Combined immunostimulatory monoclonal antibodies extend survival in an aggressive transgenic hepatocellular carcinoma mouse model

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TRANSLATIONAL RELEVANCE

Advanced hepatocellular carcinoma remains an unmet need in cancer therapy. Immunostimulatory monoclonal antibodies anti-PD-1, B7-H1 (PD-L1), CD137 and OX40 are under intensive development in clinical trials in which these novel agents are tested as monotherapies for cancer patients with very encouraging results. This study provides evidence for synergistic therapeutic effects of a combination of these antibodies against autochthonous liver cancer arising in c-myc transgenic mice. Evidence for a more intense and active immune infiltrates in the spontaneous tumors and interference with immune tolerance establishment provide a strong rationale for testing these feasible combination strategies in hepatocellular carcinoma patients.
ABSTRACT

Purpose: Immunostimulatory monoclonal antibodies (ISmAbs) that unleash antitumor immune responses are showing efficacy in cancer clinical trials. Anti-B7-H1 (PD-L1) monoclonal antibodies (mAbs) block a critical inhibitory pathway in T cells, while anti-CD137 and OX40 mAbs provide T-cell costimulation. A combination of these ISmAbs (anti-CD137 + anti-OX40 + anti-B7-H1) was tested using a transgenic mouse model of multifocal and rapidly progressing hepatocellular carcinoma (HCC), in which c-myc drives transformation and cytosolic ovalbumin (OVA) is expressed in tumor cells as a model antigen.

Experimental Design: Flow-cytometry and immunohistochemistry were used to quantify tumor-infiltrating lymphocytes (TILs) elicited by treatment and assess their activation status and cytolytic potential. Tolerance induction and its prevention/reversal by treatment with the combination of ISmAbs were revealed by in-vivo killing assays.

Results: The triple combination of ISmAbs extended survival of mice bearing HCCs in a CD8-dependent fashion and synergized with adoptive T cell therapy using activated OVA-specific TCR-transgenic OT-1 and OT-2 lymphocytes. Mice undergoing therapy showed clear increases in tumor infiltration by activated and blastic CD8+ and CD4+ T lymphocytes containing perforin/granzyme B and expressing the ISmAb-targeted receptors on their surface. The triple combination of ISmAbs did not result in enhanced OVA-specific cytotoxic T lymphocyte (CTL) activity but other antigens expressed by cell lines derived from such HCCs were recognized by endogenous TILs. Adoptively transferred OVA-specific OT-1 lymphocytes into tumor-bearing mice were rendered tolerant, unless given the triple mAb therapy.
Conclusion: Extension of survival and dense T cell infiltrates emphasize the translational potential of combinational immunotherapy strategies for HCC.
INTRODUCTION

Immunotherapies with cytokines, vaccines and T cell adoptive therapy have been tested in early clinical trials for HCC with minor impact on the course of the disease (1-4), with the exception of a pilot study using adoptive T cell therapy (5). Immunostimulatory monoclonal antibodies (ISmAbs) have recently emerged as a new therapeutic tool in oncology (6, 7). Their overall mode of action is the enhancement of the weak ongoing immune responses present in cancer patients. Two types of ISmAbs can be categorized depending on the receptors bound on immune system cells: agonist monoclonal antibodies (mAbs) for activating receptors (7) and antagonist mAbs for coinhibitory receptors (8).

Antagonist ISmAbs directed to coinhibitory receptors are attracting much attention following approval of an anti-CTLA-4 mAb (9) to treat metastatic melanoma, and the unprecedented clinical responses elicited by antibodies directed to PD-1 (CD279) (10) and B7-H1 (PD-L1 or CD274) in melanoma, renal cell carcinoma and lung cancer (11).

Treatment of transplantable mouse models with agonist antibodies as monotherapies has shown clear signs of efficacy in the case of anti-CD137 (12), anti-OX40 (13), anti-CD40 (14) and anti-GITR (15) mAbs. Beyond monotherapies, these agents can be used in combinatorial approaches, in which synergy is often observed against transplantable tumors (7, 16). Moreover, synergy has also been observed on carcinogen-induced sarcomas using a combination that included anti-CD40 and anti-CD137 mAbs (17).
Spontaneous carcinomas which arise in oncogene-transgenic mice are highly resistant to immunotherapy approaches (18, 19) and are likely to represent a more predictive model for translational research. To date no signs of efficacy have been reported beyond early pre-cancer stages of development (20). A model of multifocal HCC has been generated in which transgenic human c-myc and cytosolic chicken ovalbumin (OVA) are under the transcriptional control of a tet-off system (21). If deprived of doxycycline around birth, such mice develop lethal multifocal HCC clearly seen upon microscopic examination at three weeks of age. Almost 100% of such mice die of massive tumor burden within the first 10 weeks of life. Importantly, deprivation of doxycycline later in life does not result in such rapid and aggressive malignancies and tumors occur with lower penetrance and higher latency. It has been demonstrated in this model, that resting OVA specific CD8⁺ OT-1 T cells are rendered tolerant upon adoptive transfer into HCC-bearing mice (21). Strains of c-myc transgenic mice that do not co-express OVA developed tumors with similar penetrance and age onset (21). As various treatment attempts using immunization strategies have failed in these mice (21 and data not shown), we tested a combination of ISmAbs targeting CD137, OX40 and B7-H1 (PD-L1) administered at three weeks of age. Surprisingly, the survival of such mice was clearly extended and the presence of an abundant and activated T cell infiltrate in tumor nodules was observed.
MATERIALS AND METHODS

Mice. For tumor induction experiments, c-myc OVA mice were crossed with tTALAP transgenic mice to generate c-myc-OVA-tTALAP double transgenic mice (c-myc OVA tg+) maintaining the presence of doxycycline in drinking water during pregnancy as described (21). Doxycycline was removed at birth, to induce the expression of the c-myc oncogene and ovalbumin in the liver. Rag1−/− transgenic mice and T cell receptor transgenic mice specific for H-2Kb OVA257-264 (OT-1) and H-2IAβ OVA323-339 (OT-2) were purchased from Jackson Laboratories (Bar Harbor, Maine USA). C57Bl/6 mice were purchased from Harlan Laboratories (Udine, Italy). All animal procedures were conducted under institutional guidelines that comply with national laws and policies (study number 054/10).

Reagents and cell lines. EL-4 thymoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, USA). OVA transfected EL-4 cell line, EG-7, was a kind gift from Dr. Claude Leclerc (Institut Pasteur, Paris, France). MC38 was provided by Dr. Karl E. Hellström (Seattle, USA). These cell lines were authenticated by Idexx Radil (Columbia, MO, USA. Case 6592-2012). Hepa 1.6 hepatoma cell line was a kind gift of Dr. Rubén Hernández (CIMA, Pamplona, Spain) and MC38OVA was generated by lentiviral tranfection (Sancho D. et al. manuscript in preparation) and provided by Dr. Sancho (Madrid, Spain). Hybridomas, antibody production and generation of JMJ cells are described in supplementary methods.
Adoptive T cell therapy and antibody treatment. Spleens from OT-1 and OT-2 transgenic mice were mechanically disrupted and cell suspensions were cultured with 5μg/ml of the cognate OVA peptides (described in supplementary methods) for 48h (OVA\textsuperscript{257-264} and OVA\textsuperscript{323-339}, respectively). 2x10\textsuperscript{6} OT-1 cells and 2x10\textsuperscript{6} OT-2 cells were i.v. transferred to c-myc OVA tg+ mice on day 21 or 28 after birth, depending on the experiment. For survival experiments, a dose of 2x10\textsuperscript{4} IU of i.p. IL-2 was administered to mice transferred with activated OT-1 and OT-2 on day 21. For treatment with mAbs, Combo3 (100 μg of each anti-CD137, anti-OX40 and anti-B7-H1) or control rat IgG was administered i.p on the indicated days. For depletion experiments, 3 doses of 200 μg of anti-CD4 or anti-CD8β mAbs were i.p. injected starting one day before Combo3 treatment onset and every three days.

Phenotypic analyses of tumor infiltrating lymphocytes. For analysis of tumor infiltrating lymphocytes, mice were treated with two doses of control rat IgG, the single immunostimulatory mAbs, pairs of immunostimulatory mAbs or the Combo3 (anti-CD137, anti-OX40 and anti-B7-H1) antibody combination and sacrificed on the indicated days. Livers were excised, weighted and enzymatically disrupted with DNase I and collagenase D (both from Roche, Madrid, Spain) for 15 minutes at 37°C. To obtain unicellular cell suspensions, livers were mechanically disrupted and passed through a 70-μm cell strainer (BD Falcon, BD Bioscience, San Agustín de Guadalix, Spain) pressing with a plunger. To remove non-monoruclear cells, unicellular cell suspensions were pelleted, resuspended in a 35% Percoll gradient and centrifuged for 10 min, 500g at room temperature. Erythrocytes were lysed with ACK buffer (Gibco, NY, USA). Single cell suspensions were treated with FcR-Block in a PBS-based buffer containing 10% of FCS to
avoid unspecific staining. Flow cytometry and immunohistochemistry analysis were performed as described in supplementary methods.

**Statistical analysis.** Prism software (Graph Pad Software, La Jolla, CA, USA) was used to analyze tumor infiltrating lymphocytes, specific lysis and IFNγ production between groups by applying unpaired Student’s t-tests or U-Mann–Whitney tests. Survival curves were analyzed by Kaplan-Meyer plots and log rank tests. *P*-values < 0.05 were considered significant.
RESULTS

Expression of target molecules for anti-CD137, OX40 and B7-H1 (PD-L1) mAbs in the tumor microenvironment of a transgenic c-myc-driven hepatocellular carcinoma model.

Immunotherapy with immunostimulatory monoclonal antibodies (ISmAbs) needs the target molecules to be expressed at the cell surface as a requirement for biological effects. CD137 and PD-1 are not constitutive surface proteins on T cells but become expressed following antigen-elicited activation (22, 23). OX40 is also up-regulated from dim levels of expression to high levels upon T cell activation (24). Indeed, we confirmed with OT-1 (CD8+) and OT-2 (CD4+) TCR transgenic T cells that, if these lymphocytes are cultured in the presence of their cognate ovalbumin peptides, they acquire bright expression of PD-1, B7-H1 (PD-L1), CD137 and OX40 (Supplementary Fig. S1A).

In order to explore the potency of the therapeutic effects of ISmAbs directed to such targets (CD137, OX40 and B7-H1) in vivo, we selected an aggressive model of spontaneous HCC: c-myc OVA tg+ transgenic mice (21). In these mice, concurrent c-myc and ovalbumin expression can be induced specifically in the liver in a time dependent manner. In the C57Bl/6 background, if doxycycline is removed from the drinking water at birth, mice develop multifocal tumors resembling human HCCs within 2-3 weeks (Supplementary Fig. S2A). OVA-encoding mRNA is detectable at day 5 following doxycycline deprivation from the mother’s drinking water (Supplementary Fig. S2B). Carcinomas progress rapidly with tumor cells that characteristically express the OVA protein in the cytoplasm, while the surrounding healthy liver cells do not express OVA protein by immunohistochemistry (21).
Cell suspensions from the livers of such mice at 3 weeks of age show that FACS-gated CD8$^+$ and CD4$^+$ lymphocytes express PD-1 and B7-H1 at bright levels. We also documented that CD4$^+$ T cells expressed CD137 and OX40, while the expression on CD8$^+$ T cells was barely detectable or absent (Supplementary Fig. S1B).

Moreover, immunohistochemical data indicated B7-H1 expression on stromal and endothelial cells in situ (I. Gütgemann et al. manuscript in preparation). Hence, concomitant expression of the target molecules for three ISmAbs at tumor microenvironment led us to test these ISmAbs in combination as an immunotherapy strategy for autochthonous liver tumors.

_A combination of anti-CD137, anti-OX40 and anti-B7-H1 (PD-L1) mAbs extends survival and synergizes with adoptive T cell therapy._

To assess the therapeutic effects of these combinations, c-myc OVA tg+ mice were deprived of doxycycline at birth to induce HCCs and treated on day 21 and 25 of extra-uterine life with two doses of 100 $\mu$g of anti-CD137 (1D8), anti-OX40 (OX86) and anti-B7-H1 (10B5) mAbs (Combo3). Of note, mice at this age have histological evidence of multiple tumor nodules (Supplementary Fig. S2A). As can be seen in Fig. 1A, mice undergoing Combo3 treatment had a significantly extended survival if compared to those treated with control antibody, and 2 out of 10 mice were still alive 15 months later.

In the same experiment, we also treated a group of mice on day 21 with $2 \times 10^6$ activated OT-1 cells plus $2 \times 10^6$ activated OT-2 cells i.v. followed by $2 \times 10^4$ IU of IL-2 i.p. This group of mice did not experience any improvement in terms of overall survival (Fig. 1A). However, if mice...
received both such adoptive T cell transfer and the Combo3 regimen, survival was dramatically extended in comparison to the Combo3 group. In this group, 4 out of 11 mice were alive 15 months after treatment.

Fig. 1B shows representative data of ultrasound examinations performed on mice from each experimental treatment group at week 6. Photographs of the abdomen show liver enlargement and abdominal distension with less abdominal distension in treated animals.

Next, we set up an independent experiment to analyse the contribution of each antibody given separately versus the Combo3 regimen. As can be seen in Fig. 2A, 10 out of 37 (27%) mice survived in the Combo3 group. In contrast, none of the groups treated with single antibodies showed any statistically significant improved survival. Nonetheless, anti-B7-H1 mAb monotherapy showed a marginal survival benefit with 2 out of 17 (11.8%) mice alive at the end of this experiment.

We have recently carried out a clinical trial with the anti-CTLA-4 mAb tremelilumab in advanced HCC patients with signs of clinical activity (3 partial responses in 17 patients evaluated) (25). As a consequence, we tested two doses of 100 μg of anti-CTLA-4 mAb in the transgenic mice with no evidence for any contribution to extended survival either as a single agent or when given concomitantly on top of the Combo3 regimen (Combo4) (Supplementary Fig. S3).

To explore the immune cell requirements for the therapeutic activity observed upon Combo3 treatment we selectively depleted from the animals CD8β+ T lymphocytes or CD4+ T cells at the time of therapy instigation (Fig. 2B). Only CD8+ T cells were absolutely required to extend survival in this setting.
Infiltration of hepatocellular carcinomas by activated CD4$^+$ and CD8$^+$ T lymphocytes upon combinational treatment with ISmAbs.

If the mechanism of action is cellular immunity, stronger T cell infiltrates would be expected in treated c-myc OVA tg$^+$ mice. For this reason, livers from mice under treatment were thoroughly examined by histology and immunohistochemistry. Indeed, Combo3 treatment resulted in more intense tumor infiltration by CD3$^+$ T lymphocytes (Fig. 3A). Immunohistochemical staining results showing livers from mice which had only received one dose on day 28 and which were sacrificed on day 34 are shown in Fig. 3A. Interestingly, the tumor nodules show intense lymphocytic infiltrates, whereas normal surrounding non-neoplastic tissue shows less increase in T-cells (Fig. 3B). Slightly increased apoptotic tumor cells were observed in mice undergoing immunotherapy, indicating cytolytic attack by the lymphocytic infiltrate. Ki-67 staining demonstrated proliferating CD3$^+$ lymphocytes in addition to tumor cells (data not shown).

In parallel, cell suspensions from these livers were analyzed by immunofluorescence and flow cytometry. As can be observed in Fig. 4A, Combo3 led to a higher content of CD8$^+$ and CD4$^+$ T lymphocytes.

A separate set of experiments, comparing Combo3 and control antibody given on days 21 and 25 (administered as in Fig. 1), confirmed the higher lymphocyte content with comparable organ weight (Fig. 4B). More detailed FACS analyses shown in Figs. 4C and D, clearly indicate that CD8$^+$ and CD4$^+$ T cells readily infiltrated these tumors. These were both increased in number and also showed an enlarged (blastic) phenotype (Supplementary Fig. S4A and Figs. 4C and D). Accordingly, CD8$^+$ and CD4$^+$ T lymphocytes exhibited a higher intracellular content of granzyme B (Figs. 4C and D). It is of interest, that infiltrating T lymphocytes instigated by
Combo3 showed brighter surface expression of CD137, OX40 and PD-1. This is very important because surface expression permits the continuous effect of the ISmAb combination, in contrast with the control treated mice, in which expression of CD137 was almost absent on the infiltrating CD8+ T lymphocytes. OX40 was also expressed at much lower levels in the control group than in the Combo3 group.

Given the fact that depletion of CD8+ T lymphocytes spoiled the therapeutic activity of Combo3, we studied the expression of cytolytic effector mechanisms in this subset of TILs. Expression was analysed in Combo3 treated mice in comparison to the corresponding doublets and control antibody. As can be seen in Supplementary Fig. S5, perforin and granzyme B were stimulated by all the doublets with a clear further enhancement in the triple combination. Surface FasL is also slightly up-regulated while TRAIL is not. As a potential drawback, this regimen also elicited infiltrates of Foxp3+ regulatory CD4+ T cells (Tregs) that were also more abundant than in the control group (Supplementary Fig. S4B), albeit CD8+/Treg ratios were still clearly increased upon Combo3 treatment (Supplementary Fig. S4B).

**HCC-derived cell lines express OVA and are recognized by OVA-specific CD8+ T lymphocytes.**

Explanting tumors allowed for the establishment of continuously growing cell lines that died off if exposed to doxycycline in culture as expected for transformed cells in this transgenic model (data not shown). After repeated attempts, several cell lines named JMJ (JMJ7 and JMJ9) were established from a mouse suffering bulky liver tumors with slightly different morphologies by phase-contrast microscopy (Supplementary Fig. S6A). These cells expressed EpCAM and B7-H1, albeit with dim surface levels of MHC class I molecules (Supplementary Fig. S6B). However, both B7-H1 and the MHC class I and II antigen presenting molecules were up-
regulated when these cell lines were exposed in culture to interferon γ (IFNγ) for 48h (Supplementary Fig. S6B).

RT-PCR profiling of these cells and lysates from liver explants showed mRNA expression of albumin and HNF4 (indicating hepatocyte lineage) (Supplementary Fig. S6C). Ovalbumin and human c-myc genes were also expressed. More importantly, as shown in Supplementary Fig. S6D, OT-1 T cell blasts readily killed JMJ cells in chromium release assays, supporting adequate presentation of the immunodominant OVA epitope by tumor cells, even without IFNγ preincubation.

**HCC-bearing mice tolerize activated OT-1 T cell blasts unless the triple combination of ISmAbs is given.**

None of the JMJ cell lines grafted as tumors either in immunocompetent or immunodeficient \( \text{Rag1}^{-/-} \) mice (Fig. 5A). However, small ∼5x5 mm pieces of tumor if surgically implanted under a skin flap of \( \text{Rag1}^{-/-} \) immunodeficient mice rendered progressive lethal tumors that were larger than 15 mm of diameter by day 30 (Fig. 5B). However, in immunocompetent syngeneic mice, tumors were rejected in every experiment, indicating that an antitumor T-cell response was responsible for this observation.

In fact, if WT naïve mice were implanted with such HCC explants these mice developed OVA (SIINFEKL)-specific in vivo killing activity when challenged 7 days later. Interestingly, if the same experiment is performed in c-myc OVA tg+ mice, the in vivo killing ability is not observed, indicating induction of T-cell tolerance (Fig. 5C).
To explore if the Combo3 treatment can interfere with T-cell tolerance, we performed the experiments shown in Fig. 5D. Activated OT-1 lymphocytes were adoptively transferred into c-myc-OVA tg+ tumor bearing mice on day 21 followed by Combo3 or control antibody treatments on days 21 and 25. No *in vivo* killing activity by these transferred OT-1 cells was observed unless Combo3 had been administered.

*Tumor infiltrating T lymphocytes elicited by the ISmAb combination treatment recognize JMJ cell lines.*

Specific T cell responses require antigen. If tolerated against OVA, we theorized that other antigens were recognized by endogenous tumor-infiltrating CTLs. Indeed, isolated tumor infiltrating lymphocytes released IFNγ, when cocultured with irradiated JMJ7 cells, while they did not when co-cultured with control cell lines (Fig. 6A). Moreover, gated CD8+ tumor infiltrating lymphocytes (TILs) from Combo3 treated mice degranulated and expressed surface CD107a+ upon exposure to JMJ7 cells (Fig. 6B). An additional series of similar experiments with immunomagnetically isolated CD8+ TILs from Combo3-treated mice indicated that these lymphocytes recognized JMJ cells while the control cells were not recognized including MC38 cells expressing OVA (Supplementary Fig. S7A). Similar observations were made with CD8 splenocytes from the same mice (Supplementary Fig. S7B).

Our next goal was to find out if such lymphocytes would recognize H-2Kb and H-2Db fitting epitopes derived from OVA, human c-myc or the transcriptional regulator of bacterial origin (tTA). Our hypothesis was that these transgenes could be immunogenic and activate TILs from Combo3 treated mice. However, none of the corresponding synthetic peptides elicited IFNγ.
production, including subdominant epitopes of OVA, sequences of human c-myc dissimilar to mouse c-myc and the transcriptional regulator of bacterial origin (tTA) (Fig. 6C). At this point of time the identity of the recognized peptides is unclear, although c-myc transcriptional targets are likely candidates (26).

In summary, we have identified a potent triple combination of ISmAbs that can surpass established tumor immune tolerance mechanisms in such a way that survival of otherwise rapidly progressing multifocal HCC tumor bearing mice is extended. Upon treatment we found an efficient endogenous T-cell response against tumor cells.
DISCUSSION

Our study shows that a combination of ISmAbs achieves partial efficacy in an aggressive and T cell-tolerizing mouse model of hepatocellular carcinoma (21). The rationale was that blockade of the B7-H1-PD-1 interaction would transiently release a key T cell repressor system (27), while agonist anti-CD137 and OX40 mAbs would enhance the antitumor T cell response (13, 22). The improved survival with only two doses of the mAbs given on days 21 and 25 is quite remarkable. Repeated treatments for longer periods of time are problematic, as rat and hamster immunoglobulins are immunogenic in mice and elicit neutralizing antibodies. In the past, treatment of animals injected subcutaneously with tumor cell lines has been successful, although this seems not to reflect the situation in patients. Possible reasons include the organ and tumor specific microenvironment that develops when a solid tumor grows over a period of time in a given organ (28). To better predict clinical potential, preclinical evidence of immunotherapy efficacy on spontaneous transgenic tumors is to be considered in conjunction with that on orthotopically implanted tumors (29).

The rapid progression and technical demands of this tumor model prevent testing of sequential or different dosage treatments using ISmAbs, although this question remains relevant regarding clinical trial designs (30). To our knowledge, this is the first report of combined ISmAb efficacy in a spontaneous autochthonous tumor model arising in oncogene-transgenic mice. A related work includes a combination of anti-DR5, anti-CD137 and anti-CD40 mAbs that has shown partial efficacy in carcinogen-induced sarcomas (17). In addition, an anti-CD40 mAb has shown activity against transgenic pancreatic ductal adenocarcinomas in combination with gemcitabine through an effect mediated by macrophages without T lymphocyte involvement (31).
Oncogene transgenic mice are thought to pose much more serious difficulties to immunotherapy than transplanted models, as they tend to be less immunogenic (32) and more tolerogenic (33). In the model studied here, multiple independent carcinoma foci develop at the same time (21) and demonstrate the HCC typical dense neovascular microenvironment. In these tumors antigen-specific CD8+ immunity is downmodulated and tumors progress at a fast pace with T-cell tolerance representing the major hurdle (21). Triple combination of ISmAbs makes sense since synergistic effects of the combined treatment consisting of anti-CD137 plus anti-B7-H1 (34, 35) mAbs or anti-CD137 plus anti-OX40 mAbs (36) have been reported in mouse models of transplantable tumors. As expected from the mechanism of action of these three antibodies, we observe an increase in T cell infiltrates in tumor tissue composed of both activated CD4+ and CD8+ T lymphoblasts. Indeed, CD8+ T lymphocytes are an absolute requirement to achieve an extended overall survival. Furthermore, CD8+ TILs express more intensely the perforin-granzyme B cytolytic machinery upon Combo3. The effector mechanisms of tumor cell destruction are likely to involve both cytolytic granules and FasL. Of note, JMJ cells express functional Fas and IFNγR and therefore these molecules could play a synergistic role in destroying the tumor as previously reported for immunotherapy with anti-CD137 mAb (37).

Our goal was to boost CTLs with anti-CD137 and both CD8+ and CD4+ T lymphocytes with anti-OX40, while at the same time removing PD-1 negative influences on lymphocyte activation. The reported functional effects of anti-OX40 mAb repressing regulatory T cells would also be an advantage (38). Individual contributions of each mAb to the overall therapeutic effects are difficult to tease out, as each antibody may modify the biological activity and receptor expression of the other partner antibodies in the combination.
The important role of CD4+ T cells in antitumor immunity is a matter of active research (39, 40). In our model, depletion of CD4-expressing T lymphocytes did not hamper the observed treatment benefit. However, we show that CD4+ cells are infiltrating the tumors and bear the receptors to be stimulated by the combination of ISmAbs. It will be important to identify in future experiments the exact role of CD4+ T cells and the cell presenting antigen to such CD4+ T cells, as tumor cells poorly express MHC class II molecules unless exposed to IFNγ. CD4+ T lymphocytes can cooperate with the elicitation of more powerful CTLs and also mediate direct antitumor effects (41). It should be noted that in our depletion experiments we also eliminate pro-tumoral Tregs and that might be a confounding factor to interpret the results upon depletion of CD4+ lymphocytes.

Selective expression of OVA in the tumor tissue allowed two types of experiments: (i) combinations with antigen-specific adoptive T cell therapy and (ii) studies on the T cell tolerance-inducing microenvironment in tumor bearing transgenic mice.

A clear synergy was observed between the triple combination of ISmAbs and adoptive transfer of activated CD8+ and CD4+ T lymphocytes recognizing OVA. Previous studies have demonstrated that infiltration by OVA specific T-cells in the c-myc OVA tg+ model is not sufficient for tumor regression, as these T-cells are rendered tolerant within the tumor microenvironment (21). Here, we clearly demonstrate that combined ISmAb therapy prevents tolerization of adoptively transferred OVA-specific T cells and is able to improve survival of the mice. In spite of their tolerizing ability, tumors were immunogenic, as tumor explants were rejected in immunocompetent syngeneic mice, whereas tumors did engraft in Rag1-/- immunodeficient mice.
Cultured tumor infiltrating lymphocytes retrieved following treatment degranulated and released IFNγ in the presence of cell lines derived from c-myc OVA tg+ tumors but not in the presence of control tumor cell lines. As we could not identify antigenic determinants from the three transgenes (OVA, the tTA transcriptional regulator or human c-myc), the identity of the antigens recognized by endogenous TILs still remains to be elucidated.

A more detailed picture of the function of the infiltrating lymphocytes would demand in-vivo microscopy or in-vivo imaging (42, 43). It is clear that treatment increases the number of activated effector TILs but we know little about their performance in vivo. Future research is aiming at visualizing and compare the liver tumor microenvironment upon therapy using fluorescent lymphocytes.

To date ISmAbs as those in Combo3 have not been studied in patients with HCC. We have performed a clinical trial with the anti-CTLA-4 mAb tremelimunab in advanced HCC with signs of clinical activity and evidence for an enhancement of immune responses anti-hepatitis C virus (25). In our model, we did not observe efficacy with two 100 μg doses of anti-CTLA-4 mAb as monotherapy and addition of anti-CTLA-4 mAb to the triple combination.

At present, a phase I clinical trial with anti-PD-1 mAb for HCC is ongoing (NCT01658878), whose results are eagerly awaited. Combination with adoptive T cell therapy is more difficult to be translated to patients since no suitable TCRs or chimeric antigen receptors (CARs) are yet available for HCC antigens; and TILs are extremely difficult to culture from HCC tissue (Alfaro C et al. unpublished data). Therefore, it is extremely encouraging that endogenous CD8 and CD4 T-cell responses are observed in this model system, where hepatic tumors progress rapidly and render T cells tolerant.
Anti-CD137 mAb triggers liver inflammation in healthy mice and human patients (44, 45). This could both be advantageous and deleterious, because HCC patients could be prone to suffer from hepatic side effects, while they also may benefit from the inflammatory response in this organ (44, 46). Here, we found no evidence of adverse autoimmune hepatitis in the surrounding liver tissue, based on immunohistochemical findings at the doses tested. In fact, T cell infiltrates were much more intense in the malignant tissue than in the surrounding normal liver tissue.

Overall, our experiments provide promising results for further preclinical and clinical trials using combinations of more than two ISmAbs in human primary and metastatic liver cancer, showing evidence for synergistic efficacious immunotherapy in mice bearing aggressive hepatocellular carcinomas.
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FIGURE LEGENDS

Figure 1.

Survival benefit of c-myc OVA tg+ mice developing hepatocellular carcinomas upon combined treatment with anti-CD137, OX40 and B7-H1 ISmAbs, and its enhancement by adoptive T cell therapy. (A) Kaplan-Meyer survival follow-up of c-myc OVA tg+ mice whose cages were deprived of doxycycline in drinking water at birth and were treated on day 21 and 25 of life either with a combination of anti-CD137, OX40 and B7-H1 (Combo3) or control rat IgG. The indicated mice received adoptive T cell therapy (ACT) with both 2x10^6 activated OT-1 and 2x10^6 activated OT-2 cells complemented with 2x10^4 IU of IL-2 i.p. (B) Representative ultrasound images at 6 weeks of age from mice in each treatment group with the HCC contour marked by a black line and providing the estimated area. Photographs of the abdominal regions of these mice are provided. Fraction of surviving mice per group is indicated in the legend. *P* values refer to rat IgG treated control group analyzed by log rank test. **, *P* < 0.01; ***, *P* < 0.001.

Figure 2.

The triple combination of ISmAbs is efficacious and dependent on CD8+ lymphocytes, whereas each mAb given separately does not extend survival. (A) Experiments performed as in Fig. 1A but in this case 100 μg of individual antibodies were dosed to the indicated groups. (B) Mice treated as in Fig. 1A were depleted with anti-CD8β or anti-CD4 mAbs given on days 20, 23 and 26. Fractions of surviving mice at day 250 (A) and 150 (B) in the indicated groups are provided.
in the legend. \( P \) values were analysed by log rank test. ns, non significant; * \( P < 0.05 \); ***, \( P < 0.001 \).

**Figure 3.**

**Immunohistochemistry studies of the livers of mice undergoing treatment with the triple combination or single ISmAbs.** (A) Histological images (H&E and the indicated monostaining) of the livers from c-myc OVA tg+ mice analysed at week 5 after receiving on day 28 treatment with one dose of indicated mAb or its combination. Immunostainings were performed for CD3 and TUNEL\(^+\) apoptotic nuclei. The bars represent 50 μm and these images (400X magnification) are representative of multiple microscopic fields from five or six livers analyzed each. (B) Quantitative analysis of the number of CD3\(^+\) T lymphocytes in the tumor versus non tumoral liver from the indicated treatment groups automatically counted from whole liver tissue sections. Data show the mean ± SD of five or six mice per group. #, absolute number of cells. **, \( P < 0.01 \).

**Figure 4.**

**The ISmAb triple combination attains more numerous and robust T lymphocyte infiltrates in HCCs.** (A) Absolute numbers of CD3\(^+\), CD8\(^+\) and CD4\(^+\) lymphocytes from cell suspensions retrieved from individual livers of mice treated at week 4 (day 28) as in Fig. 3 with the indicated antibodies. (B) Liver weights and absolute numbers of CD45\(^+\) leukocytes per gram of tissue in the liver of c-myc OVA tg+ mice treated with control antibody or the triple combination (Combo3) as in Fig. 1 on days 21 and 25 of life. (C and D) Flow cytometry results of samples as in B, analyzed for CD8\(^+\) (C) and CD4\(^+\) (D) percentages, frequency of lymphoblastic cells.
according to FSC/SSC and the percentage of positive cells and MFI for CD137, OX40, PD-1 and granzyme B immunostainings, as indicated in the corresponding graphs. A pool of three independent experiments (with 2-6 mouse per group in each experiment) is shown in B, C and D. #, absolute numbers. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, non significant.

Figure 5.

The ISmAb triple combination therapy acting on CTLs determines the immunonogenic versus tolerogenic behaviour of HCC arising in c-myc OVA tg+ mice. (A) Failure to graft of JMJ cells in C57Bl/6 Rag1−/− and WT mice. Numbers of grafting attempts with $10^6$ cells per mouse are plotted. Both JMJ7 and 9 subcultures were tested with identical negative grafting results. (B) Individual follow up of the size of HCC explants (∼5x5 mm) onto C57Bl/6 Rag1−/− and WT mice. (C) Conventional in vivo killing assay against SIINFEKL-loaded splenocytes in syngeneic mice with implanted s.c. tumor cubes following the time course indicated in the time flow scheme. (D) Doxycycline deprived c-myc OVA tg+ mice were treated on day 21 with $2x10^6$ activated OT-1 T cells and with control rat antibody or Combo3 on days 21 and 25. On day 28, as indicated in the time flow scheme an in vivo killing assay measuring CTL activity against SIINFEKL was performed. Data were summarized from three independent experiments (with 2-3 mice per group in each experiment) and displayed as a dot graph. **, $P < 0.01$.

Figure 6.

TILs in HCC tumors recognize specific tumor antigens in JMJ cells. (A) TILs isolated from tumor bearing mice of 4-week of age which had been treated with Combo3 on days 21 and 25 were cultured with irradiated JMJ7 and MC38 cells that had been IFNγ pretreated for 48h to
upregulate MHC-I and II expression. Concentration of IFNγ in the supernatants was measured 72h later. n=5 mice, repeated twice. (B) TILs as in A, restimulated for 5h to assess CD107a surface expression by flow cytometry on gated CD8+ T lymphocytes. The graph shows a pool of two experiments (with 3-5 mice per group in each). (C) TILs isolated as in A in a separate experiment, were pooled and restimulated in vitro with JMJ7 or the indicated soluble 8-9 mer synthetic peptides predicted to fit H-2Kb and H-2Db antigen presenting molecules in the sequences of chicken OVA, human-c-myc and tTA proteins. **, P < 0.01; ***, P < 0.001.
Figure 1

A

B

Rat IgG (0/10)
Combo3 (2/10)
ACT + Rat IgG (0/10)
ACT + Combo3 (4/11)

---

ACT + Combo3
No tumor

---

Combo3
11.18 mm²

---

Rat IgG
42.42 mm²

---

Week 6
Figure 3

A

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B

#CD3+ cells

- **Tumor**
- Non tumor

**

**
Figure 6

A

B

C

OVA

Human c-myc

tTA

JMJD7

25-7-264

32-3-339

25-32-176-183

55-62

5-13

72-80

84-91

123-131

117-125

184-192

276-284

12-20

125-133

IFN-γ (pg/ml)

1500

1000

500

0

10⁻⁵

10⁻⁴

10⁻³

10⁻²

10⁻¹

10⁰

10ⁱ

10⁲

10⁳

Rat IgG

%CD107⁺/CD8⁺

MC38

PMA

Ionomycin

JMJD7

**

***
Combined immunostimulatory monoclonal antibodies extend survival in an aggressive transgenic hepatocellular carcinoma mouse model


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