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Green tea polyphenol epigallocatechin-3-gallate suppresses melanoma growth by inhibiting inflammasome and IL-1β secretion

Lixia Z. Ellis [a], Weimin Liu [a], Yuchun Luo [a], Miyako Okamoto [a], Dovina Qu [a], Jeffrey H. Dunn [a], Mayumi Fujita [a,b,*]

[a] Department of Dermatology, University of Colorado School of Medicine, Aurora, CO 80045, USA
[b] Denver Veterans Affairs Medical Center, Denver, CO 80220, USA

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ABSTRACT

Epigallocatechin-3-gallate (EGCG), the major polyphenolic component of green tea, has been demonstrated to possess anti-inflammatory, antioxidant, anti-mutagenic and anti-carcinogenic properties. The anti-melanoma effect of EGCG has been previously suggested, but no clear mechanism of action has been established. In this study, we demonstrated that EGCG inhibits melanoma cell growth at physiological doses (0.1–1 μM). In the search for mechanisms of EGCG-mediated melanoma cell suppression, we found that NF-κB was inhibited, and that reduced NF-κB activity was associated with decreased IL-1β secretion from melanoma cells. Since inflammasomes are involved in IL-1β secretion, we investigated whether IL-1β suppression was mediated by inflammasomes, and found that EGCG treatment led to downregulation of the inflammasome component, NLRP1, and reduced caspase-1 activation. Furthermore, silencing the expression of *NLRP1* abolished EGCG-induced inhibition of tumor cell proliferation both *in vitro* and *in vivo*, suggesting a key role of inflammasomes in EGCG efficacy. This paper provides a novel mechanism for EGCG-induced melanoma inhibition: inflammasome downregulation → decreased IL-1β secretion → decreased NF-κB activities → decreased cell growth. In addition, it suggests inflammasomes and IL-1β could be potential targets for future melanoma therapeutics.

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1. Introduction

Melanoma is the most deadly type of skin cancer and its prevalence continues to rise [1], making it crucial to search for effective therapeutics. It is increasingly recognized that cancer, including melanoma, represents an inflammatory condition [2]. Human melanoma cells secrete many inflammatory cytokines and chemokines that are associated with cancer invasiveness and aggressiveness [3]. Therefore, targeting inflammation may provide us with a new line of therapeutics for melanoma. Among the many inflammatory mediators involved in melanoma development and progression, IL-1β has been shown to be one of the critical cytokines mediating tumor growth, progression, immunosuppression and chemoresistance [4,5]. In metastatic melanoma cells, IL-1β is constitutively secreted and activated, mediating macrophage chemotaxis, angiogenesis, and sustained melanoma growth [6]. IL-1β is synthesized as an inactive precursor (pro-IL-1β) which is cleaved to the biologically active and secreted form by caspase-1 [7]. Caspase-1, in turn, is regulated by a multi-protein complex termed the inflammasome. The inflammasomes are responsible for the recruitment and activation of caspase-1 [8]. Each inflammasome complex consists of a nucleotide oligomerization domain-like receptor (NLR) component, an ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)) component, and caspase-1. Assembly of the inflammasomes is triggered by pathogen or danger-associated molecular patterns, which cause oligomerization of NLRs. Oligomerized NLRs interact with ASC, which in turn cleaves pro-caspase-1 into active caspase-1 [9]. NLRP (NACHT-, LRR- and pyrin-domain-containing protein) 1 and NLRP3 inflammasomes are two of the best characterized human inflammasomes. NLRP1 contains a CARD domain that can directly interact with inflammatory caspases-1, and thus ASC may not be required for NLRP1-mediated caspase-1 activation [10]. In melanoma cells, NLRP3 inflammasome is constitutively assembled and activated, suggesting its importance in tumorigenesis [6].

(−)-Epigallocatechin-3-gallate (EGCG) is the major polyphenol component of green tea responsible for its biological effects. It has been shown to inhibit various inflammatory enzymes and cytokines, including iNOS, COX2, MMPs, IL-6, IL-8, IL-12 and TNFα, all of which are induced by secreted active IL-1β [11–13]. It has

Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; CARD, caspase recruitment domain; EGCG, epigallocatechin-3-gallate; IL, interleukin; NLR, NOD-like receptor; NLRP, NACHT-, LRR- and pyrin-domain-containing protein; NOD, nucleotide oligomerization domain; shRNA, short hairpin RNA.

* Corresponding author at: Department of Dermatology, University of Colorado School of Medicine, Mail Stop #8127, 12801 E 17th Ave., Room 4124, Aurora, CO 80045, USA. Fax: +1 303 724 4048.
E-mail address: mayumi.fujita@ucdenver.edu (M. Fujita).

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also been suggested that EGCG has an anti-tumor effect in melanoma cells, but previous reports have utilized doses of EGCG (20–100 μM) much higher than the physiological concentrations [14,15]. Consuming 5–6 cups of green tea a day leads to a serum concentration of EGCG of only about 1 μM [16], and drinking up to 8–16 cups of green tea a day is known to be safe [17].

In this study we investigated whether physiological concentrations of EGCG (0.1–1 μM) can inhibit melanoma cells. We present evidence that physiological doses of EGCG have an inhibitory effect on the proliferation of human metastatic melanoma cell lines. Furthermore, we demonstrated that EGCG suppresses NF-κB activity and reduces IL-1β secretion. The decreased IL-1β is associated with downregulation of NLRP1, a component of the inflammasomes, and reduced caspase-1 activation. The inhibitory effect of EGCG on tumor proliferation was abolished by silencing *NLRP1*, suggesting a key role of inflammasomes in the tumor-inhibitory effect of EGCG in human melanoma cells.

2. Materials and methods

2.1. Cell culture and EGCG treatment

Human metastatic melanoma cell lines 1205Lu and HS294T were obtained from the American Type Culture Collection. 1205Lu cells were maintained in RPMI 1640 medium (Mediatech, Inc.), and HS294T cells in Dulbecco's modified Eagle's medium (Mediatech, Inc.). Both media were supplemented with 10% fetal bovine serum (FBS, Mediatech, Inc.), 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine. Cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere.

EGCG (⩾95% purity, Sigma–Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 100 mM (45.837 mg/ml). EGCG was then further diluted in phosphate-buffered saline (PBS) to varying concentrations (0.1, 1, or 10 μM, which are 0.046, 0.46 and 4.6 μg/ml, respectively) prior to use in cell culture. Due to the short half-life of EGCG *in vitro*, EGCG was added every 8–10 h. DMSO diluted in PBS (0.01% DMSO, corresponding to the amount contained in 10 μM EGCG) was used for vehicle control.

2.2. Cell viability assay

About 4×10^3–5×10^3 cells/well were plated in flat-bottom 96-well plates and treatment with EGCG (0.1, 1, or 10 μM) was started 4 h after plating. Cells were incubated for up to 96 h. Cell viability was determined by the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega).

2.3. Lactate dehydrogenase (LDH) cytotoxicity assay

2.5×10^4 Cells/well were plated in 24-well plates and treatment with EGCG (0.1, 1, or 10 μM) was started 4 h after plating. Cells were incubated for up to 96 h. Supernatants and lysates were then transferred to 96-well plates to complete the assay. Cytotoxicity was determined using the Cytotox96® Non-Radioactive Cytotoxicity Assay (Promega). Cytotoxicity was calculated as follows: %cytotoxicity = 100% × (experimental LDH − spontaneous LDH)/(maximum LDH − spontaneous LDH).

2.4. NF-κB activity determination

2.5×10^4 Cells/well were plated in 24-well plates. After an overnight incubation, cells were transfected with a control vector, pMetLuc2-Reporter (Clontech) or an NF-κB vector, pNFκB-MetLuc2 Reporter (Clontech) in Opti-MEM® (Invitrogen). After 4 h, medium was changed to supplemented RPMI 1640, and cells were treated with EGCG (0.1, 1, or 10 μM). EGCG was added every 8 h. After 24 h, supernatants were collected and luciferase activity was measured using the Ready-to-Glow™ Secreted Luciferase Reporter Assay (Clontech).

2.4.1. IL-1β and IL-18 production and secretion determinations

1.2×10^4 Cells/well were plated in 48-well plates. After an overnight incubation, medium was changed to Opti-MEM® (Invitrogen), and EGCG (0.1, 1, or 10 μM) was added every 8 h. After 24 h, supernatants were collected to assess secreted IL-1β. To assess synthesized IL-1β, cells were lysed with 0.5% Triton X-100 in PBS and subjected to a freeze–thaw cycle.

Supernatants and cell lysates were analyzed using the Human IL-1β/IL-1F2 DuoSet ELISA Development System (R&D Systems) and Human IL-18 ELISA Kit (MBL International Corporation).

2.4.2. Western blotting analysis

Cells were incubated with EGCG (1 μM) for 24 h. EGCG was added every 8 h. Supernatants were collected for analysis of secreted protein. Cells were lysed on ice in 1X SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) supplemented with protease inhibitor mixture (Roche Applied Science). Lysates were heated to 95 °C for 5 min. About 20 μg lysates were then loaded into SDS–PAGE gel (Invitrogen), and proteins were separated by electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes (0.4 μm) in 25 mM Tris, 192 mM glycine, and 20% methanol at 60 V for 1.5 h. Blots were incubated with primary antibodies at 4 °C overnight, followed by incubation with secondary antibodies. Blots were then developed with horseradish peroxidase substrate (West Femto Solution from Thermal Fisher Scientific, Inc.) and analyzed using Gel-Doc 200 (Bio-Rad).

2.4.3. Short hairpin RNA (shRNA) transfection

1205Lu cells were transfected with shRNA Lentiviral Particles against control and *NLRP1* (Santa Cruz Biotechnology, Inc.) in supplemented RPMI 1640 containing 5 μg/ml Polybrene (Santa Cruz Biotechnology, Inc.). Following overnight transduction, cells were incubated in supplemented RPMI 1640 with 1 μg/ml puromycin to select for stable clones expressing transduced shRNA. Transfected 1205Lu cells were maintained in supplemented RPMI 1640 with 1 μg/ml puromycin at 37 °C.

2.4.4. In vivo study

Six-week-old female athymic nu/nu mice were obtained from the National Cancer Institute. Animals were kept under specific pathogen-free conditions, according to National Institutes of Health Animal Care Guidelines. Experimental protocols were approved by the Institutional Animal Care and Use Committee of University of Colorado Denver. 1205Lu cells transfected with control shRNA or *NLRP1* shRNA were re-suspended in Matrigel (BD Biosciences) 1:1 diluted with PBS. Mice were injected subcutaneously with the melanoma cells, with two implantations (1×10^6 cells/implantation) in each mouse. Tumors were allowed to grow to a size of 100 mm^3, at which time mice were randomized into two groups and treated with vehicle (10% DMSO in saline) or EGCG (20 mg/kg dissolved in 10% DMSO in saline) intraperitoneally daily for 17 days. Tumor size was evaluated three times a week by caliper measurements using the following formula: tumor volume = (longest diameter) × (shortest diameter)^2/2. Relative tumor growth was calculated by tumor volume of treated mice divided by tumor volume at the initiation of therapy.

2.4.5. Statistics

Statistically significant differences were determined using Student's unpaired *t*-test. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. EGCG inhibits proliferation of human metastatic melanoma cells in vitro

Two different human metastatic melanoma cell lines, 1205Lu and HS294T cells, were treated with varying concentrations of EGCG (0.1, 1, or 10 μM) for 24, 48, 72, and 96 h, followed by cell viability/proliferation analysis. In both 1205Lu (Fig. 1A) and HS294T (Fig. 1B) cells, cell proliferation was significantly inhibited by increasing concentrations of EGCG in a dose-dependent manner ($^*p < 0.05$ relative to control, $*$ denotes every value below it is significantly different from control). In addition, LDH levels were not significantly different from control following treatment with different concentrations of EGCG (data not shown), suggesting that EGCG suppresses melanoma cell growth *in vitro* via a mechanism other than cell death.

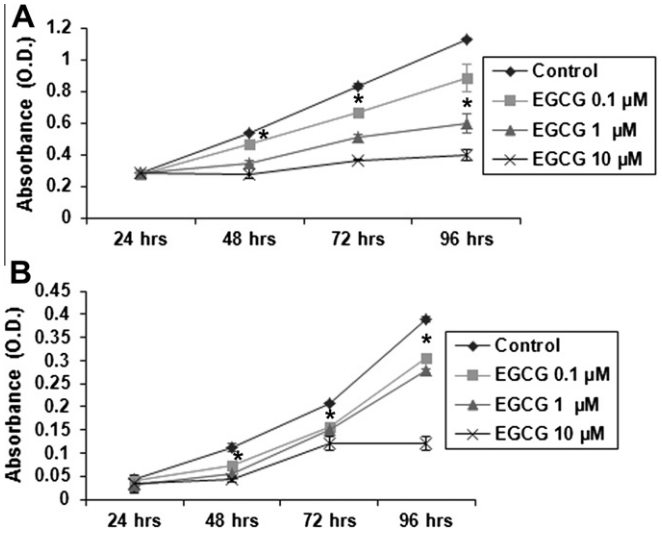

Fig. 1. The effects of EGCG on human metastatic melanoma cell proliferation *in vitro*. 1205Lu (A) and HS294T (B) cells were treated with varying doses of EGCG for up to 96 h and cell viability was determined. Data are presented as mean ± SEM ($n = 3$) and the data shown are representative of two separate experiments. $^*p < 0.05$ relative to control, * denotes every value below it is significantly different from control.

3.2. NF-κB activity is suppressed by EGCG in human metastatic melanoma cells

Since NF-κB plays a major role in melanoma cell proliferation [18], the activity of NF-κB was assessed following EGCG treatment. Both melanoma cell lines, 1205Lu cells and HS294T cells, after being transfected with pNFκB-MetLuc2 Reporter Vector, were treated with varying concentrations of EGCG (0.1, 1, or 10 μM) followed by NF-κB activity determination. In both transfected 1205Lu (Fig. 2A) and HS294T (Fig. 2B) cells, treatment with EGCG led to dose-dependent inhibition of NF-κB activity ($^*p < 0.05$).

3.3. EGCG reduces IL-1β secretion but not production, and downregulates NLRP1 inflammasome in human metastatic melanoma cells

IL-1β is constitutively secreted by metastatic melanoma cells and plays a significant role in tumor development and progression [6]. Because IL-1β signaling leads to activation of NF-κB, we determined whether EGCG can decrease the activity of IL-1β. As indicated earlier, secreted IL-1β is the active form. 1205Lu cells and HS294T cells were treated with different concentrations of EGCG (0.1, 1, 2 or 10 μM) for 24 h, and IL-1β levels were determined in both lysates and supernatants. Whereas EGCG treatment did not change the synthesis of IL-1β, it significantly decreased the secretion of IL-1β in melanoma cells (1205Lu cells represented in Fig. 3A and B, and HS294T cells in Fig. 3C and D, $^*p < 0.05$), suggesting that the secretion mechanism was altered by EGCG treatment.

As IL-1β secretion is tightly controlled by inflammasomes, we investigated inflammasome components (NLRP1, NLRP3, ASC and caspase-1) following EGCG treatment. 1205Lu cells were treated with 1 μM EGCG for 24 h, and lysates and supernatants were subjected to western blot analysis. EGCG treatment led to decreased levels of NLRP1 in both lysates and supernatants (Fig. 3E). Furthermore, this downregulation of NLRP1 was shown to be specific, as NLRP3 and ASC levels were not changed. To determine whether the decreased NLRP1 was associated with functional impairment of inflammasome, the levels of its downstream molecule, caspase-1, were determined. Full-length caspase-1 (p45) is cleaved to p10 to be active. As shown in Fig. 3E, EGCG reduced active p10 in the cell lysates and supernatants, suggesting that EGCG-mediated NLRP1 reduction was accompanied by decreased inflammasome function.

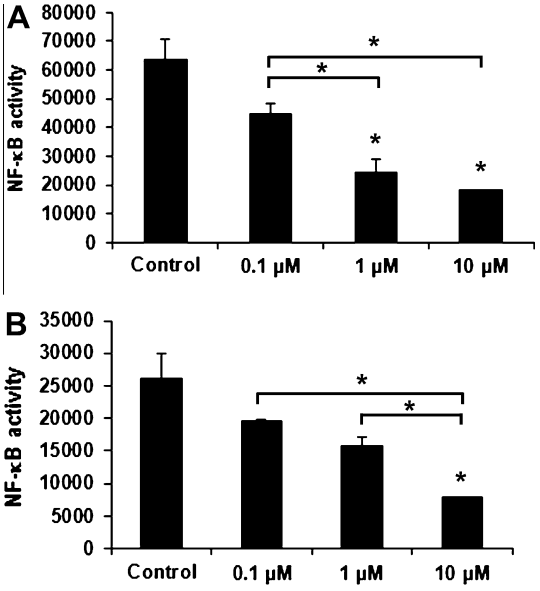

Fig. 2. The effects of EGCG on NF-κB activities in human metastatic melanoma cells *in vitro*. 1205Lu (A) and HS294T (B) cells were treated with varying doses of EGCG for 24 h and NF-κB activity was determined. Data are presented as mean ± SEM ($n = 3$) and the data shown are representative of two separate experiments. $^*p < 0.05$, * above the bars represents the difference relative to control, whereas the * above the lines represents the difference between the two bars under the lines.

Because active caspase-1 processes pro-IL-1β and pro-IL-18 to the bioactive forms, we investigated levels of synthesized and secreted IL-18 in melanoma cells in response to EGCG. Intracellular levels of IL-18 were found to be $\geqslant$100 times less than those of IL-1β in melanoma cells (1205Lu and HS294T cells), and EGCG did not change the intracellular IL-18 levels. In addition, secreted IL-18 was undetectable in these cells (data not shown), suggesting a role of inflammasome-mediated IL-1β but not IL-18 in the tumor-inhibitory effect of EGCG in human melanoma cells.

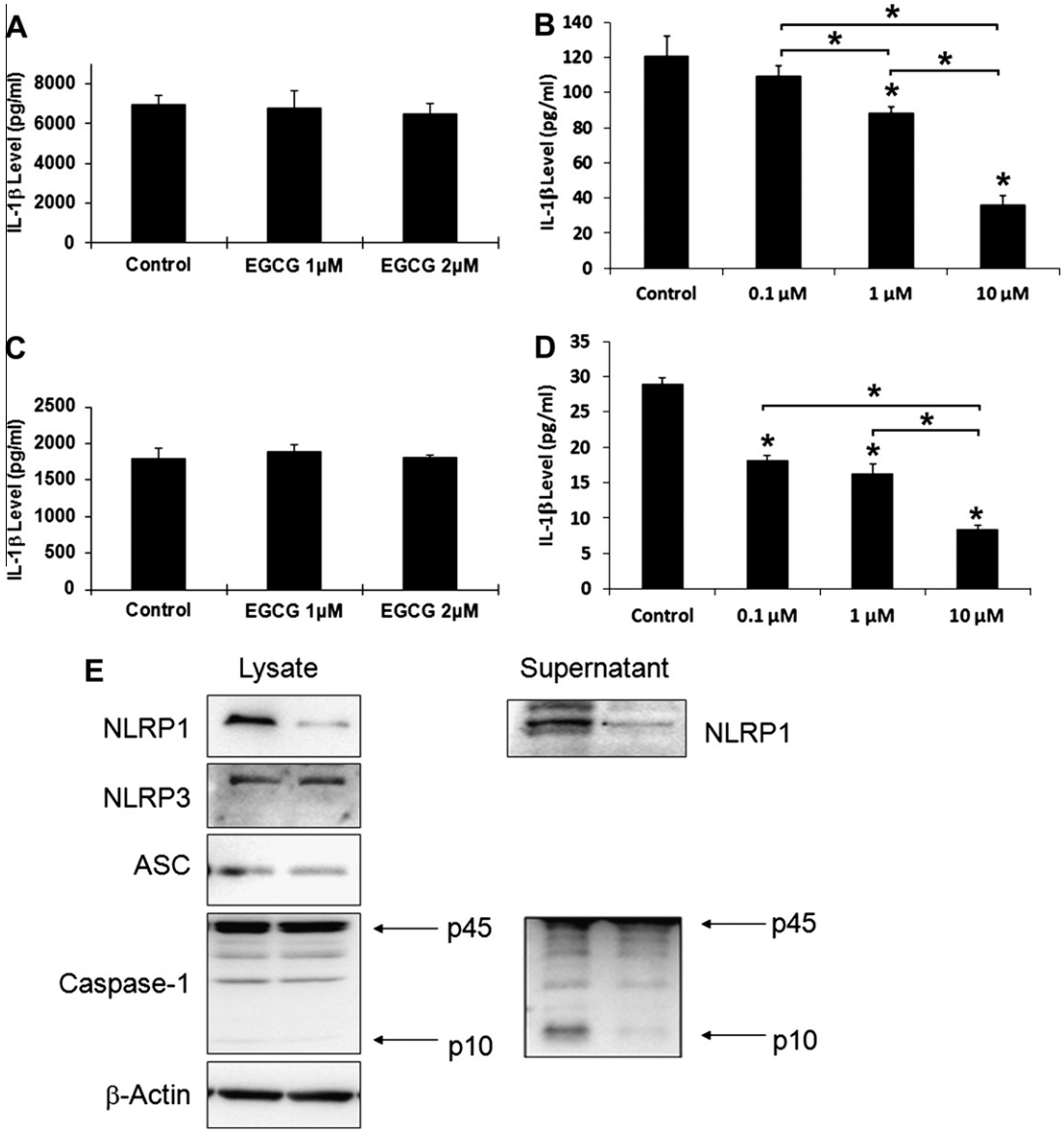

Fig. 3. The effects of EGCG on IL-1β and inflammasome components in human melanoma cells. (A–D) Effects of varying concentrations of EGCG on IL-1β levels in the lysates and supernatants of 1205Lu cells (A and B) and HS294T cells (C and D). Data are presented as mean ± SEM ($n = 3$) and the data shown are representative of two separate experiments. $^{*}p < 0.05$, * above the bars represents the difference relative to control, whereas the * above the lines represents the difference between the two bars under the lines. (E) Effects of 1 μM EGCG on the levels of NLRP1, NLRP3, ASC and caspase-1 in 1205Lu cell lysates and supernatants. Cells were treated for 24 h. The data shown are representative of two separate experiments.

3.3.1. Growth inhibition by EGCG is abolished after NLPR1 knockdown

To determine whether EGCG-induced inhibition of proliferation is due to inflammasome downregulation, 1205Lu cells were transfected with *NLRP1* shRNA to silence *NLRP1* (Fig. 4A). The transduced 1205Lu-*NLRP1*-shRNA cells no longer showed growth inhibition by EGCG, suggesting that the inhibitory effect of EGCG was inflammasome-dependent (Fig. 4B and C, $^{*}p < 0.05$ relative to control, $*$ denotes every value below it is significantly different from control).

We then determined whether the same effect can be observed *in vivo*. Nude mice were injected with 1205Lu-control-shRNA cells or 1205Lu-*NLRP1*-shRNA cells and treated with EGCG at 20 mg/kg intraperitoneally (i.p.) daily. This dose is physiologic as plasma EGCG concentration measured an hour after i.p. injection of 10 mg/kg EGCG was 138 ± 44 ng/ml (0.3 ± 0.1 μM) [19]. EGCG treatment significantly suppressed tumor growth in mice injected with 1205Lu-control-shRNA cells, but not in mice injected with 1205Lu-*NLRP1*-shRNA cells, confirming that the inhibition of tumor growth by EGCG requires inflammasomes (Fig. 4D, $^{*}p < 0.05$ relative to vehicle-shControl).

4. Discussion

Our recent work indicates that melanoma, especially metastatic melanoma, represents an autoinflammatory state characterized by constitutive activation of inflammasomes and IL-1R signaling [6]. Given the strong link between inflammation and melanoma, and the role of IL-1β in tumor growth, strategies that can inhibit IL-1β secretion would be logical candidates for melanoma therapeutics. In this study, we demonstrated that EGCG, a potent anti-inflammatory chemical, may be one of these candidate molecules. Using doses that are practical, in the 0.1–1 μM range, we found that EGCG decreases metastatic melanoma cell growth, both *in vitro* and *in vivo*. In addition, IL-1β secretion is decreased, likely

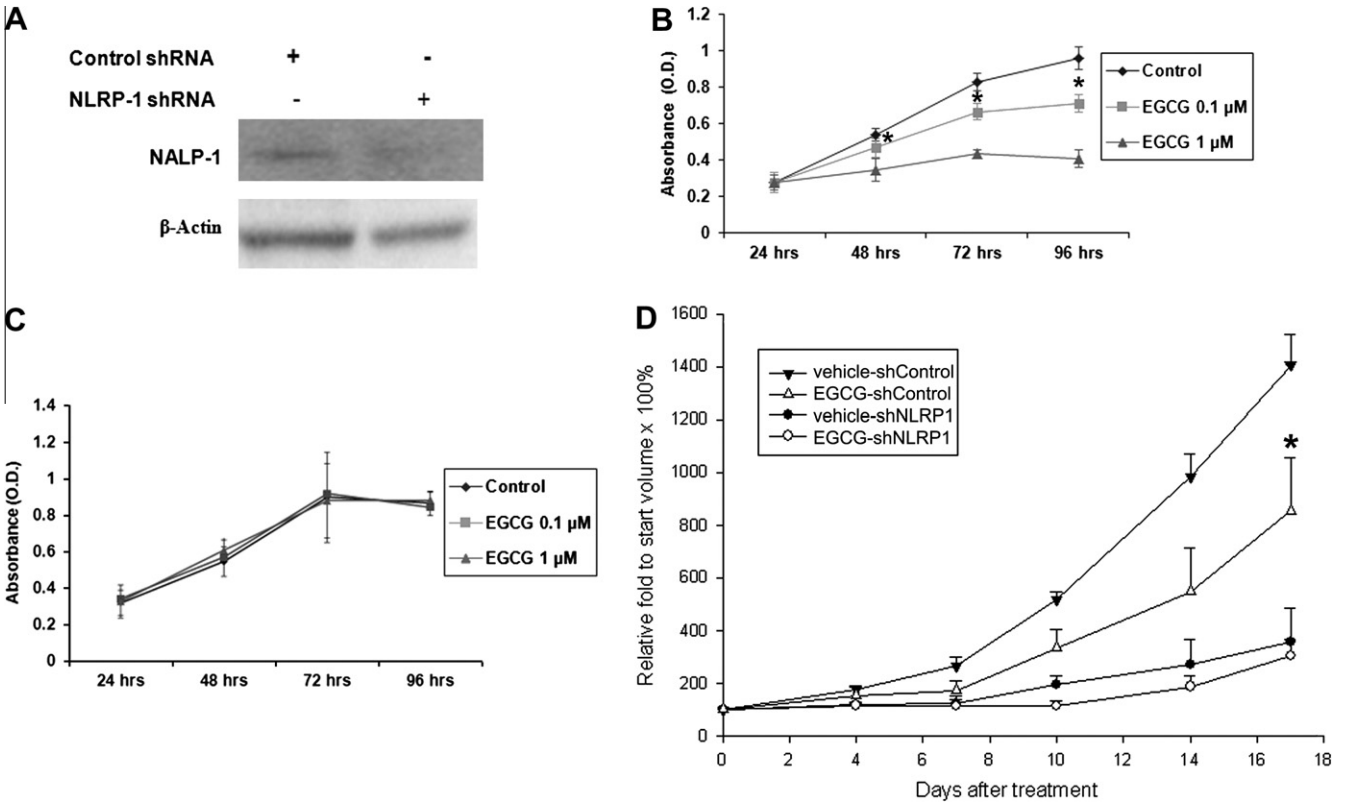

Fig. 4. The effects of EGCG on 1205Lu cells after knocking down NLRP1. (A) NLRP1 protein expression in 1205Lu cells transfected with control and *NLRP1* shRNA. (B and C) 1205Lu cells transfected with control shRNA (1205Lu-control-shRNA) (B) and *NLRP1* shRNA (1205Lu-NLRP1-*shRNA*) (C) were treated with varying doses of EGCG for up to 96 h and cell viability was determined. Data are presented as mean ± SEM ($n = 3$) and the data shown are representative of two separate experiments. $^{*}p < 0.05$ relative to control, * denotes every value below it is significantly different from control. (D) Effects of EGCG *in vivo* on xenograft tumor growth injected with 1205Lu-control-shRNA or 1205Lu-*NLRP1*-shRNA cells. Mice were treated with 20 mg/ml EGCG intraperitoneally daily. Vehicle-shControl, mice injected with 1205Lu-control-shRNA and treated with vehicle; EGCG-shControl, mice injected with 1205Lu-control-shRNA and treated with EGCG; Vehicle-shNLRP1, mice injected with 1205Lu-*NLRP1*-shRNA and treated with vehicle; EGCG-shNLRP1, mice injected with 1205Lu-*NLRP1*-shRNA and treated with EGCG. Data are presented as mean ± SEM ($n = 8$) and the data shown are representative of two separate experiments. $^{*}p < 0.05$ (1205Lu-control-shRNA mice treated with vehicle vs. 1205Lu-control-shRNA mice treated with EGCG).

due to specific downregulation of inflammasome molecule, NLRP1, and subsequent inhibition of caspase-1 activity. Furthermore, NLRP1 downregulation was demonstrated to play an important role in EGCG-induced growth inhibition since silencing *NLRP1* abolished the EGCG effects on growth. The reduced secretion of IL-1β likely contributes to inactivation of NF-κB, which then leads to decreased cell proliferation. To our knowledge, this is the first publication demonstrating the effect of EGCG on inflammasomes in cancer research. The only other report which examined the impact of EGCG on inflammasomes was a study which demonstrated preventive effects of EGCG in lupus nephritis mice via NLRP3 inhibition [20]. Our study did not demonstrate NLRP3 inhibition; this discrepancy could result from different cell types (renal cortex cells vs. melanoma cells) and different doses of EGCG (120 mg/kg vs. 20 mg/kg in our study).

The link between inflammasomes and melanoma is only recently beginning to be appreciated. In addition to above-mentioned constitutive expression of inflammasomes in melanoma, it was reported that inflammasomes in the melanoma tumor microenvironment diminishes the endogenous antitumor immune response and facilitates tumor growth [21]. These data further support inflammasome activity as a putative target for melanoma treatment.

A few other studies have also investigated the effect of EGCG in melanoma cells. Nihal and colleagues demonstrated induction of apoptosis by EGCG [22], which is in discordance with our data which showed that reduced cell viability is due to decreased cell growth but not cell death. This discrepancy could be explained by the higher concentrations of EGCG used in their study (2.2–22 μM). In addition, even though both studies used the HS294T melanoma cells, characteristics of cells may differ depending on the number of passages. Indeed, Ravindranath and colleagues recently demonstrated differential effects of green tea catechins on different melanoma cell lines [14]. To make matters more complex, EGCG has been shown to block apoptosis in other melanoma cell lines [23].

The mechanisms by which EGCG inhibits inflammasomes are unclear. Previous studies have identified a 67-kDa laminin receptor as a cell surface receptor for EGCG [24], which could play a role in the pathway leading to inflammasome downregulation. Since EGCG is a potent antioxidant [25], modulation of the redox balance in melanoma tumor cells could also potentially lead to changes in inflammasomes.

Developing EGCG into a practical therapeutic agent may require an interdisciplinary approach in order to modify the structure of EGCG and improve its potency and pharmacokinetic properties. For example, one of the methylation products, 7-OMe EGCG, was shown to have equal efficacy but increased bioavailability when compared to EGCG [26]. Given the aggressive nature of melanomas, effective therapies may most likely involve a combinational approach. In fact, EGCG has been shown to have a stronger anti-melanoma effect when used in conjunction with vitamin A [27], vorinostat [28], interferon [29], DNA vaccination [30], dacarbazine [31], and even red light [32]. This combinational treatment will likely decrease the doses required for either medication, and consequently decrease the adverse effects associated with these therapies.

In conclusion, this research not only proposes a novel treatment for melanoma, but also provides mechanistic data that can be applied to various other inflammatory and malignant disorders in which IL-1β and inflammasomes play a major role.

Acknowledgments

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