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Title: Targeting the EP1 receptor reduces Fas ligand expression and increases the anti-tumor immune response in an *in vivo* model of colon cancer.

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Short Title: Targeting the EP1 signaling pathway reduces colon tumor cell growth *in vivo.*

Key words: PGE_2, EP1 receptor, immune suppression, cancer

Abbreviations used: Cyclooxygenase (COX); Prostaglandin E2 (PGE_2); Fas ligand (FasL); Cytotoxic T cell (CTL); regulatory T cell (Treg); Tumor-associated macrophage (TAM).

Article Category: Research Article; Cancer Cell Biology

Novelty and Impact Statement: This paper demonstrates that the EP1 receptor is a potential target for the treatment of established colon tumors. The anti-tumorigenic effects of targeting the EP1 receptor *in vivo* include reduced expression of FasL, reduced regulatory T cell infiltration and an improved anti-tumor immune response.

Abstract:

Despite studies demonstrating that inhibition of cyclooxygenase-2 (COX-2)-derived prostaglandin E_2 (PGE_2) has significant chemotherapeutic benefits *in vitro* and *in vivo*, inhibition of COX enzymes is associated with serious gastrointestinal and cardiovascular side-effects, limiting the clinical utility of these drugs. PGE_2 signals through four different receptors, (EP1 –EP4), and targeting individual receptor(s) may avoid these side-effects, whilst retaining significant anti-cancer benefits. Here we show that targeted inhibition of the EP1 receptor in the tumor cells and the tumor microenvironment resulted in the significant inhibition of tumor growth *in vivo*. Both dietary administration and direct injection of the EP1 receptor-specific antagonist, ONO-8713, effectively reduced the growth of established CT26 tumors in BALB/c mice, with suppression of the EP1 receptor in the tumor cells alone less effective in reducing tumor growth. This anti-tumor effect was associated with reduced FasL expression and attenuated tumor-induced immune suppression. In particular, tumor infiltration by $CD4^+CD25^+Foxp3^+$ regulatory T cells was decreased while the cytotoxic activity of isolated splenocytes against CT26 cells was increased. $F4/80^+$ macrophage infiltration was also decreased, while there was no change in macrophage phenotype. These findings suggest that the EP1 receptor represents a potential target for the treatment of colon cancer.

Introduction

Numerous studies have demonstrated a link between chronic inflammation and cancer. One such inflammatory mediator is prostaglandin E2 (PGE_2) [1]. PGE_2 is derived from arachidonic acid as a result of the activity of cyclooxygenases (COXs). Numerous mouse models of cancer have demonstrated that COX-2-derived PGE_2 promotes tumor growth [2, 3], with increased expression of COX-2 and PGE_2 being found in various human malignancies. Moreover inhibition of COX-2-derived PGE_2 has significant chemotherapeutic benefits *in vitro* and *in vivo* [1, 4-6, 7]. However, despite these anti-cancer benefits, inhibition of COX enzymes has been found to be associated with serious gastrointestinal and cardiovascular side-effects [4, 7], limiting the clinical utility of these drugs.

PGE_2 activates four different G-protein-coupled receptors – EP1, EP2, EP3 and EP4, with targeting of the receptors offering the potential of anti-neoplastic activity with fewer side-effects. Although most studies to date have identified the EP2 and EP4 receptors as being responsible for the tumor-promoting effects of PGE_2 [8], the EP1 receptor may also be an effective target against colon cancer. Human colon cancer cells express the EP1 receptor *in vivo* [9, 10], while EP1 receptor knockout mice have significantly fewer azoxymethane (AOM)-induced aberrant crypt foci (ACF) [11] and colon cancer development [12]. Furthermore, ONO-8711, a selective EP1 antagonist, significantly reduced AOM-induced ACF and intestinal polyp formation in APC^{Min} mice [11, 13]. Moreover, Kitamura *et al* showed that the EP1 and EP4 receptor subtypes may have separate intrinsic roles and, to some extent, contribute to different aspects of colon tumorigenesis [14]. The EP4 antagonist was found to be more effective at reducing polyp size, whereas the EP1 antagonist was more effective at reducing polyp number [14]. Targeting the EP1 receptor was also shown not to affect prostacyclin production in human endothelial cells [15], important given that inhibition of prostacyclin

production by COX-2 selective inhibitors was shown to be one of the major contributors to the cardiovascular side-effects of these drugs [16].

Despite these promising findings, the mechanisms by which the EP1 receptor promotes tumorigenesis are unclear. Signalling through the EP1 receptor on colon tumor cells was recently shown by us to upregulate expression of Fas ligand (FasL/CD95L) *in vitro*, and may represent one potential mechanism [9]. We and others have shown that expression of FasL or its receptor Fas on tumor cells promotes tumor growth *in vivo* [17, 18]. However, whether induction of tumor-expressed FasL in response to signalling through the EP1 receptor occurs *in vivo* is unclear. Moreover, the EP1 receptor is expressed by both tumor cells and multiple immune cell types. Thus, the pro-tumorigenic effects of the EP1 receptor could be due to PGE_2 signalling through EP1 not on the tumor cells directly but rather on immune cells in the tumor microenvironment. For instance, PGE_2 suppresses the effector functions of helper T (Th) cells, cytotoxic T cells (CTLs) and natural killer (NK) cells, and enhances the accumulation of regulatory T (Treg) cells [19]. PGE_2 also plays a role in the differentiation of monocytes towards an immunosuppressive or 'M2-like' phenotype [20]. Such tumor-associated macrophages (TAM) can play an important role in tumor progression. Whether signalling through the EP1 receptor suppresses the anti-tumor immune response *in vivo* is unknown but has been explored in the present study.

The findings of this study suggest that the EP1 receptor is a potential therapeutic target for the treatment of colon cancer. Blocking EP1 receptor signalling in established tumors was found to inhibit tumor growth *in vivo*. Suppression of tumor growth required inhibition of EP1 receptor signalling in both the tumor cells and non-tumor cells in the tumor microenvironment, and was associated with a reduction in FasL expression, reduced Treg cell infiltration and an improved anti-tumor immune response.

Materials and Methods

Chemicals

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The EP1 receptor antagonist ONO-8713 was a generous gift from Ono Pharmaceuticals Co. Ltd., Osaka, Japan.

Mice and Tumor Model

Female BALB/c mice (4-6 wk) were obtained from Harlan UK Ltd. (Oxon, United Kingdom) and maintained in a pathogen-free environment in the animal facility of University College Cork. Animal experiments were performed in accordance with institutional guidelines using an Animal Research Committee-approved protocol. CT26 cells, a murine colon cancer cell line of BALB/c origin, was kindly provided by Dr. Stephen Todryk (Northumbria University, UK). Cells were maintained *in vitro* at 37°C in a 5% CO_2 humidified atmosphere in DMEM supplemented with 100µg/ml streptomycin, 100U/ml penicillin and 10% fetal bovine serum. To establish s.c. tumors, mice were injected into the right flank with $2.0x10^5$ tumor cells re-suspended in 100µl PBS. Tumor growth was monitored by regular measurement of tumor length (a) and width (b) using a Vernier calliper, and the volume was calculated as ½ ($a \times b^2$). Animals were sacrificed after 48 days.

Generation of FasL$^{Low/negative}$ and EP1 $^{Low/negative}$ colon cancer cells

Cells were transfected with lentiviral particles containing 3 target-specific shRNAs against FasL (sc-35358-V), EP1 (sc-40170-V) or control lentiviral particles containing scrambled shRNA (sc-108080) (Santa Cruz Biotechnology, Santa Cruz, CA), according to the manufacturers' instructions. Briefly, cells were seeded in 24-well plates at a concentration of $2x10^5$ cells/ml. Cells were infected 24hrs later with lentiviral particles in the presence of

5µg/ml polybrene. Cells were cultured in selection medium containing puromycin until resistant clones could be identified. Resistant clones were selected by limiting dilution. Knockdown of FasL and EP1 expression was determined by real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blotting. The clones with the lowest level of FasL or EP1 expression were designated as $CT26^{FasL\ shRNA}$ and $CT26^{EP1\ shRNA}$, respectively, while the clone transfected with scrambled RNA was designated as $CT26^{scr\ shRNA}$.

Proliferation assay

Cell proliferation was measured by resazurin reduction [21]. Cells were seeded at $2x10^5$cells/ml in 96-well plates. After incubation for 24h, media supplemented with 44µM resazurin was added and resazurin reduction to resorufin measured fluorometrically using a GENios plate reader (TECAN, Grodig, Austria) and Xfluor spreadsheet software. Results obtained were expressed in fluorescence units (FU) and the percentage viability calculated as follows: (FU treated/ FU control) x 100.

Tumor cell and tumor-associated macrophage isolation

Tumors were sliced into 1-3 mm^3 pieces and incubated for 1hr at 37°C with collagenase/dispase solution (Roche Diagnostics, Mannheim, Germany). After washing in PBS, a single cell suspension was obtained by passing the cells through a cell strainer (Benton Dickonson, Franklin Lakes, NJ). Tumor-associated macrophages were isolated by seeding the cell suspension on 24 well plates at a concentration of $0.5x10^5$ cells/ml. Cells were washed 3hr later with PBS to remove non-adherent cells. The adherent population was characterised by immunofluorescence and morphological criteria.

Splenocyte isolation

Single cell suspensions were obtained by mechanical disruption with a syringe plunger in RPMI 1640 medium supplemented with 10% FCS. The suspensions were then passed through a cell strainer and red cells lysed using red cell lysis buffer. Cells were then washed and resuspended in complete media.

Autologous mixed lymphocyte-tumor reaction.

Splenocytes were stimulated with IL-2 and irradiated CT26 tumor cells (6.2 Gy at 3 Gy/min) at a ratio of 12:1. After 5 days in culture, the *in vitro* stimulated splenocytes were collected and tested for their cytotoxic activity against CT26 cells using the Ziva Tox Ultrasensitive Cytotoxicity Assay (Jaden BioScience Inc., San Diego, CA), according to the manufacturer's instructions. Briefly, BrdU was added for the last 4hr of incubation, cells were fixed, washed and incubated with stringency solution. Cells were then incubated with anti-BrdU antibody conjugate solution, washed, followed by incubation with preparation solution prior to addition of the CDP*Star®Chemiluminescent substrate. Chemiluminescense was detected using a Glomax multi-detection system luminometer (Promega, Madison, WI).

Flow Cytometry.

Single cell suspensions from tumor tissue were prepared. Monoclonal antibodies to CD8, F4/80 (BD Biosciences, New Jersey, USA), CD4 and CD25 (eBioscience, San Diego, USA) were used to label the cells for phenotypic analysis. Antibodies to the transcription factor Foxp3 (eBioscience, San Diego, USA) were used to label permeabilized cells. Debris and dead cells were excluded from flow cytometric analysis using a selection gate on forward scatter and side scatter cellular properties. The frequency of Treg cells was assessed by gating $CD4^+$ cells only and subsequently plotting $CD25^+$ cells against $Foxp3^+$ cells. Cell populations were assessed using the Accuri C6 Flow Cytometer System and CFlow commercial software.

Western blotting.

Tumor cells were lysed for 1hr on ice in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150mM NaCl and 1% Triton-X 100, supplemented with complete protease inhibitors (Roche Diagnostics). Equal amounts of protein were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked for 1hr at room temperature with 5% non-fat dry milk in PBS containing 0.1% Tween-20. Membranes were probed overnight at 4°C with anti-FasL specific antibody (Abcam, Cambridge, UK), or anti-EP1, anti-EP2, anti-EP3 or anti-EP4 specific antibodies (Cayman Chemical, Ann Arbor, MI). Membranes were washed and incubated with the appropriate secondary antibody conjugated with HRP (Dako Corp.,Carpinteria, CA, USA). Results were visualised by chemiluminescence detection (Millipore, Billerica, MA). As an internal control, all membranes were subsequently stripped of the first antibody and reprobed with anti-β-actin-specific antibody (Sigma-Aldrich).

RT-PCR

Total cellular RNA was isolated using the GenElute Mammalian Total RNA Mini kit according to the manufacturer's instructions. cDNA was synthesised using the SuperScript Vilvo kit (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed using an Applied Biosystems PRISM 7500 PCR system (Applied Biosystems, Foster City, CA, USA) and Syber Green Jumpstart Taq ReadyMix. All samples were run in triplicate and relative quantitation calculated using the $2^{-\Delta\Delta Ct}$ method.

PGE_2 ELISA

Tumor cells were isolated and seeded at $0.5x10^5$ cells/ml. After 24hrs supernatants were harvested and PGE_2 levels determined in triplicate by ELISA (Arbor Assays, Ann Arbour, MI).

Statistical Analysis

Means with SEM are represented in each graph. Statistical analysis was performed using GRAPHPAD PRISM version 5.0 for Windows (GraphPad Software, San Diego, CA). P-values were calculated using the unpaired Student's t-test with $p< 0.05$ considered statistically significant.

Results

Blocking EP1 receptor suppresses tumor growth in vivo.

In vivo studies to date on the role of the EP1 receptor in colon tumorigenesis have investigated whether suppressing EP1 receptor signalling prior to tumor development, either using an EP1 receptor antagonist or EP1 receptor knockout mice, is effective in preventing tumorigenesis [11, 12]. However, whether targeting the EP1 receptor has therapeutic potential for the treatment of established tumors is unknown. To investigate this, BALB/c mice were injected subcutaneously with CT26 cells. Beginning either 20 days (n=8) after tumor cell inoculation, at which time all mice had palpable tumors, or after 27 days (n=8), the specific EP1 receptor antagonist ONO-8713 was administered orally as a powder mixed into their daily feed at 1000ppm. 1000ppm was selected based on the findings of a preliminary study using 500ppm and 1000ppm (supplemental Fig.S1). ONO-8713 is a potent second generation EP1 receptor antagonist [22]. Consistent with having a *Ki* binding value for the EP1 receptor of 0.3nM and a *Ki* value of greater than 1000nM for the other EP and IP receptor subtypes, it has been shown not to have agonistic or antagonistic actions on the other prostanoid receptors [22]. Oral administration was used since this was successful in reducing ACF formation and intestinal polyp formation in APC^{Min} mice [11]. Moreover, therapeutically this is an ideal means of drug administration. Food consumption was monitored every second day, with no difference between the groups seen. Since there was the possibility that the antagonist would not reach the site of tumor inoculation, ONO-8713 was administered by direct injection into the tumors of one group of mice (n=5) three times a week (30mg/kg per injection) beginning at day 27. Although twice weekly injection of established tumors was used in the preliminary study (supplemental Fig.S1), this was increased to thrice weekly to determine if this would result in a greater reduction in tumor growth.

As shown in figure 1*A*, blocking the EP1 receptor suppressed tumor growth *in vivo*. Beginning ONO-8713 administration at day 20 (when the average tumor size was 0.26cm^3) significantly suppressed tumor growth by 65% (p=0.023). Delaying the start of administration until day 27 when the tumors were larger (average size 0.5cm^3) also reduced tumor growth (42% reduction), although this was not significant. Direct injection of established tumors (average size 0.5cm^3) with ONO-8713 suppressed tumor growth by 78% (p=0.023), with three of the five established tumors actually regressing in size. There was extensive necrosis, however, present in these injected tumors.

This reduction in tumor growth *in vivo* could be due to suppression of the EP1 receptor either on the tumor cells or non-tumor cells in the tumor microenvironment. To evaluate this and to control for the effect of EP1 receptor expression by tumor cells, the EP1 receptor was suppressed in CT26 tumor cells prior to s.c. inoculation. Numerous clones were generated and the clone with the greatest reduction was selected for *in vivo* analysis (Fig.1*B*). Suppressing tumor expression of the EP1 receptor had no effect on tumor development but did result in a decrease in tumor growth (20%) *in vivo* (Figure 1*A*), suggesting that inhibition of tumor cell growth *in vivo* is predominantly due to effects of the EP1 receptor antagonist on the host, rather than on the tumor cells directly. Indeed the e*x vivo* growth of tumor cells isolated from the tumor-bearing mice was unaffected by EP1 receptor antagonism (Fig.1*C*). However, CT26$^{\text{EP1 shRNA}}$ cells still expressed the EP1 receptor (Fig.1*B*); albeit at a much lower level. This lower level of expression may still be capable of transducing a pro-tumorigenic signal.

CT26 cells secrete PGE_2 and express all four EP receptors (supplemental Fig.S2). Changes in the level of PGE_2 in the tumors could thus affect signalling through the other receptors. To determine whether the reduced tumor growth correlated with changes in the level of PGE_2, endogenous PGE_2 levels in the tumors were determined by ELISA (Fig.1*D*). Although PGE_2

levels were slightly lower in treated tumors, there was no significant difference between the groups, suggesting that the reduced tumor growth was not due to alterations in PGE_2-mediated signalling through the other EP receptors.

Inhibition of EP1 receptor suppresses expression of FasL by colon tumors.

We have previously shown that PGE_2 signalling through the EP1 receptor increases FasL expression in human colon tumor cells [9], and that suppressing FasL expression by tumor cells reduces tumorigenesis *in vivo* [17]. Consistent with these findings, both knockdown of the EP1 receptor by shRNA or treatment with ONO-8713 suppressed FasL expression in CT26 cells *in vitro* (Fig.2*A*) and in tumors *in vivo* (Fig.2*E*). Moreover, both tumor development (Fig. 2*C*) and growth (Fig.2*D*) was significantly suppressed when the inoculated tumors had FasL expression suppressed in advance by shRNA (Fig.2*B*). A third of the mice inoculated with $CT26^{FasL\ shRNA}$ did not develop tumors (Fig.2*C*), and in the mice that did develop tumors (7/11), the growth of the tumors was significantly reduced ($p<0.007$) (Fig.2*D*). Interestingly, although the initial appearance of the tumors was delayed, by day 48 however, $CT26^{FasL\ shRNA}$ tumors that did develop were similar in size to those that were treated with ONO-8713 beginning at day 20, with no statistical difference between them.

Incorporation of ONO-8713 into the diet of $CT26^{FasL\ shRNA}$ tumor-bearing mice beginning at day 27 did not result in a further significant reduction in tumor volume (Fig.2*D*). Given that the growth of $CT26^{FasL\ shRNA}$ tumors was already greatly reduced, it was perhaps unsurprising that ONO-8713 administration did not have any additive effects. From day 36 on, however, tumor growth was halted in these mice, in contrast to the slow increase in tumor growth seen in non-treated $CT26^{FasL\ shRNA}$ tumors (Fig.2*D*). Moreover, although FasL was not reduced to the same extent in the $CT26^{EP1\ shRNA}$ clones *in vitro* compared to the ONO-8713-treated cells and the $CT26^{FasL\ shRNA}$ clones, FasL mRNA and protein levels were reduced in the $CT26^{EP1}$

^{shRNA} -derived tumors *in vivo* (Fig.2*E*). Despite this, growth of these tumors was significantly greater than that of CT26$^{FasL\ shRNA}$ tumors ($p<0.0179$). Together these findings suggest that PGE_2 may have additional effects on cells in the tumor microenvironment that are affected by ONO-8713 treatment.

Blocking EP1 receptor signalling enhances intratumoral CD8$^+$ T cells, suppresses intratumoral CD4$^+$ T cells and Treg cells, and increases CTL activity.

To assess whether blocking PGE_2-EP1 signalling affects T cell recruitment, tumors excised after 48 days were dissociated with collagenase/dispase, and single cell suspensions were analysed by flow cytometry. Infiltration of the tumors by CD4$^+$ T cells was significantly decreased in all groups on blocking EP1 receptor signalling (Fig.3*B*). In contrast, CD8$^+$ T cell infiltration was increased, although this was not significant (Fig.3*B*). Antagonising the EP1 receptor also reduced the number of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells present in the tumors, with a significant reduction occurring following oral administration of ONO-8713 beginning at day 20 and direct injection of ONO-8713 into the tumors (Fig.3*B*). In contrast, the level of Treg cells in CT26$^{EP1\ shRNA}$ tumors was increased, suggesting that EP1 receptor signalling in cells in the tumor microenvironment, rather than the tumor cells themselves, plays an important role in Treg cell recruitment and/or expansion. Given that Treg cells are potent suppressors of the anti-tumor immune response [23], failure to CT26$^{EP1\ shRNA}$ tumors to block the expansion of Treg cells may account for the enhanced growth of these tumors *in vivo* relative to ONO-8713-treated mice.

CTLs are a critical component of the immune response to tumors, with CTL activity declining with progressive tumor growth. To determine if blocking the EP1 receptor affects PGE_2-mediated suppression of CTL activity, CTL were generated from tumor-bearing mice and examined for cytotoxic activity against CT26 cells (Fig.3*C*). CTL from CT26$^{scr\ shRNA}$

tumor-bearing mice had an average CTL activity of 33%, while CTL from non-tumor-bearing mice exhibited a cytotoxicity of 85%. Cytotoxicity was significantly increased in effector cells generated from tumor-bearing mice orally administered ONO-8713 beginning at day 20 (83%) ($p=0.0031$) and directly injected with ONO-8713 (92%) ($p<0.0001$), relative to those derived from untreated $CT26^{scr\ shRNA}$ tumor-bearing mice. Although CTL generated from $CT26^{EP1\ shRNA}$ tumors (70%) ($p< 0.0001$) and tumor-bearing mice orally administered ONO-8713 beginning at day 27 (77%) ($p< 0.0001$) exhibited increased CTL activity relative to those generated from non-treated tumor-bearing mice (33%), interestingly this CTL activity was significantly less than that of the PBS control mice (85%), $p=0.0029$ and $p<0.0001$, respectively.

Blocking EP1 alters TAM infiltration but does not play a role in the polarisation of tumor-associated macrophages (TAM).

Cytokines implicated in the differentiation of macrophages towards an immunosuppressive M2-like phenotype include PGE_2 and IL-6 [20]. Given the important role played by TAM in tumor development, the effect of blocking the EP1 receptor on TAM infiltration and polarisation was determined. Analysis revealed significantly reduced levels of $F4/80^+$ cells within the tumors when administration of the antagonist began at day 20, or following direct injection of the antagonist (Fig.4*A*). In contrast, TAM levels were comparable to that of control tumors when the initiation of antagonist administration was delayed until day 27 when the tumors were larger, and when EP1 was directly suppressed in the tumor cells.

These macrophages retained characteristics of the M2-like phenotype. They were $IL\text{-}12^{low}$, $IL\text{-}6^{low}$ and nitric oxide synthase $(NOS)^{low}$ (Fig.4*B*), with no change in PGE_2 secretion (Fig. 4*C*). Although macrophages from ONO-8713 injected tumors were $IL\text{-}12^{high}$, $IL\text{-}6^{high}$, NOS^{high} and secreted significantly less PGE_2, suggestive of a more anti-tumorigenic M1-like

phenotype, this may be due to the high level of necrosis present in these injected tumors. Indeed, ingestion of necrotic cells has been shown to increase the transcription of several cytokines by macrophages [24].

Discussion

PGE_2 is the most abundant prostaglandin found in a variety of human malignancies, facilitating tumor progression by stimulating cell proliferation and angiogenesis, and suppressing the anti-tumor immune response [1, 19]. PGE_2 released from apoptotic tumor cells may also help to repopulate tumors following chemo- and radio-therapeutic regimens, with PGE_2 being shown to stimulate the growth of therapy-resistant tumor cells [25]. Despite being one of the PGE_2 receptors implicated in tumorigenesis [11, 12], the pro-tumorigenic effects of the EP1 receptor are very poorly understood. Importantly from a therapeutic standpoint, in the current study we demonstrate that targeting the EP1 receptor significantly retards the growth of established tumors *in vivo*. This inhibition of tumor growth was unlikely due to direct inhibition of PGE_2-induced tumor cell growth, as suppressing EP1 receptor expression in tumor cells alone was far less effective in reducing tumor growth *in vivo* than when multiple cell types within the tumor microenvironment were affected. Moreover, growth of the tumor cells *in vitro* was unaffected by suppression of the receptor.

One of the potential mechanisms by which the EP1 receptor mediates its pro-tumorigenic effects is upregulation of FasL expression in tumor cells [9]. Fas signalling in response to binding of FasL has been shown to have numerous pro-tumorigenic effects, with expression of Fas and FasL by malignant cells being associated with enhanced tumor growth, inflammation, metastases and apoptotic depletion of tumor-infiltrating lymphocytes *in vivo* [26, 27]. Indeed, suppressing EP1 receptor signalling was found to effectively suppress FasL expression by tumor cells *in vitro* and *in vivo*. However, despite exhibiting reduced FasL expression, the reduction in growth of CT26 $^{\text{EP1 shRNA}}$ tumors was far less than that of CT26 $^{\text{FasL shRNA}}$ tumors or ONO-8713-treated tumors. Although CT26 $^{\text{EP1 shRNA}}$ clones exhibited reduced FasL expression, the level of expression was greater than that seen in the CT26 $^{\text{FasL shRNA}}$ clones and following treatment with ONO-8713 *in vitro*. Oligomerization of FasL is

required for triggering of Fas signalling [28], and thus the threshold of FasL expression may play a role in determining whether FasL promotes tumorigenesis, with the CT26 $^{EP1\ shRNA}$ tumor cells exhibiting a level of FasL expression sufficient to mediate the pro-tumorigenic effects of FasL. Alternatively, the greater reduction in tumor growth seen in ONO-8713-treated tumors may be due to the affect of the antagonist on PGE_2/EP1 receptor signalling in cells in the tumor microenvironment.

Immune cells are a prominent component of solid tumors. Such cells, if appropriately activated, can mediate tumor rejection [20]. The EP receptors are expressed by multiple immune cell types [29], and thus the EP1 antagonist could potentially suppress EP1 receptor signaling in these cells. In our study we found that the pro-tumorigenic effects of the EP1 receptor are also immunological in nature. Blocking EP1 receptor signalling reduced the level of intratumoral $CD4^+$ T cells and increased the level of $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cell recruitment was also altered in CT26$^{EP1\ shRNA}$ tumors, suggesting that this alteration in T cell recruitment may be due to changes in secretion of T cell chemotactic factors by the tumor cells. For instance, both IL-16 and CCL5 (RANTES) have preferential effects on $CD4^+$ T cell chemotaxis [30]. Alternatively, this difference in the level of the intratumoral $CD4^+$ and $CD8^+$ T cells could be due to differences in the sensitivity of the T cell subsets to the anti-proliferative effects of PGE_2, with $CD8^+$ T cells being shown to be more susceptible to PGE_2-mediated inhibition of proliferation than CD4+ T cells [31]. Although EP2 and EP4 receptors have been shown to predominantly mediate the anti-proliferative activity of PGE_2 on lymphocyte proliferation [29], this study did not subdivide the lymphocytes into $CD4^+$ and $CD8^+$ cells.

$CD8^+$ T cells, if appropriately activated, can mediate tumor rejection, with $CD8^+$ CTL among the major anti-tumor effector mechanisms [32, 33]. Such anti-tumor activity is strongly suppressed by Treg cells [23]. Together with altered T cell infiltration, blocking EP1 receptor

signalling was also associated with reduced levels of Treg cells within the tumors and enhanced cytotoxic activity of T cells. Indeed mice with the greatest reduction in tumor growth *in vivo* also had the greatest reduction in Treg cells and the highest CTL activity. Changes in the level of Treg cells and CTL activity are likely due to effects of the antagonist on non-tumor cells in the tumor microenvironment, as the level of Treg cells remained high in CT26 $^{EP1\ shRNA}$ tumors. CTL generated from splenocytes from these mice also exhibited the least CTL activity of all the treatment groups. Indeed, this change in the level of Treg cell infiltration represents one of the major differences between ONO-8713-treated tumor cells and EP1-suppressed CT26 $^{EP1\ shRNA}$ tumor cells, suggesting that PGE_2 acts in a paracrine fashion to recruit Treg cells to tumors.

How blocking the EP1 receptor alters Treg cell infiltration and/or expansion is unclear. Induction of Treg cells and suppression of CTL activity in response to PGE_2 have previously been ascribed to activation of cAMP/protein kinase A (PKA) by EP2 and EP4 [34, 35]. The EP1 receptor has traditionally been associated with the activation of Ca^{2+} signalling through coupling to G_q and the activation of phospolipase C (PLC). A recent study, however, has shown that the EP1 receptor can also activate PKA, independently of cAMP [36], which may potentially accounting for this previously unknown role for the EP1 receptor in the induction of Treg cells in the tumor microenvironment.

The level of TAM present within the tumors was also suppressed on blocking of the EP1 receptor in the tumor microenvironment. TAM are a major constituent of the leukocyte infiltrate in solid tumors and are recruited to tumors by tumor-derived chemotactic factors [37]. Such TAM have been shown to be skewed in tumors towards an immunosuppressive or 'M2'-like phenotype by environmental cues such as PGE_2, IL-10 and IL-6 [20, 38, 39]. Skewing of TAM towards this M2 phenotype favors tumor progression by suppressing T cell proliferation, stimulating tumor cell proliferation, angiogenesis, tumor cell migration and

increasing stroma reaction. Consistent with the ability of TAM to promote tumor progression, those tumors which showed the greatest suppression of tumor growth also showed the greatest reduction in TAM. This reduction in TAM was not due to alterations in CCL2 (data not shown), suggesting other macrophage-chemotactic factors may play a more important role in this model. Moreover, although the level of PGE_2 present in the tumor microenvironment following blocking of the EP1 receptor was unaltered, the macrophages retained an M2-like phenotype, suggesting that the EP1 receptor is involved in TAM recruitment but not polarisation.

In conclusion, we have shown that the EP1 receptor mediates several of the pro-tumorigenic effects of PGE_2 and that EP1 receptor antagonism is effective in reducing the growth of established tumors. Given that we have previously shown that human colon tumors *in vivo* express the EP1 receptor [9], and that EP1 receptor antagonists inhibit chemically induced breast cancer development in rats [40] and reduce the number of skin tumors per mouse following UVB exposure [41], EP1 receptor antagonists may be good candidates as chemotherapeutic agents for not only colon, but also other cancers.

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Figure Legends

Figure 1. Inhibiting EP1 receptor signalling reduces the growth of pre-established tumors *in vivo*. *(A)* $2x10^5$ CT26 colon tumor cells were s.c. inoculated into BALB/c mice. Tumor cells inoculated and treatments are indicated. Unless otherwise indicated, ONO-8713 was administered in the basal diet. Tumor growth was monitored as described in the *Materials and Methods.* Data points represent the mean value +/- SEM and show the findings of one tumor challenge experiment. *(B)* EP1 receptor expression was suppressed in $CT26^{EP1\ shRNA}$ cells. The efficiency of suppression of EP1 receptor was assessed by RT-PCR and Western blotting. For the RT-PCR, data was normalised to β-actin and analysed using the $2^{-\Delta\Delta CT}$ method. Immunoblotting for β-actin was used as the loading control. *(C)* Proliferation of tumors cells *ex vivo* was unaffected by EP1 receptor expression or EP1 receptor signalling. Cell proliferation was determined by resazurin reduction. Proliferation was normalised to tumors cells isolated from $CT26^{scr\ shRNA}$ tumors. Data shown are the findings from three independent experiments. *(D)* Blocking EP1 receptor signalling doesn't affect PGE_2 secretion by tumor cells. Tumor cells were isolated from all tumor-bearing mice and cultured *ex vivo* for 24hr. Cell culture supernatants were harvested and the level of PGE_2 measured in triplicate by ELISA.

Figure 2. Signalling through the EP1 receptor upregulates FasL expression *in vivo*. *(A)* CT26 cells were treated with increasing concentrations of ONO-8713 for 24hr, or FasL and EP1 receptor expression was suppressed in CT26 $^{FasL\ shRNA}$ and CT26 $^{EP1\ shRNA}$ cells, respectively. FasL expression was determined by Western blotting. Expression of the housekeeping gene β-actin was used as an internal control. Results shown are representative of three independent experiments. *(B)* FasL expression was suppressed in $CT26^{FasL\ shRNA}$ cells. The efficiency of suppression of FasL was assessed by RT-PCR and Western blotting. RT-PCR data was

normalised to β-actin and analysed using the $2^{-\Delta\Delta CT}$ method. *(C)* Suppression of FasL expression reduces tumor development *in vivo*. Cells were s.c. inoculated into BALB/c mice. Mice were palpated thrice weekly to detect tumors. Data shown are the percentage of mice remaining tumor free on the indicated day. *(D)* Suppression of FasL expression reduces tumor growth *in vivo*. $2x10^5$ $CT26^{scr\ shRNA}$ or $CT26^{FasL\ shRNA}$ were s.c. inoculated into BALB/c mice. ONO-8713 was administered in the basal diet with the day of initiation of treatment indicated. Tumor growth was monitored as described in the *Materials and Methods.* Data points represent the mean value +/- SEM and show the findings of one tumor challenge experiment. (E) Blocking EP1 signalling suppresses FasL expression *in vivo*. Tumors were excised after 48 days. FasL expression in the excised tumors was analysed by RT-PCR and Western blotting. For Western blotting, 30µg of protein from all tumors per treatment group was pooled.

Figure 3. Blocking EP1 receptor signalling alters the intratumoral immune cell population. *(A)* Flow cytometric analysis revealed that suppressing EP1 receptor signalling significantly reduces the percentage of $CD4^+$ T cells and increases the percentage of $CD8^+$ T cells within the tumors. Data points represent the mean +/- SEM; n≥5 mice/group. *(B)* Representative dot plots show the gating strategy for the identification of Treg cells within the tumor tissues. Treg cells were characterised as the ratio of $CD25^+$ $Foxp3^+$ cells in the CD4 gate. Treg cell infiltration was suppressed by EP1 receptor antagonism. Data points represent the mean +/- SEM; n≥5 mice/group. *(C)* CTL activity is recovered in ONO-8713-treated tumor-bearing mice and in mice inoculated with $CT26^{EP1\ shRNA}$ tumor cells. Splenocytes were isolated and after 5 days *in vitro* stimulation with irradiated CT26 cells and IL-2, cytotoxic activity was assessed as described in the *Materials and Methods*. Data represents splenocytes from one or two mice per treatment group and is representative of three to five independent experiments. (**** $p<0.0001$; *** $p<0.001$; ** $p<0.01$).

Figure 4. Antagonising the EP1 receptor reduces macrophage infiltration but does not alter macrophage polarisation. *(A)* Flow cytometric analysis revealed that suppressing EP1 receptor signalling reduces the percentage of $F4/80^{+}$ cells within the tumors. Data points represent the mean +/- SEM; n≥5 mice/group. *(B)* The EP1 receptor doesn't play a role in TAM polarisation. Macrophages were isolated from a minimum of five excised tumors per treatment group as described in the *Materials and Methods*. RNA was isolated, pooled and changes in transcription of IL-12, IL-6 and NOS2 determined by RT-PCR. Data was normalised to β2-microglobulin and analysed using the $2^{-\Delta\Delta CT}$ method. Results represent the mean +/- SEM. *(C)* Blocking signalling through the EP1 receptor doesn't alter secretion of PGE_2 by TAM. Isolated macrophages (n=3 per group) were cultured *ex vivo* for 24hr. Cell culture supernatants were harvested and the level of PGE_2 measured in triplicate by ELISA.

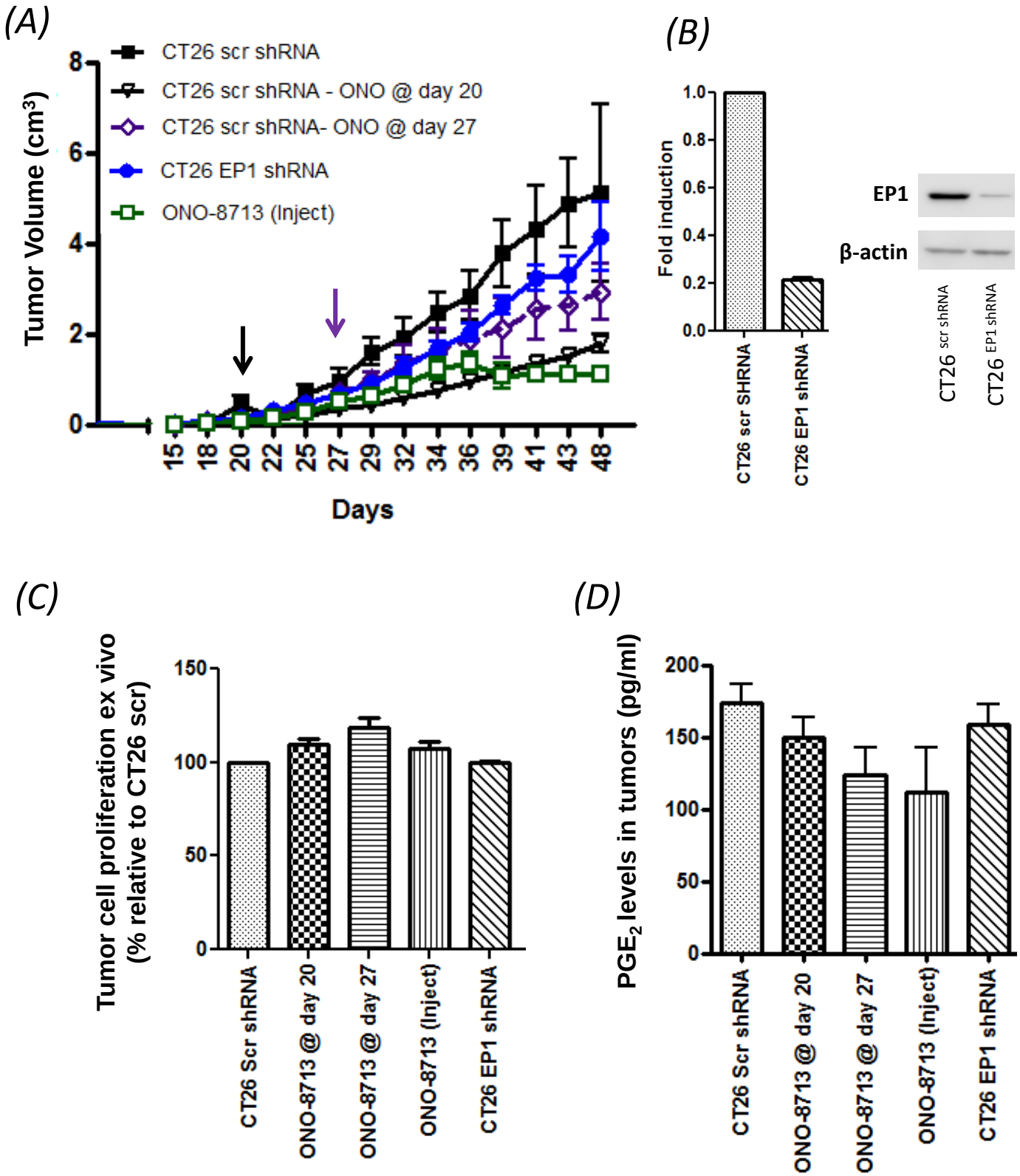

(A)
CT26 scr shRNA
CT26 scr shRNA - ONO @ day 20
CT26 scr shRNA- ONO @ day 27
CT26 EP1 shRNA
ONO-8713 (Inject)
Tumor Volume (cm³)
Days
(B)
Fold induction
CT26 scr SHRNA
CT26 EP1 shRNA
EP1
β-actin
CT26 scr shRNA
CT26 EP1 shRNA
(C)
Tumor cell proliferation ex vivo
(% relative to CT26 scr)
CT26 Scr shRNA
ONO-8713 @ day 20
ONO-8713 @ day 27
ONO-8713 (Inject)
CT26 EP1 shRNA
(D)
PGE2 levels in tumors (pg/ml)
CT26 Scr shRNA
ONO-8713 @ day 20
ONO-8713 @ day 27
ONO-8713 (Inject)
CT26 EP1 shRNA

Figure 1

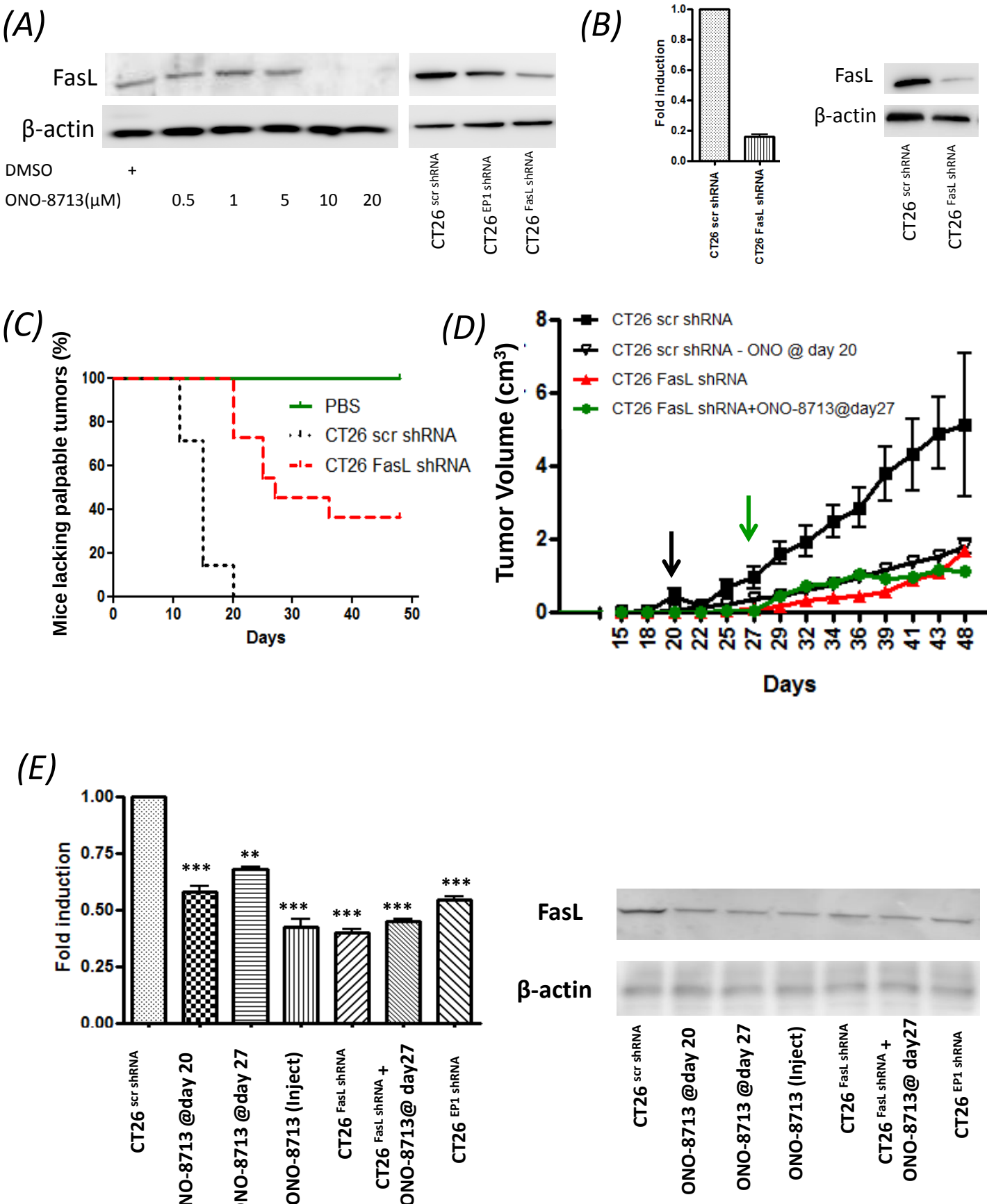

(A)
FasL
β-actin
DMSO
+
ONO-8713(μM)
0.5
1
5
10
20
CT26 scr shRNA
CT26 EP1 shRNA
CT26 FasL shRNA
(B)
Fold induction
CT26 scr shRNA
CT26 FasL shRNA
FasL
β-actin
CT26 scr shRNA
CT26 FasL shRNA
(C)
Mice lacking palpable tumors (%)
PBS
CT26 scr shRNA
CT26 FasL shRNA
Days
(D)
Tumor Volume (cm³)
CT26 scr shRNA
CT26 scr shRNA - ONO @ day 20
CT26 FasL shRNA
CT26 FasL shRNA+ONO-8713@day27
Days
(E)
Fold induction
CT26 scr shRNA
ONO-8713 @day 20
ONO-8713 @day 27
ONO-8713 (Inject)
CT26 FasL shRNA
CT26 FasL shRNA + ONO-8713@ day27
CT26 EP1 shRNA
FasL
β-actin

Figure 2

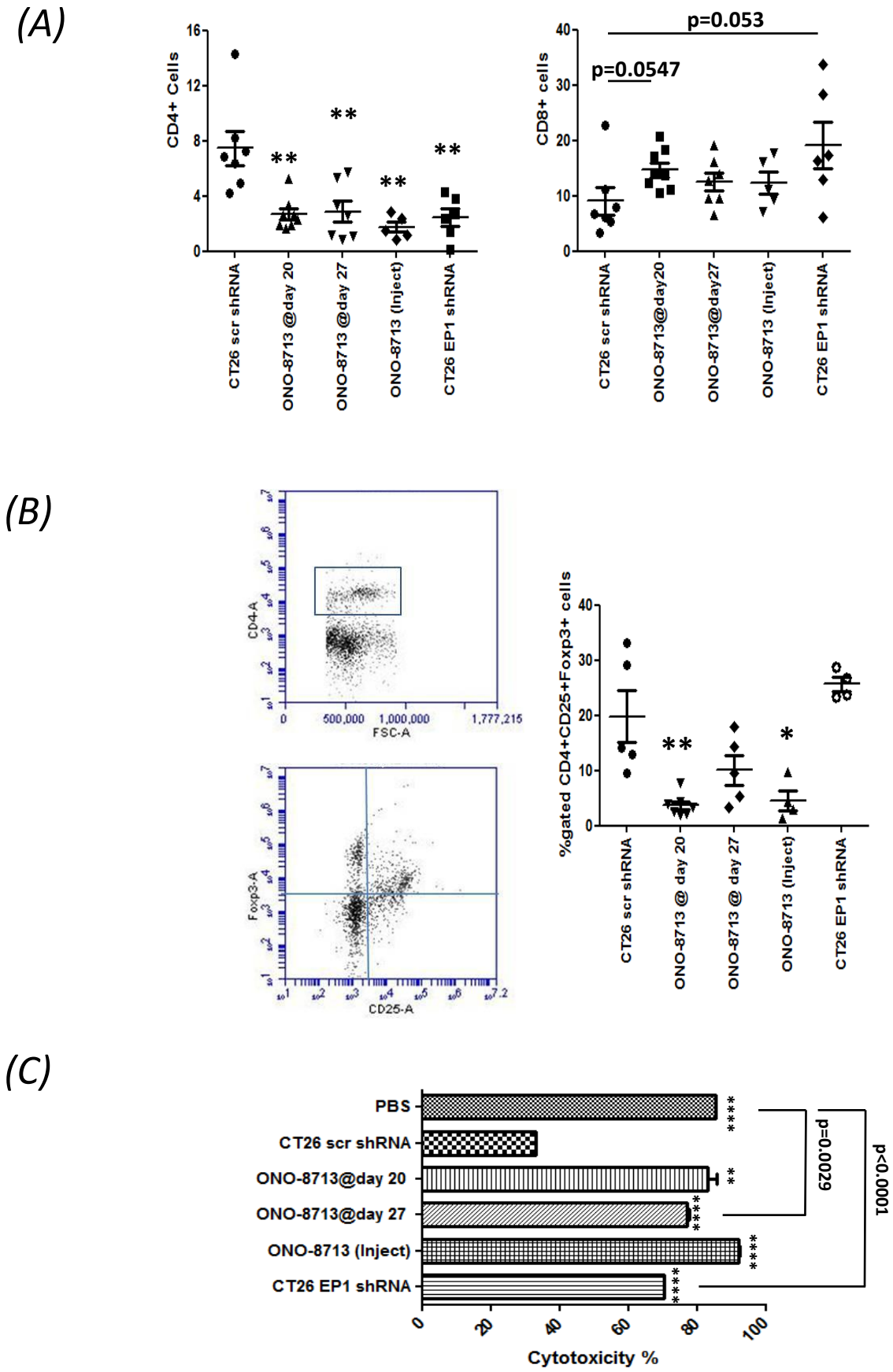
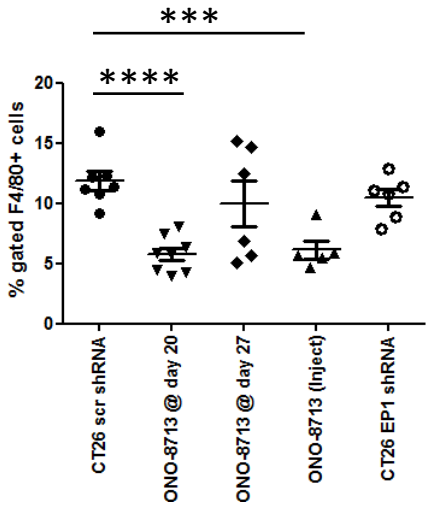
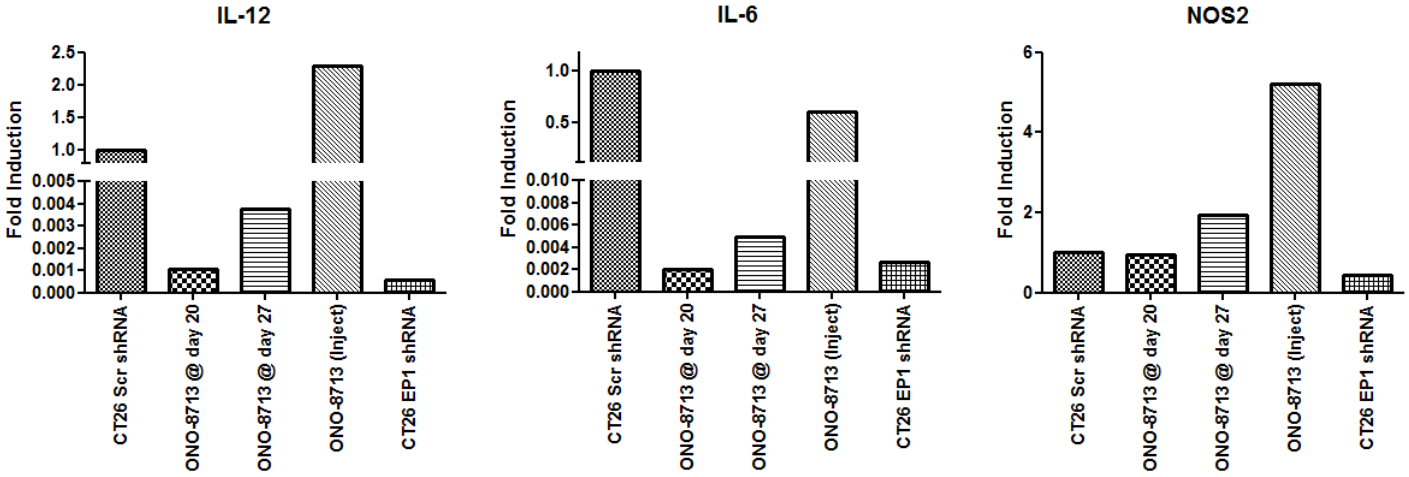

(A)
CD4+ Cells
16
12
8
4
0
**
**
**
**
CT26 scr shRNA
ONO-8713 @day 20
ONO-8713 @day 27
ONO-8713 (Inject)
CT26 EP1 shRNA
CD8+ cells
40
30
20
10
0
p=0.053
p=0.0547
CT26 scr shRNA
ONO-8713@day20
ONO-8713@day27
ONO-8713 (Inject)
CT26 EP1 shRNA
(B)
CD4-A
FSC-A
0
500,000
1,000,000
1,777,215
Foxp3-A
CD25-A
%gated CD4+CD25+Foxp3+ cells
40
30
20
10
0
**
*
CT26 scr shRNA
ONO-8713 @ day 20
ONO-8713 @ day 27
ONO-8713 (Inject)
CT26 EP1 shRNA
(C)
PBS
CT26 scr shRNA
ONO-8713@day 20
ONO-8713@day 27
ONO-8713 (Inject)
CT26 EP1 shRNA
****
**
****
****
***
p=0.0029
p<0.0001
0
20
40
60
80
100
Cytotoxicity %

Figure 3

(A)


***
****
% gated F4/80+ cells
20
15
10
5
0
CT26 scr shRNA
ONO-8713 @ day 20
ONO-8713 @ day 27
ONO-8713 (Inject)
CT26 EP1 shRNA

(B)


IL-12
Fold Induction
2.5
2.0
1.5
1.0
0.005
0.004
0.003
0.002
0.001
0.000
CT26 Scr shRNA
ONO-8713 @ day 20
ONO-8713 @ day 27
ONO-8713 (Inject)
CT26 EP1 shRNA
IL-6
Fold Induction
1.0
0.5
0.010
0.008
0.006
0.004
0.002
0.000
CT26 Scr shRNA
ONO-8713 @ day 20
ONO-8713 @ day 27
ONO-8713 (Inject)
CT26 EP1 shRNA
NOS2
Fold Induction
6
4
2
0
CT26 Scr shRNA
ONO-8713 @ day 20
ONO-8713 @ day 27
ONO-8713 (Inject)
CT26 EP1 shRNA

(C)

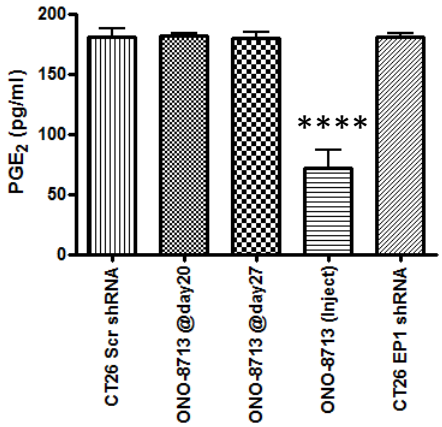
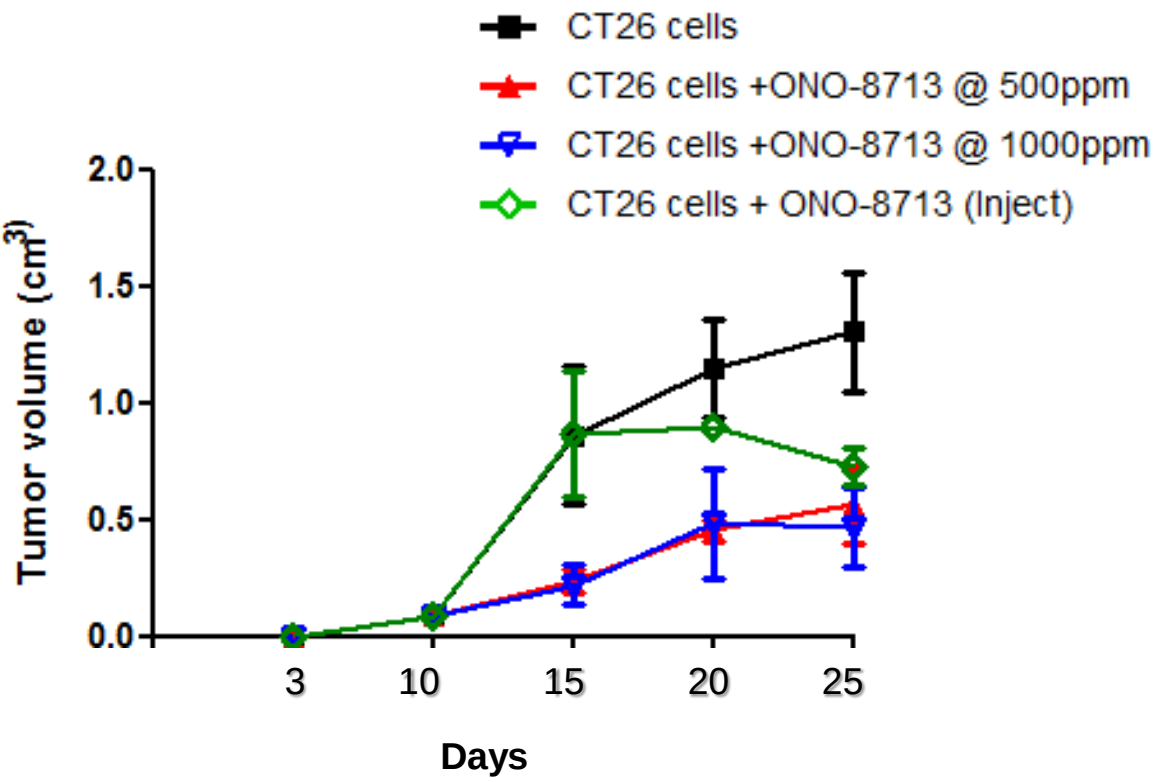

PGE2 (pg/ml)
200
150
100
50
0
****
CT26 Scr shRNA
ONO-8713 @day20
ONO-8713 @day27
ONO-8713 (Inject)
CT26 EP1 shRNA

Figure 4

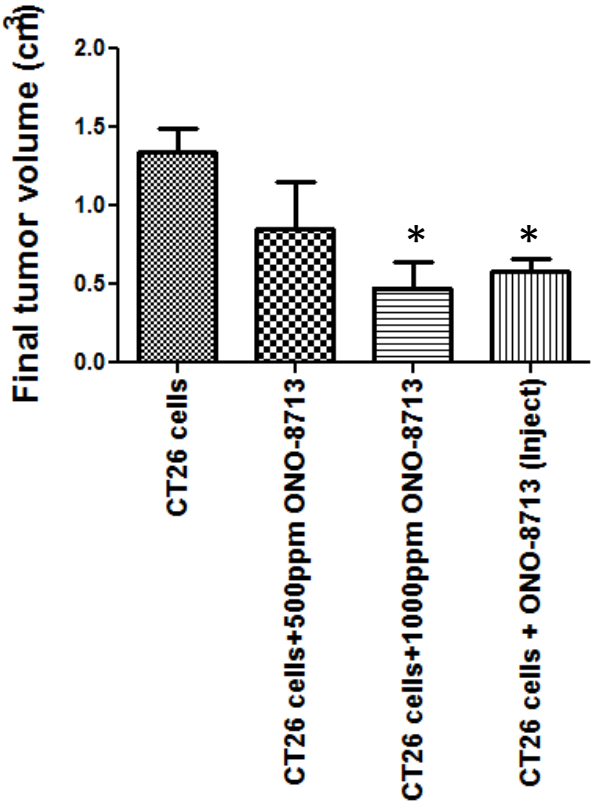
Supplementary Figure S1: ONO-8713 administration reduces the growth of pre-established tumors *in vivo*. *(A)* $4x10^5$ CT26 colon tumor cells were s.c. inoculated into BALB/c mice. ONO-8713 was administered in the basal diet at a final concentration of either 500 or 1000ppm, or directly injected into tumors twice weekly once they had reached a size of $0.5cm^3$. Tumor growth was monitored by measurement of tumor length (a) and width (b) using a vernier caliper, and the volume was calculated as ½ ($a \times b^2$). Data points represent the mean value +/- SEM; n=3 mice/group and show the findings of one tumor challenge experiment. *(B)* After 25 days animals were sacrificed and the final tumor volume was recorded.

Supplementary Figure S2: CT26 cells express all four EP receptors and secrete PGE_2. Expression of the receptors is unaffected by PGE_2. *(A)* Cells were treated with 1μM PGE_2 for 24hr and EP receptor expression detected by Western blotting. Expression of the housekeeping gene β-actin was used as an internal control. Results are representative of three independent experiments *(B)* Suppressing the EP1 receptor or FasL expression does not alter PGE_2 secretion *in vitro*. Cells were cultured for 24 hrs with or without 10μM ONO-8713 as indicated. Cell culture supernatant was collected and PGE_2 levels were determined in triplicate by ELISA.

(A)

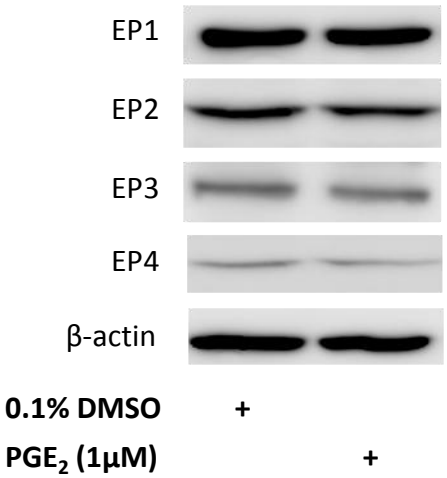


(B)



Supplementary Figure 1

(A)



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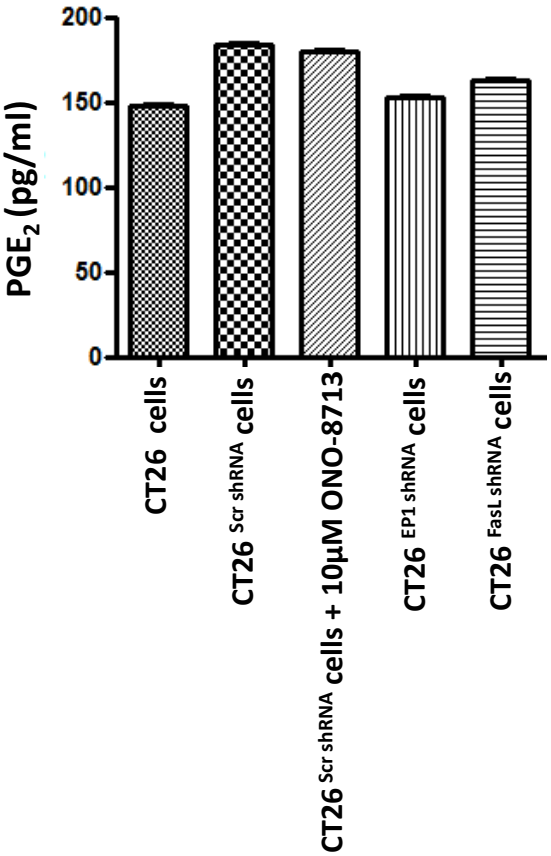

Supplementary Figure 2