IMMUNOLOGICAL INVESTIGATIONS
2023, VOL. 52, NO. 8, 966–984
https://doi.org/10.1080/08820139.2023.2261980

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RESEARCH ARTICLE

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Regulatory T Cell Inhibition by P60 Combined with Adenoviral AFP Transduced Dendritic Cells for Immunotherapy of Hepatocellular Carcinoma

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ABSTRACT

Background & Aims: Vaccination with tumor-associated antigen-pulsed dendritic cells leads to specific T-cell response against hepatocellular carcinoma. However, clinical response has been shown to be limited. High regulatory T-cell count is associated with poor prognosis and seems to mediate immune tolerance in hepatocellular carcinoma. Forkhead box P3-peptide inhibitor P60 has been shown to specifically inhibit regulatory T-cell function in murine models. Aim of this study was to investigate whether P60 can improve the immune response induced by vaccination with adenovirus-transduced dendritic cells expressing alpha-fetoprotein in subcutaneous and orthotopic murine models for hepatocellular carcinoma.

Methods: Mice developing subcutaneous or orthotopic HCC received daily treatment with P60 starting at different tumor stages. Additionally, mice were vaccinated twice with dendritic cells expressing alpha-fetoprotein.

Results: In a preventive setting prior to tumor engraftment, vaccination with alpha-fetoprotein-expressing dendritic cells significantly decreased tumor growth in a subcutaneous model ($p = .0256$), but no further effects were achieved by addition of P60. However, P60 enhanced the antitumoral effect of a vaccination with alpha-fetoprotein-expressing dendritic cells in established subcutaneous and orthotopic hepatocellular carcinoma characterized by high Treg levels ($p = .011$).

Conclusion: In this study, we showed that vaccination with alpha-fetoprotein-expressing dendritic cells in combination with a specific inhibition of regulatory T-cells by using P60 leads to synergistic tumor inhibition and prolonged survival. This emphasizes the importance of regulatory T-cells inhibition for obtaining an effective antitumoral immune response in hepatocellular carcinoma.

KEYWORDS
Alpha-fetoprotein; cancer immunotherapy; dendritic cell-based vaccination; hepatocellular carcinoma; regulatory T-cells

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the second leading cause of cancer-related deaths worldwide with an increasing global incidence (Galle et al., 2018; McGlynn et al., 2021). Over a period of one decade, the tyrosine kinase inhibitor sorafenib remained the only systemic therapy for advanced HCC. Since 2017, further tyrosine kinase inhibitors and four immune checkpoint inhibitors (ICI) have been approved for first- or second-line therapy in non-resectable HCC (Kole et al., 2020; Ziogas et al., 2020). The combination of the PD-L1 inhibitor atezolizumab with the vascular endothelial growth factor (VEGF) inhibitor bevacizumab is the first approved combined immunotherapy leading to a prolonged overall and progression-free survival compared to sorafenib (Finn et al., 2020). Despite these new systemic treatment options for HCC, clinical responses remain limited (Mahn et al., 2020; Ziogas et al., 2020).

Besides ICI, immunotherapies using dendritic cells (DC) may be promising strategies towards HCC. DC are professional antigen-presenting cells able to prime specific T-cell responses against tumor-associated antigens (TAA), such as alpha-fetoprotein (AFP) (Banchereau & Steinman, 1998). We and others showed that DC pulsed with AFP induce AFP-specific cytotoxic T-cells (CTL) towards HCC cells *in vitro* (González-Carmona et al., 2006). Tumor lysate-pulsed DC engineered to express co-stimulatory checkpoint inhibitors, such as CD40L, or cytokines, such as IL-12, resulted in decreased tumor growth in a murine HCC model (Gonzalez-Carmona et al., 2008; Vogt et al., 2014, 2021). However, DC-based immunotherapies lead to limited clinical responses in patients with HCC (Ladhams et al., 2002; Lee et al., 2005; Palmer et al., 2009). These poor therapeutic effects can be explained by various mechanisms that tumors, including HCC, develop to escape antitumoral immune response (Schmidt et al., 2012).

In HCC, the up-regulation of regulatory T-cells (Tregs) is of critical importance. Elevated Treg counts in liver, tumor tissue and peripheral blood are associated with disease progression and poor outcome in HCC (Fu et al., 2007; Shen et al., 2010). To date, several approaches to overcome immunosuppressive Treg function have been tested. However, most of these attempts lack specificity (Byrne et al., 2011). In contrast, a specific impairment of Treg function can be achieved by FOXP3-specific peptide inhibitor P60 which suppresses the nuclear translocation of FOXP3. Casares et al. showed recently that P60 inhibits immunosuppressive Treg function and increases the stimulation of CTL *in vitro*. Moreover, P60 led to an improved immune response towards colorectal cancer (CRC) and antiviral vaccines in mice (Casares et al., 2010).

In this study, we analyzed whether a specific inhibition of immunosuppressive Treg function using P60 improves the antitumoral effect of a vaccination with adenovirus-transduced DC to express murine AFP (Ad-mAFP-transduced DC) in subcutaneous (s.c.) and orthotopic murine HCC models.

Material and methods

Mice and cell lines

Eight-week-old to ten-week-old male C3H/HeN mice (Charles River, Sulzfeld, Germany) were kept under standard specific pathogen-free conditions. All animal studies were performed in agreement with the local ethics committee, State Office for Nature and

Consumer Protection in North Rhine-Westphalia, 50.203.2-BN 22. Human embryonic retinoblastoma cells, 911 (Fallaux et al., 1996) were used to propagate E1-deleted adenoviruses. Murine hepatoma cells, Hepa129 (NCI-Frederick Cancer Research and Development Center), were used for s.c. and orthotopic tumor inoculation in mice. They were stably transfected with mAFP plasmid which has been generated and described previously (Hanke et al., 2002). The 911 cells were maintained in DMEM (PAA, Germany) and the Hepa129-mAFP cells in RPMI1640 (GibcoBRL, Berlin, Germany) complemented with 10% fetal calf serum (GibcoBRL), 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma, Munich, Germany).

Adenoviral vectors

For transduction of DC, three adenoviral vectors were used: Ad-LacZ (encoding the irrelevant E. coli β-galactosidase) as control vectors, Ad-mAFP (encoding mAFP). These vectors were generated using the AdEasy system as described previously (González-Carmona et al., 2006; Gonzalez-Carmona et al., 2008).

Murine DC

Bone marrow-derived DC were generated as described by Inaba et al. with modification (Inaba et al., 1992). Briefly, bone marrow of C3H/HeN mice was depleted of erythrocytes using 0.9% ammonium chloride. For depletion of *T*- and B-lymphocytes as well as granulocytes, bone marrow was first incubated with a mixture of rat anti-mouse antibodies (CD4, CD8, Ly6G, CD45R, and MHC-II H-2Kb antibodies, Southern Biotechnology, Birmingham, AL) and next with goat anti-rat IgG conjugated to magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). These antibodies bind specifically to the Fc region of the primary antibodies. Separating columns were placed in a strong magnetic field and equilibrated with MACS buffer.

The cell suspension was then applied to the separation columns and the run was collected in tubes. DC were finally gathered by negative selection in a magnetic field.

Collected cells were cultured with GM-CSF (200 U/ml) on day 1, 3 and 5 and from day 1 with IL-4 (100 U/ml) daily (tebu-bio, Offenbach, Germany).

Adenoviral transduction of DC

On day 6 after their generation (in the presence of cytokines) DC were transduced with adenoviruses (Ad-mAFP or Ad-LacZ) at various multiplicity of infection (MOI) ratios. Transductions were performed in RPMI1640 with 2% FCS for 2 h at 37°C. DC that have been transduced with Ad-LacZ serve as control group.

FOXP3 inhibitory peptide P60

FOXP3-specific inhibitory peptide P60 (RDFQSFRKMWPFFAM) and control peptide P301 (NNTRKRIRIQRGPGR) were produced by the solid-phase method of Merrifield using the fluorenylmethyloxycarbonyl alternative as described previously (Golgher et al., 2002). For confirmation of the binding capacity of P60, surface plasmon resonance using

GST-FOXP3 and GST proteins covalently bound onto the chip was performed (Casares et al., 2010).

Tumor inoculation

HCC were induced by s.c. inoculation of 1×10^6 Hepa129 mAFP cells into the right flank of C3H/HeN mice as described previously (Fallaux et al., 1996). Tumor growth was monitored by measuring two perpendicular diameters with calipers. Mice were sacrificed when their tumors reached 1500–2000 mm^3 or animals became moribund. Orthotopic HCC were induced by inoculation of 1×10^5 Hepa129 mAFP cells into the left liver lobe after laparotomy as described previously (Schmitz et al., 2004). Mice were sacrificed when they developed clinically relevant malignant ascites or became moribund.

Vaccination protocol with DC

Mice developing s.c. or orthotopic HCC received two vaccinations with adenovirus-transduced DC. Both vaccinations were performed with DC transduced with the same adenoviral vectors (Ad-mAFP, Ad-LacZ). Treatment was performed as s.c. inoculation of 1×10^6 DC in 50 µl RPMI 1640 into the right flank of C3H/HeN-mice.

Treatment with P60

Tumor-bearing mice received daily i.p. application of 50 µg FOXP3-inhibitor peptide, P60 in saline. Non-functional peptide P301 was used as control.

Elisa

For cytokine quantification, malignant ascites one and three days after the second s.c. DC treatment was analyzed using an enzyme-linked immunosorbent assay (ELISA) (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Flow cytometry

One and three days post second s.c. DC vaccination, the immune response of the treated animals was analyzed. Malignant ascites were aspirated from tumor-bearing mice with orthotopic HCC. Cell suspensions were first stained with Fc block/rat anti-FcRII-III (from 24G2 hybridoma) in order to bind the CD16 and CD32 receptors located on the surface of monocytes, B lymphocytes, macrophages, granulocytes, NK cells, and blood platelets.

Treg cell analysis was carried out with the Treg staining kit (eBioscience), composed of FITC-conjugated anti-CD4 (clone RM4–5), PE-conjugated anti-CD25 (clone PC61.5) and APC-conjugated FOXP3 (FJK-16s), was used according to the manufacturer's instructions. Viable and dead cells were distinguished by staining with DAPI. Flow cytometry was performed on a FACSCanto II using Diva software and FlowJo7.2.2 (TreeStar Inc. Ashland, USA).

Statistical analysis

For descriptive statistics, means and standard deviations (SD) or standard errors of the mean (SEM) are given. An unpaired t-test was used to analyze statistical significance. Survival rates are presented as Kaplan–Meier curves and were compared using log-rank-test. A two-sided p-value of less than 0.05 was considered significant. Data were analyzed by using the statistical software GraphPad Instat and GraphPad Prism.

Results

Antitumoral effects of a monotherapy with P60 towards subcutaneous HCC in vivo

In this first set of experiments, we use our established subcutaneous HCC model with Hepa129-mAFP cells. In this model, we have already shown an increase of regulatory T cells ($CD4^+/FoxP3^+$) and IL-10 in untreated tumors (Vogt et al., 2014) indicating a possible role of Treg during tumor development in this model.

Three different experimental schedules were tested in order to determine the effect of Treg inhibition at different tumor stages. All tumor-bearing mice were treated daily with 50 µg P60 or P301. The first group of mice was treated starting from the day of tumor inoculation (early-stage tumor). Treatment of the two other groups was started on day 3 after tumor inoculation, when tumor volume reached $52.30 \pm 44.2\ mm^3$ (intermediate-stage tumor) or on day 5, when tumor volume reached $112.6 \pm 75.5\ mm^3$ (advanced-stage tumor), respectively, and continued until mice were sacrificed.

Regardless of starting time-point and duration of therapy, treatment with P60 alone had no significant effect on survival compared to the untreated control group ($p = .41$). However, we observed a trend of P60 to inhibit tumor growth in the group of tumor-bearing mice which were treated when tumor was more advanced reaching a mean volume of $112.6 \pm 75.53\ mm^3$. This advantage, found only in advanced tumors, was shown as prolonged median survival, but was also noticed when comparing tumor sizes on day 9 after start of treatment. However, this effect was not significantly different compared to the untreated group ($p > .05$) (Figure 1a,b).

Monotherapy with P60 has no effect on orthotopic tumor growth

We also analyzed the effect of Treg inhibition using P60 on established orthotopic HCC. Due to the faster tumor progression of the orthotopic tumors, treatment was started on day 3 after tumor inoculation (advanced tumor stage) and continued until death of mice. Again, there was no advantage observed in survival compared to the control group (median survival 22 vs. 21 days) ($p = .44$) (Figure 1c).

Preventive vaccination with ad-mAFP-transduced DC leads to delayed tumor progression and prolonged survival in orthotopic murine HCC model but is not improved by adding P60

In the next set of experiments, we tested whether the application of P60 in combination with a vaccination with DC expressing murine AFP as tumor-associated antigen (Ad-mAFP DC) had a greater effect than the application Ad-mAFP DC alone as vaccine. Vaccination with

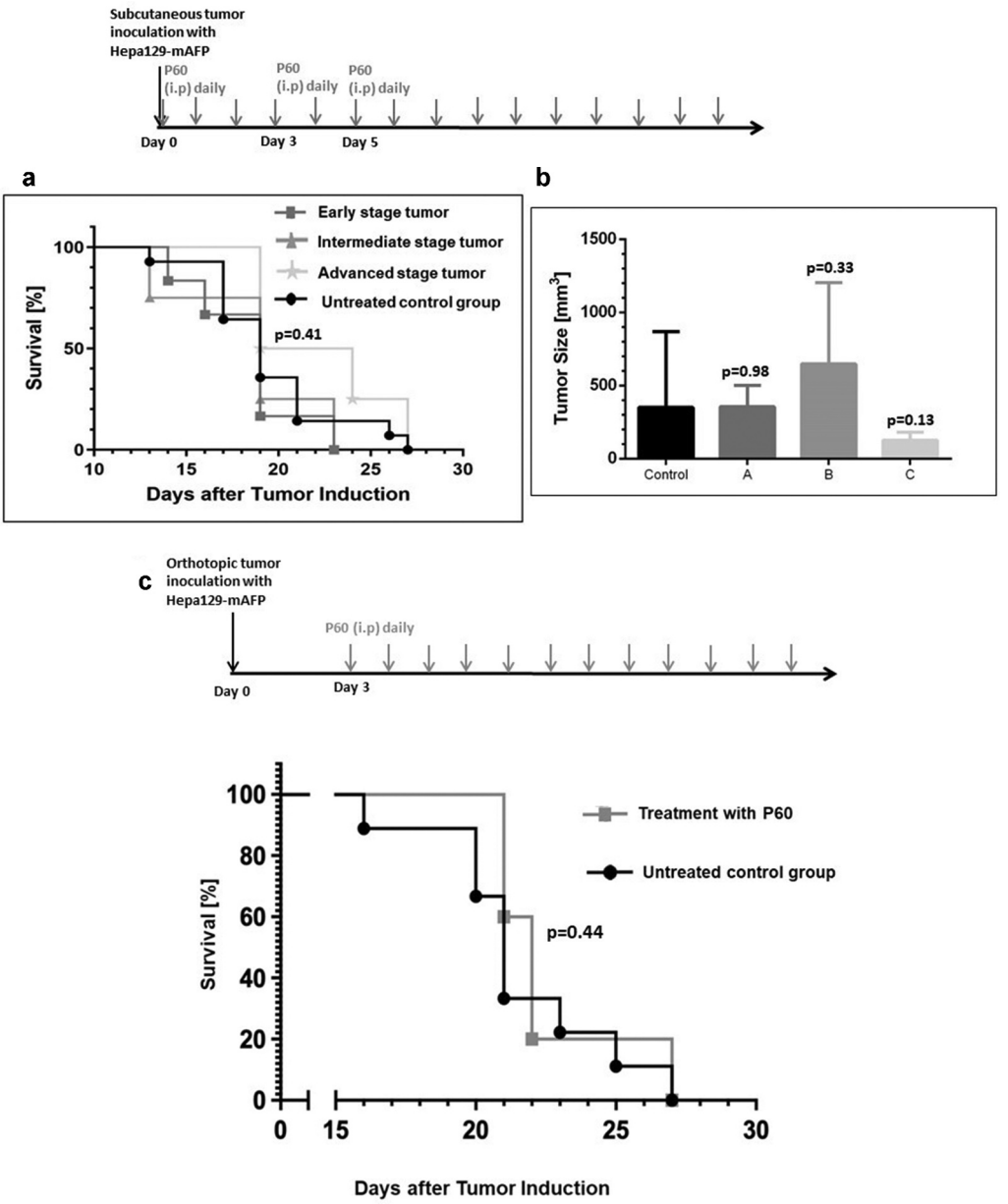

Figure 1. Treatment with FOXP3 inhibitor P60 i.P. daily in a subcutaneous and orthotopic HCC mouse model. Mice bearing subcutaneous (s.c.) mAFP-expressing tumors were treated with FOXP3 inhibitor peptide P60 intraperitoneally (i.p.) daily. (a) treatment was either started on the day of tumor inoculation (early stage tumor) (square, $n = 6$), on day 3 after tumor inoculation when tumor volume reached 52.3 $mm^3 \pm 44.19\ mm^3$ (intermediate stage tumor) (triangle, $n = 4$) or on day 5, when tumor volume reached 112.6 $mm^3 \pm 75.53\ mm^3$ (advanced stage tumor) (star, $n = 4$). Untreated control group (circle, $n = 12$). Representation of survival as Kaplan-Meier curve. (b) tumor size in mm (Kole et al., 2020) (+ SEM) on day 9 after start treatment with P60 i.P. daily. Treatment was either started again on the day of tumor inoculation (early stage tumor) (A), on day 3 after tumor inoculation, when tumor volume reached 52.3 $mm^3 \pm 44.19\ mm^3$ (intermediate stage tumor) (B) or on day 5, when tumor volume reached 112.6 $mm^3 \pm 75.53\ mm^3$ (advanced stage tumor) (c) Therapy with P60 had no influence on tumor progress and

Ad-mAFP-DC was performed on days 10 and 5 before tumor-cells inoculation. In addition, treatment with 50 µg P60 or P301 was performed daily from the day of the first vaccination until 5 days after tumor inoculation. Clinical signs of tumor growth were studied until death of mice or for a maximum of 3 months, respectively.

Mice treated with Ad-mAFP-transduced DC showed delayed signs of tumor growth and a significantly longer survival compared to the untreated group (Figure 2) ($p = .025$). However, an additional application of P60 had no effect on tumor progression or survival compared to control peptide P301 in this preventive setting.

Combined treatment with ad-mAFP-transduced DC and P60 decreases tumor growth and prolongs survival in s.C. murine pre-established HCC model

Next, combined therapy with Ad-mAFP-DC and P60 was applied in pre-established HCC. Firstly, we performed experiments in subcutaneous HCC model.

First, vaccination with Ad-mAFP- or Ad-LacZ-transduced DC was performed when tumor volume had reached $110 \pm 64\ mm^3$. Five days later, the second s.c. inoculation of adenovirus-transduced DC was performed. Daily treatment with 50 µg P60 was performed from the day of the first DC injection.

In this setting, combined P60 with Ad-mAFP led to a significantly prolonged survival compared to the untreated group ($p = .011$) or P60 with Ad-LacZ (Figure 3a). On day 13, tumor-growth in mice treated with Ad-mAFP-transduced DC and P60 was significantly decreased compared to the control group ($p = .033$). By contrast, combination treatment with the control Ad-LacZ-transduced DC and P60 had no significant effect on tumor size ($p = .110$, Figure 3b).

Treatment with adenovirus-transduced DC expressing mAFP in combination with P60 significantly prolongs survival in orthotopic murine HCC model

On days three and six after orthotopic tumor injection, tumor-bearing mice were vaccinated with Ad-mAFP- or Ad-LacZ-transduced DC. Treatment with 50 µg P60 or P301 was performed daily starting from the day of the first DC injection.

Mice bearing orthotopic tumors showed a significant prolonged survival when treated with Ad-mAFP-transduced DC and P60 compared to the untreated group (Figure 4) ($p = .016$). By contrast, tumor-bearing mice did not survive significantly longer when treated with Ad-mAFP or Ad-LacZ-transduced DC and control peptide P301 compared to the untreated control group ($p > .05$).

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survival. When treatment was started at more progressed tumor stage, tumor progress was delayed. However, this advantage was not significant compared to the untreated control group ($p = .41$). **C survival rates of orthotopic mAFP-expressing tumor-bearing mice treated with FOXP3 inhibitor P60 i.p. daily**. Treatment started, when tumor was established in the liver 3 days after tumor inoculation and continued until mice were sacrificed (square, $n = 6$). Untreated control group (circle, $n = 6$). There was no advantage observed in survival compared to the control group (median survival 22 vs. 21 days after tumor inoculation) ($P = .44$).

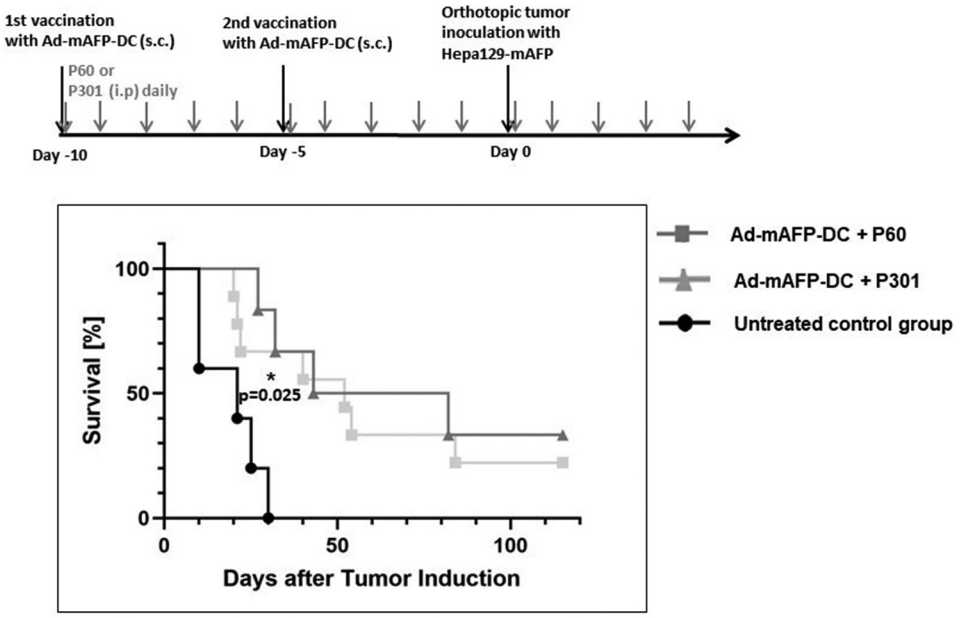

Figure 2. Survival rates of orthotopic mAFP-expressing tumor-bearing mice vaccinated with ad-mAFP-transduced dendritic cells (DC) subcutaneously (s.c.) on days 10 and 5 before tumor inoculation. In addition to vaccine, mice received daily intraperitoneally (i.p.) treatment with control peptide P301 (triangle, $n = 6$) or FOXP3 inhibitor peptide P60 (square, $n = 9$) starting on day 10 before tumor inoculation until day 5 after. Untreated control group (circle, $n = 5$). Representation as Kaplan-Meier curve. Mice treated with mAFP-expressing DC showed delayed signs of tumor growth and significantly prolonged survival compared to the untreated control group (*$p = .025$). However, an additional application of P60 had no effect on tumor progression and survival compared to control peptide P301 in this preventive setting.

Infiltration of Treg and a positive expression of IL-10 in ascites in pre- established orthotopic HCC model

To understand the differential effects of P60 when used in combination with Ad-mAFP-DC we analyzed immune parameters (infiltration of Treg and a positive expression of IL-10 in ascites) associated with these treatments. Tumor-bearing mice were treated s.c. with Ad-mAFP-DC on day 0 directly after s.c. vaccination. Ascites was obtained by peritoneal puncture.

Ascites of controlsshowed an infiltration of Treg cells $0.5 \pm 0.1\%$. The Treg amount was significantly enhanced up to $0.75 \pm 0.05\%$ in treatment group ($p = .042$) (Figure 5a). Three days after s.c. inoculation of Ad-mAFP-DC, ascites of control mice showed still an infiltration of Treg ($0.64 \pm 0.15\%$ of cells). In contrast, the amount of Treg cells had significantly decreased by 56% in the group treated with Ad-mAFP-DC ($p = .0072$) (Figure 5a,c). Correspondingly, a high amount of IL-10 was observed in the control ascites on day 0 (190 ± 115.3 pg/100 µg protein) and day 3 (363 ± 53.5 pg/100 µg protein). In the case of Ad-mAFP-DC-treated mice, IL-10 amount was significantly higher (500 ± 30.2 pg/100 µg protein) than the control group ($p = .002$). Interestingly, despite decreased amounts of Treg, the levels of IL-10

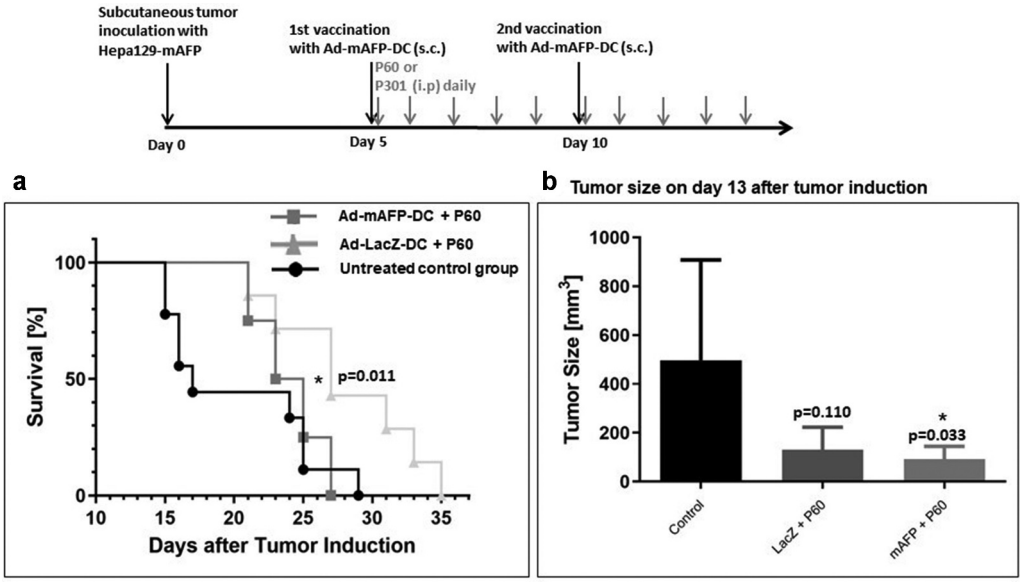

Figure 3. Mice bearing subcutaneous (s.c.) mAFP-expressing tumors were subcutaneously (s.c.) vaccinated twice with dendritic cells (DC) transduced with ad-mAFP or ad-LacZ, respectively. Additionally, mice received daily treatment with FOXP3 inhibitor peptide P60 intraperitoneally (i.p.). **a** survival rate of tumor-bearing mice vaccinated with ad-LacZ- (square, $n = 4$) or ad-mAFP-transduced DC (s.c). (triangle, $n = 7$) treated with P60 (i.p) daily starting from day 3 after tumor inoculation; untreated control group (circle, $n = 9$). Data presented as Kaplan-Meier curve. Mice treated with ad-mAFP-transduced DC and P60 showed significantly prolonged survival compared to the untreated control group (*$p = .011$). **b** tumor size on day 13 after tumor inoculation in mm (Kole et al., 2020) (+ SEM). Tumor progress was significantly delayed in mice treated with mAFP-expressing DC and P60 compared to untreated mice (*$p = .033$).

enhanced on day 3 (434 ± 50.5 pg/100 µg protein) and was similar to the controls ($p = .102$) (Figure 5b).

Discussion

In this study, we aimed to provide a proof of principle for a novel immunotherapeutic strategy with alpha-fetoprotein (AFP)-expressing dendritic cells (AFP-DC) in combination with P60, a specific inhibitor of regulatory T-cells via FoxP3, for the treatment of advanced hepatocellular carcinoma (HCC). Treatment with P60 leads to a synergistic antitumoral effect when combined with a vaccination of Ad-mAFP-transduced DC in mice with pre-established HCC. Interestingly, this synergism was strongest in more advanced tumors and in orthotopic HCC model *in vivo*, emphasizing the importance of regulatory T-cells inhibition for obtaining an effective antitumoral immune response in HCC.

DC are potent antigen-presenting cells and play an important role in the initiation of antigen-specific T-cells responses. We and others have shown in several studies that adenoviral-encoded expression of tumor-specific antigens, such as AFP, in DC promotes AFP-specific immunity by inducing tumor-specific effector cells *in vitro* and in murine cancer models (González-Carmona et al., 2006; Gonzalez-Carmona et al., 2008; Sadeghlar et al., 2020; Schumacher et al., 2004; Vogt et al., 2021). However, the clinical response to

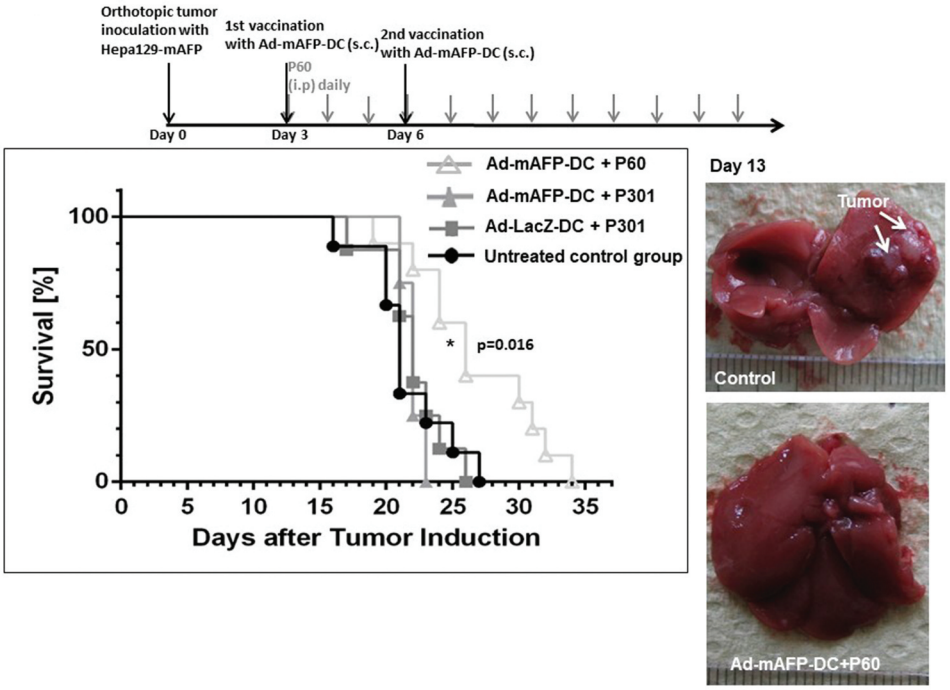

Figure 4. Survival rate of mice bearing orthotopic mAFP-expressing tumors. Mice were vaccinated twice (on days 3 and 6 after tumor inoculation) with dendritic cells (DC) transduced with ad-mAFP or ad-LacZ, respectively. Additionally, they received either daily intraperitoneal (i.p.) application of FOXP3 inhibitor peptide P60 or control peptide P301 starting from the day of the first vaccination. Mice treated with mAFP-expressing DC and P60 showed prolonged survival compared to the untreated control group. Data shown as Kaplan-Meier curve. Therapy regimen: mAFP-DC + P60 (clear triangle, $n = 10$), mAFP-DC + P301 (triangle, $n = 4$), LacZ-DC + P301 (square, $n = 5$), untreated control group (circle, $n = 7$). (*$p < .05$ mAFP-DC + P60 compared to all control groups). Significance was determined using the Mann-Whitney-test. Left: representative tumor at day 13 after induction (without treatment, control or after combined therapy with ad-mAFP-DC and P60).

DC-based immunotherapies in HCC remains limited (Ladhams et al., 2002; Lee et al., 2005; Palmer et al., 2009). In one of the largest phase II studies, reported by Palmer et al., only 28% of 35 patients with advanced HCC achieved a disease control (Palmer et al., 2009).

Multiple immune escape mechanisms have been described in HCC to date, explaining this limited effect of DC-based immunotherapies (Prieto et al., 2015). In the early tumor stage, higher levels of TAA-specific CTL are found in the tumor microenvironment of HCC. The secretion of cytokines, chemokines, and growth factors leads to the process of immune editing, which describes a change in the tumor microenvironment towards an immunosuppressive milieu (Santhakumar et al., 2020). One approach to overcome the immunosuppressive tumor milieu is the blockade of immune checkpoint with ICI (immune checkpoint inhibitors). To date, four ICI addressing either PD-1/PD-L1 or CTLA-4 have been approved for first- or second-line therapy in advanced HCC. However, all of them show limited clinical responses as monotherapy (Ziogas et al., 2020). Expression of ICI

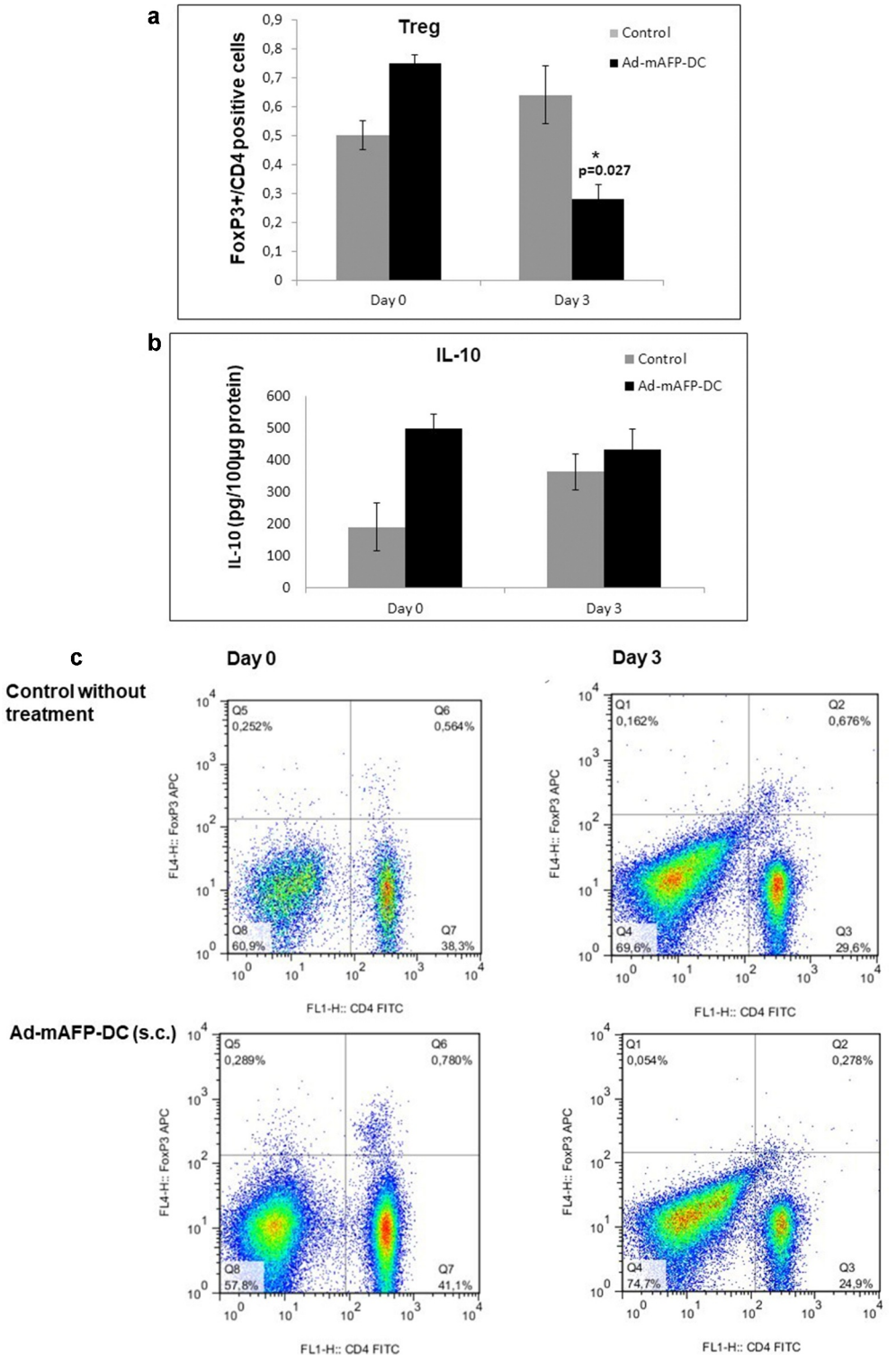

Figure 5. Infiltration of Treg and IL-10 in malignant ascites of orthotopic murine pre-established hepatocellular carcinoma model. Percentage of regulatory T-cells (Treg) in ascites was analyzed via flow cytometry. Mice were treated subcutaneously (s.c.) with Ad-mAFP-DC (dendritic cells) 3 days after

ligands is low on tumor cells and immune cells in HCC (Langhans et al., 2019). Therefore, other immune escape mechanisms and therapeutic targets must be discussed for HCC.

Treg upregulation is an immune escape mechanism of critical importance in HCC. Elevated Treg count in the peripheral blood, lymph nodes, and the tumor microenvironment of patients suffering from HCC predicts a poor prognosis (Fu et al., 2007; Shen et al., 2010). Moreover, the balance of intratumoral Treg and CTL after tumor resection correlates with the overall survival in HCC (Gao et al., 2007).

Immunosuppressive functions of Treg are diverse and many drugs targeting Treg had been developed before the exact immune inhibitory functions of Treg were established (Li et al., 2020). CD25 is one of the most investigated targets for Treg inhibition. It is part of the IL-2 receptor and expressed on Treg but also on CTL. In the tumor milieu, they compete for IL-2 and its consumption by Treg causes a deficiency for IL-2, suppressing proliferation of CTL. Treg depletion using anti-CD25 antibodies promoted an enhanced antitumoral immune response in several preclinical trials (Golgher et al., 2002; Shimizu et al., 1999). However, results from clinical trials remained equivocal (Baur et al., 2013; Jacobs et al., 2010; Morse et al., 2008; Rech et al., 2012). The major limitation of CD25 inhibitors seems to be insufficient specificity. Jacobs et al. reported a general depletion of $CD25^{+}$ cells in patients with malign melanoma treated with anti-CD25 antibodies and tumor peptide vaccine (Jacobs et al., 2010). Others found DC being modulated towards tolerance (Baur et al., 2013) and B-cells inhibited under therapy (Morse et al., 2008). Further attemps to overcome Treg inhibition were tested targeting glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) (Tran et al., 2018) and chemokine receptor 4 (CCR4) (Kurose et al., 2015) with limited response in clinical trials. Greten et al. demonstrated that low-dose cyclophosphamide reduced the frequency and suppressor function of Tregs in peripheral blood of patients with HCC. However, an activated AFP-specific T-cell response was only found in six of the 13 treated patients (Greten et al., 2010). This underlines the requirement of a specific and effective strategy to overcome Treg suppressor function to improve anticancer T-cell-response.

Zheng et al. investigated T-cell subsets in liver cancer. They showed that FOXP3 is a target specific for tumor infiltrating Treg. FOXP3 is the main transcription factor in human T-cells for Treg differentiation and function (Zheng et al., 2017). Casares et al. reported an inhibitor peptide P60 which is able to bind to FOXP3 and inhibit its nuclear translocation and homodimerization. Furthermore, P60 inhibits downregulation of the activity of the transcription factors NF- κB and NFAT by FOXP3.

First studies by Casares *et al.* found that administration of P60 *in vivo* results in controlled Treg suppression in terms of duration and intensity and enhances the immune response, in particular after a vaccination procedure (Casares et al., 2010).

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tumor inoculation. Analysis of immune cell recruitment was performed on day 0 and on day 3 after the s. c. vaccination with adenoviral transduced DC. Treg count in peritoneal ascites of mice vaccinated with Ad-mAFP-transduced DC was significantly reduced over time (a), while a positive expression of IL-10 was equal compared to the untreated control group (b). Results represented as means ± SEM of three independent experiments ($n = 4$) (*$p < .05$). Significance was determined using the Mann-Whitney test. Flow cytometry of FoxP3/CD4 positive T cells (Treg) of representative tumors after treatment with Ad-mAFP-DC on day 0 and day 3, three days after tumor induction (c).

Therefore, P60 does not deplete Treg but impairs their immunosuppressive function (Casares et al., 2010; Lozano et al., 2017; Setiawan et al., 2019). Considering that Treg depletion promotes conversion of $CD4^+$ cells into $CD4^+CD25^+FOXP3^+$ cells (Valzasina et al., 2006), functional impairment might be the preferred mode. Several preclinical trials showed improved antitumoral immune response towards cancer vaccines under therapy with P60 (Casares et al., 2010; Moreno Ayala et al., 2017).

In our study, a preventive treatment with P60 alone or a treatment of pre-established tumors with P60 alone did not protect towards an s.c. or orthotopic tumor challenge with Hepa129 mAFP cells. This is in line with the findings published by Casares et al. (2010) who reported that monotherapy with P60 did not prevent tumor growth in a murine CRC model (CT26 cells).

As expected, and in line our previous findings, a preventive vaccination with Ad-mAFP-transduced DC led to significantly prolonged survival after orthotopic tumor challenge with Hepa129-mAFP cells compared to the untreated control group. However, and contrary by the previous published work showing improved antitumoral effect of a preventive anticancer vaccination by adding P60, in our murine HCC model, additional P60 to a vaccination with Ad-mAFP-DC did not enhance the protective effect of a vaccination with Ad-mAFP-transduced DC (Casares et al., 2010). This discrepancy could be related to the different vaccination protocols. Indeed, while Casares et al. used a peptide-based immunization protocol, in this work we are using a DC-based vaccine. Probably, peptide vaccination is more sensitive to inhibition by Tregs than DC-based vaccination, which is usually more potent and thus less amenable to the effects of Tregs. Indeed, in our tumor model, Ad-mAFP-DC vaccination appears to reduce the amount of Treg within malignant ascites. Furthermore, our further experiments showed that the presence of Tregs infiltrating tumor tissue or malignant ascites may be necessary for the antitumoral effect of P60. Treg count in peripheral blood of patients with HCC increases even more, with the highest counts in advanced-stage disease (Langhans et al., 2019; Shen et al., 2010). We induced murine HCC by inoculation of Hepa129-mAFP s.c. or orthotopically in healthy C3H/HeN mice. In the subcutaneous model, we have previously shown an increase of regulatory T cells ($CD4^+$/$FoxP3^+$) and IL-10 in untreated tumors (Vogt et al., 2014) indicating a possible role of Treg during tumor development in this model. As shown from flow cytometry of malignant ascites from orthotopic HCC at days 0 and 3, we observe also an infiltration of Treg and IL-10 in ascites. Therefore, a target for P60 towards FOXP3 might be lacking before HCC development in our model, since no activated Treg are infiltrating any tumor tissue. This is possibly the reason why P60 can only develop its effect in the presence of HCC and is ineffective in a preventive setting in our tumor model. However, and contrary to our observations, preventive treatment with anti-CD25 antibody PC61 decreased tumor growth in two s.c murine HCC models using MH129 or MH134 cell lines, respectively. This effect could not be achieved when tumors were established. Immunohistological examination of the tumor tissue showed increased expression of CD4, CD25, and FOXP3 with progressing tumor growth (Nagayama et al., 2007).

Comparing the effects of P60 in established s.c. and orthotopic HCC, we observe that the antitumoral effect of a monotherapy with P60 achieved only a trend to prolong survival and decrease tumor growth when treatment with P60 was established and started at an advanced tumor stage in the s.c. tumor model. But this advantage of

a P60 monotherapy was not statistically significant compared to untreated tumor-bearing mice ($p = .41$).

For the vaccination with Ad-mAFP-transduced DC and consistent with our own preclinical and other clinical trials in HCC (Ladhams et al., 2002; Lee et al., 2005; Palmer et al., 2009) treatment with Ad-mAFP-transduced DC alone did not significantly improve outcome of s.c. or orthotopic tumor-bearing mice. However, as expected, in a therapeutic pre-established setting using Ad-mAFP-transduced DC and P60 for combined treatment of established s.c. and orthotopic HCC, we observed a synergistic effect. Despite a decrease in the levels of Tregs as a consequence of the Ad-mAFP-DC vaccine, P60 still has a beneficial effect. The mice which received two vaccinations with mAFP-expressing DC and daily application of P60 showed significantly delayed signs of tumor growth and prolonged survival compared to the untreated control group.

Previously, we were able to show that Treg upregulation limits antitumoral effect of DC-based immunotherapies (Vogt et al., 2014). Our present study provides new evidence that functional Treg inhibition can help to overcome these limitations. As far as we know, there are no comparable studies testing tumor vaccines in combination with Treg inhibition in a preclinical HCC model. Our results are in accordance with data from preclinical trials in other tumor entities. Treg inhibition in combination with antitumor vaccines led to improved immune response towards established tumors in animal models of melanoma (Unger et al., 2015) thymoma (Fujinami et al., 2016) and CRC (Ghiringhelli et al., 2004). However, our study has some limitations, including only the analysis of the frequency of Treg cells and IL-10 within malignant ascites in the orthotopic model. Further analysis of immunological mechanisms should be further analyzed in order to understand the synergistic effects of P60 in combination with Ad-mAFP-DC vaccination in pre-established HCC.

In summary, functional Treg inhibition using FOXP3 inhibitor peptide P60 can increase the antitumoral effect of a vaccination with Ad-mAFP-transduced DC by decreasing tumor growth and prolonging survival in established s.c. and orthotopic murine HCC model, when Treg are infiltrating tumor tissue. Our results suggest that a continued Treg inhibition seems to be essential for success of the therapy with P60 underlining Treg upregulation as a critical tumor escape mechanism in HCC and a promising target in combination with DC-based immunotherapy.

Acknowledgments

We thank Mr M. Gentz for excellent technical assistance during our work.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the following grants awarded to M.A. Gonzalez-Carmona and Julia Waysczak: BONFOR from the University of Bonn, grant number 109255 from the “Deutsche

Krebshilfe" (German Cancer Aid) and GO 1874/1-2 grant from"Deutsche Forschungsgemeinschaft" (DFG). Pablo Sarobe is funded by Instituto de Salud Carlos III, Fondo Europeo de Desarrollo Regional "Una manera de hacer Europa" (grant PI20/00260) and the "Murchante contra el cáncer" initiative. Additionally, the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) funded this publication, grant number 410853455 to Miroslaw Kornek.

Author contributions

JS and AV: carrying out the experiments, analysis and interpretation of data, drafting of the manuscript, study concept and design. FS, CM, RM, and TZ: acquisition of data, analysis and interpretation of data, drafting of the manuscript, study concept and design; AB, MK, NC, JL, PS, CB, HM, SM, JK, ISW, CPS: critical revision of the manuscript for important intellectual content; MG: generation of the hypothesis and supervision of the study, acquisition of data, analysis and interpretation of data, drafting of the manuscript, study concept and design. All authors contributed to the writing and revision of the manuscript. All authors approved the final version of the manuscript.

References

Banchereau, J., & Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature*, *392* (6673), 245–252. https://doi.org/10.1038/32588

Baur, A. S., Lutz, M. B., Schierer, S., Beltrame, L., Theiner, G., Zinser, E., Ostalecki, C., Heidkamp, G., Haendle, I., Erdmann, M., Wiesinger, M., Leisgang, W., Gross, S., Pommer, A. J., Kämpgen, E., Dudziak, D., Steinkasserer, A., Cavalieri, D., Schuler-Thurner, B., & Schuler, G. (2013). Denileukin diftitox (ONTAK) induces a tolerogenic phenotype in dendritic cells and stimulates survival of resting treg. *Blood*, *122*(13), 2185–2194. https://doi.org/10.1182/blood-2012-09-456988

Byrne, W. L., Mills, K. H. G., Lederer, J. A., & O'Sullivan, G. C. (2011). Targeting regulatory T cells in cancer. *Cancer Research*, *71*(22), 6915–6920. https://doi.org/10.1158/0008-5472.CAN-11-1156

Casares, N., Rudilla, F., Arribillaga, L., Llopiz, D., Riezu-Boj, J. I., Lozano, T., López-Sagaseta, J., Guembe, L., Sarobe, P., Prieto, J., Borrás-Cuesta, F., & Lasarte, J. J. (2010). A peptide inhibitor of FOXP3 impairs regulatory T cell activity and improves vaccine efficacy in mice. *Journal of Immunology (Baltimore, Md. 1950)*, *185*(9), 5150–5159. https://doi.org/10.4049/jimmunol.1001114

Fallaux, F. J., Kranenburg, O., Cramer, S. J., Houweling, A., van Ormondt, H., Hoeben, R. C., & van der Eb, A. J. (1996). Characterization of 911: A new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Human Gene Therapy*, *7*(2), 215–222. https://doi.org/10.1089/hum.1996.7.2-215

Finn, R. S., Qin, S., Ikeda, M., Galle, P. R., Ducreux, M., Kim, T.-Y., Kudo, M., Breder, V., Merle, P., Kaseb, A. O., Li, D., Verret, W., Xu, D.-Z., Hernandez, S., Liu, J., Huang, C., Mulla, S., Wang, Y., Lim, H. Y., … Cheng, A. L. (2020). Atezolizumab plus Bevacizumab in unresectable hepatocellular carcinoma. *The New England Journal of Medicine*, *382*(20), 1894–1905. https://doi.org/10.1056/NEJMoa1915745

Fu, J., Xu, D., Liu, Z., Shi, M., Zhao, P., Fu, B., Zhang, Z., Yang, H., Zhang, H., Zhou, C., Yao, J., Jin, L., Wang, H., Yang, Y., Fu, Y.-X., & Wang, F.-S. (2007). Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology*, *132*(7), 2328–2339. https://doi.org/10.1053/j.gastro.2007.03.102

Fujinami, N., Yoshikawa, T., Sawada, Y., Shimomura, M., Iwama, T., Sugai, S., Kitano, S., Uemura, Y., & Nakatsura, T. (2016). Enhancement of antitumor effect by peptide vaccine therapy in combination with anti-CD4 antibody: Study in a murine model. *Biochemistry and Biophysics Reports*, *5*, 482–491. https://doi.org/10.1016/j.bbrep.2016.02.010

Galle, P. R., Forner, A., Llovet, J. M., Mazzaferro, V., Piscaglia, F., Raoul, J.-L., Schirmacher, P., & Vilgrain, V. (2018). EASL clinical practice guidelines: Management of hepatocellular carcinoma. *Journal of Hepatology*, *69*(1), 182–236. https://doi.org/10.1016/j.jhep.2018.03.019

Gao, Q., Qiu, S.-J., Fan, J., Zhou, J., Wang, X.-Y., Xiao, Y.-S., Xu, Y., Li, Y.-W., & Tang, Z.-Y. (2007). Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *Journal of Clinical Oncology Official Journal of the American Society of Clinical Oncology*, *25*(18), 2586–2593. https://doi.org/10.1200/JCO.2006.09.4565

Ghiringhelli, F., Larmonier, N., Schmitt, E., Parcellier, A., Cathelin, D., Garrido, C., Chauffert, B., Solary, E., Bonnotte, B., & Martin, F. (2004). CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *European Journal of Immunology*, *34*(2), 336–344. https://doi.org/10.1002/eji.200324181

Golgher, D., Jones, E., Powrie, F., Elliott, T., & Gallimore, A. (2002). Depletion of CD25+ regulatory cells uncovers immune responses to shared murine tumor rejection antigens. *European Journal of Immunology*, *32*(11), 3267–3275. https://doi.org/10.1002/1521-4141(200211)32:11<3267:AID-IMMU3267>3.0.CO;2-1

Gonzalez-Carmona, M. A., Lukacs-Kornek, V., Timmerman, A., Shabani, S., Kornek, M., Vogt, A., Yildiz, Y., Sievers, E., Schmidt-Wolf, I. G. H., Caselmann, W. H., Sauerbruch, T., & Schmitz, V. (2008). CD40ligand-expressing dendritic cells induce regression of hepatocellular carcinoma by activating innate and acquired immunity in vivo. *Hepatology (Baltimore, Md)*, *48*(1), 157–168. https://doi.org/10.1002/hep.22296

González-Carmona, M. A., Märten, A., Hoffmann, P., Schneider, C., Sievers, E., Schmidt-Wolf, I. G. H., Sauerbruch, T., & Caselmann, W. H. (2006). Patient-derived dendritic cells transduced with an a-fetoprotein-encoding adenovirus and co-cultured with autologous cytokine-induced lymphocytes induce a specific and strong immune response against hepatocellular carcinoma cells. *Liver International Official Journal of the International Association for the Study of the Liver*, *26*(3), 369–379. https://doi.org/10.1111/j.1478-3231.2005.01235.x

Greten, T. F., Ormandy, L. A., Fikuart, A., Höchst, B., Henschen, S., Hörning, M., Manns, M. P., & Korangy, F. (2010). Low-dose cyclophosphamide treatment impairs regulatory T cells and unmasks AFP-specific CD4+ T-cell responses in patients with advanced HCC. *Journal of Immunotherapy (Hagerstown, Md. 1997)*, *33*(2), 211–218. https://doi.org/10.1097/CJI.0b013e3181bb499f

Hanke, P., Serwe, M., Dombrowski, F., Sauerbruch, T., & Caselmann, W. H. (2002). DNA vaccination with AFP-encoding plasmid DNA prevents growth of subcutaneous AFP-expressing tumors and does not interfere with liver regeneration in mice. *Cancer Gene Therapy*, *9*(4), 346–355. https://doi.org/10.1038/sj.cgt.7700445

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., & Steinman, R. M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *The Journal of Experimental Medicine*, *176*(6), 1693–1702. https://doi.org/10.1084/jem.176.6.1693

Jacobs, J. F. M., Punt, C. J. A., Lesterhuis, W. J., Sutmuller, R. P. M., Brouwer, H. M. L. H., Scharenborg, N. M., Klasen, I. S., Hilbrands, L. B., Figdor, C. G., de Vries, I. J. M., & Adema, G. J. (2010). Dendritic cell vaccination in combination with anti-CD25 monoclonal antibody treatment: A phase I/II study in metastatic melanoma patients. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, *16*(20), 5067–5078. https://doi.org/10.1158/1078-0432.CCR-10-1757

Kole, C., Charalampakis, N., Tsakatikas, S., Vailas, M., Moris, D., Gkotsis, E., Kykalos, S., Karamouzis, M. V., & Schizas, D. (2020). Immunotherapy for hepatocellular carcinoma: A 2021 update. *Cancers*, *12*(10), 2859. https://doi.org/10.3390/cancers12102859

Kurose, K., Ohue, Y., Wada, H., Iida, S., Ishida, T., Kojima, T., Doi, T., Suzuki, S., Isobe, M., Funakoshi, T., Kakimi, K., Nishikawa, H., Udono, H., Oka, M., Ueda, R., & Nakayama, E. (2015). Phase ia study of FoxP3+ CD4 treg depletion by infusion of a humanized anti-CCR4 antibody, KW-0761. *Clin Cancer Res*, *21*(19), 4327–4336. https://doi.org/10.1158/1078-0432.CCR-15-0357

Ladhams, A., Schmidt, C., Sing, G., Butterworth, L., Fielding, G., Tesar, P., Strong, R., Leggett, B., Powell, L., Maddern, G., Ellem, K., & Cooksley, G. (2002). Treatment of non-resectable

hepatocellular carcinoma with autologous tumor-pulsed dendritic cells. *Journal of Gastroenterology and Hepatology*, *17*(8), 889–896. https://doi.org/10.1046/j.1440-1746.2002.02817.x

Langhans, B., Nischalke, H. D., Krämer, B., Dold, L., Lutz, P., Mohr, R., Vogt, A., Toma, M., Eis-Hübinger, A. M., Nattermann, J., Strassburg, C. P., Gonzalez-Carmona, M. A., & Spengler, U. (2019). Role of regulatory T cells and checkpoint inhibition in hepatocellular carcinoma. *Cancer Immunology, Immunotherapy*, *68*(12), 2055–2066. https://doi.org/10.1007/s00262-019-02427-4

Lee, W.-C., Wang, H.-C., Hung, C.-F., Huang, P.-F., Lia, C.-R., & Chen, M.-F. (2005). Vaccination of advanced hepatocellular carcinoma patients with tumor lysate-pulsed dendritic cells: A clinical trial. *Journal of Immunotherapy (Hagerstown, Md. 1997)*, *28*(5), 496–504. https://doi.org/10.1097/01.cji.0000171291.72039.e2

Li, C., Jiang, P., Wei, S., Xu, X., & Wang, J. (2020). Regulatory T cells in tumor microenvironment: New mechanisms, potential therapeutic strategies and future prospects. *Molecular Cancer*, *19*(1), 116. https://doi.org/10.1186/s12943-020-01234-1

Lozano, T., Gorraiz, M., Lasarte-Cía, A., Ruiz, M., Rabal, O., Oyarzabal, J., Hervás-Stubbs, S., Llopiz, D., Sarobe, P., Prieto, J., Casares, N., & Lasarte, J. J. (2017). Blockage of FOXP3 transcription factor dimerization and FOXP3/AML1 interaction inhibits T regulatory cell activity: Sequence optimization of a peptide inhibitor. *Oncotarget*, *8*(42), 71709–71724. https://doi.org/10.18632/oncotarget.17845

Mahn, R., Vogt, A., Kupczyk, P., Sadeghlar, F., van Beekum, K., Hüneburg, R., Meyer, C., Toma, M., Ahmadzadehfar, H., Essler, M., Matthaei, H., Lingohr, P., Kalff, J. C., Strassburg, C. P., & Gonzalez-Carmona, M. A. (2020). Programmed cell death protein 1 (PD-1)-inhibition in hepatocellular carcinoma (HCC): A single center experience. *Scandinavian Journal of Gastroenterology*, *55*(9), 1057–1062. https://doi.org/10.1080/00365521.2020.1794539

McGlynn, K. A., Petrick, J. L., & El-Serag, H. B. (2021). Epidemiology of hepatocellular carcinoma. *Hepatology (Baltimore, Md)*, *73*(Suppl 1), 4–13. https://doi.org/10.1002/hep.31288

Moreno Ayala, M. A., Gottardo, M. F., Imsen, M., Asad, A. S., Bal de Kier Joffé, E., Casares, N., Lasarte, J. J., Seilicovich, A., & Candolfi, M. (2017). Therapeutic blockade of Foxp3 in experimental breast cancer models. *Breast Cancer Research and Treatment*, *166*(2), 393–405. https://doi.org/10.1007/s10549-017-4414-2

Morse, M. A., Hobeika, A. C., Osada, T., Serra, D., Niedzwiecki, D., Lyerly, H. K., & Clay, T. M. (2008). Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines. *Blood*, *112*(3), 610–618. https://doi.org/10.1182/blood-2008-01-135319

Nagayama, Y., Hase, W., Motoyoshi, Y., Saitoh, O., Sogawa, R., & Nakao, K. (2007). Distinct responses of two hepatocellular carcinoma cell lines of a similar origin to immunotherapies targeting regulatory or effector T cells. *Oncology Reports*, *17*(5), 1269–1273. https://doi.org/10.3892/or.17.5.1269

Palmer, D. H., Midgley, R. S., Mirza, N., Torr, E. E., Ahmed, F., Steele, J. C., Steven, N. M., Kerr, D. J., Young, L. S., & Adams, D. H. (2009). A phase II study of adoptive immunotherapy using dendritic cells pulsed with tumor lysate in patients with hepatocellular carcinoma. *Hepatology (Baltimore, Md)*, *49*(1), 124–132. https://doi.org/10.1002/hep.22626

Prieto, J., Melero, I., & Sangro, B. (2015). Immunological landscape and immunotherapy of hepatocellular carcinoma. *Nature Reviews Gastroenterology & Hepatology*, *12*(12), 681–700. https://doi.org/10.1038/nrgastro.2015.173

Rech, A. J., Mick, R., Martin, S., Recio, A., Aqui, N. A., Powell, D. J., Colligon, T. A., Trosko, J. A., Leinbach, L. I., Pletcher, C. H., Tweed, C. K., DeMichele, A., Fox, K. R., Domchek, S. M., Riley, J. L., & Vonderheide, R. H. (2012). CD25 blockade depletes and selectively reprograms regulatory T cells in concert with immunotherapy in cancer patients. *Science Translational Medicine*, *4*(134), 134ra62. https://doi.org/10.1126/scitranslmed.3003330

Sadeghlar, F., Vogt, A., Mohr, R. U., Mahn, R., van Beekum, K., Kornek, M., Weismüller, T. J., Branchi, V., Matthaei, H., Toma, M., Schmidt-Wolf, I. G. H., Kalff, J. C., Strassburg, C. P., & González-Carmona, M. A. (2020). Induction of cytotoxic effector cells towards cholangiocellular, pancreatic, and colorectal tumor cells by activation of the immune checkpoint CD40/CD40L on

dendritic cells. *Cancer Immunology, Immunotherapy CII*, *70*(5), 1451–1464. https://doi.org/10.1007/s00262-020-02746-x

Santhakumar, C., Gane, E. J., & Liu, K., & McCaughan, G.W (2020). Current perspectives on the tumor microenvironment in hepatocellular carcinoma. *Hepatology International*, *14*, 1–11.

Schmidt, N., Neumann-Haefelin, C., & Thimme, R. (2012). Cellular immune responses to hepatocellular carcinoma: Lessons for immunotherapy. *Digestive Diseases (Basel, Switzerland)*, *30*(5), 483–491. https://doi.org/10.1159/000341697

Schmitz, V., Tirado-Ledo, L., Tiemann, K., Raskopf, E., Heinicke, T., Ziske, C., González-Carmona, M. A., Rabe, C., Wernert, N., Prieto, J., Qian, C., Sauerbruch, T., & Caselmann, W. H. (2004). Establishment of an orthotopic tumour model for hepatocellular carcinoma and non-invasive in vivo tumour imaging by high resolution ultrasound in mice. *Journal of Hepatology*, *40*(5), 787–791. https://doi.org/10.1016/j.jhep.2004.01.010

Schumacher, L., Ribas, A., Dissette, V. B., McBride, W. H., Mukherji, B., Economou, J. S., & Butterfield, L. H. (2004). Human dendritic cell maturation by adenovirus transduction enhances tumor antigen-specific T-cell responses. *Journal of Immunotherapy (Hagerstown, Md. 1997)*, *27*(3), 191–200. https://doi.org/10.1097/00002371-200405000-00003

Setiawan, M. F., Rudan, O., Vogt, A., Gonzalez-Carmona, M. A., Langhans, B., Schmidt-Wolf, R., Garofano, F., Strassburg, C. P., Lasarte, J. J., Casares, N., Lozano, T., Weiher, H., & Schmidt-Wolf, I. G. H. (2019). FOXP3 inhibitory peptide P60 increases efficacy of cytokine-induced killer cells against renal and pancreatic cancer cells. *Anticancer Research*, *39*(10), 5369–5374. https://doi.org/10.21873/anticanres.13730

Shen, X., Li, N., Li, H., Zhang, T., Wang, F., & Li, Q. (2010). Increased prevalence of regulatory T cells in the tumor microenvironment and its correlation with TNM stage of hepatocellular carcinoma. *Journal of Cancer Research and Clinical Oncology*, *136*(11), 1745–1754. https://doi.org/10.1007/s00432-010-0833-8

Shimizu, J., Yamazaki, S., & Sakaguchi, S. (1999). Induction of tumor immunity by removing CD25+CD4+ T cells: A common basis between tumor immunity and autoimmunity. *Journal of Immunology (Baltimore, Md. 1950)*, *163*(10), 5211–5218. https://doi.org/10.4049/jimmunol.163.10.5211

Tran, B., Carvajal, R. D., Marabelle, A., Patel, S. P., LoRusso, P. M., Rasmussen, E., Juan, G., Upreti, V. V., Beers, C., Ngarmchamnanrith, G., & Schöffski, P. (2018). Dose escalation results from a first-in-human, phase 1 study of glucocorticoid-induced TNF receptor–related protein agonist AMG 228 in patients with advanced solid tumors. *The Journal for ImmunoTherapy of Cancer*, *6*(1), 93. https://doi.org/10.1186/s40425-018-0407-x

Unger, W. W., Mayer, C. T., Engels, S., Hesse, C., Perdicchio, M., Puttur, F., Streng-Ouwehand, I., Litjens, M., Kalay, H., Berod, L., Sparwasser, T., & Kooyk, Y. V. (2015). Antigen targeting to dendritic cells combined with transient regulatory T cell inhibition results in long-term tumor regression. *Oncoimmunology*, *4*(8), e970462. https://doi.org/10.4161/21624011.2014.970462

Valzasina, B., Piconese, S., Guiducci, C., & Colombo, M. P. (2006). Tumor-Induced Expansion of Regulatory T Cells by conversion of CD4+CD25− lymphocytes is thymus and proliferation Independent. *Cancer Research*, *66*(8), 4488–4495. https://doi.org/10.1158/0008-5472.CAN-05-4217

Vogt, A., Sadeghlar, F., Ayub, T. H., Schneider, C., Möhring, C., Zhou, T., Mahn, R., Bartels, A., Praktiknjo, M., Kornek, M. T., Toma, M., Schmidt-Wolf, I. G. H., Branchi, V., Matthaei, H., Kalff, J. C., Strassburg, C. P., & Gonzalez-Carmona, M. A. (2021). Alpha-fetoprotein- and CD40Ligand-expressing dendritic cells for immunotherapy of hepatocellular carcinoma. *Cancers*, *13*(13), 3375. https://doi.org/10.3390/cancers13133375

Vogt, A., Sievers, E., Lukacs-Kornek, V., Decker, G., Raskopf, E., Meumann, N., Büning, H., Sauerbruch, T., Strassburg, C. P., Schmidt-Wolf, I. G. H., & Gonzalez-Carmona, M. A. (2014). Improving immunotherapy of hepatocellular carcinoma (HCC) using dendritic cells (DC) engineered to express IL-12 in vivo. *Liver International Official Journal of the International Association for the Study of the Liver*, *34*(3), 447–461. https://doi.org/10.1111/liv.12284

Zheng, C., Zheng, L., Yoo, J.-K., Guo, H., Zhang, Y., Guo, X., Kang, B., Hu, R., Huang, J. Y., Zhang, Q., Liu, Z., Dong, M., Hu, X., Ouyang, W., Peng, J., & Zhang, Z. (2017). Landscape of infiltrating T cells in liver cancer revealed by single-cell sequencing. *Cell*, *169*(7), 1342–1356.e16. https://doi.org/10.1016/j.cell.2017.05.035

Ziogas, I. A., Evangeliou, A. P., Giannis, D., Hayat, M. H., Mylonas, K. S., Tohme, S., Geller, D. A., Elias, N., Goyal, L., & Tsoulfas, G. (2020). The role of immunotherapy in hepatocellular carcinoma: A systematic review and pooled analysis of 2,402 patients. *The Oncologist*, *26*(6), e1036–e1049. https://doi.org/10.1002/onco.13638