Combined Immunostimulatory Monoclonal Antibodies Extend Survival in an Aggressive Transgenic Hepatocellular Carcinoma Mouse Model

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Abstract

Purpose: Immunostimulatory monoclonal antibodies (ISmAb) that unleash antitumor immune responses are showing efficacy in cancer clinical trials. Anti-B7-H1 (PD-L1) monoclonal antibodies (mAb) block a critical inhibitory pathway in T cells, whereas 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, 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 (TIL) 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 hepatocellular carcinomas 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 hepatocellular carcinomas 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.


Introduction

Immunotherapies with cytokines, vaccines, and T-cell adoptive therapy have been tested in early clinical trials for hepatocellular carcinoma 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 (ISmAb) 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 patients with cancer. Two types of ISmAbs can be categorized depending on the receptors bound on immune system cells: agonist monoclonal antibodies (mAb) 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; ref. 10) and B7-H1 (PD-L1 or CD274) in melanoma, renal cell carcinoma, and lung cancer (11).
Activated T-cell infiltrate in tumor nodules was observed. Clearly extended and the presence of an abundant and 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 patients with hepatocellular carcinoma.

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 patients with cancer 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 patients with hepatocellular carcinoma.

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 precancer stages of development (20). A model of multifocal hepatocellular carcinoma 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 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 hepatocellular carcinoma clearly seen upon microscopic examination at 3 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 restating OVA specific CD8+ OT-1 T cells are rendered tolerant upon adoptive transfer into hepatocellular carcinoma-bearing mice (21). Strains of c-myc transgenic mice that do not coexpress OVA developed tumors with similar penetrance and age onset (21). As various treatment attempts using immunization strategies have failed in these mice (ref. 21 and data not shown), we tested a combination of 15mAbs targeting CD137, OX40, and B7-H1 (PD-L1) administered at 3 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-2IAb OVA323-339 (OT-2) were purchased from Jackson Laboratories. C57Bl/6 mice were purchased from Harlan Laboratories. 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. OVA-transfected EL-4 cell line, EG-7, was a kind gift from Dr. Claude Leclerc (Institut Pasteur). MC38 was provided by Dr. Karl E. Hellström (University of Washington, Seattle). These cell lines were authenticated by Idexx Radil (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 transfection (Sancho D. and colleagues; manuscript in preparation) and provided by Dr. Sancho (Centro Nacional de Investigaciones Cardiovasculares, 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 48 hours (OVA257-264 and OVA323-339, respectively). A total of 2 × 106 OT-1 cells and 2 × 106 OT-2 cells were intravenously transferred to c-myc OVA tg + mice on day 21 or 28 after birth, depending on the experiment. For survival experiments, a dose of 2 × 10^4 IU of i.p. IL-2 was administered to mice transferred with activated OT-1 and OT-2 on day 21 or 28. For treatment with mAbs, Combo3 (100 μg of each anti-CD137, anti-OX40 and anti-B7-H1) or control rat IgG was administered intraperitoneally on the indicated days. For depletion experiments, 3 doses of 200 μg of anti-CD4 or anti-CD8β mAbs were injected intraperitoneally starting one day before Combo3 treatment onset and every 3 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, weighed, and enzymatically disrupted with DNase I and collagenase D (both from Roche) 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) pressing with a plunger. To remove non-mononuclear cells, unicellular cell suspensions were pelleted, resuspended in a 35% Percoll gradient and centrifuged for 10 minutes, 500 × g at room temperature. Erythrocytes were lysed with ACK buffer (Gibco). Single-cell suspensions were treated with FcR-Block in a PBS-based buffer containing 10% of fetal calf serum to avoid unspecific staining. Flow cytometry and immunohistochemistry analysis were performed as described in Supplementary Methods.

Statistical analysis

Prism software (GraphPad Software) was used to analyze tumor infiltrating lymphocytes, specific lysis, and IFN-γ production between groups by applying unpaired Student t tests or U-Mann–Whitney tests. Survival curves were analyzed by Kaplan–Meier 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 ISmAbs needs the target molecules to be expressed at the cell surface as a requirement for biologic effects. CD137 and PD-1 are not constitutive surface proteins on T cells but become expressed following biologic effects. CD137 and PD-1 are not constitutive to be expressed at the cell surface as a requirement for hepatocellular carcinoma model

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 hepatocellular carcinoma: c-myc OVA tg+ mice were deprived of doxycycline at birth to induce hepatocellular carcinomas and treated on day 21 and 25 of extrauterine life with two doses of 100 μg of anti-CD137 (1D8), anti-OX40 (OX86), and anti-B7-H1 (10B5) mAbs (Combo3). Of note, mice at this age have histologic 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 with those treated with control antibody, and 2 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 × 10^6 activated OT-1 cells plus 2 × 10^6 activated OT-2 cells i.v. followed by 2 × 10^6 II-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 with the Combo3 group. In this group, 4 of 11 mice were alive 15 months after treatment.

Figure 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 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 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 tremelimumab in advanced hepatocellular carcinoma patients with signs of clinical activity (3 partial responses in 17 patients evaluated; ref. 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 combination.
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 the animals CD8\(^+\) 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.

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–Meier 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 \(2 \times 10^6\) activated OT-1 and \(2 \times 10^6\) activated OT-2 cells complemented with \(2 \times 10^4\) IU of IL-2 i.p. B, representative ultrasound images at 6 weeks of age from mice in each treatment group with the hepatocellular carcinoma 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 \(\mu\)g of individual antibodies were dosed to the indicated groups. B, mice treated as in Fig. 1A were depleted with anti-CD8\(^+\) 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 analyzed by log rank test. ns, non significant; *, \(P < 0.05\); ***, \(P < 0.001\).
Infiltration of hepatocellular carcinomas by activated CD4<sup>+</sup> and CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T lymphocytes (Fig. 3A). Immunohistochemical staining results showing livers from

![Figure 3. Immunohistochemistry studies of the livers of mice undergoing treatment with the triple combination or single ISmAbs. A, Histologic images (H&E and the indicated monostaining) of the livers from c-myc OVA tg<sup>+</sup> mice analyzed 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<sup>+</sup> apoptotic nuclei. The bars represent 50 µm, and these images (× 400 magnification) are representative of multiple microscopic fields from five or six livers analyzed each. B, quantitative analysis of the number of CD3<sup>+</sup> 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.](image-url)
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 Fig. 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 Fig. 4C and D). Accordingly, CD8+ and CD4+ T lymphocytes exhibited a higher intracellular content of granzyme B (Fig. 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 stimulation of CD8+ lymphocytes.

None of the JMJ cell lines grafted as tumors either in immunocompetent or immunodeficient Rag1-/- mice (Fig. 5A). However, small ~5 x 5 mm pieces of tumor if surgically implanted under a skin flap of Rag1-/- mice were rejected in every experiment, indicating that an anti-tumor T-cell response was responsible for this observation.

To explore whether 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+ 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 tolerized against OVA, we theorized that other antigens were recognized by endogenous tumor-infiltrating CTLs. Indeed, isolated tumor infiltrating lymphocytes released IFN-γ, when cocultured with irradiated JMJ7 cells, while they did not when cocultured with control cell lines (Fig. 6A). Moreover, gated CD8+ 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
Figure 4. The ISmAb triple combination attains more numerous and robust T lymphocyte infiltrates in hepatocellular carcinomas. 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.
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 IFN-γ 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 hepatocellular carcinoma 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), whereas 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

Figure 5. The ISmAb triple combination therapy acting on CTLs determines the immunogenic versus tolerogenic behavior of hepatocellular carcinoma 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 10⁶ 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 hepatocellular carcinoma explants (~5 x 5 mm) onto C57B6/6 Rag1−/− and WT mice. C, conventional in vivo killing assay against SIINFEKL-loaded splenocytes in syngeneic mice implanted subcutaneously with 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 2 x 10⁶ activated OT-1 T cells and with control rat antibody or Combo3 on days 21 and 25. On day 29, as indicated in the time flow scheme, an in vivo killing assay 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.
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 hepatocellular carcinoma 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 IFN$\gamma$R 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 biologic 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 IFN$\gamma$. 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

Figure 6. TILs in hepatocellular carcinoma tumors recognize specific tumor antigens in JMJ cells. A, TILs isolated from tumor-bearing mice 4 weeks of age that had been treated with Combo3 on days 21 and 25 were cultured with irradiated JMJ7 and MC38 cells that had been IFN-$\gamma$ pretreated for 48 hours to upregulate MHC-I and II expression. Concentration of IFN-$\gamma$ in the supernatants was measured 72 hours later. n = 5 mice, repeated twice. B, TILs as in (A), restimulated for 5 hours to assess CD107$\alpha$ 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-2k$^+$ and H-2d$^+$ antigen presenting molecules in the sequences of chicken OVA, human-c-myc, and tTA proteins. **, P < 0.01; ***, P < 0.001.
depletion experiments we also eliminate protumoral 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 IFN-γ 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 TIEA 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 vitro 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 comparing the liver tumor microenvironment upon therapy using fluorescent lymphocytes.

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

At present, a phase I clinical trial with anti-PD-1 mAb for hepatocellular carcinoma 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 hepatocellular carcinoma antigens; and TILs are extremely difficult to culture from hepatocellular carcinoma tissue (Alfaro C and colleagues; 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 patients with hepatocellular carcinoma 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.

Disclosure of Potential Conflicts of Interest

B. Sangro is a consultant/advisory board member of Medimmune. I. Melero has a commercial research grant and honoraria from speakers' bureau from Bristol Myers Squibb and is a consultant/advisory board member of Merck, Bristol Myers Squibb, and Medimmune. No potential conflicts of interest were disclosed by the other authors.

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