Cancer Immunology, Immunotherapy (2019) 68:379–393
https://doi.org/10.1007/s00262-018-2283-0

ORIGINAL ARTICLE

Enhanced anti-tumor efficacy of checkpoint inhibitors in combination with the histone deacetylase inhibitor Belinostat in a murine hepatocellular carcinoma model

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Received: 17 January 2018 / Accepted: 6 December 2018 / Published online: 13 December 2018
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Abstract

Immune checkpoint inhibitors are currently tested in different combinations in patients with advanced hepatocellular carcinoma (HCC). Nivolumab, an anti-PD-1 agent, has gained approval in the second-line setting in the USA. Epigenetic drugs have immune-mediated antitumor effects that may improve the activity of immunotherapy agents. Our aim was to study the therapeutic efficacy of checkpoint inhibitors (anti-CTLA-4 and anti-PD-1 antibodies) in combination with the histone deacetylase inhibitor (HDACi) Belinostat. In a subcutaneous Hepa129 murine HCC model, we demonstrated that Belinostat improves the antitumor activity of anti-CTLA-4 but not of anti-PD-1 therapy. This effect correlated with enhanced IFN-γ production by antitumor T-cells and a decrease in regulatory T-cells. Moreover, the combination induced early upregulation of PD-L1 on tumor antigen-presenting cells and late expression of PD-1 on tumor-infiltrating effector T-cells, suggesting the suitability of PD-1 blockade. Indeed, Belinostat combined with the simultaneous blockade of CTLA-4 and PD-1 led to complete tumor rejection. These results provide a rationale for testing Belinostat in combination with checkpoint inhibitors to enhance their therapeutic activity in patients with HCC.

Keywords Checkpoint inhibitors · HDAC inhibitor · Hepatocellular carcinoma · M1 macrophages · PD-1/PD-L1 expression · T regulatory cells

These results were presented as an abstract [1] and poster (poster SAT-159) at the International Liver Congress (ILC), April 11–16 2018 Paris, France.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00262-018-2283-0) contains supplementary material, which is available to authorized users.

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Abbreviations

FDA	Food and Drug Administration
HCC	Hepatocellular carcinoma
HDACi	Histone deacetylase inhibitors
PTCL	Peripheral T cell lymphoma

Introduction

More than 850,000 cases of liver cancer are diagnosed annually [2]. Hepatocellular carcinoma (HCC) accounts for 90% of these cases and is the third most common cause of cancer-related death worldwide. Different etiologies are involved in HCC development including cirrhosis, hepatitis B and C virus infection, excessive alcohol consumption and obesity [3]. For patients that are diagnosed with or reach the advanced stage, Sorafenib has been the only systemic therapy available for almost a decade [4] while only recently Regorafenib has proved effective after Sorafenib failure [5]. Immunotherapy, mainly the one based on inhibitors

of T-cell checkpoint molecules such as CTLA-4 or PD-1/PD-L1, has demonstrated in the recent years its enormous potential against cancer [6–9] and is currently used to treat several tumor types. In HCC, signs of activity were first shown with the anti-CTLA-4 antibody Tremelimumab [10]. More recently, the anti-PD-1 agent Nivolumab has shown unequivocal antitumor activity [11] leading to accelerated approval by the Food and Drug Administration (FDA) to treat patients with advanced HCC previously treated with Sorafenib. Combination of these antibodies with therapies already used in HCC have also shown promising results [12]. Moreover, different clinical trials based on monotherapies with CTLA-4 or PD-1/PD-L1 blocking antibodies or their combinations are ongoing (https://clinicaltrials.gov). However, both in other tumors [13] and in HCC [11] only a subset of patients responds to these therapies. To benefit a higher proportion of patients, several approaches combining these antibodies with new or existing therapies are being developed in the preclinical setting or tested in clinical trials [14]. Most of these combinations, nevertheless, are not supported by a strong scientific rationale.

Epigenetic drugs targeting modifications at the DNA and histone levels are a group of antitumor agents (reviewed in Ref. [15]). While the presumed mechanism of action of these compounds was a direct antitumor effect, they can simultaneously modify antitumor immunity. Thus, they may not only act at the level of tumor cells, by modulating antigen expression and the machinery responsible for their presentation and recognition by T-cells [16, 17], but they may also directly act on the immune system, activating effector cells [18, 19] and inhibiting immunosuppressive mechanisms [20, 21]. Accordingly, some of these compounds increase the efficacy of checkpoint inhibiting antibodies in different murine tumor models [22, 23]. HDACi have demonstrated promising effects by improving the antitumor efficacy of immunotherapies based on anti-CTLA-4, anti-PD-1 or their combination [22, 24]. Belinostat is an HDACi that, according to its profile, can be considered as a pan-inhibitor [25]. It has been clinically tested in patients with solid and hematological tumors [26–28], but is currently approved by the FDA (Beleodaq®) only for the treatment of peripheral T-cell lymphoma (PTCL) [29]. In HCC, although preclinical data showed an inhibitory effect of Belinostat on the growth of HCC lines [30, 31], no convincing signs of efficacy were shown in a phase I/II trial [32]. Nevertheless, as Belinostat was well tolerated, it is a good candidate for combination therapy.

Based on the potential for a true synergistic effect, we hypothesized that the combined administration of Belinostat with checkpoint inhibitors may lead to an enhanced therapeutic effect. We thus tested the therapeutic efficacy of Belinostat in combination with anti-CTLA-4 or anti-PD-1 antibodies in a preclinical model of C3H mice injected with Hepa129 HCC cells, analyzing antitumor efficacy of these combinations as well as associated immune-mediated mechanisms.

Materials and methods

Reagents

Belinostat (AMRI, Rensselaer, NY, USA) was dissolved in buffer containing 50 mg/ml L-Arginine as formulated in the commercialized i.v. form Beleodaq®. Antibodies against CTLA-4 (9D9), PD-1 (RMP1-14) or isotype control (rat IgG2a, 2A3) were purchased from BioXcell.

Mice

Eight-week-old female C3H mice were maintained in pathogen-free conditions.

Tumor cells

Hepa129 HCC cells [33] were grown in complete medium (RPMI 1640 supplemented with 10% heat-inactivated bovine serum and antibiotics). Cell stocks were generated upon cell-line receipt and early passages were used for tumor experiments. They were routinely tested for mycoplasma.

Tumor treatment experiments

Mice were injected subcutaneously with 10^6 Hepa129 tumor cells. To determine therapeutic efficacy, treatment started 1 week later, when tumors had reached 5 mm in diameter (day 0). Mice received intraperitoneal (i.p.) injections of therapeutic or control antibodies (50 µg/mouse) at days 0 and 7. Some groups received daily administration of Belinostat for 3 weeks (days 0–21), either i.p. (90 mg/kg) or orally (45 mg/kg). In experiments used to analyze intratumor immune parameters, treatment started when tumors had reached 8 mm. Tumor volume was calculated using the formula: $V = (\text{length} \times \text{width}^2)/2$. Mice were killed when tumor diameter reached 17 mm.

ELISPOT

T cells producing IFN-γ were determined by ELISPOT (BD-Biosciences) as described [34]. Briefly, splenocytes (5×10^5/well) were stimulated with 5×10^4 irradiated tumor cells, previously treated for 24 h with 500 U/ml of murine IFN-γ. The in vitro effect of Belinostat on tumor cells was tested using cells previously treated with 0.5 µM Belinostat. After 1 day of culture, the number of spot-forming cells was enumerated with an automated counter.

In vitro assays to test the effect of Belinostat

In the case of tumor cells, effect of Belinostat on acetylation was tested by culturing Hepa129 cells (10^6/well) with different Belinostat concentrations in 24-well plates for 2 h and then harvested. When measuring effect on inhibition of tumor cell growth, cells (10^4/well) were cultured for 48 h, harvested and the proportion of surviving cells was determined after staining with Trypan Blue. IC_{50} was determined using the GraphPad Prism software after plotting Belinostat concentrations and cell survival. After that, cells were routinely treated with this concentration and 24 h later they were harvested for flow cytometry or antigen presentation assays.

Belinostat effect on macrophages was tested by culturing bone marrow cells (10^6 cells/well) in 12-well plates in 3 ml in the presence of 100 ng/ml of macrophage colony-stimulating factor (m-CSF). After 5 days, cells were polarized towards M1 macrophages with IFN-γ (25 ng/ml) and LPS (100 ng/ml), M2 with IL-4 (10 ng/ml) or left untreated, with or without 0.1 µM Belinostat. One day later, cells were harvested and M1 markers CD86 and CD38 were analyzed by flow cytometry.

Western blot

In vitro cultured tumor cells were lysed in a buffer containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$ and histones extracted as described in Ref. [35]. Then, proteins were separated, transferred to nitrocellulose membranes and detected by incubating the membrane with anti-acetylated H4 histone (1/10,000 dilution; Abcam) or anti-H2A.X histone (1/10,000 dilution; Abcam) antibodies, followed by 1/10,000 dilution of HRP-conjugated goat anti-rabbit IgG (Millipore). Protein bands were detected by enhanced chemiluminescence (ECL) (Amersham).

Flow cytometry

Spleens and tumors were obtained after treatment, incubated with collagenase/DNase for 15 min and subsequently homogenized. Cells were incubated for 10 min with Fc Block™ (BD-Biosciences) and stained with specific antibodies. To analyze antigen-presenting cells (APC) and PD-L1 expression antibodies CD45-APC-Cy7, CD11c-BV570, F4/80-Pacific Blue, CD11b-APC, Ly6C-PerCP/Cy5.5 and Ly6G-PE-Cy7 were used to define dendritic cells (DC), monocytes, granulocytes and macrophages (M1 detected with anti-CD38 PerCP/Cy5.5 and M2 with anti-CD206 PE-Cy7, among CD11b and F4/80 positive cells) and anti-PD-L1-PE. Antibodies were purchased from Biolegend, except those targeting CD45, Ly6G and PD-L1 (BD-Biosciences). Analysis of lymphocyte subsets and expression of PD-1 on these cells was carried out using antibodies CD45-APC-Cy7, CD3-PE-Cy5 (AbD serotec), CD8-BV421 (Biolegend), CD4-BV570 (Biolegend), CD25-PE-Cy7 (TONBO Biosciences) and PD-1-PerCP/Cy5.5 (Biolegend). Cells were then fixed, permeabilized and finally intracellularly stained with anti-Foxp3-APC (TONBO). Tumor cells treated in vitro with Belinostat were stained with anti-H-$2K^k$-FITC (BD-Biosciences), PD-L1-PE (BD-Biosciences) and CD86-PE-Cy5 (Biolegend). Samples were acquired with a FACSCantoII flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star Inc).

Protein array

Mice (2–3/group) with 8 mm tumors received different treatments for 19 days as described above and tumors were excised, weighted, homogenized in Radioimmunoprecipitation assay (RIPA) buffer and total protein content measured using the bicinchoninic acid assay. The concentration of different cytokines, chemokines and growth factors in the samples was determined using a protein array (Quantibody Mouse Cytokine Array 5; RayBiotech) according to manufacturer's instructions.

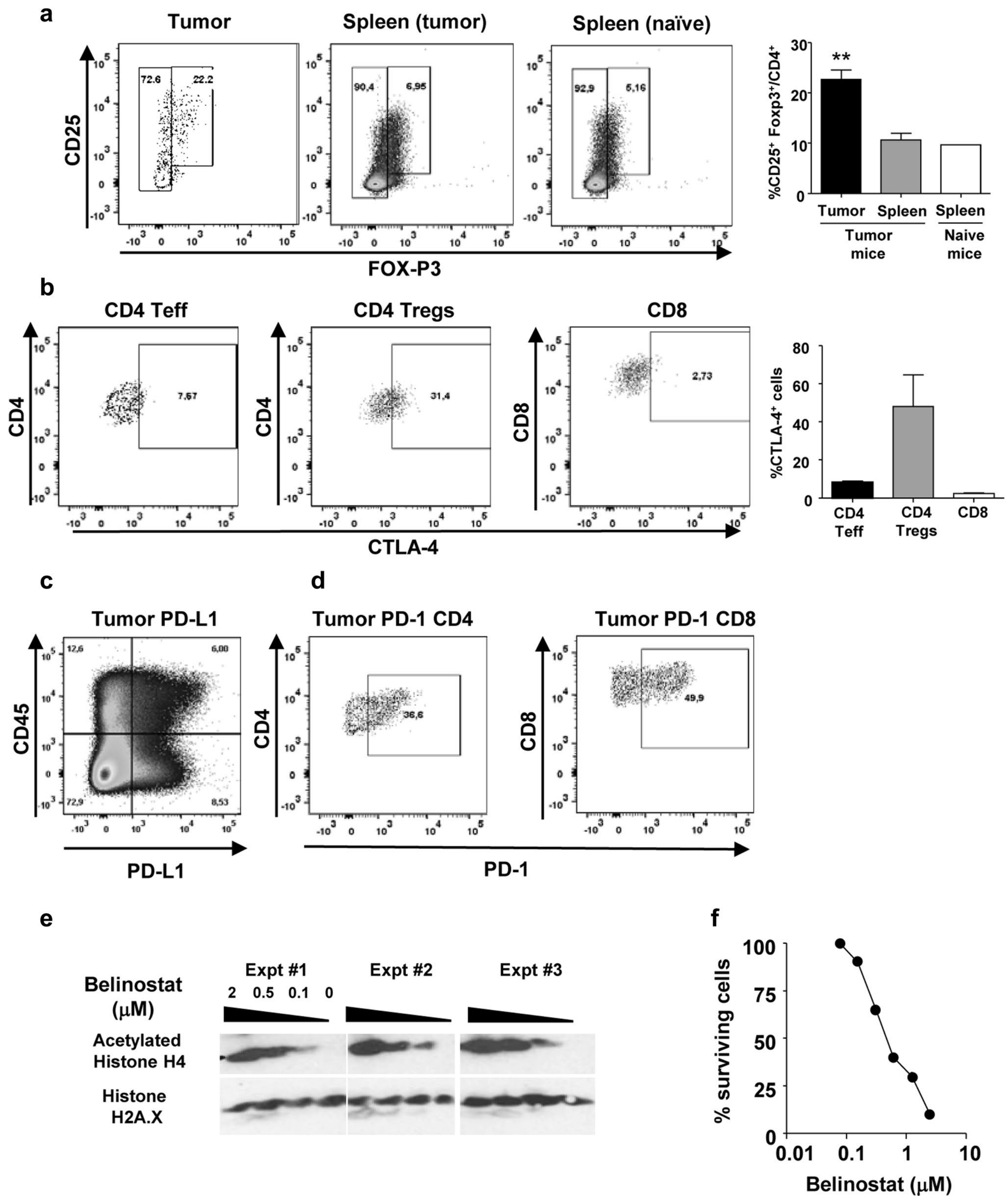
Statistical analysis

Tumor size and immune responses were analyzed using Student's *t* tests. $P < 0.05$ was taken to represent statistical significance.

Results

Expression of CTLA-4 and PD-1/PD-L1 in Hepa129 tumors and sensitivity of immune and tumor cells to Belinostat

The efficacy of combinations of Belinostat with checkpoint inhibitors in HCC was analyzed using the Hepa129 tumor model. Tumors growing after subcutaneous inoculation of Hepa129 cells contained infiltrating $CD4^+$ Tregs, at levels clearly above those observed in the spleen of these tumor-bearing animals or in the spleen of naïve mice without tumors (Fig. 1a). A significant percentage of these $CD4^+$ Tregs, as opposed to effector CD4 or CD8 T-cells, expressed CTLA-4 (Fig. 1b). As for the PD-1/PD-L1 axis, although PD-L1 was not expressed by tumor cells in vitro (data not shown), it was detected in vivo on tumor cells and on infiltrating $CD45^+$ leukocytes (Fig. 1c). Moreover, a significant proportion of CD4 and CD8 infiltrating T-cells was PD-1^+ (Fig. 1d). These features resembled some of the conditions observed in HCC patients [36–39] and suggested that they



could be amenable to treatment with checkpoint inhibiting antibodies.

Epigenetic drugs have been described to regulate properties of tumor cells. Thus, before administering Belinostat in combination with checkpoint inhibitors, we tested its capacity to modulate histone acetylation in Hepa129 tumor cells. In vitro experiments demonstrated evident histone acetylation when cells were treated with

◂**Fig. 1** Expression of CTLA-4 and PD-1/PD-L1 in Hepa129 tumors and sensitivity of tumor cells to Belinostat C3H mice were injected with 10^6 Hepa129 tumor cells and 10 days later tumors were harvested, homogenized and stained with antibodies to analyze tumor cells and infiltrating leukocytes. **a** Percentage of Tregs among total CD4 T-cells in their tumor, spleen and in the spleen of control naive mice. Representative dot plots (left) and summary of results from four mice (right) (**$p<0.01$). **b** Percentage of CTLA-4$^+$ cells in tumor effector and regulatory CD4 T-cells and in CD8 T-cells. **c** Expression of PD-L1 on infiltrating CD45$^+$ leukocytes and in CD45$^-$ tumor cells. **d** Percentage of PD-1$^+$ cells in tumor-infiltrating CD4 and CD8 T-cells. **e** In vitro effect of Belinostat on histone H4 acetylation in Hepa129 cells treated with different concentrations and analyzed 2 h later. **f** In vitro inhibitory effect of Belinostat on Hepa129 cells, corresponding to one representative experiment out of three carried out

different Belinostat concentrations (Fig. 1e). This effect was accompanied by a dose-dependent inhibition of cell proliferation in vitro. In several experiments, we observed that Belinostat inhibited growth of these cells at an IC_{50} of 0.4567 ± 0.070 µM (Fig. 1f).

Thus, the presence of the immune checkpoint targets in Hepa129 tumors and the sensitivity of hepatoma cells to Belinostat suggested this tumor model could be suitable for testing combinatorial therapies.

Belinostat improves therapeutic efficacy of anti-CTLA-4 but not of anti-PD-1 antibodies

Combination of Belinostat with checkpoint inhibiting antibodies was studied by treating mice bearing 5-mm tumors with daily i.p. administration of Belinostat for 3 weeks (days 0–21), together with antibody administration at days 0 and 7. Monotherapy with Belinostat, despite its in vitro effect, did not show any antitumor in vivo effect at the dose tested. Similarly, anti-PD-1 antibodies had a poor effect, a result that was not improved after combination with Belinostat. Anti-CTLA-4, however, clearly delayed tumor growth, an effect that was more evident when combined with Belinostat (Fig. 2a). Indeed, analysis of tumor size at day 18, when control animals treated with isotype antibodies had still a survival above 70%, showed a statistically significant antitumor effect, observed only in mice treated with anti-CTLA-4 + Belinostat (Fig. 2b). Regarding survival, all mice treated with anti-CTLA-4 + Belinostat were still alive at day 30, compared to 75% of those treated with anti-CTLA-4 alone. By contrast, less than 40% survival was observed in the remaining groups (Fig. 2c). These results strongly suggest that the antitumor effect induced by anti-CTLA-4 is improved by combination with Belinostat.

Belinostat enhances antitumor immunity and decreases Tregs in anti-CTLA-4-treated mice

To understand the immune mechanisms behind the observed effect, immune parameters were analyzed in the spleen of tumor-bearing mice subjected to a 14-day treatment, when anti-CTLA-4-treated mice showed stable tumor size while the remaining groups had growing tumors. Although CD4 and CD8 T-cell proportions did not show significant differences between groups (Fig. 3a, b), the percentage of CD4 Tregs decreased in mice treated with anti-CTLA-4 + Belinostat compared to anti-CTLA-4 alone (Fig. 3c). Antitumor immunity was tested by stimulating splenocytes with irradiated tumor cells, and using IFN-γ production as read out. While Belinostat did not enhance the immune response in mice treated with isotype control or anti-PD-1 antibodies, it significantly promoted a stronger response in mice treated with anti-CTLA-4 (Fig. 3d). Finally, Belinostat decreased the proportion of PD-1$^+$ cells in CD4 but not CD8 T-cells during anti-CTLA-4 treatment, but not in mice treated with control or anti-PD-1 antibodies (Fig. 3e, f). These results suggest that Belinostat enhances antitumor immunity while down regulating inhibitory mechanisms in animals treated with anti-CTLA-4 but not with anti-PD-1, an effect that is associated with the enhanced antitumor effect.

To test if the enhanced immune responses induced by Belinostat + anti-CTLA-4 were mediated by modulation of tumor cell antigenicity/immunogenicity, we performed additional experiments in vitro. Hepa129 cells barely expressed MHC-I, CD86 or PD-L1 in vitro, and Belinostat did not modify their levels (Supplementary Fig. 1a–c). Accordingly, tumor-specific T-cells obtained from cured mice recognized untreated and Belinostat-treated tumor cells similarly (Supplementary Fig. 1d), suggesting that mechanisms different from direct modulation of tumor cell properties are involved in the enhanced antitumor effect induced by the combination.

Enhanced antitumor effect of anti-CTLA-4 + Belinostat combination is associated with increased early infiltration of M1 macrophages and PD-L1 upregulation

To understand the mechanisms associated with the superior efficacy of anti-CTLA-4 + Belinostat, we studied intratumor immune parameters at different time points. At day 7, although the combination group had the highest proportion of CD45$^+$ infiltrating leukocytes, the difference did not reach statistical significance (Supplementary Fig. 2a, $p=0.07$; isotype vs anti-CTLA-4 + Belinostat). Regarding innate immunity mediated by APC, lower percentages of DC were found in the combination group and no differences existed in the proportion of monocytic and granulocytic cells or in the

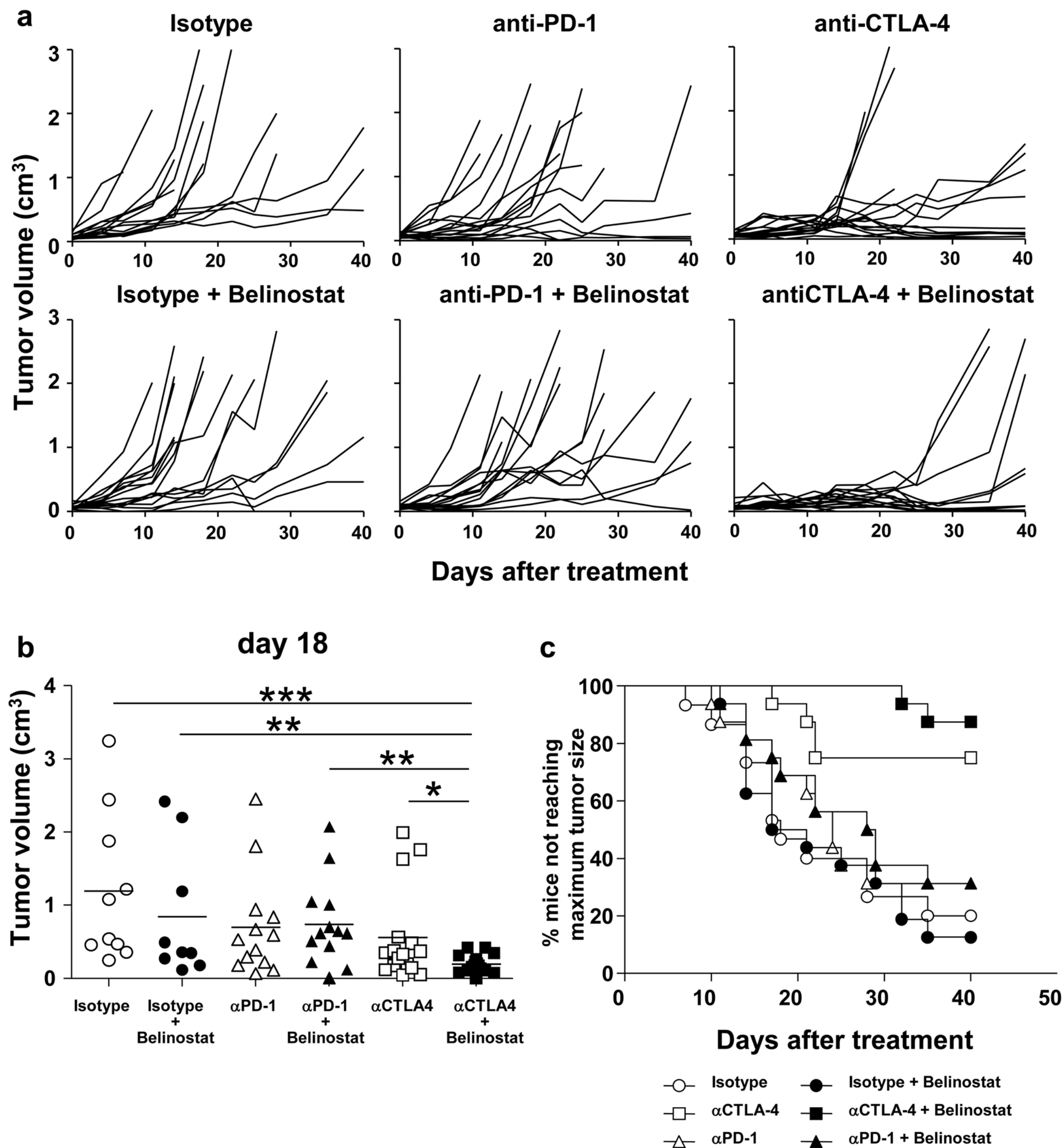


Fig. 2 Belinostat improves therapeutic efficacy of anti-CTLA-4 but not of anti-PD-1 antibodies. Mice ($n = 14$–16/group) bearing 5 mm tumors were treated with isotype control, anti-PD-1 or anti-CTLA-4 antibodies (days 0 and 7) with or without Belinostat (days 0–21). **a** Tumor growth of each individual mouse was plotted. **b** Tumor volume at day 18 ($^{*,**,***}p < 0.05$, 0.01 or 0.001, respectively). **c** Mice were killed when tumor reached 17 mm and the percentage of those not reaching this tumor size was represented

entire macrophage population. Interestingly though, a significant increase in M1 macrophages (CD38$^+$) and a trend towards less M2 macrophages (CD206$^+$) was observed in the combination group compared to anti-CTLA-4 (Fig. 4a). To study the effect of Belinostat on M1 macrophage increase, in vitro experiments were carried out. Addition of Belinostat to unpolarized or IL-4-polarized (M2) macrophages led to an increase in the percentage of macrophages expressing CD38 and CD86 M1 markers (Fig. 4b). These effects were not observed when adding Belinostat to M1-polarized macrophages (data not shown), which already had high expression of these markers.

Regarding PD-L1, despite similar levels of PD-L1 expression when considering total CD45$^+$ tumor leukocytes (Supplementary Fig. 2b), increased proportions of PD-L1$^+$ cells induced by the combination were found in most APC populations, the difference being statistically significant for DC as well as for M1 and M2 macrophages (Fig. 4c). This was accompanied by higher per cell expression levels, measured as mean fluorescence intensity. Although PD-L1 can

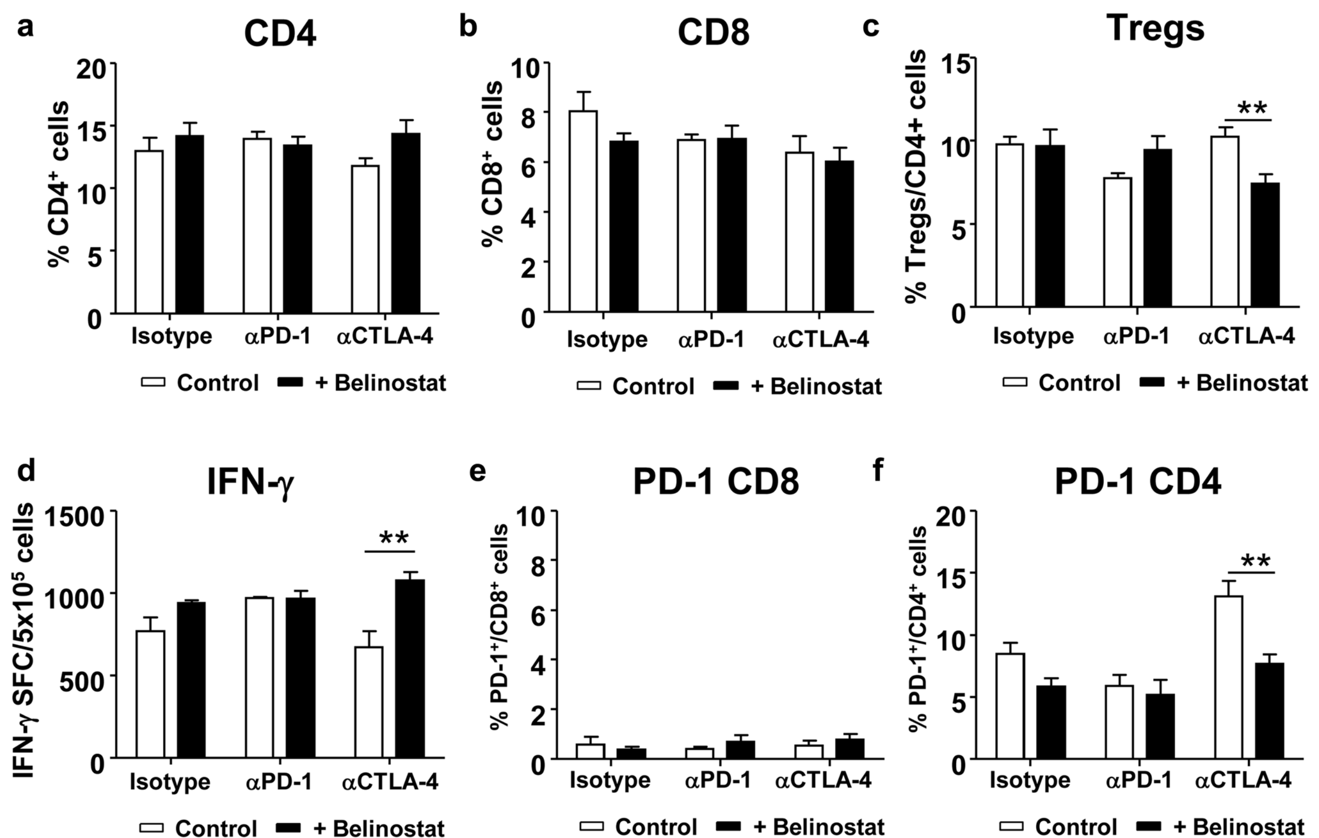


Fig. 3 Belinostat enhances antitumor immunity and decreases Tregs in anti-CTLA-4-treated mice. Mice ($n = 5$–6/group) bearing 5 mm tumors were treated with isotype control, anti-PD-1 or anti-CTLA-4 antibodies (days 0 and 7) with or without Belinostat (days 0–14) and spleens were harvested for immunological analyses. Percentages of CD4 (**a**), CD8 (**b**) and regulatory T-cells (**c**), as well as the expression of PD-1 on CD8 (**e**) and CD4 (**f**) T-cells was determined by flow cytometry. The antitumor immune response was analyzed by ELISPOT measuring IFN-γ-producing cells after stimulation of splenocytes with irradiated tumor cells (**d**) (**$p < 0.01$)

be also expressed by tumor cells, $CD45^-$ cells expressed similar PD-L1 levels irrespective of treatment (Supplementary Fig. 2c), a result in agreement with the lack of effect of Belinostat on PD-L1 expression when used in vitro with tumor cells (Supplementary Fig. 1c).

Analyses of the proportion and phenotype (PD-L1 expression) of APC subsets at later stages (days 14 or 19 after treatment) did not show relevant differences between groups (data not shown).

Anti-CTLA-4 + Belinostat combination modulates PD-1 expression in Tregs and effector T-cells associated with higher effector cytokine levels

The early differences observed in innate intratumor immunity (in terms of APC proportions and PD-L1 expression), and considering the effect that these events may have on adaptive immunity, prompted us to study tumor-infiltrating T-cells at day 7. As opposed to innate immunity, no relevant differences were detected in the proportion of CD4, CD8 or Treg cells (data not shown). By contrast, higher proportions of CD4 and CD8 T-cells were found at day 14 in Belinostat-treated mice, as compared mainly with the combination group. Moreover, mirroring the splenic cell compartment (Fig. 3), a lower proportion of Tregs were observed in the tumor compartment in the combination group compared with anti-CTLA-4, a difference that almost reached statistical significance ($p = 0.06$) (Fig. 5a). Furthermore, anti-CTLA-4 increased the proportion of $PD\text{-}1^+$ cells in all T-cell subsets. Interestingly, combination with Belinostat decreased these values both in CD4 Tregs and in CD8 T-cells, but not in effector CD4 T-cells (Fig. 5b).

Changes observed at day 14 led us to analyze these parameters at day 19, when mice treated with the combination had lower tumor burden than those treated with anti-CTLA-4 alone. The only significant change in terms of cell proportions was a decrease in CD4 T-cells (as observed at day 14), whereas the decrease in the proportion of CD8 or Tregs was no longer statistically significant (Fig. 5c). Of note, as opposed to day 14, where anti-CTLA-4 but not the combination increased PD-1 expression, day 19 was characterized by PD-1 upregulation in effector CD8 and CD4

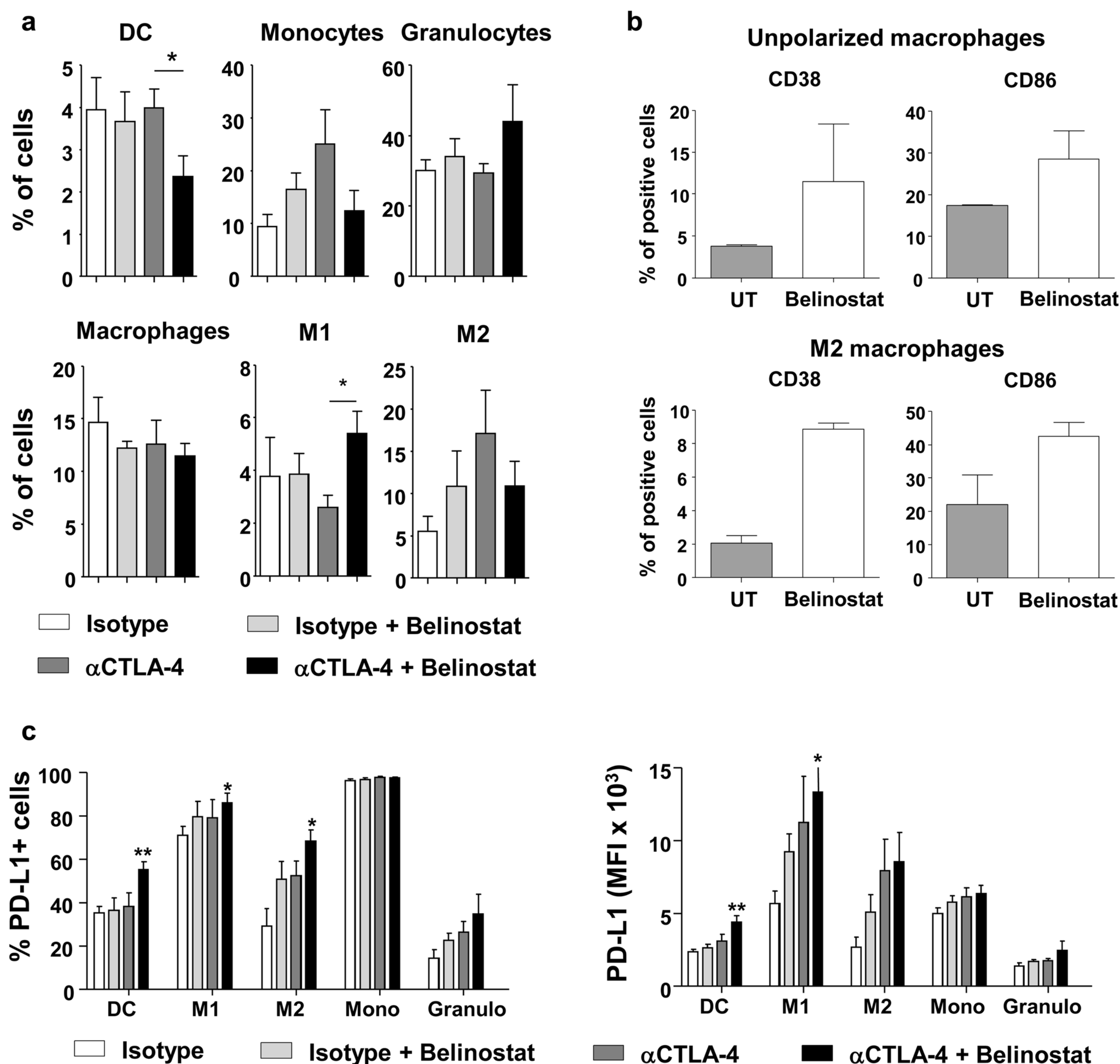


Fig. 4 Characterization of tumor-infiltrating antigen-presenting cells and expression of PD-L1. Mice ($n=5$–6/group) bearing 8 mm tumors were treated with isotype control or anti-CTLA-4 antibodies with or without Belinostat, tumors were harvested at day 7 and analyzed by flow cytometry. **a** Proportion of DC, monocytes, granulocytes and macrophages (total and M1/M2) amongst $CD45^+$ infiltrating cells. **b** Expression of M1 macrophage markers CD38 and CD86 in unpolarized or M2-polarized macrophages treated in vitro with Belinostat. Results shown are the sum of two different experiments with a single well per experiment. **c** Expression of PD-L1 in APC subsets obtained from mice shown in panel a. Results are represented as % of positive cells (left panel) or as mean fluorescence intensity (right panel) ($*p<0.05$)

T-cells induced by the combination. Simultaneously, the combination decreased the proportion of PD-1-expressing Tregs (Fig. 5d). These results indicate that the combination not only modifies the proportion of infiltrating T-cells, but also the expression of PD-1 in effector and regulatory T-cells over time.

PD-1 is an inhibitory receptor induced by Ag recognition and concomitant TCR signaling [40]. Although it has been associated with exhausted T-cells, it can be also considered an activation marker [41]. Therefore, we measured the production of cytokines in tumor tissue at day 19, coinciding with PD-1 upregulation on T-cells. These experiments

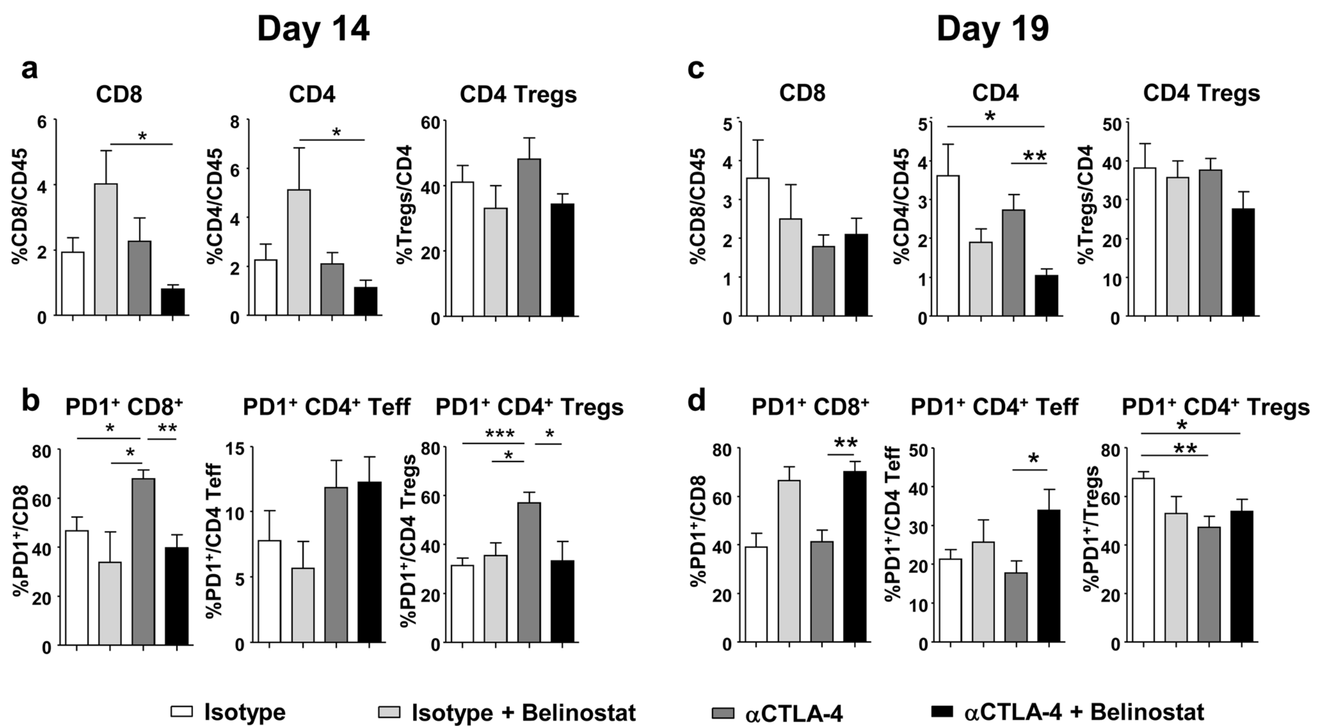


Fig. 5 Characterization of tumor-infiltrating T-cell subsets and expression of PD-1. Mice ($n = 5$–6/group) bearing 8 mm tumors were treated with isotype control or anti-CTLA-4 antibodies with or without Belinostat, tumors were harvested at days 14 (**a, b**) and 19 (**c, d**), and analyzed by flow cytometry. Graphs represent the proportion of CD8, effector and regulatory CD4 T-cells, among CD45$^+$ infiltrating cells (**a, c**) as well as the proportion of PD-1$^+$ cells in the above-mentioned subsets (**b, d**) (*$p < 0.05$; **$p < 0.01$)

showed an overall increase in cytokines associated with T-cell effector functions (e.g., IFN-γ, IL-2, TNF-α) in mice treated with the combination (Fig. 6). Interestingly, there were also enhanced levels of cytokines produced by innate immune cells and chemokines, suggesting that the higher proportion of PD-1$^+$ cells is associated with stronger immune activation.

Addition of PD-1-blocking antibodies to the anti-CTLA-4 + Belinostat combination induces complete tumor rejection

Since the strong but incomplete antitumor efficacy of the anti-CTLA-4 + Belinostat combination was associated with enhanced levels of antitumor cytokines and modulation of expression of receptors/ligands of the PD-1/PD-L1 axis, we hypothesized that simultaneous blockade of this pathway may improve the antitumor effects of the combination. Thus, tumor-bearing mice were treated with the triple combination (anti-PD-1 + anti-CTLA-4 + Belinostat).

Although we did not have previous data on the efficacy of Belinostat by oral route, pharmacokinetic studies in mice comparing Belinostat administered via different routes showed that, despite reaching different maximum plasma concentrations (C_{max}), drug elimination was rapid and the concentration remained up to 1 µM for at least 1 h—independent of the route of administration (Supplementary Fig. 3). Moreover, Steele et al. [42] had reported that histone H4 hyperacetylation in PBMCs after oral dosing was comparable to that achieved after i.v. administration. Therefore, these data prompted us to include additional groups for testing oral administration of Belinostat, both in monotherapy and in combinations. No differences were observed when Belinostat was administered i.p. or orally, neither as monotherapy nor combined with anti-CTLA-4. The triplet induced tumor rejection in all treated mice (Fig. 7a) and a 100% long-term survival in this group, as opposed to 60–70% survival in mice treated with anti-CTLA-4 + Belinostat combination or the dual (anti-PD-1 + anti-CTLA-4) checkpoint blockade (Fig. 7b).

Discussion

The immunomodulatory effects reported for epigenetic drugs [43], including HDACi, prompted us to determine if the HDACi Belinostat could improve the efficacy of immunotherapy based on checkpoints inhibitors in a murine HCC model. Although we are aware that subcutaneous tumors do not fully reflect the particular liver environment,

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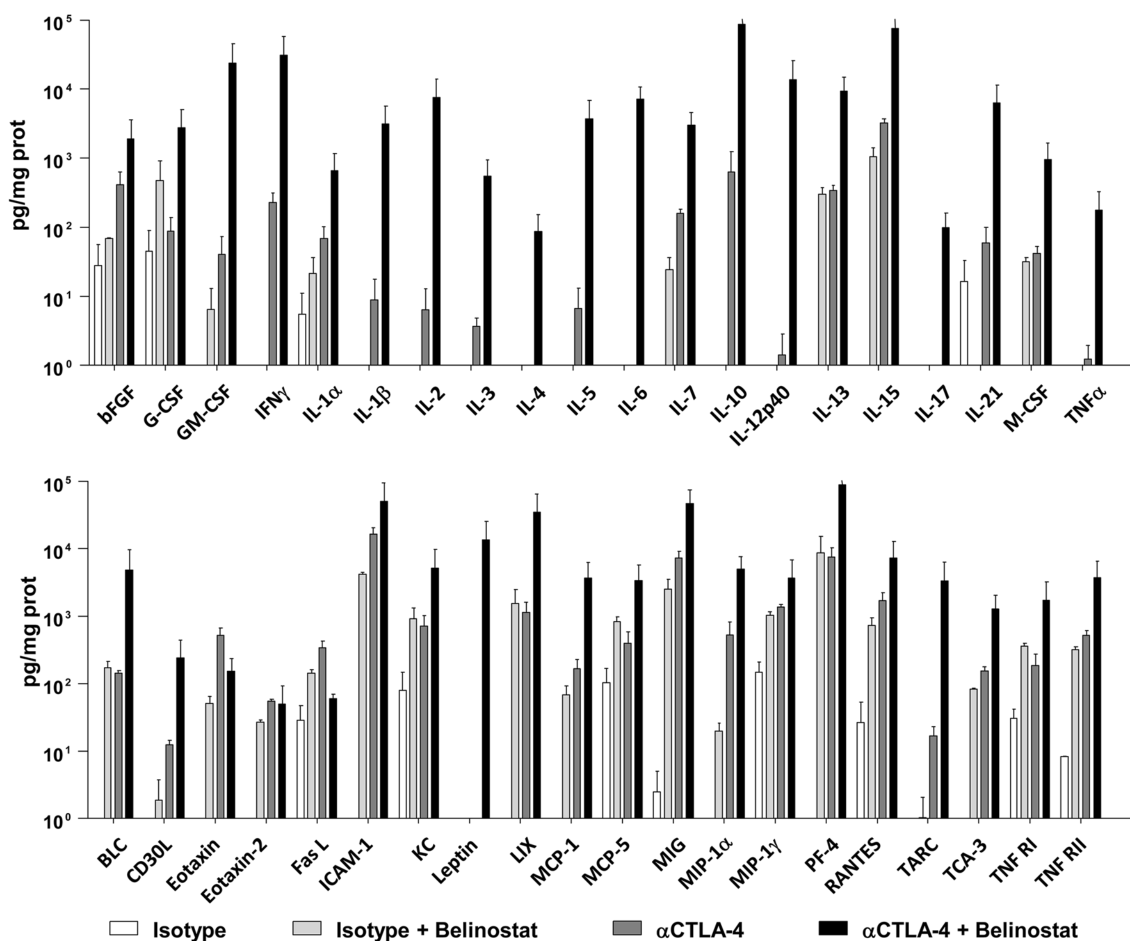

Fig. 6 Cytokines, chemokines and growth factors in tumor tissue from treated animals. Mice (2–3/group) with 8 mm tumors received different treatments for 19 days, when tumors were excised, homogenized and the content of different cytokines, chemokines and growth factors were determined using a protein array

important immune features of human HCC patients [36–39] were present in our model. Indeed, we observed a leukocyte infiltrate containing CTLA-4$^+$ Tregs and upregulation of PD-1 on T-cells. Furthermore, PD-L1 was expressed both on infiltrating and tumor cells. A predominance of macrophages (mainly of the M2 subset) over lymphocytes was also observed. This is in agreement with data on the HCC immune landscape [44], and provides evidence of the presence of immune subsets equivalent to those found in patients, that expressed the relevant targets for immunotherapy. Moreover, Hepa129 tumors have been used to analyze the efficacy of different HCC therapies. Accordingly, orthotopic implantation of this cell line has been shown to constitute a relevant HCC model [45]. Not only does it lead to a similar growth for subcutaneous and intrahepatic tumors, but also a clear expression of relevant HCC tumor markers glypican-3, 9BA12 and chondroitin sulfate in both locations [46]. In addition, subcutaneous as well as orthotopic Hepa129 tumors have shown equivalent sensitivity to certain therapies [47], including immunotherapy [33]. These data suggested that, although not directly in the liver environment, relevant features of this tumor model may be useful to test the therapeutic effect of our approach with potential relevance for HCC.

Interestingly, despite expression of CTLA-4 and PD-1/PD-L1 in the tumors, only anti-CTLA-4 and not anti-PD-1 therapy had a clear antitumor effect, which was further enhanced by Belinostat. A greater relevance of CTLA-4 and/or Tregs than PD-1/PD-L1 as immunosuppressive mechanisms at initial tumor stages may explain the superior sensitivity to anti-CTLA-4 in this setting and at the doses tested. Moreover, Belinostat only increased the therapeutic efficacy of anti-CTLA-4, associated with improved immune parameters, including increased antitumor Th1 immunity (IFN-γ) and decreased immunomodulatory mechanisms (Tregs and PD-1 expression). Epigenetic drugs may modulate tumor cell antigenicity/immunogenicity leading to improved antitumor responses [16, 17]. However, our in vitro results regarding expression of molecules involved in antigen presentation, co-stimulation, co-inhibition as well as T-cell recognition

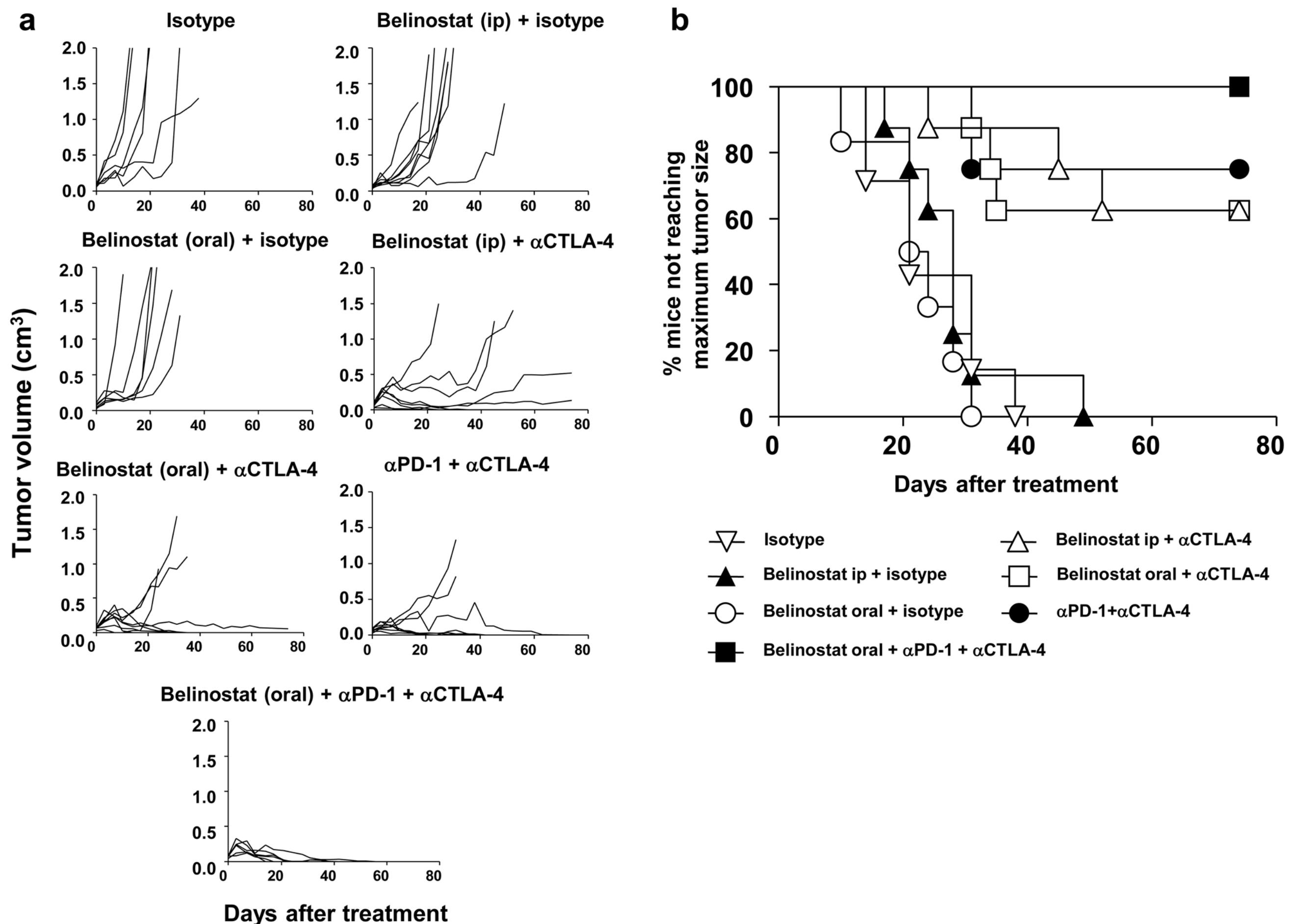

Fig. 7 Addition of PD-1-blocking antibodies to the antiCTLA-4 + Belinostat combination induces full tumor rejection. Mice (n = 6–8/group) bearing 5 mm tumors were treated with isotype control, anti-CTLA-4, anti-PD-1 + anti-CTLA-4 antibodies (days 0 and 7) with or without Belinostat (days 0–21; i.p. or oral). **a** Tumor growth of each individual mouse was measured. **b** Mice were killed when tumor reached 17 mm and the percentage of those not reaching this tumor size was represented

of tumor cells treated with Belinostat do not point to this effect as the mechanism responsible for the enhanced antitumor efficacy. In fact, it suggests that the increased efficacy of the combination therapy may be related to its effect on immune cells. Indeed, HDACi have been described as promoters of Treg function and generation [48], associated with higher expression of CTLA-4 [49]. This CTLA-4 upregulation would render Tregs more susceptible to depletion by anti-CTLA-4, thus explaining the lower Treg proportions observed in the anti-CTLA-4 + Belinostat combination but not in Belinostat monotherapy.

Analysis of intratumoral immune parameters revealed that in terms of innate immunity anti-CTLA-4 + Belinostat promoted a decrease in DC, potentially related to a higher migration of this important APC subset to secondary lymphoid organs, where they could trigger T-cell responses. Although HDACi may exert immunomodulatory effects on DC [50], no effect on DC counts was observed in mice treated only with Belinostat. Moreover, although the HDACi Trichostatin A has been described as promoter of a mixed M1/M2 phenotype [51], tumor macrophages in the combination group were characterized by higher M1 infiltrate and a trend towards lower proportion of M2. Our in vitro experiments showed an increase in M1 markers in unpolarized and M2-polarized macrophages treated with Belinostat. In vivo, monotherapy with Belinostat, as opposed to the combination, did not lead to an increase in M1 macrophages. Therefore, we postulate that the inflammatory environment created by anti-CTLA-4 antibodies probably reinforces this M1-promoting effect of Belinostat, leading to an evident increase in vivo only in the combination group.

In parallel with these events favoring an antitumor profile, counter-regulatory mechanisms such as PD-L1 upregulation were triggered. It has been reported that HDACi upregulate PD-L1 expression in melanoma cells [24], a result not observed in vitro in our HCC model. Although Belinostat

alone did not enhance PD-L1 expression in APC subsets in vivo, it promoted higher PD-L1 expression when combined with anti-CTLA-4. These results do not fully rule out a direct effect of Belinostat on these cells, but they may suggest that its effect on PD-L1 upregulation could be stronger in the more inflammatory environment induced by the combination treatment, as reported for the HDACi Romidepsin, which synergizes with IFN-γ in the induction of genes dependent on this cytokine [52].

Another interesting issue is the evolution of the adaptive immune response and associated inhibitory mechanisms induced by the combination. No significant changes in the proportion of tumor-infiltrating T-cells were found at day 14. Although enhanced antitumor immunity was observed in splenic cells (Fig. 3d), the lack of differences when analyzing global T-cell proportions in the tumor may not fully reflect this activity, requiring thus functional antigen recognition assays to determine the specificity of these T-cells. Interestingly, although anti-CTLA-4 therapy enhanced the levels of PD-1$^+$ T-cells, combination with Belinostat decreased the proportion of these cells (mainly in CD8 and Tregs), indicating that a lower number of cells was susceptible to this inhibitory mechanism and suggesting that this could be associated with the stronger antitumor immunity observed. Analysis of T-cells at day 19 (when therapy-mediated effects on tumor were clear), showed that while PD-1 levels were still lower in Tregs treated with the combination, they were increased both in effector CD4 and CD8 T-cells. PD-1 has been considered an exhaustion-related marker, but it is also associated with T-cell activation upon antigen recognition and TCR signaling [41]. Indeed, the higher proportion of PD-1$^+$ infiltrating T-cells in the combination group is associated with upregulation of effector cytokines, suggesting that this elevated PD-1 expression may be due to an enhanced activation and concomitant antitumor immunity. However, playing a role in negatively regulating T-cell activation, PD-1 upregulation may preclude full tumor rejection at this point. Indeed, although tumor growth was better controlled in the combination group at the end of treatment (days 18–20) when compared to monotherapy with anti-CTLA-4, some tumors in mice treated with the combination rebounded at later stages, once treatment was stopped (days 25–35) (Fig. 2).

Upregulation of PD-1/PD-L1 molecules by the combination therapy at different time points and the lack of complete tumor rejection led us to hypothesize that additional blockade of this pathway would increase its therapeutic effect. Using this approach all mice rejected their tumors, suggesting the relevance of blocking this non-redundant inhibitory pathway induced by the double combination. There are examples in the literature showing the pertinence of blocking PD-1/PD-L1 in combined therapies, which include anti-CTLA-4 with strategies such as vaccines [53], radiotherapy [54] or virotherapy [55]. Thus, partial responses induced by anti-CTLA-4-containing combinations, which are linked to the emergence of resistance through upregulation of PD-L1 and exhaustion of PD-1$^+$ T-cells, are improved in the triple therapy [54].

Checkpoint inhibitors have demonstrated a superior efficacy in those tumors with a higher mutational load [56], due to their increased antigenicity. In addition, it has been shown that epigenetic drugs modify tumor cells by promoting tumor antigen expression and presentation [17]. Belinostat is currently used in PTCL, a tumor with low mutational rate [57]. On the other hand, HCC has been described to possess a higher mutational load than most hematological malignancies [58], suggesting that checkpoint inhibitors, in combination with a drug that enhances tumor antigenicity, might have a superior effect.

In summary, we have demonstrated that Belinostat increases the therapeutic effect of anti-CTLA-4 antibodies in an HCC model. Moreover, triple therapy including blockade of the PD-1 inhibitory pathway induced by the double combination resulted in complete tumor rejection. These results suggest that Belinostat may improve the efficacy of single agent CTLA-4 therapy as well as combined CTLA-4 plus PD-1/PD-L1 treatment in HCC patients.

Acknowledgements Authors thank Dr. Gonzalez-Carmona for tumor Hepa129 tumor cell line and Dr. M. Hommel for manuscript revision.

Author contributions DL designed and performed experiments, acquired, analyzed and interpreted the data, and revised the manuscript. MR, LV, LS, JE and TI performed experiments and revised the manuscript. JJL, PP, VT-J, BV, GD and BS participated in the design of experiments, interpretation of the data, and revised the manuscript. PS designed experiments, analyzed and interpreted the data, and wrote the manuscript. All authors read and approved the final manuscript.

Funding This work is funded by a commercial research Grant from Onxeo. Pablo Sarobe is supported by Grants from Ministerio de Economia y Competitividad/Instituto de Salud Carlos III co-financed by European FEDER funds (PI14/00343; PI17/00249), from Fundación Bancaria La Caixa “Hepacare” project and received financial support from the “Murchante contra el cáncer” initiative. Bruno Sangro is supported by the European Commission’s 7th Framework Programme (EC FP7) Project: “Cancer Vaccine Development for Hepatocellular Carcinoma—HEPAVAC” (Grant no. 602893), by European Commission H2020, Project “Immunology and Immunotherapy of cancer: strengthening the translational aspect—HepaMUT” (Grant no. AC16/00165) and by Plan Estatal de I+D+I 2013–2016, co-financed by Instituto de Salud Carlos III—Subdirección General de Evaluación y Fomento de la investigación and Fondo Europeo de Desarrollo Regional (FEDER) (Grant no. PI16/01845).

Compliance with ethical standards

Conflict of interest Perrine Pivette, Véronique Trochon-Joseph, Bérangère Vasseur and Graham Dixon were or are employed by Onxeo. Bruno Sangro received consulting and/or lecture fees from Adaptimmune, Astra Zeneca, Bayer Healthcare, Bristol-Myers-Squibb, Med-

immune and Onxeo. All other authors declare that they have no conflict of interest.

Ethical approval All animal procedures were approved by the Animal Ethics Committee of the Universidad de Navarra (Project approval number: E1-16(149-14E2)). They were in accordance with the ethical standards and guidelines for laboratory animals of the Universidad de Navarra.

Animal source All mice were obtained from Envigo (Barcelona, Spain).

Cell line authentication Hepa129 HCC cells were a kind gift from Dr. M. Gonzalez-Carmona (Bonn, Germany). Re-authentication of cells has not been performed since receipt.

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