Polyphenol E enhances the anti-tumour 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 paediatric 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 NCI, has anticancer activity in mouse models of neuroblastoma.

Experimental design. We used three neuroblastoma models: a) transgenic TH-MYCN mouse developing spontaneous neuroblastomas b) NOD/SCID mice xenotransplanted with human SHSY5Y cells c) A/J mice transplanted with syngeneic Neuro 2A cells. Mice were randomized in control and Polyphenon E-drinking groups. Blood from neuroblastoma patients and normal controls was used to assess phenotype and function of myeloid cells.

Results. Polyphenon E reduced the number of tumour 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 tumour 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 signalling and induction of G-CSF. The proliferation of T cells infiltrating a patient metastasis was re-activated 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.

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 tumourigenesis 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 patient's intratumoural T cells. 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 antitumour immune response in neuroblastoma patients injected with therapeutic antibodies targeting the GD2 molecule. The observation that Polyphenol E significantly reduces spontaneous tumourigenesis in TH-MYCN mice indicates that the formulation could also be used to prevent recurrence after cancer remission induced by standard therapies.

Introduction

Green tea has been used for thousands of years in traditional Chinese medicine to treat human disease. Epidemiologic data demonstrates 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 anti-oxidant and anti-cancer activity. In recent years a number of reports have shown that green tea catechins inhibit tumour proliferation and induce tumour 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 tumourigenesis 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 anti-cancer effects of Polyphenon E could be mediated by the immune system.

Materials and Methods

Reagents. Polyphenon E was kindly provided by Andrew Munro, Polyphenon-pharma NY, USA. Polyphenon E contains 53% EGCG, 9% epicatechin, 11% (−)-epigallocatechin, 5% epicatechin-3-gallate, and 5% (−)-gallocatechin gallate. Therefore, 5 µg/mL Poly E contains □□□□ 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.

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

Isolation of human neuroblastoma cells from tumour

A surgically resected human neuroblastoma metastasis was briefly washed in PBS, mechanically dispersed and passed through 70µm cell strainer (BD

Biosciences) to obtain single-cell suspension. To obtain the cell line growing as monolayer cells were plated in 25ml tissue culture flasks and cultured in RPMI 1640 medium (supplemented with 20% FCS, 2 mM l-glutamine, 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 1× nonessential amino acids and 1% penicilin/streptomycin solution). FACS analysis with a GD-2 antibody showed that >90% of cells were positive to this neuroblastoma antigen.

Mouse bone marrow cultures and generation of MDSCs by co-culture with neuroblastoma cells. Bone marrow samples (BM) were harvested from the femurs and tibias of 8 weeks old C57Bl/6 (Ly5.2) or A/J mice. Total BM 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, 20 ng/ml mIL-3). To obtain bone marrow-derived MDSCs, $2.5x10^5$/ml cells were plated into 6 well plates (Falcon, BD, NJ, USA) either in RPMI plus 20% FCS supplemented with GM-CSF (40 ng/ml) and IL-6 (40 ng/ml) cytokines or in conditioned medium from neuroblastoma cells derived from tumours isolated from MYCN mice and cultured for 3-4 days until confluent. The neuroblastoma condition medium was used fresh or defrosted after storage at -80 C. Cells were maintained at 37°C in 5% CO2-humidified atmosphere for 4 days before FACS analysis. For PE treatment, ECGC at a concentration of 5□g/ml was added at day 1 and 3.

Isolation of mouse and human T cells. We isolated CD3+ T cells from the spleens of 8 weeks old C57BL/6 mice via positive selection by using CD3 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Neutrophils and

MDSC subset isolation was carried out by staining the cells with APC -Ly6G, PE-CD11B and FITC-Ly6C conjugated antibodies (all from BD, Oxford, UK) and FACS sorting. Purity of cell populations was evaluated by flow cytometry and exceeded 95%. Human PBMC cells were isolated from the blood or disaggregated tumour cells using FICOLL-Hypaque. MDSC- free PBMC were obtained by negative selection using a FITC conjugated CD66b primary antibody (BD, Oxford, UK) followed by an anti FITC microbeads. All separations were performed using Miltenyi Biotec kits and MidiMacs columns. Human PBMC and murine CD3+ splenocytes were activated for 5 days with antiCD3/28 beads (Dynabead) at a 1:1 ratio (cells: beads) following manufacturer protocol (T cell activation/expansion KIT Miltenyi Biotec). Regulatory T cells (TREG) were detected by FACS analysis using a kit, following manufacturer's protocol (eBioscience Inc. San Diego, CA, USA). Interferon gamma release was measured by ELISA after mixing CD3+ cells with monocytic or granulocytic MDSCs (T-cells/MDSCs = 2:1), which were washed after culture for 4 days with Polyphenon E-containing, or control, medium. Antibodies used for blocking experiments were: 67kDa Laminin, clone MLuC5 (Abcam, Cambridge, UK) and an isotype IGM control (Santa Cruz, CA, USA); G-CSF, IL-6 and rat IgG isotype control (Abcam, Cambridge, UK).

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 tumour penetrance in this background is 100%, whereas in

other mixed backgrounds is significantly reduced (9). Emerging tumours were dissected and snap-frozen in liquid nitrogen for subsequent analysis. SCID mice (CB17/ICR-Prkdc^{scid}/IcrCrl) were purchased from Charles River. Mice were subcutaneously injected into both flanks with 1x10^6 neuroblastoma cells. Polyphenon E (0.3%) was supplemented in the drinking water to half of the injected mice starting from the day of tumour injections. A/J mice were purchased from Charles Rivers. Tumour size was monitored with a calliper and calculated according to the formula: V=(length x width^2)/2. Immunodepletions were carried out by intra-peritoneum injections of 200□g antibody/mouse at -1, +3, +7, +15 days from tumour injections. The antibodies used for immunodepletion (CD4, CD8 and isotype control) were purchased from Bioxcell, West Lebanon, NH, USA.

Immunohistochemistry and FACS staining. Frozen sections were cut, air-dried and fixed in 4% paraformaldheyde 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^{o}C. We used a two-step detection with biotin conjugated anti-rat antibody followed by Cy2 conjugated Streptavidin (both from Jackson Immunoresearch). For FACS staining, tumours were disaggregated in PBS and collagenease (100□g/ml) at 37C for 2 hours and subjected to red blood cell lysis prior to incubation with 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 values ± SE and the significance calculated using Student's T test.

Results

The anti-cancer 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 tumour development in TH-MYCN transgenic mice exposed to oral catechins, with approximately half of mice free from tumour after 8 months, compared with complete tumour penetrance in control mice (figure 1a). Interestingly, tumour 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 (10, 11) (figure 1b). Similarly, although SHSY5Y human neuroblastoma cells were sensitive to Polyphenon E *in vitro* (IC_{50} ~ 30□g/ml, figure S1), it did not impair their growth when engrafted in immunodeficient SCID mice (figure 1c). We therefore speculated that the anticancer effects of Polyphenon E were mediated through augmentation of cellular immunity. Gene expression analysis in the emerging tumours indicated that myeloid markers such as Cd11b and Gr-1 were downregulated in mice drinking Polyphenon E (data not shown). The induction of myeloid derived suppressor cells (MDSCs) was recently identified as an important tumour evasion

mechanism (12). To visualise myeloid cells infiltrating developing neuroblastomas in TH-MYCN transgenic mice, we immunostainined tumours 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 tumour infiltrating myeloid cells (figure 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 tumour growth compared to control mice (figure 2a). This result confirmed that Polyphenon E is able to reduce tumour 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 tumours and lymphoid organs of Polyphenon E drinking mice, compared to control animals (figure 2b). This was accompanied by a significant and robust increase of single positive CD4 and double positive CD4/CD8 T cells infiltrating the tumours of Polyphenon E drinking mice, compared to control mice (figure 2c). Of note, nonconventional double positive T cells have been detected in cancer patients and display cytotoxic activity against myeloma and melanoma, the latter tumour sharing with neuroblastoma a neuroectodermal origin (13-15)

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

To assess the role of myeloid cells in tumour growth in this setting, we co-injected 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 tumour volumes in mice co-injected with myeloid cells, compared to mice injected with neuroblastoma cells alone. Of note, the tumour promoting effect was completely abolished if myeloid cells had been cultured with Polyphenon E before injection (figure 3a). We detected an increase of infiltrating T cells in tumours co-injected with Polyphenon E-treated MDSCs (figure S1a), and immune depletion of CD8, but not CD4, T cells resulted in enhanced tumour growth on administration of Polyphenon E-treated MDSCs. This observation is consistent with CD8 cells being required for the host response against tumour that is liberated by the Polyphenon E suppression of MDSC activity (figures 3b and S1b). Thus, we conclude that Polyphenon E-mediated inhibition of MDSCs tumourigenic activity requires CD8 T cells.

Polyphenon E promotes differentiation of immature myeloid cells via activation of the 67kDa 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 (Figure S2) in the presence or absence of 5μg/ml of Polyphenon E, a concentration that is not toxic to transformed or normal human and mouse cell lines *in vitro* (figure 1b and figures 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 (figure 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 (figure 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 appeared to mainly promote morphological differentiation of granulocytic MDSCs (figure 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 tumour sites (figure 4c).

A major component of Polyphenon E is epigallocatechin 3-gallate (EGCG), which has been shown to affect cell signalling *via* interaction with the 67-kDa laminin receptor (11). We investigated whether this receptor was involved in polyphenon E signalling 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, demonstrating that the 67kDa laminin receptor signalling is required for the differentiating activity of Polyphenon E (figure 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 secretion of cytokines using a commercial array. While Polyphenon E did not induce increased secretion of cytokines in granulocytic MDSCs (data not shown), three cytokines were sharply up-regulated in monocytic MDSCs: IL-16, G-CSF and IL-6 (figure S6, panel a). We focused our attention on G-CSF and IL-6, since these cytokines were previously shown to be important for 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 accumulation of monocytic MDSCs and a concurrent decrease of cells with a granulocytic phenotype (figure S6 panels b,c).

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 interferon gamma (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 pre-treated with Polyphenon E (figure 5a). Similar results were observed using granulocytic MDSCs (not shown). FoxP3-expressing regulatory T cells (Treg cells) 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. Pre-treatment 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 (figure 5b). Interestingly, incubation with Polyphenon E reduced Arginase-1 expression in 8 out of 10 bone marrow cell preparations cultured for 4 days in the presence of neuroblastoma supernatants (figure 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 tumour of neuroblastoma patients

It is still unknown whether MDSCs-like cells are induced in neuroblastoma patients. The CD66b antigen is one of several markers present on the surface of MDSCs in tumour bearing patients (20, 21), and we first determined that cells expressing MDSC markers, including CD66b, are increased in the blood of neuroblastoma patients, in comparison to age-matched controls (supplementary table 1). We had sufficient blood sample for detailed

functional analysis in one patient who had an increased number of CD66b positive cells, compared to a healthy control (figure 6a). Stimulation of PBMC (peripheral blood mononuclear cells) 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 patient's CD4 and CD8 T cells (figure 6b), indicating that myeloid cells bearing this marker possess immunosuppressive activity. To verify the potential role of tumour-infiltrating MDSCs in neuroblastoma, leucocytes were isolated from a metastasis of another patient. A large number of cells from the disaggregated tumour were positive for the myeloid markers CD11b, CD66b, CD68 and CD33 (figure S7). Leukocytes isolated by Ficoll centrifugation were then incubated with beads bearing an anti-CD3/CD28 antibody to activate tumour-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 (figure 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 tumour-infiltrating CD3+lymphocytes. The pro-proliferative effects of Polyphenon E were abrogated after cell depletion by beads linked to a CD66b antibody (figure 6d).

Discussion

Myeloid suppressor cells, or MDSCs, are myelo-monocytic cells with immunosuppressive activity induced by tumour growth. These cells have been thoroughly characterised 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, HLA-DR^{low} markers have immunosuppressive activity (22). Pharmacologically-induced differentiation, or depletion of these cells have been shown to improve the immune response in cancer patients (23, 24). Thus, it is likely that therapeutic interventions aimed at the inactivation of MDSCs could benefit patients by re-activating the antitumour immune response. Neuroblastoma is a paediatric cancer with a dismal outcome in its high-risk, metastatic form. The current treatment options are chemotherapy, autologous stem cell transplantation, surgery, and radiation therapy. 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 non-toxic drugs would be particularly advantageous in this paediatric setting. Polyphenon E is a well-defined pharmaceutical grade mixture that contains five different catechins used in cancer clinical trials funded by the National Cancer Institute.

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 asympthomatic Rai stage 0 to II chronic lymphocytic leukemia showed a decline in absolute lymphocytic counts and/or lymphoadenopathy in the majority of patients (6). Importantly, the formulation was non-toxic and very well tolerated even at very high dosages (6). More recently, a placebo controlled study in prostate cancer patients 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 pre-clinical study using mouse models of neuroblastoma, we have shown that Polyphenon E has a significant anti-cancer 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 demonstrates 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 1 and figure 6). 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 neuroblastoma patients.

The mechanism by which Polyphenon E disables MDSCs is probably the induction of terminal differentiation (figure 4). We show that G-CSF is the key

cytokine required to mediate the differentiation activity of Polyphenon E and that signalling from the 67kDa laminin receptor is implicated in MDSCs maturation. This is a notable finding since the pathways activated downstream of this receptor, when identified, could be exploited for therapeutic purposes. One important recent advance 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 promise in mouse models (28, 29). From these studies it is clear that cellular mediated cytotoxicity and immune regulatory cells are crucially involved in the therapeutic effects of immunotherapy. Myeloid cells hamper the function not only of T but also NK cells, which are known to be essential for the effect of the ch14.18 antibody (30, 31). Furthermore, it is known that tumour induced Tregs blunt the NK and CD4/CD8 T cell immune response elicited by different forms of vaccination (28, 32). Thus, we hypothesise that the increased numbers of myeloid cells in the blood of patients with neuroblastoma could interfere with the antitumour 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 regulatory T cells (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.

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Figure legends

Fig. 1. Polyphenon E prevents tumourigenesis in TH-MYCN mice.

a) Kaplan Meyer 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 standard errors. Mock indicates cells in the presence of ethanol vehicle; GM indicates cells in growth medium. c) $1x10^6$ SHSY5Y cells were injected in the flanks of SCID mice. Mice were randomized in two 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 tumour 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 showed in the graphs on the right. Asterisks indicate that the difference observed was statistically significant (CD11b $p<0.04$ and Gr-1 $p<0.05$).

Fig. 2. Polyphenon E suppresses tumour growth in a therapeutic model of neuroblastoma.

a) A/J mice were randomised in control and Polyphenon E drinking groups (6 mice each group) and injected in both flanks with $1x10^6$ Neuro 2A cells. After 3 weeks, tumours were excised and weighed. Control tumours were significantly larger than those excised from Polyphenon E drinking mice. * indicates statistical significance (p=0.024). b) Quantification of CD11b/GR-1 double positive, MDSCs-like cells in the organs of control or Polyphenon E (PE) drinking, tumour injected mice. Standard errors are indicated by the error bars and * indicates statistical significance (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 tumours of all PE drinking mice. The bar plots in the bottom show a significant increase of CD4 and double positive CD8/CD4 cells. Error bars indicate standard errors, * p=0.045; ** p=0.006.

Fig. 3. MDSCs promote neuroblastoma growth *in vivo*.

a) $5x10^5$ 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). Tumour volumes were measured at regular intervals with a caliper. Error bars indicate standard errors. The difference in tumour 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 tumour growth was significant in mice with CD8 immunodepletion (day 14, MDSC-PE isotype control *vs* MDSC-PE CD8 antibody p= 0.01).

Fig. 4. Polyphenon E modulates the MDSCs 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 (PE) or absence of Polyphenon E. Each dot is representative of a bone marrow preparation deriving from a single mouse. The asterisk indicate 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 of the panel. Error bars indicate standard errors. c) Migration of monocytic (LyG-ve/LyChigh) and granulocytic (LyG+ve/LyClow) 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 hrs 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 67kDa laminin receptor antibody. Percentages of cells with a monocytic or granulocytic phenotype are indicated in the Y axis; □LR indicates the laminin antibody; * p=0.04; ** p=0.01

Fig. 5. Polyphenon E impairs the immunosuppressive activity of MDSCs. a) Interferon-gamma produced by CD3 cells alone (control), mixed with monocytic (Ly6C-ve/Ly6G+ve) MDSCs (2:1 ratio) pre-treated with control vehicle (MDSC) or Polyphenon E (MDSC+PE). Asterisk indicates statistical significance, p=0.001. b) Modulation of Foxp3+ Treg by myeloid cells developing in neuroblastoma-conditioned medium. CD11b+ myeloid cells bearing the markers indicated on the top of the panel 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 pre-treated 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 Gapdh, as a loading control, primers, using RNAs extracted from bone marrow cells prepared from 10 mice cultured in the presence or absence of Polyphenon E.

Fig. 6. MDSC-like cells are found in the blood of neuroblastoma patients and Polyphenon E rescues 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. PBMC from the neuroblastoma patient or control individual were stained with 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 in the right side of each FACS plot.

c) Leukocytes infiltrating a resected tumour 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 our 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 in the top of the graph. Error bars indicate standard errors.