washed and pooled in HBE buffer. To count basophils and evaluate yields, an aliquot of about 1 ml of this cell suspension was transferred to a Bayer ADVIA 2120 automated hemocytometer [28]. The volume of working cell suspensions was adjusted with HBE in order to get a basophil count of 90–150 basophils/μl. Compared with hemocytometer counts of starting whole blood (30–50 basophils/μl), an average enrichment of about 1.5–3.0 times (mean = 2.4) was currently obtained. Final cell buffered culture contained a heterogeneous population of leukocytes from which basophils were electronically purified by flow cytometry as described in Section 2.4. Trypan blue exclusion test revealed that 98.5% ± 5.6 SD leukocytes were viable. Aliquots (100 μl) of cell samples were incubated at 37 °C for 10 min with an equal volume of HBE in the absence or in the presence of quercetin at the indicated final doses. Activation was performed by adding 50 μl of treated cells to 50 μl of HBC buffer containing 200 nmol/L of fMLP or 8 μg/ml of goat anti-human IgE. Resting assays were performed by incubating cells in HBC without agonists. Incubation was carried out at 37 °C for 30 min and blocked by adding 100 μl of ice-cold HBE supplemented with 2.8 mmol/L sodium-EDTA (Na<sub>3</sub>-EDTA). The samples were then put on ice and stained with monoclonal antibodies (20 min at +4 °C) [21]. Afterwards, red blood cells underwent lysis with an ammonium-buffer (NH<sub>4</sub>Cl 155 mmol/L, Na<sub>2</sub>HCO<sub>3</sub> 10 mmol/L, Na<sub>3</sub>EDTA 0.10 mmol/L, pH = 7.2) for 2 min at +4 °C, then centrifuged at 700 g and pellets were recovered and re-suspended in a PBS-buffered saline solution (pH 7.4) for flow cytometry reading.

### 2.4. Flow cytometry and data processing

By using flow cytometry and specific markers it is possible to get a virtual electronic purification of basophils at homogeneity and to investigate membrane and functional changes, a method particularly useful in pharmacological sciences. Basophil membrane markers were evaluated by flow cytometry using a five-color fluorochrome panel including CD45-APCCy7, CD123-PECy5 and HLA-DR-PECy7 as phenotyping markers and CD63-FITC and CD203c-PE as activation ones; basophils were phenotyped by gating CD45<sup>dimly</sup> leukocytes for CD123<sup>bright</sup>/HLA-DR<sup>non-expressing</sup> cells, as previously reported [21,29]. Flow analysis was performed using a 488 nm–633 nm two-laser BD FACScanto flow cytometer: the instrument had a 10,000 events/s capability, six-color detection and 0.1% sample carryover. Analysis were performed with a mean flow rate of 300–500 events/s, setting an excess limit of 50,000 events to record in the basophil gate in order to analyze the whole buffered suspension volume and have a proper estimation of cell recovery and reproducibility. Compensation followed cytometer manufacturer's instructions for an off-line

procedure by applying automated electronic algorithms and preset templates, by using biparametric logarithmic dot plots, gate-specific tubes and single-tube data analysis, and optimizing FSC threshold and fluorochrome voltage as set up parameters. Mean of fluorescence intensity (MFI) was calculated automatically by the cytometer software. Percentage of activated cells was calculated by the software considering the CD63 expressing cells (CD63-FITC<sup>positive</sup> cells) counted to the right of a threshold that was established to include the main peak of fluorescence of a sample of resting cells. In order to reduce standard deviation due to positive fluorescent cells respect to negative or dimly ones, a logarithmic scale and a coefficient of variation to measure variability dispersion were used.

### 2.5. Propidium iodide cell viability test

Propidium iodide (PI) test was performed using a modified previously described method [30]. Cells were treated with different doses of quercetin for 40 minutes at 37 °C as described, blocked with ice-cold HEPES modified buffer supplemented with 2.8 mmol/L Na<sub>3</sub>-EDTA and soon after stained with CD45-APCCy7 monoclonal antibody and with 5 μM propidium iodide dye. PI fluorescence of CD45-expressing cells (total leukocyte population including basophils) was evaluated with the BD FACScanto flow cytometer by using 536 nm excitation wavelength and 617 nm emission wavelength (FL3 or C channel). PI positive control was performed by incubating an aliquot of quercetin untreated cells with 0.1% saponin for 20 min at 37 °C. Percent of PI-negative (viable) cells was calculated by the instrument software.

### 2.6. Statistics

Data were analyzed using the software SPSS, version 11 for Windows, Chicago, IL. Descriptive statistics were obtained by plotting triplicate data and their mean values and S.E.M. for each experiment using the Sigma plot 10 software. Kolmogorov–Smirnov and Shapiro–Wilk goodness-of-fit tests were performed to determine whether the sample population followed a Gaussian distribution. Differences between quercetin-treated and untreated cells were analyzed by using a one way analysis of variance (ANOVA) followed by Fisher LSD test. The effect of the drug on the various parameters was also calculated for each experiment as a percentage with respect to the control values (taken as 100%), after subtraction of the resting (unstimulated) baseline values, according to the formula:

$$\left[ \frac{\text{Sample Quercetin} - \text{Resting}}{\text{Sample Control} - \text{Resting}} \right] * 100$$

where Sample Quercetin is the value (MFI or percent of CD63-FITC<sup>positive</sup> cells) of each quercetin-treated sample, Resting is the mean value of triplicate samples of spontaneous (constitutive) marker expression in resting cells, and Sample Control is the mean value of triplicate samples of control, quercetin untreated, activated cells. Differences of dose–effect curves between fMLP-treated and anti-IgE treated cells were analyzed by a Wilcoxon non-parametric test for paired samples (fMLP vs. anti-IgE at each drug dose). A value of  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Fluorescence behavior of activation markers

Fig. 1 describes the fluorescence behavior of CD63 and CD203c in resting, non-activated cells (panels A), and in cells which were treated with 4 μg/ml anti-IgE (panels B) or with 100 nM fMLP (panels C), resulting from the instrumental output in a typical experiment performed. Histogram distribution reflects the number of molecules expressed in the whole basophil population and hence the dynamics

of their expression/inhibition following external challenge. In this experiment, a relatively low dose of quercetin (0.1 μg/ml) was tested. In the resting condition fluorescence of both markers showed unimodal distribution, with fluorescence intensity under approximately 10<sup>3</sup> units for CD63 and under 2 × 10<sup>3</sup> units for CD203c. Very few cells (from 0.5% to 2.5% of total) appeared as small peaks to the right of the threshold line that was set to separate CD63<sup>negative</sup> (non-expressing) from CD63<sup>positive</sup> (expressing) cells. This indicates that the cell preparation and isolation procedure used caused little or no significant spontaneous activation of basophils. The fluorescence pattern of both markers was not affected by quercetin treatment (see panels A2 and A4 as compared with panels A1 and A3).

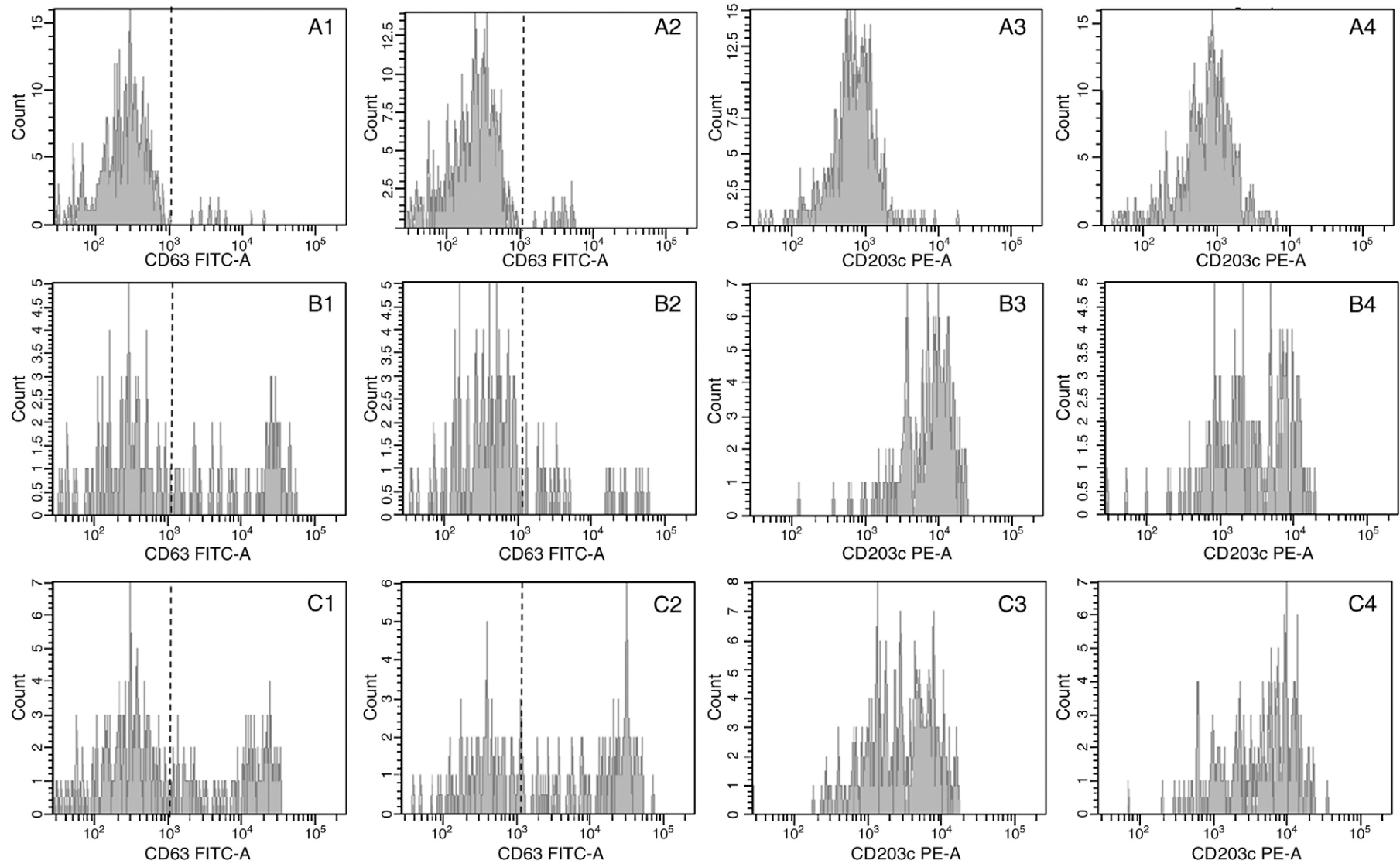
Triggering with 4 μg/ml anti-IgE caused the appearance of a peak of activated cells which up-loaded CD63 molecules on their membrane (panel B1 compared with panel A1) or caused a shift to the right of fluorescence distribution for CD203c-PE, indicating an up-regulation of this marker (panel B3 compared with panel A3). These flow cytometry patterns demonstrate that only a percentage of cells (from 15% to 35% according to the experiments and subjects) up-regulated CD63 molecules, while almost all of them increased the fluorescence of CD203c, an observation in agreement with other evidence previously reported [21,31]. Quercetin treatment markedly reduced the peak of CD63<sup>positive</sup> cells (panel B2 compared with panel B1) and inhibited the shift of CD203c fluorescence distribution (panel B4 compared with panel B3). When basophils were treated with 100 nM fMLP a pattern of fluorescence increase alike to that of anti-IgE was observed (panels C1 and C3), but a completely different effect of quercetin appeared. As a matter of fact, 0.1 μg/ml of quercetin slightly increased the peak of CD63<sup>positive</sup> cells (panel C2 compared with panel C1) and the total fluorescence distribution, as assessed by CD203c (panel C4 compared with panel C3). This cell behavior was assessed under the effect of different concentrations of quercetin and data regarding the MFI or the percent of activated cells were collected and computed in order to build up dose–response curves.

### 3.2. Dose–response effects of quercetin

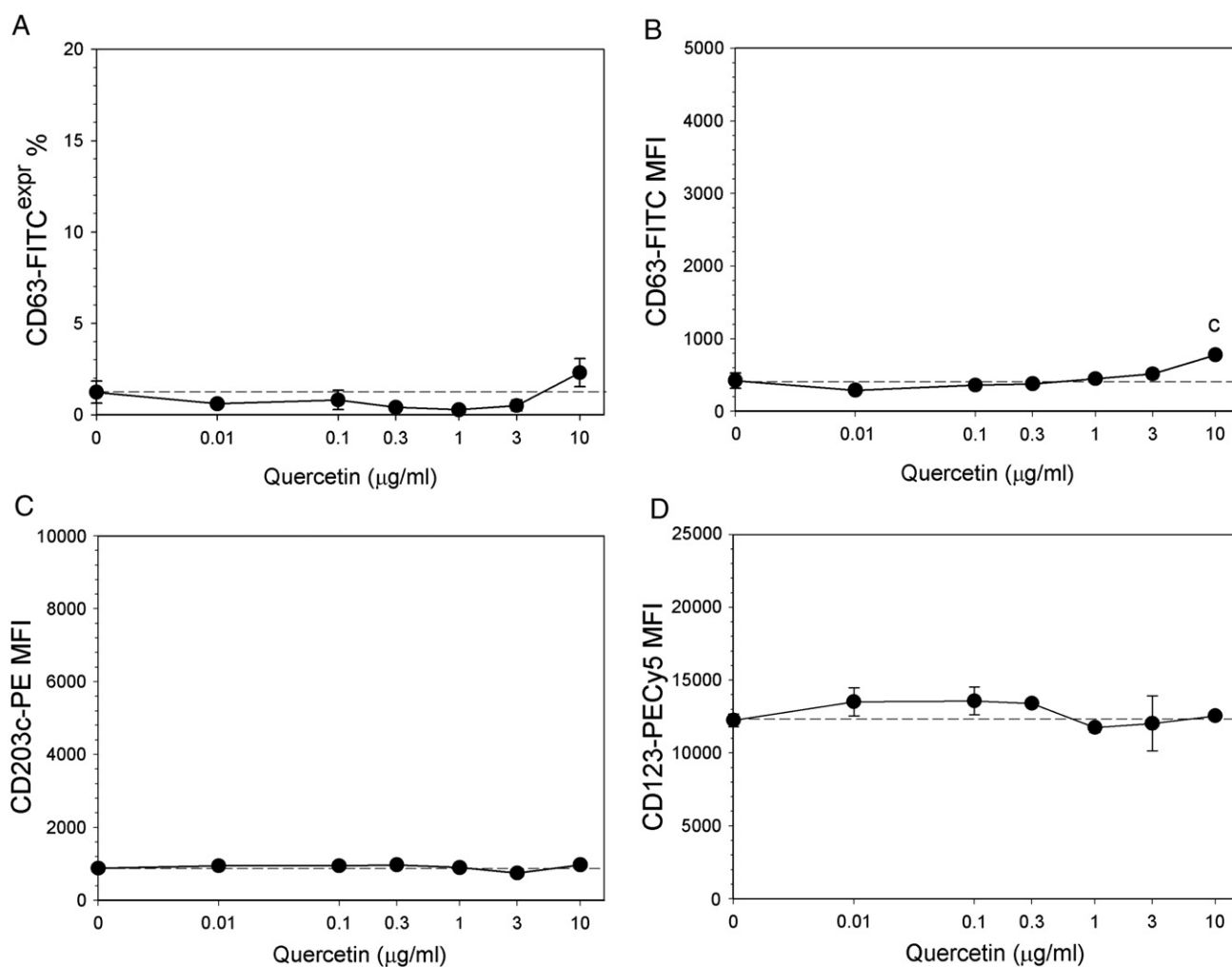
In resting, unactivated cells (Fig. 2) no significant effect of quercetin was observed, except for a small increase in the % of CD63-expressing cells (panel A) and in the CD63-MFI (panel B) when using 10 μg/ml of the compound. As shown in Fig. 3, quercetin was able to decrease CD63 (panels A and B) and CD203c up-regulation triggered by stimulation with anti-IgE in a dose-dependent fashion. The bioflavonoid exerted a very strong effect at 10 μg/ml ( $p < 0.0001$ ) and significantly decreased basophil marker expression even at 0.1 μg/ml ( $p < 0.0001$ ). A small inhibition was seen even at 0.01 μg/ml quercetin, which was significant ( $p < 0.05$ ) only in the CD203c evaluation, probably due to the lower data variability. The flavonol showed a targeted effect only on the CD63 and CD203c activation markers, whereas it did not affect the phenotyping marker CD123 (panel D). Since the latter is a specific constitutive marker of integral basophil membrane, this result might be considered as a confirmation of the lack of toxicity of quercetin.

Quercetin was also found to affect marker up-regulation following activation with chemotactic peptides (Fig. 4), though with a notably different pattern. Membrane expression of CD63 and CD203c was reduced only by quercetin doses in the microgram range, while an increase of CD63<sup>positive</sup> basophils (panel A) and of CD63-MFI (panel B) was induced by low doses of bioflavonoid. The flavonol did not affect the phenotyping marker CD123 (panel D) in this experiment too.

This distinction of effects, depending on the doses and agonists, was confirmed in a separate experiment in which the same basophil population was triggered with the two stimuli (Fig. 5): quercetin strongly inhibited CD63 and CD203c expressions following anti-IgE activation (panels A and C, respectively), while in fMLP-stimulated basophils, low doses of the compound showed a priming effect



**Fig. 1.** Fluorescence histograms of CD63-FITC (panels of columns 1 and 2) and CD203c (panels of columns 3 and 4) in resting (panels of row A), anti-IgE-treated (panels of row B) and fMLP-treated (panels of row C) basophils in the absence (panels of columns 1 and 3) or in the presence of 0.1  $\mu\text{g}/\text{ml}$  quercetin (panels of columns 2 and 4). In the x-axis increasing values of fluorescence and in the y-axis number of counted cells are reported. Evaluation of CD203c and CD63 is made on  $\text{CD45}^{\text{dim}}$  leukocytes which highly express CD123 and do not express HLA-DR. These leukocytes are recognized as pure basophils [21,29]. The vertical dashed line in panels of columns 1 and 2 was set as a threshold separating  $\text{CD63}^{\text{neg}}$  from  $\text{CD63}^{\text{pos}}$  (bright phenotype) basophils and to calculate the percentage of activated cells in each condition. Percentage of  $\text{CD63}^{\text{expressing}}$  cells in this experiment: A1 = 1.2%; B1 = 25.6%; C1 = 23.1%; A2 = 1.8%; B2 = 10.5%; and C2 = 43.1%.



**Fig. 2.** Dose response of quercetin action on the percentage of cells expressing CD63 bright phenotype (A), on CD63 MFI (B), on CD203c MFI (C) and on CD123 MFI (D) in resting, unactivated human basophils. Values are mean  $\pm$  S.E.M. of triplicate assays. Dashed line reports the level of basophil activation without quercetin. One way ANOVA *p* values: panel A, *p* = 0.067; panel B, *p* = 0.0001; panel C, *p* = 0.083; panel D, *p* = 0.639. Post-hoc (LSD) analysis of each dose is indicated as: (a) *p* < 0.05; (b) *p* < 0.01; (c) *p* < 0.001.

(panels B and D). In this experiment, even CD203c was enhanced by doses as low as 0.01–0.10 µg/ml of quercetin, though to a lesser extent than CD63. In two further experiments, quercetin was tested on basophils activated with 50 nM phorbol-12-myristate-13-acetate (PMA); in these conditions flavonol proved to be almost totally ineffective and a small inhibition was observed only at the highest doses employed (10 µg/ml) (data not shown).

### 3.3. Cell viability

Cell samples were incubated for 40 min at 37 °C with and without quercetin and their viability was assessed by PI exclusion test. In all conditions over 96.5% of leukocytes were negative to the fluorescent probe (96.88%  $\pm$  1.10 S.E.M. with 10 µg/ml quercetin, 97.02%  $\pm$  1.04 S.E.M. with 1 µg/ml quercetin, 96.76%  $\pm$  1.07 S.E.M. in control, untreated cells), indicating no toxicity of the tested compound. For technical reasons linked to fluorescence probes, this latter evaluation of viability was performed on the whole leukocyte population, but due to the lack of any toxicity on the whole population, this result can be assumed to basophils as well.

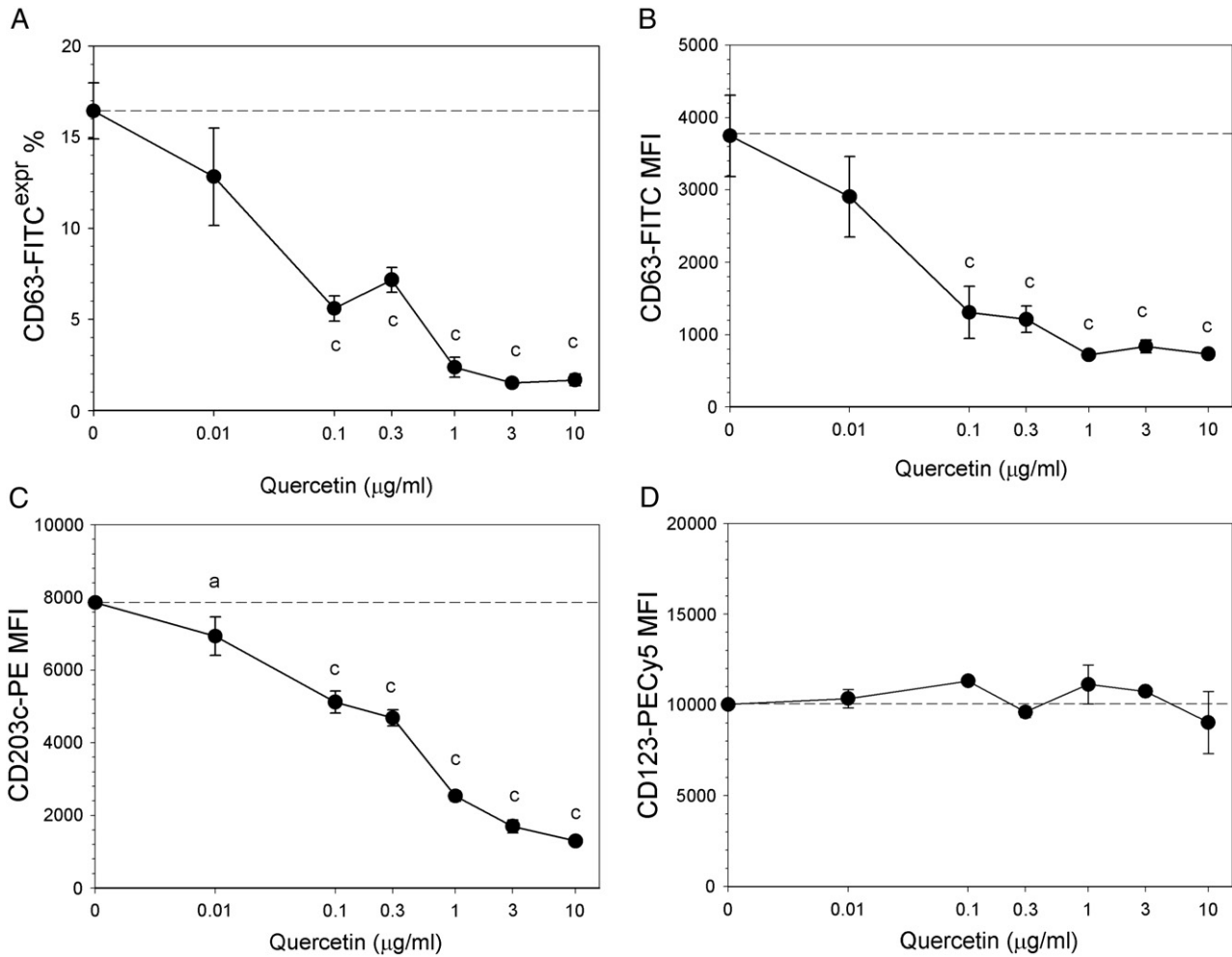
### 3.4. Summary of pooled results

The effect of the drug on the various parameters was calculated as a percentage with respect to the control values (taken as 100%), after

subtraction of the resting (unstimulated) baseline values (see [Methods](#)). This allowed the effects of the various dilutions, tested in separate experiments and standardized as a percentage of their internal control values, to be compared and statistically evaluated. [Table 1](#) reports the summary of all the experiments performed, with pooled data and statistics. The inhibition of anti-IgE treated basophils was significant at all tested doses. With fMLP, the biphasic pattern of quercetin effects (priming at low dose, inhibition at high dose) was evident using both activation markers, but priming was significant only considering CD63 up-regulation. Differences of dose–effect trends between cells treated with fMLP and with anti-IgE were analyzed by a Wilcoxon signed rank test. A value of *p* < 0.0001 was obtained considering all the activation parameters used (CD63<sup>positive</sup> cells, CD63MFI and CD203c). No significant difference was detected in the effects of quercetin on CD123 expression using the two agonists (*p* = 0.44).

## 4. Discussion

Allergies are a cause for concern due to their increasing prevalence in human population. Given their anti-allergic activity, flavonoids have the potential to counter this trend. An appropriate intake of quercetin from food and beverage or from supplemental administration could be expected to improve allergic symptoms, to aid anti-inflammatory and anti-oxidative responses and to prevent the onset



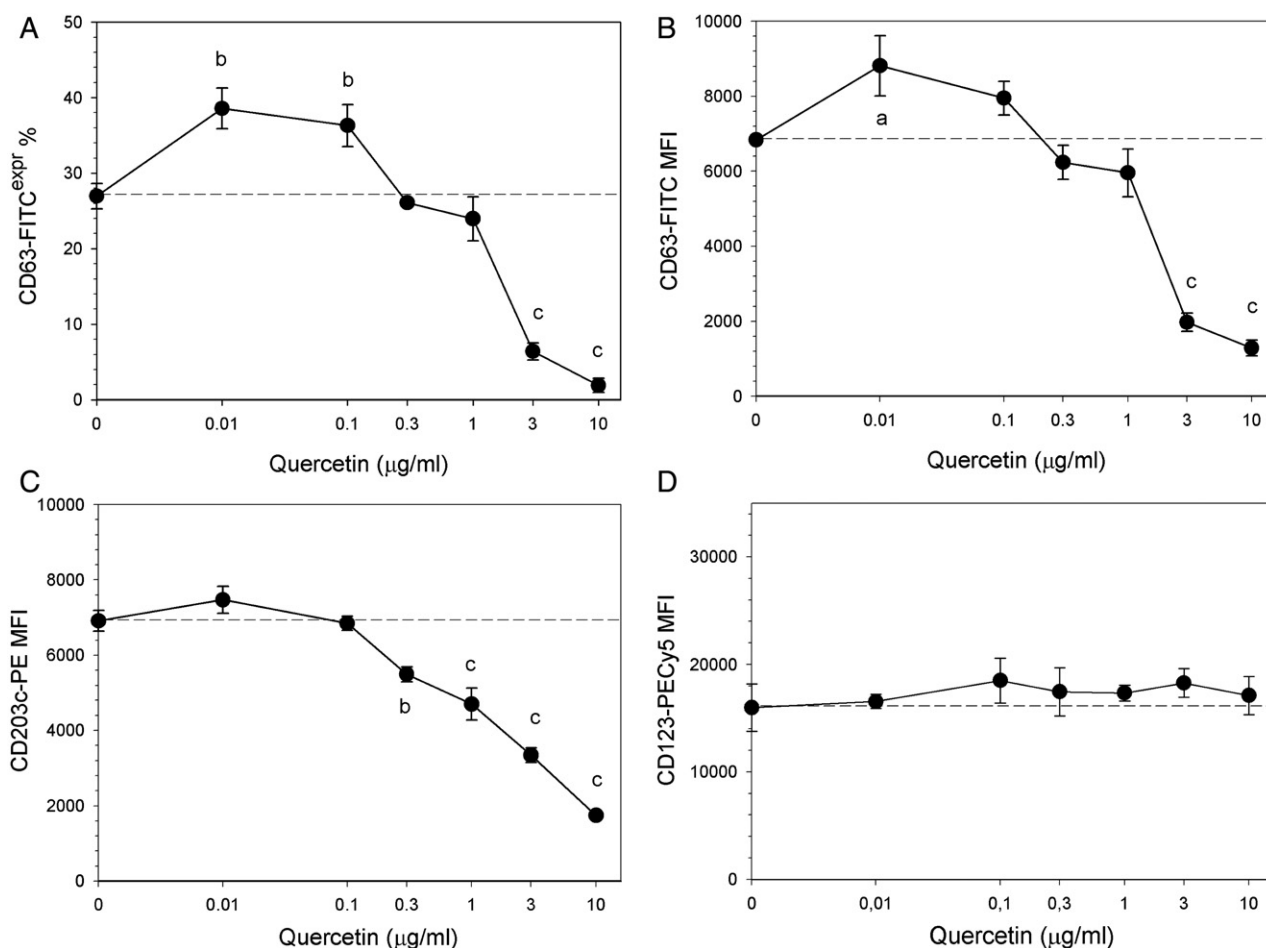
**Fig. 3.** Dose response of quercetin action upon basophil membrane markers following stimulation with 4 µg/ml goat monoclonal human anti-IgE. Values are mean  $\pm$  S.E.M. of triplicate samples of one representative experiment out of three performed. Dashed line reports the level of basophil activation without quercetin. One way ANOVA *p* values: panel A, *p* < 0.0001; panel B, *p* < 0.0001; panel C, *p* < 0.0001; panel D, *p* = 0.075. Post-hoc (LSD) analysis of each dose is indicated as: (a) *p* < 0.05; (b) *p* < 0.01; (c) *p* < 0.001.

of allergic chronic diseases. To achieve this goal, further research insights about cell signaling, e.g. quercetin intracellular targets and more information concerning the bioavailability of flavonoids, along with clinical trials to verify their effectiveness, are required. In this work, flow cytometry of membrane marker expression proved to be a sensitive approach for investigating the effect of quercetin on basophil responsiveness and allowed simultaneous assay of different doses of the drug in different conditions. The hypothesis of a stimulus-specific modulatory action of a naturally-derived bioflavonoid is intriguing as a possible therapeutic approach to allergy and inflammation.

Our results show that doses as low as 0.01–0.1 µg/ml of quercetin inhibit the activation of basophils triggered by an agonist that mimics the IgE-mediated allergic response, such as a monoclonal anti-human IgE. The doses observed to be active in this model are lower than those reported in previous works which evaluated histamine release, where quercetin was found to be effective in a dose range of approximately 1.5–15 µg/ml or higher [17,23,32]. Whether this discrepancy is due to different experimental conditions, or to a real dose-dependent dissociation of the quercetin effects on membrane markers from those on histamine release, is still to be elucidated. However, this second hypothesis seems less plausible because the CD63 expression is correlated with degranulation events and thus with histamine release [33]. The doses acting on *in vitro* anti-IgE basophil responses, detected in this work by fluorimetric marker expression, are within the range of quercetin plasma concentration, which has been found to

be reached during therapeutic intervention trials in humans [34–38]: plasma concentrations reached 431 nmol/L (0.13 µg/ml) after 1 week supplementation with 150 mg/d pure quercetin [36], 0.63 µmol/L (0.19 µg/ml) after 1 week supplementation with 80 mg/d quercetin equivalents from onions [37] and reached a maximum of 1.5 µmol/L (0.45 µg/ml) after 28 days of supplementation with high doses of quercetin (>1 g/d) [38]. Thus, the high sensitivity of the anti-IgE-mediated pathway to inhibition by quercetin reinforces its role as an anti-allergic compound. It would be interesting to investigate in further studies whether similar effects are obtained also on basophils from allergic subjects.

Using the same method, we were able to show that basophils, triggered with formylated peptides, were primed to enhance responses by the same low doses of the flavonoid inhibiting the anti-IgE-mediated triggering. The response to fMLP was down-regulated only by high concentrations of the compound, which are probably beyond the pharmacologic and nutraceutical range. The observed biphasic, or “hormetic” [39] mechanism by quercetin in basophil stimulated with formylated peptides, has not been described yet, although a biphasic behavior of quercetin was reported in cell proliferation models [40,41]. This evidence could support speculation about the role of flavonoids in the regulation of these cells *in vitro*, and possibly *in vivo* as therapeutic modulatory agents. The enhancing effect of low doses of quercetin on the fMLP mediated response may be of particular interest since basophil activity would not be



**Fig. 4.** Dose response of quercetin action upon basophil membrane markers following stimulation with 100 nM fMLP. Values are mean  $\pm$  S.E.M. of triplicate samples of one representative experiment out of four performed. Dashed line reports the level of basophil activation without quercetin. One way ANOVA *p* values: panel A, *p* < 0.0001; panel B, *p* < 0.0001; panel C, *p* < 0.0001; panel D, *p* = 0.902. Post-hoc (LSD) analysis of each dose is indicated as: (a) *p* < 0.05; (b) *p* < 0.01; (c) *p* < 0.001.

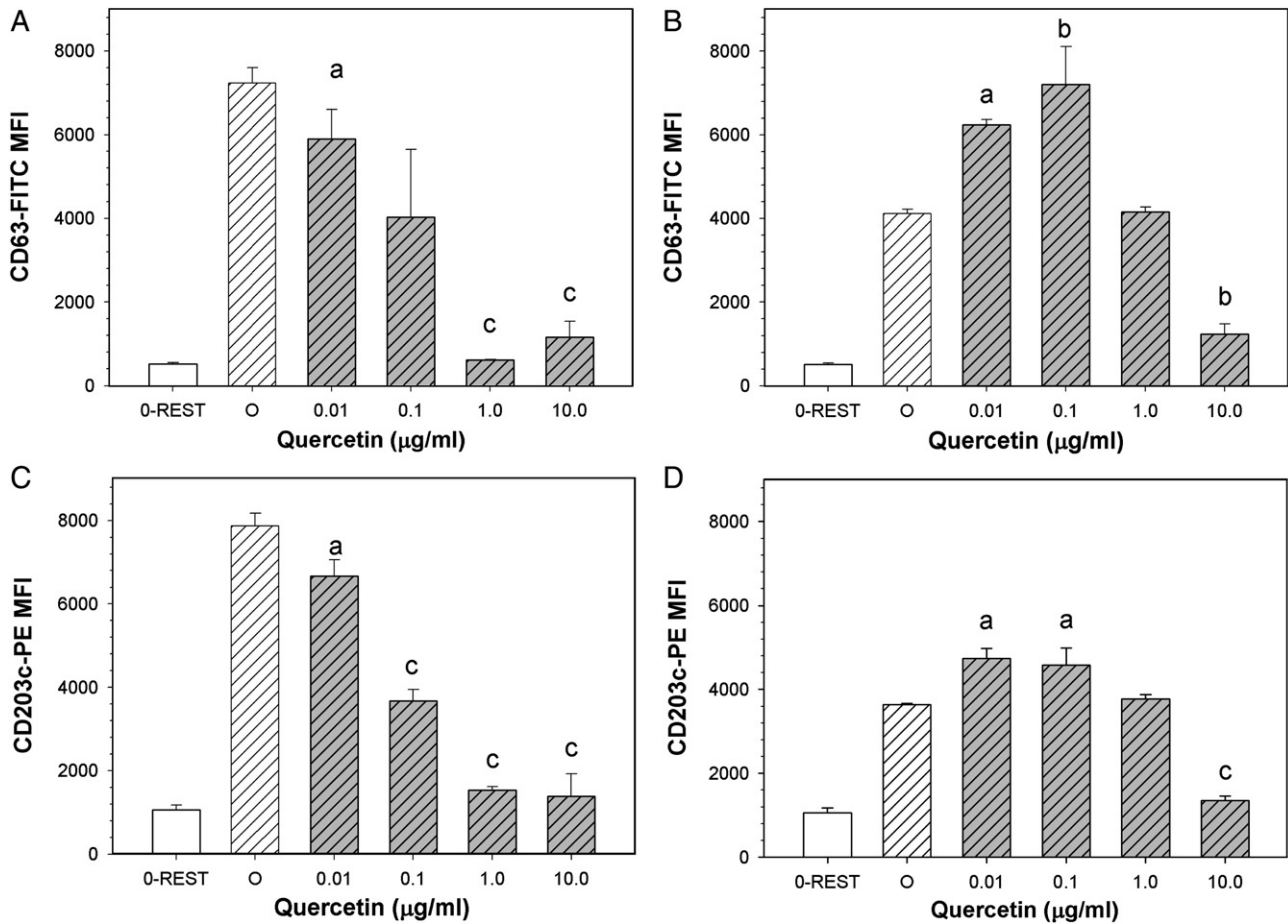
dampened but rather enhanced by this compound, when these cells are involved in an inflammatory focus triggered by bacterial products, thus preserving their signaling role in the orchestrated defense systems against infections. This interpretation would be in agreement with the evidence of an “anti-infectious” activity of quercetin [6], which could be due to the strengthening of inflammatory reactions to invading bacteria (basophil signaling, histamine and cytokine release, and phagocytosis), rather than to a direct action against microorganisms. In fact, a direct anti-microbial activity has been reported for flavonoids at higher doses, ranging from 0.5 to 2.0 µg/ml [42]. The evidence that quercetin plays a wider role in immunology and inflammation, since it affects T-lymphocytes, [13], eosinophils [43] and neutrophils [44], is in concurrence with its possible action in non-IgE-mediated reactions, such as natural immunity and inflammatory mechanisms, as it is exemplified by our fMLP model.

A possible mechanism for the inhibitory effects of quercetin is suggested by the evidence that flavonols interfere with many intracellular mediators and serine/threonine kinases such as the mitogen-activated protein kinase (MAPK) [45], and by reports describing a role for p38-MAP kinase on CD63 expression [46,47], but our data do not help to discriminate these possible pathways. However, while a role for MAPK might be proposed to explain the inhibitory effect of quercetin towards CD63 expression [20], no evidence was reported on the relationship between MAPK and CD203c, which in our conditions was markedly inhibited by the compound. Moreover, quercetin inhibits other important signaling pathways in FcεRI-IgE and formyl peptide receptors, such as mem-

brane lipid rafts downstream of receptor activation [48], phosphatidylinositol-3-kinase (PI3K) [49] and protein kinase C (PKC) [50,51]. However, inhibitors of PI3K, such as wortmannin, markedly affect CD63 expression but very little that of CD203c [24], whereas in our system both markers are inhibited by quercetin. A role for inhibition of PKC is questionable also due to the observed absence of quercetin effect on PMA-activated basophils in our testing system, which is in agreement with other previously reported evidence in macrophages [48].

The biochemical targets that would suggest a possible explanation of the priming of fMLP-dependent basophil activation by quercetin are actually a matter of speculation. Apparently, numerous candidate target proteins produce a complex reaction pattern, which is hardly predictable. Activation with fMLP triggers F-actin polymerization in basophils [52] and quercetin has been shown to affect cytoplasmic and nucleus F-actin in the micromolar dose range [53]. The existence of a complex equilibrium of G/F-actin and the existence of different F-actin pools might suggest a balancing role for quercetin in inhibiting or promoting actin polymerization depending on its molecular affinity and dose concentration [54], thus causing a biphasic (priming or desensitization) effect.

fMLP receptor has been widely described in leukocytes: this seven-transmembrane-spanning G-protein-linked receptor can be considered a prototype for chemoattractant receptors in these cells and mediates activation of MAPK cascade and intracellular cAMP [55] due to the activation of type II adenylyl cyclase. Protein kinase A is a cAMP-dependent kinase, the activation of which leads to formyl peptide



**Fig. 5.** CD63 MFI (A, B) and CD203c MFI (C, D) in basophils treated with increasing doses of quercetin. Activation was performed with 4 µg/ml goat monoclonal human anti-IgE (A, C) or 100 nM FMLP (B, D) on the same cell preparation within the same experiment. Values are mean ± S.E.M. of triplicate assays. One way ANOVA *p* values: panel A, *p* = 0.002; panel B, *p* = 0.0001; panel C, *p* < 0.0001; panel D, *p* < 0.0001. Post-hoc (LSD) analysis of each dose is indicated as: (a) *p* < 0.05; (b) *p* < 0.01; (c) *p* < 0.001.

receptor phosphorylation and desensitization. Since evidence was reported showing the inhibitory effect of quercetin on PKA [56], the enhancement of response to FMLP could be due the removal of this feed-back regulatory mechanism on the receptor. An alternative explanation is that inhibition by quercetin of small down-regulatory GTPases, such as those involved in Ras/Raf pathways [57] might remove this type of receptor desensitization, thus causing a consequent priming effect [58].

The biphasic effect of quercetin on the inflammation model found in this *in vitro* study is important in view of the use of quercetin as a bioactive functional food ingredient and/or food supplement. Many attempts have been made to test *in vivo* bioflavonoids as anti-inflammatory and anti-allergic substances [11], but the clinical evidence is inconclusive, mostly with regard to flavonoid bioavailability [3,59]. However, the higher availability of quercetin glycosides in plant extracts and whole vegetables [59,60] might provide further grounds for proposing quercetin or its conjugates as good candidates for anti-allergic, anti-inflammatory and anti-infectious compounds. Quercetin occurrence in plants, its chemical structure and bioavailability in humans are the main topics to be faced in order to assess this compound in therapy.

In summary, the stimulus specificity of quercetin effects could be extrapolated to a possible pharmacologic specificity in the regulation of inflammatory processes, according to the nature of the triggering agents and of the various molecular mediators involved. This kind of selectivity would characterize the pharmacologic profile of quercetin as compared with other conventional anti-inflammatory agents such as anti-histaminic and non-steroidal anti-inflammatory drugs

(NSAIDs). According to the findings reported in this investigation, quercetin concentration range could be critical in promoting as well as inhibiting selected phenomena of the inflammation response to

**Table 1**

Effects of quercetin on basophil activation markers, expressed as percent of control, untreated cells.

| Parameter            | Quercetin dose | Anti-IgE |            |         | FMLP   |            |          |
|----------------------|----------------|----------|------------|---------|--------|------------|----------|
|                      |                | Mean     | Std. error | P       | Mean   | Std. error | <i>p</i> |
| CD63 MFI             | 0 (control)    | 100.00   | 5.33       | –       | 100.00 | 3.97       | –        |
|                      | 0.01 µg/ml     | 82.12    | 6.93       | 0.0329  | 135.56 | 8.06       | 0.0037   |
|                      | 0.1 µg/ml      | 36.73    | 8.78       | <0.0001 | 152.13 | 14.56      | <0.001   |
|                      | 1 µg/ml        | 6.64     | 1.92       | <0.0001 | 90.27  | 4.73       | 0.4043   |
|                      | 10 µg/ml       | 9.32     | 2.50       | <0.0001 | 14.46  | 4.25       | <0.0001  |
| %CD63 positive cells | 0 (control)    | 100.00   | 4.28       | –       | 100.00 | 2.16       | –        |
|                      | 0.01 µg/ml     | 82.07    | 6.89       | 0.0352  | 137.25 | 6.60       | <0.0001  |
|                      | 0.1 µg/ml      | 32.55    | 5.41       | <0.0001 | 134.88 | 6.80       | <0.001   |
|                      | 1 µg/ml        | 3.36     | 6.53       | <0.0001 | 86.16  | 4.58       | 0.0905   |
|                      | 10 µg/ml       | 4.16     | 5.61       | <0.0001 | 16.66  | 6.60       | <0.0001  |
| CD203c MFI           | 0 (control)    | 100.00   | 3.25       | –       | 100.00 | 3.18       | –        |
|                      | 0.01 µg/ml     | 88.12    | 4.79       | 0.0190  | 118.01 | 7.29       | 0.0797   |
|                      | 0.1 µg/ml      | 49.82    | 3.86       | <0.0001 | 106.57 | 9.69       | 0.5159   |
|                      | 1 µg/ml        | 9.30     | 1.71       | <0.0001 | 72.05  | 8.91       | 0.0080   |
|                      | 10 µg/ml       | 0.20     | 2.77       | <0.0001 | 10.22  | 3.78       | <0.0001  |

The data are pooled from 3 separate experiments with anti-IgE and 3 with FMLP as agonist, performed in triplicate assays (total *N* = 9 samples for each experimental condition). ANOVA resulted significant in all parameters and agonists used (<0.001). Indicated *p* values are post-hoc analysis (LSD) of the differences between quercetin different doses and control.



environmental allergens or to bacteria. This compound is an important factor of human nutrition as well as vitamins; its bioavailability has been underestimated in the past but it can be improved by food matrix components or particular delivery forms.

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