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ORIGINAL ARTICLE

EGCG targeting efficacy of NF-κB downstream gene products is dictated by the monocytic/macrophagic differentiation status of promyelocytic leukemia cells

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Abstract Central nervous system infiltration by circulating leukemic cells and enhanced in vitro transendothelial migration of promyelocytic leukemia HL-60-derived macrophages through a blood–brain barrier model was recently demonstrated. The intrinsic molecular and signaling mechanisms involved are, however, poorly documented. Drug targeting of such translocation event performed by circulating microbes and immune cells may prevent secondary cerebral infections and development of brain pathologies. In this study, we specifically investigated the in vitro targeting efficacy of the chemopreventive and dietary-derived epigallocatechin-3-gallate (EGCG) molecule on the NF-κB-mediated transcriptional regulation of a panel of 89 biomarkers associated with promyelocytic HL-60 differentiation into macrophages. NF-κB-mediated signaling during HL-60 macrophage differentiation was reversed by EGCG, in part through reduced IκB phosphorylation and led to the inhibition of moderately to highly expressed NF-κB gene targets among which the matrix metalloproteinase (MMP)-9 and the cyclooxygenase (COX)-2. In contrast, EGCG exhibited low efficacy in reversing NF-κB-regulated genes and showed selective antagonism toward COX-2 expression while that of MMP-9 remained high in terminally differentiated macrophages. Decreased expression of the 67-kDa non-integrin Laminin Receptor in terminally differentiated macrophages may explain such differential EGCG efficacy. Our results suggest that terminally differentiated macrophage transendothelial migration associated with neuroinflammation may not be pharmacologically affected by such a specific class of flavonoid. The differentiation status of a given in vitro cell model must therefore be carefully considered for optimized assessment of therapeutic drugs.

Keywords EGCG · Leukemia · NF-κB · Macrophage differentiation · Blood–brain barrier

Abbreviations

BBB	Blood–brain barrier
ECM	Extracellular matrix
EGCG	Epigallocatechin-3-gallate
LR	Laminin receptor
MMP-9	Matrix metalloproteinase-9
NF-κB	Nuclear factor-kappa B
PMA	Phorbol 12-myristate 13-acetate

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Introduction

The identification of transcription factors, such as NF-κB, STAT3, HIF-1α, and their gene products, such as cytokines, chemokines, and chemokine receptors, have laid molecular foundation for the decisive role of inflammation in carcinogenesis [1]. More specifically, NF-κB-mediated signaling can intervene in oncogenesis through its capacity to further regulate the expression of a large number of downstream gene products involved in apoptosis, cell proliferation and

differentiation [2]. Impaired NF-κB activity has now been demonstrated not only in solid cancers but also in various types of hematologic malignancies including acute myeloid leukemia (AML), chronic myelogenous leukemia and in a subset of myelodysplastic syndromes [3, 4].

Given that NF-κB-mediated inflammation contributes to survival and proliferation of malignant cells, tumor angiogenesis, metastasis and reduced response to chemotherapy, new therapeutic strategies combining different NF-κB or proteasome inhibitors has, therefore, been proposed in adjuvant therapy for cancer [5]. Unfortunately, the chemotherapeutic treatment outcome of various hematologic disorders, including most adult acute promyelocitic leukemia (APL) and AML, remains unacceptable [6, 7]. Hence, novel avenues for the treatment of leukemia are required.

Considerable attention has recently been focused on identifying naturally occurring chemopreventive substances capable of inhibiting, retarding, or reversing multistage carcinogenesis [8]. In fact, it has been demonstrated that some edible phytochemicals alter gene expression, directly or indirectly, thereby regulating carcinogenic processes. (−)-Epigallocatechin-3-gallate (EGCG), a principal antioxidant derived from green tea, has been ascribed proteasome inhibition properties and is one of the most extensively investigated chemopreventive phytochemicals considered in clinical trials [9–11]. EGCG has been shown to block each stage of carcinogenesis by modulating the signal transduction pathways involved in cell proliferation, transformation, inflammation, apoptosis, metastasis, and invasion [12]. Moreover, its antiangiogenic properties make it a good candidate for targeting tumor-associated neovascularization [13]. Since the inclusion of antiangiogenic drugs into treatment protocols for leukemia and for hematologic malignancies is becoming an important task for future clinical studies [14, 15], we sought to investigate the in vitro anti-NF-κB molecular effects of EGCG on the monocytic/macrophagic differentiation processes using a myeloid leukemia cell model.

Among the several leukemic cell lines that have been established over the years, the human promyelocytic HL-60 leukemia cells have proven useful in understanding the process whereby immature cells differentiate into cells of distinct mature myelomonocytic lineages [16]. In particular, HL-60 cells can be induced to differentiate into mature functional monocytic/macrophagic-like cells by the tumor-promoting and protein kinase C activator phorbol 12-myristate 13-acetate (PMA, also known as TPA) [17, 18]. Macrophage differentiation has also been shown to involve secretion and activation of collagenase MMP-9 [19], a crucial matrix metalloproteinase involved in extracellular matrix (ECM) degradation during tumor metastasis and in inflammatory disorders [20].

Since an important aspect in inflammation and tumor progression is the involvement of the inflammatory response mediated by tumor-associated macrophages (TAM) [21], and since polyphenols have been suggested to regulate the anti-tumorigenic properties of TAM [22], we compared the transcriptional chemopreventive efficacy of EGCG on NF-κB targets involved in PMA-mediated signaling and in late stage terminally differentiated HL-60 macrophages.

Materials and methods

Materials

Sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Cell culture media was obtained from Life Technologies (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from Perkin Elmer (Waltham, MA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The polyclonal antibodies against IκB and phospho-IκB were purchased from Cell Signaling (Danvers, MA). The polyclonal antibody against COX-2 was from Cayman Chemical (Ann Arbor, MI). The polyclonal antibody against the 67-kDa Laminin Receptor was from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody against GAPDH was from Advanced Immunochemical Inc. (Long Beach, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). EGCG and other reagents were from Sigma-Aldrich Canada.

Cell culture

The HL-60 promyelocytic cell line was purchased from American Type Culture Collection (Manassas, VA) and maintained in Iscove's modified Dulbecco's medium (Gibco Invitrogen Cell Culture Systems, Burlington, ON) containing 20 % (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and were cultured at 37 °C under a humidified atmosphere containing 5 % CO_2. Slides of PMA-treated HL-60 cells were mounted for light microscopy and air-dried, stained with Diff-Quick (Baxter Healthcare Corp., Miami, FL) and examined for morulae. Given that numerous protocols can be found in the literature to differentiate resting HL-60 cells into "macrophage-like cells" with PMA (between 2 and 8 days with various PMA concentrations, alone or in combination with other

molecules), we wish to emphasis that we termed our cell models as follows throughout the text: The "HL-60 macrophage differentiation" condition represents the adherent subpopulation of HL-60 cells immediately harvested upon PMA treatment. We termed "terminally differentiated macrophages" those same adherent cells, which were subsequently maintained in culture for 24–48 h more.

Gelatin zymography

Gelatin zymography was used to assess the extent of proMMP-9 gelatinolytic activity as previously described for proMMP-2 [23]. Briefly, an aliquot (20 µl) of the culture medium was subjected to SDS-PAGE in a gel containing 0.1 mg/ml gelatin, a substrate that is efficiently hydrolyzed by both proMMP-2 and proMMP-9. The gels were then incubated in 2.5 % Triton X-100 and rinsed in nanopure distilled H_2O. Gels were further incubated at 37 °C for 20 h in 20 mM NaCl, 5 mM $CaCl_2$, 0.02 % Brij-35, 50 mM Tris–HCl buffer, pH 7.6, then stained with 0.1 % Coomassie Brilliant blue R-250 and destained in 10 % acetic acid, 30 % methanol in H_2O. Gelatinolytic activity was detected as unstained bands on a blue background.

Immunoblotting procedures

Proteins from control and treated cells were separated by SDS-PAGE. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 h at room temperature with 5 % non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3 % Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3 % bovine serum albumin and 0.1 % sodium azide, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1/2,500 dilution) in TBST containing 5 % non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC).

Total RNA isolation, cDNA synthesis, and real-time quantitative RT-PCR

Total RNA was extracted from cell suspensions or monolayers using TriZol reagent (Life Technologies, Gaithersburg, MD). For cDNA synthesis, 2 µg of total RNA was reverse-transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was stored at −80 °C prior to PCR. Gene expression was quantified by real-time quantitative PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). DNA amplification was carried out using an Icycler iQ5 (Bio-Rad, Hercules, CA) and product detection was performed by measuring binding of the fluorescent dye SYBR Green I to double-stranded DNA. The QuantiTect primer sets were provided by Qiagen (Valencia, CA): MMP-9 (QT00040040), COX-2 (QT00040586), β-Actin (QT01136772). GAPDH primer sets were synthesized by Biocorp (Dollard-des-Ormeaux, QC) with the following sequences: forward CCATCACCATCTTCCAGG AG and reverse CCTGCTTCACCACCTTCTTG. The relative quantities of target gene mRNA compared against two internal controls, GAPDH and β-Actin mRNA, were measured by following a ΔC_T method employing an amplification plot (fluorescence signal vs. cycle number). The difference (ΔC_T) between the mean values in the triplicate samples of target gene and those of GAPDH and β-actin mRNAs were calculated by iQ5 Optical System Software version 2.0 (Bio-Rad, Hercules, CA) and the relative quantified value (RQV) was expressed as $2_T^{-\Delta C}$.

Human NF-κB signaling targets PCR array

The Human NF-κB Signaling Targets RT^2 Profiler PCR Arrays (PAHS-225, SA Biosciences, Frederick, MD) were used according to the manufacturer's protocol. The detailed list of these key genes responsive to NF-κB signal transduction can be found on the manufacturer's website (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-225A.html). Using real-time quantitative PCR, we reliably analyzed expression of a focused panel of genes related to NF-κB downstream gene targets. Relative gene expressions were calculated using the $2_T^{-\Delta\Delta C}$ method, in which C_T indicates the fractional cycle number where the fluorescent signal reaches detection threshold. The "delta–delta" method uses the normalized ΔC_T value of each sample, calculated using a total of five endogenous control genes (*B2 M*, *HPRT1*, *RPL13A*, *GAPDH*, and *ACTB*). Fold change values are then presented as average fold change = 2(average $^{\Delta\Delta C}_{T}$) for genes in adherent PMA-differentiated macrophages relative to control HL-60 cells in suspension. Detectable PCR products were obtained and defined as requiring <35 cycles. The resulting raw data were then analyzed using the PCR Array Data Analysis Template (http://www.sabiosciences.com/pcrarraydataanalysis.php). This integrated web-based software package automatically performs all $\Delta\Delta C_T$ based fold change calculations from our uploaded raw threshold cycle data.

Statistical data analysis

Unless otherwise stated, data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t* test. Probability values of less than 0.05 were considered significant and an asterisk

identifies such significance in the figures. Error bars in all figures represent standard error means (SEM) values.

Results

EGCG reverses PMA-mediated IκB degradation in adherent HL-60 cells

Among the agents well documented to induce differentiation of HL-60 cells, the tumor-promoting agent phorbol-12-myristate-13-acetate (PMA) triggers a terminal differentiated monocytic/macrophage phenotype [17]. We have previously validated that PMA treatment of serum-starved HL-60 cells induced an adhesive phenotype accompanied by macrophage differentiation in these cells which originally remain in suspension [24]. In order to first assess whether any NF-κB signaling was involved upon PMA stimulation, we performed immunoblotting on lysates isolated from adherent cells and found that IκB expression (Fig. 1a) decreased dose-dependently, as a consequence of its prior phosphorylation, and to be almost completely degraded at 10 nM PMA (Fig. 1c, left panel). When increasing EGCG concentrations were added simultaneously to PMA, IκB degradation by PMA was prevented (Fig. 1b) and almost completely reversed at 3 μM EGCG (Fig. 1c, right panel). This evidence suggests that NF-κB signaling is involved in PMA-induced differentiation process and that NF-κB gene targets are potentially modulated.

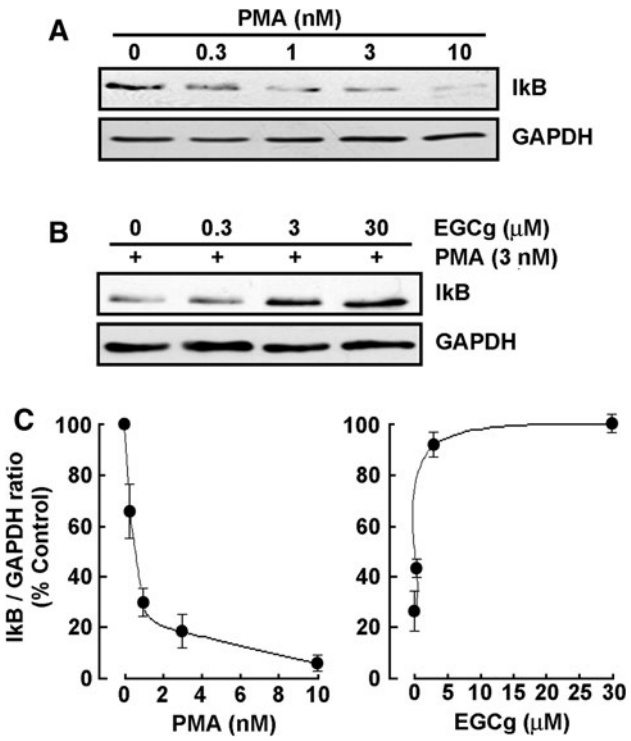

Fig. 1 EGCG reverses PMA-mediated IκB degradation in adherent HL-60 cells. **a** Serum-starved HL-60 cells were treated with increasing PMA concentrations, or **b** with combined 3 nM PMA and increasing concentrations of EGCG for 18 h. Cells that remained in suspension were discarded and lysates from the adherent macrophage-differentiated cells isolated, electrophoresed via SDS-PAGE and immunodetection of IκB and GAPDH proteins performed as described in the "Materials and methods" section. **c** Quantification was performed by scanning densitometry of the autoradiogram. Data are representative of four independent experiments and were represented as the percent (%) expression of untreated HL-60 cells in suspension (*left panel*) and as the percent (%) of 3 nM PMA/30 μM EGCG-treated adherent cells (*right panel*)

EGCG reverses PMA-induced IκB phosphorylation in adherent HL-60 cells

Iκb phosphorylation status was next investigated and the effect of EGCG assessed. PMA is shown to trigger maximal IκB phosphorylation upon 30 min treatment with PMA (Fig. 2a), which phosphorylation is subsequently followed by significant IκB degradation within the next hour (Fig. 2b). When such PMA treatment is performed in the presence of EGCG (Fig. 2c), IκB phosphorylation by PMA is significantly reduced (Fig. 2d, black bars). One may now conclude that EGCG's significant reduction in PMA-induced IκB phosphorylation may prevent p65/p50 NF-κB translocation to the nucleus that would regulate transcription of NF-κB gene targets during HL60 differentiation.

Gene array analysis reveals NF-κB gene targets are associated with macrophage differentiation status and with differential inhibitory potential of EGCG

In light of the evidence that PMA-induced macrophage differentiation involves NF-κB signalling, we used a gene array approach to explore some NF-κB downstream gene targets. Transcriptional profiling was performed on genes involved in inflammation, apoptosis, development and cell differentiation, stress response, and immune response. Among the 89 NF-κB downstream gene targets assessed, 25 % were highly (>tenfold, Table 1) induced in differentiated HL-60 macrophage-like cells when compared to vehicle-treated HL-60 cells (Fig. 3a). Among these, COX-2 and MMP-9 gene expression is specifically shown (Fig. 3a, arrows) as these two biomarkers are thought to contribute to transendothelial migration by immune cells. Anti-PMA inhibitory potential of EGCG was subsequently assessed on the gene expression of the similar NF-κB gene targets using gene array strategy. We found that ECGG inhibited, by more than 70 % approximately half of the PMA-inducible NF-κB targets assessed (Fig. 3b) including COX-2 and MMP-9. Collectively, we identified several NF-κB downstream target genes that are significantly induced during PMA-induced HL-60 differentiation into macrophages

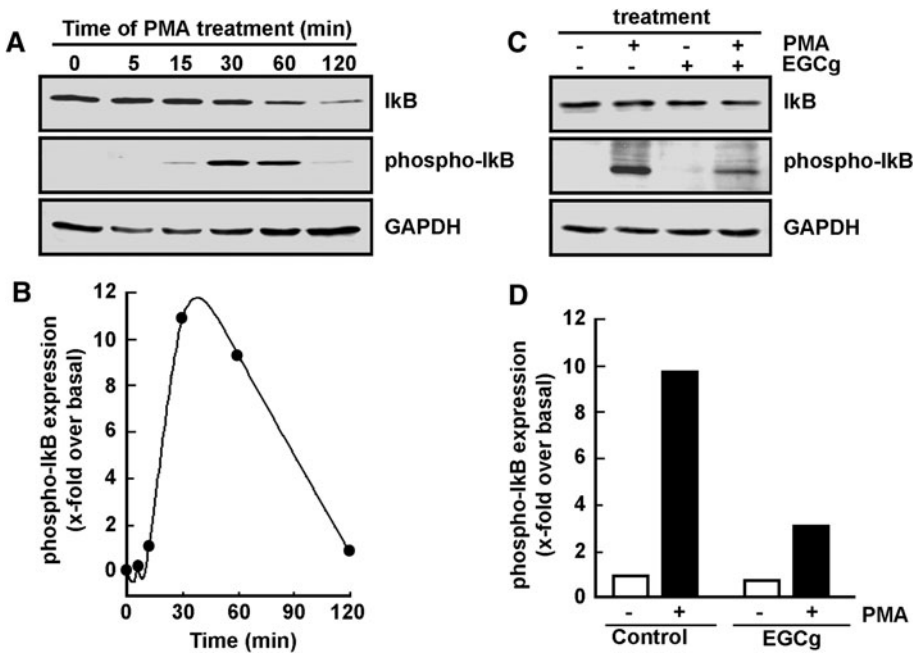

Fig. 2 EGCG reverses PMA-induced IκB phosphorylation in adherent HL-60 cells. **a** Serum-starved HL-60 cells were treated with 3 nM PMA for up to 120 min. Cells that remained in suspension were discarded and lysates from the adherent macrophage-differentiated cells isolated, electrophoresed via SDS-PAGE and immunodetection of IκB, phospho-IκB, and GAPDH proteins performed as described in the "Materials and methods" section. **b** Quantification was performed by scanning densitometry of the autoradiogram. Data are representative of two independent experiments and were represented as the *x*-fold expression over untreated HL-60 cells in suspension (time 0). The respective phospho-IkB/GAPDH ratios were blotted. **c** Similarly as in **a**, cells were treated for 30 min in the presence of 3 nM PMA, 30 μM EGCG, or a combination of both, and adherent cells harvested. **d** Quantification was performed as in **b**

and demonstrated that EGCG can very efficiently inhibit the expression of those highly induced genes as well as that of moderately induced genes (Table 2).

EGCG antagonizes COX-2 and MMP-9 biomarkers expression induced upon PMA-mediated HL-60 cell differentiation into macrophages

Given that MMP-9 is the major MMP that contributes to BBB disruption [25] and that COX-2 inhibition was demonstrated to limit BBB disruption [26], we next aimed at validating those transcriptional profiling data obtained through gene arrays (Tables 1,2) at the molecular level, and further wished to investigate the chemopreventive efficacy of EGCG on these two biomarkers' expression regulation. Serum-starved HL-60 cells were therefore treated with increasing PMA concentrations, or with combined 3 nM PMA and increasing concentrations of EGCG for 18 h. Cell lysates from the adherent PMA-differentiated cells increasingly expressed COX-2 (Fig. 4a, upper panel), while MMP-9 secreted into the conditioned media also dose-dependently increased as assessed by gelatin zymography (Fig. 4a, lower panel). Total RNA was isolated from the above-mentioned conditions and qRT-PCR performed to confirm that both MMP-9 and COX-2 transcriptional regulation (Fig. 4b) paralleled that of their respective protein expression. When increasing concentrations of EGCG were added during PMA-induced HL-60 differentiation, we found in accordance with the gene array data that both MMP-9 and COX-2 PMA-mediated induction was reversed (Fig. 4c). Interestingly, EGCG had no effect on neither MMP-9 (Fig. 4d) nor COX-2 (Fig. 4e) basal levels. Given the paralleled effects on MMP-9 gene expression and MMP-9 gelatinolytic activity, one can safely rule out the possible contribution of TIMP-1 on MMP-9. Further, quantification of MMP-9 protein levels, through ELISA measures, may be required to strengthen and complement our enzymatic and gene expression assessment.

Differential efficacy of EGCG to inhibit NF-κB gene targets between PMA-mediated HL-60 cell differentiation into macrophages and differentiated macrophages

We next sought at investigating the effect of EGCG on terminally differentiated macrophage-like cells. We treated HL-60 cells with PMA for 18 h. Media containing PMA was removed and adherent terminally differentiated cells further cultured for 24 h in serum-free media containing or

Table 1 PMA triggers transcriptional increase in NF-κB gene targets in adherent HL-60 cells

Gene name	Gene ID	Induction by PMA (*x*-fold)	Functional grouping
IL1RN	3557	556	Cytokines/chemokines
CCL5	6352	352	Differentiation, cytokines/chemokines
IL1B	3553	200	Inflammation, cytokines/chemokines
ICAM1	3383	176	Immune response
MMP9	*4318*	*137*	*Differentiation, apoptosis*
IL1A	3552	102	Inflammation, cytokines/chemokines
IL2RA	3559	71	Inflammation
INS	3630	40	Inflammation, apoptosis
CD40	958	27	Immune response
IL8	3576	24	Cytokines/chemokines
F3	2152	23	Anti-apoptosis
PDGFB	5155	22	Stress response
TNSF10	8743	20	Cytokines/chemokines
CD83	9308	15	Immune response
CCL2	6347	14	Immune response, cytokines/chemokines
MITF	4286	12	Differentiation, apoptosis
BIRC3	330	11	Apoptosis
COX2	*5743*	*10*	*Inflammation, apoptosis*
TNFRSF1B	7133	9.7	Apoptosis

Serum-starved HL-60 cells were treated with 3 nM PMA for 18 h. Total RNA was isolated from vehicle-treated cells that remained in suspension and from PMA-treated cells that adhered to the flasks (macrophage-differentiated cells). The identity of only those genes that were induced by tenfold in adherent versus suspension cells is shown and is extracted from Fig. 2a. Data are representative from two independent arrays. Italicized data were further confirmed at the protein and/or activity level

not 30 μM EGCG. Gene expression levels of key NF-κB targets were compared between adherent terminally differentiated cells and adherent PMA-induced differentiation cells using gene arrays. We found that similar transcriptional profiling characterized both cell populations with a correlation of more than 0.9 (Fig. 5a). When adherent terminally differentiated cells were then treated with EGCG, we found that only 23 % of the NF-κB gene targets were inhibited by more than 70 % (Fig. 5b, black bars) as compared to PMA-induced differentiation cells (Fig. 5b, dotted line). Interestingly, while COX-2 gene expression was still inhibitable by EGCG, that of MMP-9 was found insensitive to EGCG (Fig. 5b, arrows).

EGCG antagonizes COX-2 but not MMP-9 expression induced upon PMA-mediated HL-60 cell differentiation into macrophages

In order to further validate the gene array data obtained previously, immunoblotting and qPCR were performed to assess the impact of EGCG on COX-2 and MMP-9 protein (Fig. 6a) and gene (Fig. 6b) expression in terminally differentiated HL-60 macrophages. While EGCG efficiently inhibited COX-2 protein (Fig. 6a, upper panel) and gene expression (Fig. 6b, open circles), lack of MMP-9 inhibition by EGCG was observed in terminally differentiated cells both at the protein (Fig. 6a, lower panel) and transcriptional level (Fig. 6b, closed circle).

The 67-kDa Laminin Receptor expression is decreased in terminally differentiated HL-60 macrophages

The 67-kDa non-integrin Laminin Receptor (LR) has recently been identified as a direct cell surface receptor for EGCG [27]. We therefore tested the expression of both LR subunits during PMA-induced differentiation as well as in terminally differentiated HL-60 macrophages. We found that the 67-kDa LR expression remained unaffected, as compared to expression in non-differentiated HL-60 cells (Susp.) during PMA treatment regardless of EGCG (Fig. 7a, closed circles). In contrast, EGCG efficiently inhibited the induction of the 37-kDa LR precursor (Fig. 7a, open circle). When PMA alone was first used to terminally differentiate HL-60 cells into macrophages, the expression of both the 37- and 67-kDa LR subunits was significantly reduced (Fig. 7b).

Discussion

Molecular evidences that demonstrate the various functions of NF-κB during different tumor stages and that supports the rationale to target NF-κB in cancer prevention and therapy have recently been provided [28]. Accordingly, pharmacological targeting of NF-κB-regulated downstream gene products may reasonably be envisioned in the inhibition of inflammatory [1, 29–31] and carcinogenic

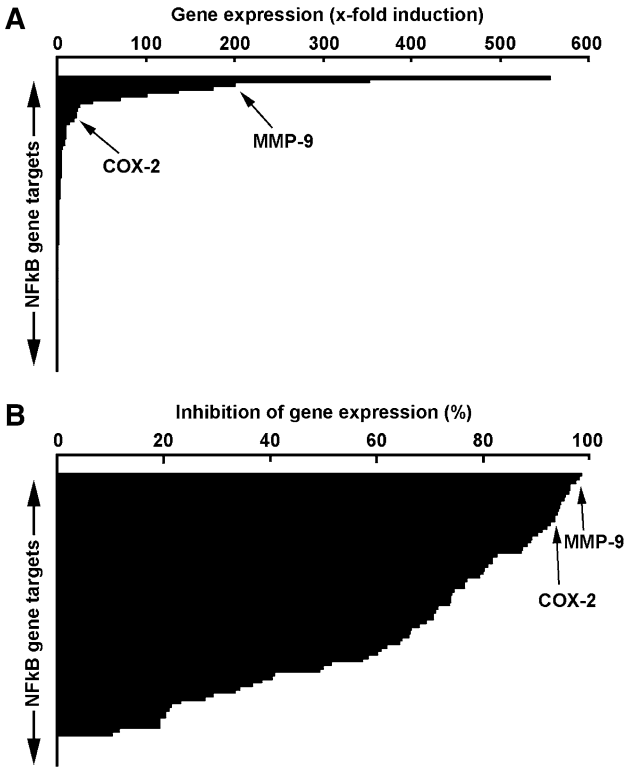

Fig. 3 Gene array analysis reveals NF-κB gene targets associated with macrophage differentiation and high inhibitory potential of EGCG. **a** Serum-starved HL-60 cells were treated with 3 nM PMA for 18 h. Total RNA was isolated from vehicle-treated cells that remained in suspension and from PMA-treated cells that adhered to the flasks (macrophage-differentiated cells). **a** Histogram representation of the 89 NF-κB gene targets array expression levels. **b** Serum-starved HL-60 cells were treated with either 30 μM EGCG or a combination of 3 nM PMA and 30 μM EGCG for 18 h. Total RNA was isolated from EGCG-treated cells that remained in suspension and from PMA-treated cells that adhered to the flasks (macrophage-differentiated cells). Histogram representation of the gene expression array levels represents the percent of gene inhibition

processes [5, 32, 33]. Recent findings in the anti-inflammatory activity of plant and diet-derived compounds, demonstrate that most of them belong to the chemical group of alkaloids, coumarins, flavonoids, polyphenols, and terpenoids [32]. Although flavonoids have been used in inflammatory pathways targeting [34, 35] and that evidence from cancer genetics and cancer genome studies supports the involvement of NF-κB in human cancer, particularly in multiple myeloma, the therapeutic potential and benefit of targeting NF-κB still remain debatable [36], partly because of the heterogeneous cellular composition and status in cell differentiation within the tumor microenvironment. As such, the recruitment and infiltration of macrophages in the tumor microenvironment activates them to support the malignant progression of cancer cells, and administration of either NF-κB-targeting drugs or COX-2 inhibitors was shown to block both inflammatory angiogenesis and tumor angiogenesis [37].

Table 2 EGCG reverses PMA-mediated IκB degradation in adherent HL-60 cells

Gene name	Gene ID	EGCG inhibition (%)	Functional grouping
MMP9	4318	99	Differentiation, apoptosis
CCR5	1234	98	Inflammation
IL1RN	3557	97	Cytokines/chemokines
CD40	958	96	Immune response
VCAM1	7412	96	Differentiation
CXCL1	2919	96	Cytokines/chemokines
CCL5	6352	95	Cytokines/chemokines, development
FASLG	356	95	Apoptosis
IL1B	3553	95	Inflammation, cytokines/chemokines,
COX2	*5743*	*95*	*Inflammation, apoptosis*
CXCL2	2920	94	Cytokines/chemokines
IL1R2	7850	94	Immune response
TNF	7124	94	Inflammation, development, stress response
CXCL10	3627	94	Inflammation
MITF	4286	93	Differentiation, apoptosis
PDGFB	5155	92	Stress response
IL2RA	3559	91	Inflammation
CCL2	6347	90	Immune response, cytokines/ chemokines
CXCL9	4283	90	Inflammation

Serum-starved HL-60 cells were treated with either 30 μM EGCG or a combination of 3 nM PMA and 30 μM EGCG for 18 h. Total RNA was isolated from EGCG-treated cells that remained in suspension and from PMA-treated cells that adhered to the flasks (macrophage-differentiated cells). The identity of only those genes which expression was inhibited by 90 % and more is shown and is extracted from Fig. 2b. Data are representative from two independent arrays. Italized data were further confirmed at the protein and/or activity level

In this study, we analyzed the transcriptional regulation of 89 NF-κB-regulated genes by assessing their expression in PMA-mediated signaling and in terminally differentiated HL-60 macrophages. The respective gene expression profiles were, not surprisingly, found induced within the cell differentiation, immune response, and inflammation functions (Table 1) and were relatively similar between PMA-treated and terminally differentiated cell populations ($r^2 = 0.91$; Fig. 4a). In contrast, high EGCG inhibitory activity was found in PMA-mediated pro-carcinogenic stimulation of HL-60 cells (Fig. 3b), while significantly lower inhibitory capacity was attributed to such flavonoid in terminally differentiated macrophages (Fig. 5b). Altogether, this supports the chemopreventive, rather than therapeutic, efficacy of EGCG and provides rational for its crucial role as a signal transduction inhibitor. Moreover, we show that the macrophagic/monocytic differentiation status significantly influences EGCG's ability to inhibit PMA-induced downstream NF-κB gene targets. The latter may, in

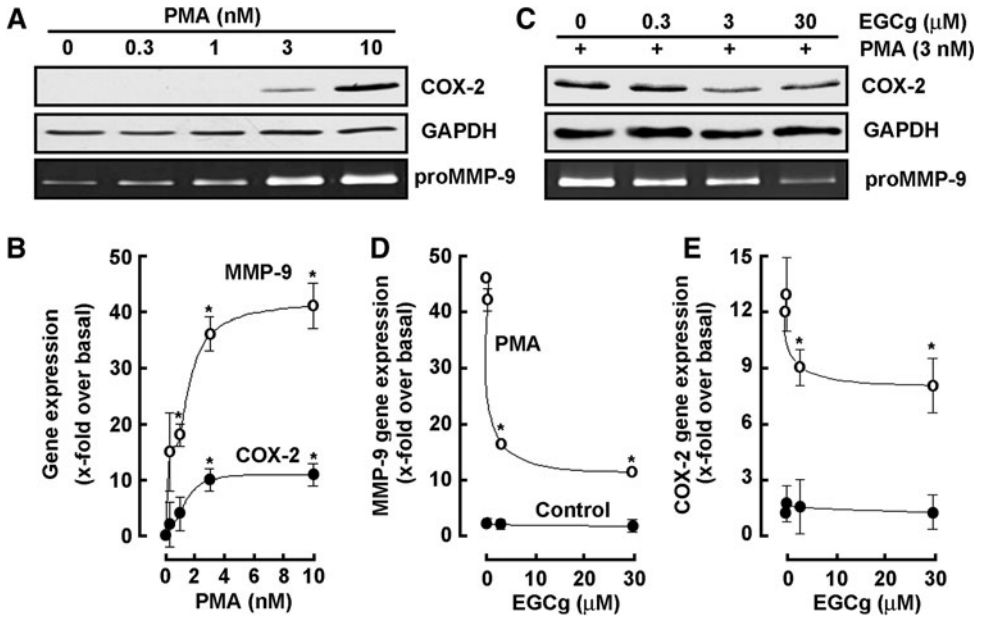

Fig. 4 EGCG antagonizes COX-2 and MMP-9 biomarkers expression induced upon PMA-mediated HL-60 cell differentiation into macrophages. **a**, **b** Serum-starved HL-60 cells were treated with increasing PMA concentrations, or **c**, **d**, **e** with combined 3 nM PMA and increasing concentrations of EGCG for 18 h. Cells that remained in suspension were discarded and lysates from the adherent macrophage-differentiated cells isolated, electrophoresed via SDS-PAGE and immunodetection of COX-2 (**a**, **c**, *upper panels*) and GAPDH (**a**, **c**, *middle panels*) proteins performed as described in the "Materials and methods" section. Conditioned media were collected and gelatin zymography (**a**, **c**, *lower panels*) performed as described in the "Materials and methods" section. Total RNA was isolated from similar conditions than in **a** and **b**, and qRT-PCR performed as described in the "Materials and methods" section to assess MMP-9 and COX-2 gene expression (**b**, **d**, **e**)

part, be explained by the downregulation of the 67-kDa LR expression, which is considered as the EGCG receptor [27]. Although significantly less efficient in terminally differentiated macrophages, the fact that EGCG is still able to abrogate the expression of some NF-κB gene targets is suggestive of pharmacological effects independent of the 67-kDa LR expression as reported elsewhere [38].

The differential targeting efficacy of EGCG is further demonstrated in our study in relation to the NF-κB downstream gene targets COX-2 and MMP-9, both considered as inflammation biomarkers [35, 39]. While COX-2 protein and gene expression is inhibited both during PMA treatment and in terminally differentiated macrophages, we show that MMP-9 in contrast can no longer be inhibited in those cells that acquired macrophagic phenotype. Given the lack of MMP-9 inhibition by EGCG in terminally differentiated macrophages (Fig. 6), our data therefore imply that EGCG may not be efficient in inhibiting the ability of macrophages to disrupt the BBB and to infiltrate the brain. While human brain microvascular endothelial cells (HBMEC) play an essential role as structural and functional components of the BBB, its disruption by the macrophage-secreted MMP-9 may therefore favor secondary cerebral infections and development of brain pathologies [40, 41]. Moreover, although some studies report pharmacological effects of EGCG at submicromolar levels, most experiments require concentrations of above 10–20 μM to demonstrate that effect. Given that in humans, tea polyphenols undergo glucuronidation, sulfation, methylation, and ring fission, the peak plasma concentration of EGCG is believed to approximate 1 μM [42]. Accordingly, IC_{50} effects in our current study were reached at ~ 3 μM or less in accordance with those plasma concentrations reported, while optimal effects were achieved at 30 μM in accordance with in vitro data reported above (i.e., 10–20 μM). Finally, unmetabolized EGCG was directly tested within an acellular system and on the activity of recombinant MMPs [43]. The authors reported an IC_{50} value for EGCG of 0.8 μM, again nicely approximating that EGCG concentration we report in this current study. Given the very close IC_{50} values calculated between cellular and acellular in vitro models, one may therefore safely consider that the impact of EGCG we evaluated may well originate from the parental non-metabolized molecule.

Only few reports documented an association between COX-2 expression and ECM degradation consequent to procarcinogenic stimulation. Among the intracellular events that could link PMA-induced signaling to COX-2 induction, NF-κB can contribute to regulate the expression of COX-2 through endoplasmic reticulum stress and, in part, through induction of the endoplasmic reticulum chaperone GRP78/BiP, which is expressed at high levels in a variety of tumors

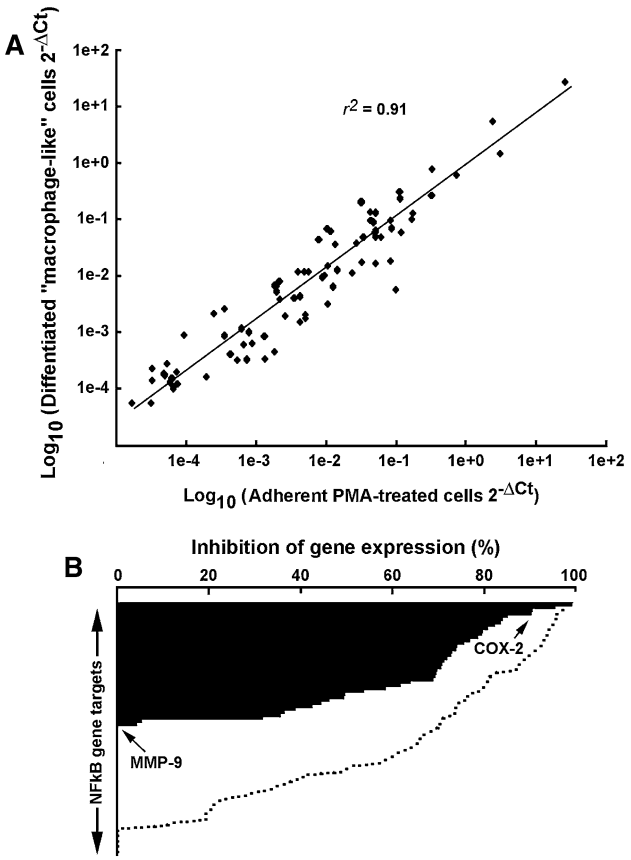

Fig. 5 Differential efficacy of EGCG to inhibit NF-κB gene targets between PMA-mediated HL-60 cell differentiation into macrophages and differentiated macrophages. **a** Total RNA was isolated from adherent cells obtained upon 3 nM PMA treatment of serum-starved HL-60 cells, and gene expression profiles compared with total RNA isolated from PMA-differentiated cells cultured for 2 days. **b** EGCG inhibitory effect was evaluated from total RNA isolated from adherent cells obtained upon 3 nM PMA treatment of serum-starved HL-60 cells (*dotted lines*, data from Fig. 3b), and gene expression profiles compared with total RNA isolated from PMA-differentiated cells cultured for 2 days and then serum-starved in the presence of 30 μM EGCG

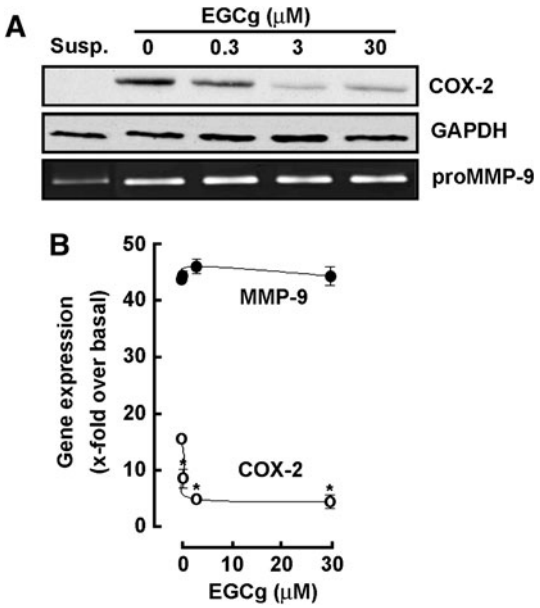

Fig. 6 EGCG antagonizes COX-2 but not MMP-9 expression induced upon PMA-mediated HL-60 cell differentiation into macrophages. Serum-starved HL-60 cells were treated for 18 h with 3 nM PMA to induce HL-60 cell differentiation and adhesion. Adherent cells were then further cultured in serum-free conditions and in the presence of increasing concentrations of EGCG for 18 h. **a** Cell lysates were electrophoresed via SDS-PAGE and immunodetection of COX-2 (*upper panels*) and GAPDH (*middle panels*) proteins performed as described in the "Materials and methods" section. Conditioned media were collected and gelatin zymography (*lower panels*) performed as described in the "Materials and methods" section. **b** Total RNA was isolated from similar conditions as described earlier, and qRT-PCR performed as described in the "Materials and methods" section to assess MMP-9 and COX-2 gene expression. Susp, untreated and non-differentiated HL-60 cells growing in suspension

and which confers drug resistance to both proliferating and dormant cancer cells [44]. As for the transcriptional control of MMP-9 expression upon PMA stimulation, there is increasing evidence that its expression can also be regulated at the levels of mRNA stability, translation, and protein secretion. The ability to modulate MMP-9 expression at multiple steps through distinct signaling pathways may be particularly important during malignant conversion, when tumor cells need to induce or maintain MMP-9 levels in response to changing environmental cues. Among the nuclear factors shown to stabilize the mRNA and augment the expression of MMP-9, the RNA-binding protein HuR has been ascribed a pivotal role in the development of tumors [20, 45], and increased nucleocytoplasmic shuttling was also reported to promote COX-2 mRNA stabilization [46]. More importantly, HuR has been found to be a key mediator in PMA-treated HL-60 cells where inhibition of HuR expression by EGCG was reported [24].

In recent years, a large number of mechanisms of action have been attributed to flavonoids commonly found in fruits, vegetables, wine, or tea as they can act as potent antioxidants and free radical scavengers [47, 48]. Flavonoids targeting of NF-κB has also been shown to inhibit in vitro brain endothelial cell tubulogenesis [49]. Accordingly, among strategies developed to jointly inhibit ECM degradation and inflammation processes in carcinogenesis, the design, synthesis and evaluation of flavonoid derivatives has not surprisingly emerged as a potent strategy to also target neurodegenerative disorders including different forms of dementia, as well as Alzheimer's disease [50, 51]. Our data support the chemopreventive potential of such a class of molecules, but prompt for caution when interpreting the data particularly regarding the differentiation status of a given in vitro experimental cell model. Finally, our data support a crucial role for MMP-9 and provide a

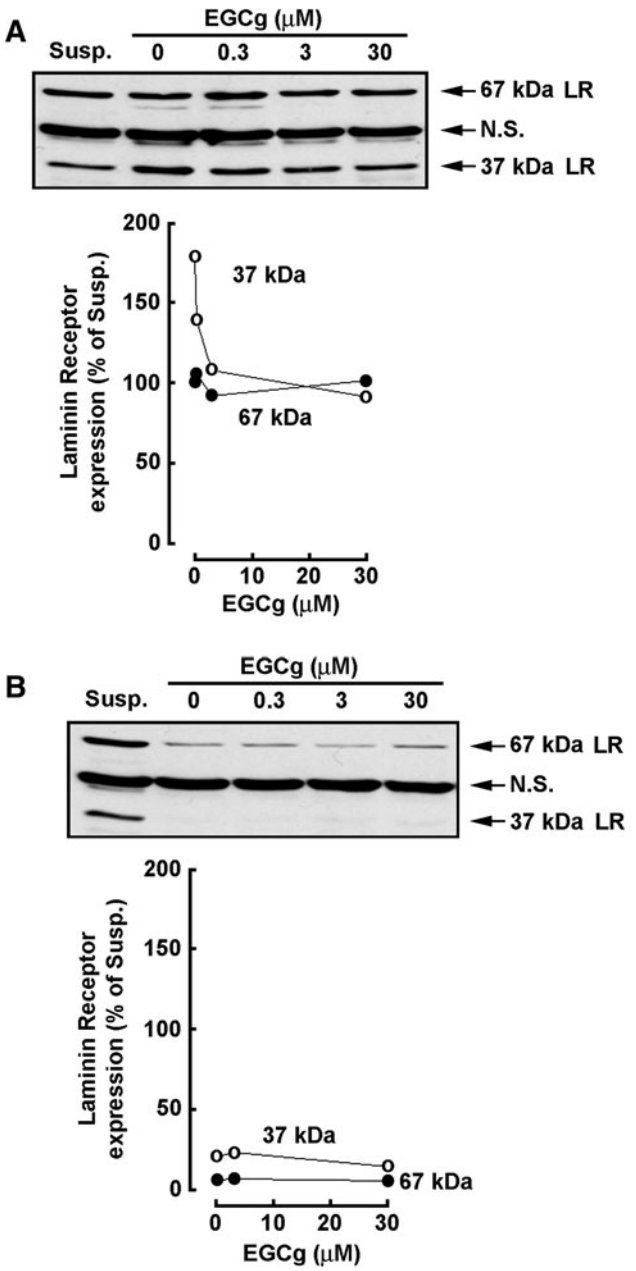

Fig. 7 The 67-kDa Laminin Receptor expression is decreased in terminally differentiated HL-60 macrophages. **a** Serum-starved HL-60 cells were treated with 3 nM PMA in the presence of increasing concentrations of EGCG to induce HL-60 cell adhesion for 18 h. **b** PMA alone was first used to terminally differentiate HL-60 cells into macrophages. Adherent cells were afterward further cultured in serum-free conditions and in the presence of increasing concentrations of EGCG for 18 h. Cell lysates from **a** and **b** were electrophoresed via SDS-PAGE and immunodetection of the 37-kDa (*open circles*)/67-kDa (closed circles) Laminin Receptor (LR) proteins performed as described in the "Materials and methods" section. Data are representative of 2 independent experiments. *NS* nonspecific immunoreactivity, *Susp* untreated and non-differentiated HL-60 cells growing in suspension

molecular rational explaining how the disruption of the BBB [52, 53], and subsequent transendothelial migration of terminally differentiated HL-60 macrophages [54], may lead to cerebral infections and development of brain pathologies.

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