Immunotherapy of hepatocellular carcinoma: is there a place for regulatory T-lymphocyte depletion?

Immunotherapy represents a potential therapeutic option for patients with hepatocellular carcinoma (HCC), especially as secondary treatment to prevent recurrence. It has been shown that a patient’s survival is directly correlated to the type and number of tumor-infiltrating immune cells, indicating that immune responses have a direct effect on the clinical course of the disease. We have assessed the potential of immunotherapy against HCC in preclinical models of low tumor burden. An antigen-specific strategy targeting α-fetoprotein, and consisting of immunization with a DNA-based synthetic vector (DNAmAFP/704), was tested on an autochthonous model of chemical hepatocarcinogenesis and led to an important (65%) reduction of the tumor burden. A nonspecific approach of CD25+ T-cell depletion by injection of PC61 antibody was also tested on an orthotopic HCC model and led to a significant protection against tumor development. Antigen-specific immunotherapy and Treg depletion are promising strategies in physiologically relevant HCC preclinical models. Future clinical trials will demonstrate if a combination of Treg depletion with an antigen-specific immunotherapy will also translate into clinical responses in HCC patients.

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world (600,000 deaths/year) and its incidence is increasing. The main etiologies of HCC are the hepatitis B and C viral infections and alcohol consumption. HCC follows a multistep process of carcinogenesis through the progressive evolution of chronic liver disease leading to extensive fibrosis and cirrhosis and, finally, HCC [1,2]. Current treatment options include liver transplantation, surgical resection and local ablative therapies. However for all except transplantation, tumor recurrence rates are up to 70% after 5 years. These recurrences are mainly due to new tumor nodules arising from the diseased parenchyma. In the context of an absence of systemic therapeutic treatment, immunotherapy represents a potentially beneficial option for HCC patients [3].

**Rational for HCC immunotherapy: clinical data**

Taken together, the following arguments build a solid rationale for HCC immunotherapy:

- HCC is an immunogenic tumor in which MHC molecules are expressed [4];

- There are a number of tumor antigens that can be targeted specifically, such as α-fetoprotein (AFP) or glypican-3, and T lymphocytes specific for these antigens are not deleted and spontaneous tumor-specific T-cell responses have been measured in untreated patients [5,6];

- Recently, it has been shown that some destructive treatments, such as radiofrequency thermal ablation, could stimulate the natural tumor-specific immune response in HCC patients and that those patients who show this enhanced response have an increased disease-free survival time [7];

- The presence of lymphocyte infiltrates in HCC has been detected and can constitute prognostic factors. In an analysis performed on tumor samples taken from more than 300 patients, Gao et al. have demonstrated that a high number of infiltrating activated cytotoxic T lymphocytes (CTLs) and a low number of infiltrating regulatory T cells (Tregs) were both associated with improved overall survival. The presence of low intratumoral Tregs in combination with high intratumoral activated CTLs was an independent prognostic marker for both overall survival and disease-free survival. This intratumoral balance of regulatory and cytotoxic T cells is therefore a promising independent predictor of recurrence and survival in HCC [8]. Frequency of circulating Treg cells has also been shown to be greater in patients with HCC, compared...
with control groups and patients with liver cirrhosis. This increased frequency of circulating Tregs has been shown to correlate with a poor survival of HCC patients [9].

In this context, trying to tip the balance towards a potent antitumor immune response by boosting the CTL response and/or blocking regulatory mechanisms is a valid strategy. To date, clinical trials of immunotherapy on HCC patients have all consisted of attempts to stimulate the antitumor immunity [10]. Among them, most have been performed on patients at an advanced stage of the disease. No clinical benefits were observed but they demonstrated the absence of toxicity of the treatments. The first trial to give promising results was a randomized trial consisting of an adoptive transfer of nonspecifically activated autologous peripheral blood mononuclear cells (PBMCs) after surgical resection. This strategy led to an increased disease-free survival time in the group of patients who received the adjuvant immunotherapy [11]. Strikingly, two strategies that gave encouraging clinical outcomes involved immunotherapy as adjuvant treatments, after surgical resection, to reduce the risk of recurrence [11,12]. This is an important observation, showing that the best chance for HCC immunotherapy is under conditions of low tumor burden.

Preclinical results
Taking these results into account, new strategies of immunotherapy should be developed as adjuvant treatments to reduce recurrence or even as preventive treatments in cirrhotic patients, since in this population the incidence of HCC is 4–5% per year. At a preclinical level, candidate strategies should be tested on clinically relevant animal models. We have developed two HCC models in which the tumor grows within the liver parenchyma. With these two models, we have tested several immunotherapy strategies in conditions of low tumor burden, including antigen-specific immunotherapy and nonspecific immunotherapy. In the orthotopic model, Hepa1.6 cells are injected in the liver of C57BL6 mice via the portal vein. Over a period of 3 weeks, the tumor cells progressively invade the whole parenchyma. Hepa1.6 cells stably expressing β-galactosidase (β-Gal) were injected into transgenic mice for which β-Gal is a self-antigen [13]. These mice were preventively vaccinated with a synthetic vector consisting of a low dose of plasmid DNA encoding β-Gal formulated with an amphiphilic block copolymer (704, also known as ICA614). This preventive vaccination gave complete protection against tumor development in five out of the six injected mice, whereas all the mice from the control group showed significant tumor burden at 3 weeks [14]. This result was confirmed with a clinically relevant antigen, AFP, in an autochthonous HCC model. In this chemical carcinogenesis model, 2-week-old male mice receive an injection of diethylnitrosamine (DEN) and subsequently develop liver tumors over a period of 8–10 months. The first nodules are visible after 5 months and they re-express AFP. DEN mice were vaccinated at 4 and 5 months with a synthetic vector consisting of plasmid DNA encoding AFP and ICA614. They were sacrificed at 8 months and the analysis of their tumor burden demonstrated that there was a reduction of 65% of the total tumor surface in the AFP immunized group [15].

Other immunotherapy strategies include nonspecific approaches to suppress the regulatory mechanisms that hinder the natural antitumor immune response. As stated earlier, the presence of infiltrating Treg cells in HCC is associated with a poor prognosis and at a preclinical level, Treg cell depletion has been shown to impair the growth of other tumors [16]. In the orthotopic model described above, the preventive depletion of Treg cells by injection of anti-CD25 antibody (PC-61, 400 µg) led to drastically reduced tumor growth, with four out of five mice presenting a normal, tumor-free liver at the time of sacrifice [Cany J, Unpublished Data]. This result supports that Treg cell depletion is a promising potential adjuvant treatment for HCC patients.

Conclusion
α-fetoprotein-specific vaccination with a potent vector and Treg depletion are two strategies of immunotherapy that have significant antitumor effects on relevant preclinical HCC models. Recent work by Greten et al. demonstrates that depletion of Treg cells by low-dose cyclophosphamide treatment of HCC patients unmasks AFP-specific T-cell responses [17]. A combination of AFP-specific vaccination with Treg depletion appears a promising candidate strategy for a clinical trial of HCC immunotherapy as an adjuvant treatment to reduce recurrence.

Financial & competing interests disclosure
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patients received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.
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