In PD-1+ human colon cancer cells NIVOLUMAB promotes survival and could protect tumor cells from conventional therapies

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ABSTRACT

Background Colorectal cancer (CRC) is one of the most prevalent and deadly tumors worldwide. The majority of CRC is resistant to anti-programmed cell death-1 (PD-1)-based cancer immunotherapy, with approximately 15% with high-microsatellite instability, high tumor mutation burden, and intratumoral lymphocytic infiltration. Programmed death-ligand 1 (PD-L1)/PD-1 signaling was described in solid tumor cells. In melanoma, liver, and thyroid cancer cells, intrinsic PD-1 signaling activates oncogenic functions, while in lung cancer cells, it has a tumor suppressor effect. Our work aimed to evaluate the effects of the anti-PD-1 nivolumab (NIVO) on CRC cells. Methods In vitro NIVO-treated human colon cancer cells (HT29, HCT116, and LoVo) were evaluated for cell growth, chemo/radiotherapeutic sensitivity, apoptosis, and spheroid growth. Total RNA-seq was assessed in 6–24 hours NIVO-treated human colon cancer cells HT29 and HCT116 as compared with NIVO-treated PES43 human melanoma cells. In vivo mice carrying HT29 xenograft were intraperitoneally treated with NIVO, OXA (oxaliplatin), and NIVO+OXA, and the tumors were characterized for growth, apoptosis, and pERK1/2/pP38. Forty-eight human primary colon cancers were evaluated for PD-1 expression through immunohistochemistry. Results In PD-1+ human colon cancer cells, intrinsic PD-1 signaling significantly decreased proliferation and promoted apoptosis. On the contrary, NIVO promoted proliferation, reduced apoptosis, and protected PD-1+ cells from chemo/radiotherapy. Transcriptional profile of NIVO-treated HT29 and HCT116 human colon cancer cells revealed downregulation of BATF2, DRAM1, FXYD3, IFIT3, MT-TN, and TNFRSF11A, and upregulation of CLK1, DCAF13, DNAJC2, MTHFD1L, PRPF3, PSMD7, and SCFD1; the opposite regulation was described in NIVO-treated human melanoma PES43 cells. Differentially expressed genes (DEGs) were significantly enriched for interferon pathway, innate immune, cytokine-mediated signaling pathways. In vivo, NIVO promoted HT29 tumor growth, thus reducing OXA efficacy as revealed through significant Ki-67 increase, pERK1/2 and pP38 increase, and apoptotic cell reduction. Eleven out of 48 primary human colon cancer biopsies expressed PD-1 (22.9%). PD-1 expression is significantly associated with lower pT stage. Conclusions In PD-1+ human colon cancer cells, NIVO activates tumor survival pathways and could protect tumor cells from conventional therapies.

INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent and deadly tumors worldwide.1,2 Despite improved knowledge, diagnostics, and screening, up to 30% of patients present with synchronous metastases3 and 40%–50% will eventually develop metastases within 3 years from diagnosis.4 Tumor microenvironment (TME) plays a crucial role in CRC development as the validated Immunoscore (CD3+ and CD8+ T cell infiltration) represents a powerful prognostic factor in localized CRC.5 Based on intrinsic gene expression, four biologically distinct consensus molecular subtypes (CMS) were described for CRC:6,7 CMS1, defined by an upregulation of immune genes, is highly associated with microsatellite instability (MSI-H). CMS2 is characterized by canonical pathway upregulation as defined by the adenoma-carcinoma sequence. Genetically chromosomal unstable tumors are associated with mutations in APC, p53, and RAS. CMS2 represents an overactivated epithelial growth factor pathway with higher expression of epidermal growth factor receptor (EGFR)/EGFR ligands and human epidermal growth factor receptor 2 overexpression. CMS3 is characterized by metabolic changes in glutaminolysis and lipidogenesis. Finally, CMS4 is featured by an activated tissue growth factor (TGF)-β pathway and

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epithelial–mesenchymal transition. The molecular character-
ization recapitulates cancer cells and TME features
but has a limited impact on therapy choice. Conventional
of metastatic disease is based on
fluoropyrimidines plus oxaliplatin or irinotecan chemothera-
plus biological agents (bevacizumab and EGFR
ing targets) according to molecular profiling. The majority of human CRC is resistant to immune-
checkpoint blockade (ICB)-based cancer immunotherapy
as only 15% shows MSI-H, presents high tumor mutation
burden (TMB), increased neoantigens and intratumoral lymphocytic infiltration. The underlying mechanism
how ICB resistance is incompletely understood. In 2017,
Food and Drug Administration (FDA) granted the anti-programmed cell death-1 (PD-1), nivolumab
(NIVO) approval for treatment of DNA mismatch repair-
deficient (dMMR) and MSI-H metastatic CRC (mCRC),
progressing after treatment with fluoropyrimidines, oxal-
platin and irinotecan. Most recently, the FDA approved
anti-PD-1 pembrolizumab (Keytruda) for first-line treat-
ment of patients with unresectable or metastatic MSI-H/
dMMR CRC. Programmed death-ligand 1 (PD-L1) acti-
vates PD-1 in melanoma cells with a tumor-promoting
effect through mammalian target of rapamycin signaling,
independent of adaptive immunity. These effects were
suppressed by anti-PD-1 mAb (aPD-1). Tumor cell
intrinsic PD-1 signaling has been reported in liver,
bladder, melanoma, thyroid, pancreatic cancer, as well as
in non-small cell lung cancer (NSCLC). Differently
from melanoma, in which PD-1 signaling activates onco-
genic functions, in lung cancer, PD-1 signaling promotes
growth inhibition. Beyond immune cell expression,
little is known about the biological significance of the
intrinsic PD-1 pathway in CRC. The role of intrinsic PD-1
expression and signaling in relation to anti-PD-1 immune
therapy was evaluated in human CRC cells.

METHODS
Reagents
Soluble PD-L1 (sPD-L1) was obtained from R&D Systems
(Minneapolis, Minnesota, USA), and nivolumab was
kindly provided by Dr Maiolino-INT-Fondazione G.
Pascale, Naples. Recombinant human IFN-γ or IFN-α
was obtained from R&D Systems. Nivolumab F(ab)2 frag-
ments were generated using Pierce F(ab)2, Preparation
Kit (cat. TS-44988, Thermo Fisher Scientific).

Cell lines
Human CRC cells (HCT116, LoVo, and SW620) were
cultured in high-glucose Dulbecco’s modified Eagle’s
medium (Gibco BRL, Grand Island, New York, USA).
HT29 and COLO205 were cultured in RPMI (Invitro-
gen, San Diego, California, USA). PES43 human melan-
oma cancer cells were cultured in Iscove’s Modified
Dulbecco’s Medium (Gibco BRL). MOLT4 (human T-cell
acute lymphocytic leukemia cells) and 8505C (human
anaplastic thyroid cancer cells) were cultured in RPMI.
Medium was supplemented with 10% fetal bovine serum
(FBS), penicillin (100 µg/mL), and streptomycin (100
µg/mL) (Invitrogen). Cells were maintained in an incub-
ator with a humidified atmosphere of 5% CO2 at 37°C
and were proved mycoplasma-free. HT29, HCT116, LoVo
SW620, and Colo205 human colon cancer cells’ molecular
characteristics are described in online supplemental

Cell growth and cytotoxicity
About 20–50×10^4 cells/well were seeded in a 6-well plate
in a medium culture containing 10% FBS. After 24 hours,
the medium was replaced and NIVO (100 nM–1µM–
10µM), sPD-L1 (1 µg/mL), and combination of NIVO
and sPD-L1 were added. Cells were counted on hemo-
cytometer. For cytotoxicity assay, 2000 cells/well were
seeded in triplicate into 96-well plates, and 24 hours later,
the cells were treated with 5-fluorouracil (5-FU), oxal-
platin (OXA), cisplatin, (CDDP), doxorubicin (Doxo)
and paclitaxel (Tax), irinotecan (IRI) plus/minus NIVO
(10 µM). Cells were incubated at 37°C with 5% CO2 for
72 hours, followed by SRB assay. The optical density
was determined at 540 nm by a microplate reader. IC_{50}
the concentration at which growth was inhibited by 50%.
Relative ratio (RR) is defined as the ratio of cytotoxic
drugs plus NIVO IC_{50}/cytotoxic drugs IC_{50}. For irradia-
tion, cells were plated in 6-well plate treated with NIVO
(1 and 10 µM) and irradiated with 2, 4, and 8 Gy dose of
6 MV X-ray of a linear accelerator. After 6 days, colonies
were counted.

Three-dimensional spheroids growth
About 70%–80% grown human colon cancer cells
(HT29, HCT116, and SW620) were detached with trypsin
and vital cells counted (Trypan blue exclusion). Further,
1×10^4 cell/mL was evaluated for sphere-forming and
drug sensitivity OXA (10 µM), NIVO (10 µM), and
combination. About 50 µL media containing treated 3000
cells was seeded into inverted Petri dish lid. At least 20
drops per dish were plated to develop hanging drops
grown for 72 hours. The images of individual spheroids
were captured via optical inverted microscope (using ×10
objective) (Zeiss, Germany) on day 3. Spheroid images
were analyzed with ImageJ software.

Flow cytometry
For cell-surface markers, cells (100,000 cells/tube) were
harvested and incubated with specific or isotype control
antibodies in the dark for 30 min at 4°C in staining buffer.
PE anti-human CD279 (PD-1) antibody was from Miltenyi
Biotec (Bergisch Gladbach, Germany), PE mouse anti-
human CD274 (clone MIH1) (PD-L1) antibody was
from BD Biosciences (San Jose, California, USA). Isotype
control antibody PE mouse IgG2b and isotype control
antibody PE mouse IgG1, k from BD Biosciences. Stained
cells were evaluated with a FACSariaIII (BD Biosciences)
and all data were analyzed using Facs-Diva software V.8.1
(BD Biosciences).
**Immunoblotting**

Cells were lysed in a whole-cell buffer containing protease and phosphatase (10 mM NaF, 10 mM Na-pyrophosphate, 1 mM Na₃VO₄) inhibitors. Rabbit monoclonal antibodies for p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2; T202/Y204), phospho-P38 MAPK (T180/Y182), P38 MAPK antibodies were from Cell Signaling (Danvers, Massachusetts, USA). Secondary antibodies include goat anti-rabbit-HRP (Jackson ImmunoResearch, West Grove, Pennsylvania, USA). Protein expression was detected with Image Acquisition using ImageQuant LAS 4000 (GE Healthcare, Pittsburgh, Pennsylvania, USA).

**RNA, cDNA, and real-time PCR**

RNA was extracted from cell lines with TRIzol Reagent (Invitrogen, Carlsbad, California, USA) following the manufacturer’s instructions. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster, California, USA) and data were collected and quantitatively analyzed on an QuantStudio 5 Real-Time PCR System with 2–∆Ct method. Relative mRNA expression was normalized with β-actin (ACTB) gene expression. Primer sequences are reported in online supplemental table 5. The primer pairs were subjected to a specificity checking process through the Primer-BLAST publicly available tool.

**Annexin V–propidium iodide (PI) assay**

Cell apoptosis was detected by Annexin V-FITC Apoptosis Detection Kit (eBioscience, Invitrogen by Thermo Fisher Scientific) according to the manufacturer’s instructions. In brief, cells were suspended in binding solution and labeled with 5 µL of annexin V-FITC and 5 µL of PI, followed by incubation at room temperature in the dark for 15 min. Analysis was carried out within 1 hour using the flow cytometer (FACS ARIAIII; BD Biosciences). Annexin V fold change is derived by the ratio control/treated cells.

**In vivo study**

Female Athymic Nude (Hsd:Athymic Nude-Foxn1nu) mice were subcutaneously injected with HT29 (5×10⁶) colon cancer cells. When tumor mass reached ~50 mm³, treatment was conducted intraperitoneally with anti-human PD-1 (NIVO) monoclonal antibody (5 mg/kg intraperitoneal, two times per week/3 weeks), oxaliplatin (OXA) (10 mg/kg intraperitoneal, two times per week/3 weeks), oxal, caliper. Tumor volume was estimated with the formula:

\[ \text{Tumor volume} = \frac{\text{length} \times \text{width}^2}{2} \]

Mean tumor volumes were evaluated for each mouse ±SEM (n=6 per group) three times/week (n=24). The experimental unit is the individual mouse and, a sample size of 24 mice was selected using the G*Power software package, for a priori repeated measures analysis of variance (ANOVA) of tumor volume across time with large effect size=0.77 based on pilot experiments, achieved a power >85%; alpha=0.05. Mice with tumor ulceration were excluded from analysis. Animals were euthanized, the tumors collected and fixed overnight in neutral pH-buffered formalin. All animal studies were performed in compliance with national regulations on the protection of animals used for scientific purposes (Italian decree n. 26 dated 3 April 2014 acknowledging European Directive 2010/63/EU) with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and with the principle of the ‘3Rs’ (Replacement, Reduction and Refinement), Italian Ministry of Health permission (147/2017-PR 13/02/2017). Researchers were not blinded to group identity and randomization of animal groups was done when appropriate.

**Immunohistochemistry (IHC)**

In vivo studies

Tumor histology was evaluated through H&E staining on a Dako autostainer (Agilent). Three micrometer sections from formalin-fixed, paraffin-embedded (FFPE) HT29 tumor were incubated with the appropriate serum designed for blocking endogenous mouse IgG and non-specific background in mouse tissues (Rodent Block M; Biocare Medical), and then incubated overnight at 4°C using primary antibodies: anti-Ki-67 (1:75, Ki-67 Antigen (Dako Omnis) Clone MIB-1); cleaved caspase-3 antibody (1:250, Monoclonal Rabbit IgG Clone #269518 anti-human cleaved caspase-3 (Asp175) antibody); mouse monoclonal anti-human PD-1 (1:50 [NAT105] Abcam); phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (1:400, D13.14.4E, XP Rabbit mAb #4370- CST), phospho-p38 MAPK (Thr180/Tyr182) (1:400, D3F9, XP Rabbit mAb #4511-CST). Anti-PD-1, predilute, NAT105 (Cell marque) in a Benchmark XT (Ventana Medical Systems) was used for section from patients with colon cancer. The IHC staining was evaluated in at least 10 consecutive not overlapping high-power field (HPF) ×400 magnification (0.237 mm²/field) in at least five areas using an Olympus BX51 microscope (Olympus, Tokyo, Japan). Stained sections were independently evaluated by expert pathologist/researchers blinded to initial assessments.

**Patients’ study**

FFPE tissue blocks derived from 48 surgically collected colon cancer tumor samples were obtained. Sections of 4 µm were obtained, placed on slides with permanent positive charged surface, and processed as above. The samples were incubated overnight at 4°C using primary antibody (CD279/PD1 polyclonal antibody, #PA5-20350 dilutions 1:50) (Thermo Fisher Scientific).

**Statistical analysis**

SPSS software (V.20.0) were used for statistical analysis. Data were expressed as mean±SD or SEM as stated in figure legends. The continuous variables were compared using an unpaired Student’s t-test or a Mann-Whitney U test if the variables were not normally distributed. For multiple groups comparison, Kruskal-Wallis test followed by Dunn’s multiple test was used to determine significantly
different groups. For multiple groups comparison, repeated measures ANOVA (RMANOVA) with Tukey’s HSD post hoc test was used to determine treatment effect over time. The $\chi^2$ test was used to compare the frequency and percentage in categorical variables. Spearman’s rank correlation was used to evaluate correlation between two continuous variables. Linear regression analysis was used to model the relationship between two variables. P<0.05 was considered to indicate a statistically significant difference.

**RESULTS**

**Human colon cancer cells express functional PD-1 that is not regulated by IFN-α/γ. PD-1 blockade increases human colon cancer cell growth**

PD-1 and PD-L1 levels were evaluated in human colon cancer cells. PD-1 but not PD-L1 was detectable in HT29, HCT116, LoVo, SW620, and Colo205 cells (figure 1A and online supplemental figure 1). HT29, HCT116, and LoVo significantly overexpressed PD-1 as compared with SW620 and Colo205 cells. As PD-L1 is IFN-induced, the effect of type I (IFN-α) and type II (IFN-γ) interferons (IFNs) was studied on PD-1 and PD-L1 expression. Neither IFN-α nor IFN-γ induced PD-1, while both IFN-α and IFN-γ increased PD-L1 expression in HT29 and HCT116 cells, at protein (figure 1B(i)) and at transcriptional level (figure 1B(ii))

To evaluate the effect of cell-intrinsic PD-1 signaling on apoptosis and cell growth, human colon cancer cells HT29, HCT116, and LoVo were treated with sPD-L1 and NIVO. As shown in figure 1C, soluble PD-L1 (sPD-L1) increased apoptosis in HT29, HCT116, and LoVo by 4.7±1.6-fold, 6.3±2.4-fold, and 3.4±0.6-fold, respectively, while NIVO rescued cells from apoptosis (online supplemental figure 2). In figure 1D, NIVO increased cell growth (1.6-fold, 2.5-fold, 1.4-fold post 72 hours in HT29, HCT116, and LoVo, respectively), while sPD-L1 reduced it. Low doses of NIVO (0.1–1 µM) also increased the proliferation of HT29, HCT116, and LoVo cells (online supplemental figure 3). Interestingly, NIVO did not affect cell growth in SW620 and Colo205 human colon cancer cells expressing lower PD-1 (online supplemental figure 4). To exclude the Fc receptors engagement on tumor-associated macrophages, Fc receptors and the efficacy of the anti-PD-1 F(ab)2 were evaluated. The Fc receptors were minimally detected or undetectable in HCT116, LoVo, and SW620. The unique FcγRI detected in HT29 is FcγRII (CD32). Moreover, anti-PD-1/F(ab)2 fragment modified cell growth as NIVO (online supplemental figure 5A,B). To dissect the downstream pathway following PD-1 engagement, (p)-ERK1/2 and (p)-P38 were evaluated in HT29, HCT116, and PES43 cells. Six-hour NIVO (10 µM) induced pERK in HT29 and HCT116 cells, while 6-hour NIVO (10 µM) reduced pERK in human melanoma cell PES43 (figure 1E). Moreover, 6-hour NIVO (10 µM) increased pP38 in HT29, while, as expected for K-RAS mutant cell HCT116, NIVO did not affect pP38 (figure 2C). Conversely, 6-hour NIVO (10 µM) reduced...
**Figure 1** Human colon cancer cells express functional PD-1 that is not regulated by IFN-α/γ. PD-1 blockade increases human colon cancer cell growth. (A) Percentages (mean±SD) of PD-1 and PD-L1 surface protein expression on five colon cancer cells (HT29, HCT116, LoVo, SW620, Colo205), as determined by flow cytometry. Bar charts show combined results from at least three independent experiments. PES43 (human melanoma cancer cell line) and MOLT4 (human T-cell acute lymphocytic leukemia cell line) were used as PD-1 positive control and 8505C (human anaplastic thyroid cancer cell line) was used as PD-L1 positive control. *P value <0.05; **p value <0.01; ***p value <0.001. Student’s t-test was used. (B) Histograms represent changes in (i) % fluorescence intensity by flow cytometry (mean±SD) and (ii) mRNA expression indicated as 2∆Ct for PD-1 and PD-L1 in HT29, HCT116, and LoVo cells treated with IFN-γ (50 IU/mL) for 48 hours and IFN-α (3000 IU/mL) for 24 hours. Bar graphs represent the average of at least three experiments. P value >0.05 ns (not significant); *p value <0.05. Student’s t-test was used. (C) HT29, HCT116, and LoVo cells were treated with sPD-L1 (1 µg/mL) or sPD-L1 +NIVO (10 µM) for 24 hours. Cell apoptosis rates were detected through Annexin V and propidium iodide (PI) dual staining method. Relative fold change of apoptotic cells is shown in the histogram (mean±SD). Bar graphs represent the average of at least two experiments. P value >0.05 ns; *p value <0.05. Student’s t-test was used. (D) HT29, HCT116, and LoVo growth curves following NIVO (10 µM), sPD-L1 (1 µg/mL), or combination sPD-L1 +NIVO treatment for 24, 48, and 72 hours. All data are representative of at least two experiments. P value >0.05 ns; *p value <0.05; **p value<0.01; ***p value <0.001. Student’s t-test was used. (E) Immunoblot analysis (representative of n=2 independent experiments) of phosphorylated (p) and total ERK1/2 and P38 in HT29, HCT116, and PES43 cell lines treated with NIVO (10 µM) for 15 min, 6–18 hours. The numbers above the gel lanes represent the relative protein level, which was determined from the band intensity using ImageJ software, and normalized relative to the total protein. IFN, interferon; NIVO, nivolumab; sPD-L1, soluble PD-L1.
Figure 2  PD-1 blockade reduces the effect of chemo/radiotherapy on human colon cancer cells. (A) HT29, HCT116, and LoVo cells were treated with NIVO (1 µM), OXA (40 µM), 5-FU (50 µM), or NIVO +5-FU/OXA for 24 hours. Annexin V/PI analysis was performed. Bar graphs represent relative fold change of apoptotic cells obtained from at least two independent experiments±SD. P value >0.05 ns (not significant); *p value <0.05; **p value <0.01; ***p value <0.001. (B) (i) HT29 and HCT116 cells were exposed to 2–4–8 Gy plus/minus NIVO (1–10 µM) for 1 week. Survival curves for radiation plus NIVO treatment in HT29 and HCT116 cells. Data represent means obtained from two experiments±SD. P value >0.05 ns; *p value <0.05; ***p value <0.001. Student’s t-test was used. (ii) Representative images of a colony formation assay (left) and quantification data (right) for HT29 and HCT116 cells treated with NIVO (1 µM) and F(ab)_2 (1µM). Data are representative of two experiments±SD. *P value <0.05; **p value <0.01. Student’s t-test was used. (C) Spheroids growth of HT29, HCT116, and SW620 72 hours treated with OXA (10 µM), NIVO (10 µM), and combination. Images were obtained at optical inverted microscope (using ×10 objective) (Zeiss, Germany) on day 3. Spheroid images were typically analyzed with ImageJ software. Grouped dot plot express spheroid area (mean±SD). For each data point, at least nine spheroids were analyzed. Data are representative of three experiments±SD. 5-FU, 5-fluorouracil; NIVO, nivolumab; OXA, oxaliplatin; PI, propidium iodide.
pP38 in PES43. These data suggest that PD-1 inhibited proliferation by suppressing P38 and ERK signaling.

To further investigate the role of downstream signaling in PD-1-mediated cell proliferation, HT29 and HCT116 cell growth was evaluated in the presence of MEK/ERK1/2, AKT, or P38 inhibitors (online supplemental figure 6). As shown in online supplemental figure 6, the kinase inhibitors significantly reverted NIVO-induced cell growth in HT29 cells (upper panel). In HCT116, both AKT and MEK/ERK1/2 inhibitors reverted NIVO-induced cell growth, while as anticipated, the P38 inhibitor did not affect proliferation (lower panel).

**NIVO protects colon cancer cells from chemo/radiotherapy and potentiates spheroids growth**

To investigate the effect of PD-1 signaling on chemotherapy, human colon cancer cells were treated with 5-FU, OXA, CDDP, DOXO, IRI, and TAX in the presence of NIVO for 72 hours. As summarized in table 1, NIVO reduced the efficacy of chemotherapy, increasing the 5-FU (3.1-fold, 2.2-fold, and 1.9-fold), OXA (2.8-fold, 4.8-fold, and 15.8-fold), and IRI (2.2-fold, 1.7-fold, and 2.9-fold) IC₅₀ in HT29, HCT116, and LoVo, respectively (table 1). In addition, as shown in table 1, NIVO increased the IC₅₀ for CDDP, DOXO, and TAX. Conversely, in SW620 cells, low PD-1 expressing NIVO did not affect chemosensitivity. As PD-1 signaling increased cell proliferation and survival in melanoma, cytotoxicity was evaluated in human melanoma PES43 cells. PES43 are more sensitive to DOXO and TAX in the presence of NIVO, as previously reported for ovarian cancer cells. The percentage of apoptosis was reduced when NIVO was added to 5-FU and OXA in HT29, HCT116, and LoVo cells (figure 2A and online supplemental figures 7,8).

HCT116 and HT29 cells were exposed to 2–4–8 Gy of X-ray in the presence of NIVO (figure 2B(i)). In HT29 and HCT116 cells, NIVO increased resistance to radiation, promoting cell growth (1.3-fold and 1.7-fold at 4 and 8 Gy in HT29 and 1.6-fold, 1.8-fold, and 2.2-fold at 2, 4, and 8 Gy in HCT116) (figure 2B(i)). Treatment with NIVO-F(ab)₂ (1 μM) achieved the same increase in clonal efficiency obtained with NIVO (figure 2B(ii)).

Moreover, to reproduce the three-dimensional interaction of tumor cells and the relative drug sensitivity, the human colon cancer cells HT29, HCT116, and SW620 were tridimensionally cultured in spheroids, and NIVO/NIVO plus OXA efficacy was evaluated. As observed in figure 2C, NIVO treatment (10 μM) enhanced spheroid mean area in HT29 (p < 0.001), while OXA significantly reduced the area of spheroid in HT29, HCT116, and SW620 (p < 0.001). NIVO rescued OXA effect on HT29 and HCT116 spheroid growth but not on SW620 cells, low PD-1-expressing cells.

**NIVO-induced differential gene expression in human CRC cells**

RNA-Sequencing was conducted on HT29 and HCT116 treated with NIVO, sPD-L1, and combination sPD-L1+NIVO for 6–24 hours and compared with PES43 cells. The aim was to identify, among the differentially expressed genes (DEGs), treatment-affected genes in colon cancer cells that are counter-regulated in melanoma cells. As highlighted by the principal component analysis (PCA) in figure 3A, HCT116, HT29, and PES43 cells displayed independent clustering in which the first two components explained ~86% of variability, suggesting a marked difference in global gene expression profiles among the three cell lines. After data normalization, when the PCA is performed on cell lines, clusters are clearly distinct at 6 and 24 hours of treatment for HT29, HCT116, and PES43 with an explained variability of ~35.4% for HCT116, ~24.9% for HT29, and ~59.3% for PES43 (online supplemental figure 9). As shown by the

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**Table 1** The IC₅₀ (mean±SEM) values for 5-fluoruracil, oxaliplatin, cisplatin, doxorubicin, paclitaxel, and irinotecan with or without nivolumab treatment in HT29, HCT116, LOVO, SW620, and PES43 cell lines.

<table>
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<tr>
<th></th>
<th>HT29 (PD-1: 23%)</th>
<th>HCT116 (PD-1: 31%)</th>
<th>LOVO (PD-1: 22%)</th>
<th>SW620 (PD-1: 15%)</th>
<th>PES43 (PD-1: 20%)</th>
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<td>Oxaliplatin +NIVO</td>
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<td>20.9±0.95</td>
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<td>Paclitaxel +NIVO</td>
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Statistically significant: *p<0.05; **p<0.01; ***p<0.001.

RR, resistance ratio (IC₅₀ of chemotherapy+NIV/IC₅₀ of chemotherapy).
Figure 3  Differentially expressed genes (DEGs) in NIVO-treated human colon cancer and melanoma cells. (A) Principal component (PC) analysis was performed for the samples using the gene expression values. Clustering of HCT116 cells (red dots), HT29 cells (green dots), and PES43 cells (blue dots) treated with PBS, NIVO (10 µM), sPD-L1 (1 µg/mL), and NIVO +sPD-L1. (B) Volcano plots of genes differentially expressed in NIVO-treated PES43, HT29, and HCT116 for 6 hours (upper panel) and 24 hours (lower panel) in pairwise comparisons versus untreated cells. The log2 fold change difference is represented on the x-axis and –log10 of corrective p value (q-value) is represented on the y-axis. Each point represents a gene. Red points indicate genes called as differentially expressed (DE) at adjusted p value (adjP) ≤0.05. (C) Comparison of the DEGs on HCT116, HT29, and PES43 cells on stimulation with NIVO for 6 hours (left panel) and 24 hours (right panel) using Venn diagrams. (D) Heat maps of genes differentially expressed in NIVO-treated PES43, HT29, and HCT116 for 6 hours (left panel) and 24 hours (right panel). NIVO, nivolumab; sPD-L1, soluble PD-L1.
Volcano plots in figure 3B, we identified 8306, 618, and 3846 genes significantly affected, respectively, in PES43, HT29, and HCT116 after 6 hours of NIVO treatment, while 7312, 1147, and 5410 were significantly modified in PES43, HT29, and HCT116 after 24 hours of NIVO treatment. In PES43, a robust increase in NIVO-upregulated genes was detected as compared with HT29 and HCT116 cells (figure 3B). Similar differences were observed in DEGs induced by sPD-L1 (online supplemental figure 10A) or NIVO +PD-L1 (online supplemental figure 10B) after 6 (online supplemental figure 10A,B, upper) and 24 hours (online supplemental figure 10A,B, lower) in PES43, HT29, and HCT116. We focused on the genes commonly regulated among HT29, HCT116, and PES43, identifying 149 and 299 genes shared after 6 and 24 hours of treatment with NIVO, respectively, as depicted in the Venn diagram in figure 3C (online supplemental figure 11). Among genes commonly modulated at 6 and 24 hours of treatment with NIVO, 19 (NIVO-6 hours) and 67 (NIVO-24 hours) genes revealed an inverse correlation (based on logFC) between colon cancer cells and PES43 (figure 3D). As shown in figure 3D, 6 hours after NIVO, six interesting genes were upregulated in PES43 and downregulated in HT29 and HCT116 (BATE2, DRAM1, FXYD3, IFIT3, MTIF3, TNFRSF11A) and seven genes were downregulated in PES43 and upregulated in HT29 and HCT116 (CLK1, DCAF13, DNAJC2, MTHFD1L, PRPF3, PSMD7, SCFD1) (figure 3D and online supplemental table 3). After 24 hours of NIVO, gene regulation is generally milder. Commonly affected genes were upregulated in PES43 and downregulated in HT29 and HCT116 (AMIGO2, CLDN12, DRAM1, DUSP5, GRB10, GULP1, HSPA5, RESF1, CDKN2B, DLGAP1-A52, EGR1, NDRG1, SAMD9) and 24 genes were downregulated in PES43 and upregulated in HT29 and HCT116 (CLK1, DCAF13, DNAJC2, MTHFD1L, PRPF3, PSMD7, SCFD1) (figure 3D and online supplemental table 3). Of note, PD-L1 expression is observed in TME and to PES43 a less aggressive phenotype, with tumor suppressor reduction and regulating genes of innate immune response, type I-IFN signaling, and cytokine-mediated signaling pathway (figure 4, upper panel). These features, although less clearly designed, are kept at 24 hours of treatment (figure 4, lower panel).

qRT-PCR validation was conducted on 12 genes differentially expressed between HT29 and HCT116 versus PES43 after 6 hours of NIVO treatment (BATE2, DRAM1, FXYD3, IFIT3, TNFRSF11A, CLK1, DCAF13, DNAJC2, MTHFD1L, PRPF3, PSMD7, SCFD1) and 12 genes affected by 24-hour NIVO treatment (DRAM1, DUSP5, EGR1, PAPI, GNE, MAGED1, NT5D2, PIA54, PRR11, THRA, UBE4B, USP5). qPCR profiles were consistent with the patterns of expression revealed by the RNA-Seq (online supplemental figure 12A,B). Linear regression analysis for the 24 selected DEGs/cell line between RNA-seq (x axes) and qRT-PCR (y axes) revealed correlations ranging from 0.78 to 0.85 for Pearson’s correlation at P value <0.0001 (online supplemental figure 12C).

NIVO potentiates in vivo growth of HT29 tumors
The NIVO effect was evaluated in a HT29 xenograft model. HT29 cells were inoculated in CD1 athymic mice and, when the tumors reached 50 mm³, mice were randomized in four groups and treated twice a week with intraperitoneal PBS (n=6), NIVO (n=6), OXA (n=6), and combination NIVO +OXA (n=6) for 3 weeks. Tumor histology was evaluated with H&E (online supplemental figure 13). Consistent with the in vitro experiments, NIVO significantly increased tumor growth by 1.88-fold (figure 5A and online supplemental figure 13). As shown in figure 5A, at day 34, the mean tumor volume (mm³) was 1059.5±117.4 in OXA-treated mice, 3102.5±184.8 in NIVO-treated mice, and 1645.7±261.6 in OXA +NIVO-treated mice, 2.9-fold and 1.6-fold more than the OXA-treated mice, respectively, suggesting that PD-1 blockade increases tumor growth and reduces the efficacy of chemotherapy. Equally, NIVO increased tumor mass in comparison with the control group (figure 5B). Tumors were further characterized for Ki-67, cleaved-caspase-3, pERK1/2 and pP38, PD-1 expression. NIVO significantly increased the fraction of Ki-67 expressing cells (p=0.023) as compared with the untreated and to the combination with OXA (p=0.027) (figure 5C). Moreover, NIVO and NIVO plus OXA significantly reduced the percentage of apoptotic cells as revealed through cleaved caspase-3 detection (1.62%±1.89% and 0.76%±0.64% vs 6.8±3.3; p=0.010 and p=0.002, respectively). As shown in figure 5C, although not statistically significant, NIVO-treated tumors expressed higher pP38 as compared with untreated tumors. Interestingly, OXA-treated tumors displayed significantly low of pP38 positive cells (p=0.0061), while pP38 positive cells increased in NIVO plus OXA-treated tumors (p=0.0047) suggesting higher cell proliferation within the NIVO-treated tumors. In addition, pERK1/2 significantly increased in OXA plus NIVO as compared with the NIVO-treated tumors (figure 5C). Although not statistically significant, PD-1 reduction was detected in both NIVO and NIVO +OXA-treated tumors (figure 5C).

PD-1 is expressed in human CRC
The Cancer Genome Atlas (TCGA) database demonstrated that the PDCD1 gene encoding PD-1 was widely transcribed in 17 cancers including CRC (online supplemental figure 14). However, colon cancer tissues include infiltrated lymphocytes. Forty-eight paraffin-embedded colon cancer samples were analyzed for PD-1 expression through IHC. PD-1 expression is observed in TME and cancer cells (online supplemental figure 15). Clinical-pathologic features showed that size of primary tumors is significantly associated with PD-1 expression (p=0.041) (table 2). Of note, PD-1 expression was mainly retrieved
in colon cancer in the mucinous histology \((p=0.007)\) (online supplemental figure 16).

**DISCUSSION**

Herein, PD-1 expression of human colon cancer cells is functional and inhibition through NIVO treatment results in a protective effect-promoting growth, reducing apoptosis and chemo/radio sensitivity in vitro and in vivo. PD-1 expression regulation has been extensively studied as DNA methylation, histone modifications, glycosylation/fucosylation and ubiquitination were reported. While these studies mainly refer to lymphocytes, the PD-1 expression control in epithelial neoplastic cells is poorly defined. Likely, gene copy number, epigenetic alterations, and microenvironment-derived stimulant regulate PD-1 expression in human cancer cells. As c-Fos binds to the AP-1-binding site and activates Pdcd1 transcription in tumor-infiltrating T cells, Fos reduction was retrieved among the genes regulated in NIVO-treated colon cancer cells. Also, FOXO4 and FOXO3 were regulated by NIVO treatment in human colon cancer cells, mechanism previously reported for FOXO1 in antigen-specific CD8+ T cells during chronic lymphocytic choriomeningitis virus infection. PD-1 expression is also regulated through...
proteosomal degradation; herein, 24 hours of NIVO treatment increased the UBA1 (ubiquitin-like modifier activating enzyme 1) and UBE4B (Ubiquitination factor E4B) in HT29 and HCT116.

Transcriptional profiles identify NIVO-modulated genes regulated in opposite direction between colon cancer and melanoma cells. As previously reported in melanoma, liver, and pancreatic cancer, PD-L1 induction of PD-1 pathway promotes tumor cell growth. On the contrary, in NSCLC, PD-L1 induction of PD-1 pathway has a tumor suppressor function. We have previously shown that in human melanoma PES43 cells NIVO or pembrolizumab reduced cells growth and inhibited the phosphorylation of ERK1/2, P38 MAPK, AKT, and 4EBP1. In contrast, we have shown here that proliferative and survival signaling are induced after NIVO in colon cancer cells. In HT29 and HCT116 human colon cancer cells, PD-1 signaling suppresses cell growth through p38, AKT, and MEK/ERK1/2 pathways. To identify gene expression pathways involved, total RNAseq was evaluated following NIVO treatment in HT29, HCT116 human colon cancer cells and compared with NIVO-treated human melanoma cell PES43. In colon cancer cells, a clear downregulation of BATF2, IFIT3, and TNFRSF11A and induction of CLK1, DCAF3, DNAJC2, MTHFD1L, PRF3, PSMD7, and SCFD1 was reported, while 6 hours of NIVO treatment upregulated BATF2, IFIT3, and TNFRSF11A in PES43 cells. Interestingly, IFIT3 (interferon-induced protein with tetratricopeptide repeats 3) is an interferon-induced protein and TNFRSF11A, TNF receptor superfamily member 11a, regulates NF-κB, JNK, ERK1/2, p38, and AKT/PKB. BATF2 is a tumor suppressor gene, thus BATF2 overexpression and CLK1 downregulation suggest that NIVO reduces aggressiveness in PES43 and potentiates it in human colon cancer cells. MAGED1, a member of the melanoma antigen family significantly overexpressed in melanoma and colon cancer, is clearly reduced by NIVO in PES43 cells while upregulated in human colon cells. EGR1, among the most induced gene in PES43 and repressed in colon cancer cells, is a Zinc-finger transcription factor implicated in the regulation of cell growth and metastasis targeting MMP9 and/or MDM. Thus, it appears that 6 hours of NIVO treatment impacts on gene expression conferring to HT29 and HCT116, a more aggressive phenotype, and to PES43, a less aggressive phenotype, with tumor suppressor reduction and regulating genes of innate immune response, type 1-IFN signaling and cytokine-mediated signaling pathways. Consistently, PD-1 blockade by NIVO enhanced colon cancer cell growth in vitro and in vivo independently of adaptive immunity. In support of that, blockade of colon cancer-intrinsic PD-1 promoted chemo-radio resistance and impaired apoptosis. Chemotherapy and radiotherapy might potentiate in vivo the efficacy of ICBs by increasing immunogenicity following cellular death (immunogenic cell death, ICD). In triple negative breast cancer, a short-term induction with doxorubicin or cisplatin magnifies the response to anti-PD-1 treatment. NIVO, nivolumab; OXA, oxaliplatin; PD-1, programmed cell death-1.

Figure 5  PD-1 blockade accelerated the growth of subcutaneous HT29 tumors and decreased efficacy of chemotherapy. Tumor growth curve (caliper tumor volumes±SEM) (A) and tumor weight (grams) (B) of HT29 tumor subcutaneously implanted in CD1 athymic mice treated as indicated. (C) Immunohistochemistry (IHC) for Ki67, cleaved caspase-3, pP38, pERK1/2, PD-1. Representative microphotographs (left) show localization of selected markers. The scale bars at the bottom of the figure indicate 50 μm for ×400 magnification. P value <0.05 was considered statistically significant for Kruskal-Wallis test followed by Dunn’s multiple comparison. Bar graph (right) illustrates quantification of immunohistochemistry staining from collected tumors (means±SD). NIVO, nivolumab; OXA, oxaliplatin; PD-1, programmed cell death-1.
### Table 2  The relationship between PD-1 expression and clinical-pathologic features of human colon cancer

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and enriches immune-related genes, including T-cell cytotoxicity and JAK-STAT pathway activation.\textsuperscript{48} Comparably, radiotherapy has been shown to induce immunogenic cell death, resulting in phagocytosis of tumor cells, processing of tumor antigens, and priming of CD8 + T cells.\textsuperscript{49} At variance from these studies, our observations provide the first report in which targeting PD-1 results in colon cancer radio/chemo resistance. Although larger validation is needed, tumor-cell intrinsic PD-1/total PD-1 expression could represent a potential biomarker for ICB selection in patients with CRC. As 'proof of concept', intrinsic PD-1 expression was retrieved in a subset of patients with colon cancers, mainly in the neoplastic cells, associated with lower pT stage. PD1-blocking treatments in colon cancer PD-1 expressing would enhance tumor cell growth, promoting tumor progression disease. These effects need to be considered in the evaluation of immunotherapy efficacy and in the features of colon cancer immunoresistance.

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### Contributors

CI designed the experiments, carried out in vitro and in vivo experiments, analyzed and interpreted data, drafted and revised the manuscript. DR analyzed and interpreted RNA-sequencing data, read and approved this manuscript. MN, LP, GR, CD, GG, FA, S Santaga, AMT, CR, and REA performed the technical work and analyses, read and approved this manuscript. PM provided nivolumab, read and approved this manuscript. CDA, AL, and AB carried out in vivo experiments, read and approved this manuscript. CDA, ADM, and FT carried out immunohistochemistry, read and approved this manuscript. FL and NP suggested on the design of experiments. RP, NN, and RMM participated in conception and design of experiments, read and revised the manuscript. S Scala devised the project and designed the experiments, analyzed, and interpreted data, wrote, revised, approved the manuscript and is guarantor of this manuscript.

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### Competing interests

None declared.

### Patient consent for publication

Not applicable.

### Ethics approval

Animal experimentation was done according to the Italian national law (Legislative Decree 26/2014) and Directive 2010/63/EU on the protection of animals used for scientific purposes. Ethical approval was obtained from the Italian Ministry of Health.

### Provenance and peer review

Not commissioned; externally peer reviewed.

### Data availability statement

Data are available on reasonable request.

### Supplemental material

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### ORCID id

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### REFERENCES

Supplementary Material and Methods

Reagents

SB203580 (P38 MAPK inhibitor), Triciribine (AKT inhibitor) and Selumetinib (MEK/ERK inhibitor) were from Selleckchem (Houston, TX, USA).

Cell growth

2000 cells/well were seeded in triplicate into 96-well plates and 24 hours later cells were treated with NIVO (10 μM) in the presence or absence of Selumetinib (Selu – 500nM), Triciribine (10uM), SB293580 (10uM). Cells were incubated at 37 °C with 5% CO2 for 24, 48, 72 h, followed by SRB assay. The optical density values were determined at 540 nm by a microplate reader.

In silico studies

Study of PD-1 expression genes in CRC was performed by the online tools GEPIA (http://gepia.cancer-pku.cn/index.html). GEPIA is a web server for analyzing the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from the TCGA and the GTEx projects using a standard processing pipeline.
Supplementary Figure Legend

Supplementary Fig. 1. Human colon cancer cells express functional PD-1 not regulated by IFN-α/γ. Flow cytometry plots of PD-1 and PD-L1 surface protein expression on 5 colon cancer cells (HT29, HCT116, LoVo, SW620, COLO205). PES43 (human melanoma cancer cell line) and MOLT-4 (human T-cell acute lymphocytic leukemia cell line) were used as PD-1 positive control and 8505C (human anaplastic thyroid cancer cell line) was used as PD-L1 positive control.

Supplementary Fig. 2. PD-L1 induces apoptosis in the human colon cancer cells. Flow cytometry data showing cell apoptosis in the HT29, HCT116 and LoVo cells treated with sPD-L1 (1µg/mL) or sPD-L1+NIVO (10µM) for 24h. Cell apoptosis rates were detected by FACS analysis with AnnexinV and PI dual staining method.

Supplementary Fig. 3. Nivolumab promotes proliferation in colon cancer cells. HT29, HCT116, LoVo growth curves following NIVO (0.1-1 µM), sPD-L1 (1µg/mL) or combination NIVO+sPD-L1 treatment for 24, 48, 72h. All data are representative of at least 2 experiments. P-value >0.05 n.s. (not significant), p > 0.05*, P-value < 0.05; **, P-value < 0.01; *** P-value < 0.001. Student t test was used.

Supplementary Fig. 4. Nivolumab blockade did not affect cell growth in low PD-1 expressing human colon cancer cells. Representative images of growth curve of SW620 and COLO205 cancer cell lines treated with NIVO (0.1-1-10 µM).

Supplementary Fig. 5. Nivolumab F(ab)2 promotes human colon cancer cells proliferation. (A) Flow cytometry percentages (mean ± SD) of FcγRs expression FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) in colon cancer cells (HT29, HCT116, LoVo, SW620, COLO205); (B) Representative images of growth curve (lower) of HT29 and HCT116 colon cancer cell lines treated with NIVO (0.1 - 1µM) toward F(ab)2 fragments for 24, 48, 72h. P-value >0.05 n.s. (not significant), * P-value < 0.05, ** P-value < 0.01.

Supplementary Fig. 6. Nivolumab promotes human colon cancer cell growth through pAKT, pERK and pP38 signaling. HT29 and HCT116 colon cancer cell proliferation at 72 hours in the presence of NIVO (10µM) plus or minus Selumetinib (Selu – 500nM), Triciribine (10µM), SB293580 (10µM) compared to control. Data are representative of at least 3 experiments. P-value >0.05 n.s. (not significant), *, P-value < 0.05; **, P-value < 0.01 vs CTRL; §, P-value < 0.05; §§§ P-value < 0.001 vs NIVO. Student t test was used.
Supplementary Fig. 7. Nivolumab reduces the effect of chemotherapy on human colon cancer cells. HT-29, HCT116 and LoVo cells were treated with NIVO (1uM), OXA(40uM), 5-FU (50uM) or NIVO+5-FU/OXA for 24h. Annexin V/PI analysis was performed. Representative dot plots of PI and Annexin V double staining on the HT29, HCT116, LoVo cells. P-value >0.05 n.s. (not significant), P-value < 0.05; **, P-value < 0.01; *** P-value < 0.001. Student t test was used.

Supplementary Fig. 8. Nivolumab F(ab)2 reduces the effect of chemotherapy on human colon cancer cells. Flow cytometry data showing cell apoptosis in the HT29, HCT116 and LoVo cells treated with F(ab)2 (1uM), OXA(40uM), 5-FU (50uM) or F(ab)2 +5-FU/OXA for 24h. Cell apoptosis rates were detected by FACS analysis with AnnexinV and PI dual staining.

Supplementary Fig. 9. Differentially expressed (DE) genes between NIVO-treated and control groups in human colon cancer and melanoma cells. Principal component (PC) analysis was performed for the samples using the gene expression values. Clustering of PES43 cells (A), HT29 cells (B) and HCT116 cells (C) treated with PBS, NIVO 10uM, sPD-L1 (1ug/ml) and NIVO+sPD-L1 for 6-24h.

Supplementary Fig. 10. Differentially expressed (DE) genes between NIVO-treated and control groups in human colon cancer and melanoma cells. Volcano plots of genes differentially expressed in PD-L1-treated (A) and NIVO+PD-L1-treated (B) PES43, HT29 and HCT116 for 6h (upper panel) and 24h (lower panel) in pairwise comparisons vs. untreated cells. Red points indicate genes called as differentially expressed (DE) at adjusted p value (adjP)<0.05.

Supplementary Fig. 11. Differentially expressed (DE) genes between NIVO-treated and control groups in human colon cancer and melanoma cells. Comparison of the differentially expressed genes on HCT116, HT29 and PES43 cells upon stimulation with PD-L1 (upper panel) and NIVO-PD-L1 (lower panel) for 6h (left panel) and 24h (right panel) using Venn diagrams.

Supplementary Fig. 12. Validation of RNAseq NIVO-treated genes at 6-24hours by qRT-PCR. (A) RT-qPCR analysis was performed to assay the relative expression profiles of 12 genes differentially expressed in PES43, HCT116 and HT29 after 6 hours NIVO: BATF2, DRAM1, FXYD3, IFIT3, TNFRSF11A, CLK1, DCAF13, DNAJC2, MTHFD1L, PRPF3, PSMD7, SCFD1. (B) RT-qPCR analysis was performed to assay the relative expression profiles of 12 genes differentially expressed in HT29, HCT116 and PES43 after 24 hours NIVO: DRAM1, DUSP5, EGR1, DPP9, GNE, MAGED1, NT5DC2, PIAS4, PRR11, THRA, UBE4B, USP5. (C) Pearson correlation between log2 fold changes in gene expression between 6-24 hours NIVO-
treated and control for PES43, HCT116 and HT29, as determined by qRT-PCR and RNA-seq (P<0.05).

**Supplementary Fig13. HT29 tumor Hematoxylin and eosin staining.** (A) Photograph of FFPE H&E slides from each tumor. The scale bar at the bottom of the figure indicate 10mm. (B) Representative H&E microphotographs of HT29 tumor for each group. The scale bars at the bottom of the figure indicate 100µm and 50µm for 200x and 400x magnification, respectively.

**Supplementary Fig14. PD-1 is expressed in human colorectal cancer** Dot plots showing the expression levels of PDCD-1 in various kinds of clinical tumor tissues based on data from TCGA.

**Supplementary Fig15. PD-1 is expressed in TME and cancer cells.** Microphotographs showed (a) negative tumor (Black arrow) with positive TME for PD-1 expression (White arrow), (b) low and (c) moderate intrinsic carcinoma cells PD-1 staining (Black arrow).

**Supplementary Fig16. PD-1 is mainly retrieved in mucinous adenocarcinoma.** Relationship between PD-1 expression (%) and mucinous adenocarcinoma (MA) or not otherwise specified type (NOS).
**Supplementary Table 1.** Colon cancer cell lines classified by the MSI and CIMP, and mutation status of cancer critical genes

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor localization</th>
<th>Molecular characteristics</th>
<th>TP53</th>
<th>KRAS</th>
<th>BRAF</th>
<th>PIK3CA</th>
<th>PTEN</th>
<th>MSI</th>
<th>CIMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>colon</td>
<td>p.R273H</td>
<td>WT</td>
<td>p.V600E; T1195</td>
<td>WT</td>
<td>WT</td>
<td>MSS</td>
<td>CIMP+</td>
<td></td>
</tr>
<tr>
<td>LOVO</td>
<td>colon</td>
<td>WT</td>
<td>p.G13D; V14A</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>MSI</td>
<td>CIMP-</td>
<td></td>
</tr>
<tr>
<td>SW620</td>
<td>descending colon</td>
<td>p.R273H; p.P309S</td>
<td>p.G12V</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>MSS</td>
<td>CIMP-</td>
<td></td>
</tr>
<tr>
<td>COLO205</td>
<td>colon</td>
<td>p.Y107fs; Y103fs</td>
<td>WT</td>
<td>p.V600E</td>
<td>WT</td>
<td>WT</td>
<td>MSS</td>
<td>CIMP+</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: MSI, microsatellite instability; MSS, microsatellite stable; CIMP, CpG island methylator phenotype; wt, wild type.

**Supplementary Table 2.** The Consensus Molecular Subtypes of colon cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cancer cell-adapted CMS classifier (Nearest Template Prediction)</th>
<th>Distance to CMS1</th>
<th>Distance to CMS2</th>
<th>Distance to CMS3</th>
<th>Distance to CMS4</th>
<th>Prediction</th>
<th>P-value*</th>
<th>FDR (FDR &lt; 0.2)</th>
<th>CMS group</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td></td>
<td>0.68</td>
<td>0.75</td>
<td>0.66</td>
<td>0.76</td>
<td>CMS3</td>
<td>0.01</td>
<td>0.02</td>
<td>CMS3</td>
</tr>
<tr>
<td>HCT116</td>
<td></td>
<td>0.67</td>
<td>0.74</td>
<td>0.76</td>
<td>0.61</td>
<td>CMS4</td>
<td>0.001</td>
<td>0.002</td>
<td>CMS4</td>
</tr>
<tr>
<td>LOVO</td>
<td></td>
<td>0.61</td>
<td>0.75</td>
<td>0.72</td>
<td>0.61</td>
<td>CMS1</td>
<td>0.001</td>
<td>0.002</td>
<td>CMS1</td>
</tr>
<tr>
<td>SW620</td>
<td></td>
<td>0.74</td>
<td>0.74</td>
<td>0.78</td>
<td>0.66</td>
<td>CMS4</td>
<td>0.01</td>
<td>0.02</td>
<td>CMS4</td>
</tr>
<tr>
<td>COLO205</td>
<td></td>
<td>0.70</td>
<td>0.71</td>
<td>0.72</td>
<td>0.78</td>
<td>CMS1</td>
<td>0.7</td>
<td>0.7</td>
<td>No label</td>
</tr>
</tbody>
</table>
**Supplementary Table 3.** Log (FC) and function of Differential Expressed Genes (DEGs) after 6h NIVO in human colon cancer and melanoma cells

<table>
<thead>
<tr>
<th>GENE</th>
<th>Log FC</th>
<th>Functional properties</th>
<th>Reported functional studies in cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BATF2</td>
<td>1.12</td>
<td>Suppressor of activator protein 1 transcription factor AP-1</td>
<td>BATF2 deficiency promotes progression in human colorectal cancer via activation of HGF/PI3K/AKT signaling</td>
</tr>
<tr>
<td>DRAM1</td>
<td>0.38</td>
<td>DNA damage regulated autophagy modulator 1</td>
<td>Decreased DRAM1 expression was associated with poor prognosis in non-small cell lung carcinoma</td>
</tr>
<tr>
<td>FXYD3</td>
<td>0.62</td>
<td>Small regulatory protein associated with Na, K-ATPase as a subunit of the Na-K-ATPase</td>
<td>Reduced FXYD3 expression is correlated with the presence of lymph node metastasis in colon cancer</td>
</tr>
<tr>
<td>IFIT3</td>
<td>1.15</td>
<td>IRF genes are rapidly induced by interferon (IFN) treatment and viral infection</td>
<td>IFIT3 increased p21 protein level by downregulating c-Myc, a repressor of p21 in the cell</td>
</tr>
<tr>
<td>MT-TN</td>
<td>0.62</td>
<td>Mitochondrially Encoded tRNA-Asn</td>
<td>N/A</td>
</tr>
<tr>
<td>TNFRSF11A</td>
<td>2.47</td>
<td>Provides instructions for making a protein called receptor activator of NF-κB (RANK).</td>
<td>Reduced TNFRSF11A mRNA expression is associated with poor prognosis in colon carcinoma</td>
</tr>
<tr>
<td>CLK1</td>
<td>-2.12</td>
<td>Protein kinase that specifically regulates alternative splicing of SR (Serine and arginine-rich) protein</td>
<td>CLK1 inhibition decreased cell viability, proliferation, invasion and migration in gastric cancer</td>
</tr>
<tr>
<td>DCAF13</td>
<td>-0.58</td>
<td>RNA binding protein and can be a substrate receptor for CUL-4-AIP1 complexes with ubiquitin-protein ligase complex</td>
<td>DCAF13 overexpression was associated with poor survival in hepatocellular carcinoma</td>
</tr>
<tr>
<td>DNAJC2</td>
<td>-0.61</td>
<td>The protein is capable of forming a heterodimeric complex that associates with ribosomes, acting as a molecular chaperone for nascent polypeptide chains as they exit the ribosome.</td>
<td>DNAJC2 increased expression is correlated with gastric cancer progression</td>
</tr>
<tr>
<td>MTHFD1L</td>
<td>-0.37</td>
<td>Enzyme involved in the de novo synthesis of purines and thymidylate and support cellular methylation reactions</td>
<td>MTHFD1L silencing reduced proliferation, invasion and migration in colorectal cancer</td>
</tr>
<tr>
<td>PRPF3</td>
<td>-0.42</td>
<td>Spliceosomal component essential for pre-mRNA processing</td>
<td>PRPF3 knockdown in colon cancer reduced growth</td>
</tr>
<tr>
<td>PSMD7</td>
<td>-0.33</td>
<td>It forms a dimer with PSMD14 that functions in the removal of attached ubiquitin chain</td>
<td>PSMD7 downregulation induced apoptosis and suppressed tumorigenesis of esophageal squamous cell carcinoma</td>
</tr>
<tr>
<td>SCFD1</td>
<td>-0.50</td>
<td>SCFD1 associates with the trafficking of extracellular matrix like collagen and skeletal development in zebrafish</td>
<td>High SCFD2 mRNA expression is correlated with poorer breast cancer prognosis</td>
</tr>
</tbody>
</table>

### Supplementary Table 4. Log (FC) and function of Differential Expressed Genes (DEGs) after 24h NIVO in human colon cancer and melanoma cells

<table>
<thead>
<tr>
<th>GENE</th>
<th>Log FC</th>
<th>Functional properties</th>
<th>Reported functional studies in cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMIGO2</td>
<td>0.93</td>
<td>Transmembrane protein that promotes survival of enterobacterial granule proteins</td>
<td>Silencing of AMIGO2 decreased cell adhesion and migration and inhibited tumour growth in human gastric adenocarcinoma[21]</td>
</tr>
<tr>
<td>CLDN12</td>
<td>-0.80</td>
<td>Integral membrane proteins and components of tight junction strands</td>
<td>Anti-tumor effect in breast cancer[21]</td>
</tr>
<tr>
<td>DRAM1</td>
<td>0.41</td>
<td>Lyposomal modulator of autophagy</td>
<td>Decreased DRAM1 expression was associated with poor prognosis in non-small-cell lung carcinoma[22]</td>
</tr>
<tr>
<td>DUSP5</td>
<td>-0.86</td>
<td>Phosphatase activity toward ERK</td>
<td>Overexpressed in human gastric and colorectal carcinoma[23]</td>
</tr>
<tr>
<td>GRB10</td>
<td>0.91</td>
<td>Negatively regulates insulin/IGF signaling and directly phosphorylated by the mTOR complex 1 (mTORC1)</td>
<td>mTORC1 regulates PI3K/Akt/mTORC1 in bladder, breast or brain cancer[24]</td>
</tr>
<tr>
<td>GULP1</td>
<td>0.80</td>
<td>Adopter protein necessary for the engulfment of apoptotic cells by phagocytes</td>
<td>Potential tumor suppressor in ovarian cancer and urothelial carcinoma of the bladder[25]</td>
</tr>
<tr>
<td>HSPA5</td>
<td>0.68</td>
<td>Molecular chaperone involved in the unfolded protein response</td>
<td>Correlated with poor prognosis in gastric cancer, breast cancer, prostate cancer, and renal cell carcinoma, or with favorable prognosis in esophageal, colorectal, and lung-thymic cancers[26]</td>
</tr>
<tr>
<td>RESF1</td>
<td>0.76</td>
<td>Nuclear protein</td>
<td>Distinct in different cancer types[26]</td>
</tr>
<tr>
<td>CDKN2B</td>
<td>-1.34</td>
<td>Cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK4/6 kinases</td>
<td>Tumor suppressor in a variety of human malignancies[27]</td>
</tr>
<tr>
<td>DGAP1-AS2</td>
<td>1.49</td>
<td>Cytoskeletal long non-coding RNA</td>
<td>Upregulated DGAP1-AS2 is correlated with poor prognosis of glioma patients[28]</td>
</tr>
<tr>
<td>EGR1</td>
<td>1.74</td>
<td>Cytoskeletal protein involved in cell growth, hormone response, differentiation, hypoxia response and DNA damage response</td>
<td>Egr-1 prevented head and neck squamous cell carcinoma (HNSCC) metastasis through downregulation of MMP9 and/or MDM2[29]</td>
</tr>
<tr>
<td>NDRG1</td>
<td>-0.70</td>
<td>Cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK4/6 kinases</td>
<td>Lower NDRG1 gene expression correlated with survival and poor prognosis in colon cancer[29]</td>
</tr>
<tr>
<td>SAMD9</td>
<td>-1.33</td>
<td>Regulates cell proliferation and apoptosis</td>
<td>Overexpression of SAMD9 suppresses tumorigenesis and progression during non small cell lung cancer[30]</td>
</tr>
<tr>
<td>ARCC10</td>
<td>-0.51</td>
<td>ATP-dependent transporter probably involved in cellular detoxification through lysosomal anion excretion</td>
<td>Overexpression of ARCC10 is associated with overall survival of colorectal cancer patients[31]</td>
</tr>
<tr>
<td>COPZ1</td>
<td>-0.30</td>
<td>Involved in the Golgi apparatus and endoplasmic reticulum lipid traffic, endosome maturation, and autophagy</td>
<td>APA2A1 deletion impairs the viability of thyroid tumor cells, leads to short in autophagy, ER stress, UPS and apoptosis, reduces tumor growth, activates type I IFN pathway in thyroid cancer[33]</td>
</tr>
<tr>
<td>DPP9</td>
<td>-0.45</td>
<td>Restoration of phosphatidylserine (PS) from diphosphatidylglycerol in the PS-rich cell surface membrane</td>
<td>Identified among DEGs in liver cancer[34]</td>
</tr>
<tr>
<td>DPP9</td>
<td>-0.45</td>
<td>Restores the membrane potential by proteolytic cleavage of cytoplasmic substrates such as mitochondria, necroptosis and peptide hormones</td>
<td>DPP9 increases chemoresistance and is an Indicator of Poor Prognosis in Colorectal Cancer[35]</td>
</tr>
<tr>
<td>GNE</td>
<td>0.79</td>
<td>Catalase is a rate-limiting key step in the scut acid biosynthetic pathway</td>
<td>Loss of GNE indices autophagic processes in pancreatic cancer cells[36]</td>
</tr>
<tr>
<td>GYS1</td>
<td>-0.97</td>
<td>Adduction of glucose monomers to the growing glycoprotein molecule</td>
<td>GYS1 induces gliogenesis and promotes tumor progression via the NF-κB pathway in Clear Cell Renal Carcinoma[37]</td>
</tr>
<tr>
<td>MIRN181</td>
<td>-0.28</td>
<td>Involved in the regulation of the intracellular response to DNA damage</td>
<td>Involved in the response of the intracellular response to DNA damage[38]</td>
</tr>
<tr>
<td>MID3G</td>
<td>0.65</td>
<td>Involved in the regulation of the intracellular response to DNA damage</td>
<td>Involved in the response of the intracellular response to DNA damage[38]</td>
</tr>
<tr>
<td>MAGED1</td>
<td>1.34</td>
<td>Secretory vesicle protein complex involved in Golgi apparatus and endoplasmic reticulum lipid traffic, endosome maturation, and autophagy</td>
<td>MAGED1 has been reported to be significantly overexpressed in esophageal carcinoma, colon cancer, melanoma, prostate cancer, breast cancer and lung cancer[39]</td>
</tr>
<tr>
<td>MVK</td>
<td>-0.47</td>
<td>Catalase conversion ERR1011, a mitochondrial enzyme, into methylmalonyl-CoA</td>
<td>Pathway methylmalonyl-CoA metabolism contributes to tumorigenesis[40]</td>
</tr>
<tr>
<td>NSD2L</td>
<td>-0.42</td>
<td>Involved in cholesterol biosynthesis</td>
<td>Cholesterol Pathway Inhibitors TGIF-β-Signaling to Promote Basal Differentiation in Pancreatic Cancer[41]</td>
</tr>
<tr>
<td>NTSC2</td>
<td>-0.90</td>
<td>Hydrolysis activity and S- nucleotidase activity</td>
<td>Up-regulated in CRC tissues and cell lines and CRC patients correlate with poor overall survival[42]</td>
</tr>
<tr>
<td>PIA54</td>
<td>-0.35</td>
<td>Functions as an E3 type small ubiquitin-like modifier (SUMO) ligase, stabilizing the interaction between UBE2L2 and the substrate, and as a SUMO-activating factor.</td>
<td>PIA54 is highly expressed in pancreatic cancer cells inducing cell growth genes[43]</td>
</tr>
<tr>
<td>PRR11</td>
<td>-0.43</td>
<td>Involved in cellular response to damage</td>
<td>PRR11 promoted the growth and progress of colorectal cancer[44]</td>
</tr>
<tr>
<td>SFB2</td>
<td>-0.27</td>
<td>Involved in pro-IFNγ signaling as a component of the signaling factor IFNβ complex</td>
<td>SFB2 is associated with poor progression-free survival in prostate cancer patients[45]</td>
</tr>
<tr>
<td>THRA</td>
<td>-0.95</td>
<td>Nuclear hormone receptor with high affinity receptor for thyroid hormones, including thyroid hormone receptors and thyroxine</td>
<td>Upregulated THRA1 in human colorectal cancer patients is correlated with Wnt-β-catenin activity[46]</td>
</tr>
<tr>
<td>TOM1</td>
<td>-0.83</td>
<td>Adapter protein needed for the maturation of phosphatases</td>
<td>TOM1 gene represents a multiple myeloma risk allele[47]</td>
</tr>
<tr>
<td>UBA1</td>
<td>-0.45</td>
<td>Catalyzes the first step in ubiquitin conjugation</td>
<td>UBA1 knockdown effectively inhibits colon tumor growth and mediates tumor regression in a human colorectal xenograft model[48]</td>
</tr>
<tr>
<td>UBE4B</td>
<td>-0.55</td>
<td>Member of E3 ubiquitin ligase involved in multubiquitin chain assembly</td>
<td>High UBE4B expression may be related to a poor clinical outcome in RCC patients[49]</td>
</tr>
<tr>
<td>USP5</td>
<td>-0.63</td>
<td>Ubiquity-specific protease that cleaved preferentially branched ubiquitins</td>
<td>Upregulated USP5 correlated with CRC stages and the overall survival of CRC patients and conferred chemotherapeutics resistance[50]</td>
</tr>
<tr>
<td>XO7</td>
<td>-0.54</td>
<td>Mediates nuclear export of various cytoplasmic cargo proteins</td>
<td>Elevated cytoplasmic expression of XPO7 in ovarian cancer is strongly associated with poor overall patient survival[51]</td>
</tr>
</tbody>
</table>
Supplementary Table 5. Primer Sequences for SYBR Green RT-qPCR.

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>FWD Sequence (5’ → 3’)</th>
<th>REV Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>TAGCACAGCCTGGATAGCAA</td>
<td>GAGAAATCTGGGACACACACC</td>
</tr>
<tr>
<td>BATF2</td>
<td>AGACCCCAAGGAGAACAA</td>
<td>CAGGGCGAGGTTCTTT</td>
</tr>
<tr>
<td>CDKN2B</td>
<td>AACGGGAGCAAGGAAAGGTCT</td>
<td>TGTGCGCAGGTACCCTCGAA</td>
</tr>
<tr>
<td>CLK1</td>
<td>GTGCCTGACATGGGGAGAGGC</td>
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References


Supplementary Figure 1

The figure shows the distribution of PD-1 and PD-L1 expression in various cell lines: HT29, HCT116, LOVO, SW620, COLO205, MOLT4, and 8505C. The x-axis represents the count of cells, while the y-axis represents the proportion of cells expressing PD-1 or PD-L1. Two conditions are compared: control (light gray) and positive staining (dark gray). The data suggest that positive staining is more prevalent in some cell lines compared to the control.
Supplementary Figure 2

**HT29**

<table>
<thead>
<tr>
<th>PI</th>
<th>AnnexinV FITC</th>
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<td>CTR</td>
<td>Early apoptosis</td>
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<tr>
<td>PD-L1</td>
<td>Early apoptosis</td>
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<tr>
<td>PD-L1+Irinotecan</td>
<td>Early apoptosis</td>
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**HCT116**

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<td>Early apoptosis</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Early apoptosis</td>
</tr>
<tr>
<td>PD-L1+Irinotecan</td>
<td>Early apoptosis</td>
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</table>

**LOVO**

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<td>CTR</td>
<td>Early apoptosis</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Early apoptosis</td>
</tr>
<tr>
<td>PD-L1+Irinotecan</td>
<td>Early apoptosis</td>
</tr>
</tbody>
</table>
Supplementary Figure 3

**HT29**

**HCT116**

**LOVO**

Cell Number

CTRL  
NIVO 0.1µM  
NIVO1µM  
PD-L1  
PD-L1 +NIVO 0.1µM  
PD-L1 +NIVO1µM

0 24h 48h 72h

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Supplementary Figure 4
Supplementary Figure 5

Part A

- Y-axis: % Positive cells
- X-axis: HT29, HCT116, LOVO, SW620, Colo205
- Legend: CD16, CD64, CD32

Part B

- HT29 and HCT116 graphs
- Y-axis: Cell number
- X-axis: 0, 24h, 48h, 72h
- Legends: CTRL, NIVO 0.1µM, NIVO 1µM, F(ab)2 NIVO 0.1µM
- Statistical symbols: ***, ns

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Supplemental material

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Supplementary Figure 6

**HT29**

- CTRL
- INH p38
- INH MEK
- INH AKT
- NIVO
- NIVO+INH p38
- NIVO+INH MEK
- NIVO+INH AKT

**HCT116**

- CTRL
- INH p38
- INH MEK
- INH AKT
- NIVO
- NIVO+INH p38
- NIVO+INH MEK
- NIVO+INH AKT

Cell proliferation at 72h (% of control)
Supplementary Figure 7
Supplementary Figure 10A

6h PD-L1

PES43

HT29

HCT116

24h PD-L1
Supplementary Figure 10B

PES43  HT29  HCT116

6h NIVO+ PD-L1

24h NIVO+ PD-L1
Supplementary Figure 11

**PD-L1 6h**

HCT116: 557, 153, 2095, 1793, 4607
HT29: 37, 66, 71, 43, 49

**PD-L1 24h**

HCT116: 384, 68, 71, 43, 49
HT29: 11, 1, 0, 0, 0

**NIVO + PD-L1 6h**

HCT116: 1989, 153, 1793, 1844, 2529
HT29: 37, 66, 71, 43, 49

**NIVO +PD-L1 24h**

HCT116: 1989, 153, 1793, 1844, 2529
HT29: 37, 66, 71, 43, 49
Supplementary Figure 12A

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Supplementary Figure 12B
Supplementary Figure 12C

- **PES43**
  - Log Fold Change RNA-seq vs Log Fold Change qRT-PCR
  - Pearson r = 0.7977
  - p < 0.0001

- **HCT116**
  - Log Fold Change RNA-seq vs Log Fold Change qRT-PCR
  - Pearson r = 0.8310
  - p < 0.0001

- **HT29**
  - Log Fold Change RNA-seq vs Log Fold Change qRT-PCR
  - Pearson r = 0.7796
  - p < 0.0001
Supplementary Figure 13

A

CTRL | NIVO | OXA | OXA+NIVO

B

200X | 400X

SCALEBAR 400X: 50 µm  SCALEBAR 200X: 100 µm
Supplementary Figure 15
Supplementary Figure 16

Histotype

PD-1 cancer intrinsic expression %

MA   NOS

P = 0.0007

*  **
In PD-1+ human colon cancer cells NIVOLUMAB promotes survival and could protect tumor cells from conventional therapies

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In Brief
Intrinsic PD-1 is expressed and functional in human colon cancer cells. NIVOLUMAB promotes human colon cancer proliferation, reduces apoptosis, protects cells from Chemo/Radiotherapy in vitro and in vivo.