

# **Regulation of intra-tumoral T cell immunity in liver cancer**

**Guoying (Estella) Zhou**

**周国影**

*To my family members who are no longer with us*



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# **Regulation of Intra-tumoral T Cell Immunity in Liver Cancer**

Regulatie van intra-tumorale T cel immuniteit in lever kanker

## **Thesis**

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by command of the  
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by

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# CHAPTER 1

## **General introduction and outline of the thesis**

### **Review: Immune suppressor cells and checkpoints in liver cancer**

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*In preparation*

## 7 Aims and outline of this thesis

In the current thesis we investigate some of the abovementioned immune suppressive mechanisms in the tumor microenvironment of liver cancers with the goal to identify new and potentially promising immunotherapeutic targets to overcome the intra-tumoral immune inhibition and enhance anti-tumor reactivity of tumor-infiltrating T cells in patients with liver cancer. We study how to reduce the immunosuppressive capacity of pro-tumor regulatory T cells, and how to activate anti-tumor functions of effector T cells in hepatocellular carcinoma, cholangiocarcinoma and liver metastasis of colorectal cancer by manipulating co-inhibitory and co-stimulatory pathways. For this purpose, we use leukocytes isolated from resected liver tumors, tumor-free liver tissues and peripheral blood collected from patients that undergo liver tumor resection, and perform flow cytometry analyses and *in vitro* immune cell culture assays. The ultimate aim of these studies is to provide new immunotherapeutic approaches to treat patients with primary liver cancer or CRC liver metastasis.

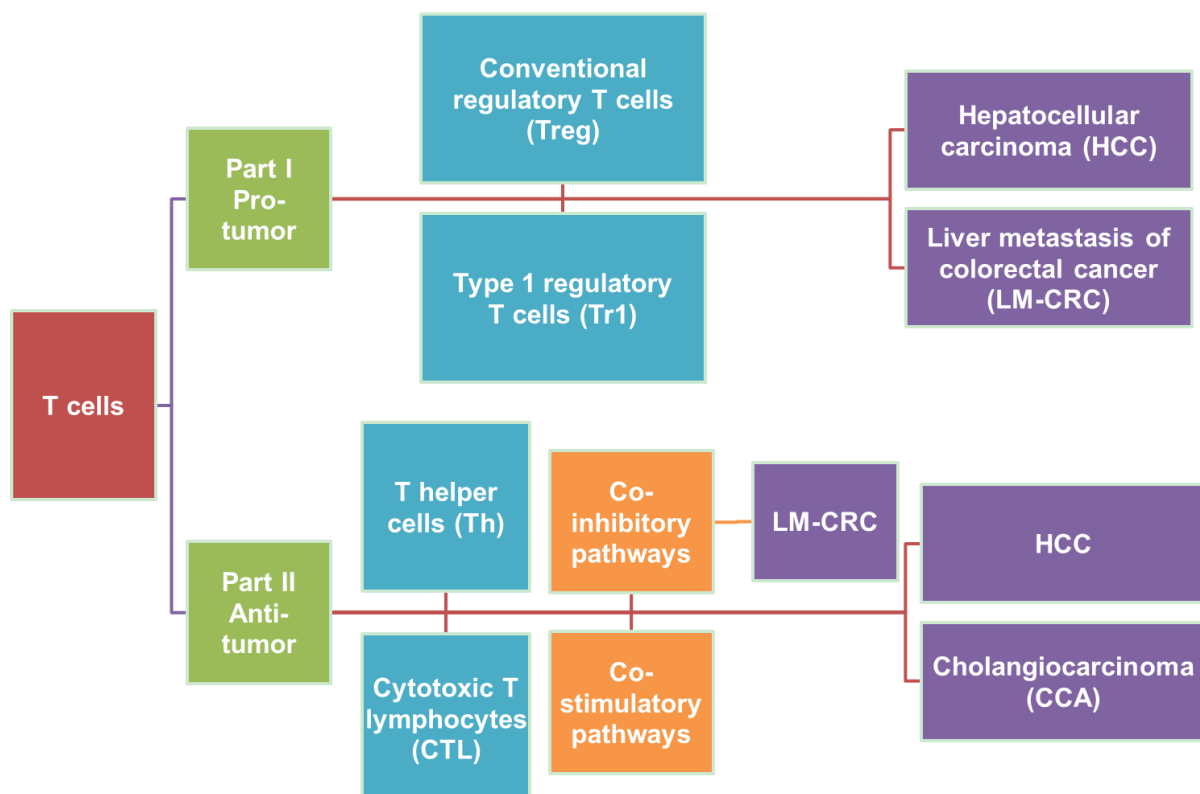
**Part I** focuses on two types of pro-tumor T cells, immunosuppressive conventional regulatory T cells and type 1 regulatory T cells. We study how to abrogate the immune suppression exerted by these cells in the tumor microenvironment (Figure 4). In **Chapter 2**, we study the phenotype and suppressive capacity of conventional regulatory T cells in hepatocellular carcinoma and liver metastasis of colorectal cancer, and demonstrate that stimulation of these cells via the co-stimulatory molecule GITR in combination with blockade of the co-inhibitory molecule CTLA4 can completely alleviate *ex vivo* inhibition of effector T cell functions by human liver tumor-derived regulatory T cells. In **Chapter 3**, we identify an intra-tumoral population of IL-10-producing Type 1 regulatory T cells in hepatocellular carcinoma and liver metastasis of colorectal cancer, and show in co-cultures that plasmacytoid dendritic cells enhance IL-10 production by these cells through engagement of their co-stimulatory molecule inducible T cell costimulator (ICOS). In **Chapter 4**, we challenge the use of anti-CD25 antibodies to deplete regulatory T cells from human tumors and propose the risk of depleting CD25-expressing non-Treg cells that may be important for anti-tumor immunity.

**Part II** focuses on two types of anti-tumor T cells, CD4<sup>+</sup> T helper cells and CD8<sup>+</sup> cytotoxic T cells. We study how to invigorate effector functions of tumor-infiltrating T cells by targeting co-inhibitory and co-stimulatory immune checkpoint pathways (Figure 4). In **Chapter 5**, we investigate which co-inhibitory pathways suppress intra-tumoral helper and cytotoxic T cells in hepatocellular carcinoma, and demonstrate that blocking PD-L1, TIM3, or LAG3 increases anti-tumor antigen responses of tumor-infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells in *ex vivo* assays. Importantly, combining antibody against PD-L1 with antibodies against TIM3, LAG3, or



CTLA4 further increases *ex vivo* functions of tumor-infiltrating T cells. In **Chapter 6**, we examine co-inhibitory molecules in liver metastasis of colorectal cancer with mismatch repair-proficient type, and compare the immune cell infiltration and expression of co-inhibitory molecules in LM-CRC with those in primary CRC and peritoneal metastasis of colorectal cancer. We show that antibody blockade of LAG3 or PD-L1 enhances *ex vivo* functions of tumor-infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells from LM-CRC. In **Chapter 7**, we analyze the composition and characteristics of immune infiltrates in cholangiocarcinoma, and test the effect of blocking co-inhibitory and activating co-stimulatory pathways on *ex vivo* effector functions of tumor-infiltrating T cells from cholangiocarcinoma. In **Chapter 8**, we demonstrate that agonistic targeting of the co-stimulatory molecule GITR by GITR ligand or anti-GITR antibody promotes *ex vivo* functions of T cells isolated from hepatocellular carcinoma.

A summary of the work presented in this thesis, as well as the importance and implications of these studies as a whole are discussed in **Chapter 9**.



**Figure 4. Outline of the thesis**

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# **PART I**

## **Regulation of intra-tumoral suppressor T cells in liver cancers**



# CHAPTER 2

## **GITR engagement in combination with CTLA-4 blockade completely abrogates immunosuppression mediated by human liver tumor-derived regulatory T cells *ex vivo***

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## **ABSTRACT**

In liver cancer tumor-infiltrating regulatory T cells (Ti-Treg) are potent suppressors of tumor-specific T-cell responses and express high levels of the Treg-associated molecules cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR). In this study, we have evaluated the capacity of GITR-ligation, CTLA-4-blockade and a combination of both treatments to alleviate immunosuppression mediated by Ti-Treg. Using *ex vivo* isolated cells from individuals with hepatocellular carcinoma (HCC) or liver metastases from colorectal cancer (LM-CRC) we show that treatment with a soluble form of the natural ligand of GITR (GITRL), or with blocking antibodies to CTLA-4, reduces the suppression mediated by human liver tumor-infiltrating CD4<sup>+</sup>Foxp3<sup>+</sup> Treg, thereby restoring proliferation and cytokine production by effector T cells. Importantly, combined treatment with low doses of both molecules exhibited stronger recovery of T cell function compared with either treatment alone. Our data suggest that in patients with primary and secondary liver cancer both GITR-ligation and anti-CTLA-4 mAb can improve the anti-tumor immunity by abrogating tumor-infiltrating Treg mediated suppression.

**Keywords:** Hepatocellular carcinoma (HCC), liver metastases from colorectal cancer (LM-CRC), regulatory T cells (Treg), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR).

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## Introduction

Hepatocellular carcinoma, the sixth most frequent tumor in the world <sup>1</sup> and the third most common cause of cancer-related death, <sup>2</sup> is an aggressive tumor with poor prognosis. HCC accounts for most of primary liver malignancies. However colorectal cancer (CRC), the third most common cancer worldwide, commonly metastasizes to the liver, which is the leading cause of CRC mortality. <sup>3, 4</sup> The current therapeutic options for HCC and liver metastasis from CRC (LM-CRC) include surgery (resection and liver transplantation) and local (ablative) therapy. Unfortunately, less than 20% of HCC patients are eligible for curative procedures <sup>5</sup> because most of them have advanced disease at the time of diagnosis. Furthermore, over 50% of LM-CRC patients that undergo surgery develop recurrence within 2 years. <sup>6, 7</sup> Chemotherapy has showed limited efficacy and provides a survival advantage of only 2.3-2.8 months in advanced HCC. <sup>8, 9</sup> An attractive alternative to these current therapeutic options is immunotherapy, which is based on the sensitivity, specificity and memory of the immune system. However, immune regulation in the liver and tumor environment may contribute to tumor outgrowth and limit the efficacy of immunotherapeutic strategies. <sup>10, 11</sup> In support of this we and others have described the presence of regulatory CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in liver tumors, that suppress local anti-tumor immunity and that are associated with poor patient prognosis. <sup>12-16</sup> Liver Ti-Treg are characterized by high expression of glucocorticoid-induced tumor necrosis factor receptor related gene (GITR), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and inducible T cell co-stimulator (ICOS). <sup>12</sup> These molecules are important for the immunosuppressive function of Treg and can be targets for immunotherapeutic interventions. Abrogation of Ti-Treg function may allow the induction of anti-tumor immunity at the tumor site and improve the outcome of immunotherapy aimed to activate anti-tumor responses. In advanced melanoma, CTLA-4-blocking has demonstrated a survival advantage <sup>17, 18</sup> and CTLA-4-blockade was shown to induce antiviral immunity in HCC-patients infected with hepatitis C virus (HCV). <sup>19</sup> However, only limited tumor-responses were reported, emphasizing the need to improve this strategy. Combining several candidate-immunomodulators may achieve synergistic immunoregulatory activity.

In this study we have evaluated *ex vivo* the capacity of GITR-ligation, CTLA-4-blockade and a combination of both to alleviate immunosuppression mediated by Ti-Treg isolated from patients with primary and secondary liver cancer.

## Results

***GITR<sup>+</sup>CTLA-4<sup>+</sup> Treg accumulate in liver tumors and have an increased suppressive capacity***

In order to confirm our previous finding showing that activated CD4<sup>+</sup>Foxp3<sup>+</sup>Treg are sequestered at the liver tumor site, we analyzed Treg in lymphocytes isolated from fresh liver tumors, tumor-free liver (TFL) tissues, and peripheral blood (PB) in a new cohort of HCC and LM-CRC patients by flow cytometry. Treg were present in all the three compartments analyzed, but were significantly more concentrated in the tumor areas compared with TFL ( $p = 0.0004$ ) and blood ( $p < 0.0001$ ) (Fig. 1A). We also corroborate in this new cohort that Ti-Treg are more suppressive than circulating Treg by analyzing their impact on T cell proliferation of autologous CD4<sup>+</sup>CD25<sup>-</sup> T cells stimulated with CMV-activated dendritic cells (DC). Ti-Treg showed a stronger suppression of T cell proliferation compared with blood Treg ( $p = 0.0005$ ) (Fig. 1B). In addition, we analyzed the surface expression level of GITR and intracellular expression of CTLA-4 (Figure 1C). GITR expression was significantly higher on tumor Treg than on Treg isolated from TFL ( $p = 0.0005$ ) and blood ( $p = 0.0002$ ). Tumor Treg were also distinguishable from blood and TFL Treg by their elevated intracellular expression of CTLA-4, which is a key negative regulator of T-cell activation ( $p = 0.0004$  and  $0.0018$  respectively). Moreover, we found that a big proportion of Ti-Treg expressed both molecules, in contrast with blood or TFL derived Tregs that have a very low proportion of double positive cells (Figure 1D). Thus, Ti-Treg derived from liver tumors express high levels of GITR and CTLA-4 and have an enhanced suppressive capacity.

### ***GITR engagement reduces suppressive capacity of Ti-Treg***

Soluble GITRL (sGITRL) was able to lower T cell suppression by Ti-Treg derived from liver tumors of patients with HCC or LM-CRC (Figure 2). CD4<sup>+</sup>CD25<sup>-</sup> effector T cells proliferated robustly in response to autologous DC activated with CMV. This proliferative response, as well as the production of TNF $\alpha$  was not affected by the addition of 10 or 20  $\mu\text{g/ml}$  of sGITRL. However, GITRL induced an increased amount of IFN $\gamma$  suggesting a limited direct effect on CD4<sup>+</sup>CD25<sup>-</sup> T cells. CMV-specific T cell proliferation and cytokine production were inhibited by Ti-Treg derived from both groups of patients (Figure 2A). Importantly, the addition of 10  $\mu\text{g/ml}$  of sGITRL significantly reduced the suppression mediated by Ti-Treg on CMV-specific T cells, recovering both T-cell proliferation ( $57 \pm 17\%$  vs  $69 \pm 20\%$  proliferating CD4<sup>+</sup> T cells,  $p = 0.0005$ ) and cytokine production by proliferating cells (TNF $\alpha$ :  $42 \pm 25\%$  vs  $63 \pm 28\%$ ,  $p < 0.0001$ ; IFN $\gamma$ :  $44 \pm 24\%$  vs  $80 \pm 37\%$ ,  $p = 0.0001$ ) (Figure 2A-B). However, 10  $\mu\text{g/ml}$  of sGITRL did not abrogate suppression of T cell proliferation completely, and a higher dose of sGITRL (20  $\mu\text{g/ml}$ ) could not further restore antigen-specific T cell proliferation nor cytokine production (Figure 2 A-B), and induced T cell death instead in most patients investigated (data not shown). Thus, sGITRL is able to partially decrease T cell suppression mediated by Ti-Treg isolated from liver cancer patients.



***CTLA-4 blockade prevents T cell suppression mediated by Ti-Treg in a dose-dependent manner***

To address the impact of CTLA-4 in the suppression mediated by Ti-Treg we used a neutralizing antibody for CTLA-4 in our in vitro model of T cell proliferation. The anti-CTLA-4 antibody at a concentration of 10 µg/ml did not affect T cell proliferation or TNF $\alpha$  and IFN $\gamma$  production in the absence of Treg, however treatment with 20 µg/ml of anti-CTLA-4 antibody induced an increase in the production of IFN $\gamma$  (Figure 3B), suggesting a direct effect of this antibody on CD4+CD25- effector T cells at this concentration. Anti-CTLA-4 treatment reduced the suppression mediated by Ti-Treg in a dose-dependent manner (Figure 3A). Recovery of T cell function was observed in both T-cell proliferation ( $52.8 \pm 22.5$  % when Ti-Treg were present vs  $69.3 \pm 23$  % in the presence of Ti-Treg and 10 µg/ml of anti-CTLA-4,  $p = 0.008$ , vs  $78.6 \pm 19.9$  % with 20 µg/ml of anti-CTLA-4 antibody,  $p = 0.008$ ) and cytokine production by proliferating cells (TNF $\alpha$ :  $43 \pm 23$  % in the presence of Ti-Treg vs  $67 \pm 18$  % with Ti-Treg and 10 µg/ml of anti-CTLA-4 antibody,  $p = 0.025$ , and  $83 \pm 42$  % with Ti-Treg and 20 µg/ml of anti-CTLA-4 antibody,  $p = 0.008$ ; IFN $\gamma$ :  $44 \pm 26$  % vs  $59 \pm 33$  %,  $p = 0.48$ , vs  $101 \pm 69$  %,  $p = 0.014$ , respectively) (Figure 3 A-B). Because T cell proliferation was not recovered completely with 20 µg/ml of anti-CTLA-4 antibody, as opposed to (near) full recovery of cytokine production, we were interested in investigating the effect of increasing the concentration of anti-CTLA-4 antibody further. Therefore, another set of antibody blocking experiments was performed with 40 and 80 µg/ml of anti-CTLA-4 antibody, which showed that higher concentrations of the neutralizing antibody for CTLA-4 resulted in complete recovery of T cell proliferation (Figure 4).

***GITRL and anti-CTLA-4 antibody combination additively abolish T cell suppression mediated by Ti-Treg***

We next asked whether a low dose of GITRL combined with a low dose of neutralizing anti-CTLA-4 antibody could fully recover T cell function in the presence of Ti-Treg. Blood derived CFSE-labeled CD4+CD25- stimulated with autologous DC activated with CMV were cultured in the presence of Ti-Treg and treated with 10 µg/ml of GITRL and anti-CTLA-4 antibody, either as monotherapy or in combination. Monotherapy with GITRL or anti CTLA-4 antibody induced partial recovery of T cell proliferation and cytokine production as observed in the experiments mentioned above (Figure 5, 2 and 3). In comparison with the effect of each treatment alone, the combination of low doses of anti-CTLA-4 and GITRL resulted in marked enhancement of T cell proliferation and cytokine production (Figure 5 A-B). Importantly, the combination of low doses of both molecules provided similar effects as those observed with a

high dose of anti-CTLA-4 antibody, restoring T cell proliferation and TNF $\alpha$  production completely.

***Combination of GITRL and anti-CTLA-4 antibody restored tumor-specific T cell response.***

To investigate whether the combination of GITRL and anti-CTLA-4 antibody is able to reestablish tumor-specific T cell responses, we used blood derived CFSE-labeled CD4+CD25- T cells stimulated with DC cultured with Tumor Lysates (TL-DC). Cells were co-cultured in the presence of Ti-Treg and treated with 10  $\mu$ g/ml of GITRL and 10  $\mu$ g/ml of anti-CTLA-4 antibody. All cell types and the TL were autologous. After one week of culture we observed that Ti-Treg suppressed efficiently cell proliferation and cytokine production of T cells against TL (Figure 6). However when the cells were treated with both GITRL and anti-CTLA-4 antibodies the tumor specific response can be completely restored.

**Discussion**

New treatment modalities are required to prolong survival in patients with liver cancer. Immunotherapy is an attractive option for HCC patients and patients with liver metastasis of CRC because increasing evidence suggests that immune responses play an important role in the control of these types of cancer. It has been reported that the composition of the tumor infiltrating lymphocytes (TILs) is associated with the prognosis of both diseases, for example increased numbers of Treg are correlated with disease progression<sup>20-24</sup> and recurrence.<sup>25</sup> Furthermore Treg frequencies have been associated with increased numbers of intra-tumoral macrophages,<sup>13, 26</sup> circulating myeloid-derived suppressor cells (MDSC),<sup>27</sup> and with a compromised CD8 T cell response.<sup>14, 15</sup> Altogether these observations suggest that depletion or inhibition of Treg and concomitant stimulation of effector cells may be effective to reduce recurrence and prolong survival in patients with liver cancer. Indeed, depletion of CD25+ T cells in patients or animals models can result in induction of tumor-specific responses.<sup>28, 29</sup> Moreover, in a small clinical trial in advanced HCC patients, treatment with low doses of cyclophosphamide resulted in reduced numbers of Treg and unmasking of circulating tumor-specific CD4+ T cell responses.<sup>30</sup>

We previously reported the presence of highly activated CD4+Foxp3+ Treg in liver tumors of individuals with HCC or LM-CRC.<sup>12</sup> These tumor-infiltrating Treg are characterized by the expression of high levels of GITR and CTLA-4, which are important molecules for their regulatory function and which can be targets for immunotherapy.<sup>12, 16</sup> GITR activation by its ligand acts as a co-stimulatory molecule for effector T cells that confers them less

susceptible to Treg mediated suppression, whereas engagement of GITR expressed by Treg transiently inhibits their suppressive capacity (reviewed in <sup>31</sup>). The GITR agonist antibody DTA-1 represents a very effective antitumor therapy in murine tumor models by increasing antitumor CD4+ and CD8+ T-cell effector functions as well as destabilizing and causing apoptosis of Tregs in the tumor microenvironment. <sup>32, 33</sup> Importantly, our findings support a role for GITR engagement as (part of) an immunotherapy for liver cancer patients, since treatment with sGITRL reduced Treg mediated inhibition of T cell functions. However, the observed effect was only partial and could not be enhanced by dose escalation. Indeed, a high dose of sGITRL had a cytotoxic effect on effector T cells in our in vitro system. In preclinical animal models, treatment with agonistic antibodies for GITR has been shown to cause anaphylaxis upon repeated doses. <sup>34</sup> These observations warrant careful design and dosing strategies of drugs engaging GITR to prevent serious side effects during immunotherapy for patients with liver tumors.

On the other hand, CTLA-4 is constitutively expressed at high levels in CD4+Foxp3+ Treg and is a negative regulator of T-cell activation. By binding the B7 molecules CD80 and CD86, CTLA-4 reduces the T-cell stimulatory capacity of antigen-presenting cells such as dendritic cells (DC). <sup>35</sup> Therefore, by using monoclonal antibodies (mAb) which block CTLA-4, it is possible to alleviate inhibition of DC function by Treg and thereby restore conventional T-cell proliferation after activation. <sup>36</sup> Recent studies have demonstrated that anti-CTLA-4 mAb administration augments clinical anti-tumor responses and improves survival in patients with metastatic melanoma <sup>18, 37</sup> resulting in FDA approval of Ipilimumab, an anti-CTLA-4 antibody, for the treatment of unresectable or metastatic melanoma. The promising clinical activity of anti-CTLA-4 mAb in melanoma encourages to explore its therapeutic applicability in other malignancies, especially because it does not require specific targets expressed on tumor cells. However, there is scarce evidence for efficacy in liver cancer. Only one clinical trial using the anti-CTLA-4 mAb tremelimumab in HCC patients infected with HCV has been conducted, but it showed a partial tumor response in merely 17% of patients. <sup>19</sup> Even though the clinical response was limited, the results point to a potential use for anti-CTLA-4 mAb in immunotherapeutic regimens for these patients. In our study 20 µg/ml of anti-CTLA-4 antibody (BNI3) showed a 60% recovery of T cell proliferation, whereas a concentration of 10 µg/ml only provided a recovery of 35% of T cell proliferation. Higher doses were able to recover T cell functions completely. However, therapy with anti-CTLA-4 mAb ipilimumab has induced life-threatening adverse events, <sup>17, 38, 39</sup> which is a major limitation in exploring higher doses for the treatment. The current clinically approved dosing for anti-CTLA-4 antibody (Ipilimumab) is 3 mg/kg which results in a steady state serum concentration (C<sub>min</sub>) of about

10 µg/ml,<sup>18, 40</sup> and an overall response rate of 4% in patients with advanced melanoma with 3% of patients experiencing severe immune-related adverse events. A higher dose of Ipilimumab of 10 mg/kg (Cmin 32 µg/ml) showed a better response rate of 11% but also higher frequency (15%) of severe adverse events.<sup>18</sup>

Based upon results from murine models, it has previously been suggested that GITR stimulation and CTLA-4 inhibition can be combined to enhance the induction of immune responses and might even work synergistically.<sup>41</sup> Importantly, combination of low doses of anti-CTLA-4 antibody and GITRL resulted in a complete recovery of *in vitro* T cell proliferation and TNFα production. Even though the mechanisms by which these molecules exert their immunomodulatory effects is still elusive, it has been suggested that they can act either by abrogating the immunosuppressive function of Treg or by rendering effector T cells resistant to Treg mediated suppression. In our experiments we observed that both molecules had limited direct effects on effector T cells, only inducing an increase in the production of IFNγ, but this effect was not evident for TNFα and T cell proliferation, suggesting that both mechanisms can play a role in abrogation of the suppression mediated by Treg. It remains to be seen whether combination therapy of anti-CTLA-4 mAb and GITRL has a more beneficial safety profile compared to treatment with a high dose of either compound alone, but our data show that the combination of both treatments at low doses exhibited stronger inhibition of *ex vivo* Treg-mediated suppression compared with either treatment alone, suggesting that this combination may be more effective to induce anti-tumor immunity in patients with liver cancer.

In conclusion our data suggest that in patients with primary and secondary liver cancer both GITR-ligation and anti-CTLA-4 mAb can improve antigen-specific T cell responses in the tumors by protecting against tumor-infiltrating Treg mediated suppression of their function. However, as part of an immunotherapeutic strategy, combining low doses of both drugs may be at least as effective as monotherapy with higher doses of either of the drugs.

## **Materials and Methods**

### ***Patients***

Between June 2011 and November 2013 a total of 28 individuals who were eligible for surgical resection of HCC (n=10) or LM-CRC (n=18) were enrolled. Paired samples of fresh liver tumor tissue and tumor-free liver tissue (TFL) obtained at the maximum distance from the tumor (>1cm), were used for isolating tumor-infiltrating lymphocytes (TILs) and intra-hepatic lymphocytes. In addition, peripheral blood was collected. None of the patients was

treated with chemotherapy or radiation prior to resection. The clinical characteristics of the patients are summarized in table 1. The study was approved by the local ethics committee and all patients in the study gave informed consent before tissue donation.

### ***Cell preparation***

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation. Single cell suspensions from TFL and tumor tissue were obtained by tissue digestion. Fresh tissue was cut into small pieces and digested with 0.5 mg/ml of collagenase (Cat. C5138, Sigma-Aldrich) and 0.2 mg/ml of DNase I (Cat. 10104159001, Roche), for 30 minutes at 37 °C. Cell suspensions were filtered through 100 and 70 µm pore cell-strainers (BD, biosciences) and mononuclear cells (MNC) were obtained by Ficoll density gradient centrifugation. Viability was determined by trypan blue exclusion.

### ***Flow cytometry analysis***

PBMC and MNC isolated from TFL or tumor tissue were analyzed for expression of surface and intracellular markers using the following anti-human antibodies: PE-labeled anti-IFN $\gamma$  (B27, BD Biosciences) and anti-CD25 (BC96) from ebiosciences; APC-labeled anti-FoxP3 (236A/E7) and anti-TNF $\alpha$  (Mab11) from e-biosciences; APC-H7-labeled anti-CD4 (SK3, BD Biosciences); PeCy7-labeled anti-CD3 (UCTH1); eFluor $\text{®}$ 450-labeled anti-CD127 (eBioRDR5) from e-biosciences. Cells were incubated with the antibodies 10 min at room temperature in the dark, then washed and fixed with 1% paraformaldehyde. For intracellular cytokine staining, cells were permeabilized using the BD Cytofix/cytoperm fixation/permeabilization kit, and for FoxP3, cells were treated with the FoxP3 staining buffer set from e-biosciences. Dead cells were excluded by using the LIVE/DEAD fixable dead cell stain kit with aqua fluorescent reactive dye (Invitrogen). Cells were analyzed using a FACSCanto II system (BD Biosciences).

### ***Suppression assay***

Myeloid dendritic cells (mDC) were isolated from PBMC by positive selection (BDCA-1 DC isolation kit, Cat. 130-090-506, Miltenyi Biotec). mDC were cultured overnight with 10 µg/ml of Cytomegalovirus antigens (CMV, Cat. EL-01-02, Microbix biosystems, Canada). Autologous CD4 $^{+}$ CD25 $^{-}$  cells were isolated from PBMCs that were kept overnight at 4 °C in medium supplemented with 10% of bovine fetal serum, by magnetic sorting (Cat. 130-091-301, Miltenyi Biotec). CD4 $^{+}$ CD25 $^{-}$  T cells were labeled with 0.1 µM of carboxyfluorescein diacetate succinimidyl ester (CFSE, C34554, Invitrogen) and co-cultured with the autologous mDC activated with CMV, at a ratio of 1 to 10 for 5 days in round-bottom 96-well plates with

at least  $5 \times 10^4$  CD4+CD25<sup>-</sup> T cells. Proliferation was measured by dilution of CFSE. CD4+CD25<sup>+</sup> Treg were isolated by magnetic sorting as previously described.<sup>12</sup> Treg were added at 1:5 ratio versus CD4+CD25<sup>-</sup> T cells, and co-cultured for 5 days. Treg were labeled with CellTrace Violet (Invitrogen) in order to be excluded from proliferating T cells.<sup>12</sup> Furthermore, after co-culture T cells were re-stimulated overnight with autologous monocyte-derived DC activated with CMV, in the presence of brefeldin A and monensin (BD Biosciences). Proliferation and cytokine production were analyzed by flow cytometry and reported as relative proliferation or relative cytokine production where the numbers of proliferating and cytokine-producing cells induced by CMV-DCs were considered 100%. In some experiments azide-free and low endotoxin soluble GITRL (R and D systems, Cat. 6987-GL/CF), anti-CTLA-4 neutralizing (BNI3, Beckman Coulter, Cat. IM2070) or isotype control (IgG2a, Biolegend) antibodies were added to the co-cultures. Monocyte-derived DC were obtained by culturing monocytes with 10 ng/ml IL-4 and 50 ng/ml GM-CSF for 5 days, then immature DC were activated with CMV as described previously for mDC.

#### ***Tumor-specific T cell proliferation and suppression assay***

Tumor lysates (TL) were generated from freshly dissected tumors by five cycles of freezing and thawing in phosphate buffered saline, followed by filtration (0.2  $\mu$ m). mDC isolated from PBMC were cultured overnight with media or 20  $\mu$ g/mL of autologous TL in the presence of 10 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Miltenyi Biotec) and 0.5  $\mu$ g/mL of polyinosinic:polycytidylic acid (InvivoGen, San Diego, CA). CD4+CD25<sup>-</sup> cells were isolated from PBMC that were kept overnight at 4°C in medium supplemented with 10% of bovine fetal serum, by magnetic sorting (Miltenyi Biotec). CD4+CD25<sup>-</sup> T cells were labeled with 0.1  $\mu$ M of CFSE (Invitrogen) and co-cultured with autologous mDC cultured with media or TL, at a ratio of 1:10 for 5 days in round-bottom 96-well plates with at least  $5 \times 10^4$  CD4+CD25<sup>-</sup> T cells. Proliferation was measured by dilution of CFSE. CD4+CD25<sup>+</sup> Treg were isolated by magnetic sorting as described above. Treg were added at 1:5 ratio versus CD4+CD25<sup>-</sup> T cells, and co-cultured for 5 days. Treg were labeled with CellTrace Violet (Invitrogen) in order to be excluded from proliferating T cells. Furthermore, after co-culture T cells were re-stimulated by PMA (40ng/mL) and ionomycin (1 $\mu$ g/mL) for 5 hours in the presence of Brefeldin (Sigma, 5 $\mu$ g/mL) during the last 4 hours. Proliferation and cytokine production were analyzed by flow cytometry and reported as fold increase of specific T cell proliferation or cytokine production, calculated by dividing the percentage of proliferation or cytokine production (TNF $\alpha$  or IFN $\gamma$ ) in the TL condition by that in the control condition (media cultured DC). In all three experiments soluble GITRL (R and D systems, 10  $\mu$ g/mL),

neutralizing anti-CTLA-4 (BNI3, Beckman Coulter, 10 µg/mL) or isotype control (IgG2a, Biologend, 10 µg/mL) antibodies were added to the co-cultures.

### **Statistical analysis**

All data set distributions were analyzed for normality using the Shapiro-Wilk normality test. The differences between paired groups of data were analyzed according to their distribution by either paired t-test or Wilcoxon matched pairs test using GraphPad Prism Software (version 5.0). P-values less than 0.05 were considered statistically significant (\*P<0.05; \*\*P<0.01; \*\*\*P<0.001).

**Table 1: Patient characteristics**

	<b>HCC (n=10)</b>	<b>LM-CRC (n=18)</b>
Sex (male/female)	8 / 2	12 / 6
Age (years)	55.1 ± 6.8	67.4 ± 1.9
Race (Caucasian/Asian/African)	9 / 0 / 1	18 / 0 / 0
ALT (units/L)	48.2 ± 8.6	27.9 ± 3.6
Bilirubin (µmol/L)	14.0 ± 4.5	8.7 ± 3.4
Prothrombin time (INR)	1.2 ± 0.03	1.0 ± 0.01
Liver fibrosis (metavir score) F0-F1 / F2 / F3-F4-cirrhosis	9 / 0 / 1	18 / 0 / 0
Stage of disease (TNM)	St I n=7 St II n=3	St IVa n=18

Etiology of liver disease in HCC patients: 6 no known liver disease, 1 hemochromatosis, 1 porphyria, 1 hepatitis B virus, 1 cirrhosis ECI

INR= international normalized ratio

Where applicable: mean ±SEM

## Figure legends

**Figure 1. Tumor-infiltrating Treg are potent suppressors of T cell responses, and they are characterized by the expression of higher levels of CTLA-4 and GITR.** (A) The proportions of Treg (CD3+CD4+CD25+FoxP3+) among CD4 T cells were analyzed by flow cytometry in tumor, tumor-free liver tissue (TFL) and peripheral blood from patients with HCC or LM-CRC. Differences were analyzed by Wilcoxon matched pairs test. (B) CFSE-labeled CD4+CD25- T cells from PB were stimulated with autologous CMV-activated mDC (CMV-DC) for 5 days. Autologous blood or tumor derived Treg from HCC-patients or LM-CRC patients were added in a ratio 1:5. Inhibition of T cell proliferation by Treg was determined by flow cytometry and reported as percentage of suppression of T cell proliferation. Data analyzed by paired t-test. (C) Differential expression of surface GITR and intracellular CTLA-4 was measured on Treg present in blood, TFL and tumor tissue. Differences were analyzed by Wilcoxon matched pairs test for GITR and by paired t-test for CTLA-4. (D) FACS analysis of the co-expression of CTLA-4 and GITR by Treg from blood, TFL and tumor tissue. One representative patient and the collective data analyzed by Wilcoxon matched pairs test. HCC (closed symbols) or LM-CRC (open symbols). Values are also depicted as means  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**Figure 2. GITR engagement partially abrogates suppression mediated by tumor-infiltrating Treg.** CD4+CD25- effector T cells were isolated from peripheral blood and labeled with CFSE, and co-cultured during 5 days with autologous mDC activated with CMV antigens. Autologous tumor Treg from HCC-patients (closed symbols) or LM-CRC patients (open symbols) were added in the absence or presence of different concentrations of soluble GITRL (sGITRL). T cell proliferation (A) and cytokine (IFN $\gamma$  and TNF $\alpha$ ) production by proliferating cells (B) were measured by flow cytometry after re-stimulation with CMV-activated Mo-DC. Values are also depicted as means  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Differences were analyzed by paired t-test.

**Figure 3. CTLA-4 blockage decreases T cell suppression mediated by tumor-infiltrating Treg in a dose dependent manner.** CFSE-labeled CD4+CD25- T cells isolated from PB were stimulated with autologous CMV-DC and cultured with autologous tumor derived Tregs from HCC-patients (closed symbols) or LM-CRC (open symbols) in the absence or presence of 10 or 20  $\mu$ g/ml of blocking anti-CTLA-4 mAb or an irrelevant isotype control antibody. Cell proliferation (A) and cytokine production by proliferating cells (B) were analyzed by flow cytometry after re-stimulation with CMV-activated Mo-DC. Values are also



depicted as means  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . All data were analyzed by paired t-test.

**Figure 4. High doses of anti-CTLA-4 mAb can completely abrogate T cell suppression mediated by tumor-infiltrating Treg.** The effect of higher doses of anti-CTLA-4 mAb on Treg-mediated suppression was tested using lymphocytes isolated from three different LM-CRC patients. T cell proliferation analysis revealed that higher doses of the neutralizing anti-CTLA-4 antibody are able to completely abolish the suppression mediated by tumor-infiltrating Treg.

**Figure 5. GITRL and CTLA-4 blockage additively abolish T cell suppressive capacity of tumor-infiltrating Treg.** Blood-derived CFSE-labeled CD4<sup>+</sup>CD25<sup>-</sup> T cells from HCC-patients or LM-CRC patients were co-cultured with autologous CMV-DC for 5 days. Autologous tumor derived Treg were added in a ratio 1:5. Cells were treated with 10  $\mu$ g/ml of anti-CTLA-4 mAb or GITRL or a combination of both. Cell proliferation and cytokine production were analyzed by flow cytometry after re-stimulation with CMV-activated Mo-DC. (A) Depicts a representative experiment showing T cell proliferation, IFN $\gamma$  and TNF $\alpha$  production after co-culture and re-stimulation. (B) Collective data of five patients tested (three LM-CRC and two HCC) showing the relative T cell proliferation or cytokine production (IFN $\gamma$  and TNF $\alpha$ ) by proliferating cells. Values are means  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Comparison between groups was made by paired t-test.

**Figure 6. Treatment with GITRL and anti-CTLA-4 antibody can recover *ex vivo* anti-tumor T-cell immunity.** Blood mDC activated with autologous TL were used to stimulate CFSE-labeled autologous peripheral CD4<sup>+</sup>CD25<sup>-</sup> T cells for one week. In some cultures autologous Ti-Treg were added and cells were treated with 10  $\mu$ g/ml of an isotype control antibody or with a mixture of 10  $\mu$ g/ml of GITRL and 10  $\mu$ g/ml of anti-CTLA-4 antibody. T cell proliferation and cytokine production were analyzed by flow cytometry after re-stimulation with PMA and ionomycin. Proliferation and cytokine production are reported as fold increase of specific T cell proliferation or cytokine production, calculated by dividing the percentage of proliferation or cytokine production (TNF $\alpha$  or IFN $\gamma$ ) in the mDC + TL condition by that in the control condition without TL (medium DC). (A) A representative analysis from one patient and (B) Collective data from three different patients tested. HCC (closed symbols) or LM-CRC (open symbols).

Figure 1.

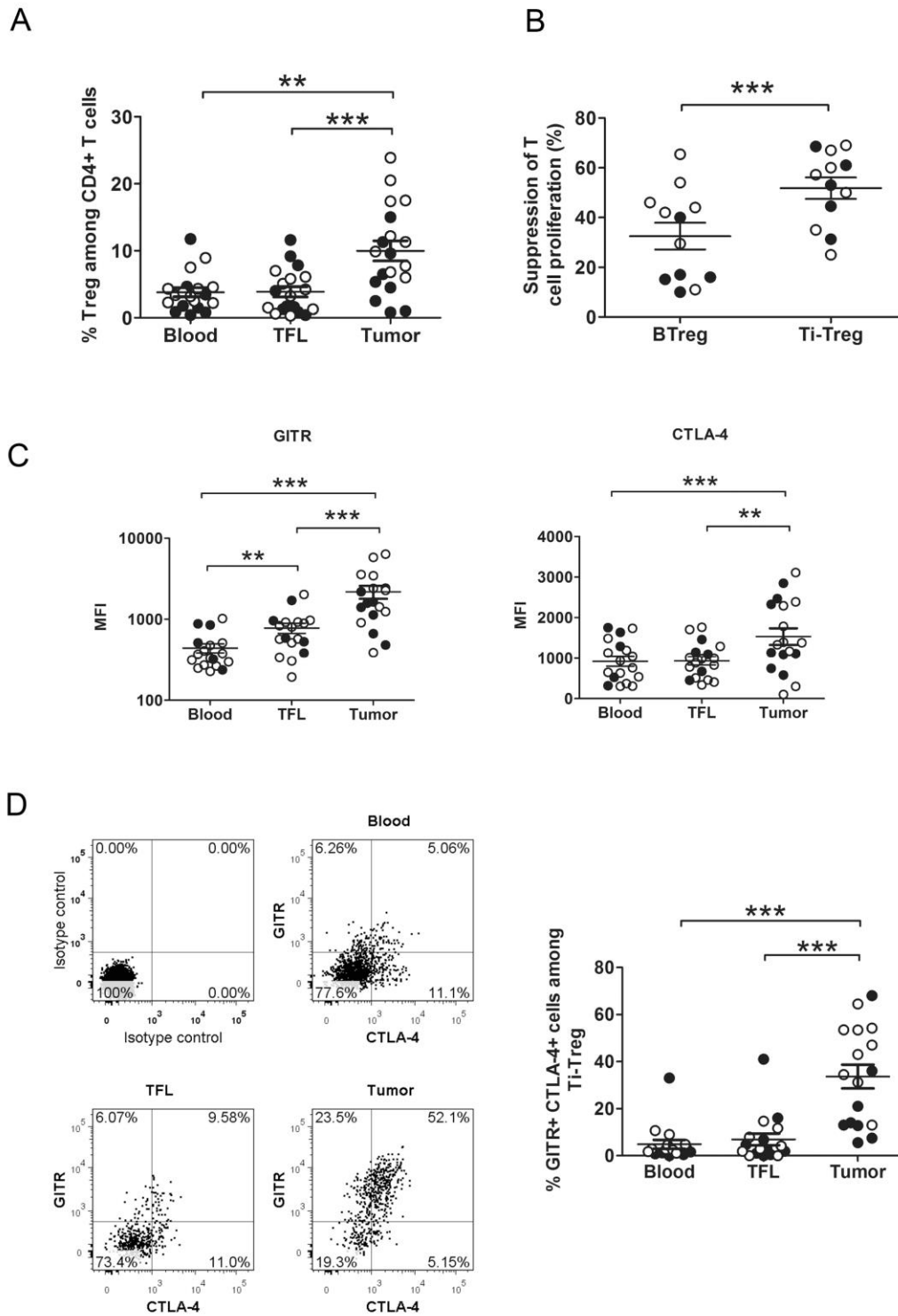


Figure 1

Figure 2.

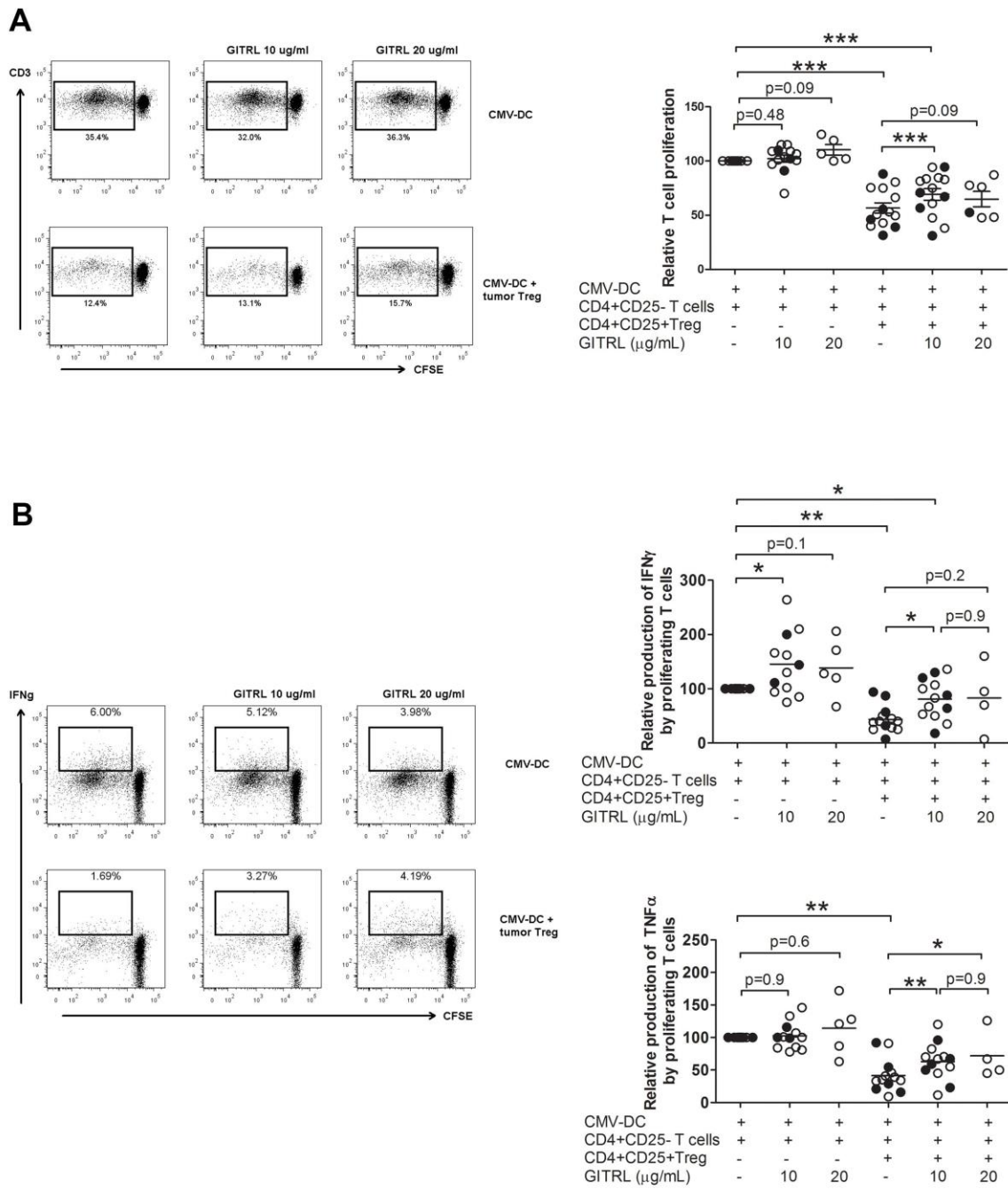


Figure 2

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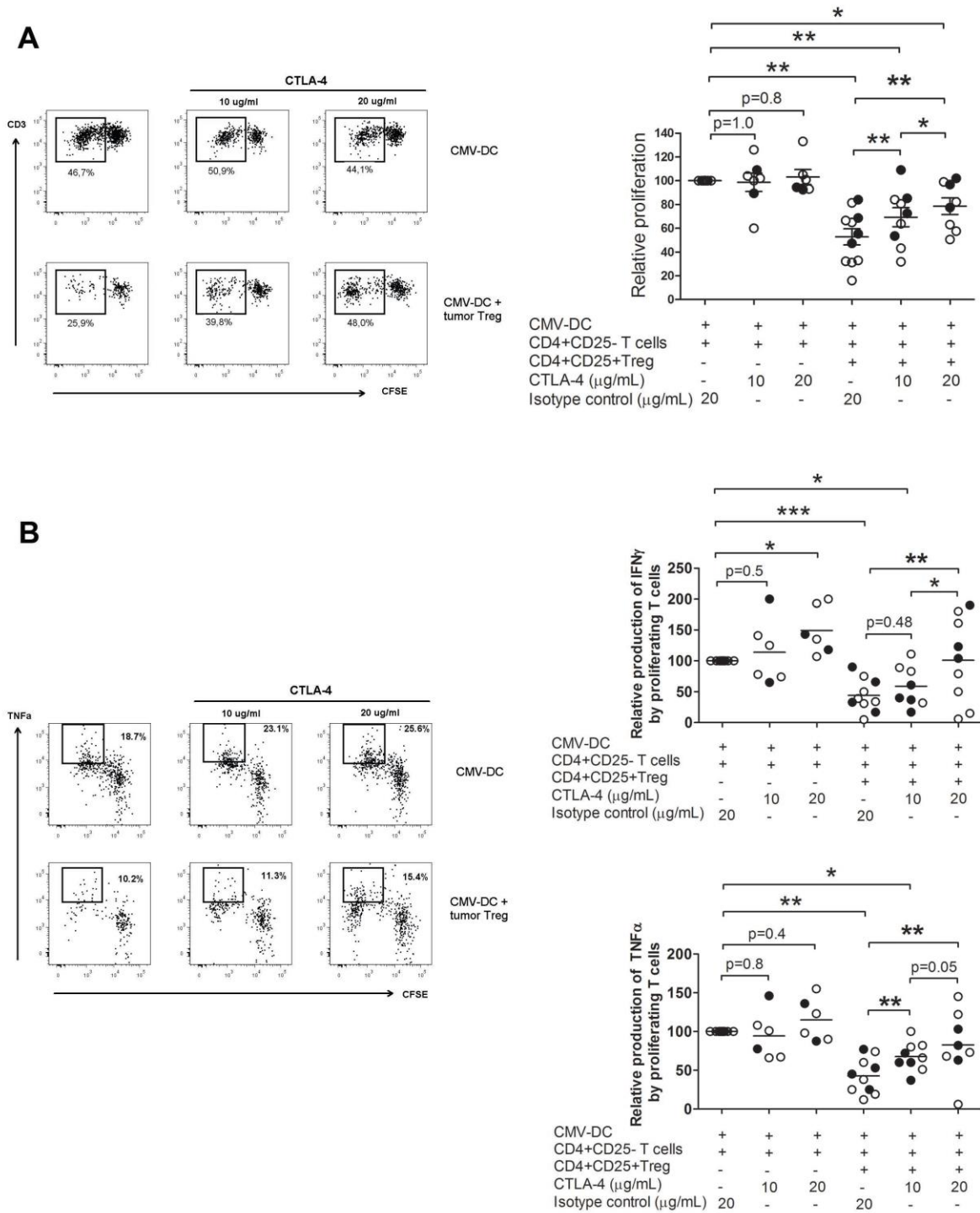


Figure 3

Figure 4.

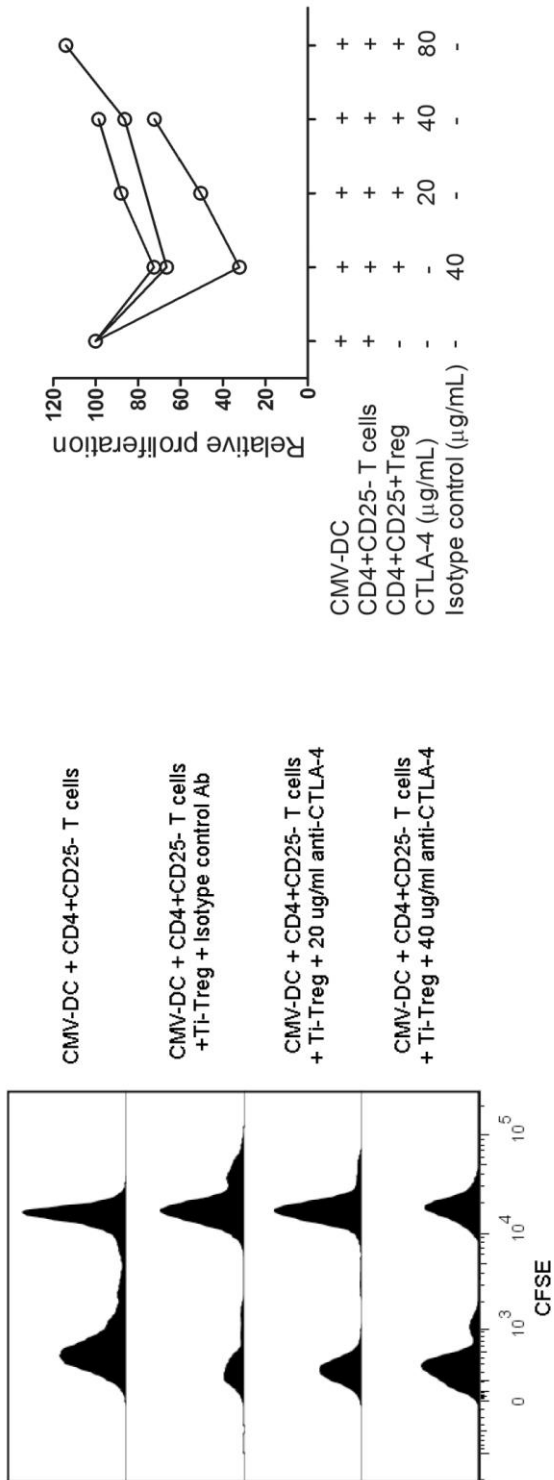


Figure 4

Figure 5.

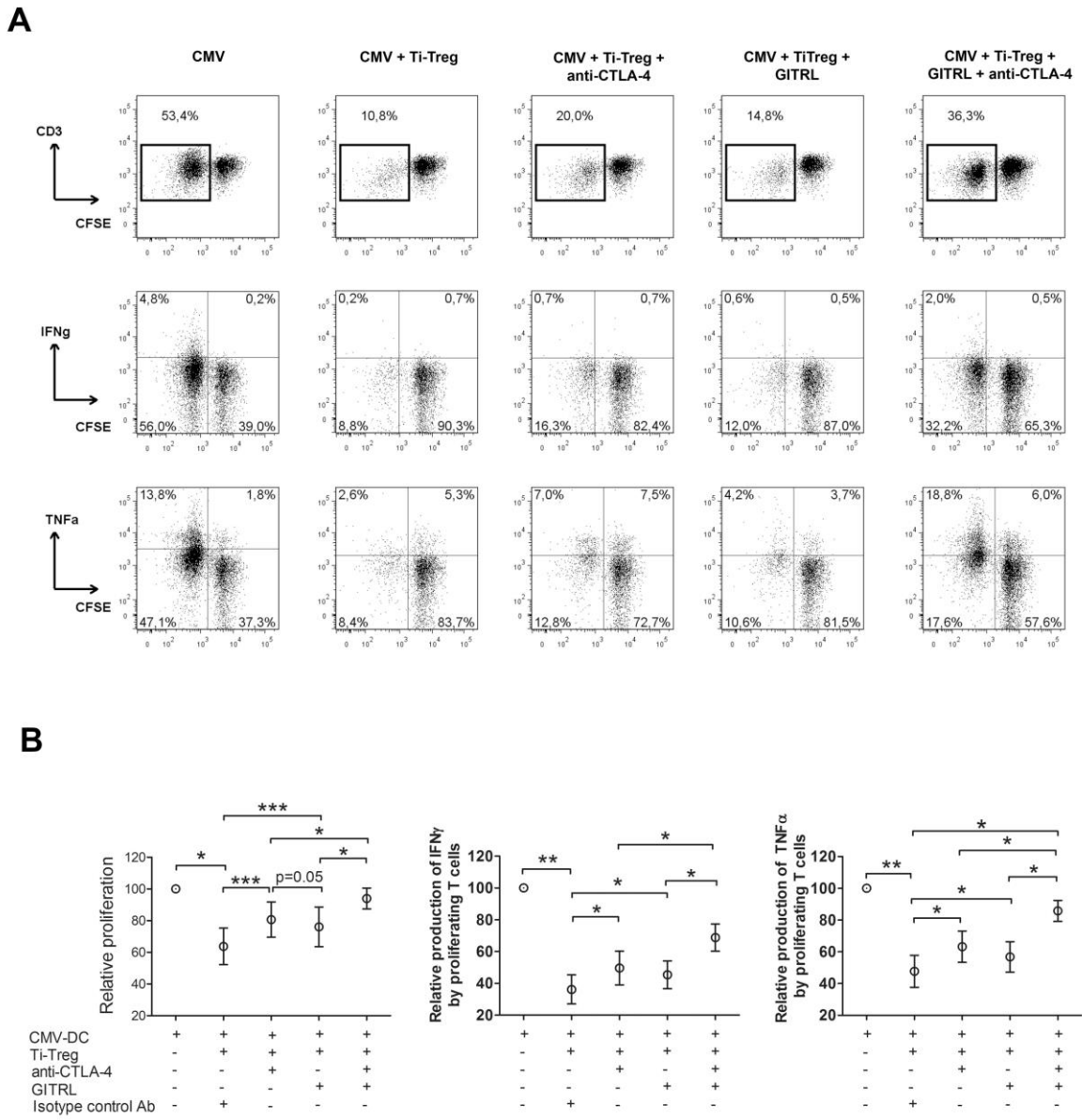


Figure 5

Figure 6.

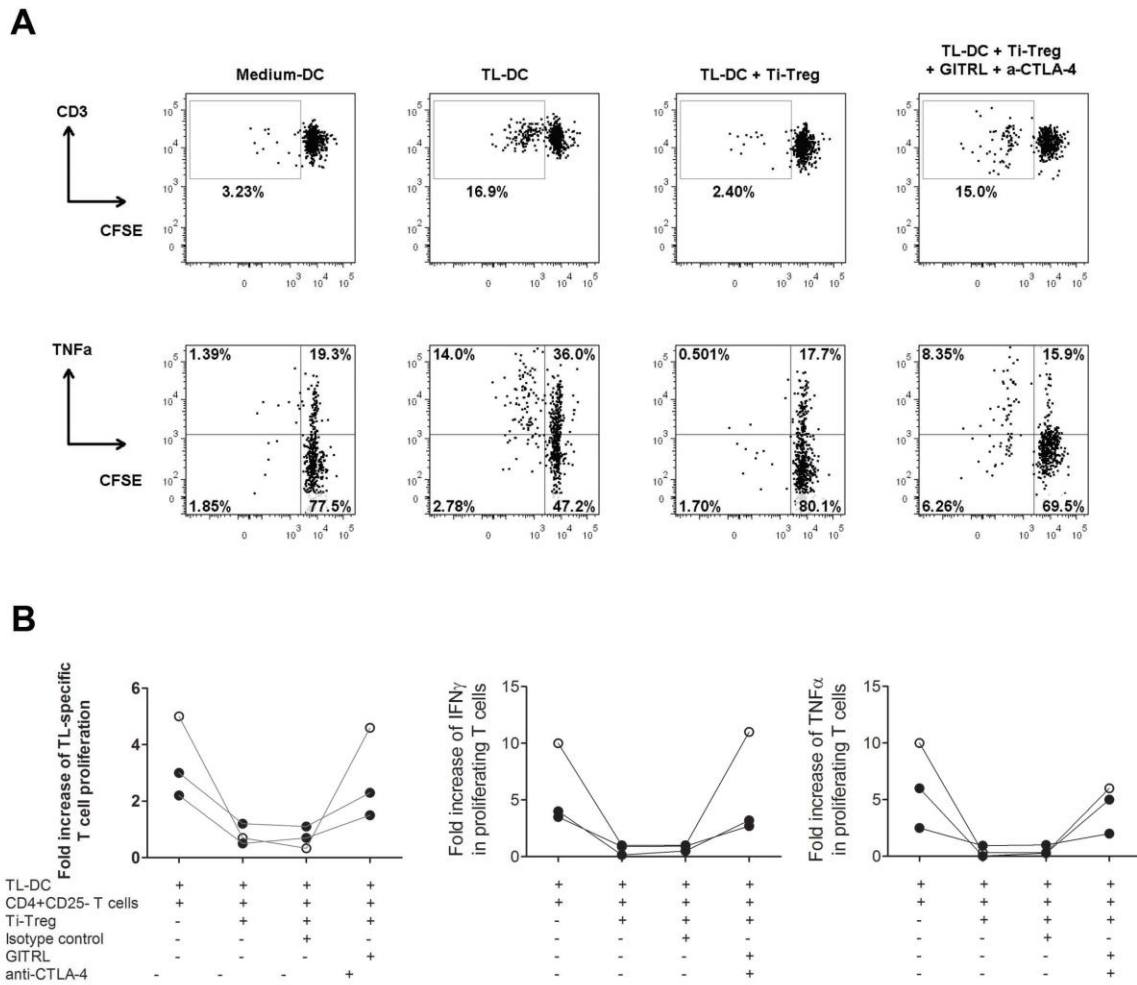


Figure 6

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## Chapter 2

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# CHAPTER 3

## **Tumor-infiltrating plasmacytoid dendritic cells promote immunosuppression by Tr1 cells in human liver tumors**

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## **ABSTRACT**

CD4<sup>+</sup> type 1 T regulatory (Tr1) cells have a crucial role in inducing tolerance. Immune-regulation by these cells is mainly mediated through the secretion of high amounts of IL-10. Several studies have suggested that this regulatory population may be involved in tumor-mediated immune-suppression. However, direct evidence of a role for Tr1 cells in human solid tumors is lacking. Using *ex vivo* isolated cells from individuals with hepatocellular carcinoma (HCC; n=39) or liver metastases from colorectal cancer (LM-CRC; n=60) we identify a CD4<sup>+</sup>FoxP3<sup>+</sup>IL-13<sup>+</sup>IL-10<sup>+</sup> T cell population in tumors of individuals with primary or secondary liver cancer that is characterized as Tr1 cells by the expression of CD49b and the lymphocyte activation gene 3 (LAG-3) and strong suppression activity of T cell responses in an IL-10 dependent manner. Importantly, the presence of tumor-infiltrating Tr1 cells is correlated with tumor infiltration of plasmacytoid dendritic cells (pDCs). pDCs exposed to tumor-derived factors enhance IL-10 production by Tr1 cells through up-regulation of the inducible co-stimulatory ligand (ICOS-L). These findings suggest a role for pDCs and ICOS-L in promoting intra-tumoral immunosuppression by Tr1 cells in human liver cancer, which may foster tumor progression and which might interfere with attempts of immunotherapeutic intervention.

**Keywords:** Hepatocellular carcinoma; colorectal cancer liver metastasis; ICOS-L; IL-10; Immunotherapy, Tr1 cells.

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## Introduction

The two most common types of cancer affecting the liver are HCC and LM-CRC.<sup>1,2</sup> For the majority of patients curative treatments are not available and alternative treatments like immunotherapy have so far shown limited efficacy.<sup>3,4</sup> One of the main obstacles for immunotherapy is the immunosuppressive environment within tumors.<sup>5,6</sup> In support of this, we recently described the accumulation of CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (Tregs) that are potent suppressors of anti-tumor immunity at the tumor site of patients with liver cancer.<sup>7,8</sup> However, other types of inhibitory T cells may also be involved in local immunosuppression inside the tumor.

Tr1 cells were initially described in individuals who developed long-term tolerance after allogeneic transplantation.<sup>9</sup> Since then Tr1 cells have proven to be important in promoting and maintaining tolerance in autoimmunity, allergy and transplantation.<sup>10</sup> There is experimental evidence suggesting a putative role for Tr1 cells in tumor escape from immune surveillance.<sup>11,12</sup> However, the absence of a defined cell surface signature and the reliance on a cytokine profile to distinguish Tr1 cells from other T cell subsets complicated their identification and study.<sup>9</sup> It was shown that exposure of dendritic cells to tumor-derived factors favors the induction of Tr1-like cells from naïve CD4<sup>+</sup> T cells.<sup>13</sup> Additionally, tumor-infiltrating lymphocytes (TILs) of patients with head and neck squamous cell carcinoma were shown to contain more Tr1 precursors than peripheral blood.<sup>14</sup> These findings suggest that this regulatory T cell population could be involved in tumor-mediated immune-suppression. However, direct evidence for the presence of Tr1 cells and their role in solid tumor development remained elusive. Now, using recently described surface markers for the identification of Tr1 cells,<sup>15</sup> we report that TILs in patients with liver cancer contain a subset of suppressive CD4<sup>+</sup>Foxp3<sup>-</sup> T cells with the phenotypic and functional characteristics of Tr1 cells. In addition, we provide evidence suggesting that intra-tumoral immunosuppression by Tr1 cells is promoted by tumor-infiltrating pDCs.

## Results

### ***IL-10 producing CD4<sup>+</sup>Foxp3<sup>-</sup> T cells accumulate in human liver tumors***

In order to characterize the CD4<sup>+</sup> T cell subsets present in liver tumors we compared pair wise the cytokine profile of CD4<sup>+</sup> TILs isolated from individuals with HCC or liver metastasis from CRC (LM-CRC) to the cytokines produced by CD4<sup>+</sup> T cells from the tumor-free area of the liver (TFL) and peripheral blood (Figure 1 and Supplementary Figure 1). Freshly isolated single cell suspensions were activated with PMA and Ionomycin in the presence of protein

transporter inhibitors and analyzed by flow cytometry. Compared to TFL and blood, CD4+ TILs contained significantly higher frequencies of IL-10-producing cells in both groups of patients (Figure 1A). In contrast, the frequencies of IFN $\gamma$ , TNF $\alpha$  and IL-13 producing CD4+ T cells were similar in TFL and tumors.

To further characterize these IL-10-producing CD4+ T cells, we analyzed their expression of IL-13 and FoxP3, and we observed that the majority of IL-10+ cells did not produce IL-13 nor expressed FoxP3 (Figure 1B, C and D). These cells were also negative for IL-4 (Supplementary Figure 1B). Therefore, only a minor proportion of tumor-infiltrating IL-10-producing CD4+ T cells correspond to FoxP3+ Tregs or Th2 cells. Instead, the large majority of IL10-producing CD4+ T cells are FoxP3-negative and IL-13-negative, and these cells are enriched at the tumor site (Figure 1E). Moreover, an important proportion of these cells produced IFN $\gamma$  without significant differences between blood, TFL and tumor (Supplementary Figure 1C).

***Tumor-infiltrating CD4<sup>+</sup>FoxP3<sup>-</sup>IL-10<sup>+</sup> T cells are potent suppressors of T cell function in an IL-10 dependent mechanism***

To investigate the functional properties of tumor-infiltrating CD4<sup>+</sup>FoxP3<sup>-</sup>IL-13<sup>-</sup>IL-4<sup>-</sup>IL-10<sup>+</sup> cells, we isolated CD4<sup>+</sup>CD25<sup>-</sup> T cells, which are FoxP3<sup>-</sup> (Supplementary Figure 2A), from TILs and activated them with antibodies to CD3 and CD46<sup>16</sup> or ICOS<sup>17</sup>, two co-stimulatory molecules that have been described to stimulate IL-10 production (Supplementary Figure 2B). Importantly, we activated these cells for only 24-48 hours to prevent de novo generation of IL10-producing cells from naïve T cells. Similar to what we had observed upon short-term stimulation with PMA and ionomycin, this stimulation also revealed higher proportions of IL10-producing CD4<sup>+</sup> cells in tumor tissue than in TFL or blood, (Supplementary Figures 2C-D). After activation IL-10<sup>+</sup> cells were enriched by magnetic sorting (Supplementary figure 3). We investigated the immunosuppressive potential of tumor-infiltrating IL-10 producing CD4<sup>+</sup>CD25<sup>-</sup> T cells by assessing their capacity to suppress T-cell proliferation and cytokine production in vitro (Figure 2A, B). CFSE-labeled PBMCs from healthy donors were stimulated with PHA and co-cultured in the presence of the IL-10<sup>+</sup> enriched fraction (IL-10<sup>high</sup>) or the remaining fraction (IL-10<sup>low</sup>). Notably, both cell fractions suppressed proliferation and cytokine production of responder CD3<sup>+</sup> T cells (Figure 2A, B), but the degree of suppression differed considerably. Whereas the IL-10<sup>low</sup> fraction suppressed moderately (20.4  $\pm$  5.5 %; mean  $\pm$  SE), the IL-10<sup>high</sup> fraction strongly suppressed T cell proliferation (60.2  $\pm$  9.2 %, p=0.005). No difference in suppression between cells stimulated with CD46 or anti-ICOS antibodies was observed (Supplementary Figure 3B). Similar findings were observed

in the setting of CMV-specific CD4<sup>+</sup> T cell responses (Supplementary Figure 4). To investigate whether the suppression was mediated by IL-10, we administered a neutralizing anti-IL-10R antibody to the co-cultures. As expected, suppression by the high IL-10 producing CD4<sup>+</sup> T cells was prevented when IL-10R was blocked, in all patients tested (Figure 2C). Thus, these data show that liver tumors are infiltrated by IL-10 producing CD4<sup>+</sup>FoxP3<sup>-</sup> T cells which are potent suppressors of T cell responses in an IL-10 dependent manner. The limited suppression observed when the IL-10<sup>low</sup> fraction was added to the T-cell culture is likely a consequence of IL-10 producing cells remaining in this fraction after enrichment of the IL-10<sup>high</sup> fraction by magnetic sorting (Supplementary Figure 5A), and could also be blocked by neutralizing anti-IL-10R antibodies (data not shown). In support of this explanation, there is a positive correlation ( $p = 0.023$ ) between the frequencies of IL-10<sup>+</sup> cells present in the IL-10 high or low fractions obtained after magnetic sorting and their degree of T cell suppression observed in the co-cultures (Supplementary Figure 5B).

***Tumor-infiltrating CD4<sup>+</sup>FoxP3<sup>-</sup>IL-10<sup>+</sup> T cells display phenotypic characteristics corresponding to Tr1 cells***

A recent study has identified that CD49b and LAG-3 are stably and selectively co-expressed on Tr1 cells.<sup>15</sup> Because Tr1 cells are functionally characterized by the production of high levels of IL-10 and T-cell suppressive capacity, we examined the expression of these markers on the tumor-infiltrating IL-10<sup>+</sup>CD4<sup>+</sup> T cells that we described above. Notably, the liver tumor-infiltrating CD4<sup>+</sup>FoxP3<sup>-</sup> T cells that produced the highest amounts of IL-10 co-expressed CD49b and Lag-3 (Figure 2D) and we observed that CD4<sup>+</sup>FoxP3<sup>-</sup>CD49b<sup>+</sup>LAG-3<sup>+</sup> T cells were selectively enriched in the tumor bed in both types of liver tumors (Figure 2 E and F). Therefore, taken together we conclude that the majority of the CD4<sup>+</sup>FoxP3<sup>-</sup>IL-10 producing cells infiltrating liver tumors correspond to Tr1 cells and that they are strongly immunosuppressive in an IL-10-dependent manner.

***Tumor-exposed pDCs promote immune suppression through activation of Tr1 cells***

Given that pDCs have been reported to prime CD4<sup>+</sup>IL-10-producing T regulatory cells through ICOSL,<sup>18</sup> and in ovarian<sup>19,20</sup> and breast cancer<sup>21</sup> expand CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs, as well as stimulate production of IL-10 in these cells, we explored tumor infiltration of pDCs in our patients. We observed that pDCs infiltrate liver tumors (Figure 3A and supplementary figure 6A), and in both groups of patients liver tumors contained higher frequencies compared to TFL. We hypothesized that in liver tumors infiltrating pDCs may promote the differentiation and/or activation of tumor-infiltrating Tr1 cells, as has been observed for CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs.<sup>22</sup> Supporting this hypothesis, the frequencies of tumor-infiltrating pDCs

and those of Tr1 cells as determined by flow cytometry, showed a highly significant positive correlation (Figure 3B). Also, by immunohistochemistry analysis we observed co-localization of CD303+ pDCs and LAG-3+ cells in the tumor milieu of both types of liver tumors (Figure 3C, D) which was substantiated by a significant positive correlation in co-localization (Pearson  $r = 0.78$ ;  $p = 0.03$ ) after counting multiple microscopic fields (200x magnifications, 2 independent observers). Such correlation was absent in TFL ( $r = -0.2$ ;  $p = 0.49$ ), and moreover less LAG-3+ and CD303+ cells were detected by immunohistochemistry in TFL than in tumors (per microscopic field of 200x magnification: LAG-3+ cells  $3.4 \pm 1.0$  versus  $6.6 \pm 1.9$  (mean  $\pm$  SEM); and CD303+ cells  $1.2 \pm 0.4$  versus  $5.3 \pm 1.6$ ). Furthermore, analysis of the expression of ICOS-L in total cell suspensions obtained from tumors of HCC and LM-CRC patients demonstrated that the main cell population expressing this molecule corresponded to CD123+ pDCs (Figure 3E), while Tr1 in tumors express high levels of ICOS (Figure 2D). We therefore hypothesized that pDCs in liver tumors may induce or activate Tr1 cells through ICOSL in liver tumors.

To test this hypothesis, pDCs isolated from blood of healthy donors were exposed to lysates of tumor or TFL and then used to stimulate autologous naïve CD4+ T cells. pDCs exposed to tumor lysates (TL-pDCs) up-regulated ICOS-L expression (Figure 4A), whereas TFL lysates (TFLL) induced a significant down-regulation of ICOS-L. In contrast, exposure of pDCs to tumor lysate or TFL lysate did not affect expression of the co-stimulatory molecule CD83 and CD86 was up-regulated only in TL-pDCs (supplementary figure 6B). pDCs induced Tr1 from naïve CD4+ T cells and stimulated IL-10 production in these cells (Figures 4B, C). Interestingly, in parallel with their increased expression of ICOS-L, TL-pDCs induced higher numbers of IL-10 producing CD4+FoxP3-CD49+LAG-3+ Tr1 cells from naïve CD4+ T cells compared to pDCs treated with TFL lysate (TFLL-pDCs)(Figure 4B), but their capacity to induce CD49b and LAG-3 expression did not differ significantly (Figure 4C). The ability of TL-pDCs to promote IL-10 production by Tr1 cells was dependent on ICOS-ICOS-L co-stimulation, as it was blocked by addition of a neutralizing antibody against ICOS-L (Figure 4B). Furthermore, there is a positive correlation ( $r = 0.75$ ,  $p = 0.006$ ) between the level of expression of ICOS-L on pDCs after exposure to medium, TFLL or tumor lysate, and the frequency of IL-10-producing Tr1 cells induced in the co-culture (Figure 4D). In contrast, the expression of CD49b and LAG-3 was not affected by ICOS-L blocking (Figure 4C). Thus, ICOS-ICOSL signaling seems to be unnecessary for the induction of the Tr1 phenotype by pDCs, but it is critical for the production of IL-10.

## Discussion



At the time of diagnosis the majority of HCC patients are not candidates for curative therapy, and in CRC patients with liver metastasis there is a high rate of recurrence after treatment.<sup>1, 2</sup> For these groups of patients immunotherapy aimed at stimulating the local anti-tumor immune response might present an attractive alternative. However, immunotherapeutic attempts have so far shown poor clinical responses and this might be related to the inhibition of tumor-specific immunity by immune regulatory mechanism present in the tumor microenvironment.<sup>3, 8</sup> Detailed insight into the complex nature of intra-tumoral immune regulation is essential for the design of immunotherapeutic strategies, and the findings of this study may contribute to development of such strategies.

TILs in liver tumors are composed mainly of CD4<sup>+</sup> T cells that are hypo-responsive to tumor antigens.<sup>7</sup> We previously showed that an important fraction of the CD4<sup>+</sup> T cells in these tumors correspond to CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs that are potent suppressors of anti-tumor immunity.<sup>7, 8</sup> There is accumulating evidence for the role of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in the development and progression of cancer.<sup>23, 24</sup> Several studies have shown that the presence of increased numbers of these cells in different tumors contributes to the suppression of antitumor immunity and correlates to decreased survival.<sup>23-26</sup> Over the years, several types of Tregs have been identified (reviewed in 9). Whereas CD4<sup>+</sup>FoxP3<sup>+</sup> Tregs are probably the best characterized Treg type, Tr1 cells were until recently a subset of T cells that lacked a defined cell surface signature, and could therefore only be characterized by the production of high levels of IL-10, low levels of IL-2, variable levels of IFN $\gamma$  and the absence of IL-4 and absence of high and constitutive expression of FoxP3.<sup>9</sup> The reliance on a cytokine profile to distinguish Tr1 cells complicated their identification and study, since these cells are not the only T cell subset that secretes IL-10. Still, experimental evidence suggested that exposure of dendritic cells to tumor derived factors favors the induction of Tr1-like cells from naïve T cells,<sup>13</sup> arguing for a role of Tr1 cells in cancer immunology. Later on, in head and neck carcinoma a suppressive role for in vitro generated CD4<sup>+</sup>CD25<sup>-</sup>FoxP3<sup>low</sup>IL-10<sup>+</sup>TGFb<sup>+</sup> cells was described.<sup>27</sup> Moreover, in Hodgkin lymphoma the presence of CD4<sup>+</sup>CD25<sup>-</sup>IL-10<sup>+</sup> regulatory T cells has been described<sup>12</sup> and more recently a study described the presence of CD4<sup>+</sup>FoxP3<sup>-</sup>CD127<sup>-</sup> regulatory T cells in blood and tumors from HCC patients. Although they did not show regulatory functionality of the tumor-infiltrating CD4<sup>+</sup>FoxP3<sup>-</sup>CD127<sup>-</sup> cells, the circulating phenotypically counterpart suppressed T cell responses through an IL-10-dependent mechanism,<sup>11</sup> suggesting a Tr1-like cell type.

However, until now direct evidence for a role of Tr1 cells in human solid tumors is lacking. The recent description of co-expression of CD49b and LAG-3 as markers that can specifically identify this population of cells,<sup>15</sup> enabled us to show that the majority of liver

tumor-infiltrating IL-10 producing CD4+ T cells consists of Tr1 cells and only a minor proportion corresponded to Th2 cells or FoxP3+ Tregs. Compared to TFL, higher numbers of these Tr1 cells are present in the liver tumors of both HCC-patients and metastatic CRC-patients, indicating selective accumulation in the tumor tissues. In functional assays the tumor-infiltrating Tr1 cells demonstrated a potent suppressive activity mediated by IL-10. Therefore, these cells represent a suppressive population in the tumor environment potentially contributing to the impaired anti-tumor immunity observed in patients with liver cancer.

Our data suggest that tumor-infiltrating Tr1 cells may be activated by pDCs present in the tumor microenvironment. Using immunohistochemistry analysis pDCs could be identified in the vicinity of Lag3+ cells. Lag3 may also be expressed on CD4+Foxp3+ Tregs<sup>9</sup>, but by flow-cytometry we observed that on the average only 6% of HCC or LM-CRC-derived CD4+Foxp3+ Tregs express Lag3 (data not shown). Therefore, this co-localization, together with the correlation between the frequencies of tumor-infiltrating pDCs and Tr1 cells observed in flow cytometric analysis (Figure 3B), suggests a possible interaction between both cell types, but further experiments are needed to confirm this *in situ* interaction. Furthermore, the main population expressing ICOSL in tumor tissues are CD123+ pDCs. In vitro exposure of immature pDCs to tumor lysates, but not lysates of normal liver tissue, conditioned pDCs to up-regulate the expression of ICOS-L and induce the production of IL-10 by Tr1 cells in a mechanism mediated by ICOS-ICOS-L signaling. There is substantial evidence suggesting that pDCs have a specialized role in the induction of peripheral tolerance by inducing IL-10-production by Tregs through ICOS-L-ICOS signaling.<sup>18, 28</sup> Both in breast and in ovarian cancer stimulation of CD4+FoxP3+ Tregs to produce IL-10 by pDCs has been described.<sup>19, 22</sup> Our results are consistent with a role for pDCs to stimulate IL10 production by Tr1 cells in the tumor microenvironment of patients with liver cancer. Altogether these data suggest that ICOS co-stimulation represents a potential target for immunotherapeutic intervention, affecting tumor-specific immunosuppression mediated by both CD4+Foxp3+ Tregs and Tr1 cells.

In summary, in patients with primary and secondary liver cancer we identified a population of tumor-infiltrating Tr1 cells that contributes to local immune suppression in an IL-10 dependent manner. pDCs may drive intra-tumoral immunosuppression by these Tr1 cells by stimulating IL-10 production via ICOS-ICOS-L signaling. Consequently, as has been shown for CD4+FoxP3+ Tregs,<sup>7, 29</sup> Tr1 cells may inhibit anti-tumor immunity at the tumor site in liver cancer and thereby promote tumor development. This knowledge is critical for the design of new immunotherapeutic interventions for patients with liver cancer and other solid cancers in

which IL-10 producing CD4+ T cells are present, and blockade of the engagement of ICOS-ICOS-L may provide alleviation of this intra-tumoral immunosuppressive mechanism.

## **Patients and Methods**

### ***Patients***

Between September 2009 and July 2013 a total of 99 individuals who were eligible for surgical resection of HCC (n=39) or LM-CRC (n=60) were enrolled. Paired samples of fresh liver tumor tissue and tumor-free liver tissue (TFL) obtained at the maximum distance (at least 1 cm) from the tumor, were used for isolating TILs and intra-hepatic lymphocytes. In addition, peripheral blood was collected. None of the patients was treated with chemotherapy or radiation prior to resection. The clinical characteristics of the patients are summarized in Table 1. The study was approved by the local ethics committee and all patients in the study gave informed consent before tissue donation.

### ***Cell preparation, flow cytometric analysis, antigen-specific T-cell activation, immunohistochemistry***

Detailed descriptions of these methods are provided in the Supplementary Information.

### ***Activation and isolation of IL-10-producing cells***

CD4+CD25- T cells were isolated from TILs of patients with liver cancer by magnetic sorting as previously reported.<sup>7</sup> Briefly, the non-CD4+ cells were removed by a magnetically labeled cocktail of antibodies followed by depletion of CD25+ cells (Cat. 130-091-301, Miltenyi Biotec). CD4+CD25- T cells were stimulated with Dynabeads that were coupled to anti-CD3 (OKT-3, Cat. 314304, Biolegend) alone or in combination with anti-CD46 (TRA-2-10, Cat.352404, Biolegend) or anti-ICOS (ISA-3; Cat. 16-9948-82, ebiosciences) antibodies using the Dynabeads antibody coupling kit (Cat. 143.11D, Invitrogen). The bead to cell ratio was 0.5:1. Cells were cultured in the presence of 250 U/mL IL-2 (Cat. 130-097, Miltenyi Biotec) for 24-48 hrs. Then, cells were magnetically sorted into IL-10<sup>low</sup> and IL-10<sup>high</sup> fractions using the secretion assay-cell enrichment kit from Miltenyi (Supplementary Figure 2).

### ***Suppression assays***

The suppressive effect of IL-10 producing CD4+ T cells was assessed by co-culture with PBMCs from healthy donors that were labeled with 0.1  $\mu$ M of carboxyfluorescein diacetate succinimidyl ester (CFSE, C34554, Invitrogen), and activated with 6  $\mu$ g/mL of phytohemagglutinin (PHA, Remel-Thermo Fisher Scientific) for 5 days. The ratio of cells was

1:10 (Tr1:PBMCs) with at least  $5 \times 10^4$  responder cells. Proliferation and cytokine production were measured by flow cytometry after re-stimulation with PMA/Ionomycin. Inhibition of T cell proliferation or cytokine production was determined by comparison with culture conditions without IL-10 producing CD4<sup>+</sup> T cells, and reported as percentage of suppression of T cell proliferation or cytokine production. To analyze the role of IL-10 in the suppressive capacity of Tr1 cells, 30 µg/ml of neutralizing anti-IL-10R antibody (3F9 Cat. 308806) or isotype-matched control antibody (both from Biolegend) were added to some co-cultures.

### ***In vitro activation and co-culture of pDCs and naïve T cells***

pDCs were enriched from PBMCs of healthy donors by positive immunomagnetic selection using anti-BDCA-4 antibodies (Cat. 130-090-532, Miltenyi Biotec). Purity of pDCs as determined by CD123 labeling was  $92 \pm 2.5$  %. pDCs were seeded at  $10^5$  cells/well in 200 µl of sRPMI medium (details can be found in Supplementary Methods) with 10% human AB serum in the presence of 10 ng/ml of IL-3 (Cat. 130-093, Miltenyi Biotec). pDCs were cultured with medium alone or in the presence of liver tissue lysates (200 µg/ml of total protein). After 18 hrs pDCs were harvested and washed. Then pDCs were stained for phenotypic analysis or co-cultured in a ratio 1:5 with autologous naïve CD4<sup>+</sup> T cells, which were purified with the naïve CD4 T cell enrichment kit from Stemcell technologies (Cat. 19155). Cells were co-cultured for 7 days. Thereafter the cells were washed and re-stimulated 5 hrs with PMA and Ionomycin. The immunophenotype of CD4<sup>+</sup> T cells and their cytokine profile were measured by flow cytometry. Tissue lysates were prepared from freshly dissected paired TFL and tumor tissues by 5 cycles of freezing and thawing in PBS, followed by filtration (0.2 µm). The total protein level was determined by BCA assay (Thermo Scientific), and the lysates were stored at -80° C before use. To determine involvement of ICOS-L in activation of Tr1, 50 µg/ml neutralizing anti-ICOS-L antibody (MIH12, Cat. 16-5889) or isotype-matched control antibody (both from e-Biosciences) were added to the co-cultures.

### ***Statistical analysis***

The differences between paired groups of data were analyzed according to their distribution by either t-test or Wilcoxon matched pairs test. Differences between different groups of patients were analyzed by either t-test or Mann Whitney test, using GraphPad Prism Software (version 5.0). P-values less than 0.05 were considered statistically significant (\*P<0.05; \*\*P<0.01; \*\*\*P<0.001).

**Table 1.** Patient characteristics

	<b>HCC (n = 39)</b>	<b>LM-CRC (n = 60)</b>
Sex (male/female)	25/14	37/23
Age (years)	62 ± 2	65 ± 1
Race (Caucasian/Asian/African)	35/3/1	59/0/1
ALT (units/L)	66 ± 16	33 ± 5
Bilirubin (µmol/L)	16 ± 4	8 ± 1.0
Prothrombin time (INR)	1.1 0.01	1.0 ± 0.01
Liver fibrosis (metavir score) F0-F1 / F2 / F3-F4-cirrhosis	19/10/10	60/0/0
Stage of disease (TNM)	St I n = 16 St II n = 23	St IVa n = 55 St IVb n = 5

Etiology of liver disease in HCC patients: 17 no known liver disease, 5 hemochromatosis, 4 NASH, 4 alcohol related liver disease, 1 porphyria, 6 hepatitis B virus, 2 hepatitis C virus.

INR = international normalized ratio.

Where applicable: mean ± SEM.

## Figure legends

**Figure 1. Accumulation of IL-10-producing CD4<sup>+</sup>FoxP3<sup>-</sup> T cells in liver tumors.** PBMCs or MNCs isolated from tissues of HCC (n= 8-9) and LM-CRC (n= 5-14) patients were stimulated in vitro for 5 hours with PMA/Ionomycin in the presence of protein transport inhibitors. IFN $\gamma$ , TNF $\alpha$ , IL-13 and IL-10 were measured by intracellular staining by flow cytometry. (A) The percentages of cytokine-producing cells among total CD3<sup>+</sup>CD4<sup>+</sup> T cells in blood, tumor-free area of the liver (TFL) and tumor. (B) IL-13 expression in viable tumor-derived CD3<sup>+</sup>CD4<sup>+</sup>IL-10<sup>+</sup> T cells of HCC and LM-CRC patients stimulated with PMA/Ionomycin. FoxP3 and IL-10 expression in CD3<sup>+</sup>CD4<sup>+</sup> T cells isolated from HCC (C) or LM-CRC tumors (D). (E) Frequencies of CD4<sup>+</sup>CD3<sup>+</sup>IL-13<sup>-</sup>FoxP3<sup>-</sup>IL-10<sup>+</sup> T cells among CD4<sup>+</sup> T cells. Red dots correspond to HCC and blue open dots are for LM-CRC (displayed as LMC in graphs). Values are means  $\pm$  SEM, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

**Figure 2. Tumor-infiltrating CD4<sup>+</sup>FoxP3<sup>-</sup>IL-10<sup>+</sup> T cells are potent suppressors of T cell function and their phenotype corresponds to Tr1 cells.** Tumor-infiltrating CD4<sup>+</sup>CD25<sup>-</sup> T cells were activated with anti-CD3/CD46 or anti-CD3/ICOS antibodies for 24-48 hrs, then stained for IL-10 and magnetically sorted into IL-10<sup>low</sup> and IL-10<sup>high</sup> fractions, which were both co-cultured at a 1:10 ratio with CFSE-labeled PBMCs from healthy donors stimulated with phytohemagglutinin (PHA) for 5 days. (A) T cell proliferation and TNF $\alpha$  production measured by flow cytometry in PHA-stimulated PBMCs cultured alone or in the presence of IL-10<sup>low</sup> or IL-10<sup>high</sup> fractions of tumor infiltrating CD4<sup>+</sup>CD25<sup>-</sup> T cells. (B) Collective analysis of the percentages of suppression of T cell proliferation and TNF $\alpha$  production from 8 patients. (C) Effect of blocking IL-10R on the suppressive capacity of CD4<sup>+</sup>IL-10<sup>high</sup> cells. Cells were cultured as described above in the presence of 30  $\mu$ g/ml of neutralizing anti-IL-10R antibody or an irrelevant isotype control antibody. (D) Expression of CD49b and LAG-3 on tumor-infiltrating CD4<sup>+</sup> T cells activated with antibodies to CD3 and ICOS for 24 hrs. Cells were gated on viable CD3<sup>+</sup>CD4<sup>+</sup> T cells and FoxP3<sup>+</sup>CD127<sup>-</sup> Tregs were excluded from the analysis. Histograms show the expression of IL-10 and ICOS in different populations based on the expression of CD49b and LAG-3. (E) CD49b and LAG-3 expression in blood, TFL and TILs isolated from a representative patient with HCC. Cells were gated on viable CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>-</sup> T cells. (F) Collective percentages of CD49b<sup>+</sup>LAG-3<sup>+</sup> cells within CD4<sup>+</sup>FoxP3<sup>-</sup> T cells in 21 patients analyzed (HCC n= 8 and LM-CRC n= 13). HCC (red dots) and LM-CRC (blue open dots).

**Figure 3. Plasmacytoid DCs are enriched at the tumor site and correlate with the frequencies of Tr1 cells.** (A) Percentages of pDCs (CD123+HLA-DR+LIN-) among CD45+ leukocytes from paired samples of TFL and tumor tissue from 78 patients tested (HCC = 27 and LM-CRC = 51). Tumor leukocytes contain significantly higher numbers of pDCs than TFL (on the average  $0.74 \pm 0.6$  % pDCs in HCC tumors and  $1.01 \pm 0.9$  % in LM-CRC tumors, compared to  $0.45$  % and  $0.75 \pm 0.6$  % in TFL, respectively). (B) Pearson correlation analysis between the frequencies of tumor-infiltrating pDCs and CD4+FoxP3-CD49b+LAG-3+ Tr1 cells in liver tumors (n = 15). Red dots are HCC and blue open dots represent LM-CRC. (C, D) Immunohistochemistry analysis shows co-localization of CD303+ pDCs (red brown) and LAG-3+ cells (blue) in LM-CRC (C) and HCC (D) tumors. Magnification 200x. An insert at higher magnification showing the close localization of CD303+ and Lag-3+ cells in HCC is displayed in D. (E) Expression of ICOSL and CD123 analyzed by flow cytometry in total cell suspensions from liver tumors.

**Figure 4. Tumor-derived pDCs induce the production of IL-10 by Tr1 cells through ICOS-ligand-ICOS signaling.** (A) Blood pDCs isolated from healthy donors after overnight culture in the presence of lysates from TFL (TFLL-pDCs) or tumor tissue (TL-pDCs) were analyzed for the expression of ICOS-L. pDCs exposed to tissue lysates were used to stimulate autologous naïve CD4+ T cells. The expression of IL-10 (B) and CD49b and Lag-3 (C) were analyzed on CD4+ T cells after co-culture with pDCs and re-stimulation with PMA/Ionomycin. IL-10 production was analyzed in CD3+CD4+FoxP3-CD49b+LAG-3+ T cells. To evaluate the impact of ICOS-ICOS-L signaling, cells were co-cultured in the presence of 50 ug/ml of control isotype antibody or anti-ICOS-L neutralizing antibody. Values are means  $\pm$  SEM, \*p < 0.05, \*\*p < 0.01. Red dots represent HCC lysates and blue open dots LM-CRC lysates. (D) Pearson correlation analysis of the expression of ICOSL on pDCs cultured with medium, TFLL or TL, and the percentage of IL-10+ Tr1 cells detected after co-culture.

Figure 1.

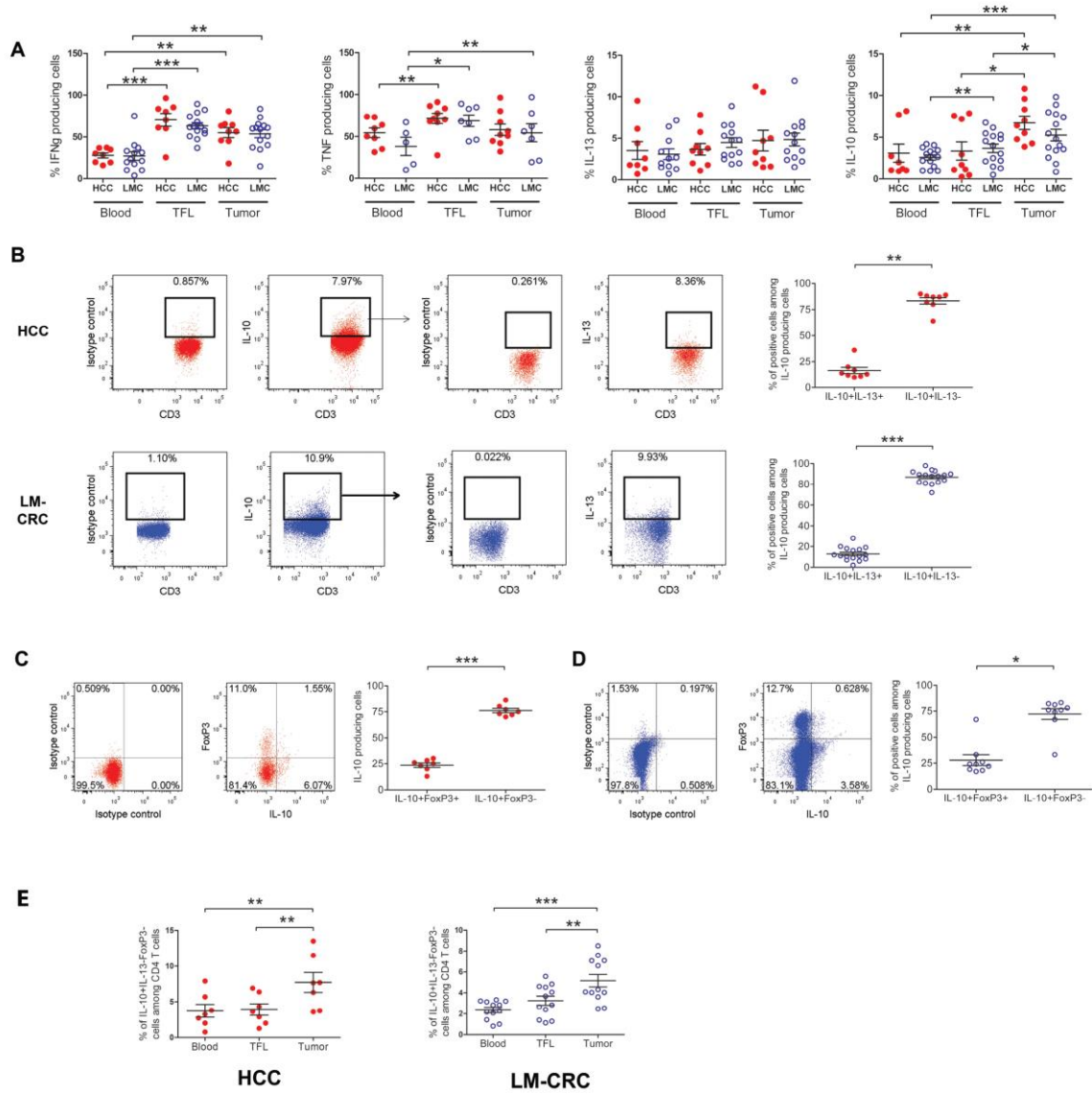


Figure 1



Figure 2.

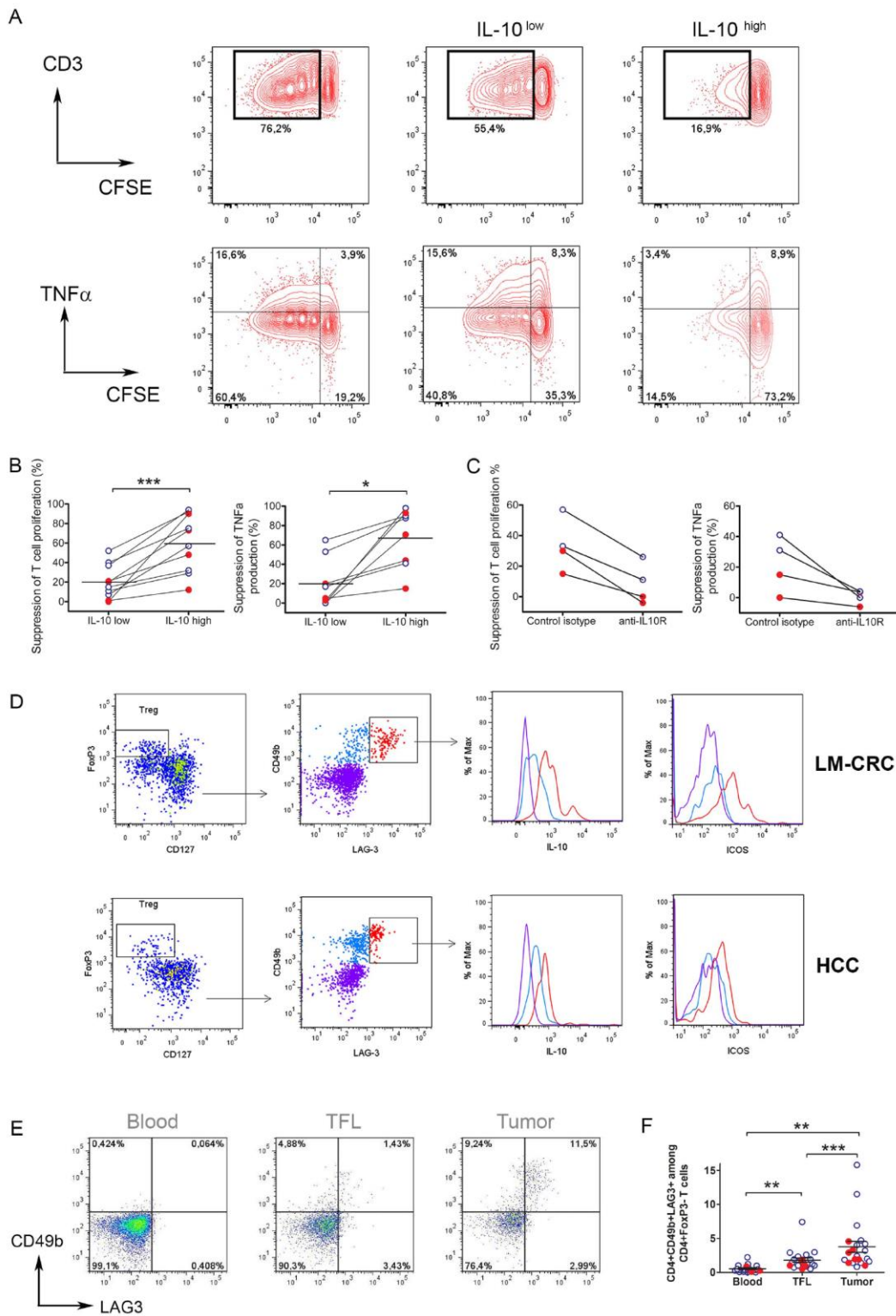


Figure 2

Figure 3.

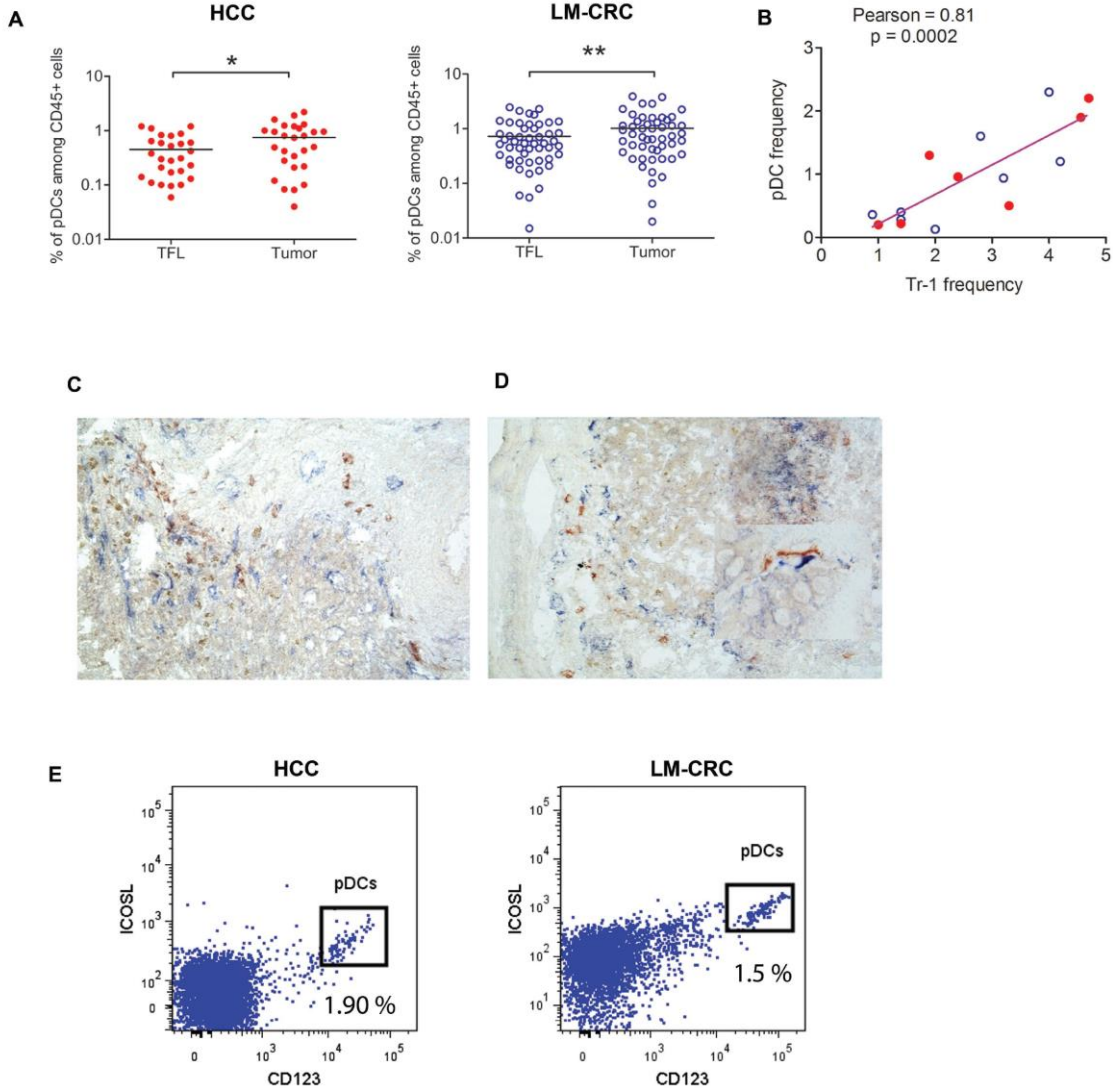


Figure 3

Figure 4.

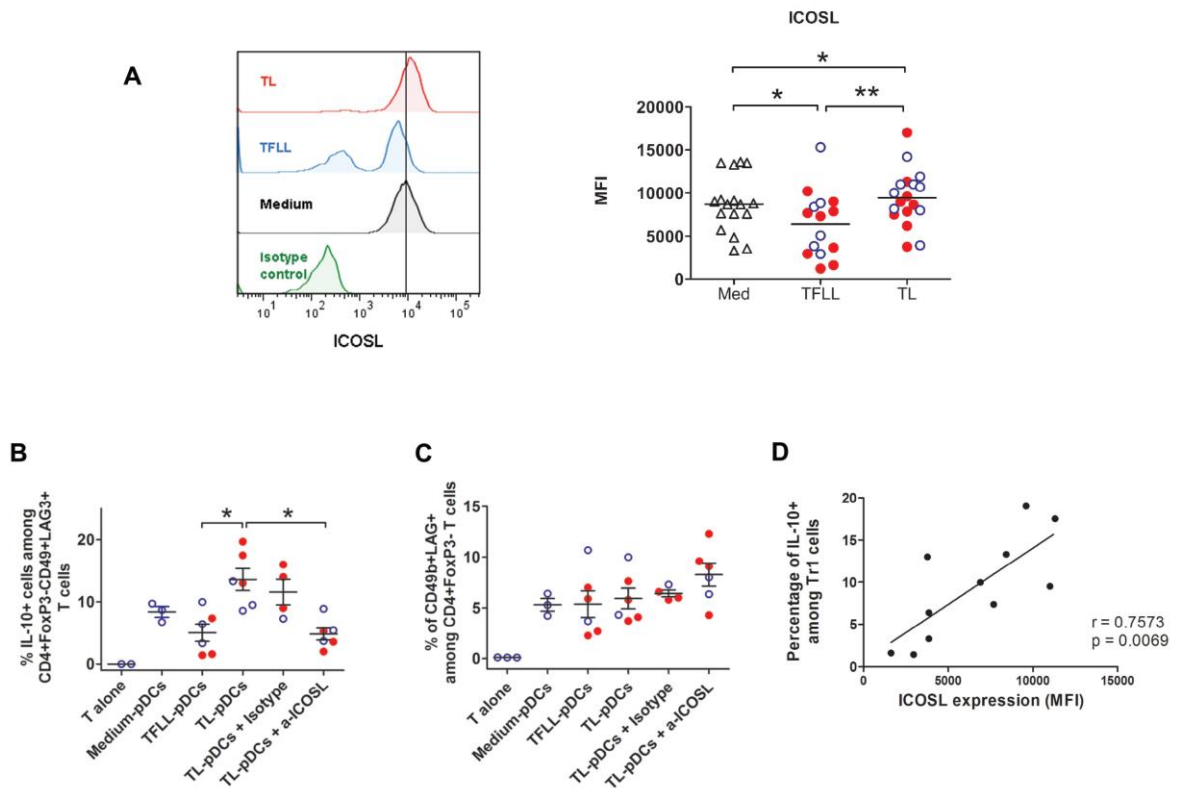
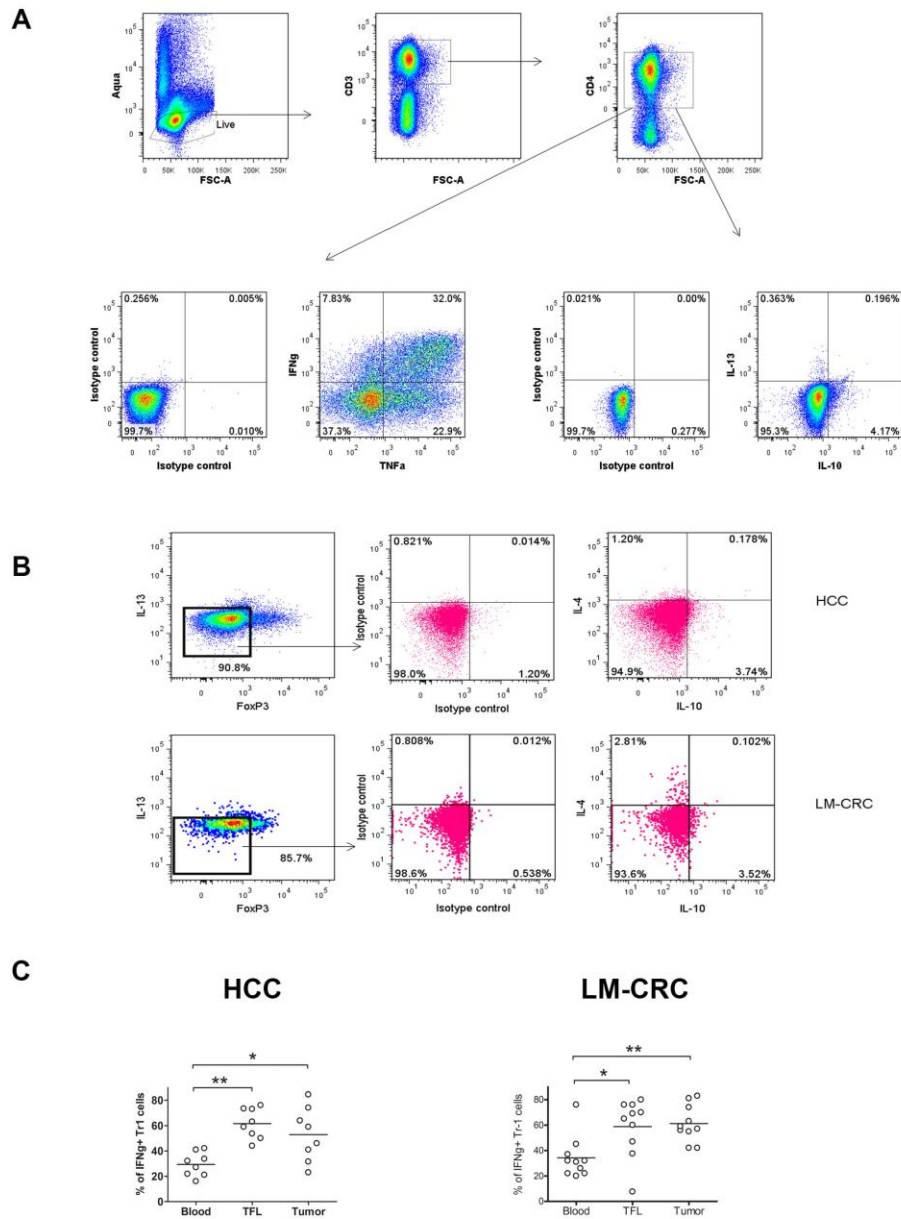


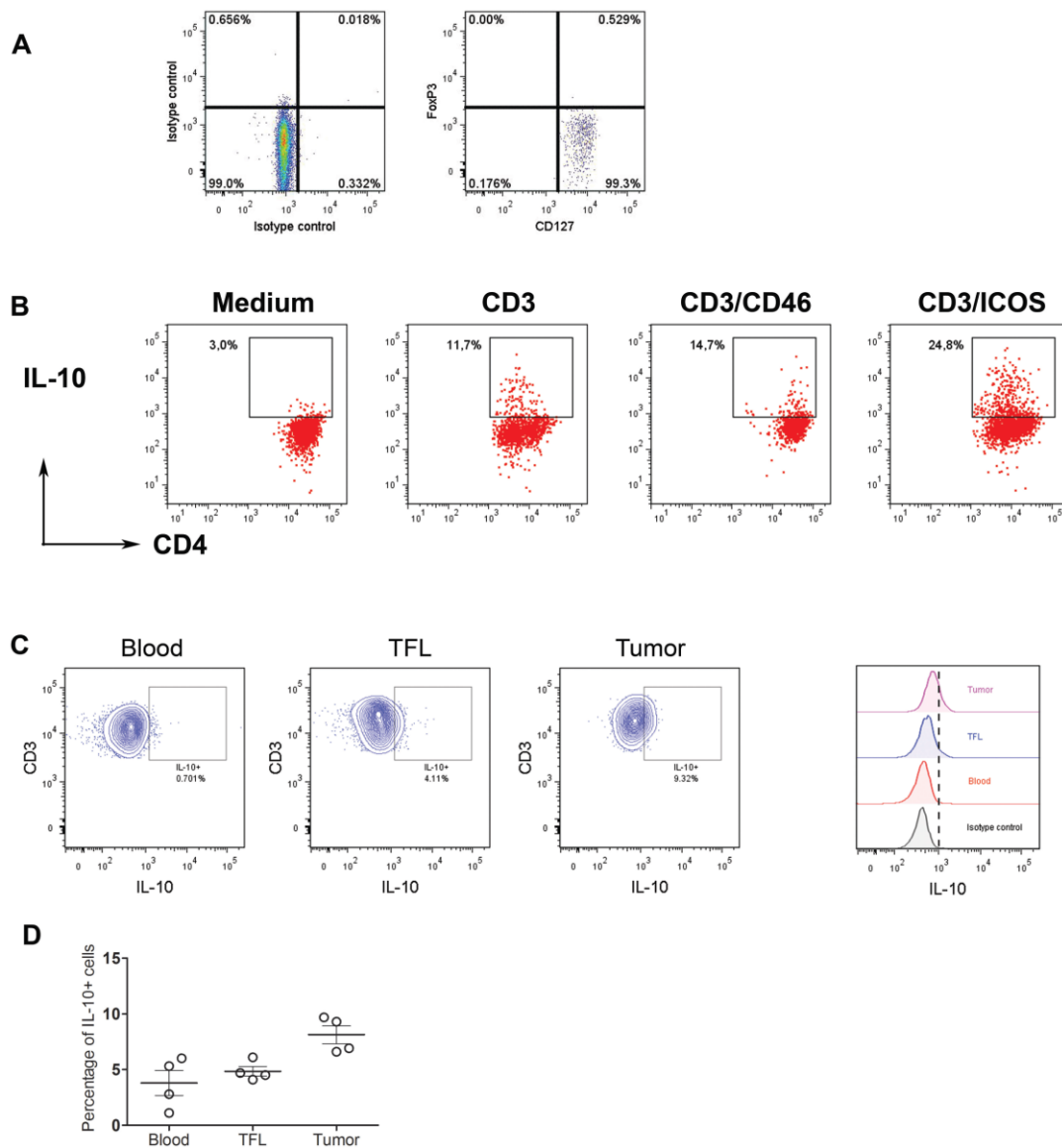
Figure 4

Supplementary Figure 1.



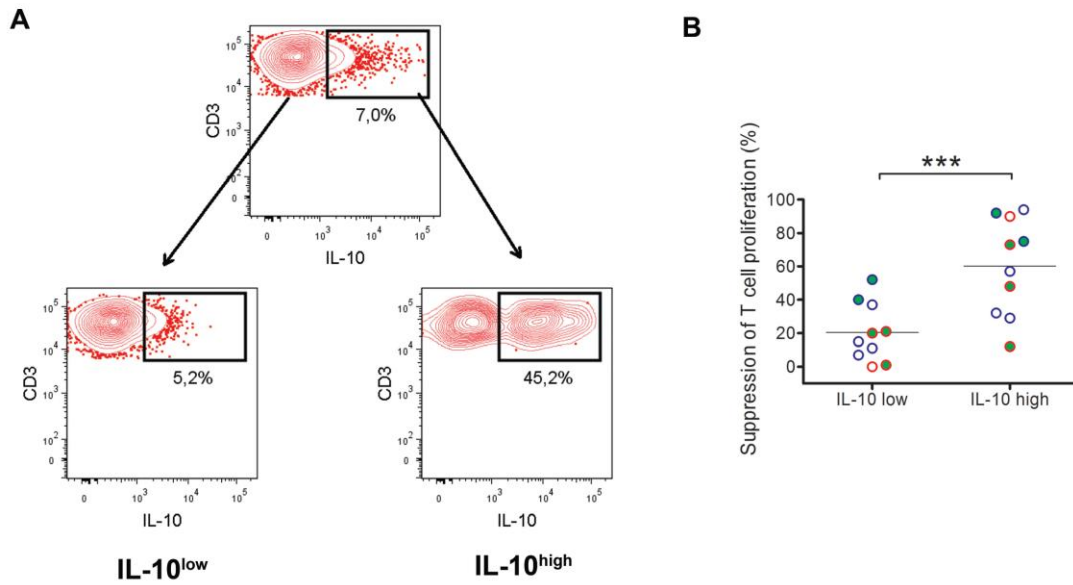
**Supplementary figure 1. Cytokine analysis of CD4+ T cells from patients with liver cancer.** (A) Gating strategy for the analysis of intracellular cytokines by flow cytometry in MNCs suspensions isolated from blood, TFL or tumor tissue stimulated by PMA/ionomycin. Viable cells were gated using LIVE/DEAD fixable dead cell stain kit (Aqua). IFN $\gamma$ , TNF $\alpha$ , IL-13 and IL-10 accumulation was analyzed in CD3+CD4+ T cells. (B) Two of five patients analyzed for the expression of IL-4. Cells were gated on viable CD3+CD4+IL-13-FoxP3- T cells. (C) Expression of IFN $\gamma$  in Tr1 cells (CD3+CD4+IL-13-FoxP3-).

## Supplementary Figure 2.



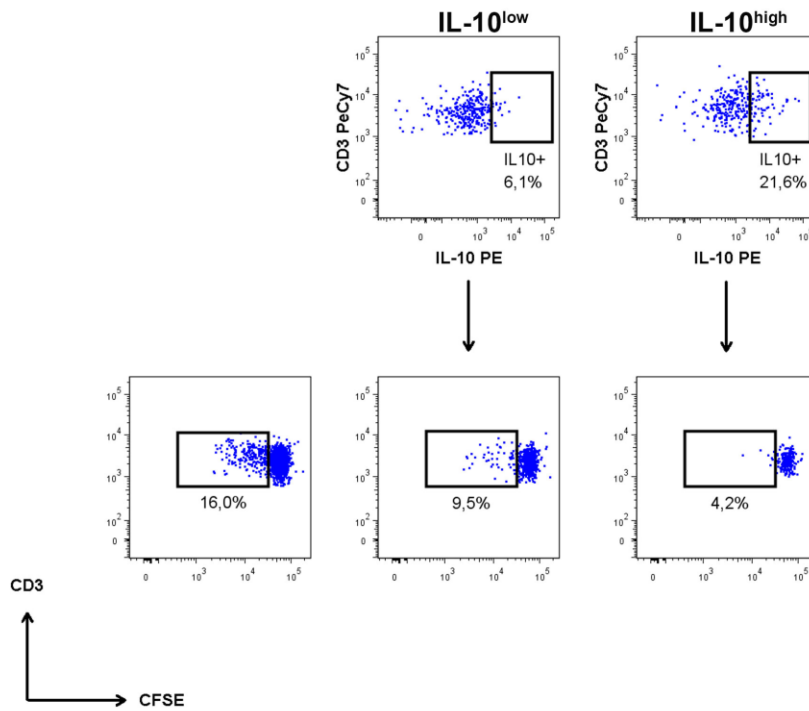
**Supplementary figure 2. Stimulation of tumor-infiltrating CD4+CD25- IL-10-producing cells.** (A) CD4+CD25- cells isolated from TILs of individuals with HCC or LM-CRC are FoxP3 negative and CD127 positive, and (B) were activated with beads-coupled antibodies to CD3 only, CD3 and CD46, or CD3 and ICOS in the presence of 250 U/ml of IL-2 for 24 hrs. Then cells were stained for IL-10 using a secretion assay and measured by flow cytometry. (C) CD4+CD25- T cells isolated from blood, TFL and tumor were activated with beads-coupled antibodies to CD3 and CD46 in the presence of IL-2 during 48 hrs and the frequency of IL-10+ cells was analyzed by flow cytometry. (D) Analysis of the frequency of IL-10+ cells among CD4+CD25- T cells activated with anti-CD3 and anti-CD46 antibodies (as described in C) in four patients.

**Supplementary Figure 3.**



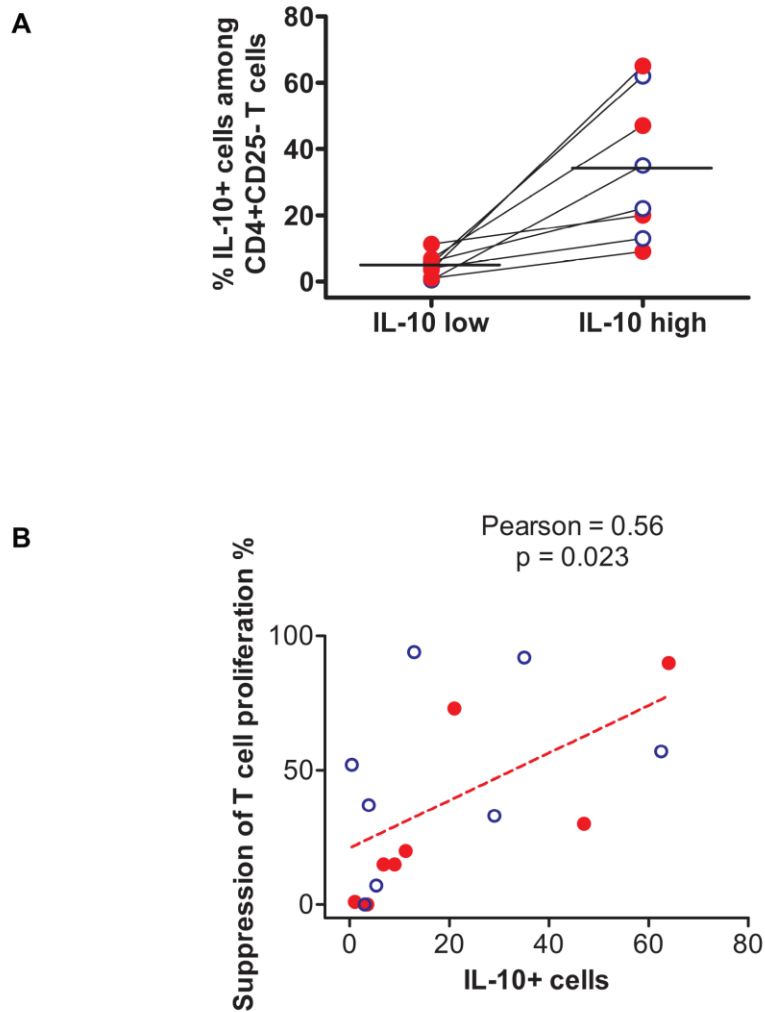
**Supplementary figure 3. Isolation and functionality of tumor-infiltrating CD4+CD25- IL-10-producing cells.** (A) CD4+CD25- cells isolated from TILs of individuals with HCC or LM-CRC were activated with beads-coupled antibodies to CD3 only, CD3 and CD46, or CD3 and ICOS in the presence of 250 U/ml of IL-2 for 24 hrs. Then cells were stained for IL-10 using a secretion assay and measured by flow cytometry. After activation and staining, cells were magnetically sorted based on the expression of IL-10. (B) Comparison of T cell suppression by tumor-infiltrating CD4+CD25- T cells stimulated with either CD3/ICOS (white dots) versus stimulation with CD3/CD46 (green dots). Red circles: CD4+CD25- T cells from HCC, blue circles: CD4+CD25- T cells from LM-CRC. No difference in suppressive activity was observed between the two protocols for IL-10 enrichment.

## Supplementary Figure 4.



**Supplementary figure 4. Tumor-infiltrating Tr1 cells efficiently suppress antigen-specific T cells.** CFSE-labeled CD4+CD25<sup>-</sup> T cells from peripheral blood were stimulated with autologous mDCs that were activated with CMV antigens. Cells were co-cultured for 5 days in the absence or presence of autologous IL-10<sup>low</sup> or IL-10<sup>high</sup> CD4<sup>+</sup> T cells isolated from tumor tissue. The Tr1:CD4+CD25<sup>-</sup> T cell ratio was 1:10. Proliferation was measured by flow cytometry. A representative analysis of one patient is shown.

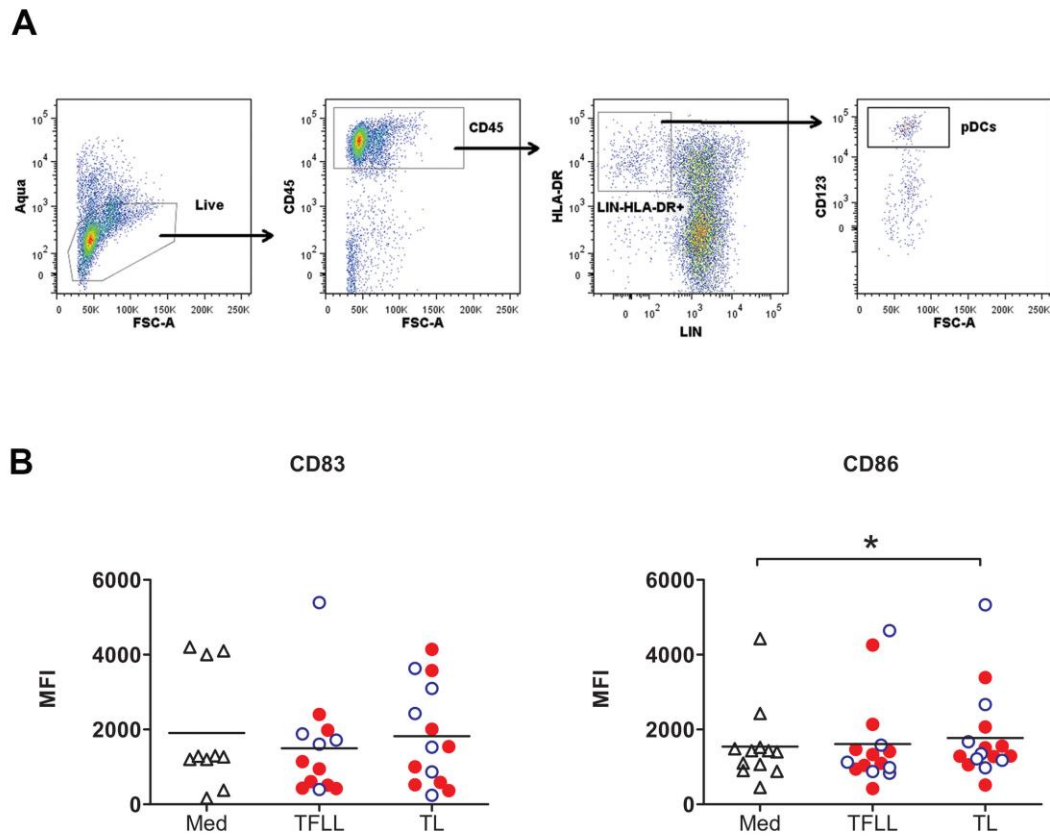
Supplementary Figure 5.



**Supplementary figure 5. The suppressive capacity of tumor-infiltrating CD4+CD25- T cells depends on the numbers of IL10-producing cells.** (A) Tumor-infiltrating CD4+CD25- T cells activated with beads-coupled antibodies to CD3 and ICOS or CD46 were stained and magnetically sorted as described in supplementary figure 3. The percentage of IL-10+ cells in each fraction was measured by flow cytometry. (B) Correlation analysis between the frequencies of IL-10+ cells detected in each fraction and the amount of suppression of PHA-stimulated CD3+ T cell proliferation observed. Red dots HCC samples and blue open dots LM-CRC samples.



## Supplementary Figure 6.



**Supplementary figure 6. Analysis of plasmacytoid DCs by flow cytometry.** A) Flow cytometric staining for pDCs in freshly isolated cells from tumor tissue. Cells first were gated on viable cells that were HLA-DR<sup>+</sup> and Lineage<sup>-</sup>, then pDCs were identified by the expression of CD123. B) Expression of CD83 and CD86 measured by flow cytometry on pDCs exposed to tissue lysates from HCC (red dots) or LM-CRC (blue open dots) or medium (trinangles).

## **Supplementary Material and Methods**

### **Cell preparation**

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. Single cell suspensions from TFL and tumor tissue were obtained by tissue digestion. Fresh tissue was cut into small pieces and digested with 0.5 mg/ml of collagenase (Cat. C5138, Sigma-Aldrich) and 0.2 mg/ml of DNase I (Cat. 10104159001, Roche), for 30 minutes at 37 °C. Cell suspensions were filtered through 100 and 70 µm pore cell-strainers (BD Biosciences) and mononuclear cells (MNCs) were obtained by Ficoll density gradient centrifugation. Viability was determined by trypan blue exclusion.

### **Cytokine analysis**

Single cell MNCs isolated from blood, TFL or tumor were resuspended at 1 million per mL in sRPMI medium (RPMI 1640 medium supplemented with 2mM glutamine, 50 U/mL of penicillin, 50 ug/mL of streptomycin, 0.1 mM MEM nonessential amino acids, 10 mM Hepes buffer and 0.1 mM sodium pyruvate) with 10% fetal calf serum (FCS). Cells were stimulated with 50 ng/mL of PMA and 1 ug/mL of ionomycin (both from Sigma-Aldrich) for 5 hrs in the presence of brefeldin A (GolgiPlug, BD) and monensin (GolgiStop, BD) during the last 3 hrs. Then intracellular cytokine accumulation was analyzed by flow cytometry.

**Flow cytometry antibodies**

Anti-human antibodies used for flow cytometry: FITC-labeled anti-Lineage (CD3, CD14, CD16, CD19, CD20, CD56) cocktail-1 (Cat. 340546, BD Biosciences); Alexa Fluor 488-labeled anti-CD49b (AK-7, Cat. 314308, Biolegend), PE-labeled anti-IL-10 (JES3-9D7, Cat. 501404, Biolegend), anti-ICOS-L (MIH12, Cat. 12-5889, e-biosciences), anti-IFN $\gamma$  (B27, Cat. 559327, BD Biosciences) and anti-IL-13 (JES10-5A2, Cat. 559328, BD Biosciences); PerCP-labeled anti-HLA-DR (L243 Cat.347402, BD Biosciences) and anti-LAG-3 (FAB2319C, R & D Systems); PerCP-Cy5.5-labeled anti-IL-4 (8D4-8, Cat. 561234, BD Biosciences); APC-labeled anti-FoxP3 (236A/E7 Cat. 17-4777) and anti-TNF $\alpha$  (Mab11, Cat. 17-7349) from e-biosciences and anti-CD83 (HB15e, Cat. 305312, Biolegend); APC-H7-labeled anti-CD4 (SK3, Cat. 641398, BD Biosciences); PeCy7-labeled anti-CD3 (UCTH1, Cat. 25-0038), anti-CD123 (6H6, Cat. 25-1239), and anti-ICOS (ISA-3, Cat. 25-9948) from e-biosciences; eFluor $\text{\textcircled{R}}$ 450-labeled anti-CD45 (HI30, Cat. 48-0459), anti-CD127 (eBioRDR5, Cat. 48-1278) and anti-CD25 (BC96, Cat.48-0259) from e-biosciences, and V450-labeled anti-CD86 (FUN-1, Cat. 560357) and anti-IL-13 (JES10-5A2, Cat. 561158) from BD biosciences.

**Suppression of antigen-specific T cells by Tr1 cells**

Myeloid DCs (mDCs) were isolated from PBMCs by positive selection (BDCA-1 DC isolation kit, Cat. 130-090-506, Miltenyi Biotec). Dendritic cells were suspended in sRPMI 1640 medium supplemented with 10% of human AB serum and cultured overnight at 37 °C in the presence of 10  $\mu$ g/ml of Cytomegalovirus antigens (CMV, Cat. EL-01-02, Microbix biosystems, Canada). The CMV antigens is a preparation that contains proteins from all the virus cycle including early and late proteins, nuclear, cytoplasmic, structural and non-structural proteins. CMV-activated

DCs were co-cultured with autologous CFSE-labeled CD4<sup>+</sup>CD25<sup>-</sup> T cells isolated from PMBCs at a ratio of 1:10 for 5 days in the presence or absence of IL-10 producing CD4<sup>+</sup> T cells. Co-cultures were performed in round-bottom 96-well plates with at least  $5 \times 10^4$  responder T cells. Proliferation and cytokine production were measured by flow cytometry after re-stimulation with autologous monocyte-derived DC, which were activated with CMV antigens, in the presence of brefeldin A and monensin. Inhibition of T cell proliferation was determined by comparison with culture conditions without suppressive T cells and reported as percentage of suppression of T cell proliferation. Monocyte-derived DCs were obtained by culturing autologous monocytes with 10 ng/ml IL-4 and 50 ng/ml GM-CSF for 5 days, then immature DCs were activated with CMV antigens as described previously for mDCs.

### **Immunohistochemistry**

Frozen sections (6  $\mu$ m) from liver tissues were fixed with cold acetone for 5 minutes. Unspecific binding was quenched by pre-incubation with PBS containing 10 % goat serum and 5 % of bovine serum albumin for 30 minutes. Endogenous peroxidase blockage and the secondary reagents used to detect the primary antibodies were from the EnVision+ System (Dako). The sections were labelled with 10  $\mu$ g/ml of anti-CD303 antibody (clone 104C12.08, Dendritics) followed by incubation with anti-mouse IgG antibodies conjugated to HRP- labelled polymer (Cat. K4005). The enzyme-linked secondary reagent was visualized with 3-amino-9-ethylcarbazole (AEC). Thereafter the sections were incubated with 10  $\mu$ g/ml of anti-LAG-3 antibody (clone 17B4, Enzo Life Sciences) followed by an anti-mouse IgG antibodies conjugated with AP (Cat. D0314, Dako) and visualized with Fast blue. Positive cell were counted in different microscopic fields of 200 times magnification of several tumors by two investigators.

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# **PART II**

## **Regulation of intra-tumoral effector T cells in liver cancers**





# CHAPTER 5

## **Antibodies against immune checkpoint molecules restore functions of tumor-infiltrating T cells in hepatocellular carcinomas**

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## ABSTRACT

**Background & Aims:** Ligand binding to inhibitory receptors on immune cells, such as programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA4), downregulates the T-cell-mediated immune response (called immune checkpoints).

Antibodies that block these receptors increase anti-tumor immunity in patients with melanoma, non-small cell lung cancer, and renal cell cancer. Tumor-infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells in patients with hepatocellular carcinoma (HCC) have been found to be functionally compromised. We analyzed HCC samples from patients to determine if these inhibitory pathways prevent T-cell responses in HCCs and to find ways to restore their anti-tumor functions.

**Methods:** We collected HCC samples from 59 patients who underwent surgical resection from November 2013 through May 2017, along with tumor-free liver tissues (control tissues) and peripheral blood samples. We isolated tumor-infiltrating lymphocytes (TIL) and intra-hepatic lymphocytes. We used flow cytometry to quantify expression of the inhibitory receptors PD-1, hepatitis A virus cellular receptor 2 (TIM3), lymphocyte activating 3 (LAG3), and CTLA4 on CD8<sup>+</sup> and CD4<sup>+</sup> T cells from tumor, control tissue, and blood; we studied the effects of antibodies that block these pathways in T-cell activation assays.

**Results:** Expression of PD-1, TIM3, LAG3, and CTLA4 was significantly higher on CD8<sup>+</sup> and CD4<sup>+</sup> T cells isolated from HCC tissue than control tissue or blood. Dendritic cells, monocytes, and B cells in HCC tumors expressed ligands for these receptors. Expression of PD-1, TIM3, and LAG3 was higher on tumor-associated antigen (TAA)-specific CD8<sup>+</sup> TIL, compared with other CD8<sup>+</sup> TIL. Compared to TIL that did not express these inhibitory receptors, CD8<sup>+</sup> and CD4<sup>+</sup> TIL that did express these receptors had higher levels of markers of activation, but similar or decreased levels of granzyme B and effector cytokines. Antibodies against CD274 (PD-L1), TIM3, or LAG3 increased proliferation of CD8<sup>+</sup> and CD4<sup>+</sup> TIL and cytokine production in response to stimulation with polyclonal antigens or TAA. Importantly, combining antibody against PD-L1 with antibodies against TIM3, LAG3, or CTLA4 further increased TIL functions.

**Conclusions:** The immune checkpoint inhibitory molecules PD-1, TIM3, and LAG3 are up-regulated on TAA-specific T cells isolated from human HCC tissues, compared to T cells from tumor-free liver tissues or blood. Antibodies against PD-L1, TIM3, or LAG3 restore responses of HCC-derived T cells to tumor antigens, and combinations of the antibodies have additive effects. Strategies to block PD-L1, TIM3, and LAG3 might be developed for treatment of primary liver cancer.

**Keywords:** immunotherapy; GPC3; MAGEC2; galectin 9

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## Introduction

Liver cancer represents the second most common cause of cancer-related mortality worldwide, and hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer.<sup>1,2</sup> The current treatment options for HCC, such as surgical resection, liver transplantation and radiofrequency ablation, are curative only for patients with early stage disease. Unfortunately, the majority of HCC patients are not eligible for curative procedures because of late diagnosis and thus have poor prognosis.<sup>3</sup> HCC is highly resistant to chemotherapy, but immunotherapy may be an attractive therapeutic option for HCC, since an inflammatory tumor microenvironment is associated with improved survival.<sup>4,5</sup> As reported in our earlier studies, intra-tumoral accumulation of activated regulatory T cells (Treg) is one of the suppressive mechanisms that contribute to HCC immune evasion.<sup>6-8</sup> However, inhibitory receptor-ligand pathways (called immune checkpoints) may additionally contribute to suppression of anti-tumor immunity in the tumor microenvironment.

There is evidence that tumor-infiltrating T cells upon chronic stimulation by tumor antigens lose their effector functions and their ability to kill tumor cells, which is accompanied by a progressive increase in the diversity and amount of inhibitory receptors expressed on them, including PD-1, TIM3, LAG3, CD244, CD160, CTLA4 and BTLA.<sup>9-12</sup> Upon interaction with their respective ligands expressed on tumor cells and tumor-infiltrating antigen-presenting cells (APC), these receptors suppress T cell responses to tumor antigens. Blocking the interactions of PD-1 and CTLA4 with their respective ligands has been shown to be a promising novel therapeutic approach for several types of human malignancies such as advanced melanoma, non-small cell lung cancer and renal cell cancer, which improved anti-tumor T cell responses and provided unprecedented clinical benefits for patients.<sup>13-18</sup>

We and others have observed that tumor-infiltrating CD4<sup>+</sup> T helper cells (Th) and CD8<sup>+</sup> cytotoxic T cells (CTL) of HCC patients are functionally compromised.<sup>6,19</sup> Recent studies have shown that the PD-1/PD-L1 inhibitory interaction mediates suppression of CD8<sup>+</sup> tumor-infiltrating lymphocytes (TIL),<sup>20-22</sup> while the TIM3/galectin-9 (GAL-9) interaction mediates CD4<sup>+</sup> TIL dysfunction<sup>23</sup> in HCC patients. Nevertheless, the expression of these and other inhibitory receptors on tumor-infiltrating T cells, and their functional relevance to both helper and cytotoxic T cell responses to tumor antigens in the HCC microenvironment have not been studied thoroughly. It is also unclear whether combined blockade of inhibitory pathways can additively increase the effector function of HCC-specific T cells.

In this study, we aimed to systematically determine which inhibitory pathways participate in the suppression of T cell responses in the HCC microenvironment. We used paired samples

of leukocytes isolated from resected tumors, tumor-free liver tissues (TFL) and peripheral blood of HCC patients, to measure the expression of five inhibitory receptors on CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL. We also performed *ex vivo* functional assays to determine whether blockade of inhibitory interactions can invigorate the functionality of tumor-infiltrating T cells. We found that inhibitory receptors PD-1, TIM3, LAG3 and CTLA4 were up-regulated on tumor-infiltrating T cells; and interestingly, PD-1, TIM3 and LAG3 were selectively increased on tumor-associated antigen (TAA)-specific CD8<sup>+</sup> TIL. Blocking PD-L1, TIM3 or LAG3 enhanced *ex vivo* proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> TIL and effector cytokine production, and combined blockade of PD-L1 with TIM3, LAG3 or CTLA4 further enhanced *ex vivo* TIL responses to polyclonal stimuli and TAA.

## **Patients and Methods**

### ***Patients***

Fifty-nine patients who were eligible for surgical resection of HCC were enrolled in the study from November 2013 to May 2017. Paired fresh tissue samples from tumors and surrounding (minimum 1cm distance from the tumor) TFL were obtained, and tumor-infiltrating leukocytes and intra-hepatic leukocytes were isolated respectively. Peripheral blood from the same patients was also collected on the day of resection. None of the patients received chemotherapy or immunosuppressive treatment at least three months before surgery. The clinical characteristics of the patients are summarized in Supplementary Table 1. Eighty-five percent of patients included in the study are Caucasians. The study was approved by the local ethics committee, and all the patients signed the informed consent before tissue and blood donation.

### ***Cell preparation***

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation. Single cell suspensions from tumors and TFL were obtained by tissue digestion as described previously.<sup>6-8</sup> Briefly, fresh tissues were cut into small pieces and digested with 0.5 mg/mL of collagenase IV (Sigma-Aldrich, St. Louis, MO) and 0.2 mg/mL of DNase I (Roche, Indianapolis, IN) for 20-30 minutes at 37 °C. Cell suspensions were filtered through 100 µm pore cell strainers (BD Biosciences, Erembodegem, Belgium) and mononuclear leukocytes were obtained by Ficoll density gradient centrifugation. Viability was determined by trypan blue exclusion.

***Ex vivo mRNA-encoded full-length tumor antigen-specific T cell stimulation assay***

PBMC or TIL of HCC patients were labeled with 0.1  $\mu$ M carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen), after which 100,000 cells in RPMI medium supplemented with 10% human AB serum, 2mM L-glutamine, 50 mM Hepes Buffer, 1% penicillin-streptomycin, 5mM Sodium Pyruvate and 1% minimum essential medium non-essential amino acids (MEM NEAA) were transferred to each well of 96-well round-bottom culture plate. Glypican-3 (GPC3) mRNA-, MAGEC2 mRNA- or (as negative controls) eGFP mRNA-transfected or mock-electroporated autologous CD40-activated B cells (B cell blasts) in the same medium were added at a PBMC/TIL : B cell ratio of 1:1. TIL were co-cultured with B cell blasts (200  $\mu$ l/ well) in the presence or absence of 10  $\mu$ g/ml neutralizing monoclonal antibodies against human PD-L1, TIM3, LAG3 or CTLA4, or combinations of these antibodies, or isotype control antibodies. After seven days, CFSE-labeled cells were harvested, and stained with CD8, CD4, CD3 antibodies. Dead cells were excluded by using the LIVE/DEAD fixable dead cell stain kit with aqua fluorescent reactive dye, and T cell proliferation was determined based on CFSE dilution by flow cytometry analysis.

***Detailed description of other methods is provided in the Supplementary Information.***

**Results**

***Increased expression of inhibitory receptors on tumor-infiltrating CD4<sup>+</sup> T helper cells and CD8<sup>+</sup> cytotoxic T cells from HCC patients.***

We first compared the expression levels of five inhibitory receptors (PD-1, TIM3, LAG3, CTLA4 and BTLA) on CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL, in paired samples of leukocytes freshly isolated from surgically resected tumors, TFL and peripheral blood from HCC patients. We excluded Foxp3<sup>+</sup> Treg from CD4<sup>+</sup> T cells because we focused on anti-tumor effector T cell populations. PD-1, TIM3 and CTLA4 were expressed on significantly higher proportions of Th and CTL in tumors than in TFL and blood, whereas LAG3 was expressed on significantly higher proportions of CTL in tumors than in TFL and blood (Fig. 1A-C). Compared to the other investigated inhibitory receptors PD-1 was expressed by most tumor-infiltrating Th and CTL and with highest median fluorescence intensity (MFI) (Supplementary Fig. 1). BTLA was expressed at negligible levels, and we did not observe any significant difference between its expression in the tumor and that in TFL. Therefore, we focused on the other four receptors in the rest of our study.

Since PD-1 was the highest expressed receptor on TIL, we analyzed co-expression of PD-1 and the other three receptors on TIL. Interestingly, we observed co-expression of TIM3,

LAG3 or CTLA4 with PD-1 on tumor-infiltrating Th and CTL, but there were also tumor-infiltrating Th and CTL expressing only TIM3, LAG3 or CTLA4 but not PD-1 (Fig. 1D, E). In addition, Supplementary Fig. 2 illustrates that there were significant, although weak, positive correlations between the percentages of PD-1, TIM3, LAG3 or CTLA4 positive Th in tumors and those in blood, and between the percentages of LAG3 or CTLA4 positive CTL in tumors and those in blood. These data indicate that the expression of inhibitory receptors on TIL is partially reflected by their expression on circulating T cells in HCC patients.

***Increased expression of PD-1, TIM3 and LAG3 on TAA-specific CD8<sup>+</sup> TIL.***

To study the expression of these inhibitory receptors on TAA-specific CTL, we used HLA-A\*0201 dextramers loaded with GPC3 and MAGEC2 peptides (Fig. 2A). These TAA are among the most frequently expressed TAA in HCC tumors in the Western-European area.<sup>24</sup> TAA dextramer-binding CTL were detectable in tumors from seven out of thirteen HLA-A2<sup>+</sup> patients, and in blood from three out of twelve patients (Supplementary Table 3). The resected tumors of the patients with TAA dextramer-binding CTL expressed the corresponding TAA (confirmed by immunohistochemistry staining, data not shown). Fig. 2B presents the percentages of CTL binding to the dominant epitope-HLA-A\*0201-dextramer complex of these patients, which ranged from 0.11%–8.0% in tumors and from 0.23%–0.87% in blood. Representative histograms of the expression of inhibitory receptors on TAA dextramer-binding versus TAA dextramer negative CD8<sup>+</sup> TIL are presented in Fig. 2C; and Fig. 2D summarizes the comparison of the expression of inhibitory receptors between CTL that did and did not recognize the dominant TAA epitope in each patient. Interestingly, expression of PD-1, TIM3 and LAG3, but not CTLA4, was significantly increased on TAA-specific CD8<sup>+</sup> TIL, compared to TAA dextramer negative CD8<sup>+</sup> TIL (MFI data are shown in Supplementary Fig. 3). Likewise, CD8<sup>+</sup> TIL that recognized non-dominant peptides demonstrated enhanced expression of inhibitory receptors in the same patients (data not shown).

***Tumor-infiltrating antigen-presenting cells from HCC patients express inhibitory ligands.***

We further analyzed the expression levels of inhibitory ligands PD-L1 (for PD-1), GAL-9 (for TIM3), MHC-II (for LAG3), CD86 and CD80 (for CTLA4) on APC subsets within tumor tissues by flow cytometry. The major human APC subsets, CD45<sup>+</sup>BDCA1<sup>+</sup>CD19<sup>-</sup> myeloid dendritic cells (mDC), CD45<sup>+</sup>CD14<sup>+</sup> monocytes and CD45<sup>+</sup>CD19<sup>+</sup> B cells, were all found in HCC tumors. The percentages of mDC and B cells were higher in tumor-infiltrating CD45<sup>+</sup> leukocytes than in TFL-infiltrating CD45<sup>+</sup> leukocytes, but cell numbers isolated per gram of

tissue did not differ (Fig. 3A, B). All five ligands were found to be expressed on tumor-infiltrating APC (Fig. 3C, D). The MFI of PD-L1 and GAL-9 on mDC and monocytes, and MHC-II on monocytes were significantly higher in the tumor than in TFL (Supplementary Fig. 4). In a separate study using immunohistochemistry, we found that PD-L1 and GAL-9 were also expressed on tumor cells in HCC tissues.<sup>25</sup> Together, these data suggest the suppression of tumor-infiltrating T cells by these inhibitory interactions in tumors of HCC patients.

***Tumor-infiltrating T cells expressing inhibitory receptors display an activated status.***

Given the increased expression of inhibitory receptors, tumor-infiltrating T cells in human HCC may be dysfunctional. Alternatively, expression of inhibitory receptors can be the consequence of local T cell activation. To study these two possibilities, we examined the activation status, cytotoxic effector molecule expression and cytokine production profile of tumor-infiltrating T cells expressing inhibitory receptors. We first compared the *ex vivo* activation status of tumor-infiltrating Th and CTL that do or do not express inhibitory receptors, using the activation markers HLA-DR and CD69. PD-1<sup>+</sup> cells were significantly more activated than PD-1<sup>-</sup> cells in both T cell populations (Fig. 4A). So were TIM3<sup>+</sup>, LAG3<sup>+</sup> and CTLA4<sup>+</sup> cells compared with TIM3<sup>-</sup>, LAG3<sup>-</sup> or CTLA4<sup>-</sup> cells, respectively. Despite their activated status, the expression of cytotoxic effector molecule granzyme B was comparable or reduced in inhibitory receptor positive CD8<sup>+</sup> TIL compared with respective receptor negative cells (Fig. 4B).

Furthermore, we stimulated TIL with PMA and ionomycin in order to assess effector cytokine production. After exposure to PMA and ionomycin for five hours, PD-1, TIM3, LAG3 and CTLA4 were not distinctly up-regulated on TIL (data not shown). The percentages of IFN- $\gamma$  and TNF- $\alpha$  producing cells in inhibitory receptor positive cells were comparable or reduced compared with those in respective receptor negative cells, in both CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL (Fig. 4C). Together, these data indicate that tumor-infiltrating T cells that express inhibitory receptors are activated but do not have enhanced functionality.

***PD-L1, TIM3 or LAG3 blockade increases ex vivo proliferation and effector cytokine production of tumor-infiltrating T cells.***

Given that the inhibitory receptors PD-1, TIM3, LAG3 and CTLA4 are up-regulated on HCC tumor-infiltrating T cells and their respective ligands are expressed on tumor-infiltrating APC subsets, we tested whether blocking the PD-1/PD-L1, TIM3/GAL-9, LAG3/MHC-II or CTLA4/CD80/CD86 pathways can enhance the functionality of tumor-infiltrating T cells. We first stimulated CFSE-labeled total tumor-infiltrating mononuclear leukocytes with CD3/CD28



beads, in the presence or absence of 10 µg/ml neutralizing antibodies against human PD-L1, TIM3, LAG3, CTLA4, or combinations of these neutralizing antibodies, or isotype control antibodies (mIgG1 and mIgG2a). After four days, T cell proliferation was measured by flow cytometry (Fig. 5A), and IFN-γ secretion in culture supernatant was quantified by ELISA. Compared with the control condition without neutralizing antibody, treatment with anti-PD-L1, anti-TIM3 or anti-LAG3 significantly enhanced the proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> TIL (Fig. 5B); these three antibodies also significantly increased IFN-γ secretion (Fig. 5C). Treatment with anti-CTLA4 significantly enhanced CD4<sup>+</sup> TIL proliferation. Interestingly, combined blockade of PD-L1 with CTLA4 further enhanced the proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> TIL, while PD-L1 blockade together with LAG3 blockade further enhanced CD8<sup>+</sup> TIL proliferation and IFN-γ secretion, and combining PD-L1 blockade with TIM3 blockade further enhanced all three types of function compared to single PD-L1 blockade (Fig. 5D, E).

***Combining PD-L1 blockade with TIM3, LAG3 or CTLA4 blockade additively increases ex vivo responses of tumor-infiltrating T cells to HCC tumor antigens.***

To test the effects of blocking these four inhibitory pathways on tumor-specific T cell immunity, we used two tumor antigen-specific T cell stimulation assays.

In the first assay, we stimulated TIL with pooled HLA-A\*0201-restricted GPC3 and MAGEC2 peptides, in the presence or absence of antibody blocking inhibitory pathway or combinations of blocking antibodies. After seven days, expression levels of intracellular IFN-γ, TNF-α, activation-induced CD137 (an accepted marker for detection of antigen-specific T cell responses<sup>26</sup>) and surface CD107a (as a measure for cytotoxic degranulation) in CD8<sup>+</sup> TIL were analyzed after re-stimulation with pooled GPC3 and MAGEC2 peptides (Fig. 6A). Compared to the stimulation with control peptide, seven HLA-A2<sup>+</sup> patients showed specific CD8<sup>+</sup> TIL responses to pooled GPC3 and MAGEC2 peptides. A summary of data (Fig. 6B) illustrates that blocking PD-L1, TIM3, LAG3 or CTLA4 increased tumor-specific CD137, CD107a expression, IFN-γ and/or TNF-α production of CD8<sup>+</sup> TIL, but large variations between individual patients were observed, ranging from no effect to higher than two-fold increases of CD8<sup>+</sup> TIL responses. Interestingly, PD-L1 blockade in combination with TIM3, LAG3 or CTLA4 blockade further enhanced tumor-specific CD8<sup>+</sup> TIL responses compared to single PD-L1 blockade in part of the patients.

In the second assay, autologous B cell blasts expanded from patient PBMC by three-week culture in the presence of trimeric CD40 ligand and rhIL-4 and then transfected with mRNA encoding GPC3 or MAGEC2 by electroporation, served as APC. B cell blasts expressed MHC-II, CD80, CD86,<sup>27</sup> PD-L1 and GAL-9 (data not shown). To enable antigen presentation

to CD4<sup>+</sup> T cells, the mRNA constructs we used encode these TAA fused to the transmembrane and luminal regions of dendritic cell lysosome-associated membrane protein (DCLamp), which is a targeting signal for the endo-lysosomal compartment, resulting in peptide loading in MHC-II in addition to MHC-I.<sup>28</sup> To validate this assay, we first co-cultured an HLA-A\*0201-restricted GPC3-specific CD8<sup>+</sup> T cell clone<sup>29</sup> or human T cells transfected with a human T cell receptor (TCR) recognizing MAGEC2 presented by HLA-A\*0201,<sup>30</sup> with GPC3 mRNA- or MAGEC2 mRNA-transfected B cell blasts expanded from PBMC of an HLA-A2<sup>+</sup> healthy donor. B cell blasts pulsed with the GPC3 FVGEFFTDV peptide or the MAGEC2 ALKVDVEERV peptide served as positive controls. Fig. 7A reveals clear IFN- $\gamma$  response of the GPC3-specific CD8<sup>+</sup> T cell clone to GPC3 mRNA-transfected B cell blasts and GPC3 peptide, and clear IFN- $\gamma$  response of MAGEC2-specific human T cells to MAGEC2 mRNA-transfected B cell blasts and MAGEC2 peptide, but the T cells did not respond to eGFP mRNA-transfected or mock-electroporated B cell blasts (negative controls) or to non-cognate antigens.

Next, we co-cultured CFSE-labeled PBMC from an HCC patient whose resected tumor expressed GPC3 strongly and MAGEC2 weakly (confirmed by immunohistochemistry staining), with GPC3 mRNA-, MAGEC2 mRNA-, eGFP mRNA-transfected or mock-electroporated autologous B cell blasts, and measured T cell proliferation after seven days. Fig. 7B reveals evident specific responses of CD4<sup>+</sup> and CD8<sup>+</sup> T cells to GPC3, and weaker responses to MAGEC2, but not to eGFP. These results confirm that TAA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses can be accurately detected and quantified using this T cell stimulation assay.

We co-cultured CFSE-labeled TIL with GPC3 mRNA-, MAGEC2 mRNA-, eGFP mRNA-transfected or mock-electroporated autologous B cell blasts, in the presence or absence of antibody blocking inhibitory pathway or combinations of blocking antibodies, and measured T cell proliferation after seven days. Representative proliferation data of CD4<sup>+</sup> and CD8<sup>+</sup> TIL from one patient whose resected tumor expressed MAGEC2 (confirmed by immunohistochemistry staining) are presented in Fig. 7C. In six patients both GPC3- and MAGEC2-specific TIL proliferative responses were observed, and in three patients either GPC3- or MAGEC2-specific responses were observed. In these nine patients, 6.6%  $\pm$  3.8% (mean  $\pm$  SEM) of CD4<sup>+</sup> and 3.9%  $\pm$  1.5% (mean  $\pm$  SEM) of CD8<sup>+</sup> proliferating TIL were observed in response to these TAA. A summary of data from these nine patients (Fig. 7D) demonstrates that blocking PD-L1, TIM3, LAG3 or CTLA4 increased tumor-specific proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> TIL in most but not all patients. Furthermore, PD-L1 blockade in combination with TIM3, LAG3 or CTLA4 blockade enhanced proliferation of both CD8<sup>+</sup> and

CD4<sup>+</sup> TIL in more patients and further enhanced TIL proliferation compared to single PD-L1 blockade.

## Discussion

The purpose of this study was to determine whether inhibitory pathways lead to suppression of T cell responses in the HCC microenvironment, and to examine whether blocking these inhibitory pathways can restore the functions of tumor-infiltrating T cells. Overall, the results of our study demonstrate that inhibitory pathways involving the receptors PD-1, TIM3, LAG3 and CTLA4 participate in intra-tumoral suppression of CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL in HCC. Single blockade of PD-L1, TIM3, LAG3, or CTLA4 could recover TIL responses to tumor antigens, but large differences in effects were observed between individual patients. Importantly, combining PD-L1 blockade with TIM3, LAG3 or CTLA4 blockade further enhanced the responsiveness of TIL and was effective in more patients.

Previous studies have revealed increased expression of PD-1,<sup>20, 21, 31</sup> TIM3<sup>23</sup> and LAG3<sup>32</sup> on either CD4<sup>+</sup> or CD8<sup>+</sup> TIL in HCC patients, but to our knowledge expression of CTLA4 and BTLA as well as co-expression of all these inhibitory receptors on both CD4<sup>+</sup> and CD8<sup>+</sup> TIL in HCC patients have not been studied before. Here, we demonstrate that tumor-infiltrating Th have significantly increased PD-1, TIM3 and CTLA4 expression; whereas CTL have significantly increased PD-1, TIM3, LAG3 and CTLA4 expression, compared with their counterparts in TFL and blood (Fig. 1C). Similar to melanoma,<sup>33</sup> BTLA was not up-regulated on tumor-infiltrating T cells. A previous study suggested that HBV positive HCC patients had more intra-tumoral TIM3<sup>+</sup> T cells compared to HBV negative HCC patients,<sup>23</sup> but we did not find any difference in the expression of inhibitory receptors between HCC patients with HBV or HCV infection or other etiologies for HCC (e.g. alcoholic liver disease). There was also no difference between patients with cirrhotic or non-cirrhotic liver (data not shown). Using HLA-A\*0201 dextramers loaded with GPC3 or MAGEC2 peptides, we are the first to demonstrate that PD-1, TIM3 and LAG3 are selectively up-regulated on tumor-infiltrating TAA-specific CD8<sup>+</sup> T cells from HCC patients (Fig. 2D, supplementary Fig. 3). Similarly, we observed that circulating TAA-specific CD8<sup>+</sup> T cells express relatively high levels of the same inhibitory receptors, which extends previous observation showing high PD-1 expression on circulating TAA-specific CD8<sup>+</sup> T cells in HCC patients.<sup>34, 35</sup> The selectively increased expression of these inhibitory receptors on TAA-specific TIL together with the expression of their respective ligands on tumor-infiltrating APC suggests that they may be involved in the regulation of tumor antigen-specific responsiveness of tumor-infiltrating T cells.

Given that expression of individual inhibitory receptor on T cells can be induced by T cell activation,<sup>36, 37</sup> but expression of increasing numbers of inhibitory receptors often coincides with gradual loss of T cell functions,<sup>9, 38, 39</sup> we investigated the activation status, cytotoxic effector molecule expression and cytokine production profile of tumor-infiltrating T cells in relation to the expression inhibitory receptors. Interestingly, CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL expressing either inhibitory receptor displayed a more activated status (Fig. 4A); however, they displayed comparable or reduced levels of granzyme B, IFN- $\gamma$  and TNF- $\alpha$  (Fig. 4B,C). Together with our data showing selectively enhanced expression of inhibitory receptors on TAA-specific TIL, these findings indicate that, in accordance with recent studies in other human cancers,<sup>11, 33, 39</sup> HCC tumor-infiltrating T cells that express inhibitory receptors are probably tumor-reactive T cells, which may continue to up-regulate the expression of inhibitory receptors in response to chronic stimulation by tumor antigens to prevent further over activation. Of note, PD1<sup>+</sup>TIM3<sup>+</sup> T cells displayed the lowest level of granzyme B, IFN- $\gamma$  and TNF- $\alpha$ , which suggests that PD-1 and TIM3 play an important role in T cell suppression in HCC microenvironment.

We performed polyclonal and tumor-specific functional assays to test the effect of blocking these inhibitory pathways on the functions of tumor-infiltrating T cells *ex vivo*. Because we were interested in and measured the net effect of blocking antibody intervention on CD4<sup>+</sup> and CD8<sup>+</sup> TIL function in a context that reflected the tumor microenvironment as much as possible, we used total tumor-infiltrating mononuclear leukocytes in all our functional assays which contained both T cells expressing inhibitory receptors and APC subsets expressing inhibitory ligands. As a consequence, tumor-infiltrating Treg and probably other types of suppressor cells were also present. Our data indicate that the *ex vivo* proliferative function of both CD4<sup>+</sup> and CD8<sup>+</sup> TIL and IFN- $\gamma$  secretion in response to polyclonal stimuli were enhanced significantly by blocking PD-L1, TIM3 or LAG3, while blocking CTLA4 only significantly enhanced CD4<sup>+</sup> TIL proliferation (Fig. 5B,C). Similar effects of blocking the PD-1/PD-L1 pathway on CD8<sup>+</sup> TIL and blocking the TIM3/GAL-9 pathway on CD4<sup>+</sup> TIL in HCC patients have been shown before,<sup>20, 21, 23, 31</sup> but we are the first to demonstrate that blocking PD-L1, LAG3 or TIM3 can enhance the functional responsiveness of both CD4<sup>+</sup> and CD8<sup>+</sup> TIL in HCC patients. Even more importantly, we found that combining anti-PD-L1 antibody with anti-TIM-3, anti-LAG-3 or anti-CTLA-4 antibody further enhanced the functionality of CD4<sup>+</sup> or CD8<sup>+</sup> TIL compared to single PD-L1 blockade (Fig. 5D,E).

The effects of inhibitory receptor-ligand blockade on responses of tumor-infiltrating T cells to tumor antigens were studied in two different assays. In the first TAA peptide assay, activation-induced CD137, cytotoxic degranulation marker CD107a, and effector cytokine

IFN- $\gamma$  and TNF- $\alpha$  levels were variably increased in CD8<sup>+</sup> TIL in the presence of single anti-PD-L1, anti-TIM3, anti-LAG3 or anti-CTLA4 antibody (Fig. 6B). Importantly, combined blocking treatments further enhanced these effects in part of the patients compared to single anti-PD-L1 antibody. In the second assay, we used autologous CD40-activated B cell blasts transfected with mRNA encoding full-length GPC3 or MAGEC2 to measure responses of CD4<sup>+</sup> and CD8<sup>+</sup> TIL to the natural repertoire of TAA-derived peptides irrespective of patient HLA typing.<sup>40</sup> Tumor antigen-specific proliferation of CD8<sup>+</sup> and CD4<sup>+</sup> TIL was increased up to four-fold in the presence of single anti-PD-L1, anti-TIM3, anti-LAG3 or anti-CTLA4 antibody, but the responses to single blockade were also variable between individual patients (Fig. 7D). Combined treatments of anti-PD-L1 with either anti-TIM3, anti-LAG3 or anti-CTLA4 antibody enhanced proliferation of both CD8<sup>+</sup> and CD4<sup>+</sup> TIL in more patients compared to single PD-L1 blockade. A recent phase 1 clinical trial of tremelimumab (CTLA4 blockade) has reported a manageable safety profile and a response rate of 17.6% in 17 HCV-related advanced HCC patients.<sup>41</sup> Very recently in a phase 1/2 clinical trial, nivolumab (PD-1 blockade) has demonstrated an objective response rate of 20%, disease control with stable disease for  $\geq 6$  months in 37%, and encouraging overall survival in 214 advanced HCC patients in the dose-expansion phase regardless of HCC etiology. In addition, liver toxicity was limited and manageable in the majority of patients, while symptomatic treatment-related adverse events were comparable in patients with and without HCV or HBV infection.<sup>42</sup> These studies are the first to show safety and efficacy of immune checkpoint blockade in HCC patients. Further clinical trials should be performed to verify whether blockade of TIM3 and LAG3 has clinical efficacy in HCC patients, and whether combined targeting of inhibitory pathways improves clinical responses.

In some of the patients we could isolate sufficient TIL to determine the expression of inhibitory receptors directly *ex vivo* in addition to performing the *ex vivo* polyclonal or mRNA-encoded TAA-specific functional assays. In both assays, the high-responding tumor-infiltrating T cells (those with > median % increase of TIL responses in the presence of PD-L1, TIM3, LAG3 or CTLA4 blockade) displayed higher frequencies of PD-1, TIM3 or LAG3-expressing cells than low or non-responding tumor-infiltrating T cells (those with < median % increase of TIL responses), respectively (Supplementary Fig. 5 & 6), suggesting that the inter-individual variations in TIL responses to single immune checkpoint blockade may be related to the differences in the expression levels of the corresponding inhibitory receptor on TIL. Interestingly, the expression levels of several investigated inhibitory receptors on tumor-infiltrating T cells correlated with those on circulating T cells (Supplementary Fig. 2). Although correlations were weak, these findings support the idea that the expression of

inhibitory receptor on circulating T cells may become useful as biomarkers to predict clinical response of individual HCC patient to checkpoint blockade therapy.

Our study has several limitations: 1) the effects of immune checkpoint blockade on tumor antigen-specific responses have been studied in limited numbers of patients, because patient samples are limited and these assays comparing many conditions require large numbers of TIL; 2) in some types of experiments, not all the conditions could be performed in every patient sample, due to limited numbers of isolated cells; 3) we included only two TAA (GPC3 and MAGEC2) in our study, but these antigens are among the most prevalent HCC-specific antigens,<sup>24</sup> they are selectively expressed on tumor cells and have been proven to be immunogenic,<sup>29, 43</sup> 4) our patients all underwent liver resection, and therefore are not representative for the whole HCC patient population; 5) in our patient cohort, there are more non-viral hepatitis patients than viral hepatitis patients, which is different from Asian HCC patient cohorts.

In summary, we conclude that PD-1, TIM3 and LAG3 are up-regulated on TAA-specific TIL in HCC patients. While blocking individual inhibitory pathway has variable effects, combining PD-L1 blockade with TIM3, LAG3 or CTLA4 blockade revitalizes *ex vivo* tumor-specific TIL responses in most patients and additively enhances the effects compared to single PD-L1 blockade. Therefore, our study provides evidence that PD-1/PD-L1, TIM3 and LAG3 are promising immunotherapeutic targets for the most common primary liver cancer, and suggests that the aforementioned combined targeting of immune checkpoints may result in superior clinical responses in more patients than targeting a single checkpoint.

## Figure legends

**Figure 1. Expression of inhibitory receptors on CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL in tumors, TFL, and blood.** PBMC and mononuclear leukocytes isolated from tumors and TFL were stained with antibodies against PD-1, TIM3, LAG3, CTLA4 and BTLA. (A) Representative dot plots of inhibitory receptor expression and appropriate isotype controls on CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>-</sup> TIL and (B) CD3<sup>+</sup>CD8<sup>+</sup> TIL. (C) The percentages of inhibitory receptor positive cells among CD4<sup>+</sup> Th or CD8<sup>+</sup> CTL in tumors, TFL and blood. Values of individual patients are depicted, and lines show medians; \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. (D) Representative dot plots of co-expression of inhibitory receptors on CD4<sup>+</sup>Foxp3<sup>-</sup> TIL and CD8<sup>+</sup> TIL. (E) The mean percentages of co-expression of inhibitory receptors among CD4<sup>+</sup>Foxp3<sup>-</sup> TIL (n=12) or CD8<sup>+</sup> TIL (n=22).

**Figure 2. Expression of PD-1, TIM3 and LAG3 is increased on TAA-specific CD8<sup>+</sup> TIL.** PBMC and TIL of HLA-A2<sup>+</sup> patients were stained with HLA-A\*0201 dextramers loaded with GPC3 FVGEFFTDV, GPC3 FLAELAYDL, MAGEC2 ALKVDVEERV, or MAGEC2 LLFGLALIEV peptides. (A) Representative dot plots of TAA dextramer staining compared to negative control. CD8<sup>+</sup> and CD8<sup>+</sup>CD4<sup>+</sup> TIL were gated within CD56<sup>-</sup>CD14<sup>-</sup>CD19<sup>-</sup> viable cells. (B) The percentages of dextramer-binding CD8<sup>+</sup> T cells in tumors of seven patients and in blood of three patients with detectable dextramer-binding CD8<sup>+</sup> T cells. Lines show medians. For each patient, the value of either the only or the dominant dextramer-binding population is depicted. (C) Representative histograms of inhibitory receptor expression on GPC3 FVGEFFTDV dextramer positive (blue line) versus dextramer negative (red line) CD8<sup>+</sup> TIL. (D) The percentages of PD-1, TIM3, LAG3 or CTLA4 positive cells in TAA dextramer positive versus TAA dextramer negative CD8<sup>+</sup> T cells in tumors and blood. For each patient, the value of either the only or the dominant dextramer-binding population is depicted. \* p < 0.05, \*\* p < 0.01. (B)(D) Blue symbols: GPC3 FVGEFFTDV positive; green symbol: GPC3 FLAELAYDL positive; red symbols: MAGEC2 LLFGLALIEV positive.

**Figure 3. Tumor-infiltrating antigen-presenting cells express inhibitory ligands.** Expression of inhibitory ligands PD-L1, GAL-9, MHC-II, CD86 and CD80 was measured by flow cytometry. (A) The percentages of B cells, mDC and monocytes within CD45<sup>+</sup> cells gated from tumors, TFL and blood. Lines depict medians; \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. (B) The absolute numbers of B cells, mDC and monocytes isolated per gram of tumor and TFL tissues. Values of individual patients are presented, lines depict medians. (C) Representative histograms of inhibitory ligand expression on tumor-infiltrating B cells, mDC and monocytes. Upper panel: ligand staining, lower panel: isotype control. (D) The

percentages of inhibitory ligand positive cells within tumor-infiltrating B cells, mDC or monocytes in individual patients are presented; lines depict medians.

**Figure 4. Tumor-infiltrating T cells expressing inhibitory receptors display a more activated status.** TIL were stained *ex vivo* with antibodies against surface activation markers HLA-DR and CD69 and stained with an antibody against the cytotoxic effector molecule Granzyme B intracellularly. (A) The percentages of HLA-DR<sup>+</sup> or CD69<sup>+</sup> cells in CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>-</sup> TIL or CD3<sup>+</sup>CD8<sup>+</sup> TIL which do or do not express PD-1, TIM3, LAG3 or CTLA4 are presented. (B) The percentages of Granzyme B<sup>+</sup> cells in CD8<sup>+</sup> TIL which do or do not express inhibitory receptors. (C) TIL were exposed to PMA and ionomycin at 37°C for five hours, in the presence of protein transport inhibitor brefeldin during the last four hours, followed by intracellular cytokine staining. The percentages of cytokine-producing cells in TIL which do or do not express inhibitory receptors are presented. Values of individual patients are depicted, and lines show means. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

**Figure 5. PD-L1, TIM3 or LAG3 blockade increases *ex vivo* proliferation and cytokine production of CD4<sup>+</sup> and CD8<sup>+</sup> TIL in response to polyclonal stimuli.** CFSE-labeled TIL were stimulated with CD3/CD28 beads, in the presence or absence of 10  $\mu\text{g/ml}$  neutralizing antibodies. (A) Dot plots show the proliferation (CFSE-low) of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> TIL after stimulation with CD3/CD28 beads, without addition of neutralizing antibody. Scatter plot shows the percentages of the proliferating (CFSE-low) CD4<sup>+</sup> and CD8<sup>+</sup> TIL of individual patients, without addition of neutralizing antibody. (B) (D) Effects of blocking inhibitory interactions on CD4<sup>+</sup> and CD8<sup>+</sup> TIL proliferation ( $n=10$ ). Since the proliferation differed between patients, the results are reported as relative proliferation percentages calculated by dividing the percentages of proliferating (CFSE-low) T cells in the conditions with neutralizing antibody or with combinations of antibodies or with isotype controls, by the percentages in the condition with only CD3/CD28 beads. Values are depicted as means with SEM, \*  $p < 0.05$ , \*\*  $p < 0.01$ . (C) (E) IFN- $\gamma$  accumulation in culture supernatant was quantified by ELISA. Values are depicted as medians with interquartile range ( $n=10$ ).

**Figure 6. Combining PD-L1 blockade with TIM3, LAG3 or CTLA4 blockade additively increases *ex vivo* responses of CD8<sup>+</sup> TIL to TAA peptide stimulation.** TIL of HLA-A2<sup>+</sup> patients were stimulated with pooled GPC3 FVGEFFTDV, GPC3 FLAELAYDL, MAGEC2 ALKVDVEERV and MAGEC2 LLFGLALIEV peptides (1  $\mu\text{g/ml}$  of each), in the presence or absence of 10  $\mu\text{g/ml}$  neutralizing antibodies. Intracellular cytokine production, CD137 and surface CD107a expression were analyzed after restimulation with pooled HCC peptides. Restimulation with HCV antigen peptide served as negative control for HCV negative



patients (including patients negative for both HCV and HBV), while restimulation with HBV antigen peptide served as negative control for HBV negative patients. (A) Representative dot plots of CD137, CD107a, IFN- $\gamma$  and TNF- $\alpha$  expression in CD3<sup>+</sup>CD8<sup>+</sup> TIL. (B) CD8<sup>+</sup> TIL responses to TAA peptides of seven patients, which are reported as fold increase calculated by dividing the percentages of CD137<sup>+</sup>, CD107a<sup>+</sup>, IFN- $\gamma$ <sup>+</sup> or TNF- $\alpha$ <sup>+</sup> cells in the conditions with neutralizing antibody or with combinations of antibodies, by the percentages in the condition with only pooled HCC peptides. Not all four read-outs were detectable in every patient, therefore results of four to seven patients are presented for each type of response. Each line and each color represent one patient.

**Figure 7. Combining PD-L1 blockade with TIM3, LAG3 or CTLA4 blockade additively increases *ex vivo* proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> TIL in response to TAA presented by mRNA-transfected autologous B cell blasts.** (A) HLA-A\*0201-restricted GPC3 FVGEFFTDV-specific CD8<sup>+</sup> T cell clone or human T cells transfected with a TCR recognizing MAGEC2 ALKVDVEERV were co-cultured with HLA-A2<sup>+</sup> B cell blasts transfected with GPC3 mRNA or MAGEC2 mRNA. EGFP mRNA- or mock- (without mRNA) electroporated B cell blasts served as negative controls, while HLA-A2<sup>+</sup> B cell blasts pulsed with 1  $\mu$ g/ml FVGEFFTDV peptide or ALKVDVEERV peptide served as positive controls. IFN- $\gamma$  accumulation in culture supernatant was quantified by ELISA after 24 hours. (B) CFSE-labeled PBMC from an HCC patient were co-cultured with GPC3 mRNA-, MAGEC2 mRNA-, eGFP mRNA- or mock-electroporated autologous B cell blasts. CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation was measured by flow cytometry. (C) (D) CFSE-labeled TIL were co-cultured with GPC3 mRNA-, MAGEC2 mRNA-, eGFP mRNA- or mock-electroporated autologous B cell blasts, in the presence or absence of 10  $\mu$ g/ml neutralizing antibodies. (C) Representative dot plots of CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation in response to eGFP mRNA-transfected (negative control) and MAGEC2 mRNA-transfected autologous B cell blasts in the absence or presence of neutralizing antibody. (D) Proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> TIL of nine patients in response to GPC3 and/or MAGEC2. For each patient, the data of either the only or the average antigen-induced TIL response are presented. Since the proliferative responses to TAA differed between patients, the results are reported as fold increase of antigen-specific T cell proliferation calculated by dividing the percentages of proliferating (CFSE-low) T cells in response to GPC3 or MAGEC2 in the conditions with neutralizing antibody or with combinations of antibodies, by the percentages in the condition without neutralizing antibody.

Figure 1.

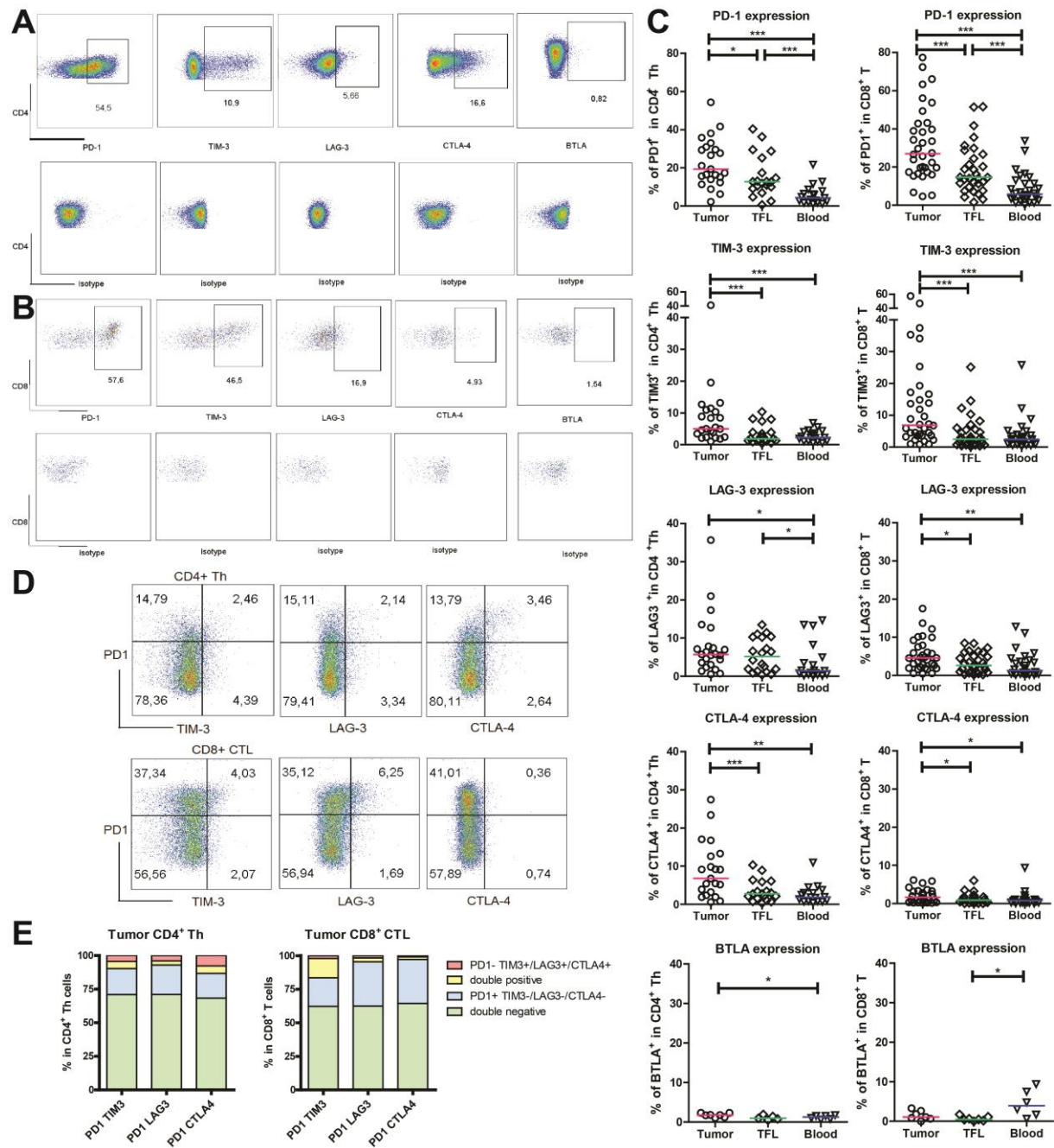
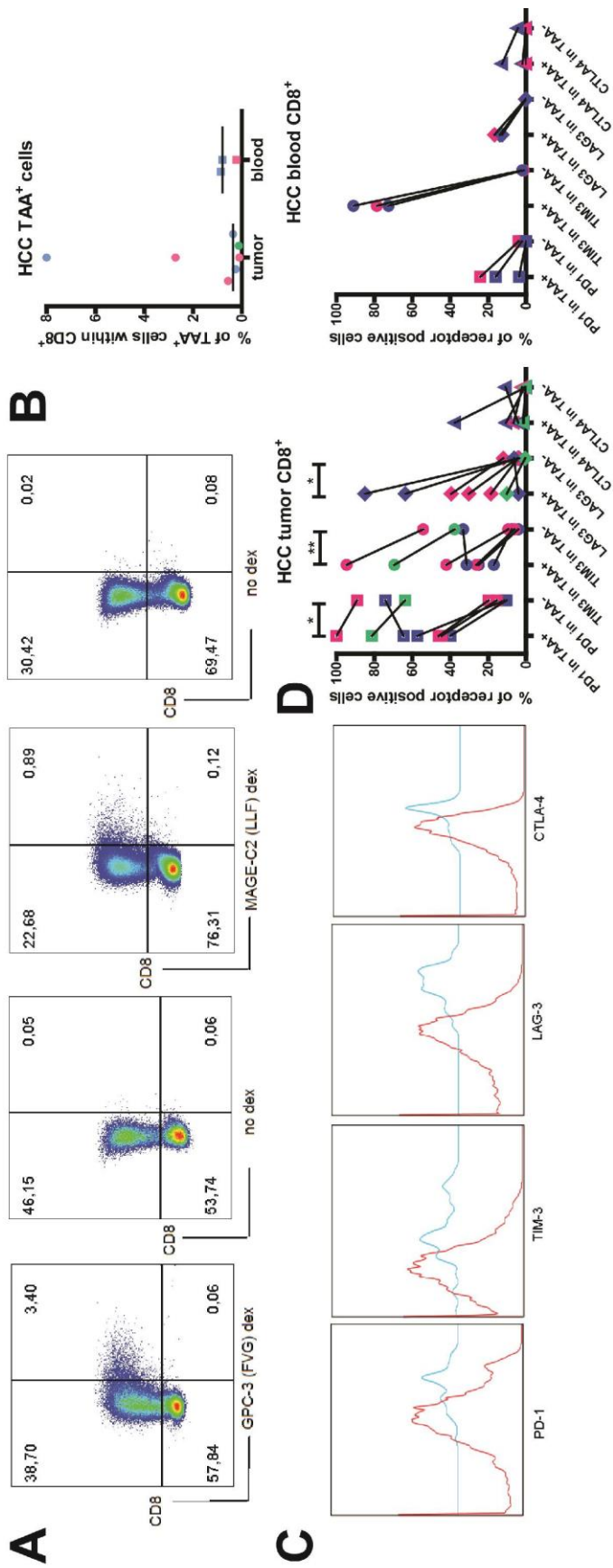


Figure 2.



**Figure 3.**

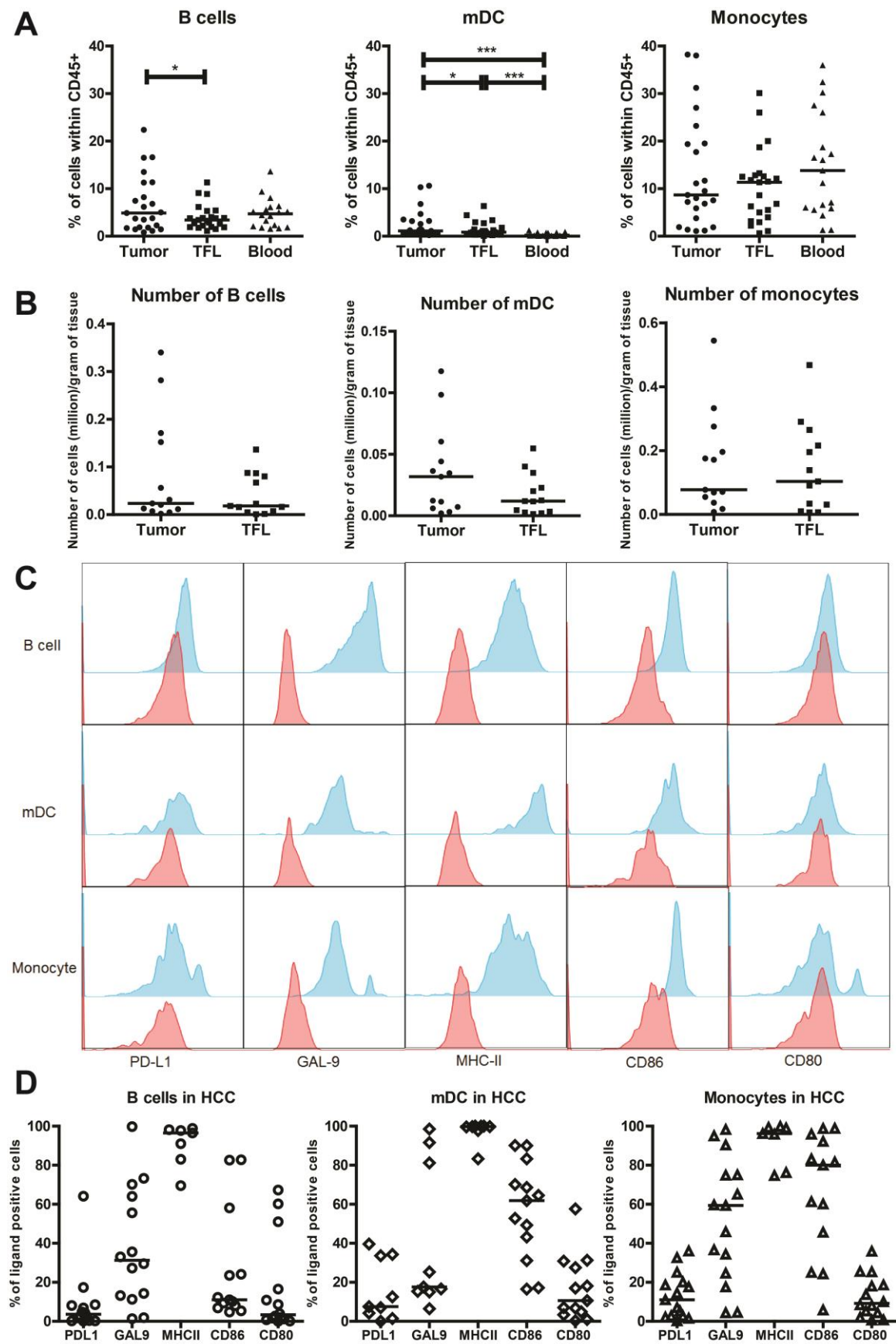


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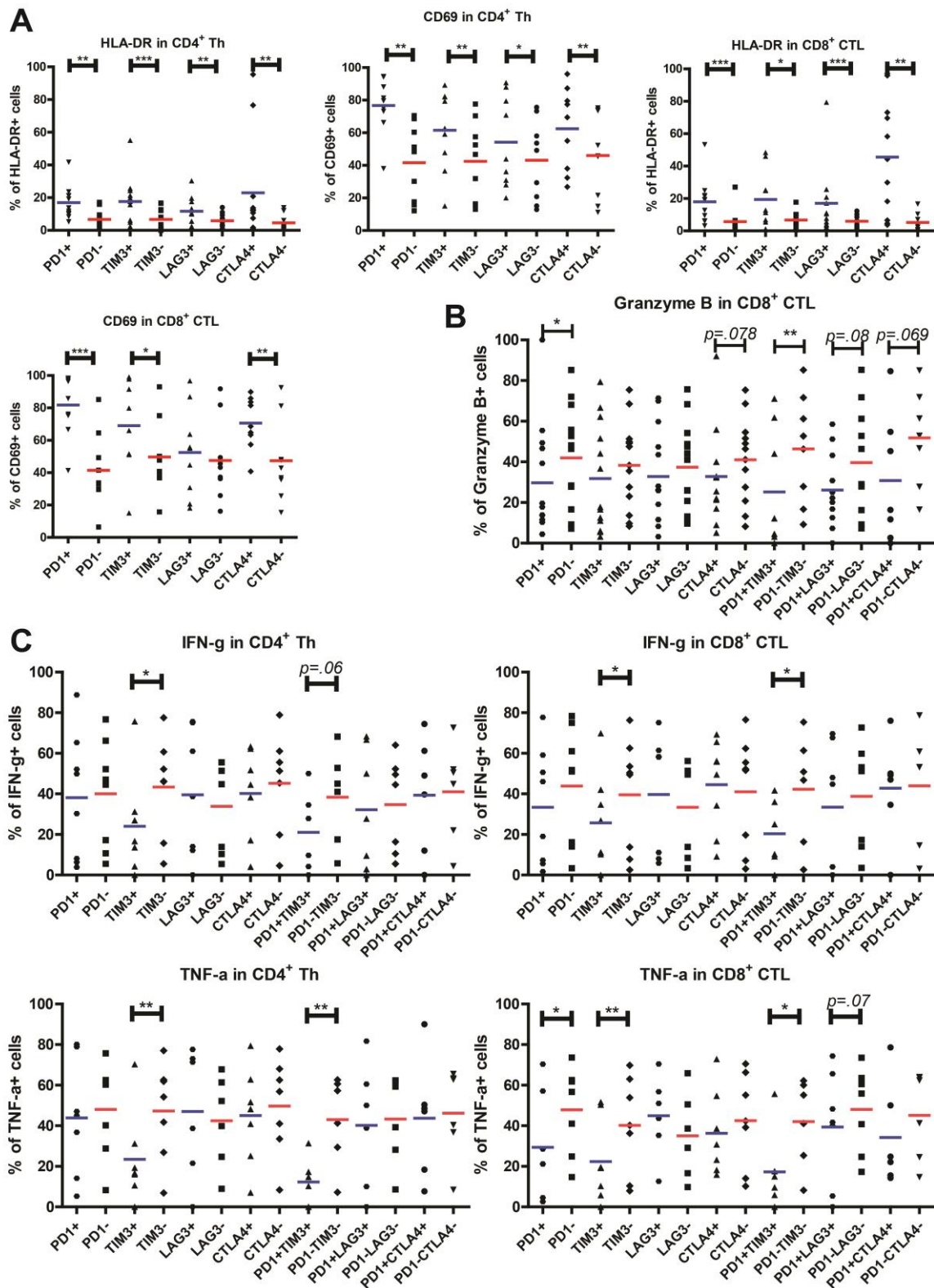
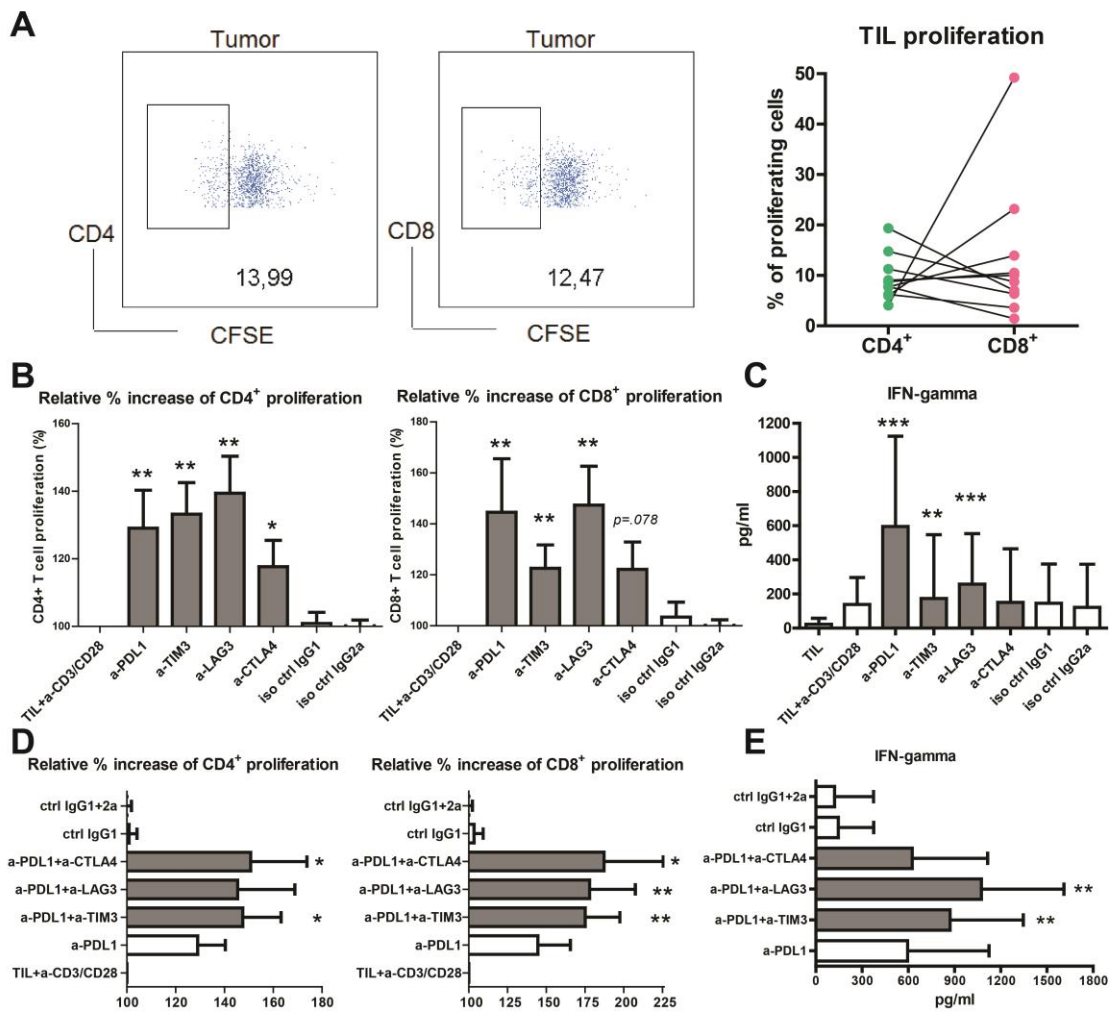


Figure 5.



**Figure 6.**

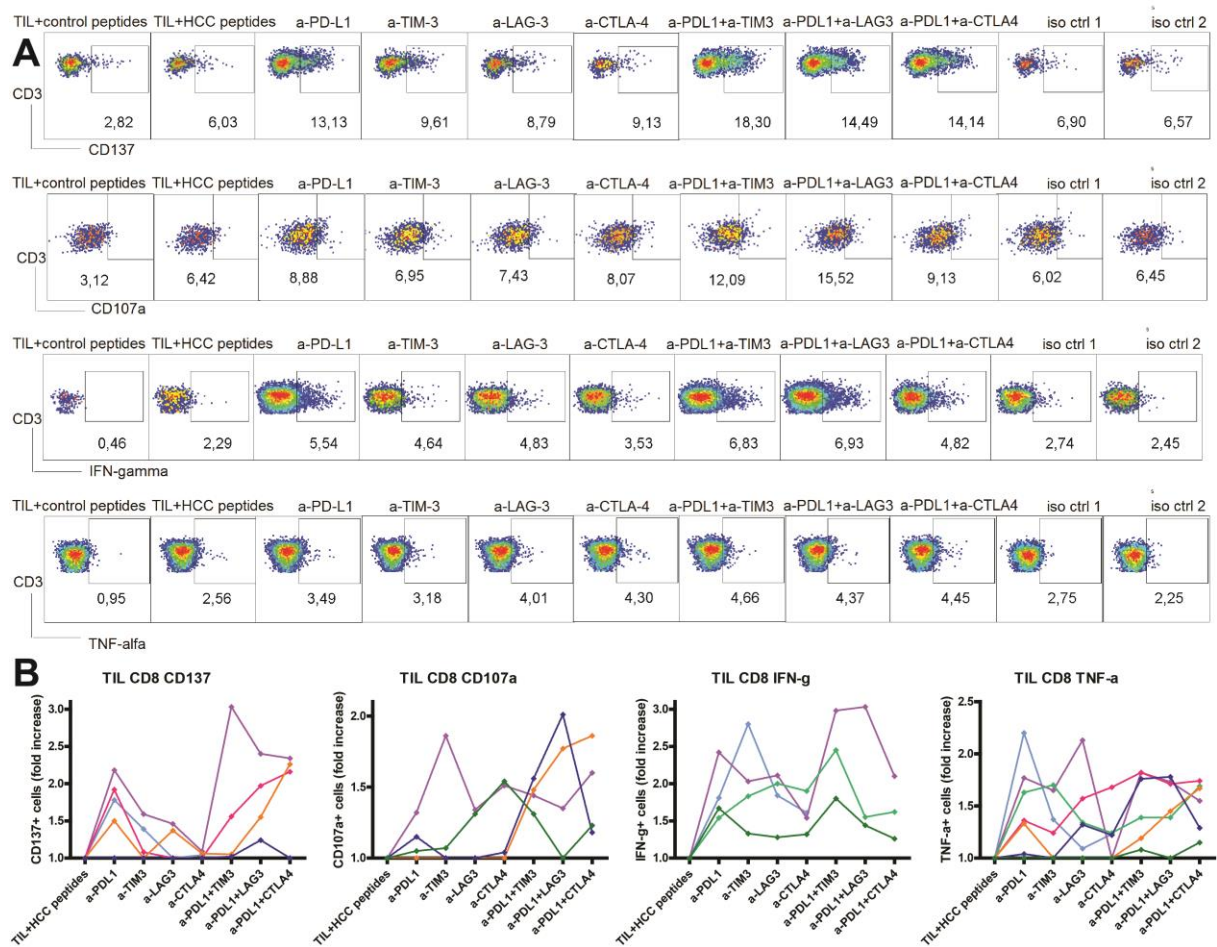
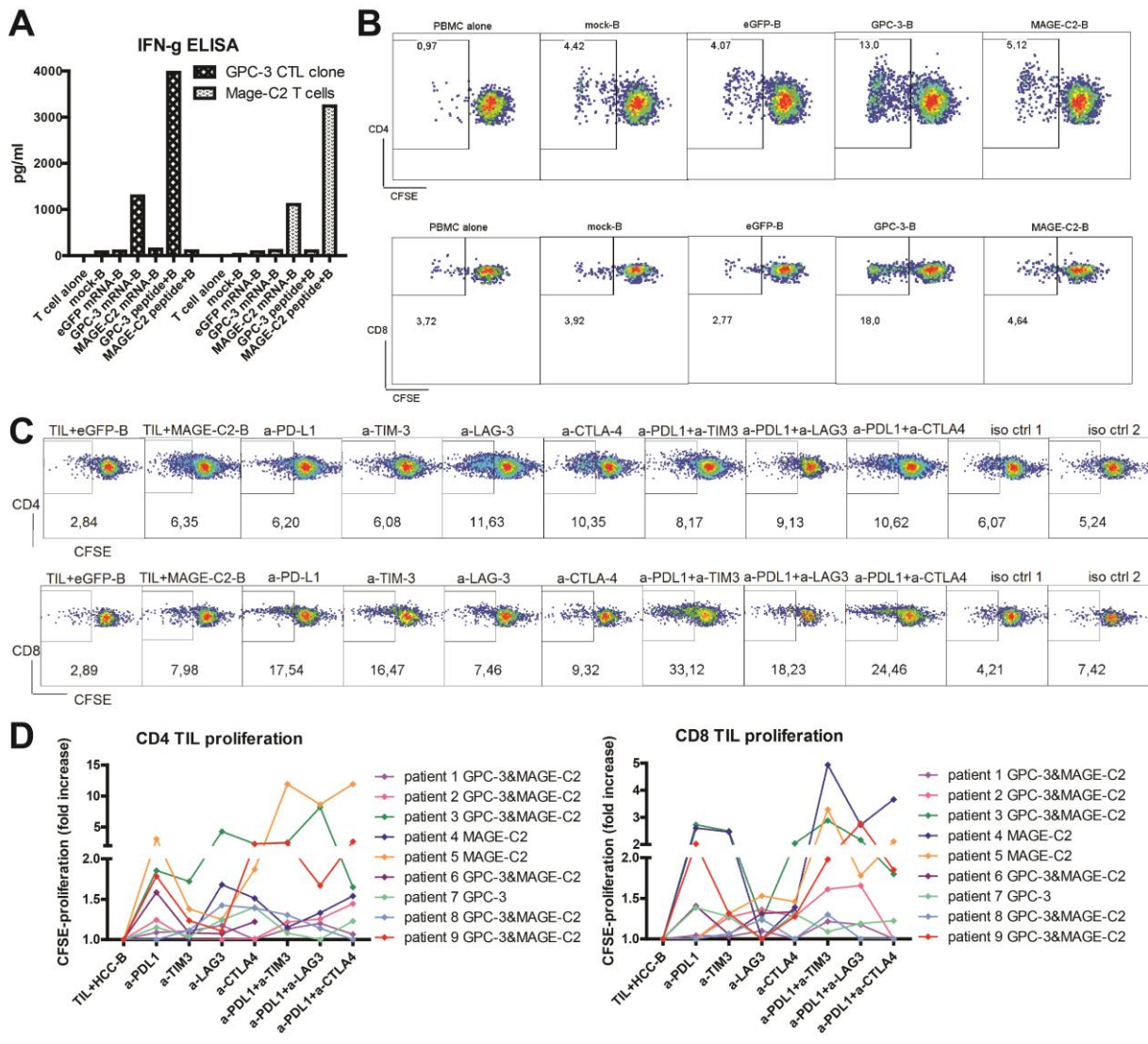


Figure 7.





### Supplementary figure legends

**Supplementary Figure 1. Expression of inhibitory receptors on CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL in the tumor, TFL, and blood.** Median fluorescence intensity of inhibitory receptors PD-1, TIM3, LAG3, CTLA4 and BTLA within (A) CD4<sup>+</sup> Th and (B) CD8<sup>+</sup> CTL from the tumor, TFL and blood. Values of individual patients are depicted, and lines show medians; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

**Supplementary Figure 2. Correlation of the expression of inhibitory receptors on T cells between the tumor and the blood.** Spearman correlation analysis demonstrates (A) positive correlations of the percentages of PD-1, TIM3, LAG3 or CTLA4 positive cells in CD4<sup>+</sup> Th between the tumor and the blood, and (B) positive correlations of the percentages of LAG3 or CTLA4 positive cells in CD8<sup>+</sup> CTL between the tumor and the blood.

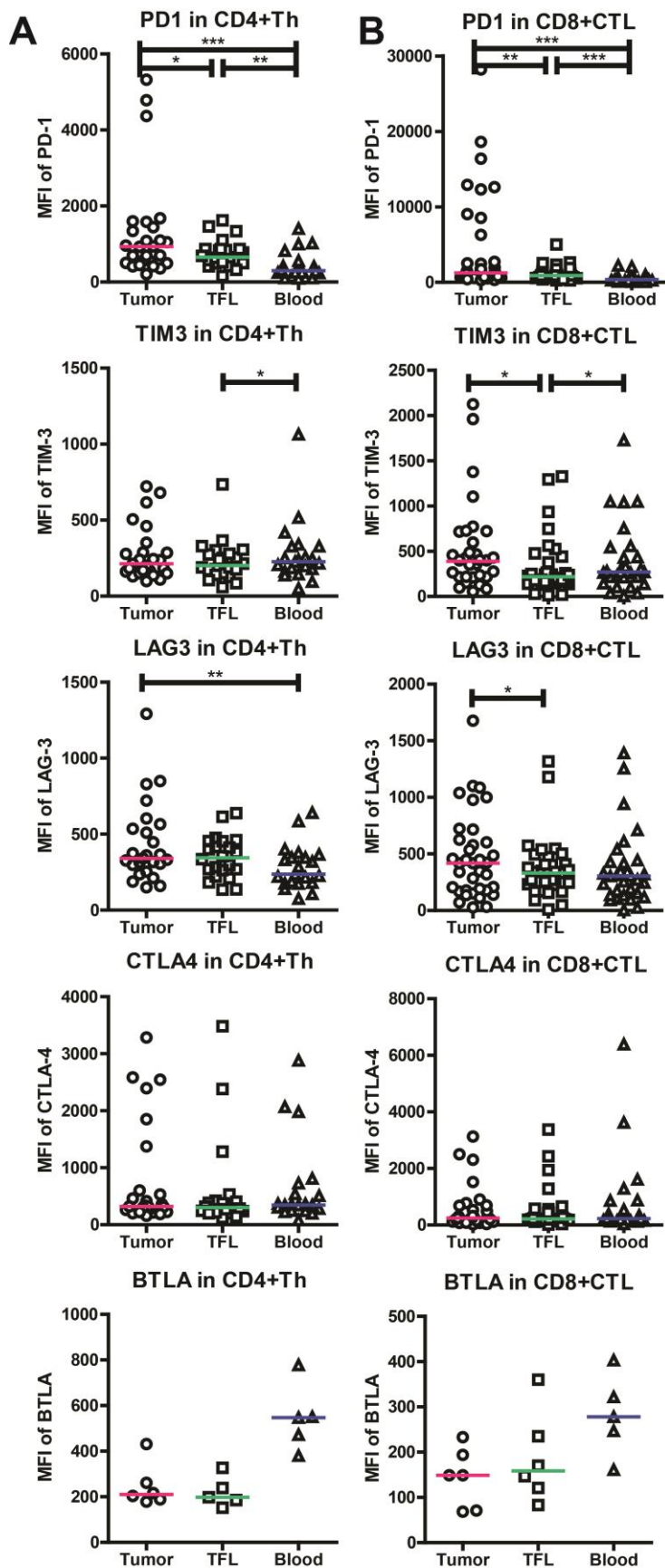
**Supplementary Figure 3. Expression of PD-1, TIM3 and LAG3 is increased on HCC TAA-specific CD8<sup>+</sup> TIL.** Median fluorescence intensity of PD-1, TIM3, LAG3 and CTLA4 in TAA dextramer positive versus TAA dextramer negative CD8<sup>+</sup> T cells in the tumor and the blood. For each patient, the value of either the only or the dominant dextramer-binding population is depicted; \*  $p < 0.05$ . Blue symbols: GPC-3 FVGEFFTDV positive; green symbols: GPC-3 FLAELAYDL positive; red symbols: MAGE-C2 LLFGLALIEV positive.

**Supplementary Figure 4. Expression of inhibitory ligands on antigen-presenting cells in the tumor, TFL and blood.** (A) Median fluorescence intensity of inhibitory ligands PD-L1, MHC-II, GAL-9, CD80 and CD86 and (B) the percentages of cells expressing inhibitory ligands within B cells, myeloid dendritic cells (mDC) and monocytes from the tumor, TFL and blood. Values of individual patients are shown; lines depict (A) means or (B) medians; \*  $p < 0.05$ , \*\*  $p < 0.01$ .

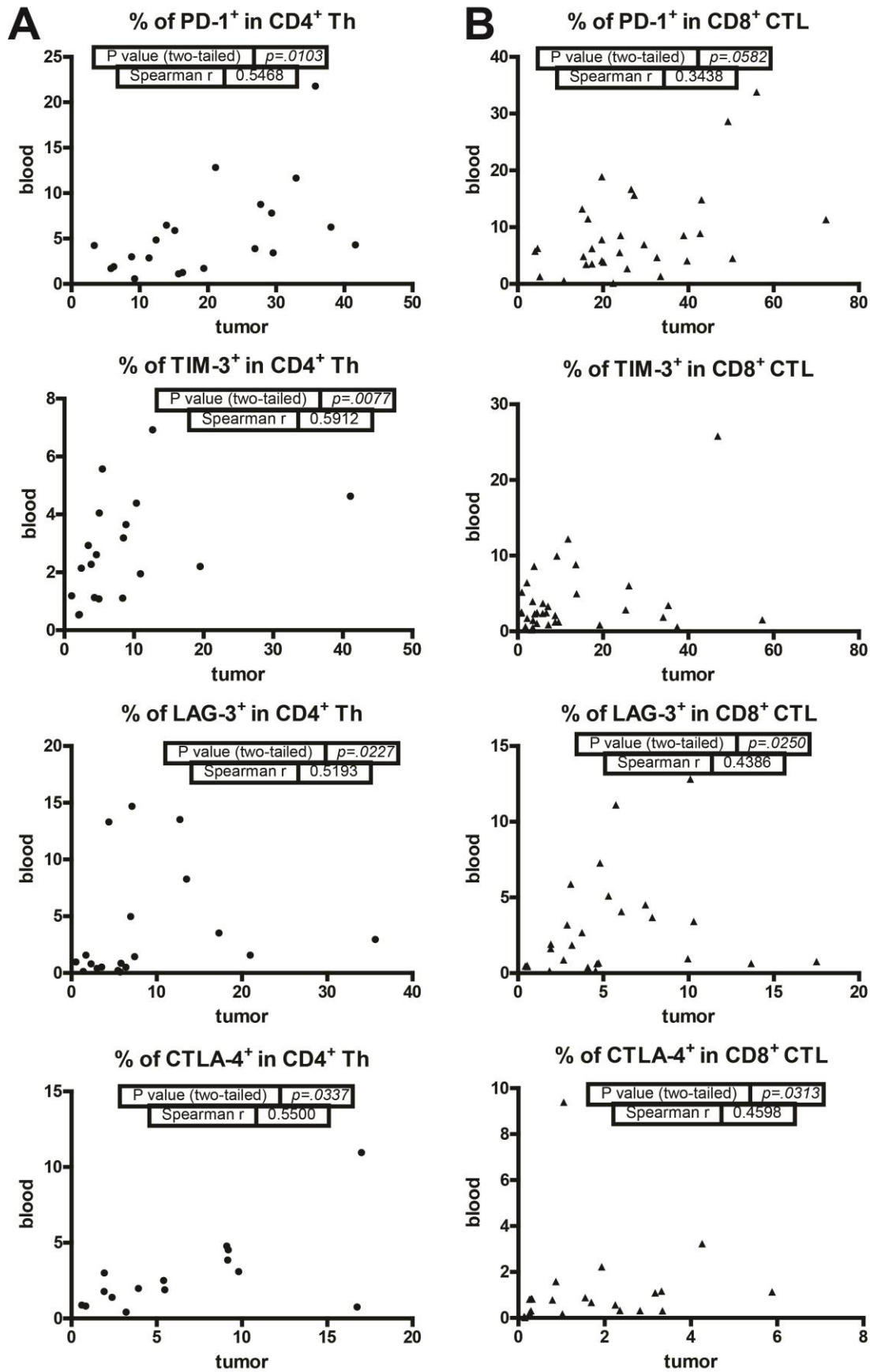
**Supplementary Figure 5. Association between the expression of inhibitory receptor and the effect of blocking antibody on TIL proliferation in polyclonal T cell activation assay.** The percentages of inhibitory receptor positive CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>-</sup> TIL or CD3<sup>+</sup>CD8<sup>+</sup> TIL are shown, comparing high-responding TIL (n=4) and low or non-responding TIL (n=4) in the four conditions with neutralizing antibody. High-responding TIL are those whose relative % increase of TIL proliferation in the presence of a neutralizing antibody is above the median % increase of all the patients, and low or non-responding TIL are those whose relative % increase of TIL proliferation is below the median level. Values are depicted as means with SEM, \*  $p < 0.05$ .

**Supplementary Figure 6. Association between the expression of inhibitory receptor and the effect of blocking antibody on TIL proliferation in mRNA-encoded full-length tumor antigen-specific T cell stimulation assay.** The percentages of inhibitory receptor positive CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>-</sup> TIL or CD3<sup>+</sup>CD8<sup>+</sup> TIL are shown, comparing high-responding TIL (n=3) and low or non-responding TIL (n=4) in the four conditions with neutralizing antibody. High-responding TIL are those whose fold increase of TIL proliferation in the presence of a neutralizing antibody is above the median fold increase of all the patients, and low or non-responding TIL are those whose fold increase of TIL proliferation is below the median level. Values are depicted as means with SEM.

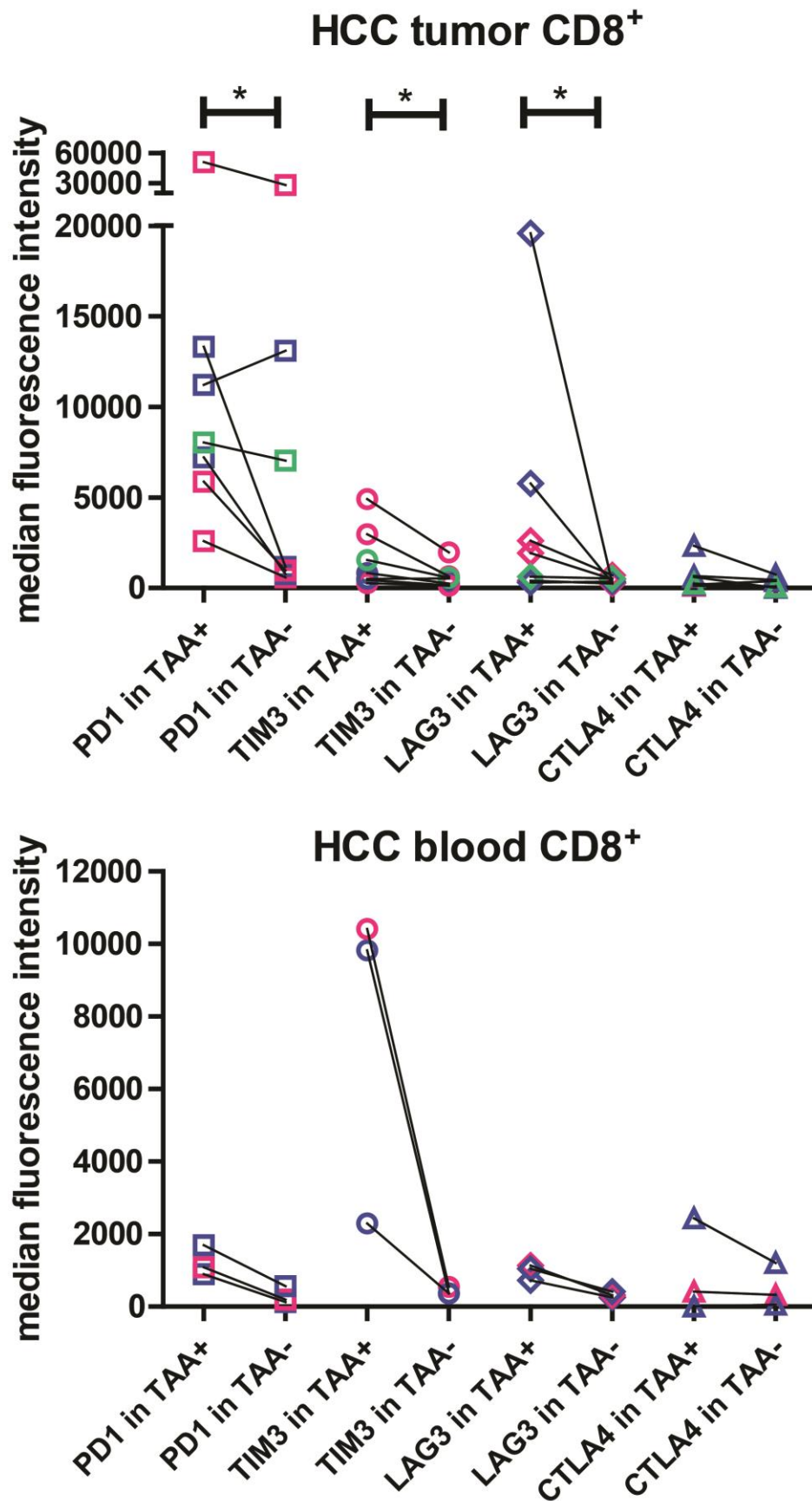
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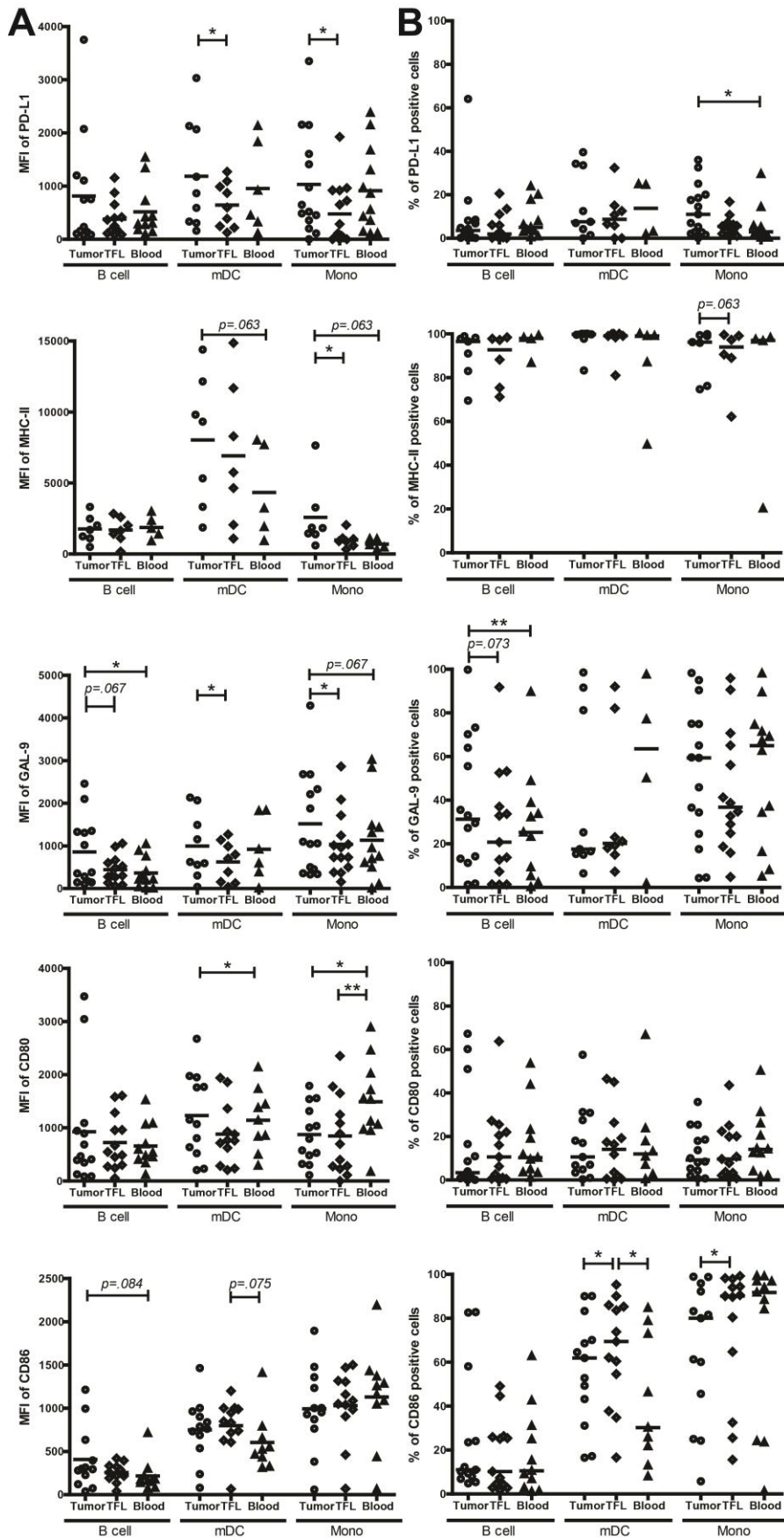
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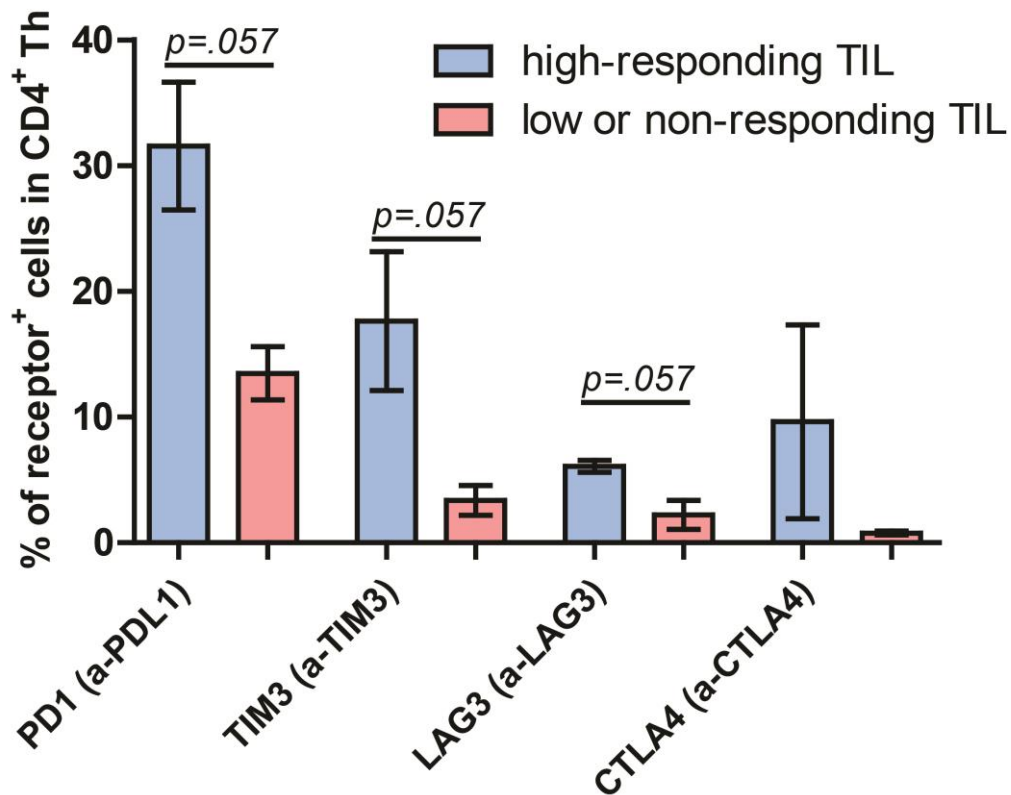
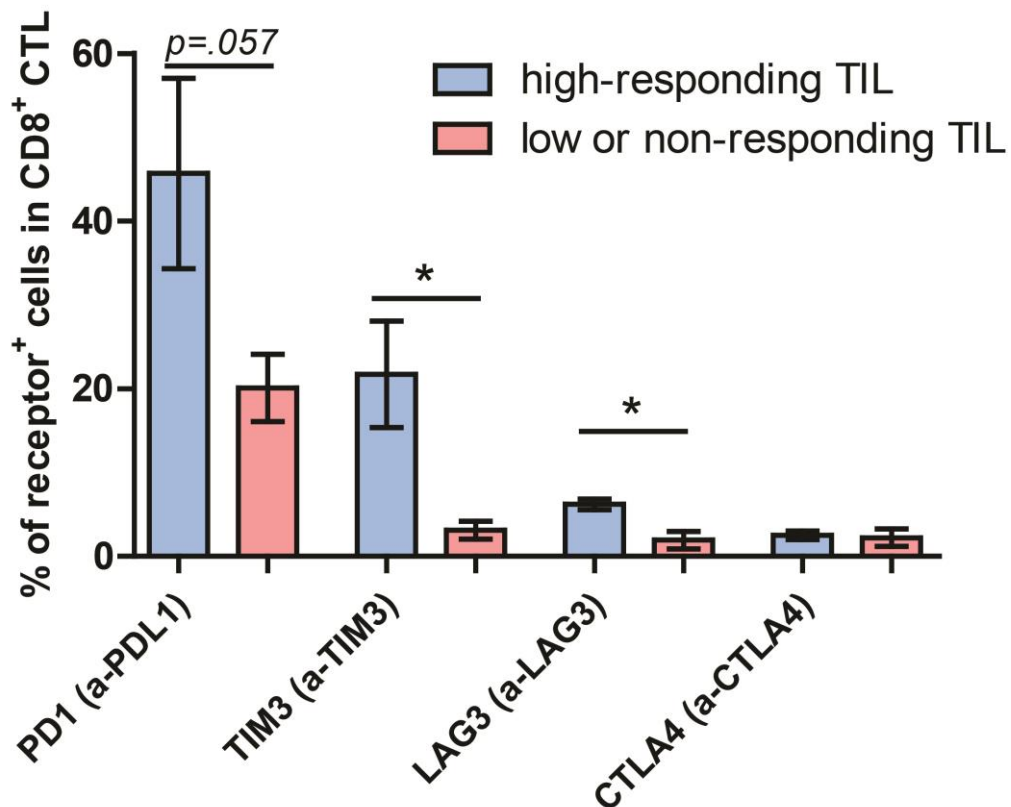
Supplementary Figure 3.



Supplementary Figure 4.

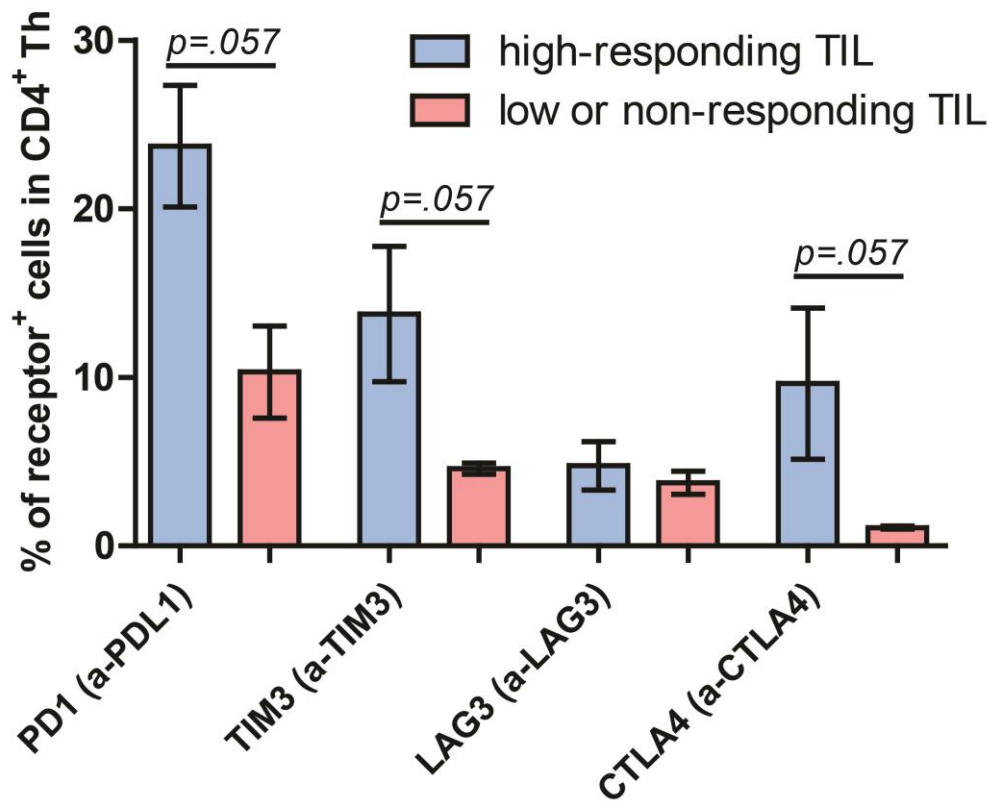


Supplementary Figure 5.

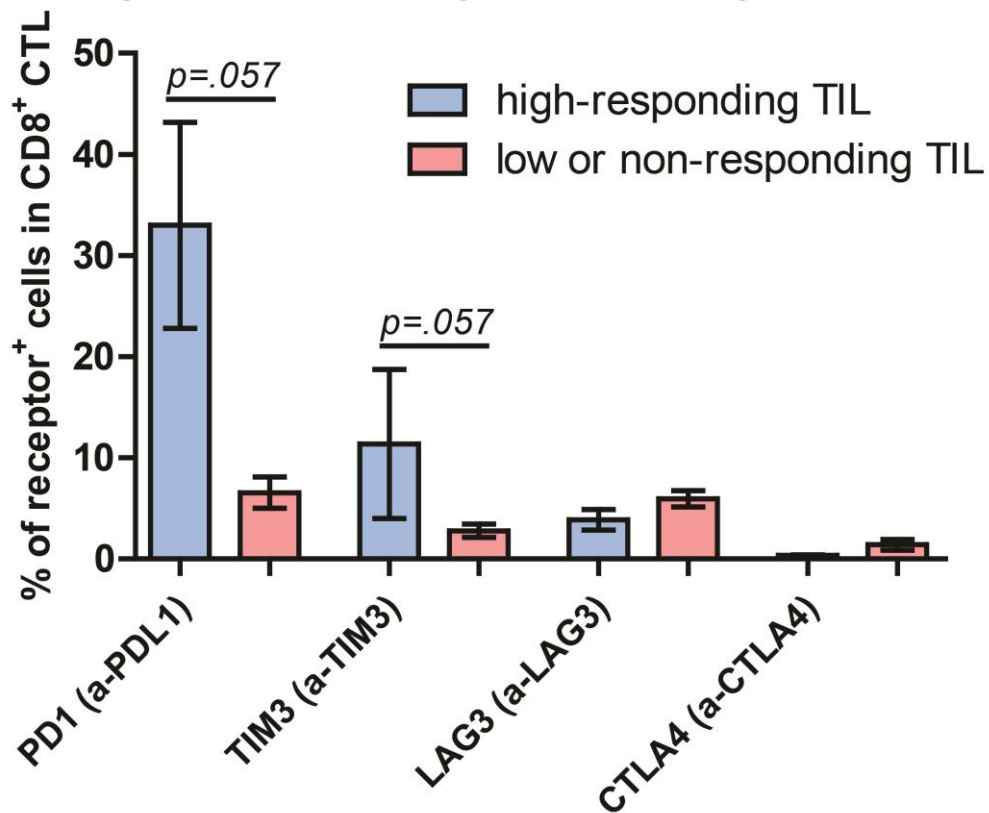
**CD4<sup>+</sup> TIL proliferation in response to checkpoint blockade****CD8<sup>+</sup> TIL proliferation in response to checkpoint blockade**

Supplementary Figure 6.

**CD4<sup>+</sup> TIL proliferation in response to checkpoint blockade**



**CD8<sup>+</sup> TIL proliferation in response to checkpoint blockade**





**Supplementary Table 1. Patient characteristics**

	HCC (n=59)
Sex (male/female)	46 / 13
Age (years)**	64.8 ± 1.8
Race (Caucasian/Asian/African)	50 / 5 / 4
ALT (units/L)**	58.7 ± 6.4
Bilirubin (µmol/L)**	16.4 ± 5.7
Protrombin time (INR)**	1.1 ± 0.02
Liver fibrosis (metavir score) F0-F1 / F2 / F3-F4-cirrhosis	28 / 4 / 27
AFP (µg/L)** <20/ 20-400/ >400/ unknown	45 / 1 / 10 / 3
Stage of disease (TNM)	St I n=28, St II n=26, St IIIa n=4, unknown n=1

Abbreviations: INR, international normalized ratio; AFP, alpha fetoprotein; TNM, tumor-node-metastasis.

Etiology of liver disease: no known liver disease (n = 23), HBV/HCV (n =10/6), alcohol-related liver disease (n = 6), HBV/HCV + alcohol (n = 3), non-alcoholic steatohepatitis (NASH)/non-alcoholic fatty liver disease (NAFLD) (n = 4/4), HBV + NASH (n = 1), porphyria (n = 1), abernathy (n=1).

\*\*Mean ± SEM.

**Supplementary Table 2. Anti-human antibodies used in flow cytometry (FACS)**

<b>Antibody</b>	<b>Clone</b>	<b>Supplier</b>	<b>Antibody</b>	<b>Clone</b>	<b>Supplier</b>
CD69 APC	L78	BD Biosciences	mIgG1-PE	P3.6.2.8.1	eBioscience
IFN-gamma-FITC	25723.11	BD Biosciences	HLA-DR-APC	LN3	eBioscience
HLA-A2-APC	BB7.2	BD Biosciences	HLA-DR-PE	LN3	eBioscience
CD19- HorizonV500	HIB19	BD Biosciences	HLA-DR-PerCP- Cy5.5	LN3	eBioscience
mIgG2a-PerCP	X39	BD Biosciences	CD14 PE-Cy7	61D3	eBioscience
CD3 PerCP- Cy5.5	UCHT1	BD Biosciences	CD3 PE-Cy7	UCHT1	eBioscience
HLA-A2-FITC	BB7.2	BD Biosciences	CD8-APC	okt-08	eBioscience
mIgG2b-FITC	27-35	BD Biosciences	CD14-eFlour450	61D3	eBioscience
mIgG1-PB	MOPC-21	BD Biosciences	IFN-gamma-Pe- Cy7	4S.B3	eBioscience
mIgG2b-PE	27-35	BD Biosciences	HLA-DR-APC- eFluor780	LN3	eBioscience
mIgG1-PerCP	X40	BD Biosciences	CD14-FITC	61D3	eBioscience
mIgG1-FITC	MOPC-21	BD Biosciences	CD279 (PD1)- PE-Cy7	J105	eBioscience
mIgG1-pacific blue	MOPC-21	BD Biosciences	CD8-efluor450	RPA-T8	eBioscience
GranzymeB-V450	GB11	BD Biosciences	CD274(B7-H1)- PE-Cy7	MIH1	eBioscience
CD137 (4-1BB)- APC	4B4-1	BD Biosciences	CD270 (HVEM)- PE	eBioHVEM- 122	eBioscience
CD19 PE	J4.119	Beckman	CD4-APC- eFluor780	OKT4	eBioscience
CD69-PE	TP1.55.3	Beckman	CD3-APC- eFluor780	SK7	eBioscience
CD3 FITC	UCHT1	Beckman	FoxP3-eFluor450	236A/E7	eBioscience
mIgG2a-PE	U7.27	Beckman	CD45 efluor 450	HI30	eBioscience
CD152 (CTLA4)- APC	L3D10	Biolegend	CD19-APC- eFluor780	HIB19	eBioscience
CD272 (BTLA)- APC	MIH26	Biolegend	CD8a-PerCp- Cy5.5	RPA-T8	eBioscience
CD3 PE	UCHT1	Biolegend	TNF $\alpha$ APC	MAB11	eBioscience
CD86-PB	IT2.2	Biolegend	CD223(LAG3)- PE	3DS223H	eBioscience
anti-TNF- $\alpha$ - PerCP-Cy5.5	Mab11	Biolegend	CD223(LAG3)- PerCP-eF710	3DS223H	eBioscience
TIM3-Brilliant- Vio421	F38-2E2	Biolegend	CD8-FITC	SK1	eBioscience
Galectin-9 PE	9M1-3	Biolegend	rat IgG2a-APC	eBR2a	eBioscience
CD80-PE-Cy7	2D10	Biolegend	mIgG1-FITC	P3	eBioscience
CD14-BV510	M5E2	Biolegend	mIgG2a-APC	eBM2a	eBioscience
CD56-BV510	HCD56	Biolegend	rat IgG2b-APC	eB149/10H5	eBioscience
HLA-DR, DP, DQ- FITC	Tü39	Biolegend	CD8-PE	RPA-T8	eBioscience
mIgG1-Pe-Cy7	MOPC-21	Biolegend	CD107a PE	H4A3	eBioscience

rat IgG2a-PE	RTK2758	Biolegend	TIM3-PE	344823	R&D systems
mlgG2a-FITC	MOPC-173	Biolegend	BDCA1-APC	AD5-8E7	Miltenyi
mlgG1-APC	MOPC-21	Biolegend			

**Supplementary Table 3. The percentages of CD8<sup>+</sup> T cells binding to different TAA-peptide-HLA-A\*0201-dextramer complexes in tumors of 13 HLA-A2<sup>+</sup> HCC patients and in blood of 12 HLA-A2<sup>+</sup> HCC patients**

Epitope	FVG	FLA	ALK	LLF	FVG	FLA	ALK	LLF
	GPC-	GPC-	MAGE-	MAGE-				
<b>TAA</b>	3	3	C2	C2	GPC-3	GPC-3	MAGE-C2	MAGE-C2
<b>Patient</b>	TIL	TIL	TIL	TIL	PBMC	PBMC	PBMC	PBMC
1	0.36%	0.32%		0.35%				
2					0.8%	0.61%		0.64%
3				0.56%				0.23%
4					0.87%			
5	0.26%	0.15%						
6								
7				0.11%				
8				2.72%				
9					NT	NT	NT	NT
10	8.00%							
11								
12								
13		0.13%						

Abbreviations: TAA, tumor-associated antigen; NT, not tested; FVG, GPC3 peptide FVGEFFTDV; FLA, GPC3 peptide FLAELAYDL; ALK, MAGEC2 peptide ALKVDVEERV; LLF, MAGEC2 peptide LLFGLALIEV; TIL, tumor-infiltrating lymphocytes; PBMC, peripheral blood mononuclear cells.

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## Supplementary Materials and Methods

### *Flow cytometric analysis*

Fresh PBMC and mononuclear leukocytes isolated from tumor and TFL were analyzed for expression of surface and intracellular markers using specific antibodies (Supplementary Table 2). Cell surface staining with fluorochrome-conjugated antibodies was *performed* in the dark at 4°C for 30 minutes, then cells were washed and resuspended in PBS with 0.2 mM EDTA and 0.5% human serum. For Foxp3, Granzyme B and CTLA4 staining, cells were fixed and permeabilized using the Foxp3 staining buffer set from eBioscience (Vienna, Austria). For intracellular cytokine staining, cells were exposed to 40 ng/mL PMA (Sigma, Zwijndrecht, The Netherlands) and 1 µg/mL ionomycin (Sigma) at 37°C for five hours in the presence of 5 µg/mL brefeldin (Sigma) during the last four hours, followed by staining of IFN-γ and TNF-α upon fixation and permeabilization using the Foxp3 staining buffer set. To identify TAA-specific T cells, PBMC and TIL of HLA-A2<sup>+</sup> HCC patients (as determined by flow cytometric analysis of binding to anti-HLA-A2 antibody) were pre-treated with protein tyrosine kinase inhibitor (PKI) dasatinib at a final concentration of 50 nM in PBS at 37°C for 30 minutes.<sup>1-3</sup> Cells were then stained with PE-labeled HLA-A\*0201/GPC3 144-152 (FVGEFFTDV), HLA-A\*0201/GPC3 522-530 (FLAELAYDL), HLA-A\*0201/MAGEC2 336-344 (ALKVDVEERV), HLA-A\*0201/MAGEC2 191-200 (LLFGLALIEV) (Immudex, Copenhagen, Denmark) or no dextramer (as a negative control) at room temperature for 10 minutes. Subsequently, cells were stained with CD8 (clone SK1), CD4, PD-1, TIM3, LAG3, CD56, CD19, CD14 antibodies at 4°C for 20 minutes and then CTLA4 antibody intracellularly using the Foxp3 staining buffer set. Dead cells were excluded by using a LIVE/DEAD fixable dead cell stain kit with aqua fluorescent reactive dye (Invitrogen, Paisley, UK). Cells were measured by a FACSCanto II flow cytometer (BD Biosciences, San Diego, USA) and analyzed using FlowJo software. Appropriate isotype control antibodies (Supplementary Table 2) were used for gating purposes.

### *Ex vivo polyclonal T cell activation assay*

All TIL cultures were performed in RPMI 1640 (Lonza, Breda, The Netherlands) supplemented with 10% human AB serum (Invitrogen), 2mM L-glutamine (Invitrogen), 50 mM Hepes Buffer (Lonza), 1% penicillin-streptomycin (Life Technologies), 5mM Sodium Pyruvate (Gibco) and 1% MEM NEAA at 37°C. TIL were labeled with 0.1 µM CFSE (Invitrogen); afterwards 100,000 cells in 200 µl medium were cultured in each well of a 96-well round-bottom culture plate and stimulated with anti-human CD3/CD28 dynabeads (Gibco-Life Technologies AS, Norway) at a cell : bead ratio of 10:1, in the presence or absence of 10

µg/ml neutralizing monoclonal antibodies against human PD-L1 (clone 5H1<sup>4</sup>), TIM3 (clone F38-2E2, Biolegend, San Diego, USA<sup>5, 6</sup>), LAG3 (clone 17B4, AdipoGen, Liestal, Switzerland<sup>7</sup>) or CTLA4 (clone BNI3, Beckman Coulter, Marseille, France<sup>8</sup>), or combinations of these antibodies, or isotype-matched control antibodies (mIgG1 and mIgG2a, Biolegend, London, UK). In preliminary experiments a cell : CD3/CD28 bead ratio of 10:1 was established to provide sub-optimal stimulation of T cell proliferation. After four days, culture supernatant was collected and secretion of IFN-γ was quantified by ELISA (Ready-SET-Go!, eBioscience). CFSE-labeled cells were harvested and stained with CD8, CD4, CD3 antibodies. Dead cells were excluded by using the LIVE/DEAD fixable dead cell stain kit with aqua fluorescent reactive dye, and T cell proliferation was determined based on CFSE dilution by flow cytometry analysis.

### ***Ex vivo tumor antigen peptide stimulation assay***

TIL of HLA-A2<sup>+</sup> HCC patients were stimulated with pooled HCC TAA peptides (GPC3 peptide FVGEFFTDV,<sup>9</sup> GPC3 peptide FLAELAYDL,<sup>10, 11</sup> MAGEC2 peptide ALKVDVEERV and MAGEC2 peptide LLFGLALIEV,<sup>12</sup> which are published immunogenic epitopes restricted by HLA-A\*0201 (1 µg/ml of each peptide), in the presence or absence of 10 µg/ml neutralizing monoclonal antibodies against human PD-L1, TIM3, LAG3 or CTLA4, or combinations of these antibodies, or isotype control antibodies. 100,000 cells in 200 µl RPMI medium supplemented with 10% human AB serum, 2mM L-glutamine, 50 mM Hepes Buffer, 1% penicillin-streptomycin, 5mM Sodium Pyruvate and 1% MEM NEAA were cultured in each well of a 96-well round-bottom culture plate. Fresh medium containing 20 IU/ml recombinant human interleukin-2 (rhIL-2, Roche) was added after two days. After seven days, cells were re-stimulated with pooled HCC TAA peptides or (as negative controls) HCV nonstructural protein 5B peptide 2594-2602 (ALYDVVTKL) or HBV core protein peptide 18-27 (FLPSDFFPSV) kindly provided by Gertine W. van Oord and Yingying Dou in our laboratory for six hours in the presence of CD107a antibody, and brefeldin (5 µg/mL, Sigma) was present during the final five hours. Re-stimulation with HCV antigen peptide served as negative control for HCV negative patients (including patients negative for both HCV and HBV), HBV antigen peptide served as negative control for HBV negative patients. Cells were then stained with CD8, CD4, CD3 antibodies, followed by staining of IFN-γ, TNF-α and CD137 upon fixation and permeabilization according to the manufacturer's instructions (eBioscience A&B fixation/permeabilization kit). Dead cells were excluded by using the LIVE/DEAD fixable dead cell stain kit with aqua fluorescent reactive dye.

### ***Expansion of human B cells by stimulation with trimeric CD40 ligand and IL-4***

Blood from HLA-A2<sup>+</sup> healthy blood bank donors or HCC patients was collected (1-2 months before surgery for HCC patients) for isolating PBMC and expanding B cells. PBMC were seeded in 6-well plates (Costar, Cambridge, USA) at a concentration of  $2-3 \times 10^6$  cells/ml in IMDM (Lonza, Breda, The Netherlands) with 10% heat inactivated human AB serum, 1% penicillin-streptomycin, 1% insulin-transferrin-selenium solution (Gibco-Invitrogen, Breda, the Netherlands), 40 IU/ml recombinant human interleukin-4 (rhIL-4, Strathmann Bioscience, Germany) and 1  $\mu$ g/ml soluble human trimeric CD40 ligand (sCD40L, kindly provided by Celldex Therapeutics, Hampton, NJ, USA). During the first seven days of culture, 1 ng/ml cyclosporine A (CsA, Novartis, Basel, Switzerland) was added to prevent T cell expansion. Residual CD3<sup>+</sup> cells were depleted by magnetic cell sorting using CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) after seven days. Since then cells were passed every 3-4 days at a concentration of  $0.5 \times 10^6$  CD19<sup>+</sup> cells/ml in the presence of rhIL-4 and sCD40L but without CsA, and after three weeks expanded B cells were cryopreserved. B cells expanded using rhIL-4 and sCD40L become B cell blasts with efficient antigen-presenting capacity<sup>13, 14</sup> and are well-suited for presentation of antigens encoded by transfected mRNA to T cells.<sup>15</sup> The purity of CD40-activated B cells (B cell blasts) was checked after staining with CD19 and CD3 antibodies and was at least 95% before freezing.<sup>13</sup>

### ***In vitro generation of eGFP, GPC3 and MAGEC2 messenger RNA***

Messenger RNA (mRNA) encoding for GPC3, MAGEC2 or eGFP were synthesized by *in vitro* transcription. The back bone vector pEtheRNA was constructed in order to maximize the level and duration of protein expression. The plasmid contains a 5' translation enhancer, a 3' RNA stabilizing sequence and a 124 residue long poly A tail. GPC3 and MAGEC2 genes were ligated into this construct and are directly followed by a sequence encoding the transmembrane and luminal regions of DCLamp, which is a targeting signal for the endolysosomal compartment, resulting in peptide loading in MHC-II. The eGFP gene was ligated into the vector without additional targeting sequences. Prior to *in vitro* transcription, the DNA plasmids were linearized with *BfuAI*. RNA synthesis was performed by eTheRNA immunotherapies NV, Niel, Belgium. During transcription a cap analog was incorporated in the mRNA. The quality of the *in vitro* transcribed mRNA was confirmed by capillary gel electrophoresis and the mRNA concentration was measured by spectrophotometry. The obtained mRNA were stored in small aliquots at -80°C till use.



***Messenger RNA electroporation***

B cell blasts were thawed and washed twice, first with serum-free IMDM and subsequently with Opti-MEM (Life technologies, Bleiswijk, The Netherlands).  $5-10 \times 10^6$  B cell blasts were resuspended in a final volume of 200  $\mu$ L of Opti-MEM containing 20  $\mu$ g of mRNA. Electroporation was performed in a 4 mm gap cuvette using a Gene Pulser Xcell™ electroporation system (Bio-Rad Laboratories, Hercules, CA). We used a square wave pulse of 600 V in a pulse time of 0.6 ms in all experiments, which we found optimal for antigen presentation to T cells. Immediately after electroporation, B cell blasts were transferred into IMDM without phenol red (Life Technologies) with 10% heat-inactivated human AB serum, 1% penicillin-streptomycin and 1% insulin-transferrin-selenium solution and incubated at 37°C. Two hours after electroporation, cells were harvested for further experiments.

***GPC3-specific CD8+ T cell clone and MAGEC2-specific human T cells***

A previously described HLA-A\*0201-restricted GPC3 (FVGEFFTDV) specific CD8<sup>+</sup> T cell clone was kindly provided by Dr. Tetsuya Nakatsura, Kashiwa, Japan.<sup>9</sup> Human T cells retrovirally transduced with a TCR that specifically recognizes MAGEC2 peptide (ALKVDVEERV) in HLA-A\*0201 as described by Straetemans T *et al.*,<sup>16</sup> were kindly provided by Dr. Reno Debets, Department of Internal Oncology, Erasmus MC. These T cells were used as responder cells for testing presentation of GPC3 and MAGEC2 by mRNA-transfected B cell blasts. GPC3-specific T cells were expanded by non-specific stimulation with 5  $\mu$ g/ml PHA and irradiated allogeneic PBMC as feeder cells, in AIM-V medium (Gibco) supplemented with 10% human AB serum, 100 IU/ml rhIL-2 and 10 ng/ml recombinant human interleukin-15. Every 3-4 days T cells were further expanded by splitting the cultures and replenishing fresh culture medium without feeder cells or PHA. After 14 days, cells were harvested for experiments or cryopreserved till further use.

***Statistical analysis***

All data set distributions were analyzed for normality by the Shapiro-Wilk normality test. Differences between paired groups of data were analyzed according to their distribution by either paired t test or Wilcoxon matched pairs test. Differences between different groups of patients were analyzed according to their distribution by either unpaired t test or Mann-Whitney test. Correlation was analyzed according to the distribution by either Pearson or Spearman correlation test. The statistical analysis was performed using GraphPad Prism Software (version 5.0). P values less than 0.05 were considered statistically significant (\* P<0.05; \*\* P<0.01; \*\*\* P<0.001).

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# CHAPTER 6

## **Blockade of LAG3 enhances responses of tumor-infiltrating T cells in mismatch repair-proficient liver metastasis of colorectal cancer**

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## ABSTRACT

**Purpose:** Liver metastasis develops in >50% of patients with colorectal cancer (CRC), and is a leading cause of CRC-related mortality. We aimed to identify which inhibitory immune checkpoint pathways can be targeted to enhance functionality of intra-tumoral T-cells in mismatch repair-proficient liver metastases of colorectal cancer (LM-CRC).

**Methodology:** Intra-tumoral expression of multiple inhibitory molecules was compared among mismatch repair-proficient LM-CRC, peritoneal metastases of colorectal cancer (PM-CRC) and primary CRC. Expression of inhibitory molecules was also analyzed on leukocytes isolated from paired resected metastatic liver tumors, tumor-free liver tissues, and blood of patients with mismatch repair-proficient LM-CRC. The effects of blocking inhibitory pathways on tumor-infiltrating T-cell responses were studied in *ex vivo* functional assays.

**Results:** Mismatch repair-proficient LM-CRC showed higher expression of inhibitory receptors on intra-tumoral T-cells and contained higher proportions of CD8<sup>+</sup> T-cells, dendritic cells and monocytes than mismatch repair-proficient primary CRC and/or PM-CRC. Inhibitory receptors LAG3, PD-1, TIM3 and CTLA4 were higher expressed on CD8<sup>+</sup> T-cells, CD4<sup>+</sup> T-helper and/or regulatory T-cells in LM-CRC tumors compared with tumor-free liver and blood. Antibody blockade of LAG3 or PD-L1 increased proliferation and effector cytokine production of intra-tumoral T-cells isolated from LM-CRC in response to both polyclonal and autologous tumor-specific stimulations. Higher LAG3 expression on intra-tumoral CD8<sup>+</sup> T-cells associated with longer progression-free survival of LM-CRC patients.

**Conclusion:** Mismatch repair-proficient LM-CRC may be more sensitive to immune checkpoint inhibitors than mismatch repair-proficient primary CRC. Blocking LAG3 enhances tumor-infiltrating T-cell responses of mismatch repair-proficient LM-CRC, and therefore may be a new promising immunotherapeutic target for LM-CRC.

**Keywords:** immunotherapy; immune checkpoint inhibitor; tumor-infiltrating lymphocyte; liver metastasis; colorectal cancer; peritoneal metastasis; LAG3; PD-1; T cell; mismatch repair

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## INTRODUCTION

Colorectal cancer (CRC) is the third most common cause of cancer-related mortality worldwide.(1-3) More than 50% of CRC patients develop metastatic disease to their liver over the course of their life,(4) and liver metastasis is a leading cause of death from CRC.(5-7) Unfortunately, surgical resection of isolated liver metastases of CRC (LM-CRC) is curative in only 20%-30% of patients,(8, 9) and systemic therapy provides limited survival benefit.(10) Patients with unresectable LM-CRC have a poor prognosis with a median survival of only two years.(11) Therefore, there is a pressing need for more effective therapeutic strategies for LM-CRC.

The immune system plays a crucial role in cancer surveillance and elimination, and antibody blockade of inhibitory immune checkpoint pathways that suppress anti-tumor T-cell immunity and assist tumor immune evasion,(12-16) has recently emerged as an attractive treatment option for multiple types of malignancies.(17-21) Targeting the PD-1/PD-L1 inhibitory pathway has resulted in objective responses in 17%-28% of advanced melanoma patients, 12%-27% of renal cell cancer patients, 10%-18% of non-small cell lung cancer patients and 20% of advanced hepatocellular carcinoma patients.(22-25) In contrast, CRC patients hardly respond to PD-1 and PD-L1 blocking antibodies(23-26), except for the minority of patients who are with mismatch repair (MMR)-deficient CRC.(27, 28) A defective MMR enzyme system occurs in 10%-20% of CRC tumors and results in microsatellite instability, which is used as a molecular marker of MMR-deficiency.(29) It has been hypothesized that the observed difference in responsiveness to PD-1/PD-L1 blockade between MMR-deficient and MMR-proficient CRC is related to the higher numbers of somatic mutations in MMR-deficient tumors, due to the reduced ability to repair DNA damage. The increased mutation rate may result in the presence of more mutation-encoded neo-antigens in the tumors, which elicit stronger anti-tumor T cell responses.(27) Indeed, MMR-deficient CRC tumors are characterized by denser CD8<sup>+</sup> T cell infiltration.(30) They also have higher expression levels of inhibitory checkpoint molecules, probably to resist immune-mediated tumor elimination.(31) Together, enhanced immune cell infiltration and upregulation of inhibitory immune checkpoints may render MMR-deficient CRC more sensitive to PD-1/PD-L1 blockade than MMR-proficient CRC.

In LM-CRC the incidence of MMR deficiency is low,(32) and it may therefore be expected that LM-CRC poorly respond to immune checkpoint inhibitors. Nevertheless, these tumors contain immune infiltrates, and increased numbers of tumor-infiltrating CD8<sup>+</sup> T cells are positively associated with overall survival and response to chemotherapy,(33, 34) indicating

that intra-tumoral T cell immunity is an important determinant of LM-CRC progression. In addition, MMR-proficient LM-CRC are immunologically distinct from primary CRC in terms of immune infiltration.(35) Moreover, the unique immune environment in the liver(36) favors immunological tolerance,(36) and one of the mechanisms used by the liver to resist immune responses is the induction of expression of inhibitory receptors on hepatic T cells(37, 38) and their ligands on hepatocytes and other liver tissue cells.(39) Previously we have observed that intra-tumoral CD8<sup>+</sup> cytotoxic T cells (CTL) and CD4<sup>+</sup> T helper cells (Th) are functionally compromised in LM-CRC,(40) and we also demonstrated that the suppression mediated by intra-tumoral regulatory T cells (Treg) in LM-CRC can be alleviated by blocking the inhibitory receptor CTLA4 and activating the stimulatory receptor GITR.(41) However, the expression and functional roles of inhibitory receptor-ligand pathways in regulating tumor-infiltrating effector T cell responses have not been studied yet in LM-CRC.

Therefore, the aim of this study was to determine whether inhibitory immune checkpoint pathways can be targeted to enhance the functionality of tumor-infiltrating lymphocytes (TIL) in MMR-proficient LM-CRC. We measured the expression of inhibitory receptors and their ligands on leukocytes isolated from paired resected metastatic liver tumors, tumor-free liver tissues (TFL) and blood of patients with LM-CRC, and compared their expression levels with those on leukocytes isolated from peritoneal metastasis of CRC (PM-CRC) and primary CRC. In addition, we studied the effects of antibody blockade of inhibitory pathways on TIL responses of LM-CRC in *ex vivo* functional assays.

## RESULTS

### ***Comparison of immune infiltrates and expression of inhibitory molecules among MMR-proficient liver metastases, peritoneal metastases and primary CRC.***

To speculate whether TIL in CRC tumors at different anatomical sites may differ in sensitivity to checkpoint inhibitors, we first compared frequencies of T cell and antigen-presenting cell (APC) subsets, as well as their expression of inhibitory molecules, between MMR-proficient LM-CRC, primary CRC, and metastases outside the liver. Two in all LM-CRC tumors and three out of twelve primary CRC tumors that we collected were MMR-deficient, whereas all eleven PM-CRC tumors were MMR-proficient (Table 1 and Supplementary Table S1). The data of the five patients with MMR-deficient tumors are shown in Supplementary Fig. S1.

We observed several interesting differences among MMR-proficient tumors from different anatomical locations. Firstly, both LM-CRC and PM-CRC contained significantly higher frequencies of CD8<sup>+</sup> CTL and significantly lower frequencies of CD4<sup>+</sup>Foxp3<sup>-</sup> Th than primary CRC, while frequencies of Treg were similar in CRC tumors at all three sites (Fig. 1A).

Secondly, CD8<sup>+</sup> CTL in LM-CRC displayed a higher frequency of PD1<sup>+</sup> cells than those in primary CRC, and also contained higher frequencies of TIM3<sup>+</sup> and LAG3<sup>+</sup> cells than those in PM-CRC, while CD4<sup>+</sup> Th in LM-CRC displayed a higher frequency of LAG3<sup>+</sup> cells than those in PM-CRC (Fig. 1B). Thirdly, CD4<sup>+</sup>Foxp3<sup>+</sup> Treg in LM-CRC contained higher frequencies of PD-1<sup>+</sup> and TIM3<sup>+</sup> cells than those in primary CRC and PM-CRC, and also displayed a higher frequency of LAG3<sup>+</sup> cells than those in PM-CRC (Fig. 1B). Finally, LM-CRC contained significantly higher frequencies of myeloid dendritic cells (mDC) and monocytes than primary CRC (Fig. 1C), which not only can present tumor antigens to T cells but also express the inhibitory ligands PD-L1 (for PD-1), galectin 9 (for TIM3), MHC class II molecules (for LAG3), CD86 and CD80 (for CTLA4) (Fig. 1D). Hierarchical clustering analysis showed that the immunological data of most LM-CRC patients clustered together as did the data of most primary CRC patients (Supplementary Fig. S2). Together, these data indicate that the CD8<sup>+</sup> CTL:Treg ratio, which is critical for immunological tumor control in primary as well as metastasized CRC,(42) is more favorable in CRC metastases compared to primary CRC. In addition, the increased expression of PD-1 on TIL suggests that TIL of LM-CRC may be more sensitive to blockade of the PD-1/PD-L1 pathway than TIL of primary CRC.

***Elevated expression of inhibitory receptors on CD8<sup>+</sup> cytotoxic T cells, CD4<sup>+</sup> T helper cells and regulatory T cells in MMR-proficient LM-CRC tumors.***

We isolated leukocytes from surgically resected metastatic liver tumors, TFL and blood of LM-CRC patients, and compared the expression levels of five inhibitory receptors (PD-1, TIM3, LAG3, CTLA4 and BTLA) on CD8<sup>+</sup> CTL, CD4<sup>+</sup>Foxp3<sup>-</sup> Th and CD4<sup>+</sup>Foxp3<sup>+</sup> Treg. When compared to TFL and blood, significantly higher proportions of CD8<sup>+</sup> CTL, Th and Treg in TIL expressed PD-1 and TIM-3. In addition, TIL contained higher frequencies of CTLA4<sup>+</sup> CTL and CTLA4<sup>+</sup> Th, while LAG3 was overexpressed on CD8<sup>+</sup> CTL in TIL when compared to TFL and blood (Fig. 2). Interestingly, the highest expression of CTLA4, which is functionally involved in the suppressive capacity of Treg,(43) and also of PD-1 and TIM3 was found on tumor-infiltrating Treg. In contrast, frequencies of BTLA<sup>+</sup> cells in intra-tumoral T cells were low, and they did not differ significantly from those in TFL and blood (Supplementary Fig. S3). Therefore, we focused on the other four receptors in the rest of this study. To investigate whether the expression of inhibitory receptors on circulating T cells had a relation with the expression on intra-tumoral T cells, we performed correlation analysis, as illustrated in Supplementary Fig. S4. There were significant positive correlations between the frequencies of PD-1<sup>+</sup> CTL and PD-1<sup>+</sup> Treg in the tumor and those in the blood, between the frequency of LAG3<sup>+</sup> Th in the tumor and that in the blood, and between the frequencies of CTLA4<sup>+</sup> Th and CTLA4<sup>+</sup> Treg in the tumor and those in the blood. These results indicate that the expression

of inhibitory receptors on circulating T cells partly reflects their expression on intra-tumoral T cells.

***Intra-tumoral antigen-presenting cells express inhibitory ligands.***

To study the expression of inhibitory ligands PD-L1, galectin 9, MHC-II molecules, CD86 and CD80 on APC in LM-CRC tissues, we measured these molecules by flow cytometry. Three major APC subsets BDCA-1<sup>+</sup>CD19<sup>-</sup> mDC, CD14<sup>+</sup> monocytes and CD19<sup>+</sup> B cells were found in all tumors. The frequency of B cells was higher in tumors than in TFL, and the frequency of mDC was higher in tumors and TFL than in the blood, whereas the frequency of monocytes was lower in tumors than in the blood (Fig. 3A). The three different tumor-infiltrating APC populations expressed the five ligands at different levels and considerable variations between individual patients were observed (Fig. 3B, C). Intra-tumoral mDC and monocytes contained higher proportions of PD-L1<sup>+</sup> and MHC-II<sup>+</sup> cells than intra-tumoral B cells. The median fluorescence intensities of five ligands in APC subsets in the tumor, TFL and blood are presented in Fig. 3D, showing that the expression of inhibitory ligands is generally not increased on tumor-infiltrating APC compared to APC in TFL. Together, the abovementioned data suggest that inhibitory interactions between T cells and APC in MMR-proficient LM-CRC are possible.

***Intra-tumoral T cells expressing inhibitory receptors show increased levels of activation markers.***

Considering that increased expression of inhibitory receptors on T cells can be induced by recent activation, or by chronic stimulation that may lead to T cell dysfunctionality, we examined the *ex vivo* activation status and *in vitro* effector cytokine production of effector T cells derived from MMR-proficient LM-CRC. First we compared the expression of activation markers HLA-DR and CD69 on intra-tumoral T cells that do or do not express inhibitory receptors. Interestingly, PD-1<sup>+</sup>, TIM3<sup>+</sup>, LAG3<sup>+</sup> or CTLA4<sup>+</sup> CTL and Th showed a more activated status than PD-1<sup>-</sup>, TIM3<sup>-</sup>, LAG3<sup>-</sup> or CTLA4<sup>-</sup> CTL and Th, respectively (Fig. 4A, B). Subsequently, we assessed effector cytokine production of tumor-derived T cells after short-term PMA and ionomycin stimulation. Despite the activated status, the frequencies of inhibitory receptor positive CTL and Th cells that produced IFN- $\gamma$  and TNF- $\alpha$  were reduced or similar to those in the respective receptor negative T cells (Fig. 4C, D). Interestingly, LAG3<sup>+</sup> CTL and Th cells did not show reduction in cytokine production. Because among all studied inhibitory receptors, tumor-infiltrating CTL and Th showed the highest expression of PD-1 (Fig. 2C, D), we analyzed co-expression of PD-1 and the other three receptors. Co-expression of PD-1 and either TIM3, LAG3 or CTLA4 was found, especially in CD8<sup>+</sup> CTL, but



expression of TIM3, LAG3 or CTLA4 without PD-1 was also observed (Supplementary Fig. S5). Similar to single receptor positive CTL and Th, double receptor positive CTL and Th showed reduced or comparable frequencies of IFN- $\gamma$  and TNF- $\alpha$  producing cells to double receptor negative cells, while LAG3<sup>+</sup>PD-1<sup>+</sup> CTL and Th did not show significant reduction in cytokine production (Fig. 4C, D). These data demonstrate that in general intra-tumoral T cells that express inhibitory receptors do not produce more effector cytokines, despite the activated status.

***Antibody blockade of LAG3 or PD-L1 boosts ex vivo proliferation and effector cytokine production of T cells derived from MMR-proficient LM-CRC.***

Because PD-1, TIM3, LAG3 and CTLA4 are upregulated on T cells in MMR-proficient LM-CRC tumors, we tested whether blockade of the PD-1/PD-L1, TIM3/galectin 9, LAG3/MHC-II or CTLA4/CD80/CD86 pathways could enhance the effector functions of tumor-derived T cells. We stimulated CFSE-labeled total tumor-infiltrating mononuclear leukocytes with CD3/CD28 beads, in the presence or absence of antagonistic antibodies against human PD-L1, TIM3, LAG3, CTLA4 or isotype controls (mIgG1 or mIgG2a). After four days of culture, T cell proliferation was measured by flow cytometry (Fig. 5A), and effector cytokine secretion in the culture supernatants was quantified by enzyme-linked immunosorbent assay. Fig. 5B illustrates that the baseline proliferation of CD8<sup>+</sup> and CD4<sup>+</sup> T cells derived from tumors was significantly lower than that of T cells from the blood of the same patients, indicating the proliferative function of intra-tumoral T cells is impaired compared to circulating T cells of patients with MMR-proficient LM-CRC. Interestingly, treatment with anti-LAG3 antibody or anti-PD-L1 antibody significantly increased the proliferation of both CD8<sup>+</sup> and CD4<sup>+</sup> TIL compared with the control condition without antagonistic antibody (Fig. 5C). These two antibodies also significantly increased IFN- $\gamma$  and TNF- $\alpha$  secretion (Fig. 5D).

***Antibody blockade of LAG3 or PD-L1 boosts ex vivo responses of T cells derived from MMR-proficient LM-CRC to autologous tumor antigens.***

To investigate whether blockade of the aforementioned inhibitory pathways can enhance tumor-specific anti-tumor T cell responses, we stimulated CFSE-labeled total tumor-infiltrating mononuclear leukocytes with autologous blood-derived mDC preloaded with autologous tumor lysates, in the presence or absence of antagonistic antibodies, and measured CD8<sup>+</sup> and CD4<sup>+</sup> T cell proliferation after six days. Increased TIL proliferation against mDC loaded with tumor lysates compared with mDC without tissue lysates was observed in all five tested patients, while loading of mDC with normal liver lysates did not increase or minimally increased TIL proliferative responses (Fig. 6A, C). Addition of anti-PD-

L1 antibody enhanced proliferative responses of TIL in all four tested patients, while treatment with anti-LAG3 antibody increased both CD8<sup>+</sup> and CD4<sup>+</sup> TIL proliferation in four out of five patients. In three out of four patients anti-LAG3 antibody boosted CD8<sup>+</sup> and/or CD4<sup>+</sup> TIL responses to higher levels than anti-PD-L1 antibody (Fig. 6A, C). After five hours of restimulation, intracellular expression of IFN- $\gamma$  and TNF- $\alpha$  in proliferating T cells was analyzed, and was found to be enhanced by both anti-LAG3 and anti-PD-L1 antibodies in most patients (Fig. 6B, D, E). Like in CD3/CD28 stimulations, anti-TIM3 and anti-CTLA4 antibodies boosted TIL responses less strongly and also in less patients.

***Higher LAG3 expression on intra-tumoral CD8<sup>+</sup> T cells is associated with longer progression-free survival of patients with MMR-proficient LM-CRC.***

In a subset of patients, we could analyze associations between the frequencies of inhibitory receptor positive TIL and patient progression-free survival after the LM-CRC resection. In survival analysis higher expression of LAG3 on CD8<sup>+</sup> TIL, CD4<sup>+</sup> Th and Treg was associated with longer time to recurrence (Fig. 7), whereas higher expression of PD-1 or TIM3 on CD8<sup>+</sup> TIL was associated with shorter time to recurrence (data not shown). In multivariable analysis only LAG3 expression on CD8<sup>+</sup> TIL was demonstrated to be an independent predictor of progression-free survival (Table 2), which supports its functional relevance to anti-cancer immunity in TIL of LM-CRC. We hypothesize that LAG3<sup>+</sup>CD8<sup>+</sup> TIL may be highly activated T cells (Fig. 4A) stimulated by tumor antigens, but subsequently inhibited by increased and continuous expression of LAG3 and interaction with its ligands on APC and tumor cells, yet with preserved effector functions (Fig. 4C), which may control tumor growth. Because the sample size is small, these results need conformation in an independent study. In one fourth of the patients we only have data of LAG3 expression on CD8<sup>+</sup> TIL but not on CD4<sup>+</sup> Th or Treg, so we did not include the latter T cell subsets in the multivariable analysis. The death events were too few to analyze overall survival of LM-CRC patients.

## **DISCUSSION**

Checkpoint inhibitors have recently emerged as attractive treatments for several types of solid cancers. However, PD-1 and PD-L1 blocking antibodies were proven unsuccessful in CRC, with the exception of MMR-deficient CRC.(23-25, 27, 28) The first aim of this study was to investigate whether the composition of the immune infiltrates and the intra-tumoral expression levels of inhibitory molecules in CRC metastases in the liver environment differ from those in primary CRC tumors and metastases outside the liver, which would suggest potential differences in sensitivity to checkpoint inhibitors among CRC tumors at different anatomical locations. The second objective was to determine whether targeting of inhibitory

checkpoint pathways by antagonistic antibodies can enhance the functionality and anti-tumor responses of tumor-infiltrating T cells in LM-CRC. Because the incidence of MMR deficiency in LM-CRC is low, we focused on MMR-proficient tumors.

This study is the first to investigate the expression levels of inhibitory receptors on TIL in LM-CRC. Similar to what we reported in hepatocellular carcinoma,(44) intra-tumoral CD8<sup>+</sup> CTL and CD4<sup>+</sup> Th in MMR-proficient LM-CRC displayed increased expression of PD-1, TIM3, CTLA4 and/or LAG3 compared to their counterparts in TFL and blood. In addition, we found selectively enhanced expression of PD-1 and TIM3 on intra-tumoral Treg (Fig. 2C-E). The elevated frequencies of inhibitory receptor positive cells in TIL together with the expression of their ligands on tumor-infiltrating APC (Fig. 3B-D) suggest that these inhibitory checkpoint pathways may be involved in intra-tumoral suppression of T cells in MMR-proficient LM-CRC.

Interestingly, we observed higher expression of PD-1 and/or TIM3 on CD8<sup>+</sup> CTL and Treg in LM-CRC than in primary CRC, while expression of LAG3, TIM3 and/or PD-1 on CD8<sup>+</sup> CTL, Th and Treg was higher in LM-CRC than in PM-CRC (Fig. 1B). We hypothesize that these differences are due to the unique tolerogenic properties of the liver environment, which induces expression of inhibitory receptors on hepatic T cells both in diseased and healthy conditions.(37, 38) Furthermore, we found increased proportions of CD8<sup>+</sup> CTL in LM-CRC and PM-CRC compared with primary CRC (Fig. 1A). The observed differences between MMR-proficient LM-CRC and MMR-proficient primary CRC are to some extent reminiscent of those found between MMR-deficient primary CRC and MMR-proficient primary CRC by Llosa *et al.* (31) They demonstrated that MMR-deficient primary CRC displayed higher infiltration with CD8<sup>+</sup> CTL as well as upregulated expression of PD-1, PD-L1, CTLA4 and LAG3 compared to MMR-proficient primary CRC, and suggested that these differences contributed to the enhanced clinical responsiveness to anti-PD-1 therapy of microsatellite unstable CRC compared with microsatellite stable CRC.(31) In addition, we found that LM-CRC contained increased frequencies of professional APC subsets (mDC and monocytes) (Fig. 1C), which may result in improved presentation of tumor antigens to intra-tumoral T cells as well as more PD-L1 and MHC-II expression in LM-CRC compared with primary CRC, because in LM-CRC mDC and monocytes expressed more PD-L1 and MHC-II than B cells (Fig. 3C). Hierarchical clustering analysis showed that the immune phenotypical data of most LM-CRC tumors clustered together as did the data of most primary CRC tumors (Supplementary Fig. S2). These data confirm those of Halama *et al.*, who demonstrated similar heterogeneity in composition of immune infiltrates between paired primary CRC tumors and liver metastases derived from the same patients.(35) We extracted and statistically analyzed their paired data

of primary CRC and LM-CRC from 16 patients and found a higher CD8<sup>+</sup> cell density in LM-CRC than in primary CRC (Supplementary Fig. S6).

Moreover, discrepancies in somatic mutations, copy number alterations, genetic and epigenetic molecular alterations between primary CRC tumors and matched liver metastases were revealed in up to a half of the cases, which may lead to higher mutational load and thereby more neo-epitopes in LM-CRC.(45-48) Together, these findings indicate major differences between liver metastases and primary CRC as well as peritoneal metastases, including differences in immune cell infiltration and inhibitory molecule expression, which may be at least partly induced by the liver environment. We therefore suggest that TIL of MMR-proficient LM-CRC may be more sensitive to PD-1/PD-L1 blockade and other checkpoint inhibitors than TIL of MMR-proficient primary CRC and CRC metastases at other anatomical locations. Nevertheless, to precisely compare the effects of checkpoint inhibitors on TIL of primary CRC and LM-CRC, experiments with TIL from paired tissues might be needed but were unfortunately not available in the current study.

To determine whether TIL expressing inhibitory receptors represent T cells that are recently activated upon recognition of tumor antigens, or are dysfunctional due to chronic antigenic stimulation, we studied their *ex vivo* activation status and *in vitro* effector cytokine production. Similar to what we reported in hepatocellular carcinoma,(44) intra-tumoral CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL expressing any of the four inhibitory receptors showed a more activated status but produced reduced or similar levels of effector cytokines than their counterparts not expressing the corresponding inhibitory receptor (Fig. 4). However, in our *ex vivo* cultures, tumor cells that may express inhibitory ligands are lacking, so the TIL might be less functional *in vivo* and need checkpoint inhibitors. Nevertheless, proliferative responses of tumor-derived T cells to CD3/CD28 stimulation were lower than those of circulating T cells (Fig. 5B). Consistent with what others reported on IFN- $\gamma$  production by CD8<sup>+</sup> TIL in primary CRC patients,(49, 50) our results demonstrate a certain degree of dysfunctionality of intra-tumoral CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL in MMR-proficient LM-CRC, which may be at least partly due to inhibitory checkpoint interactions between T cells and APC subsets in the TIL cultures, but not complete dysfunctionality.

This study is also the first to investigate the effects of antibody blockade of four inhibitory checkpoint pathways on responses of TIL isolated from MMR-proficient LM-CRC. In both polyclonal T cell activation and tumor-specific TIL stimulation assays, increased proliferation of TIL and increased production of effector cytokines were observed upon the addition of anti-LAG3 antibody or anti-PD-L1 antibody (Fig. 5 and Fig. 6) In some patients *ex vivo* anti-

tumor responses of TIL were more robustly enhanced by anti-LAG3 antibody than by anti-PD-L1 antibody (Fig. 6), suggesting that LAG3 might be the most promising target for immunotherapy of LM-CRC among all four checkpoint pathways tested in this study. However, these data need confirmation using TIL from a larger number of patients. The superiority of LAG3 blockade compared to PD-L1 blockade was more seen in tumor antigen-specific stimulation than in polyclonal stimulation. We hypothesize that the small proportion of LAG3<sup>+</sup> TIL is strongly enriched with tumor antigen-reactive T cells, which are known to constitute only a small proportion of all tumor-infiltrating T cells. In addition, we propose that PD-1 is expressed on a larger fraction of TIL which contains more non-tumor antigen-specific T cells. Although TIM3 blockade was reported to reduce T cell apoptosis and inhibit tumor growth in a mouse CT26 colon tumor model,(51) little effect was observed in our experiments using TIL derived from human LM-CRC. This result also contrasts to our recent observation that *ex vivo* responses of TIL isolated from hepatocellular carcinomas can be invigorated by TIM3 blockade.(44) This difference may relate to the lower expression of galectin 9 on APC subsets in LM-CRC compared to hepatocellular carcinomas.

Because we were interested in the net effects of checkpoint inhibitors on TIL responses in a context that reflected the LM-CRC tumor microenvironment as much as possible, we used total tumor-infiltrating mononuclear leukocytes in our functional assays, which contained both T cells expressing inhibitory receptors and APC expressing inhibitory ligands. As a consequence, tumor-infiltrating Treg,(40, 41) type 1 regulatory T cells(52) and probably other types of immune suppressor cells were also present in these assays. Therefore, the reported functional effects of checkpoint inhibitors on effector T cells in these assays may be partly indirect, mediated by effects of these antibodies on suppressor cells. The effects of checkpoint inhibitor on effector T cells in our assays might even be counteracted by its effects on suppressor cells. We found that half of Treg in LM-CRC tumors expressed PD-1, and blocking PD-1/PD-L1 interaction was shown to enhance the suppressive function of Treg isolated from liver tissues of patients with chronic hepatitis C infection.(53)

Interestingly, higher expression of LAG3 on CD8<sup>+</sup> TIL was associated with longer time to recurrence of patients with LM-CRC. We hypothesize that LAG3<sup>+</sup>CD8<sup>+</sup> TIL are cells which have recently been activated (Fig. 4A) by recognition of antigens in the tumor, and upregulate LAG3 expression in response to activation but still have functional capacity to exert effector functions and delay tumor growth (Fig. 4C). LAG3 expression is upregulated on T cells after activation and differentiation,(54, 55) and intra-tumoral T cells that recognize tumor antigens are characterized by expression of LAG3 and other inhibitory receptors.(56) Although chronic tumor antigen stimulation in the tumor microenvironment can induce T cell

exhaustion with simultaneous induction of high expression of multiple inhibitory receptors, this does not mean that all CD8<sup>+</sup> TIL which express one or more inhibitory receptors at any level are functionally exhausted. Indeed, a recent mouse study showed that tumor antigen-specific TIL which expressed LAG3 or PD-1 produced IFN- $\gamma$  in situ and had cytolytic potential.(57) Likewise, in the current study we observed that LAG3<sup>+</sup>CD8<sup>+</sup> TIL in LM-CRC were not functionally impaired (Fig. 4C). Nevertheless, they could be functionally invigorated upon antibody blockade of LAG3 (Fig. 5 and Fig. 6), indicating that interaction of LAG3 with its ligands serves as an extrinsic mechanism in the tumor microenvironment which inhibits their functionality.

Our study has several limitations: 1) Due to the finite numbers of isolated TIL, the tumor-specific stimulation assay could only be performed in a limited number of LM-CRC patients (Supplementary Table S1), neither could all the conditions be tested in every functional assay, nor could the relation between the expression of inhibitory molecules on TIL and the effects of checkpoint inhibitors on TIL responses be well analyzed. 2) We could not study paired LM-CRC, primary CRC and PM-CRC tumors from the same patients because primary and metastatic CRC tumors were resected at different time points and in different hospitals. 3) MMR-deficient tumors could not be studied well, because we received fresh tissues from only a few MMR-deficient tumors during our study.

In conclusion, increased frequencies of CD8<sup>+</sup> CTL, mDC and monocytes as well as increased inhibitory receptor expression on intra-tumoral T cells in MMR-proficient LM-CRC suggest that TIL of MMR-proficient LM-CRC may be more sensitive to immune checkpoint inhibitors than TIL of MMR-proficient primary CRC. Blockade of LAG3 and PD-L1 can both enhance *ex vivo* functions of tumor-infiltrating T cells from MMR-proficient LM-CRC. Therefore, these two inhibitory pathways may be potential immunotherapeutic targets for the most prevalent metastatic liver cancer, despite the lack of MMR deficiency. Clinical studies focusing on responses of LM-CRC to anti-LAG3 and combination with anti-PD-L1 or anti-PD-1 antibodies(12, 58) are required to conclude whether this prediction based on our preclinical study is correct.

## **PATIENTS AND METHODS**

### ***Patients and specimens***

Fifty three patients who were eligible for surgical resection of LM-CRC were enrolled in the study from November 2013 to March 2017. Another twelve patients who received surgical resection of primary CRC and another eleven patients whose PM-CRC were surgically

resected before hyperthermic intraperitoneal chemotherapy were enrolled in the study from February 2016 to December 2016. Fresh tissue samples from hepatic tumors, tumor-free liver tissues as distant as possible from the tumor (minimum 1cm distance), colorectal tumors and peritoneal tumors were obtained. Peripheral blood from LM-CRC patients was also collected on the day of resection. None of the patients received chemotherapy or immunosuppressive therapy at least three months before surgery. The clinical characteristics of the patients are summarized in Table 1. The study was approved by the local ethics committee, and signed informed consent from all patients was obtained before tissue and blood donation.

### ***Cell preparation***

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation. Single cell suspensions from tumors and tumor-free liver were obtained by tissue digestion. Briefly, fresh tissues were first cut into small pieces and then digested with 0.125 mg/mL collagenase IV (Sigma-Aldrich, St. Louis, MO) and 0.2 mg/mL DNase I (Roche, Indianapolis, IN) in Hanks' Balanced Salt solution with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Sigma, Zwijndrecht, The Netherlands) for 30 minutes at 37 °C with interrupted gently swirling. Cell suspensions were filtered through 100  $\mu\text{m}$  pore cell strainers (BD Biosciences, Erembodegem, Belgium) and mononuclear leukocytes were obtained by Ficoll density gradient centrifugation. Viability was determined by trypan blue exclusion.

### ***Flow cytometric analysis***

Fresh peripheral blood mononuclear cells (PBMC) and leukocytes isolated from tissues were analyzed for expression of surface and intracellular markers using specific antibodies (Supplementary Table S2). Cell surface staining with fluorochrome-conjugated antibodies was performed in the dark at 4°C for 30 minutes, then cells were washed and resuspended in phosphate buffered saline with 0.2 mM EDTA and 0.5% human serum. For Foxp3 and CTLA4 staining, cells were fixed and permeabilized using the Foxp3 staining buffer set (eBioscience, Vienna, Austria). For intracellular cytokine staining, cells were stimulated with 40 ng/mL PMA (Sigma, Zwijndrecht, The Netherlands) and 1  $\mu\text{g}/\text{mL}$  ionomycin (Sigma) at 37°C for five hours in the presence of 5  $\mu\text{g}/\text{mL}$  brefeldin (Sigma) for the last four hours, followed by staining of IFN- $\gamma$  and TNF- $\alpha$  upon fixation and permeabilization using the Foxp3 staining buffer set. Dead cells were excluded by using a LIVE/DEAD fixable dead cell stain kit with aqua fluorescent reactive dye (Invitrogen, Paisley, UK). Cells were measured using a FACS Canto II flow cytometer (BD Biosciences, San Diego, USA) and analyzed using

FlowJo software (version 10.0, LLC). Appropriate isotype control antibodies were used for gating purposes (Supplementary Table S2).

### ***Ex vivo polyclonal T cell activation assay***

All LM-CRC cell cultures were performed in complete medium RPMI 1640 (Lonza, Breda, The Netherlands) supplemented with 10% human AB serum (Invitrogen), 2mM L-glutamine (Invitrogen), 50 mM Hepes Buffer (Lonza), 1% penicillin-streptomycin (Life Technologies), 5mM Sodium Pyruvate (Gibco) and 1% minimum essential medium non-essential amino acids), at 37°C. TIL and PBMC from LM-CRC were labeled with 0.1  $\mu$ M carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen); afterwards  $10^5$  TIL or PBMC were cultured in 200  $\mu$ l complete medium in each well of a 96-well round-bottom culture plate and stimulated with anti-human CD3/CD28 dynabeads (Gibco-Life Technologies AS, Norway) at a cell : bead ratio of 10:1, in the presence or absence of 10  $\mu$ g/ml antagonistic monoclonal antibodies against human PD-L1 (clone 5H1, kindly provided by Dr. Haidong Dong, Mayo Clinic College of Medicine(59)), TIM3 (clone F38-2E2, Biolegend, San Diego, USA(60, 61)), LAG3 (clone 17B4, AdipoGen, Liestal, Switzerland(62)) or CTLA4 (clone BNI3, Beckman Coulter, Marseille, France(63)), or isotype-matched control antibodies (mIgG1 and mIgG2a, Biolegend, London, UK). In preliminary experiments a cell : bead ratio of 10:1 was established to provide sub-optimal stimulation of T cell proliferation. After four days, culture supernatant was collected and frozen at -20°C until secretion of IFN- $\gamma$  and TNF- $\alpha$  was quantified by enzyme-linked immunosorbent assay (ELISA, Ready-SET-Go!, eBioscience). CFSE-labeled cells were harvested and stained with CD8, CD4 and CD3 antibodies. Dead cells were excluded by using the LIVE/DEAD fixable dead cell stain kit with aqua fluorescent reactive dye, and T cell proliferation was determined based on CFSE dilution by flow cytometric analysis.

### ***Ex vivo tumor-specific T cell stimulation assay***

Tumor lysates and normal liver lysates were generated from a small piece of freshly resected metastatic liver tumor or TFL by five cycles of freezing and thawing in phosphate buffered saline, followed by filtration (0.2  $\mu$ m syringe filter), as previously described.(40, 41) Myeloid dendritic cells (mDC) were isolated from patient autologous PBMC by depletion of CD19<sup>+</sup> B cells followed by positive selection for BDCA-1 (BDCA-1 DC isolation kit, Miltenyi Biotec). mDC were cultured overnight with or without 20  $\mu$ g/mL autologous tumor lysates or normal liver lysates, in the presence of 10 ng/mL granulocyte-macrophage colony-stimulating factor (Miltenyi Biotec) and 0.5  $\mu$ g/mL polyinosinic : polycytidylic acid (InvivoGen, San Diego, CA). Simultaneously TIL isolated from LM-CRC were kept at 4°C in complete medium overnight.



Thereafter TIL were labeled with 0.1  $\mu\text{M}$  carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen), and  $10^5$  TIL were co-cultured with autologous mDC preloaded with or without tumor lysates or normal liver lysates at an mDC : TIL ratio of 1:5, in the presence or absence of 10  $\mu\text{g/ml}$  antagonistic monoclonal antibodies against human PD-L1 (clone 5H1, kindly provided by Dr. Haidong Dong, Mayo Clinic College of Medicine(59)), TIM3 (clone F38-2E2, Biolegend, San Diego, USA(44)), LAG3 (clone 17B4, AdipoGen, Liestal, Switzerland(44)) or CTLA4 (clone BNI3, Beckman Coulter, Marseille, France(63)), in 200  $\mu\text{l}$  complete medium in each well of a 96-well round-bottom culture plate. After six days, cells were restimulated with PMA (40 ng/mL) and ionomycin (1  $\mu\text{g/mL}$ ) for five hours in the presence of 5  $\mu\text{g/mL}$  brefeldin for the last four hours. Cells were then stained with CD8, CD4 and CD3 antibodies, followed by intracellular staining of IFN- $\gamma$  and TNF- $\alpha$  upon fixation and permeabilization according to the manufacturer's instructions (eBioscience A&B fixation/permeabilization kit). Dead cells were excluded by using the LIVE/DEAD fixable dead cell stain kit with aqua fluorescent reactive dye, and T cell proliferation was determined based on CFSE dilution by flow cytometric analysis.

#### ***Determination of mismatch repair status by immunohistochemistry***

Immunostainings were performed on formalin-fixed paraffin-embedded whole tissue sections (4  $\mu\text{m}$  thick) using the Benchmark Ultra autoimmunostainer (Ventana Medical Systems Inc, Roche Group, Tucson, USA) according to the manufacturer's protocols and instructions. Briefly, deparaffinization was followed by heat-induced epitope retrieval in Ultra CC1 pre-diluted buffer for 48-60 minutes at 100°C. Primary antibodies anti-MLH1 (Novocastra; Leica Microsystems B.V., Amsterdam, The Netherlands; clone ES05; dilution 1:75), anti-PMS2 (Cell Marque, Rocklin, USA; clone EPR3947, ready to use), anti-MSH2 (Cell Marque, clone G219-1129; ready to use) and anti-MSH6 (Dako, Glostrup, Denmark; clone EP49; dilution 1:75) were applied and followed by incubation (from 40 minutes to 1 hour and 32 minutes). Upon antibody incubation Ventana standard signal amplification was performed, followed by ultraWash counter-staining with one drop of Hematoxylin (for 20 minutes) and one drop of bluing reagent (for 4 minutes). Then slides were removed from the stainer, washed in water with a drop of dishwashing detergent and mounted. These immunohistochemical stainings detected the presence or absence of the protein products of the MMR genes MLH1, PMS2, MSH2 and MSH6. The pattern of their loss provides information about which gene is not functioning properly. IHC staining was evaluated under a light microscope as follows: nuclear expression of all MMR proteins indicates an MMR-proficient tumor status, loss of nuclear expression of any of the proteins indicates an MMR-deficient tumor status.

### ***Statistical analysis***

The distributions of all continuous data set were analyzed for normality by the Shapiro-Wilk normality test. Differences between paired groups of data were analyzed by either paired t test or Wilcoxon matched pairs test according to their distribution. Differences between different groups of patients were analyzed by either unpaired t test or Mann-Whitney test according to their distributions. Correlation was analyzed by either Pearson or Spearman correlation test according to their distributions. These statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). Hierarchical clustering was analyzed by one minus Pearson correlation using GENE-E (Broad Institute). Progression-free survival (time to recurrence) was calculated from the date of LM-CRC surgery to the date of event (LM-CRC recurrence), or the date of last follow-up. Patients lost to follow-up were censored as of the last day of follow-up. Survival curves were estimated by the Kaplan-Meier method. The Breslow test was used to assess differences between survival curves of different groups. For multivariable analysis, the Cox proportional Hazard regression analysis was used. These statistical analyses were performed using SPSS Statistics 21 (IBM). P values less than 0.05 were considered statistically significant (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ).

**Table 1. Patient characteristics**

	LM-CRC (n=53)		PM-CRC (n=11)		primary CRC (n=12)	
Gender (female/male)	16 / 37		4 / 7		5 / 7	
Age (years)**	66.3 ± 3.3		56.9 ± 3.8		63.4 ± 3.4	
Stage of disease (TNM)	Stage IV n=53		Stage IV n=11		Stage I n=3, Stage II n=4, Stage III n=4, Stage IV n=1	
Tumor status	MMR	MMR-deficient n=2, MMR-proficient n=51	MMR	MMR-deficient n=0, MMR-proficient n=11	MMR	MMR-deficient n=3, MMR-proficient n=9

Abbreviations: CRC, colorectal cancer; LM-CRC, liver metastasis of CRC; PM-CRC, peritoneal metastasis of CRC; TNM, tumor-node-metastasis; MMR, mismatch repair.

\*\*Mean ± standard error of the mean.

**Table 2. Multivariable Cox proportional Hazard regression analysis of progression-free survival of patients with MMR-proficient LM-CRC**

Variables	P	HR	95% CI for HR	
	value		Lower	Upper
PD-1 on CD8 <sup>+</sup> TIL	.160	2.418	.706	8.283
TIM3 on CD8 <sup>+</sup> TIL	.774	1.183	.375	3.732
LAG3 on CD8 <sup>+</sup> TIL	<b>.032</b>	<b>.351</b>	<b>.135</b>	<b>.912</b>

Abbreviations: TIL, tumor-infiltrating lymphocytes; HR, hazard ratio; CI, confidence interval.

The hazard ratio is interpreted as the chance of recurrence occurring in the “> median” group to the chance of recurrence occurring in the “< median” group.

## Figure legends

### Figure 1. Comparison of immune infiltrates and inhibitory molecule expression among MMR-proficient liver metastases (LM), peritoneal metastases (PM) and primary CRC.

(A) The frequencies of CD8<sup>+</sup> CTL, CD4<sup>+</sup>Foxp3<sup>-</sup> Th and CD4<sup>+</sup>Foxp3<sup>+</sup> Treg within CD3<sup>+</sup> TIL from LM-CRC, primary CRC and PM-CRC. (B) The frequencies of inhibitory receptor positive cells within CD8<sup>+</sup> CTL, Th and Treg in LM-CRC, primary CRC and PM-CRC. (C) The frequencies of B cells, mDC and monocytes (Mono) within CD45<sup>+</sup> cells from LM-CRC, primary CRC and PM-CRC. (D) The frequencies of inhibitory ligand positive cells within tumor-infiltrating B cells, mDC and monocytes from LM-CRC, primary CRC and PM-CRC. Values of individual patients are shown, and lines depict medians. Differences were analyzed by unpaired t test or Mann-Whitney test; \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

### Figure 2. Expression of inhibitory receptors on CD8<sup>+</sup> CTL, CD4<sup>+</sup> Th and CD4<sup>+</sup> Treg in the tumor, TFL and blood of MMR-proficient LM-CRC.

PBMC and leukocytes isolated from LM-CRC tumors and TFL were stained with antibodies against PD-1, LAG3, TIM3 and CTLA4. (A) (B) Representative dot plots of inhibitory receptor expression on (A) CD3<sup>+</sup>CD8<sup>+</sup> CTL and (B) CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>-</sup> Th in the tumor, TFL and blood; the gates were made according to appropriate isotype controls. (C) (D) (E) The frequencies of inhibitory receptor positive cells within (C) CD8<sup>+</sup> CTL, (D) CD4<sup>+</sup>Foxp3<sup>-</sup> Th and (E) CD4<sup>+</sup>Foxp3<sup>+</sup> Treg in the tumor, TFL and blood. Values of individual patients are shown, and lines depict medians. Differences were analyzed by paired t test or Wilcoxon matched pairs test; \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

### Figure 3. Intra-tumoral antigen-presenting cells of MMR-proficient LM-CRC express inhibitory ligands.

Expression of inhibitory ligands PD-L1, galectin 9 (GAL-9), MHC-II, CD86 and CD80 was measured by flow cytometry. (A) The frequencies of CD19<sup>+</sup> B cells, BDCA1<sup>+</sup>CD19<sup>-</sup> mDC and CD14<sup>+</sup> monocytes (Mono) within CD45<sup>+</sup> cells derived from tumors, TFL and blood. Values of individual patients are presented, lines depict medians. (B) Representative histograms of inhibitory ligand stainings and isotype controls on tumor-infiltrating mDC, monocytes and B cells. (C) The frequencies of inhibitory ligand positive cells within tumor-infiltrating B cells, mDC and monocytes in individual patients are presented; lines depict medians. (D) The median fluorescence intensities (MFI) of inhibitory ligands on B cells, mDC and monocytes derived from tumors, TFL and blood of LM-CRC patients. Values of individual patients are shown, and lines depict medians. Differences were analyzed by paired t test or Wilcoxon matched pairs test; \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

**Figure 4. Tumor-infiltrating T cells expressing inhibitory receptors show increased expression of activation markers.** TIL from MMR-proficient LM-CRC were stained *ex vivo* with antibodies against surface activation markers HLA-DR and CD69. (A) (B) The frequencies of HLA-DR<sup>+</sup> or CD69<sup>+</sup> cells in (A) CD8<sup>+</sup> CTL and (B) CD4<sup>+</sup> Th that do or do not express PD-1, TIM3, LAG3, or CTLA4 are presented (n=9-11). Lines show medians, whiskers depict minimum to maximum. Differences were analyzed by paired t test or Wilcoxon matched pairs test. (C) (D) TIL from MMR-proficient LM-CRC were stimulated with PMA and ionomycin at 37°C for five hours, in the presence of protein transport inhibitor brefeldin for the last four hours, followed by intracellular cytokine staining. The frequencies of cytokine-producing cells in (C) CD8<sup>+</sup> CTL and (D) CD4<sup>+</sup> Th that do or do not express inhibitory receptors are presented (n=7-12). Lines show medians, whiskers depict Min to Max, boxes indicate the 25<sup>th</sup> to 75<sup>th</sup> percentiles. Differences were analyzed by paired t test or Wilcoxon matched pairs test; \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

**Figure 5. Antibody blockade of LAG3 or PD-L1 boosts *ex vivo* proliferation and cytokine production of intra-tumoral T cells from MMR-proficient LM-CRC in response to polyclonal stimuli.** CFSE-labeled TIL from LM-CRC patients were stimulated with CD3/CD28 beads for four days, in the presence or absence of 10 µg/ml antagonistic antibodies. (A) Representative dot plots of CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> TIL proliferation in response to CD3/CD28 beads (a-CD3/CD28) in the presence or absence of antagonistic antibodies (a-) or isotype controls (iso ctrl). Dotplots indicated by “TIL” show proliferative responses in the absence of CD3/CD28 beads. In all other conditions, CD3/CD28 beads were added. (B) The percentages of proliferating cells (CFSE-low) within CD8<sup>+</sup> and CD4<sup>+</sup> T cells derived from the tumor or blood in response to CD3/CD28 beads without addition of any antagonistic antibody. Values of individual patients are presented. (C) Effects of antibody blockade of inhibitory interactions on CD8<sup>+</sup> and CD4<sup>+</sup> TIL proliferation (n=7-9). Because the proliferative responses differed between patients, the results are reported as relative proliferation in the presence of antibodies compared to baseline proliferation, which was calculated by dividing the percentages of proliferating (CFSE-low) T cells in the presence of antagonistic antibody or isotype control antibody by the percentages in the control condition with only CD3/CD28 beads. Values are depicted as means with standard error of the mean. (D) IFN-γ and TNF-α accumulation in culture supernatants was quantified at day four by enzyme-linked immunosorbent assay. Values are depicted as medians with interquartile range (n=10-11). Differences were analyzed by paired t test or Wilcoxon matched pairs test; \* p < 0.05, \*\* p < 0.01.

**Figure 6. Antibody blockade of LAG3 or PD-L1 boosts *ex vivo* responses of intra-tumoral T cells from MMR-proficient LM-CRC to autologous tumor antigens.** Blood mDC loaded with autologous tumor lysates were used to stimulate CFSE-labeled TIL, in the presence or absence of 10 µg/mL antagonistic antibodies. After six days T cell proliferation and intracellular cytokine production were analyzed after re-stimulation with PMA and ionomycin. (A) (B) Representative dot plots of T cell proliferation, IFN-γ and TNF-α expression in CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> TIL, in response to autologous mDC pre-loaded with tumor lysates (TIL+mDC+tumor lysate), in the presence or absence of antagonistic antibodies (a-). TIL responses to mDC that were not pre-loaded with tumor lysates (TIL+mDC) served as controls to determine non-antigen-specific TIL proliferation and cytokine production. (C) (D) (E) Collective data of five patients tested. Each line and each color represent one patient. The results are reported as net tumor-specific responses, calculated by subtracting the percentages of proliferating (CFSE-low) T cells or IFN-γ<sup>+</sup> or TNF-α<sup>+</sup> proliferating T cells in the control condition (mDC without tissue lysates) from the percentages in the conditions with tumor lysates (TL) in the absence or presence of antagonistic antibody. In two experiments an additional control was included, in which TIL were stimulated with blood mDC pre-loaded with normal liver lysates (NL), which did not lead to increased TIL responses.

**Figure 7. Kaplan-Meier curves of progression-free survival (time to recurrence) in relation to LAG3 expression on intra-tumoral T cells in MMR-proficient LM-CRC.** The cutoff values to divide the patients into two groups are the median percentages of LAG3<sup>+</sup> cells in tumor-infiltrating CD8<sup>+</sup> CTL, CD4<sup>+</sup>Foxp3<sup>-</sup> Th or CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells. For determination of the p values the Breslow test was used.

Figure 1.

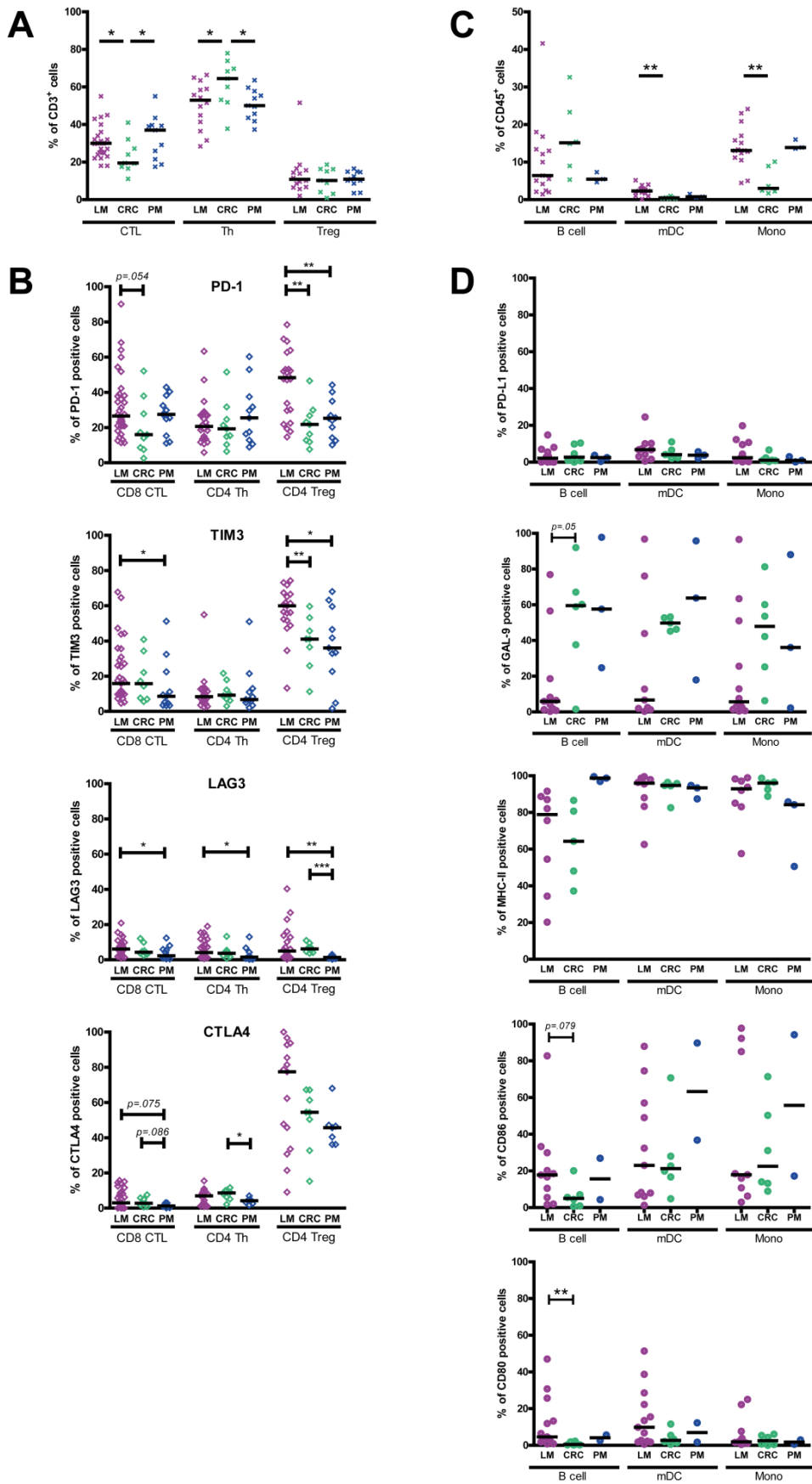
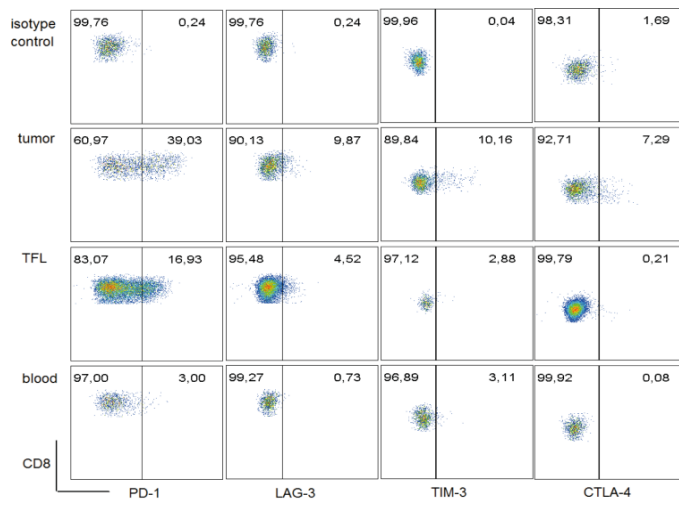
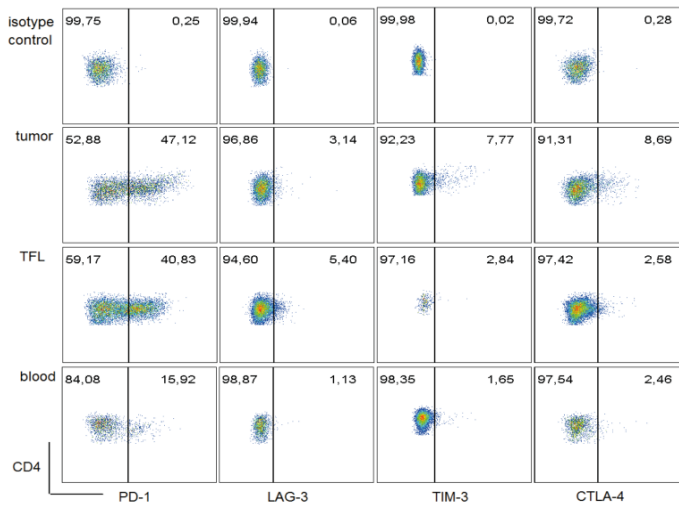


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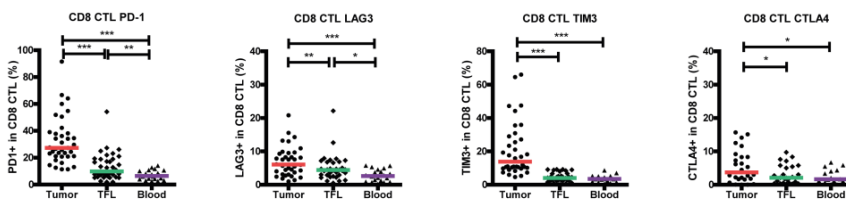
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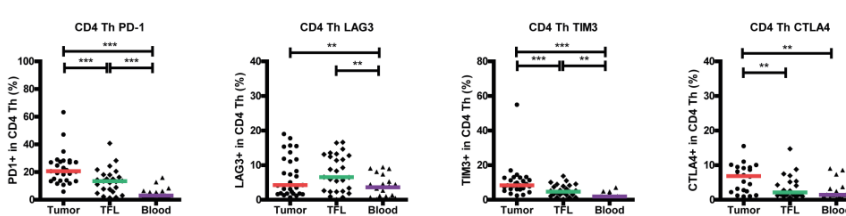
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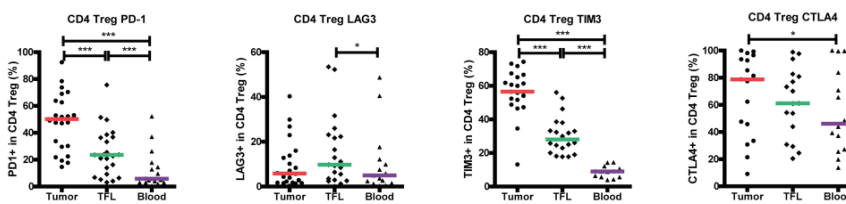




Figure 3.

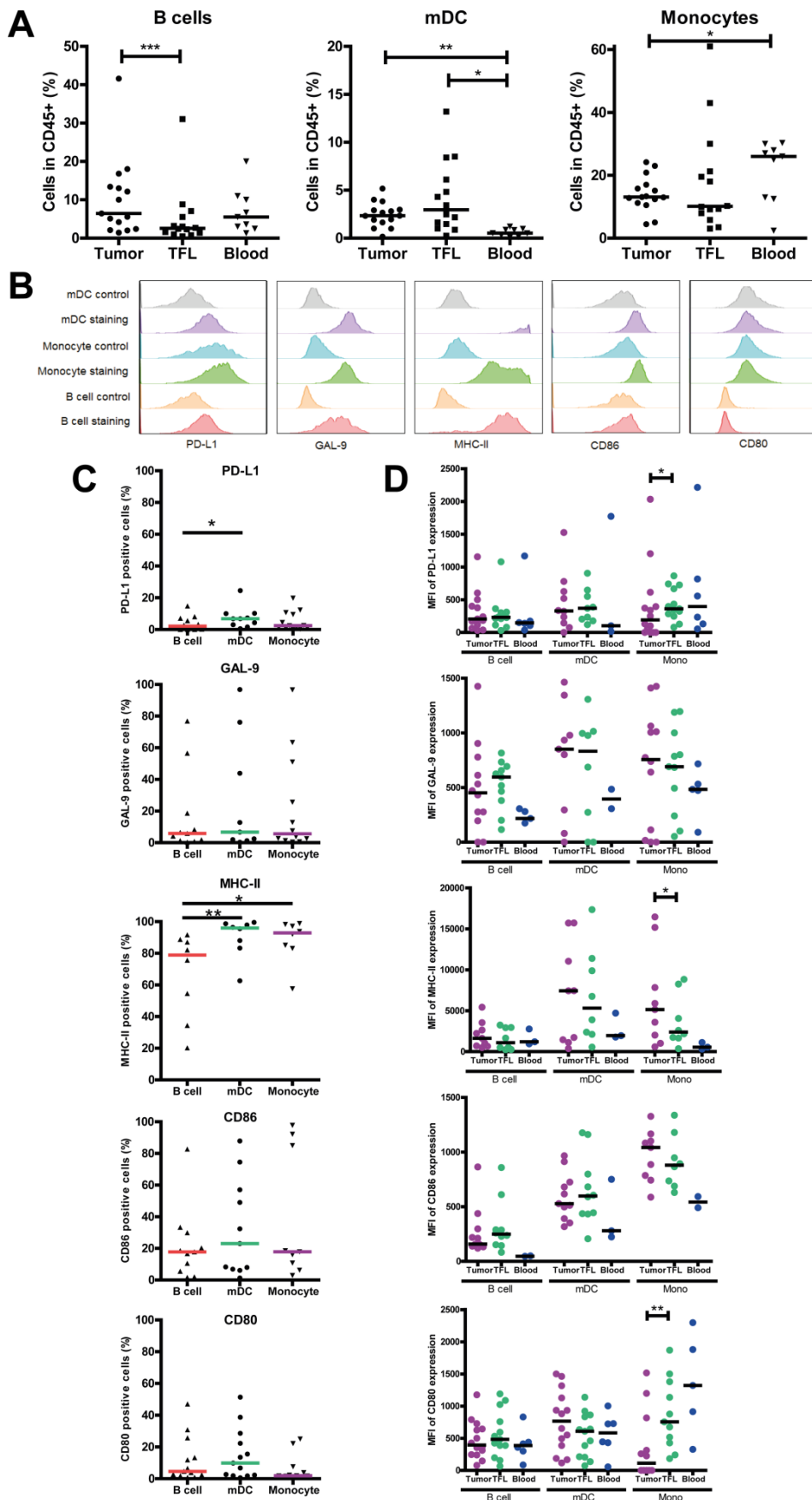


Figure 4.

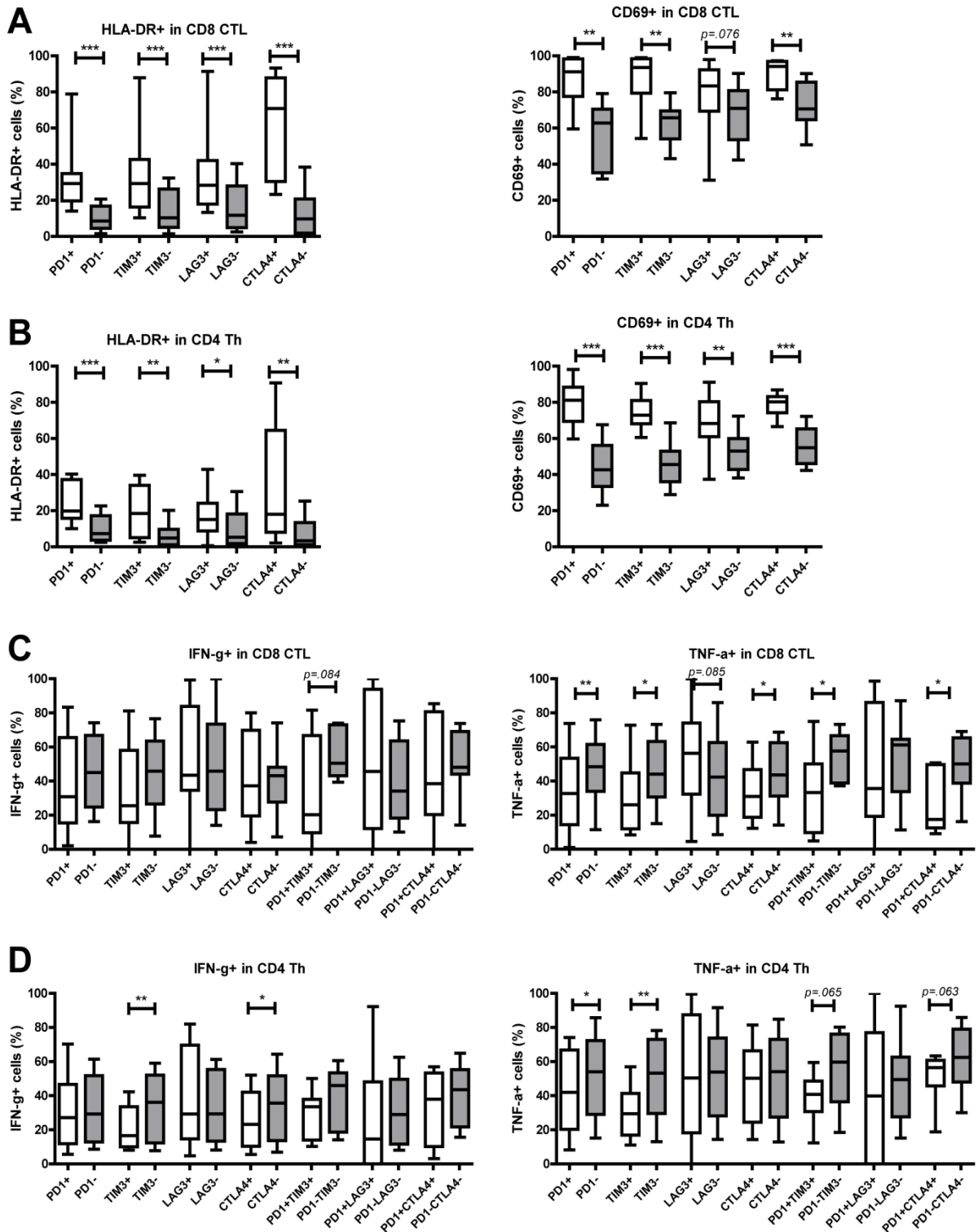


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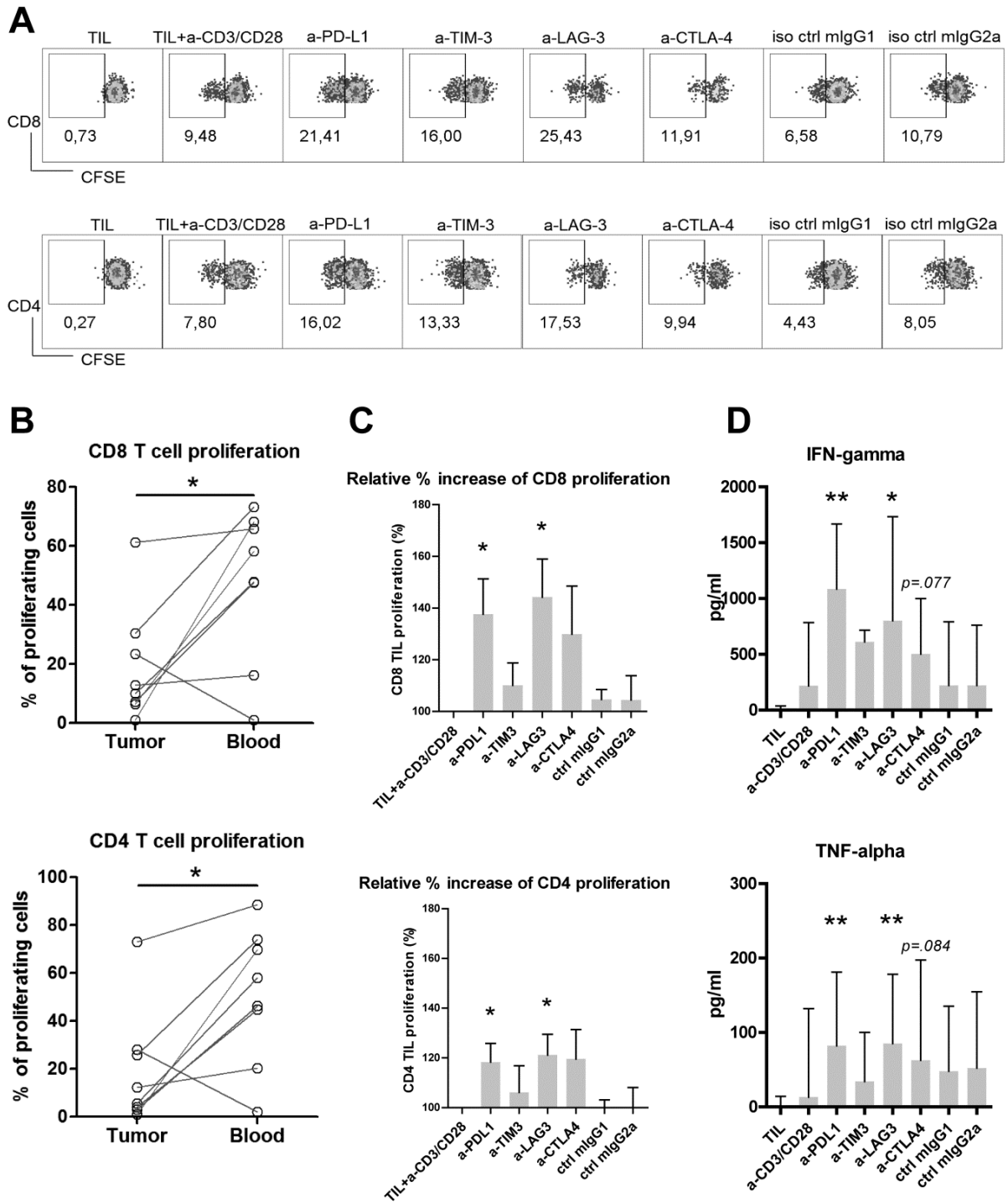


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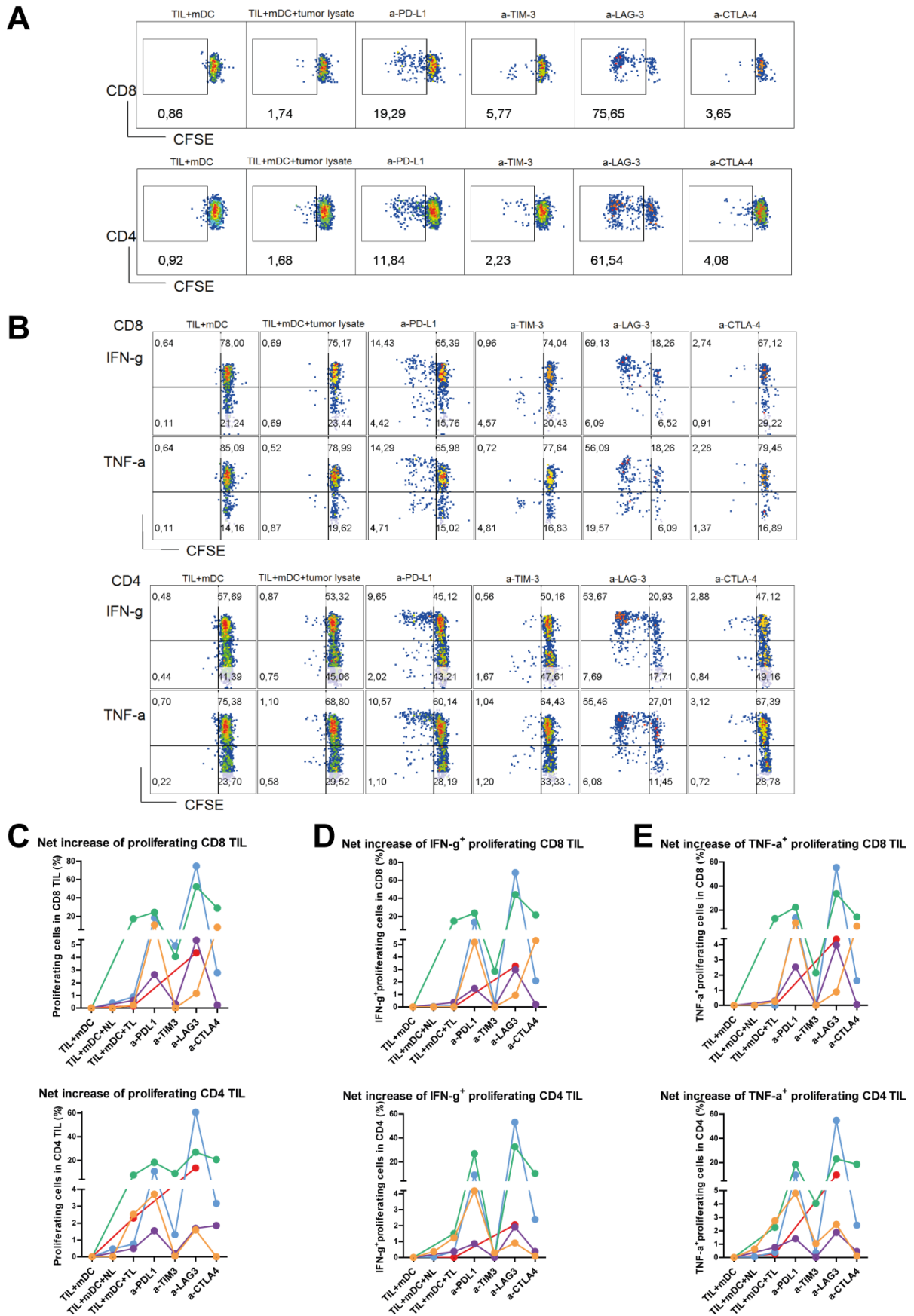
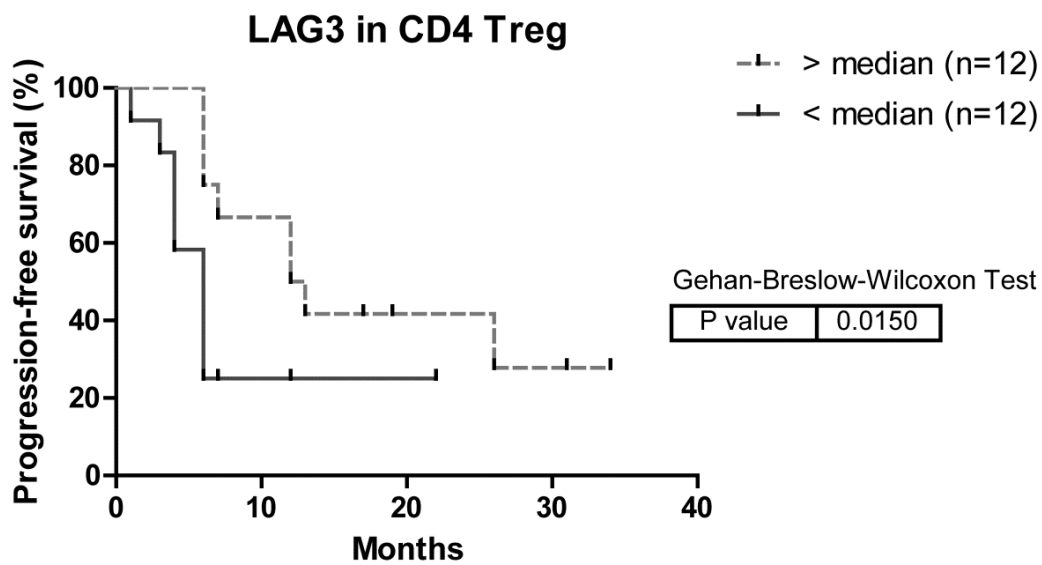
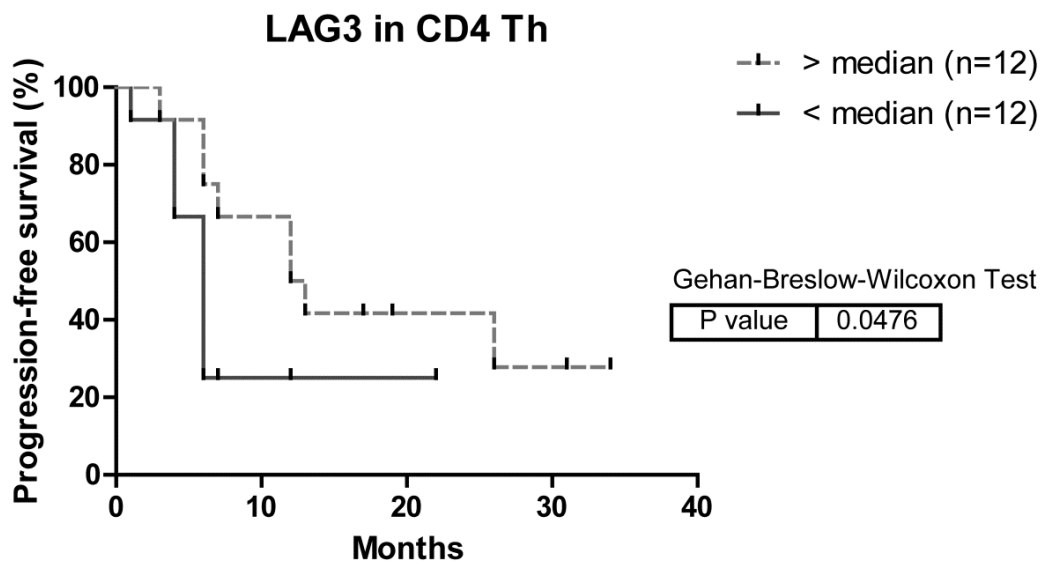
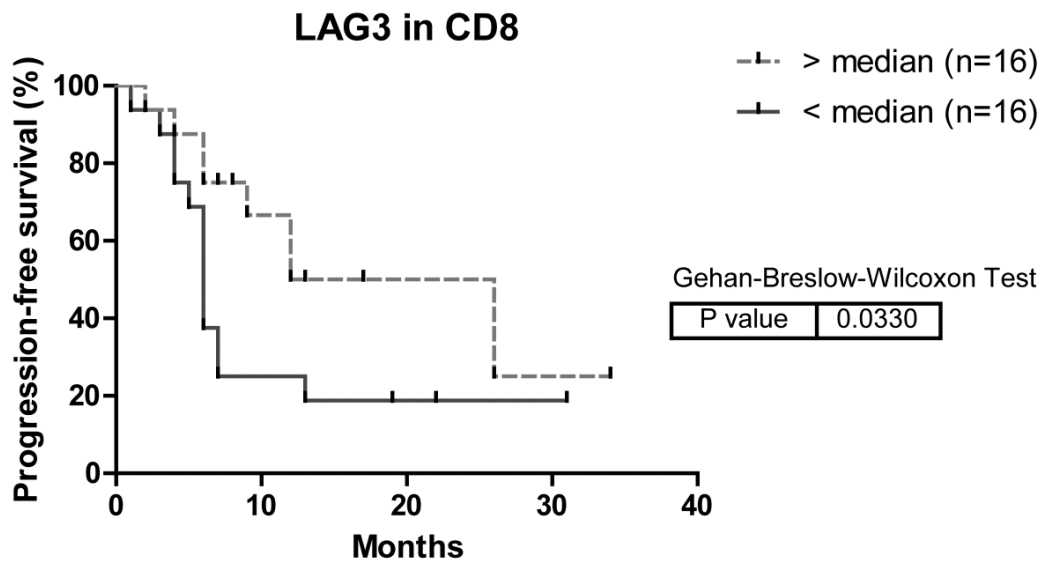


Figure 7.



### Supplementary figure legends

**Supplementary Figure S1. Immune infiltrates and inhibitory molecule expression in MMR-deficient liver metastases (LM) and primary CRC.** (A) The frequencies of CD8<sup>+</sup> CTL, CD4<sup>+</sup>Foxp3<sup>-</sup> Th and CD4<sup>+</sup>Foxp3<sup>+</sup> Treg within CD3<sup>+</sup> TIL from LM-CRC and primary CRC. (B) The frequencies of inhibitory receptor positive cells within CD8<sup>+</sup> CTL, Th and Treg in LM-CRC and primary CRC. (C) The frequencies of B cells, mDC and monocytes (Mono) within CD45<sup>+</sup> cells from primary CRC. (D) The frequencies of inhibitory ligand positive cells within tumor-infiltrating B cells, mDC and monocytes from primary CRC. Values of individual patients are shown, and lines depict medians.

**Supplementary Figure S2. Hierarchical clustering analysis of MMR-proficient liver metastases, peritoneal metastases and primary CRC.** Heatmap illustrating the frequencies of CD8<sup>+</sup> CTL, CD4<sup>+</sup> Th and CD4<sup>+</sup> Treg within CD3<sup>+</sup> TIL, the frequencies of inhibitory receptor positive cells within CD8<sup>+</sup> CTL, CD4<sup>+</sup> Th and CD4<sup>+</sup> Treg, the frequencies of B cells, mDC and monocytes within CD45<sup>+</sup> cells, and the frequencies of inhibitory ligand positive cells within tumor-infiltrating B cells, mDC and monocytes, across LM-CRC, PM-CRC and primary CRC tumors. Rows represent different parameters. Columns represent individual patients clustered by one minus pearson correlation (LM-CRC: n=5, primary CRC: n=6, PM-CRC: n=2). Due to limited numbers of TIL isolated from each tumor sample we could measure all the different phenotypic markers only in TIL isolated from limited numbers of tumors. (A) Absolute values: 0%-100%. (B) Relative values: row minimum (min) to row maximum (max). Grey: missing values.

**Supplementary Figure S3. Expression of BTLA on CD8<sup>+</sup> CTL, CD4<sup>+</sup> Th and CD4<sup>+</sup> Treg in the tumor, TFL and blood of MMR-proficient LM-CRC.** (A) (B) Representative dot plots of BTLA expression on CD3<sup>+</sup>CD8<sup>+</sup> CTL and CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>-</sup> Th in the tumor, TFL and blood; the gates were made according to appropriate isotype controls. (C) (D) (E) The frequencies of BTLA positive cells within CD8<sup>+</sup> CTL, CD4<sup>+</sup> Foxp3<sup>-</sup> Th and CD4<sup>+</sup> Foxp3<sup>+</sup> Treg in the tumor, TFL and blood. Values of individual patients are shown, and lines depict medians. Differences were analyzed by paired t test or Wilcoxon matched pairs test; \* p < 0.05.

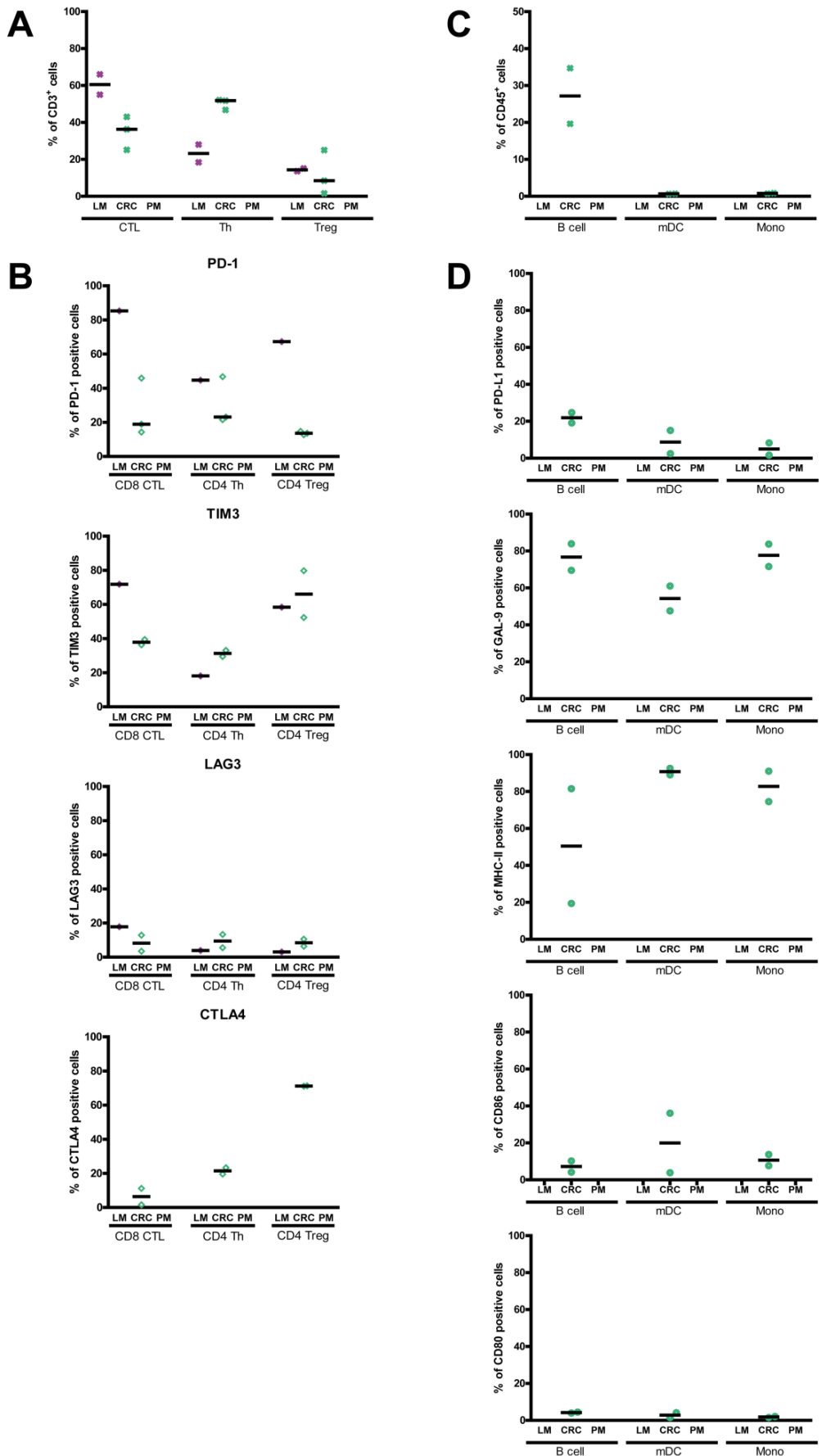
**Supplementary Figure S4. Correlation of inhibitory receptor expression on T cells between the tumor and the blood of patients with mismatch repair-proficient LM-CRC.** Spearman correlation analysis demonstrates (A) positive correlation of the frequencies of PD-1<sup>+</sup> cells in CD8<sup>+</sup> CTL and CD4<sup>+</sup> Treg between tumors and blood, (B) positive correlations of the frequencies of LAG3<sup>+</sup> cells in CD4<sup>+</sup> Th between tumors and blood, and (C) positive

correlations of the frequencies of CTLA4<sup>+</sup> cells in CD4<sup>+</sup> Th and CD4<sup>+</sup> Treg between tumors and blood. Correlations were analyzed by Spearman correlation test.

**Supplementary Figure S5. Co-expression of inhibitory receptors on tumor-derived T cells in MMR-proficient LM-CRC.** The mean frequencies of co-expression of PD-1 with TIM3, LAG3 or CTLA4 in CD8<sup>+</sup> CTL (n=20) and CD4<sup>+</sup> Th (n=10).

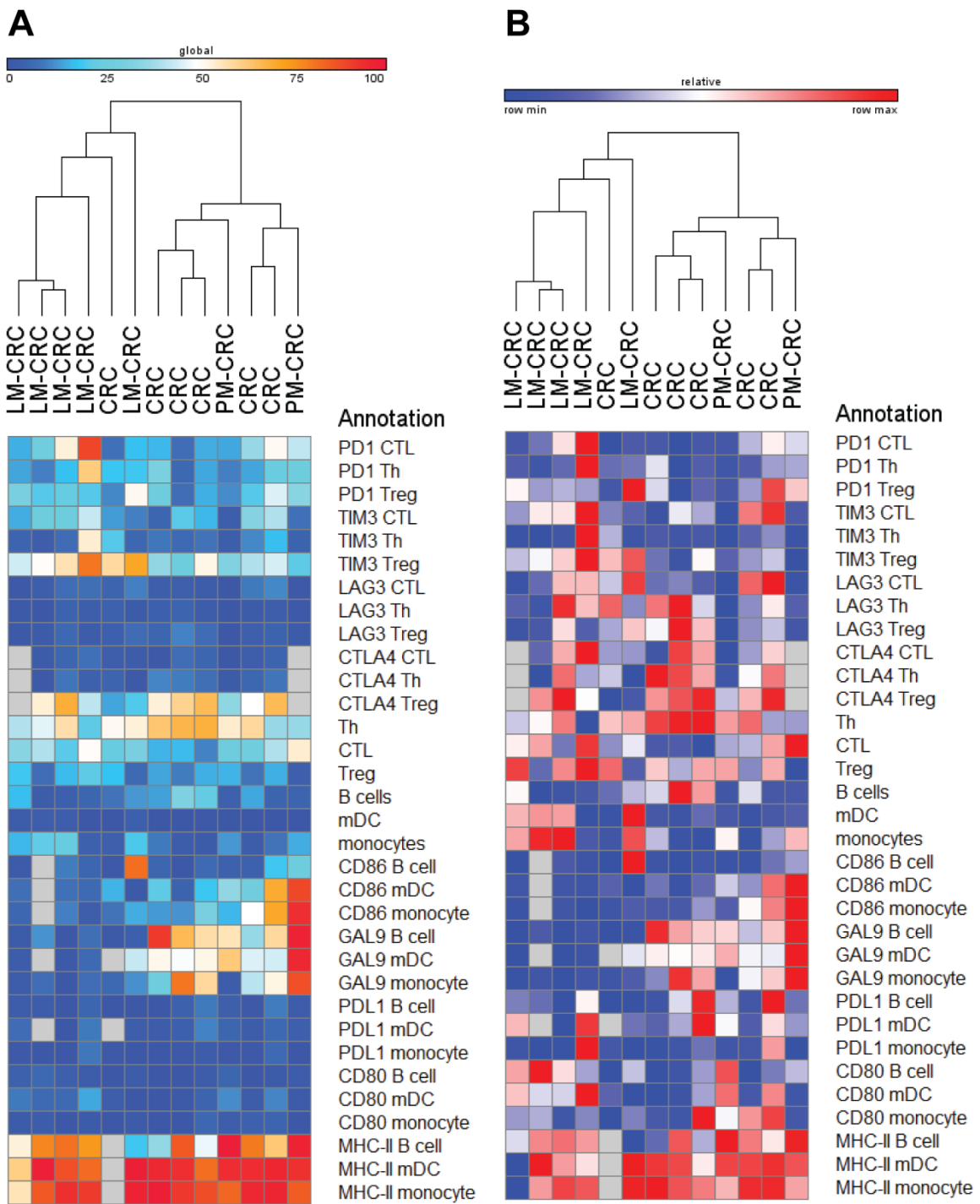
**Supplementary Figure S6. T cell densities in primary CRC tumors and liver metastases.** CD3<sup>+</sup>, CD8<sup>+</sup> and granzyme B<sup>+</sup> cell densities in paired primary CRC and LM-CRC of 16 patients. The data were reanalyzed after being extracted from Halama *et al.* Table 1.(35) Values of individual patients are shown, and lines depict means. Differences were analyzed by paired t test or Wilcoxon matched pairs test; \* p < 0.05.

Supplementary Figure S1.



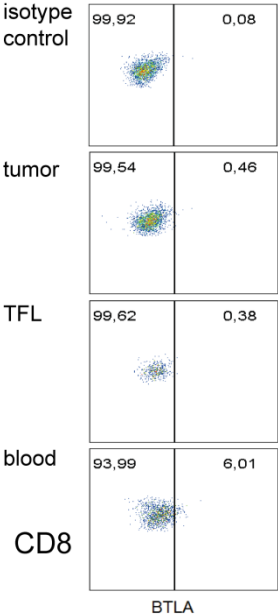


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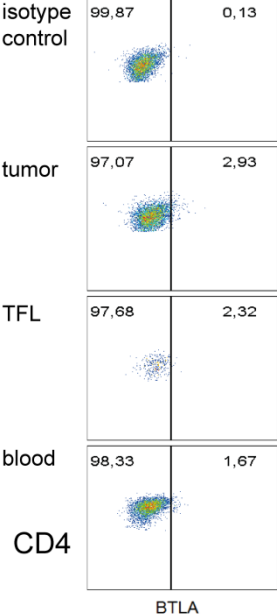


Supplementary Figure S3.

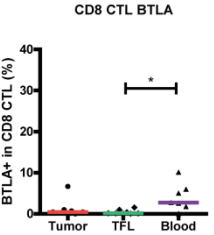
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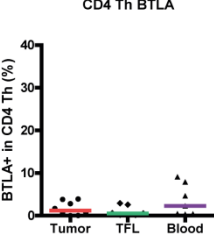
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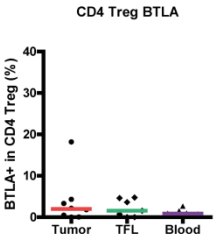
**C**



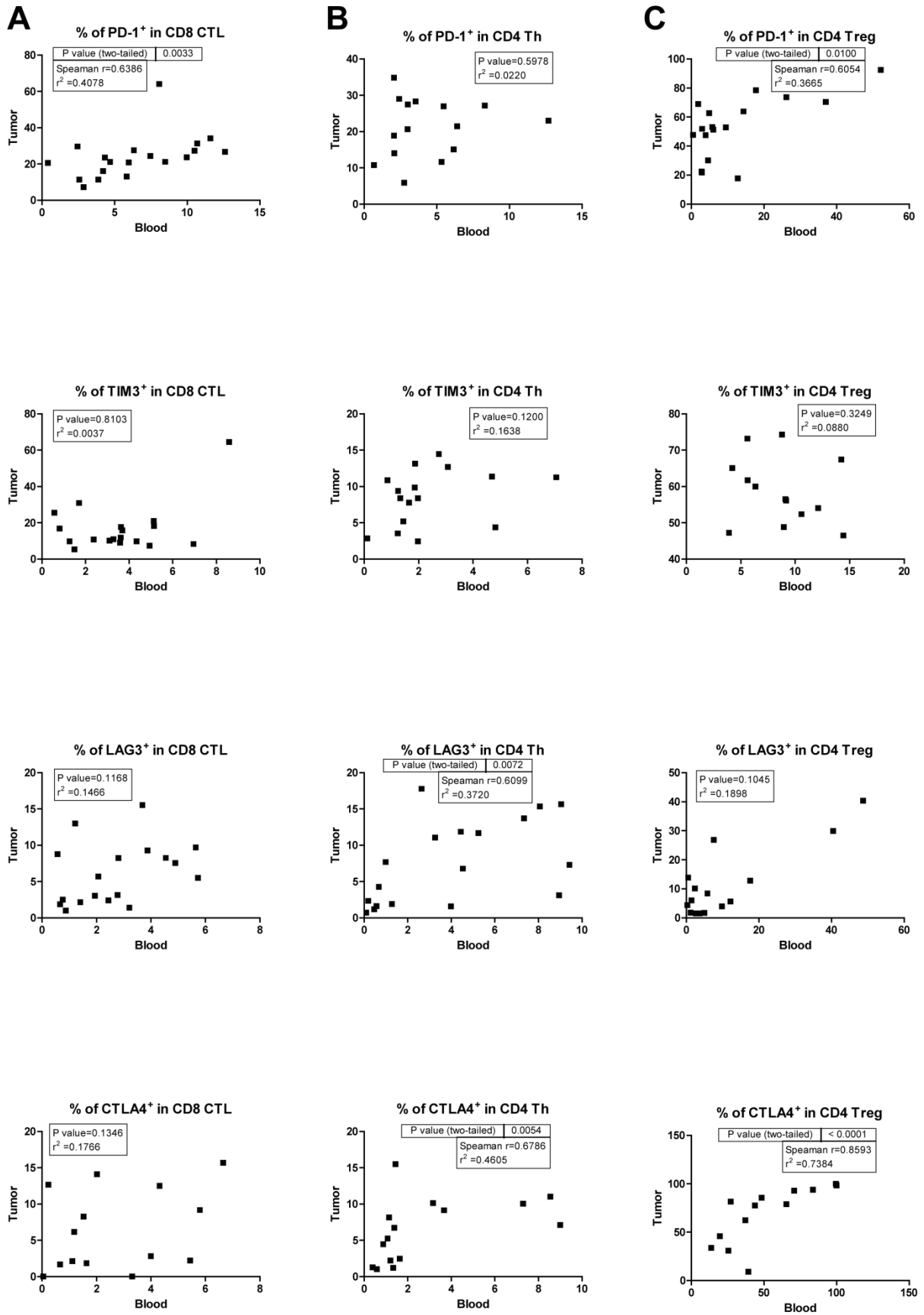
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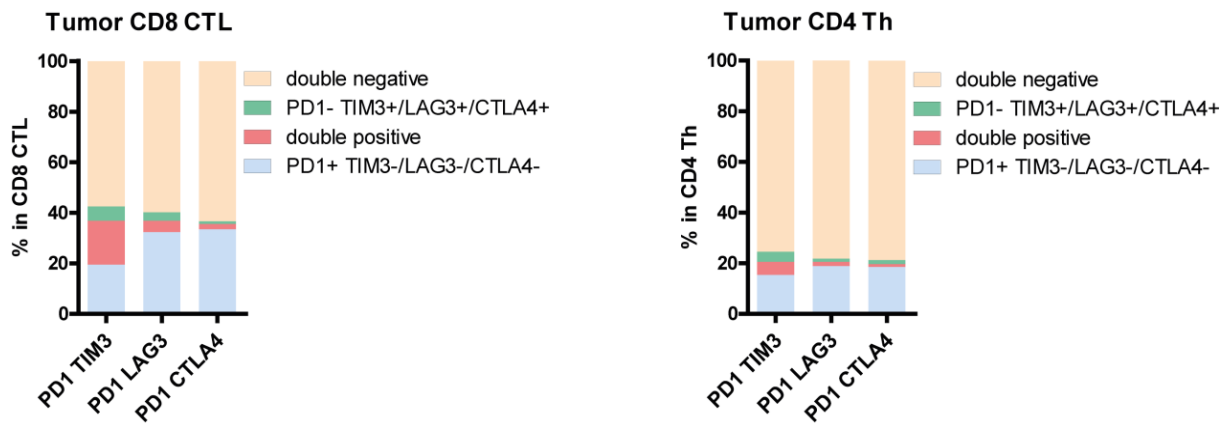
**E**



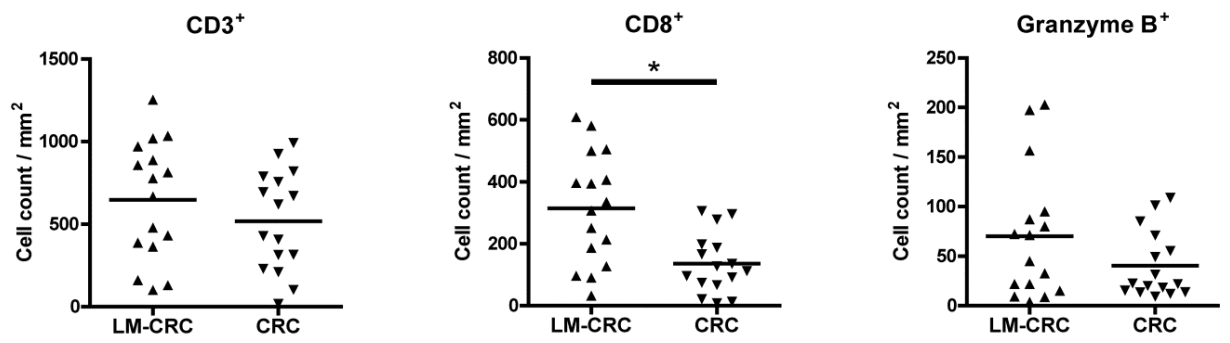
Supplementary Figure S4.



**Supplementary Figure S5.**



**Supplementary Figure S6.**



**Supplementary Table S1. Numbers of patient samples used in each type of experiments in the study**

Tumor type and number of samples	MMR status and number of samples	T cell or APC subsets Figure 1	Inhibitory receptors Figure 1, Figure 2	Inhibitory ligands Figure 1, Figure 3	Activation or cytokine Figure 4	Polyclonal T cell activation assay Figure 5	Tumor-specific T cell stimulation assay Figure 6
<b>53 LM-CRC</b>	1 deficient	■					
	1 deficient	■	■				
	1 proficient						■
	1 proficient			■	■		
	1 proficient	■	■			■	
	1 proficient	■	■	■			
	1 proficient	■	■	■	■		■
	2 proficient	■	■				■
	2 proficient	■	■				■
	4 proficient					■	
	5 proficient	■	■				
	6 proficient			■			
	11 proficient	■	■				
	15 proficient	■	■		■		
	<b>11 PM-CRC</b>	2 proficient	■	■	■		
9 proficient		■	■				
<b>12 primary CRC</b>	1 deficient	■	■	■			
	2 deficient	■	■				
	3 proficient	■	■				
	6 proficient	■	■	■			

Abbreviations: CRC, colorectal cancer; LM-CRC, liver metastasis of CRC; PM-CRC, peritoneal metastasis of CRC; MMR, mismatch repair.

**Supplementary Table S2. Antibodies used in flow cytometry**

Antibody	Clone	Supplier	Antibody	Clone	Supplier
CD69 APC	L78	BD Biosciences	CD8-PE	RPA-T8	eBioscience
IFN-gamma-FITC	25723.11	BD Biosciences	HLA-DR-APC	LN3	eBioscience
CD137 (4-1BB)-APC	4B4-1	BD Biosciences	HLA-DR-PE	LN3	eBioscience
CD14-PerCP	MOP9	BD Biosciences	HLA-DR-PerCP-Cy5.5	LN3	eBioscience
CD3 PerCP-Cy5.5	UCHT1	BD Biosciences	CD3 PE-Cy7	UCHT1	eBioscience
CD19 APC-H7	SJ25C1	BD Biosciences	CD8-APC	okt-08	eBioscience
mIgG2b-FITC	27-35	BD Biosciences	IFN-gamma-Pe-Cy7	4S.B3	eBioscience
mIgG1-PB	MOPC-21	BD Biosciences	HLA-DR-APC-eFluor780	LN3	eBioscience
mIgG2b-PE	27-35	BD Biosciences	CD279 (PD1)-PE-Cy7	J105	eBioscience

mIgG1-PerCP	X40 MOPC- 21	BD Biosciences	CD8-efluor450	RPA-T8	eBioscience
mIgG1-FITC	21 MOPC- 21	BD Biosciences	CD274(B7-H1)-PE-Cy7	MIH1 eBioHVEM- 122	eBioscience
mIgG1-pacific blue	21	BD Biosciences	CD270 (HVEM)-PE		eBioscience
mIgG2a-PerCP	X39	BD Biosciences	CD4-APC-eFluor780	OKT4	eBioscience
CD69-PE	TP1.55.3	Beckman	CD3-APC-eFluor780	SK7	eBioscience
CD3 FITC	UCHT1	Beckman	FoxP3-eFluor450	236A/E7	eBioscience
CD45 FITC	J33	Beckman	CD45 efluor 450	HI30	eBioscience
mIgG2a-PE	U7.27	Beckman	CD8a-PerCp-Cy5.5	RPA-T8	eBioscience
CD272 (BTLA)-APC	MIH26	Biolegend	CD223(LAG3)-PE CD223(LAG3)-PerCP- eF710	3DS223H	eBioscience
CD3 PE	UCHT1	Biolegend		3DS223H	eBioscience
CD86-PB	IT2.2	Biolegend	CD8-FITC	SK1	eBioscience
anti-TNF- $\alpha$ -PerCP- Cy5.5	Mab11	Biolegend	rat IgG2a-APC	eBR2a	eBioscience
Galectin-9 PE	9M1-3	Biolegend	mIgG1-FITC	P3	eBioscience
CD80-PE-Cy7	2D10	Biolegend	mIgG2a-APC	eBM2a	eBioscience
CD152 (CTLA4)-APC	L3D10	Biolegend	rat IgG2b-APC	eB149/10H5	eBioscience
HLA-DR, DP, DQ- FITC	Tü39 MOPC- 21	Biolegend	mIgG1-PE	P3.6.2.8.1	eBioscience
mIgG1-Pe-Cy7	21	Biolegend	BDCA1-APC	AD5-8E7	Miltenyi R&D systems
rat IgG2a-PE	RTK2758 MOPC- 173	Biolegend	TIM3-PE	344823	
mIgG2a-FITC	MOPC- 21	Biolegend			
mIgG1-APC	21	Biolegend			

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# CHAPTER 9

## General discussion and summary

Liver cancer represents the second most common cause of cancer-related mortality worldwide. Hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer, cholangiocarcinoma (CCA) second. Liver metastasis of colorectal cancer (LM-CRC) is the most common secondary liver cancer and a leading cause of death from CRC. The current therapeutic options for all three types of liver cancer are very limited. Hence, there is a pressing need for novel and effective treatments. The goals of this thesis are: 1) to determine whether regulatory T cells and co-inhibitory immune checkpoint pathways contribute to the immunosuppression within the tumor microenvironment in liver cancers, 2) and to identify potential promising targets to alleviate these immune suppressive mechanisms, thereby enhancing anti-tumor functions of tumor-infiltrating T cells in patients with liver cancer. In this chapter we will summarize and discuss the main findings in this thesis and propose suggestions for future research.

In **Chapter 1**, we review the current knowledge on immune suppressive mechanisms in the tumor microenvironment of HCC, CCA and LM-CRC, summarize the results of clinical studies to overcome these immune suppressive mechanisms, suggest alternative therapeutic approaches to abrogate these mechanisms based on the described novel insights from preclinical studies, and point out the current gaps in our knowledge on immune suppressive mechanisms in the tumor microenvironment of liver cancer. We discuss 1) suppressive immune cell subsets such as conventional regulatory T cells and type 1 regulatory T cells that can suppress effector T cell functions, 2) co-inhibitory pathways between T cells expressing co-inhibitory receptors and other immune cells and tumor cells expressing the ligands for these receptors, 3) intra-tumoral enzymes generating immune suppressive metabolites, 4) inhibition of migration of immune effector cells into the tumors, which leads to a paucity of immune cells inside liver cancers.

### **Regulation of intra-tumoral suppressor T cells in liver cancers**

**Part I** focuses on conventional regulatory T cells (Treg) and type 1 regulatory T cells (Tr1). We have studied how to abrogate the immune suppression exerted by these cells in the tumor microenvironment (Figure 1).

In **Chapter 2**, we show that treatment with a soluble form of the natural ligand of the co-stimulatory receptor GITR, or a blocking antibody to co-inhibitory receptor CTLA4, reduced *ex vivo* suppression of effector CD4<sup>+</sup> T cells mediated by CD4<sup>+</sup>Foxp3<sup>+</sup> Treg isolated from HCC and LM-CRC tumors, thereby restoring effector T cell proliferation and cytokine production. Importantly, a combination of low doses of both treatments exhibited a stronger recovery of effector T cell function in the presence of tumor-derived Treg compared with

either treatment alone. Our data suggest that in patients with HCC or LM-CRC both GITR-ligation and anti-CTLA-4 antibody can improve antigen-specific T cell responses in the tumors by decreasing tumor-infiltrating Treg-mediated suppression. However, as part of an immunotherapeutic strategy, combining low doses of both drugs may be at least as effective as monotherapy with higher doses of either drug.

In **Chapter 3**, we identified a population of tumor-infiltrating Tr1 cells that contributes to local immune suppression in an IL-10 dependent manner in HCC and LM-CRC. Importantly, the presence of tumor-infiltrating Tr1 cells is correlated with tumor infiltration of plasmacytoid dendritic cells. Plasmacytoid dendritic cells may drive intra-tumoral immunosuppression by Tr1 cells through stimulating IL-10 production by Tr1 cells via ICOS/ICOS-ligand signaling. Therefore, Tr1 cells may inhibit anti-tumor immunity in HCC and LM-CRC and thereby foster tumor progression. Blockade of the engagement of ICOS on Tr1 cells and ICOS-ligand on plasmacytoid dendritic cells may provide alleviation of this intra-tumoral immunosuppressive mechanism, and provide a new potential immunotherapeutic approach for patients with solid cancers in which IL-10-producing CD4<sup>+</sup> T cells are present, .

In **Chapter 4**, we propose that CD25 is a less attractive target for cancer immunotherapy in humans than originally suggested by Arce Vargas et al.,(1) because CD25 is not solely expressed on conventional Treg, but also on CD4<sup>+</sup>Foxp3<sup>low</sup>CD45RA<sup>-</sup> non-Treg in tumors, tumor-free tissues, lymph nodes and peripheral blood of HCC and CRC patients. CD25 antibody-mediated depletion of these cells may counteract the enhancement of anti-tumor immunity by Treg depletion. In addition, based on the observed reduction of CD16 (FcγR III) expression on intra-tumoral NK cells in cancer patients, the capacity of antibody-dependent cell-mediated cytotoxicity (ADCC) to deplete immune cells in human tumors may be more limited than previously expected. Therefore, CD25-depleting antibodies might not be appropriate for intra-tumoral Treg depletion in humans. Future research to develop Treg-depleting antibody therapies should focus on other targets than CD25.

Higher frequencies of Treg have been observed in HCC and LM-CRC tumors compared to paired tumor-free liver tissues and blood in Part I, and the same finding is also described for CCA tumors in Part II of this thesis. In addition, lower frequencies of CD8<sup>+</sup> cytotoxic T cells, natural killer T cells and natural killer cells as well as lower expression of the cytotoxic effector molecules perforin and granzyme B in CD8<sup>+</sup> T cells in HCC and LM-CRC tumors have previously been reported by us,(2) and are reported in Part II of this thesis for CCA tumors. These data indicate that the composition of immune infiltrates in liver tumors has immunosuppressive properties. It has been reported that the composition of tumor-infiltrating

lymphocytes is associated with the prognosis of liver cancers, for instance increased numbers of intra-tumoral Treg are associated with disease progression and recurrence.(3-8) Moreover, higher number of intra-tumoral Treg has been associated with increased numbers of intra-tumoral macrophages, circulating myeloid-derived suppressor cells, and with a compromised intra-tumoral CD8<sup>+</sup> T cell response.(9-13) Altogether these observations suggest that depletion or inhibition of intra-tumoral Treg and concomitant stimulation of immune effector cells may be effective to reduce tumor growth and recurrence as well as prolong survival of patients with liver cancer.

Until recently direct evidence for a role of Tr1 cells in human solid tumors was lacking. The recent description of co-expression of CD49b and LAG3 as markers that can specifically identify this population of cells,(14) enabled us to show that the majority of liver tumor-infiltrating IL-10-producing CD4<sup>+</sup> T cells consists of Tr1 cells and only a minor proportion corresponds to Th2 cells or Foxp3<sup>+</sup> Treg. Our data demonstrate a possible interaction between Tr1 cells and plasmacytoid dendritic cells by which tumor-infiltrating Tr1 cells may be activated by plasmacytoid dendritic cells present in the tumor microenvironment. The main population expressing ICOS-ligand in tumor tissues are CD123<sup>+</sup> plasmacytoid dendritic cells. There is substantial evidence indicating that plasmacytoid dendritic cells have a specialized role in the induction of peripheral tolerance by inducing IL-10 production by conventional Treg through ICOS-ICOS-ligand signaling.(15, 16) Stimulation of conventional Treg to produce IL-10 by plasmacytoid dendritic cells has been described in breast cancer and ovarian cancer.(17, 18) Our results are consistent with a role for plasmacytoid dendritic cells to stimulate IL-10 production by Tr1 cells in the tumor microenvironment of patients with liver cancer. Altogether these data suggest that ICOS co-stimulation represents a potential target for immunotherapeutic intervention, affecting tumor-specific immunosuppression mediated by both Treg and Tr1 cells.

In addition, other issues have already been discussed in the previous chapters.

### **Regulation of intra-tumoral effector T cells in liver cancers**

**Part II** focuses on CD8<sup>+</sup> cytotoxic T cells (CTL) and CD4<sup>+</sup> T helper cells (Th). Here, we have studied how to invigorate effector functions of tumor-infiltrating T cells by targeting co-inhibitory and co-stimulatory immune checkpoint pathways (Figure 1).

In **Chapter 5**, we conclude that co-inhibitory receptors PD-1, TIM3 and LAG3 are upregulated on tumor antigen-specific CD8<sup>+</sup> T cells isolated from patient HCC tumor tissues. Myeloid dendritic cells, monocytes and B cells in HCC tumors express ligands for these

receptors. Compared with tumor-infiltrating CD8<sup>+</sup> CTL and CD4<sup>+</sup> Th that did not express these co-inhibitory receptors, tumor-infiltrating CTL and Th that did express these receptors showed higher levels of activation markers, but similar or decreased levels of granzyme B and effector cytokines. While blocking a single co-inhibitory pathway had variable effects on *ex vivo* functional responses of HCC-derived T cells, combining antibody against PD-L1 with antibodies against TIM3, LAG3 or CTLA4 revitalized *ex vivo* tumor-specific responses of HCC-derived CD8<sup>+</sup> and CD4<sup>+</sup> T cells in most patients and additively enhanced these effects compared to single PD-L1 blockade. Overall, the aforementioned combined targeting of co-inhibitory checkpoints is a promising immunotherapeutic strategy for HCC, and may result in superior clinical responses in more patients than targeting a single co-inhibitory checkpoint.

In **Chapter 6**, we demonstrate higher proportions of CD8<sup>+</sup> T cells, myeloid dendritic cells and monocytes within immune cells isolated from mismatch repair (MMR)-proficient LM-CRC compared to immune cells derived from MMR-proficient primary CRC, suggesting an increased cytotoxic and antigen-presenting capacity of immune infiltrates in LM-CRC. In addition, we observed increased expression of co-inhibitory receptors on intra-tumoral T cells in MMR-proficient LM-CRC, which suggests that tumor-infiltrating T cells in MMR-proficient LM-CRC may be more sensitive to immune checkpoint inhibitors than tumor-infiltrating T cells in MMR-proficient primary CRC. Antibody blockade of LAG3 or PD-L1 enhanced *ex vivo* effector functions of CD8<sup>+</sup> and CD4<sup>+</sup> T cells derived from MMR-proficient LM-CRC, with LAG3 blockade showing the most robust effects on tumor-specific responses. In addition, higher LAG3 expression on intra-tumoral CD8<sup>+</sup> T cells associated with longer progression-free survival of LM-CRC patients. Overall, LAG3 may be a new promising immunotherapeutic target for LM-CRC, even for MMR-proficient tumor type. Clinical studies focusing on responses of LM-CRC to anti-LAG3 antibody treatment are recommended.

In **Chapter 7**, we analyzed the composition and characteristics of intra-tumoral immune infiltrates in CCA. We found decreased numbers of cytotoxic lymphocytes and increased numbers of Treg in CCA tumors compared with paired tumor-free liver tissues. While Treg infiltrate into the tumors, the majority of CD8<sup>+</sup> CTL and CD4<sup>+</sup> Th are sequestered at the tumor margin. Furthermore, intra-tumoral CD8<sup>+</sup> T cells show reduced expression of cytotoxic molecules compared with T cells in tumor-free liver tissues and blood. Expression of co-inhibitory receptors PD-1 and CTLA4 as well as co-stimulatory receptor GITR are upregulated on tumor-infiltrating T cells compared to T cells in tumor-free liver tissues and blood. Stimulation of GITR by the soluble ligand or blocking PD-1 by a therapeutic human antibody enhanced the effector functions of CCA-derived CD8<sup>+</sup> and CD4<sup>+</sup> T cells *ex vivo*,

which indicates that these two molecules are potential targets for immunotherapy of CCA patients.

In **Chapter 8**, we evaluated a proof of concept that agonists of co-stimulatory molecules may be effective for HCC immune therapy. We observed the highest GITR expression on CD4<sup>+</sup>Foxp3<sup>hi</sup>CD45RA<sup>-</sup> activated Treg in HCC tumors, although GITR expression on activated Treg in tumor-free liver tissues and blood was also higher than on CD4<sup>+</sup> Th and CD8<sup>+</sup> CTL in the same compartments. Furthermore, addition of recombinant GITR-ligand or humanized agonistic antibody to GITR to CD8<sup>+</sup> and CD4<sup>+</sup> T cells isolated from HCC tumors invigorated their responses to stimulations with polyclonal antigens and tumor antigens. Agonistic targeting of GITR may therefore be a potential strategy for single or combinatorial immunotherapy in HCC.

Interestingly, PD-1-expressing cells are increased in CD8<sup>+</sup> CTL, Th and Treg in HCC, LM-CRC and CCA tumors, while TIM3-expressing cells are increased in CD8<sup>+</sup> CTL and Treg, while CTLA4-expressing cells are increased in CD8<sup>+</sup> CTL and Th, and GITR-expressing cells are increased in Treg in all liver tumors compared with paired tumor-free liver tissues and blood, but LAG3 is not upregulated in CCA tumors (some data are not shown in the thesis).

In HCC, co-inhibitory receptors PD-1, TIM3 and LAG3 are selectively upregulated on intra-tumoral tumor antigen-specific CD8<sup>+</sup> T cells. The selective increased expression of these co-inhibitory receptors on tumor antigen-specific T cells together with the expression of their respective ligands on antigen-presenting cells in HCC tumors suggests that these co-inhibitory pathways may be involved in the regulation of tumor antigen-specific responsiveness of intra-tumoral T cells.

In both HCC and LM-CRC, tumor-infiltrating CD8<sup>+</sup> CTL and CD4<sup>+</sup> Th that expressed PD-1, TIM3, LAG3 or CTLA4 displayed higher levels of activation markers, compared with their counterparts that did not express the corresponding co-inhibitory receptor, except for CD69 expression on LAG3<sup>+</sup>CD8<sup>+</sup> versus LAG3<sup>-</sup>CD8<sup>+</sup> T cells. However, the tumor-infiltrating T cells expressing either co-inhibitory receptor displayed comparable or lower levels of effector cytokines and/or cytotoxic molecules. Expression of co-inhibitory receptors on T cells can be induced by recent T cell activation upon recognition of tumor antigens,(19, 20) but continuous expression of increasing numbers of co-inhibitory receptors often coincides with gradual loss of effector T cell functions due to chronic antigenic stimulation.(21-23) Our findings indicate that, in accordance with recent studies in other human cancers,(23-25) tumor-infiltrating T cells that express co-inhibitory receptors are probably tumor-reactive T cells, which continue to upregulate the expression of these receptors in response to chronic



stimulation by tumor antigens to prevent further over activation, and thereby are subsequently inhibited by the interactions of these receptors with their ligands on tumor cells or antigen-presenting cells.

In all the *in vitro* functional assays of HCC, LM-CRC and CCA, we used total tumor-infiltrating mononuclear leukocytes which contained both T cells expressing co-inhibitory receptors and antigen-presenting cells expressing their ligands, because we were interested in the net effects of antibody interventions on intra-tumoral T cell responses in a context that resembled the tumor microenvironment as much as possible. Consequently, intra-tumoral Treg, Tr1 cells and probably other types of immune suppressor cells were also present. Therefore, the reported functional effects of antibodies on effector T cells in these assays may be partly indirect, mediated by the effects of these antibodies on immune suppressor cells. A limitation of our *in vitro* assays is the lack of tumor cells which can also express ligands for co-inhibitory receptors and exert other unknown immunosuppressive effects. Therefore tumor-infiltrating T cells might be more suppressed *in vivo* than in our *ex vivo* culture models.

In HCC, targeting either CTLA4 or GITR has been shown to both reduce the immunosuppression mediated by tumor-infiltrating Treg (Part I) and restore anti-tumor functions of tumor-infiltrating effector T cells (Part II). Although the mechanisms by which these molecules exert their immunomodulatory effects are still elusive, our results suggest that they can act either by abrogating the suppressive function of Treg or by rendering effector T cells resistant to Treg-mediated suppression.

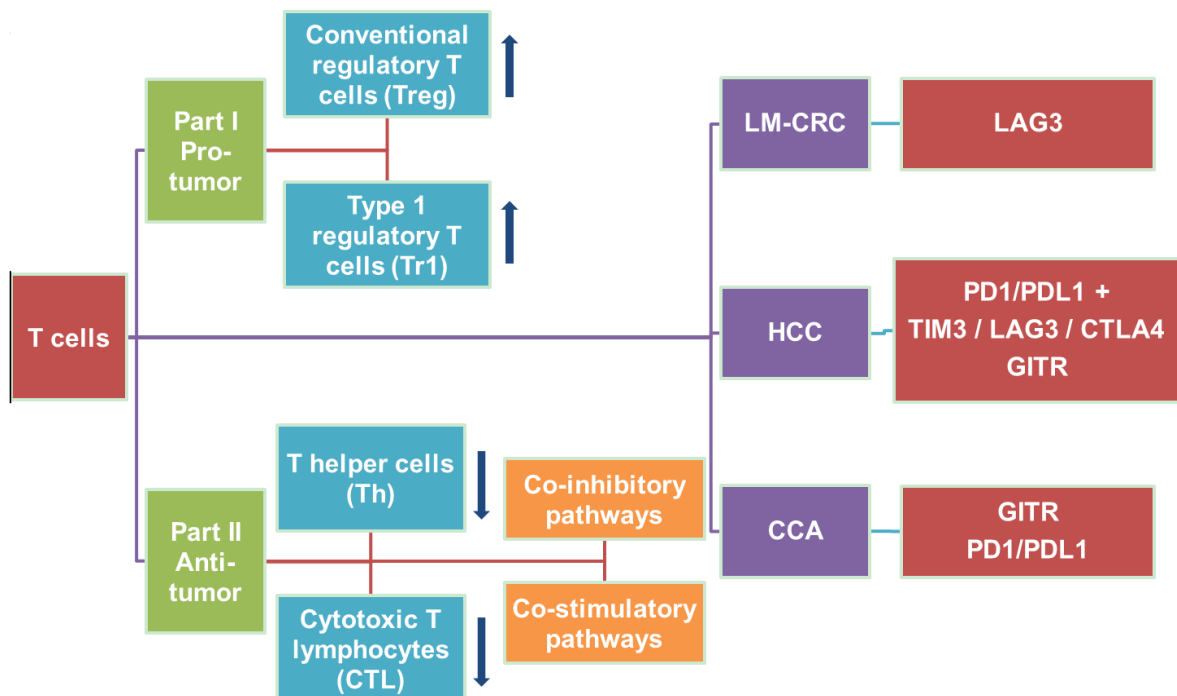
Additionally, other issues have already been discussed in the previous chapters.

### **Overall conclusions and future perspectives**

The ultimate aim of this thesis is to provide new immunotherapeutic approaches to treat patients with primary liver cancer or CRC liver metastasis. Our studies show that, increased proportions of Treg and Tr1 cells and decreased proportions of cytotoxic lymphocytes in immune infiltrates within liver tumors, as well as elevated expression of several co-inhibitory receptors on tumor-infiltrating T cells and the presence of their ligands in human liver tumors, both contribute to an immunosuppressive tumor microenvironment in liver cancers. Co-inhibitory pathways CTLA4-CD80/CD86, PD-1-PD-L1, TIM3-galectin 9, LAG3-MHC class II and co-stimulatory pathways GITR-GITR-ligand and ICOS-ICOS-ligand play an important role in regulating intra-tumor T cell immunity in patients with HCC, CCA or LM-CRC. This thesis provides new clues for immunotherapeutic interventions to overcome the abovementioned immune suppressive mechanisms and to reinforce the anti-tumor reactivity,

by demonstrating that combined blockade of PD-1/PD-L1 and TIM3, LAG3 or CTLA4 as well as stimulation of GITR are effective in reinvigorating tumor-derived T cells in HCC, antibody against LAG3 has similar potential for LM-CRC, while agonistic antibody of GITR and blocking antibody of PD-1/PD-L1 are suggested for CCA.

Currently, several clinical trials exploring the effects of anti-PD-1 antibodies in CCA patients are ongoing. Clinical trials investigating whether combination treatments of anti-PD-1/PD-L1 antibodies and anti-CTLA4 antibodies result in improved clinical efficacy are also ongoing in HCC and CCA patients. In addition, GITR agonistic antibody combined with PD-1 and/or CTLA4 antagonistic antibody, CD134 agonistic antibody combined with PD-1 and/or CTLA4 antagonistic antibody, CD134 agonistic antibody in combination with CD137 agonistic antibody are being tested in HCC patients.(26)[ClinicalTrials.gov] However, clinical trials on immunotherapy with anti-TIM3 and anti-LAG3 antibodies have not been initiated in patients with liver cancer. Moreover, clinical studies on LAG3 antagonistic antibody for patients with LM-CRC and clinical studies on GITR agonistic antibody for patients with CCA are lacking. Further research on co-expression of different immune checkpoints and clinical efficacy of combinatorial immunotherapies in patients with liver cancer is definitely needed to improve the clinical outcome.



**Figure 1. Summary of the main findings of the thesis**

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## Chapter 9

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# CHAPTER 10

**Dutch Summary**

**Nederlandse samenvatting**

Leverkanker is de op één na meest voorkomende oorzaak van kanker gerelateerde mortaliteit in de wereld. Hepatocellulair carcinoom (HCC) is de meest frequent voorkomende primaire leverkanker, cholangiocarcinoom (CCA) de tweede. In de lever gemetastaseerde colorectaal kanker (LM-CRC) is de meest voorkomende secundaire leverkanker, en de voornaamste doodsoorzaak van patiënten met colorectaal kanker (CRC). De huidige therapeutische mogelijkheden voor alle drie deze typen van leverkanker zijn heel beperkt. Vandaar dat er een dringende behoefte bestaat aan nieuwe en effectieve behandelmethoden. De doelen van dit proefschrift zijn: 1) om te bepalen of regulatoire T-cellen en co-inhibitoire immuun checkpoint routes bijdragen aan het immunosuppressieve micromilieu in levertumoren, en 2) om potentiël veelbelovende factoren te identificeren die deze immuun suppressieve mechanismen onderdrukken en daardoor de anti-tumor functie van de in de tumor infiltrerende T-cellen in patiënten met leverkanker versterken. In dit hoofdstuk zullen we de belangrijkste bevindingen samenvatten en bediscussiëren en suggesties geven voor toekomstig onderzoek.

In **hoofdstuk 1** geven we een overzicht van de actuele kennis over immunosuppressieve mechanismen in het tumor micromilieu van HCC, CCA en LM-CRC. We vatten de resultaten van klinische studies samen die proberen deze immunosuppressieve mechanismen te onderdrukken, we stellen alternatieve therapeutische benaderingen voor om deze mechanismen op te heffen gebaseerd op beschreven nieuwe inzichten van preklinische studies en we wijzen op actuele hiaten in onze kennis over immunosuppressieve mechanismen in het tumor micromilieu van leverkanker. We bediscussiëren 1) suppressieve immuun cel subsets zoals conventionele regulatoire T-cellen en type 1 regulatoire T-cellen die de functie van effector T-cellen kunnen onderdrukken, 2) co-inhibitoire interacties tussen T-cellen die co-inhibitoire receptoren tot expressie brengen en andere immuun cellen en tumor cellen, die de liganden voor deze receptoren tot expressie brengen, 3) enzymen in tumoren die metabolieten genereren die het immuunsysteem onderdrukken, 4) het verhinderen van de migratie van immuun effector cellen naar de tumor, wat leidt tot een tekort aan immuun cellen in leverkanker.

### **Regulatie van intra-tumorale suppressieve T-cellen in leverkanker**

**Deel I** focust op conventionele regulatoire T-cellen (Treg) en type 1 regulatoire T-cellen (Tr1). We hebben bestudeerd hoe de immuun suppressie die wordt uitgeoefend door deze cellen kan worden onderdrukt in het tumor micromilieu (figuur 1).

In **hoofdstuk 2** laten we zien dat een oplosbare vorm van het natuurlijke ligand van de co-stimulatoire receptor GITR en/of een blokkerende antistof tegen de co-inhibitoire receptor

CTLA4 de *ex vivo* suppressie van effector CD4<sup>+</sup> T-cellen door CD4<sup>+</sup>Foxp3<sup>+</sup> Treg uit HCC en LM-CRC tumoren vermindert, waardoor de celdeling en cytokine productie van deze effector T-cellen wordt hersteld. Belangrijk is hierbij dat een combinatie van lage doses van beide behandelingen een sterker herstel van de functie van effector T cellen laat zien in aanwezigheid van uit de tumor geïsoleerde Treg dan iedere behandeling afzonderlijk. Onze data suggereren dat behandeling van HCC en LM-CRC patiënten met GITR-ligatie of antistoffen tegen CTLA4 de antigeen specifieke T-cel responsen kan verbeteren in tumoren door vermindering van de suppressie door in de tumor geïnfiltreerde Treg. Echter, als immunotherapeutische strategie kan een combinatie van een lage dosis van beiden medicijnen even effectief zijn als een monotherapie met een hoge dosis van één van deze medicijnen.

In **hoofdstuk 3** hebben we een populatie van in de tumor geïnfiltreerde Tr1 cellen geïdentificeerd die bijdraagt aan lokale immuun suppressie op een IL-10 afhankelijke manier in HCC en LM-CRC. De aantallen van Tr1 cellen in deze tumoren zijn positief gecorreleerd aan de aantallen in de tumor geïnfiltreerde plasmacytoïde dendritische cellen. We hebben aanwijzingen gevonden die erop wijzen dat plasmacytoïde dendritische cellen via ICOS/ICOS-ligand interacties de IL-10 productie van Tr1 cellen stimuleren en hierdoor bijdragen aan de door de Tr1 veroorzaakte intra-tumorale immuun suppressie. Dus Tr1 cellen kunnen mogelijk anti-tumor immuniteit onderdrukken in HCC en LM-CRC en daardoor de tumorprogressie bevorderen. Het blokkeren van de interactie van ICOS op de Tr1 cel en ICOS-ligand op de plasmacytoïde dendritische cel kan dit intra-tumorale immunosuppressieve mechanisme verminderen en een nieuwe potentiële immunotherapeutische benadering bieden voor patiënten met solide tumoren waarin IL-10-producerende CD4<sup>+</sup> T-cellen aanwezig zijn.

In **hoofdstuk 4** suggereren we dat CD25 een minder aantrekkelijk doelwit is voor immunotherapie bij kanker bij mensen dan oorspronkelijk werd gesuggereerd door Arce Vargas et al., omdat CD25 niet alleen tot expressie komt op conventionele Treg, maar ook op CD4<sup>+</sup>Foxp3<sup>laag</sup>CD45RA<sup>-</sup>non-Treg die voorkomen in tumoren, tumorvrije weefsels, lymfeklieren en perifeer bloed van HCC en CRC patiënten. CD25 antistof-gemedieerde depletie van deze cellen kan de verhoging van anti-tumor immuniteit door Treg-depletie tegengaan. Doordat intra-tumorale NK-cellen in kankerpatiënten minder CD16 (FcyR III) tot expressie brengen, kan bovendien het vermogen om immuun cellen in menselijke tumoren te depletieren door antistof-afhankelijke celgemedieerde cytotoxiciteit (ADCC), beperkter zijn dan eerder was verwacht. Daarom zijn CD25-depletierende antistoffen mogelijk niet geschikt voor intra-tumorale Treg-depletie bij mensen. Toekomstig onderzoek met betrekking tot

Treg-depletie met behulp van antistoffen zou zich moeten richten op andere doelwitten dan CD25.

### **Regulatie van intra-tumorale effector T cellen in levertumoren**

**Deel II** focust op CD8<sup>+</sup> cytotoxische T lymfocyten (CTL) en CD4<sup>+</sup> T helper cellen (Th). In dit gedeelte hebben we bestudeerd hoe effector functies van tumor-infiltrerende T cellen kunnen worden versterkt door targetting van co-inhibitoire en co-stimulerende immuun checkpoint pathways (Figuur 1).

In **Hoofdstuk 5** concluderen we dat de co-inhibitoire receptoren PD-1, TIM3 en LAG3 verhoogd tot expressie komen op tumor antigen-specifieke CD8<sup>+</sup> CTL geïsoleerd uit humane HCC-tumor weefsels. Myeloïde dendritische cellen, monocyt en B cellen in HCC tumoren brengen de liganden van deze receptoren tot expressie. Vergeleken met tumor-infiltrerende CD8<sup>+</sup> CTL en CD4<sup>+</sup> Th zonder co-inhibitoire receptoren, brengen de tumor-infiltrerende CTL en Th met deze receptoren, meer activatie markers tot expressie, maar is de hoeveelheid granzyme B en effector cytokines die ze tot expressie brengen, is vergelijkbaar of verminderd. Het blokkeren van één co-inhibitoire pathway had variabele effecten op *ex vivo* functionele responsen van HCC tumor infiltrerende lymphocyten, maar het combineren van een antagonistisch PD-L1 antistof met een antagonistisch TIM3, LAG3 of CTLA4 antilistof, versterkte *ex vivo* tumor-specifieke responsen van CD8<sup>+</sup> en CD4<sup>+</sup> T cellen geïsoleerd uit HCC in de meeste patiënten, en dit effect ervan was groter in vergelijking met alleen blokkering van PD-L1. We concluderen dat de eerdergenoemde combinatie therapie van co-inhibitoire checkpoint antistoffen een veelbelovende immunotherapeutische strategie is voor HCC, die zou kunnen resulteren in een superieure klinische respons in een groter percentage van de patiënten vergeleken met monotherapie met één co-inhibitoire checkpoint antistof.

In **Hoofdstuk 6** laten we zien dat immuun cellen geïsoleerd uit DNA mismatch repair (MMR)-proficiënte LM-CRC tumoren hogere proporties CD8<sup>+</sup> T cellen, myeloïde dendritische cellen en monocyt en bevatten dan immuun cellen geïsoleerd uit MMR-proficiënte primaire CRC tumoren. Dit suggereert een verhoogde cytotoxische en antigen-presenterende capaciteit van immuun infiltraten in LM-CRC. Daarnaast zagen we verhoogde expressie van co-inhibitoire receptoren op intra-tumorale T cellen in MMR-proficiënte LM-CRC, wat suggereert dat de tumor-infiltrerende T cellen in MMR-proficiënte LM-CRC gevoeliger zijn voor immuun checkpoint antagonisten dan tumor-infiltrerende T cellen in MMR-proficiënte primaire CRC. Blokkade van LAG3 of PD1 door antistoffen verbeterde *ex vivo* de effector functies van CD8<sup>+</sup> en CD4<sup>+</sup> T cellen verkregen uit MMR-proficiënte LM-CRC tumoren. Van deze twee liet



blokkade van LAG3 de meest robuuste effecten zien op tumor-specifieke T-cel responsen. Bovendien was hogere LAG3 expressie op intra-tumorale CD8<sup>+</sup> T cellen geassocieerd met langere progressie-vrije overleving in LM-CRC patiënten. Alles bijeengenomen zou LAG3 een nieuwe en veelbelovende immunotherapeutische target kunnen zijn voor LM-CRC, zelfs voor het MMR-proficiënte tumor type. Klinische studies naar het effect van anti-LAG3 antilistof behandeling op LM-CRC wordt aangeraden om deze bevindingen te bevestigen.

In **Hoofdstuk 7** hebben we de samenstelling en karakteristieken van intra-tumorale immuun cel infiltraten in CCA geanalyseerd. In de CCA tumoren vonden we verlaagde aantallen cytotoxische lymfocyten en verhoogde aantallen Treg in vergelijking met de gepaarde tumorvrije leverweefsels. Waar Treg in de tumoren infiltreren, is het merendeel van de CD8<sup>+</sup> CTL en CD4<sup>+</sup> Th juist te vinden in het overgangsg gebied tussen tumorvrij leverweefsel en de tumor. Daarnaast laten de intra-tumorale CD8<sup>+</sup> T cellen verminderde expressie van cytotoxische moleculen zien vergeleken met T cellen in het tumorvrije leverweefsel en bloed, en is zowel de expressie van de co-inhibitoire receptoren PD-1 en CTLA4 als de co-stimulerende receptor GITR hoger in de tumor-infiltrerende T cellen dan op de T cellen uit het tumorvrije leverweefsel en bloed. Stimulatie van GITR door het oplosbare ligand of het blokkeren van PD-1 door een therapeutische humane antistof, verbeterden de effector functies *ex vivo* van CD8<sup>+</sup> en CD4<sup>+</sup> T cellen verkregen uit CCA tumoren, wat aangeeft dat deze twee moleculen potentiële targets zijn voor immunotherapeutische behandeling van CCA patiënten.

In **Hoofdstuk 8** onderzochten we het concept dat agonisten van co-stimulerende moleculen een effectieve immunotherapeutische behandeling kunnen bieden voor HCC. Geactiveerde CD4<sup>+</sup>FoxP3<sup>hoog</sup>CD45RA<sup>-</sup> Treg hadden de hoogste GITR expressie van alle immuuncellen in HCC tumoren. Ook in tumorvrij leverweefsel en bloed was de expressie van GITR hoger op geactiveerde Treg dan op CD4<sup>+</sup> Th en CD8<sup>+</sup> CTL in dezelfde compartimenten. Toevoeging van recombinant GITR-ligand of een gehumaniseerde agonistische antistof tegen GITR aan CD8<sup>+</sup> en CD4<sup>+</sup> T cellen geïsoleerd uit HCC tumoren verhoogde hun responsen op polyclonale of tumorantigen-specifieke stimulatie. Dus agonistische targeting van GITR zou een potentiële strategie zijn voor mono- of combinatie immunotherapie van HCC.

### **Algemene conclusies en toekomstperspectieven**

Het uiteindelijke doel van dit proefschrift is om nieuwe immunotherapeutische methoden te identificeren voor de behandeling van patiënten met primaire leverkanker of CRC levermetastasen. Onze studies tonen aan dat verhoogde aantallen Treg- en Tr1-cellen en verlaagde aantallen cytotoxische lymfocyten in immuun-infiltraten in tumoren bijdragen aan

een immunosuppressief tumor-micromilieu in levertumoren, evenals een verhoogde expressie van verschillende co-inhiberende receptoren op in de tumor geïnfiltreerde T-cellen en de aanwezigheid van hun liganden in de levertumoren. Beide factoren dragen bij aan een immunosuppressief tumor-micromilieu in leverkanker. De co-inhiberende routes CTLA4-CD80/CD86, PD-1/PD-L1, TIM3-galectine 9, LAG3-MHC klasse II en de co-stimulerende routes GITR-GITR-ligand en ICOS-ICOS-ligand spelen een belangrijke rol in de regulatie van intra-tumorale T-cel immuniteit bij patiënten met HCC, CCA of LM-CRC. Dit proefschrift biedt nieuwe suggesties voor immunotherapeutische interventies om de bovengenoemde immunosuppressieve mechanismen te onderdrukken en om de anti-tumor reactiviteit van in de tumoren geïnfiltreerde T cellen te verbeteren. Er wordt aangetoond dat gecombineerde blokkade van PD-1/PD-L1 en TIM3, LAG3 of CTLA4, evenals stimulatie van GITR, effectief zijn in het stimuleren van T-cellen afkomstig uit HCC tumoren. Antistoffen tegen LAG3 bieden een vergelijkbare mogelijkheid voor LM-CRC, terwijl agonistische antistoffen tegen GITR en antistoffen die de interactie tussen PD-1/PD-L1 blokkeren worden voorgesteld voor CCA.

Momenteel lopen er verschillende klinische onderzoeken naar de effecten van anti-PD-1-antistoffen bij CCA-patiënten. Klinische studies bij HCC en CCA patiënten die onderzoeken of een combinatie van een anti-PD-1/PD-L1-antistof met een anti-CTLA4-antistof leidt tot verbeterde klinische werkzaamheid zijn ook lopend. Bovendien worden in HCC patiënten de combinatie van een GITR-agonistische antistof met een PD-1 en/of CTLA4-antagonistische antistof, evenals een CD134-agonistische antistof gecombineerd met een PD-1 en/of CTLA4-antagonistische antistof, en een CD134-agonistische antistof in combinatie met een CD137-agonistische antistof getest. Echter, klinische trials met anti-TIM3 en anti-LAG3-antistoffen zijn niet gestart bij patiënten met leverkanker. Bovendien ontbreken in patiënten met LM-CRC klinische studies naar antagonistische antistoffen tegen LAG3, en in patiënten met CCA ontbreken klinische studies met GITR-agonistische antistoffen. Verder onderzoek bij patiënten met leverkanker naar co-expressie van verschillende immuun checkpoints en klinische werkzaamheid van gecombineerde immunotherapieën is absoluut noodzakelijk om de klinische uitkomst te verbeteren.

**Vertaald door Patrick Boor en Lisanne Noordam**

# **APPENDIX**

**Acknowledgements**

**PhD portfolio**

**List of publications**

**About the author**

## PhD portfolio

<b>Name of PhD student</b>	Guoying Zhou
<b>Department</b>	Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam
<b>PhD period</b>	October 2013 - April 2018
<b>Promotor</b>	Prof. Dr. Marco J. Bruno
<b>Co-promotor</b>	Dr. Jaap Kwekkeboom

### **General courses**

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Biomedical Research Techniques course

Annual Course on Molecular Medicine

Workshop on Microsoft Excel 2010: Advanced

Basic Introduction Course on SPSS

Biomedical English Writing Course for MSc and PhD-students

Advanced Immunology course

Workshop Presenting Skills for junior researchers

Basic and Translational Oncology course

Workshop on Photoshop and Illustrator CS6 for PhD-students and other researchers

Course on Scientific Integrity

Advanced course on Applications in flow cytometry

Daniel den Hoed Day (Erasmus MC Cancer Institute Research Day)

Erasmus MC PhD Day

Erasmus MC Liver Day

Erasmus MC Molecular Medicine Day

**Conferences – Oral presentations**

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2015 European Congress of Immunology (ECI), Vienna, Austria

2016 The Erasmus Medical Center Molecular Medicine Day, Rotterdam, the Netherlands

2016 Dutch Association for Gastroenterology (NVGE) meeting, Veldhoven, the Netherlands

2016 Cancer Immunotherapy (CIMT) Annual Meeting, Mainz, Germany

2016 International Liver Cancer Association (ILCA) Annual Conference, Vancouver, Canada

2017 Dutch Association for Gastroenterology (NVGE) meeting, Veldhoven, the Netherlands

2017 Dutch Tumor Immunology Meeting (DTIM), Breukelen, the Netherlands

2017 The Erasmus Medical Center Cancer Institute Research Day, Rotterdam, the Netherlands

**Conferences – Invited talks**

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2017 Liver Metastases Research Network (LMRN) Congress, Rotterdam, the Netherlands

2017 Hong Kong Society for Immunology (HKSI) Annual General Meeting and Scientific Meeting, Hong Kong, China

2018 Best Publication Award of the Erasmus Medical Center Molecular Medicine Day, Rotterdam, the Netherlands

2018 Top 3 Battle of NVH (Dutch Association for Hepatology) Young Hepatologist Award, Veldhoven, the Netherlands

**Conferences – Poster presentations**

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2014 Dutch Society for Immunology (NVVI) Anniversary Congress, Efteling, the Netherlands

2015 Cancer Immunotherapy (CIMT) Annual Meeting, Mainz, Germany

2016 Cancer Immunotherapy (CIMT) Annual Meeting, Mainz, Germany

2016 International Liver Cancer Association (ILCA) Annual Conference, Vancouver, Canada

2017 The Erasmus Medical Center Molecular Medicine Day, Rotterdam, the Netherlands

## Appendix

2017 The annual meeting of the Federation of Clinical Immunology Societies (FOCIS), Chicago, USA

2017 Dutch Tumor Immunology Meeting (DTIM), Breukelen, the Netherlands

2017 CRI-CIMT-EATI-AACR International Cancer Immunotherapy Conference, Mainz, Germany

2018 Cancer Immunotherapy (CIMT) Annual Meeting, Mainz, Germany

### **Scientific grants and awards**

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2015 Association for Cancer Immunotherapy (CIMT) travel grant and free conference admission

2015 Erasmus Trustfonds travel grant

2016 Dutch Society for Immunology (NVVI) travel grant

2016 Erasmus Trustfonds travel grant

2017 Dutch Association for Hepatology (NVH) travel grant

2018 Best Publication Award of the Erasmus Medical Center Molecular Medicine Day

2018 NVH (Dutch Association for Hepatology) Young Hepatologist Award and free conference admission

### **Teaching activities**

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2015 Supervising master thesis: Hannah Schutz

2016 Supervising master thesis: Remco Erkens

### **Other activities**

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Reviewer of articles for *Medicine* and *Canadian Journal of Gastroenterology and Hepatology*

2016 Selected for United European Gastroenterology (UEG) basic science course, *Hot topics in experimental gastrointestinal cancer*, Munich, Germany

## List of publications

1. **Guoying Zhou**, L Noordam, D Sprengers, M Doukas, P Boor, A van Beek, R Erkens, S Mancham, D Grünhagen, A Menon, J Lange, P Burger, A Brandt, B Galjart, C Verhoef, J Kwekkeboom, M Bruno. Blockade of LAG3 enhances responses of tumor-infiltrating T cells in mismatch repair-proficient liver metastasis of colorectal cancer. *Oncoimmunology*. 2018 in press.
2. **Guoying Zhou**, D Sprengers, P Boor, M Doukas, H Schutz, S Mancham, A Pedroza-Gonzalez, W Polak, J de Jonge, M Gaspersz, H Dong, K Thielemans, Q Pan, J IJzermans, M Bruno, J Kwekkeboom. Antibodies Against Immune Checkpoint Molecules Restore Functions of Tumor-infiltrating T cells in Hepatocellular Carcinomas. *Gastroenterology*. 2017 Oct;153(4):1107-1119.
3. **Guoying Zhou**, D Sprengers, R Erkens, S Mancham, M Doukas, L Noordam, P Boor, R van Leeuwen, B Groot Koerkamp, W Polak, J de Jonge, J IJzermans, M Bruno, J Kwekkeboom. Abrogation of the immunosuppressive tumor microenvironment in cholangiocarcinoma by targeting PD-1 or GITR. *Under submission*.
4. A Pedroza-Gonzalez, **Guoying Zhou**, S Pal Singh, P Boor, Q Pan, D Grunhagen, J de Jonge, T Khe Tran, C Verhoef, J IJzermans, H Janssen, K Biermann, J Kwekkeboom, D Sprengers. GITR engagement in combination with CTLA-4 blockade completely abrogates immunosuppression mediated by human liver tumor-derived regulatory T cells ex vivo. *Oncoimmunology*. 2015 May 29;4(12).
5. A Pedroza-Gonzalez, **Guoying Zhou**, E Vargas-Mendez, P Boor, S Mancham, C Verhoef, W Polak, D Grunhagen, Q pan, H Janssen, G Garcia-Romo, K Biermann, E Tjwa, J IJzermans, J Kwekkeboom, D Sprengers. Tumor-infiltrating plasmacytoid dendritic cells promote immunosuppression by Tr1 cells in human liver tumors. *Oncoimmunology*. 2015 Mar 19;4(6).
6. A van Beek, **Guoying Zhou\***, L Noordam\*, P Boor, S Bucktrout, S ter Borg, P Doornebosch, M Doukas, D Sprengers, J Kwekkeboom. Concerns about targeting tumor-infiltrating regulatory T cells with Fc-optimized anti-CD25 antibodies in humans. *Under submission*.
7. K Sideras, A Pedroza-Gonzalez, A Vasaturo, K Biermann, S Mancham, A Nigg, H Stoop, **Guoying Zhou**, C Verhoef, S Sleijfer, D Sprengers, J Kwekkeboom, M Bruno. Prognostic value of intra-tumoral CD8<sup>+</sup>/FoxP3<sup>+</sup> lymphocyte ratio in patients with resected colorectal cancer liver metastasis. *Journal of Surgical Oncology*. 2018 in press.
8. K Sideras, R de Man, S Harrington, **Guoying Zhou**, H Schutz, A Pedroza-Gonzalez, K Biermann, S Mancham, B Hansen, B Takkenberg, Q Pan, J IJzermans, S Sleijfer, D Sprengers, H Dong, J Kwekkeboom, M Bruno. Circulating levels of PD-L1 and Galectin-9 are associated with patient survival in Hepatocellular Carcinoma independent of their intra-tumoral expression levels. *Under submission*.
9. **Guoying Zhou\***, H Wang\*, L Luo\*, A Yuan, J Kou, G Yang, M Wang, J Wu, B von Blomberg, J Crusius, S Morré, A Peña, B Xia. Serological Screening for Celiac Disease in Adult Chinese Patients with Diarrhea Predominant Irritable Bowel Syndrome. *Medicine (Baltimore)*. 2015 Oct;94(42).

## About the author

Guoying Zhou was born on January 6<sup>th</sup> 1988, in Shunde, Foshan, Guangdong Province, China. She was raised by her beloved parents Huabi Wu and Guanlun Zhou in her hometown.

In 2006, she graduated from high school and started to study medicine (seven-year program) in Wuhan University, Hubei Province. During her medical training, she conducted clinical rotations in Zhongnan Hospital, and performed her master research under supervision of Prof. Dr. Bing Xia at the department of Gastroenterology and Hepatology. In 2007 she also started to learn French, and in 2010 and 2012 she was twice selected for a half-year exchange internship with a scholarship in Brabois Hospital, Lorraine University, Nancy, France.



After obtaining her medical degree and passing the National Medical Licensing Examination in China in 2013, she started her PhD research on tumor immunology at the department of Gastroenterology and Hepatology, Erasmus University Medical Center, Rotterdam, the Netherlands. Under supervision of Prof. Dr. Marco Bruno and Dr. Jaap Kwekkeboom, she completed several translational studies on immunotherapy for hepatocellular carcinoma, cholangiocarcinoma and liver metastasis of colorectal cancer.



