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Clinical Cancer Research

Polyphenol E Enhances the Antitumor Immune Response in Neuroblastoma by Inactivating Myeloid Suppressor Cells

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Abstract

Purpose: Neuroblastoma is a rare childhood cancer whose high risk, metastatic form has a dismal outcome in spite of aggressive therapeutic interventions. The toxicity of drug treatments is a major problem in this pediatric setting. In this study, we investigated whether Polyphenon E, a clinical grade mixture of green tea catechins under evaluation in multiple clinical cancer trials run by the National Cancer Institute (Bethesda, MD), has anticancer activity in mouse models of neuroblastoma.

Experimental Design: We used three neuroblastoma models: (i) transgenic TH-MYCN mouse developing spontaneous neuroblastomas; (ii) nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice xenotransplanted with human SHSY5Y cells; and (iii) A/J mice transplanted with syngeneic Neuro 2A cells. Mice were randomized in control and Polyphenon E–drinking groups. Blood from patients with neuroblastoma and normal controls was used to assess the phenotype and function of myeloid cells.

Results: Polyphenon E reduced the number of tumor-infiltrating myeloid cells, and inhibited the development of spontaneous neuroblastomas in TH-MYCN transgenic mice. In therapeutic models of neuroblastoma in A/J, but not in immunodeficient NOD/SCID mice, Polyphenon E inhibited tumor growth by acting on myeloid-derived suppressor cells (MDSC) and CD8 T cells. *In vitro*, Polyphenon E impaired the development and motility of MDSCs and promoted differentiation to more neutrophilic forms through the 67 kDa laminin receptor signaling and induction of granulocyte colony-stimulating factor. The proliferation of T cells infiltrating a patient metastasis was reactivated by Polyphenon E.

Conclusions: These findings suggest that the neuroblastoma-promoting activity of MDSCs can be manipulated pharmacologically *in vivo* and that green tea catechins operate, at least in part, through this mechanism. *Clin Cancer Res; 19(5); 1116–25. ©2012 AACR.*

Introduction

Green tea has been used for thousands of years in traditional Chinese medicine to treat human diseases. Epidemiologic data shows that Asian populations that consume significant quantities of green tea show a reduced incidence of cancer (1). Green tea catechins are natural polyphenolic formulations endowed with antioxidant and anticancer activity. In recent years, a number of reports have shown that green tea catechins inhibit tumor proliferation and induce tumor cell apoptosis *in vivo* and *in vitro* (2). A small placebo-controlled randomized study in subjects with high-grade prostate intraepithelial neoplasia showed that there was a significant reduction in the development of prostate cancer in patients taking 600 mg catechins per day (3). Therapeutic concentrations may be achieved by accumulation in target tissues, but are associated with minimal toxicity during prolonged treatments (1, 3, 4). The mechanisms of action have not been clarified, although it has been proposed that catechins modulate key molecular drivers of cell proliferation and survival, including cyclin D1 (5). Polyphenon E is a green tea catechin formulation, which has been shown to prevent tumorigenesis in cancer clinical trials (3, 5–7). To further explore efficacy and mechanism, Polyphenon E was supplemented in the drinking water (0.3% w/v) of neuroblastoma prone TH-MYCN transgenic mice (8). Unexpectedly, we found that the anticancer effects of Polyphenon E could be mediated by the immune system.

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Translational Relevance

In spite of its low frequency, neuroblastoma is a malignancy that accounts for 15% of all oncological childhood deaths. Current treatments include heavy chemotherapy, radiation, and surgery, which often cause long-lasting adverse effects. Polyphenol E is an orally available, clinical grade catechin formulation, which, in adult clinical trials, has shown no toxicity or adverse effects. In this study, we show that the catechin mixture significantly inhibits tumorigenesis in mouse models of neuroblastoma by inactivating cancer-induced suppressive myeloid cells. Polyphenol E caused maturation of myeloid cells, inhibition of their immunosuppressive effects, and reactivation of intratumoral T cells of the patient. These results suggest that oral administration of Polyphenon E could be particularly useful to enhance the efficacy of cancer immunotherapy. Specifically, we predict that Polyphenon E should augment the antitumor immune response in patients with neuroblastoma injected with therapeutic antibodies targeting the GD2 molecule. The observation that Polyphenol E significantly reduces spontaneous tumorigenesis in TH-MYCN mice indicates that the formulation could also be used to prevent recurrence after cancer remission induced by standard therapies.

Materials and Methods

Reagents

Polyphenon E was kindly provided by Andrew Munro (Polyphenon-pharma). Polyphenon E contains 53% epigallocatechin 3-gallate (EGCG), 9% epicatechin, 11% (−)-epigallocatechin, 5% epicatechin-3-gallate, and 5% (−)-gallocatechin gallate. Therefore, 5 μg/mL Polyphenon E contains 2.75 μg/mL EGCG, 0.45 μg/mL epicatechin, 0.55 μg/mL (−)-epigallocatechin, and 0.25 μg/mL (−)-gallocatechin gallate. The same batch of the formulation was used throughout this study. The Polyphenol E powder was dissolved into 100 mL of drinking water freshly prepared every other day.

Antibodies used for blocking experiments were: 67 kDa laminin, clone MLuC5 (Abcam), and an isotype IGM control (Santa Cruz); granulocyte colony-stimulating factor (G-CSF), IL-6, and rat IgG isotype control were also purchased from Abcam.

Cell lines

The human neuroblastoma cell line SH-SY5Y was obtained from the American Type Culture Collection, mouse neuroblastoma Neuro 2A cells were kindly provided by Dr. Steven Hart (UCL Institute of Child Health, London, United Kingdom), and maintained in Dulbecco's modified Eagle's medium supplemented with 4500 mg/L glucose, 10% fetal calf serum (FCS) and 2 mmol/L glutamine, and 1% penicillin/streptomycin. Primary mouse neuroblastoma cells were isolated from tumor emerging from MYCN mice and cultured *in vitro* for several passages in RPMI-1640 supplemented with 20% FCS, 2 mmol/L L-glutamine, 0.1 mmol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, 1× nonessential amino acids, and 1% penicillin/streptomycin.

Isolation of human neuroblastoma cells from tumor

A surgically resected human neuroblastoma metastasis was briefly washed in PBS, mechanically dispersed, and passed through a 70 μm cell strainer (BD Biosciences) to obtain single-cell suspension. Cells were plated in 25 mL tissue culture flasks and cultured in RPMI-1640 medium (supplemented with 20% FCS, 2 mmol/L L-glutamine, 0.1 mmol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, 1× nonessential amino acids, and 1% penicillin/streptomycin solution). Fluorescence-activated cell sorting (FACS) analysis with a GD-2 antibody showed that more than 90% of cells were positive to this neuroblastoma antigen.

Mouse bone marrow cultures and generation of MDSCs by coculture with neuroblastoma cells

Bone marrow samples were harvested from the femurs and tibias of 8-week-old C57Bl/6 (Ly5.2) or A/J mice. Total bone marrow cells were maintained in StemSpan serum-free medium (StemCell Technologies) supplemented with 1% penicillin/streptomycin and full cytokine cocktail (100 ng/mL mSCF, 100 ng/mL mFlt3L, 100 ng/mL hIL-11, and 20 ng/mL mIL-3). Myeloid derived suppressor cells were obtained by plating 2.5×10^5 bone marrow cells in RPMI supplemented with 20% FCS, granulocyte macrophage colony-stimulating factor (40 ng/mL) and IL-6 (40 ng/mL); alternatively, bone marrow cells were plated in the presence of conditioned medium from neuroblastoma cells isolated from MYCN transgenic mice. The neuroblastoma conditioned medium was used fresh or defrosted after storage at −80°C. Cells were maintained at 37°C in 5% CO_2 humidified atmosphere for 4 days before FACS analysis. Polyphenon E was used at a concentration of 5 μg/mL at day 1 and 3 of culture of bone marrow cells.

Isolation of mouse and human T cells

We isolated CD3+ T cells from splenocytes of 8 week old C57BL/6 mice via positive selection using a biotinylated CD3 antibody (Miltenyi Biotec). Neutrophils and MDSC subset isolation was carried out by staining the cells with allophycocyanin (APC)-Ly6G, phycoerythrin (PE)-CD11B, and fluorescein isothiocynate (FITC)-Ly6C–conjugated antibodies (all from BD) and FACS sorting. Purity of cell populations was evaluated by flow cytometry and exceeded 95%. Human peripheral blood mononuclear cells (PBMC) were isolated from the blood or disaggregated tumor cells using FICOLL-Hypaque. PBMC were depleted of MDSCs by negative selection using a FITC-conjugated CD66b antibody followed by anti-FITC microbeads. All separations were conducted using Miltenyi Biotec kits and MidiMacs columns.

Human PBMC and murine CD3+ splenocytes were activated for 5 days with anti-CD3/28 beads (Dynabeads,

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Invitrogen) at a 1:1 ratio (cells:beads) following manufacturer's protocol. Regulatory T cells (Tregs) were detected by FACS analysis using a kit, following manufacturer's protocol (eBioscience Inc.). IFN-γ release was measured by ELISA after mixing CD3+ cells with monocytic or granulocytic MDSCs (T-cells/MDSCs = 2:1).

Mouse models of neuroblastoma

Mice of the CBA background with expression of human MYCN in the neuroectoderm, prone to develop neuroblastomas, were given 0.3% Polyphenon E in the drinking water. We observed that tumor penetrance in this background is 100%, whereas in other mixed backgrounds is significantly reduced (9). Emerging tumors were dissected and snap-frozen in liquid nitrogen for subsequent analysis. Severe combined immunodeficient (SCID) mice (CB17/ICR-Prkdc^{scid}/IcrCrl) were purchased from Charles River. Mice were subcutaneously injected into both flanks with 1×10^6 neuroblastoma cells. Polyphenon E (0.3%) was supplemented in the drinking water to half of the injected mice starting from the day of tumor injections. A/J mice were purchased from Charles Rivers. Tumor size was monitored with a caliper and calculated according to the formula: $V = (\text{length} \times \text{width}^2)/2$. Immunodepletions were carried out by intraperitoneal injections of 200 μg antibody per mouse at −1, +3, +7, and +15 days from tumor injections. The antibodies used for immunodepletion (CD4, CD8 and isotype control) were purchased from Bioxcell.

Immunohistochemistry and FACS staining

Frozen sections were cut, air-dried, and fixed in 4% paraformaldehyde in PBS. After fixation, sections were washed several times in PBS followed by blocking with 5% horse serum in PBS and overnight incubation with rat anti-mouse CD11b antibody (BD Pharmingen) or rat anti-mouse GR-1 (Bioscience) in a humidified chamber at 4°C. We used a 2-step detection with biotin-conjugated anti-rat antibody followed by Cy2-conjugated streptavidin (both from Jackson Immunoresearch). For FACS staining, tumors were disaggregated in PBS and collagenease (100 μg/mL) at 37°C for 2 hours and subjected to red blood cell lysis before incubation with the antibodies.

Statistical analysis

Kaplan–Meier surviving curves and log-rank test were used to compare the survival of TH-MYCN mice treated with Polyphenon E. All other results are presented as mean ± SE and the significance calculated using Student *t* test.

Results

The anticancer activity of Polyphenon E in mouse models of neuroblastoma requires the immune system and is associated with impaired infiltration of myeloid cells

There was a significant decrease in tumor development in TH-MYCN transgenic mice exposed to oral catechins, with approximately half of mice free from tumor after 8 months, compared with complete tumor penetrance in control mice (Fig. 1A). Interestingly, tumor cells derived from TH-MYCN mice were only killed *in vitro* by concentrations of Polyphenon E that were considerably higher than plasma concentrations in rodents drinking up to 0.5% of Polyphenon E, suggesting that the effects could not be directly mediated (refs. 10, 11; Fig. 1B). Similarly, although SHSY5Y human neuroblastoma cells were sensitive to Polyphenon E *in vitro* ($IC_{50} \sim 30$ μg/mL; Supplementary Fig. S1), it did not impair their growth when engrafted in SCID mice (Fig. 1c). We therefore speculated that the anticancer effects of Polyphenon E were mediated through augmentation of cellular immunity. Gene expression analysis in the emerging tumors indicated that myeloid markers such as Cd11b and Gr-1 were downregulated in mice drinking Polyphenon E (data not shown). The induction of MDSCs was recently identified as an important tumor evasion mechanism (12). To visualize myeloid cells infiltrating and developing neuroblastomas in TH-MYCN transgenic mice, we immunostainined tumors emerging from the different treatment groups. There was a significant decrease in cells bearing the myeloid markers CD11b or Gr-1 in Polyphenon E–drinking mice, suggesting that the green tea formulation inhibits tumor-infiltrating myeloid cells (Fig. 1D). To confirm this hypothesis in a therapeutic model of neuroblastoma with a proficient immune system, A/J mice were administered Polyphenon E orally and injected subcutaneously with syngeneic Neuro 2A cells. Polyphenon E–drinking mice showed a significant inhibition of tumor growth compared with control mice (Fig. 2A). This result confirmed that Polyphenon E is able to reduce tumor growth in therapeutic as well as prophylactic settings and that this effect is dependent on functional cellular immunity. Enumeration by flow cytometry showed that CD11b/Gr-1 double-positive myeloid cells were generally less abundant in the tumors and lymphoid organs of Polyphenon E–drinking mice, compared with control animals (Fig. 2B). This was accompanied by a significant and robust increase of single-positive CD4 and double-positive CD4/CD8 T cells infiltrating the tumors of Polyphenon E–drinking mice, compared with control mice (Fig. 2C). Of note, nonconventional double-positive T cells have been detected in patients with cancer and display cytotoxic activity against myeloma and melanoma, the latter tumor sharing with neuroblastoma a neuroectodermal origin (13–15)

Promotion of neuroblastoma growth *in vivo* by tumor-induced myeloid cells is antagonized by Polyphenon E, which requires the activity of CD8 T cells

To assess the role of myeloid cells in tumor growth in this setting, we coinjected Neuro 2A cells with bone marrow cells derived from A/J mice and cultured in the presence of neuroblastoma supernatants (to induce MDSCs) with or without Polyphenon E. After 5 days in culture, these cells were about 90% positive for the CD11b marker (data not shown). We observed a significant and large increase of

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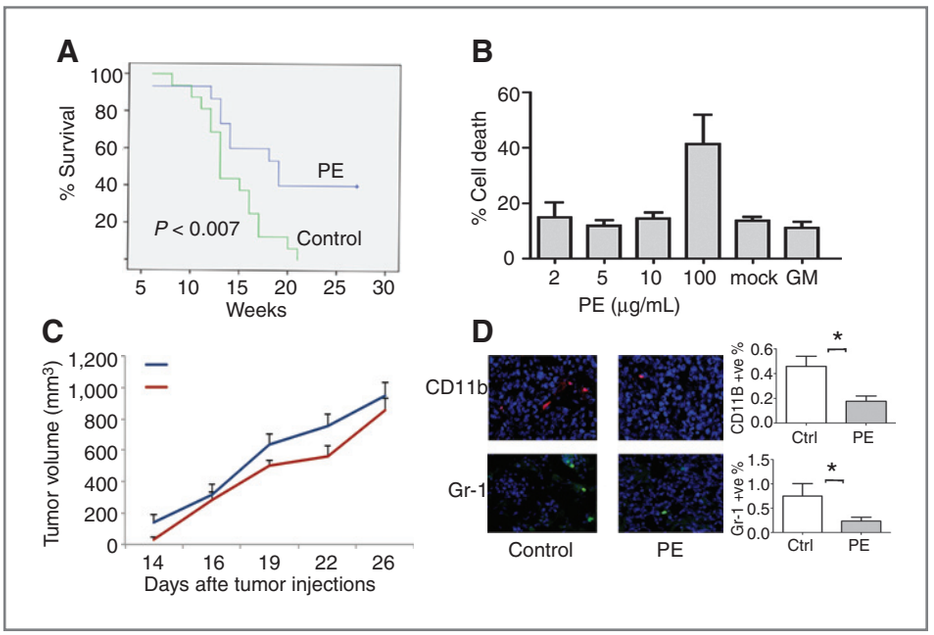

Figure 1. Polyphenon E prevents tumorigenesis in TH-MYCN mice. A, Kaplan–Meier survival curves of mice homozygous for the TH-MYCN transgene with (indicated by PE, $n = 15$) or without (indicated by control, $n = 16$) 0.3% Polyphenon E in the drinking water. B, Trypan blue dye exclusion assay to assess killing of primary neuroblastoma cells by Polyphenon E *in vitro*. Primary mouse (from TH-MYCN transgenic mice) neuroblastoma cells were cultured in the presence of increasing concentrations of Polyphenon E, as indicated. Values in the *y*-axis indicate percentages of cell death. Error bars indicate SEs. Mock indicates cells in the presence of ethanol vehicle and GM indicates cells in growth medium. C, a total of 1×10^6 SHSY5Y cells were injected in the flanks of SCID mice. Mice were randomized in 2 groups of 10 mice each, one of which had Polyphenon E in the drinking water for the duration of the experiment (4 weeks). D, immunofluorescence analysis of tumor sections from TH-MYCN transgenic mice, treated or untreated with Polyphenon E, showing the presence of infiltrating myeloid cells positive to the markers CD11b (red) or Gr-1 (green). The quantification of the experiment is shown in the graphs on the right. Asterisks indicate that the difference observed was statistically significant (CD11b $P < 0.04$ and Gr-1 $P < 0.05$).

tumor volumes in mice coinjected with myeloid cells compared with mice injected with neuroblastoma cells alone. Of note, the tumor-promoting effect was completely abolished if myeloid cells had been cultured with Polyphenon E before injection (Fig. 3A). We detected an increase of infiltrating T cells in tumors coinjected with Polyphenon E–treated MDSCs (Supplementary Fig. S1A), and immune depletion of CD8, but not CD4, T cells resulted in enhanced tumor growth on administration of Polyphenon E–treated MDSCs. This observation is consistent with CD8 cells being required for the host response against tumor that is liberated by the Polyphenon E suppression of MDSC activity (Fig. 3B and Supplementary Fig. S1B). Thus, we conclude that Polyphenon E–mediated inhibition of tumorigenic activity of MDSCs requires CD8 T cells.

Polyphenon E promotes differentiation of immature myeloid cells via activation of the 67 kDa laminin receptor and secretion of G-CSF

To investigate in more detail whether green tea catechins affect the growth and/or development of MDSCs, we cultured neuroblastoma-induced MDSCs (Supplementary Fig. S2) in the presence or absence of 5 μg/mL of Polyphenon E, a concentration that is not toxic to transformed or normal human and mouse cell lines *in vitro* (Fig. 1B and Supplementary Figs. S3 and S4). In the presence of Polyphenon E, there was a decrease in the number of cells with a monocytic phenotype (CD11b+ve/Ly6G-ve/Ly6Chigh), whereas cells bearing granulocytic markers (CD11b+ve/Ly6G+ve/Ly6Clow) were increased in number, suggesting that the catechin formulation could induce a change in the phenotype of MDSCs (Fig. 4A). Consistent with these observations, unsorted myeloid cells from bone marrow cells cultured for 4 days in neuroblastoma supernatants and exposed to Polyphenon E were morphologically more differentiated than vehicle-treated cells, with many cells displaying multilobed nuclei characteristic of mature neutrophils (Fig. 4B). When we separated by FACS sorting the cells with monocytic (CD11b+ve/Ly6G-ve/Ly6Chigh) or granulocytic (CD11b+ve/Ly6G+ve/Ly6Clow) markers, their morphology was consistent with the molecular markers, and Polyphenon E seemed to mainly promote morphologic differentiation of granulocytic MDSCs (Supplementary Fig. S5). Of note, myeloid cells with monocytic markers migrated towards neuroblastoma cells in a Transwell assay significantly better than cells with granulocytic markers, suggesting that Polyphenon E, by shifting the balance towards a granulocytic phenotype, could also hinder the migration of MDSCs at tumor sites (Fig. 4C).

A major component of Polyphenon E is EGCG, which has been shown to affect cell signaling via interaction with the 67 kDa laminin receptor (11). We investigated

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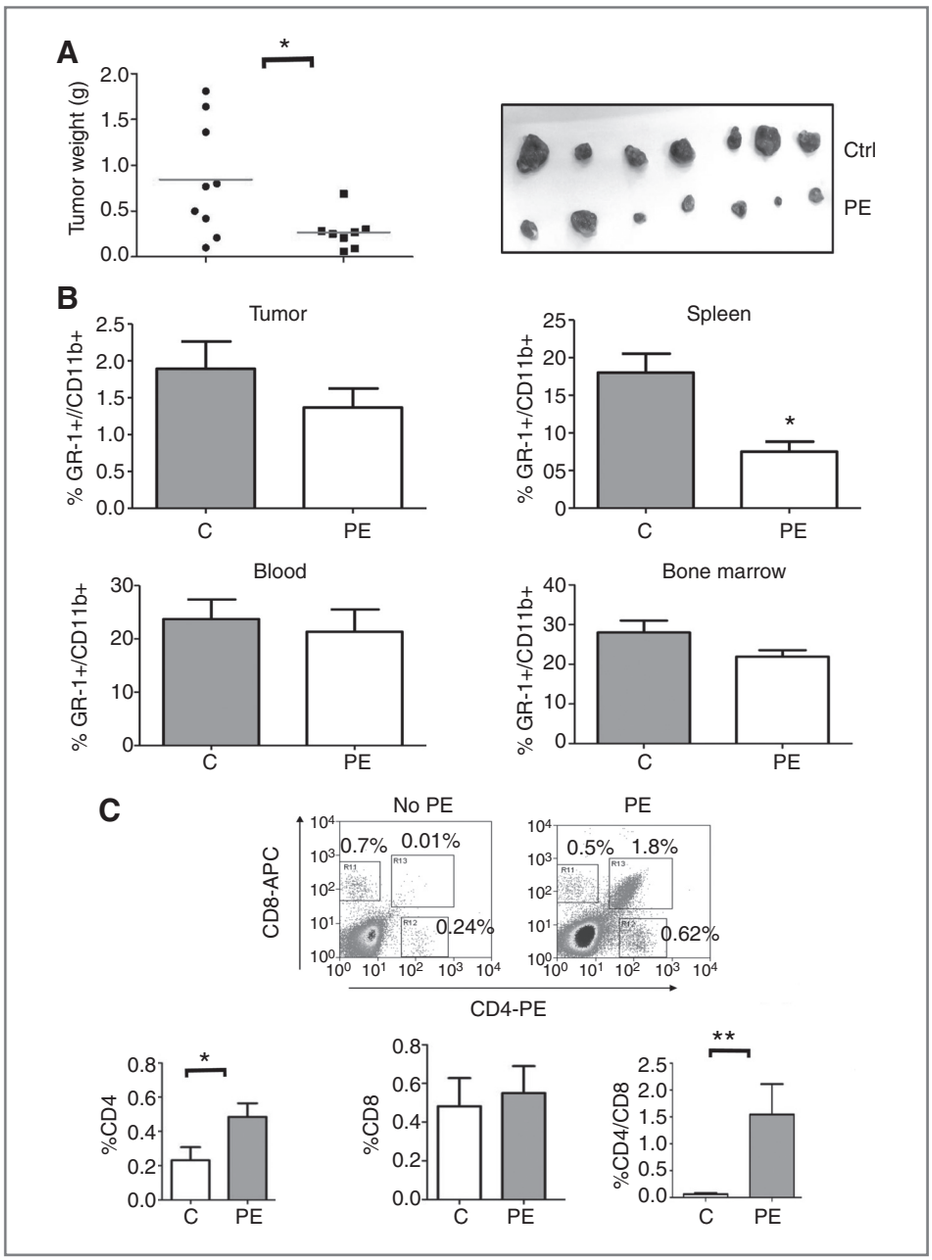

Figure 2. Polyphenon E suppresses tumor growth in a therapeutic model of neuroblastoma. A, A/J mice were randomized in control and Polyphenon E–drinking groups (6 mice each group) and injected in both flanks with 1×10^6 Neuro 2A cells. After 3 weeks, tumors were excised and weighed. Control tumors (C) were significantly larger than those excised from Polyphenon E–drinking mice. *, $P = 0.024$. B, quantification of CD11b/GR-1 double-positive, MDSC-like cells in the organs of control or Polyphenon E–drinking, tumor-injected mice. SEs are indicated by the error bars. *, $P = 0.005$. C, FACS plot showing CD4- and CD8-positive lymphocytes infiltrating the neuroblastomas of control or Polyphenon E–drinking mice. The double-positive cells were detected in the tumors of all Polyphenon E–drinking mice. The bar plots in the bottom show a significant increase of CD4- and double-positive CD8/CD4 cells. Error bars indicate SEs; *, $P = 0.045$; **, $P = 0.006$. PE, Polyphenon E.

whether this receptor was involved in Polyphenon E signaling by culturing bone marrow cells in the presence or absence of a blocking antibody or an isotype-matched control. The specific antibody completely reversed the changes in molecular markers induced by the catechins, showing that the 67 kDa laminin receptor signaling is required for the differentiating activity of Polyphenon E (Fig. 4D).

We next investigated a potential mechanism for how Polyphenon E altered the differentiation of myeloid cells. We separated, by FACS sorting, the monocytic or granulocytic MDSCs obtained after culturing bone marrow cells with neuroblastoma supernatants, with or without Polyphenon E. Next, we assessed the secretion of cytokines using a commercial array. While Polyphenon E did not induce increased secretion of cytokines in granulocytic MDSCs (data not shown), 3 cytokines were sharply upregulated in monocytic MDSCs: IL-16, G-CSF, and IL-6 (Supplementary Fig. S6A). We focused our attention on G-CSF and IL-6, as these cytokines were previously shown to be important for the differentiation and proliferation of myeloid cells (16, 17). We validated the Polyphenon E–induced secretion of the G-CSF and IL-6 by ELISA (not shown). Blocking antibodies directed against G-CSF, but not IL-6, reverted the effect of Polyphenon E on myeloid cell maturation, causing an

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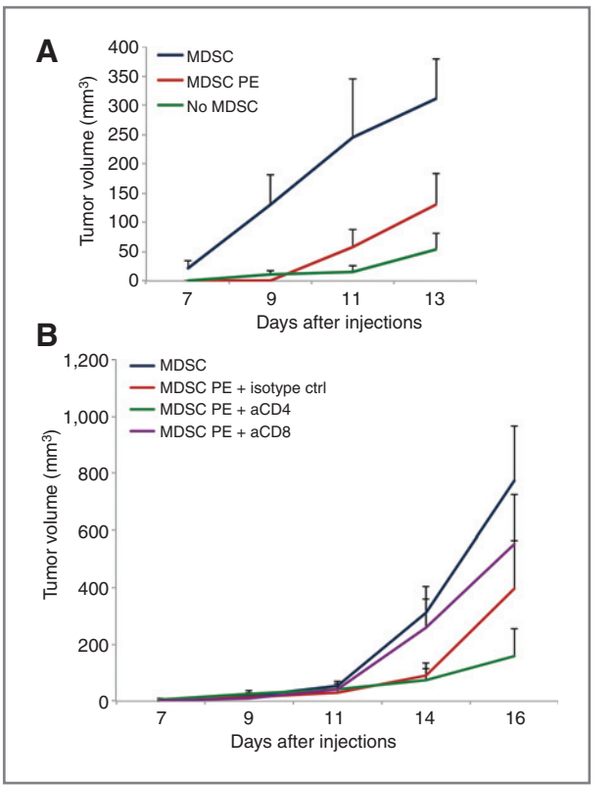

Figure 3. MDSCs promote neuroblastoma growth *in vivo*. A, a total of 5×10^5 Neuro 2A cells were injected into both flanks of A/J mice (5 mice each group) alone or mixed with the same number (1:1) of naïve or Polyphenol E-treated MDSCs (indicated by MDSC PE). Tumor volumes were measured at regular intervals with a caliper. Error bars indicate SEs. The difference in tumor growth at day 13 was significant (MDSC vs. no MDSC; $P = 0.001$; MDSC vs. MDSC-PE; $P = 0.02$). B, A/J mice (6 mice per group) were injected as described above but subjected to immunodepletion with CD4 or CD8 antibodies. The difference in tumor growth was significant in mice with CD8 immunodepletion (day 14, MDSC-PE isotype control vs. MDSC-PE CD8 antibody; $P = 0.01$).

accumulation of monocytic MDSCs and a concurrent decrease of cells with a granulocytic phenotype (Supplementary Fig. S6B and S6C).

Polyphenon E antagonizes the immunosuppressive activity of myeloid cells

To assess whether catechins not only affected the differentiation but also the immunosuppressive activity of myeloid cells, we separated vehicle or Polyphenon E-treated monocytic MDSCs (CD11b+/Ly6G-ve/Ly6Chigh) by flow cytometric sorting and mixed them with CD3+ splenic T lymphocytes. Secretion of IFN-γ was determined after stimulation with anti-CD3 and anti-CD28 antibodies. As expected, monocytic MDSCs drastically reduced the release of IFN-γ by T cells. However, there was a large and significant rescue of IFN-γ secretion when MDSCs were pretreated with Polyphenon E (Fig. 5A). Similar results were observed using granulocytic MDSCs (not shown). FoxP3-expressing Tregs have been shown to be induced by and mediate the immunosuppressive activity of MDSCs (18). Both monocytic and granulocytic myeloid populations significantly enhanced the formation of Tregs (CD4+/CD25+/Foxp3+) when mixed with total CD3+ splenocytes. Pretreatment with Polyphenon E significantly impaired the ability of myeloid cells to induce Treg cells, further suggesting that green tea catechins negatively modulate the immunosuppressive function of myeloid cells (Fig. 5B).

Interestingly, incubation with Polyphenon E reduced arginase-1 expression in 8 of 10 bone marrow cell preparations cultured for 4 days in the presence of neuroblastoma supernatants (Fig. 5C), indicating that the formulation also negatively modulates a gene critically required for the immunosuppressive function of MDSCs (19).

Myeloid cells with immunosuppressive activity are found in the blood and metastatic tumor of neuroblastoma patients

It is still unknown whether MDSC-like cells are induced in patients with neuroblastoma. The CD66b antigen is one of several markers present on the surface of MDSCs in tumor-bearing patients (20, 21), and we first determined that cells expressing MDSC markers, including CD66b, are increased in the blood of patients with neuroblastoma, in comparison with age-matched controls (Supplementary Table S1). We had sufficient blood sample for detailed functional analysis in one patient who had an increased number of CD66b-positive cells, compared with a healthy control (Fig. 6A). Stimulation of PBMCs with beads linked to CD3/CD28 antibodies, induced proliferation of control, but not patient, T lymphocytes. Interestingly, depletion of CD66b cells increased the proliferation of CD4 and CD8 T cells of the patient (Fig. 6B), indicating that myeloid cells bearing this marker possess immunosuppressive activity. To verify the potential role of tumor-infiltrating MDSCs in neuroblastoma, leucocytes were isolated from a metastasis of another patient. A large number of cells from the disaggregated tumor were positive for the myeloid markers CD11b, CD66b, CD68, and CD33 (Supplementary Fig. S7). Leukocytes isolated by Ficoll centrifugation were then incubated with beads bearing an anti-CD3/CD28 antibody to activate tumor-infiltrating lymphocytes, in the presence or absence of Polyphenon E. After 5 days in culture, there was a 3-fold increase in the percentage of CD8 T cells in the presence of Polyphenon E, but not when myeloid cells were depleted using beads linked to a CD66b antibody (Fig. 6C). Furthermore, incubation with Polyphenon E increased the incorporation of ^{3}[H]-thymidine with respect to vehicle-treated cells, suggesting that the green tea formulation stimulated the proliferation of tumor-infiltrating CD3+lymphocytes. The pro-proliferative effects of Polyphenon E were abrogated after cell depletion by beads linked to a CD66b antibody (Fig. 6D).

Discussion

MDSCs are myelomonocytic cells with immunosuppressive activity induced by tumor growth. These cells

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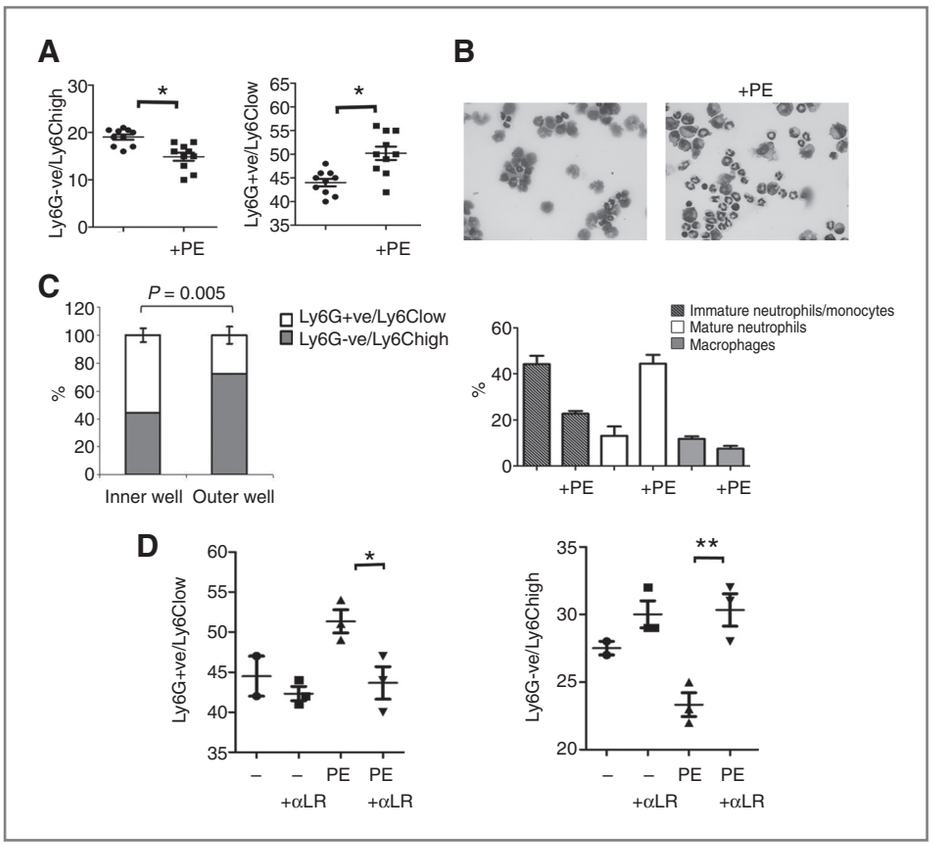

Figure 4. Polyphenon E modulates the MDSC phenotype. A, dot plot showing the percentage of CD11b^{high} bone marrow cells with a monocytic or granulocytic phenotype developing in neuroblastoma conditioned medium in the presence or absence of Polyphenon E. Each dot is representative of a bone marrow preparation deriving from a single mouse. The asterisk indicates that the difference is statistically significant ($P = 0.05$). B, differentiation quick staining of bone marrow cells cultured for 4 days in the presence of neuroblastoma supernatants with or without Polyphenon E. Percentages of immature and differentiated myeloid cells are quantified in the bar plot at the bottom. Error bars indicate SEs. C, migration of monocytic (LyG-ve/LyChigh) and granulocytic (LyG+ve/LyClow) cells towards neuroblastoma cells. Myeloid cells were placed in the inner well of a Transwell (Costar) divided from the outer well by a cell permeable membrane. Neuroblastoma cells served as attractants and were placed in the outer well. Following an incubation period of 3 hours, the cells in the inner and outer well were stained with antibodies and enumerated by FACS. D, the molecular changes induced by Polyphenon E are blocked by a 67 kDa laminin receptor antibody. Percentages of cells with monocytic or granulocytic phenotypes are indicated in the *y*-axis; αLR indicates the laminin antibody; *, $P = 0.04$; **, $P = 0.01$.

have been thoroughly characterized in the mouse and are distinguished in granulocytic and monocytic MDSCs, according to the presence of specific markers: monocytic MDSCs are CD11b+ve/Ly6G-ve/Ly6Chigh, whereas granulocytic MDSCs are CD11b+ve/Ly6G+ve/Ly6Clow. There are no uniform markers for human MDSCs, although it has been observed that lineage-negative (Lin−) myeloid cells bearing Cd11b, CD33, and various combinations of CD66b, CD14, CD15, and HLA-DR^{low} markers have immunosuppressive activity (22). Pharmacologically induced differentiation or depletion of these cells has been shown to improve the immune response in patients with cancer (23, 24). Thus, it is likely that therapeutic interventions aimed at the inactivation of MDSCs could benefit patients by reactivating the antitumor immune response. Neuroblastoma is a pediatric cancer with a dismal outcome in its high-risk, metastatic form. The current treatment options are chemotherapy, autologous stem cell transplantation, surgery, and radiotherapy. While the prognosis of children with localized neuroblastoma is very good, about 40% of patients with metastatic, high-risk neuroblastoma succumb to the disease in spite of aggressive treatments (25). The heavy chemotherapy regimens result in short- and long-term complications; therefore, the use of nontoxic drugs would be particularly advantageous in this pediatric setting. Polyphenon E is a well-defined pharmaceutical grade mixture that contains 5 different catechins used in cancer clinical trials funded by the National Cancer Institute (Bethesda, MD). A total of 15 active or recruiting phase I and II clinical trials with the agent are now going on (5). The result of a phase I trial of daily oral Polyphenon E in patients with asymptomatic Rai stage 0 to II chronic lymphocytic leukemia showed a decline in absolute lymphocytic counts and/or lymphoadenopathy in a majority of patients (6). Importantly, the formulation was

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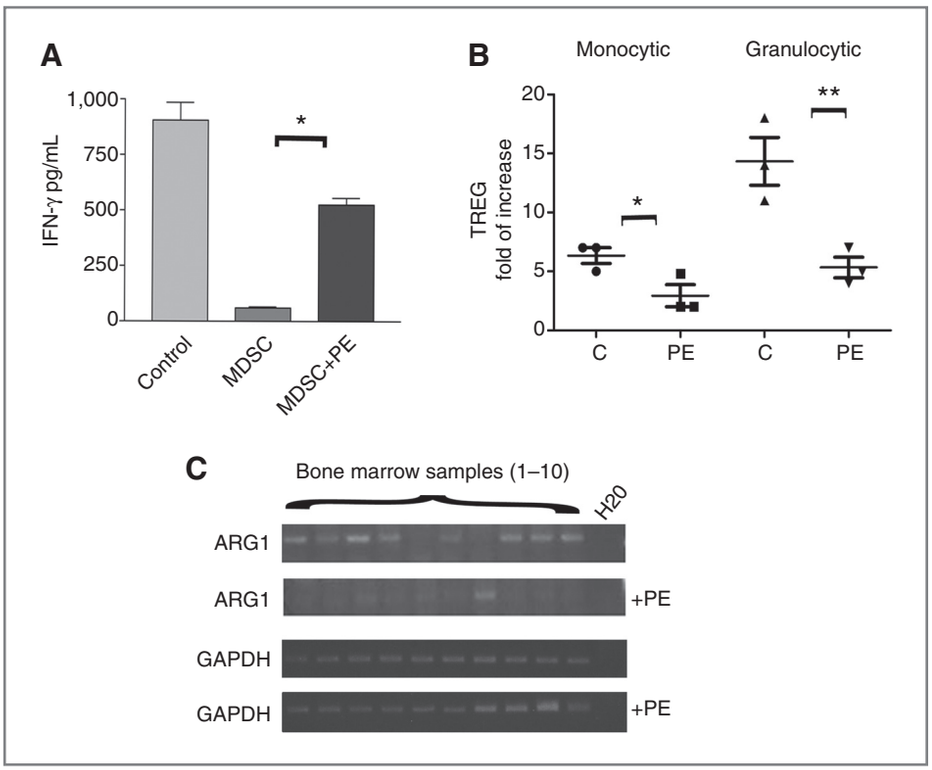

Figure 5. Polyphenon E impairs the immunosuppressive activity of MDSCs. A, IFN-γ produced by CD3 cells alone (control), mixed with monocytic (Ly6C-ve/Ly6G+ve) MDSCs (2:1 ratio) pretreated with control vehicle (MDSC) or Polyphenon E (MDSC+PE). $P = 0.001$. B, modulation of Foxp3+ Tregs by myeloid cells developing in neuroblastoma conditioned medium. CD11b+ myeloid cells bearing the markers indicated on the top were FACS sorted and mixed at a 1:1 ratio with purified CD3+ splenocytes. CD4/CD25/Foxp3 Tregs, developed in the presence or absence of myeloid cells pretreated with vehicle (C) or Polyphenon E (PE), were enumerated by flow cytometry. Values in the *y*-axis indicate the fold of Tregs increase over control (i.e., Tregs developing in the absence of myeloid cells). *, $P = 0.05$; **, $P = 0.01$. C, expression of arginase-1 is reduced in myeloid cells induced by neuroblastoma supernatants treated with Polyphenon E. Reverse transcriptase PCR analysis was carried out with arginase-1 (ARG1) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a loading control, and primers, using RNAs extracted from bone marrow cells prepared from 10 mice cultured in the presence or absence of Polyphenon E.

nontoxic and very well tolerated even at very high dosages (6). More recently, a placebo-controlled study in patients with prostate cancer before prostatectomy showed favorable changes in serum prostate-specific antigen and a decrease in Gleason score between biopsy and surgical specimens in the Polyphenon E arm, although the differences were not statistically significant (26).

In this preclinical study using mouse models of neuroblastoma, we have shown that Polyphenon E has a significant anticancer activity in both prophylactic (MYCN model) and therapeutic (AJ model) settings, suggesting that the formulation could be potentially used in neuroblastoma children as a chemopreventive agent during cancer remission or during therapy in combination with chemotherapeutic drugs. Importantly, our study shows that the anticancer effect is only significant in the context of a functioning immune system and that it occurs via inhibition of MDSCs. Another important observation in our study is the drastic increase of immunosuppressive cells bearing myeloid markers in a small cohort of children with neuroblastoma (Supplementary Table S1 and Supplementary Fig. S6). While this finding needs to be confirmed in a larger cohort of patients, to our knowledge, this is the first documentation of MDSC-like cells in patients with neuroblastoma. The mechanism by which Polyphenon E disables MDSCs is probably by the induction of terminal differentiation (Fig. 4). We show that G-CSF is the key cytokine required to mediate the differentiation activity of Polyphenon E and that signaling from the 67 kDa laminin receptor is implicated in MDSC maturation. This is a notable finding, as the pathways activated downstream of this receptor, when identified, could be exploited for therapeutic purposes. One important recent advancement in the treatment of neuroblastoma has been the development of therapeutic antibodies against the GD2 molecule. A variety of murine and hybrid murine/human antibodies have been developed, and a recent clinical trial has shown a substantial clinical effect of the ch14.18 antibody (27). Vaccination with GD2 mimotope plasmids or neuroblastoma cells transfected with cytokines have also shown promising results in mouse models (28, 29). From these studies, it is

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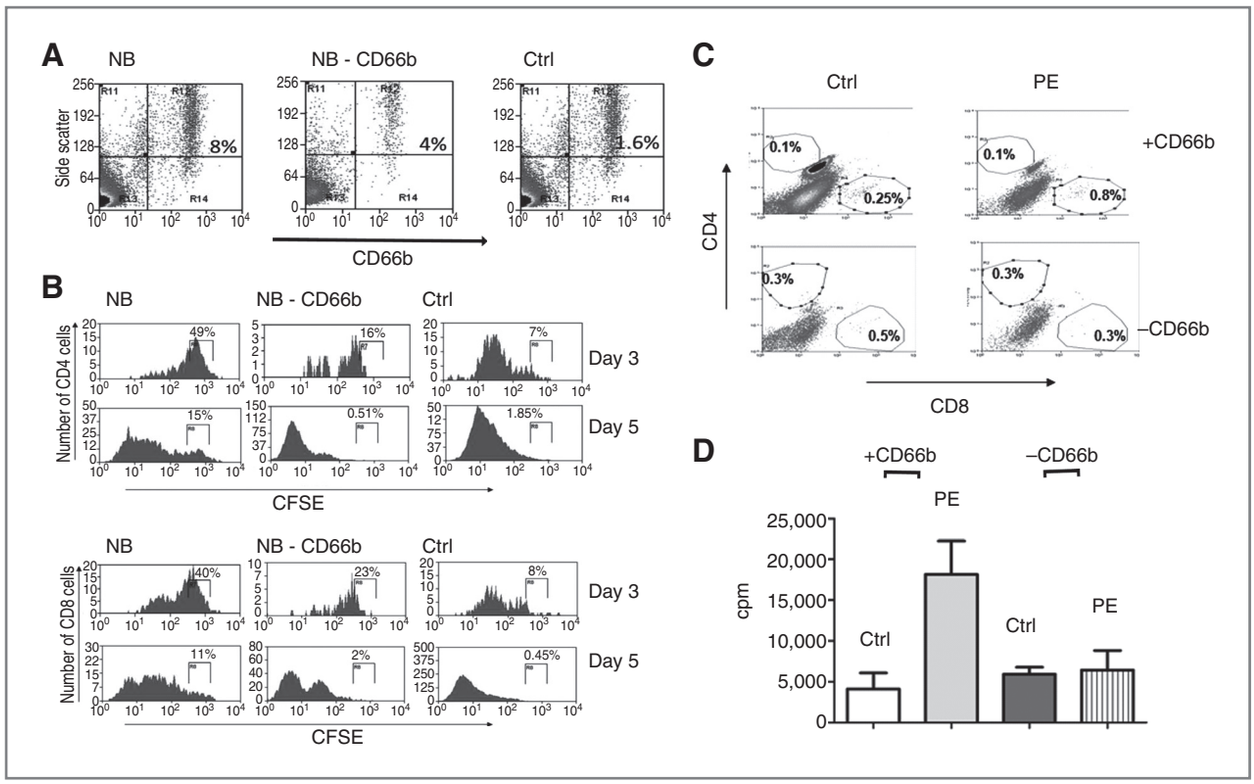

Figure 6. MDSC-like cells are found in the blood of patients with neuroblastoma and Polyphenon E rescues the proliferation of infiltrating T cells in human neuroblastoma. A, FACS plots showing the percentages of CD66b cells in the PBMC layer of a neuroblastoma patient (indicated by NB), before or after column depletion (indicated by NB -CD66b), and a normal healthy control (indicated by ctrl). B, T-cell proliferation assay. PBMCs from the patient with neuroblastoma or control individual were stained with carboxyfluorescein succinimidyl ester (CFSE) and cultured for the indicated days in the presence of CD3/CD28 beads. Cells were then stained with CD4 or CD8 antibodies and subjected to flow cytometry analysis. Percentages of resting T cells are indicated on the right side of each FACS plot. C, leukocytes infiltrating a resected tumor were isolated by Ficoll gradient and put in contact with beads linked to a CD3/CD28 agonist antibody in the presence or absence of Polyphenon E, with or without depletion of myeloid cells with beads conjugated to a CD66b antibody. After 4 days, percentages of CD4- and CD8-positive cells were scored by FACS analysis. D, infiltrating leukocytes were stimulated with an agonist CD3/CD28 antibody and incubated for 48 hours with ^{3}[H]-thymidine, in the presence (indicated by PE) or absence (indicated by Ctrl) of Polyphenon E with or without depletion of myeloid cells with a CD66b antibody, as indicated on the top of the graph. Error bars indicate SEs.

clear that cellular-mediated cytotoxicity and immune regulatory cells are crucially involved in the therapeutic effects of immunotherapy. Myeloid cells hamper the function of not only T, but also natural killer (NK), cells, which are known to be essential for the effect of the ch14.18 antibody (30, 31). Furthermore, it is known that tumor-induced Tregs blunt the NK and CD4/CD8 T-cell immune response elicited by different forms of vaccination (28, 32). Thus, we hypothesize that the increased numbers of myeloid cells in the blood of patients with neuroblastoma could interfere with the antitumor immune response and explain the failure of a significant proportion of patients to respond to immunotherapy (27). Our observation that Polyphenon E has a negative effect on MDSCs and Tregs suggests that the formulation could be helpful in the context of neuroblastoma immunotherapy and warrants the investigation of the effects of catechins in further clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Santilli, I. Piotrowska, S. Cantilena, M. D'Alicarnasso, D. Morgenstern, N. Himoudi, J. Anderson, A.J. Thrasher, A. Sala
Writing, review, and/or revision of the manuscript: J. Anderson, A.J. Thrasher, A. Sala
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Cantilena
Study supervision: A.J. Thrasher, A. Sala

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