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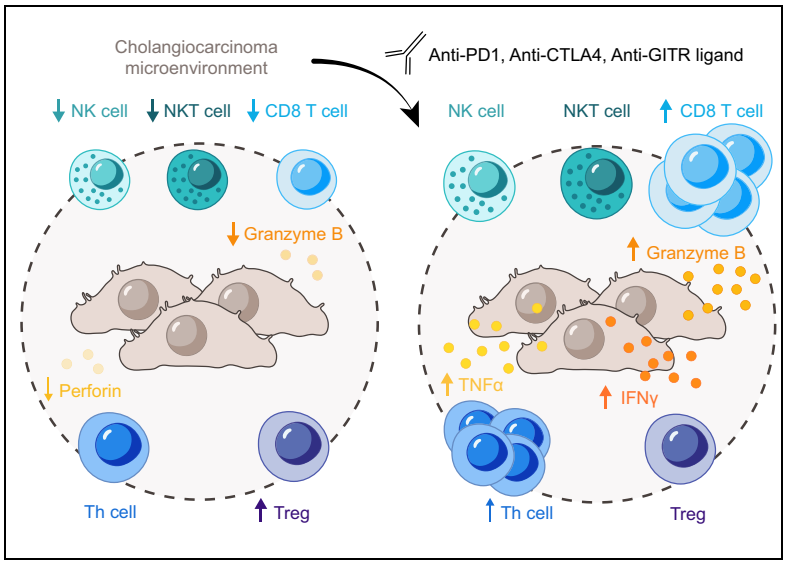
Reduction of immunosuppressive tumor microenvironment in cholangiocarcinoma by *ex vivo* targeting immune checkpoint molecules

Graphical abstract



Highlights

- NK cells and cytotoxic T cells infiltrate poorly into cholangiocarcinoma.
- Regulatory T cells accumulate in cholangiocarcinoma.
- PD1, CTLA4 and GITR are over-expressed on tumor-infiltrating T cells in cholangiocarcinoma.
- Blocking PD1 or CTLA4 or stimulating GITR enhances effector functions of tumor-infiltrating T cells in cholangiocarcinoma.

Authors

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Lay summary

The defense functions of immune cells are suppressed in cholangiocarcinoma tumors. Stimulating or blocking "immune checkpoint molecules expressed on tumor-infiltrating T cells can enhance the defense functions of these cells. Therefore, these molecules may be promising targets for therapeutic stimulation of immune cells to eradicate the tumors and prevent cancer recurrence in patients with cholangiocarcinoma.

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Reduction of immunosuppressive tumor microenvironment in cholangiocarcinoma by *ex vivo* targeting immune checkpoint molecules

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Background & Aims: Cholangiocarcinoma is an aggressive hepatobiliary malignancy originating from biliary tract epithelium. Whether cholangiocarcinoma is responsive to immune checkpoint antibody therapy is unknown, and knowledge of its tumor immune microenvironment is limited. We aimed to characterize tumor-infiltrating lymphocytes (TILs) in cholangiocarcinoma and assess functional effects of targeting checkpoint molecules on TILs.

Methods: We isolated TILs from resected tumors of patients with cholangiocarcinoma and investigated their compositions compared with their counterparts in tumor-free liver (TFL) tissues and blood, by flow cytometry and immunohistochemistry. We measured expression of immune co-stimulatory and co-inhibitory molecules on TILs, and determined whether targeting these molecules improved *ex vivo* functions of TILs.

Results: Proportions of cytotoxic T cells and natural killer cells were decreased, whereas regulatory T cells were increased in tumors compared with TFL. While regulatory T cells accumulated in tumors, the majority of cytotoxic and helper T cells were sequestered at tumor margins, and natural killer cells were excluded from the tumors. The co-stimulatory receptor GITR and co-inhibitory receptors PD1 and CTLA4 were over-expressed on tumor-infiltrating T cells compared with T cells in TFL and blood. Antagonistic targeting of PD1 or CTLA4 or agonistic targeting of GITR enhanced effector molecule production and T cell proliferation in *ex vivo* stimulation of TILs derived from cholangiocarcinoma. The inter-individual variations in TIL responses to checkpoint treatments were correlated with differences in TIL immune phenotype.

Conclusions: Decreased numbers of cytotoxic immune cells and increased numbers of suppressor T cells that over-express co-inhibitory receptors suggest that the tumor microenvironment in cholangiocarcinoma is immunosuppressive. Targeting GITR, PD1 or CTLA4 enhances effector functions of tumor-infiltrating T cells, indicating that these molecules are potential immunotherapeutic targets for patients with cholangiocarcinoma.

Lay summary: The defense functions of immune cells are suppressed in cholangiocarcinoma tumors. Stimulating or blocking "immune checkpoint" molecules expressed on tumor-infiltrating T cells can enhance the defense functions of these cells. Therefore, these molecules may be promising targets for therapeutic stimulation of immune cells to eradicate the tumors and prevent cancer recurrence in patients with cholangiocarcinoma.

Introduction

Liver cancer is the second most common cause of cancer-related mortality worldwide.[1] Cholangiocarcinoma (CCA) accounts for 10% of primary liver cancers and the incidence is significantly increasing. CCA is an aggressive hepatobiliary malignancy originating from the biliary tract epithelium with features of cholangiocyte differentiation.[2] It is classified into the following types according to its anatomic location along the biliary tree: intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA).[2,3] The median overall survival after diagnosis is 24 months and 5-year survival rate is around 10%.[4] The current treatment options for CCA are very limited. Surgical resection is potentially curative, but only 10% of patients are eligible for surgical resection and it is associated with a high recurrence rate (>50%).[2] Liver transplantation is a curative option for selected patients with pCCA but not with iCCA or dCCA.[3] The therapeutic effect of chemotherapy for advanced CCA is disappointing.[2] Therefore,

Keywords: Cholangiocarcinoma; Liver cancer; Tumour-infiltrating lymphocyte; T cell; Co-stimulatory; Co-inhibitory; Immunotherapy; Immune checkpoint.
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more effective therapies for curing CCA and preventing recurrence are urgently needed.

Cancer immunotherapy aims at stimulating the immune system to combat cancer. T cells are critical immune cells in immune responses against cancer. $CD8^+$ cytotoxic T cells that recognize tumor antigens, can kill tumor cells. $CD4^+$ T helper (Th) lymphocytes that recognize tumor antigens, can provide help to $CD8^+$ T cells, to macrophages to phagocytose tumor cells, and to B cells to produce antibodies against tumor cells. Upon antigen recognition, T cell activation is tightly regulated by 2 types of co-signaling receptor-ligand interactions which are also called 'immune checkpoint pathways', which either co-stimulate or co-inhibit T cells. T cells infiltrating into tumors generally express high levels of co-inhibitory receptors, while tumor cells and intratumoral antigen-presenting cells can express ligands for these co-inhibitory receptors.[5,6] Blocking co-inhibitory programmed cell death protein 1 (PD1)/programmed cell death 1 ligand 1 (PDL1) checkpoint interaction by specific antibodies has recently shown unprecedented and durable therapeutic effects, resulting in long-term patient survival in several types of advanced cancer, including hepatocellular carcinoma (HCC).[7] In early phase trials the safety and preliminary efficacy of agonistic targeting of co-stimulatory receptors, including GITR, for cancer immunotherapy are being addressed.[8] However, whether CCA is responsive to checkpoint antibody therapy is as yet unknown, and knowledge of the immune microenvironment in CCA tumors is still very limited.

Nevertheless, some recent data suggest that CCA tumors might be sensitive to immune checkpoint therapy. First, studies have revealed variable numbers of $PD1^+$ lymphocytes and variable PDL1 expression in CCA tissues,[9,10] and both are associated with the clinical course of the disease,[11–13], suggesting that the PD1/PDL1 pathway may be an interesting immunotherapeutic target for CCA. Secondly, in order for checkpoint antibody therapy to be effective, T cells must recognize epitopes of tumor antigens on tumor cells and infiltration of T cells into the tumor is needed.[14] While the majority of CCA tumors contain a limited number of mutations and are therefore expected to express few neoantigens,[15] a recent study has demonstrated that tumor-infiltrating lymphocytes (TILs) in CCA contain tumor antigen-reactive T cells and that adoptive transfer of enriched populations of tumor-reactive T cells induced tumor regression.[16] Thirdly, immune checkpoint therapy is generally only effective in tumors with pre-existing T cell infiltrates,[17] and the limited number of studies available have shown $CD8^+$ and $CD4^+$ T cell as well as dendritic cell infiltrations in CCA tumors.[12,18,19] However, PDL1 expression and defective HLA class I antigen expression by iCCA tumor cells, as well as the presence of regulatory T cells (Tregs) and alternatively activated (M2) macrophages in CCA tumors suggest an immunosuppressive tumor microenvironment which contains immune infiltrates with T cells that can recognize the tumor cells but are immunosuppressive.[12,20] All these data are based on immunohistochemistry staining of CCA tissues. However, knowledge of phenotypic and functional characteristics of intratumoral lymphocytes in patients with CCA and of expression of immune checkpoint molecules other than PD1 and PDL1, and whether targeting immune checkpoint molecules can improve the functions of CCA TILs is lacking.

Therefore, the objectives of this study were: i) to isolate TILs from tumors of patients with CCA and comprehensively investigate their composition and characterize them phenotypically in comparison with their counterparts in tumor-free liver (TFL) tissues and blood from the same patients; ii) to identify which co-stimulatory and co-inhibitory molecules are over-expressed on TILs compared with their counterparts in TFL and blood; iii) to determine whether targeting of these immune checkpoint molecules can improve *ex vivo* functions of TILs in patients with CCA.

Materials and methods

Study population

In this study, we focused on iCCA and pCCA. Twenty-six patients who underwent surgical resection of iCCA (n = 24) or pCCA (n = 2) in Erasmus Medical Center from 2011 to 2019 and 3 patients with iCCA in Academic Medical Center from 2017 to 2018, and 1 patient with pCCA who underwent liver transplantation in Erasmus Medical Center in 2019 were included in the study. Matched fresh tissue samples from tumor and TFL as distant as possible from the tumor (≥1 cm distance) were collected. Peripheral blood of patients with CCA was also obtained just before surgery. None of the patients received chemotherapy or immunosuppressive therapy at least 3 months prior to surgery. All patients were Caucasian. The study was approved by the local ethics committee, and signed informed consent from all patients was obtained before tissue and blood donation. The clinical characteristics of the patients are summarized in Table 1.

Immunohistochemistry

Formalin fixed, paraffin embedded tissue samples from 27 patients with iCCA (n = 25) and pCCA (n = 2) who underwent surgical resection in the period from 2011 to 2018, were retrieved from the archive of the Department of Pathology, Erasmus Medical Center. None of the patients received pretreatment before surgery. The clinical characteristics of the patients are summarized in Table 2, of which 20 patients are also included in Table 1.

Table 1. Characteristics of patients for flow cytometry and functional data.

	CCA (n = 30)
Sex (female/male)	17/13
Age (years)**	62.0 ± 2.4
Cirrhosis/no	3/27
Etiology	NAFLD = 2/NASH = 1/PSC = 2/alcohol abuse = 1/HBV = 1/unknown = 23

CCA, cholangiocarcinoma; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PSC, primary sclerosing cholangitis; PBC, primary biliary cirrhosis; HBV, hepatitis B virus infection; unknown = without identified underlying liver disease.
** Mean ± standard error of the mean.

Table 2. Characteristics of patients for immunohistochemistry data.

	CCA (n = 27)
Sex (female/male)	16/11
Age (years)**	61.9 ± 2.4
Cirrhosis/no	4/23
Etiology	NAFLD = 2/PSC = 1/PBC = 1/alcohol abuse = 3/HBV = 1/unknown = 19

CCA, cholangiocarcinoma; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PSC, primary sclerosing cholangitis; PBC, primary biliary cirrhosis; HBV, hepatitis B virus infection; unknown = without identified underlying liver disease.
** Mean ± standard error of the mean.

Further detailed protocols are described in supplementary materials and methods.

Results

Poor infiltration of cytotoxic immune cells but accumulation of regulatory T cells in cholangiocarcinoma

To compare the composition of immune cells in the tumor, TFL and blood of patients with CCA, we analyzed the proportions of $CD19^+$ B cells, $BDCA1^+CD19^-$ myeloid dendritic cells (mDCs) and $CD14^+$ monocytes/macrophages, $CD3^+CD56^-$ T cells, $CD3^+CD56^+$ natural killer T (NKT) cells, and $CD3^-CD56^+$ natural killer (NK) cells within $CD45^+$ leukocytes freshly isolated from these 3 compartments by flow cytometry (Fig. 1). Proportions of mDCs were increased in the solid tissues, while proportions of $CD14^+$ cells were decreased in solid tissues, compared with the blood, but no differences were found between the tumor and TFL (Fig. 1B-D). However, proportions of NKT cells and NK cells were decreased in the tumor compared with TFL, whereas proportions of $CD3^+CD56^-$ T cells in tumors were increased (Fig. 1F-H). In TFL about half of the T cells were $CD8^+$ T cells (Fig. 1I). However, T cells in the tumor were made up of significantly less $CD8^+$ T cells, but more $CD4^+Foxp3^+$ Tregs and $CD4^+Foxp3^-$ Th cells than in TFL (Fig. 1J-K). These data demonstrate that CCA tumors show characteristics of an immunosuppressive microenvironment with reduced proportions of cytotoxic immune effector cells, but increased proportions of regulatory T cells compared to surrounding non-tumorous liver tissues.

NK cells are excluded from tumors, cytotoxic and Th cells are sequestered around the tumors but Tregs infiltrate into the tumors

Since flow cytometry analysis of isolated TIL does not discriminate their localization in the tumor bed or at the tumor margin, we performed immunohistochemistry stains for Foxp3 (a marker of Tregs), CD8, CD4 and NKp46 (a marker of NK cells) and we counted the numbers of stained cells to assess their localization in CCA tissues (Fig. 2A). $Foxp3^+$ cells were observed both within the tumor and at the tumor margin (Fig. 2A, F), but the vast majority of $CD8^+$ T cells was sequestered at the tumor margin (Fig. 2B, E). Consequently, in the tumor the ratio of $Foxp3^+$ cell density to $CD8^+$ cell density was highest (Fig. 2G) compared to that at the tumor margin and in TFL. Since the CD4 antibody also stained sinusoidal cells it was difficult to count $CD4^+$

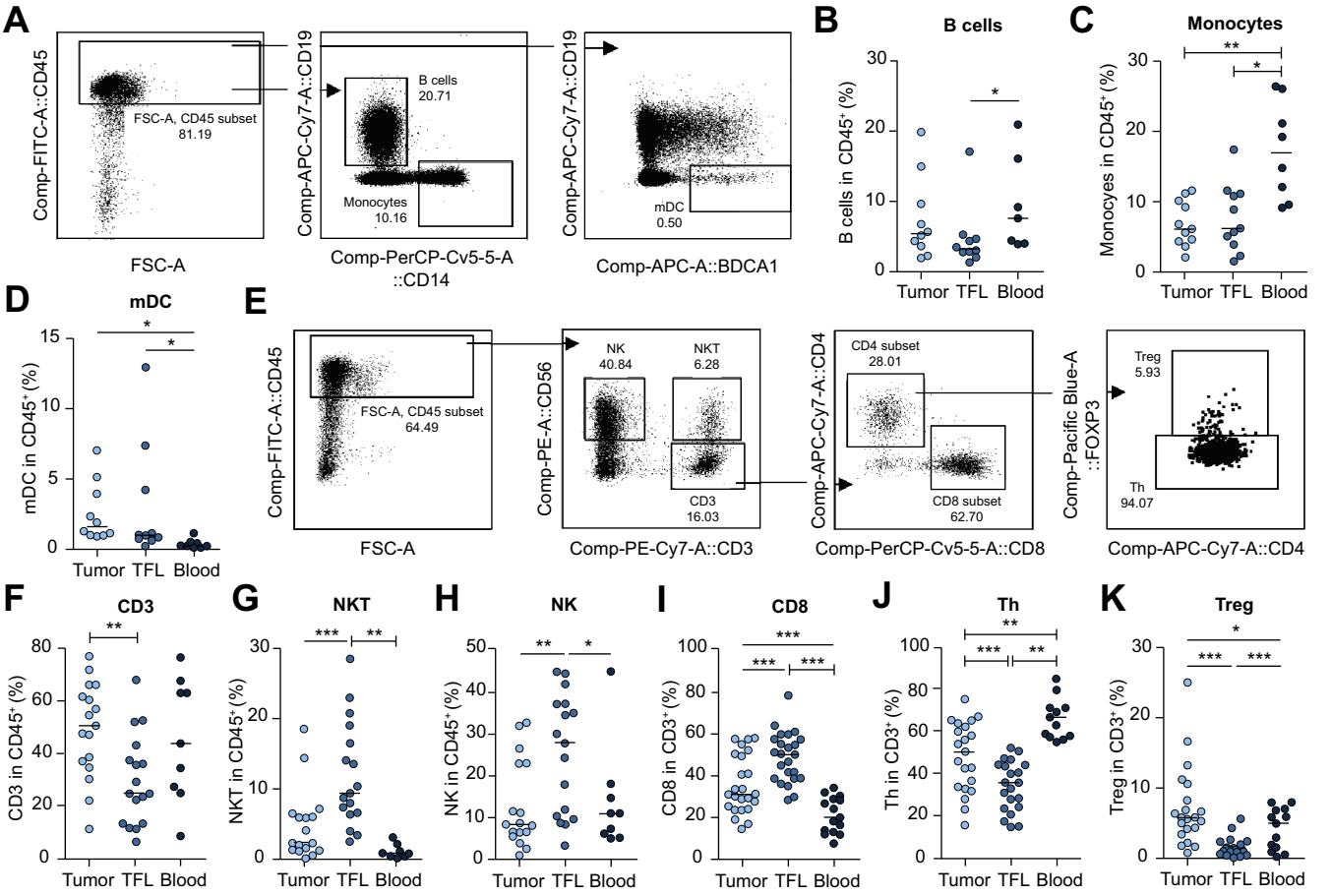

Fig. 1. Proportions of lymphocyte subpopulations and antigen-presenting cell subpopulations in immune cells from matched tumors, TFL and blood of patients with CCA. (A) Representative FACS dot plots show the gating strategies and the frequencies of (B) $CD19^+$ B cells, (C) $CD14^+$ monocytes/macrophages and (D) $BDCA1^+CD19^-$ mDCs within live $CD45^+$ cells. (E) Representative FACS dot plots show the gating strategies. The frequencies of (F) $CD3^+CD56^-$ T cells, (G) $CD3^+CD56^+$ NKT cells, (H) $CD3^-CD56^+$ NK cells within live $CD45^+$ cells. The frequencies of (I) $CD3^+CD8^+$ T cells, (J) $CD3^+CD4^+Foxp3^-$ Th cells and (K) $CD3^+CD4^+Foxp3^+$ Tregs within $CD3^+$ T cells. Values of individual patients are presented, lines depict medians. Differences were analyzed by paired *t* test ($\dashv\vdash$) or Wilcoxon matched pairs test (–); $^*p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001. CCA, cholangiocarcinoma; mDCs, myeloid dendritic cells; NK, natural killer; TFL, tumor-free liver; Th, helper T; Tregs, regulatory T cells.

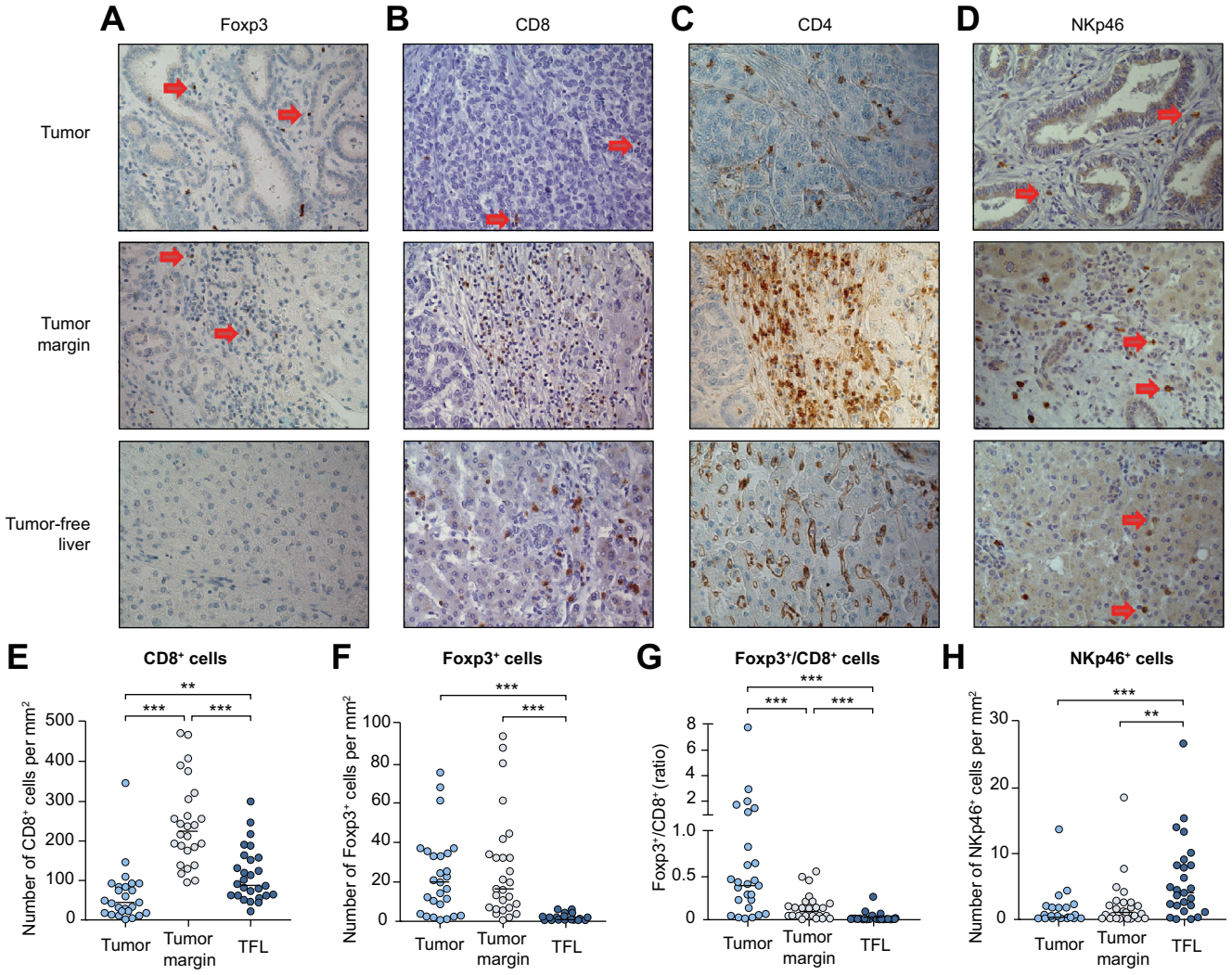

Fig. 2. Lymphocyte infiltration in the tumor, tumor margin and TFL of patients with CCA. Representative high-power fields (400x magnification) show the immunohistochemistry staining of (A) Foxp3, (B) CD8, (C) CD4, (D) NKp46 in CCA tumor, tumor margin and TFL areas. Brown color (in some pictures indicated by red arrows) indicates positive staining. (E-H) The cell densities are depicted per square millimeter in tumors, tumor margin or TFL areas. Values of individual patients are presented, lines depict medians. Differences were analyzed by Wilcoxon matched pairs test; $^{**}p$ <0.01, $^{***}p$ <0.001. CCA, cholangiocarcinoma; TFL, tumor-free liver.

lymphocytes, but from the distributions observed in the tissue sections it became clear that most CD4$^+$ lymphocytes were also sequestered at the tumor margin (Fig. 2C). In addition, the numbers of NKp46$^+$ cells were lower inside the tumor and at the tumor margin than in TFL areas (Fig. 2D, H). These data demonstrate that the majority of CD8$^+$ T cells and CD4$^+$ Th cells are sequestered at the tumor margin, NK cells are excluded from the tumors, but Tregs can infiltrate into the tumors of patients with CCA.

Activation status and cytotoxic molecule expression of lymphocytes in patients with cholangiocarcinoma

To characterize TILs and compare them with lymphocytes from paired TFL and blood of patients with CCA, we measured the expression of several activation markers and cytotoxic molecules by immune effector cells (CD4$^+$Foxp3$^-$ Th, CD8$^+$ T cells, NK cells and NKT cells) and by CD4$^+$Foxp3$^+$ Tregs (Fig. 3). The percentages of CD8$^+$ T cells expressing the proliferation marker Ki-67 and expressing the IL-2 receptor alpha chain (CD25, a marker of activation), were slightly higher in the tumor than in TFL, but this difference was not observed for the activation marker HLA-DR. The percentages of CD69$^+$ cells were higher within all T cell subsets in the solid tissue compartments than in blood, probably because CD69 is not only a T cell activation marker, but also a marker of tissue-resident T cells.[21,22] Nevertheless, Tregs in tumors showed enhanced CD69 expression compared to those in TFL. Interestingly, lower percentages of CD8$^+$ T cells expressed the cytotoxic effector molecules granzyme B and perforin in the tumor than in TFL (Fig. 3E, F). In addition, lower proportions of NK cells and NKT cells in the tumor expressed perforin than in TFL and blood (Fig. 3E, F and Fig. S1). Together, these data suggest that immune effector cells within the tumor microenvironment do not show strong phenotypic signs of activation, but the tumor-infiltrating cytotoxic lymphocytes show signs of dysfunctionality in CCA.

Expression of co-stimulatory and co-inhibitory receptors on T cells in patients with cholangiocarcinoma

To study the relevance and significance of immune checkpoint interactions in CCA, we first measured the expression of

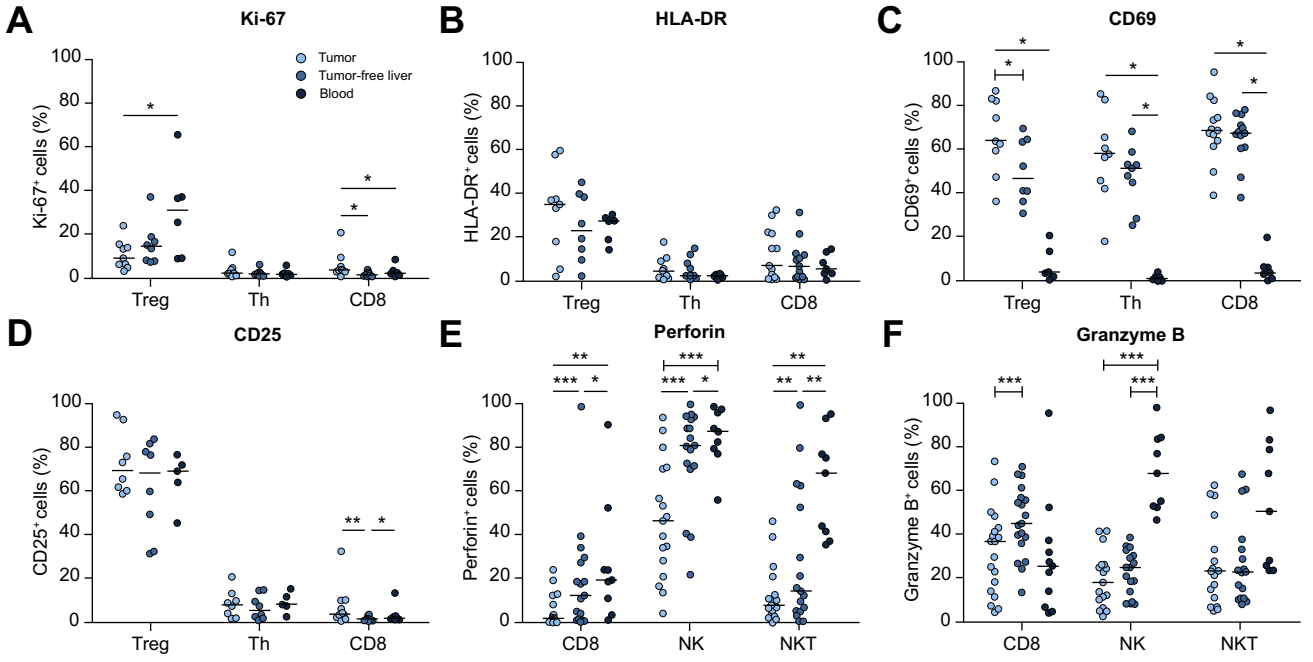

Fig. 3. Expression of activation markers and cytotoxic molecules by lymphocytes. The frequencies of (A) Ki-67$^+$, (B) HLA-DR$^+$, (C) CD69$^+$, (D) CD25$^+$ cells within Tregs, Th and T cells, as well as (E) perforin$^+$ and (F) granzyme B$^+$ cells within CD8$^+$ T cells, NK cells and NKT cells in matched tumors, TFL and blood of patients with CCA. Values of individual patients are shown, lines depict medians. Differences were analyzed by paired *t* test (|—|) or Wilcoxon matched pairs test (—); **p* <0.05, ***p* <0.01, ****p* <0.001. CCA, cholangiocarcinoma; NK, natural killer; TFL, tumor-free liver; Th, helper T; Tregs, regulatory T cells.

co-stimulatory receptors GITR (TNFRSF18) and inducible T cell co-stimulator (ICOS), as well as co-inhibitory receptors PD1, cytotoxic T-lymphocyte associated protein 4 (CTLA4), lymphocyte activating 3 (LAG3), B and T lymphocyte associated (BTLA), CD160 and CD224 on Tregs, Th and CD8$^+$ T cells (Fig. 4A). In all 3 compartments, Tregs displayed significantly higher expression of GITR, ICOS, CTLA4 and PD1 than Th and CD8$^+$ T cells (Fig. 4-B-E, J-M; p <0.05 for all comparisons). In addition, Tregs from the tumors showed higher frequencies of cells expressing co-stimulatory receptor GITR as well as of cells expressing co-inhibitory receptors PD1 and CTLA4 than those from paired TFL and blood (Fig. 4B, D-E). GITR and PD1 expression levels were also enhanced on Tregs from the tumors compared to their counterparts in TFL and blood (Fig. 4J, M). Th and CD8$^+$ T cells also showed higher frequencies of cells expressing GITR, PD1 and CTLA4 than those from paired TFL and blood, but only PD1 displayed enhanced expression levels (median fluorescence intensity) on individual Th and CD8+ T cells. Furthermore, no or minimal upregulation of co-inhibitory receptors BTLA, LAG3, CD160 or CD244 was observed in the tumor (Fig. 4F-I, N-Q). These data suggest that GITR, PD1 and CTLA4 may be involved in regulating T cell functions in the CCA tumor microenvironment (Fig. S2). Co-expression of co-inhibitory receptors PD1 and CTLA4 was observed on 32% of Tregs, but only on 4% of Th cells and 1.4% of CD8$^+$ T cells in tumors (Fig. S3).

Expression of co-stimulatory and co-inhibitory ligands in cholangiocarcinoma

Because GITR, PD1 and CTLA4 were upregulated by TILs in CCA, we measured the expression of their respective ligands GITRL, PDL1, CD86 and CD80 on 3 subsets of antigen-presenting cells: B cells, mDCs and CD14$^+$ monocytes/macrophages (Fig. 5A). CD86 and CD80 were expressed on mDCs and CD14$^+$ cells in all compartments, while CD80 was also expressed on B cells (Fig. 5D-E, H-I). However, PDL1 was expressed at relatively low levels on all 3 antigen-presenting cell subsets, including those in tumors, while GITRL expression was lowest (Fig. 5B-C, F-G). For co-inhibitory pathways to suppress T cells, both the receptor and the ligand are needed. CD86 and CD80 are known to be mainly expressed on antigen-presenting cells, while PDL1 can also be expressed on tumor cells. Therefore, we performed immunohistochemical staining for PDL1 on CCA tissues. Immunohistochemistry showed that in tumors PDL1 was not only expressed on leukocytes, but also on tumor cells, while in TFL weak expression was observed on hepatocytes. We observed PDL1 expression on tumor cells in CCA tumors in 18 out of 27 patients and on hepatocytes in TFL in 20 out of 27 patients, in either a diffuse or a patchy pattern (Fig. 5J-K).

Effector functions of tumor-infiltrating T cells are enhanced by targeting GITR, PD1 or CTLA4

Given that tumor-infiltrating CD8$^+$ T cells showed signs of dysfunctionality and GITR, PD1 and CTLA4 were over-expressed on TIL in CCA, we evaluated the effects of stimulating GITR by soluble GITRL, blocking PD1 by nivolumab and blocking CTLA4 by ipilimumab on *ex vivo* functional responses of tumor-infiltrating T cells (Fig. 6). Unfractionated CFSE-labeled tumor-infiltrating immune cells, including T cells expressing checkpoint receptors and antigen-presenting cells expressing their ligands, were stimulated with anti-CD3/CD28 beads. After 4 days, variable baseline proliferation of CD4$^+$ and CD8$^+$ TILs was observed for individual patients (Fig. 6C). CD4$^+$ and CD8$^+$ TIL proliferation (Fig. 6D-E) as well as production of interferon (IFN)-γ and granzyme B (Fig. 6F-H) were increased in the presence of GITRL. Nivolumab increased CD8$^+$ TIL proliferation as well as secretion of IFN-γ, tumor necrosis factor-alpha (TNF-α) and granzyme B. Ipilimumab increased CD4$^+$ and CD8$^+$ TIL proliferation as well as production of IFN-γ. Together, these data indicate that the

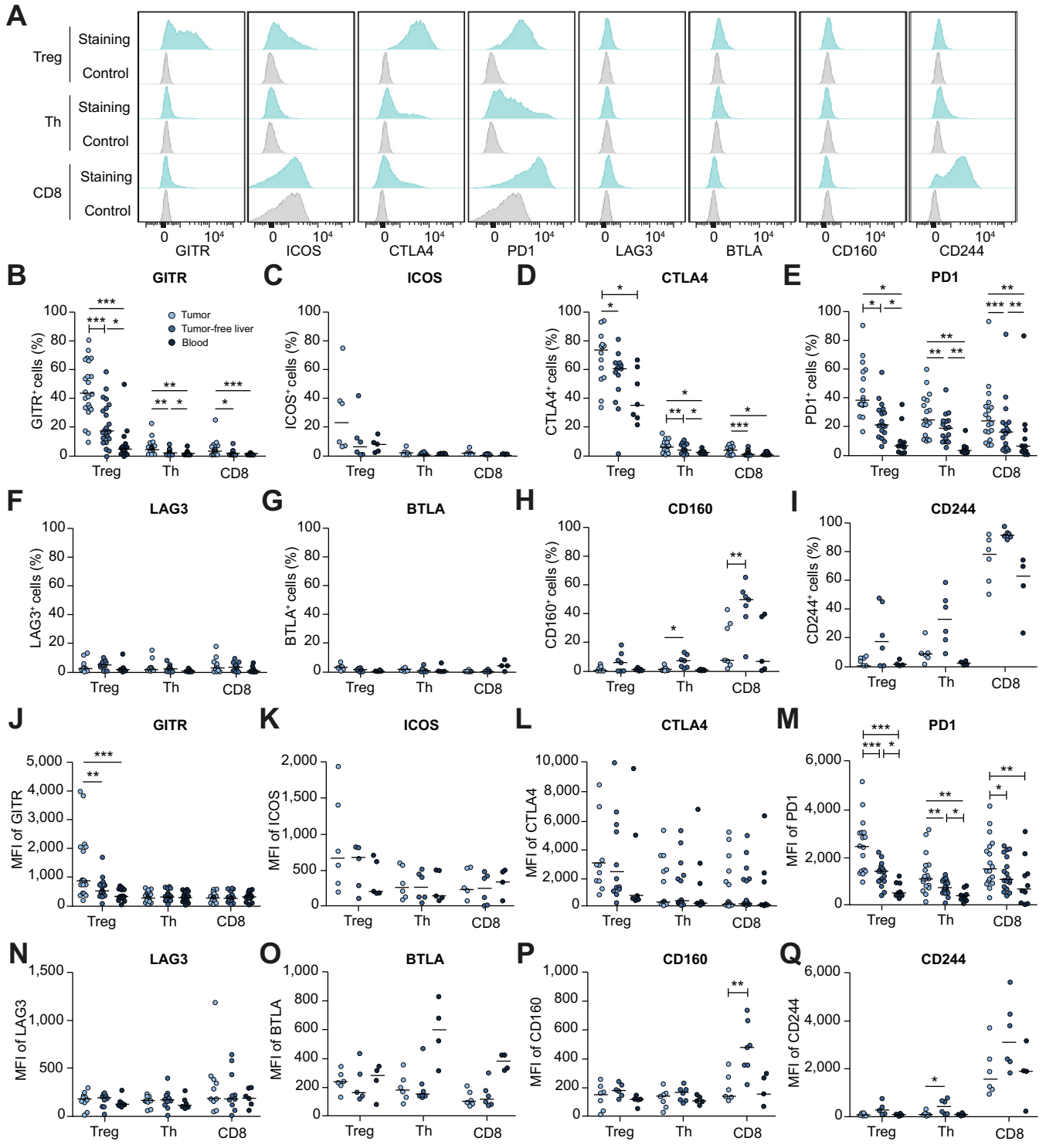

Fig. 4. Expression of co-stimulatory and co-inhibitory receptors on T cells in matched tumors, TFL and blood. (A) Representative histograms of receptor expression on Tregs, Th and CD8$^+$ T cells isolated from tumors, and fluorescence minus one controls. (B-I) The frequencies of receptor positive cells within Tregs, Th cells and CD8$^+$ cells in tumors, TFL and blood of patients with CCA. (J-Q) MFI of receptors. Values of individual patients are shown, lines depict medians. Differences were analyzed by paired *t* test (⊢⊣) or Wilcoxon matched pairs test (—); $^*p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001. CCA, cholangiocarcinoma; MFI, median fluorescence intensity; Th, helper T; Tregs, regulatory T cells. (This figure appears in colour on the web.)

effector functions of tumor-infiltrating T cells of patients with CCA can be modestly enhanced by targeting GITR, PD1 or CTLA4.

In some of the patients we could isolate sufficient TILs to determine the expression of cytotoxic molecules, co-stimulatory and co-inhibitory receptors and ligands directly *ex vivo* in addition to performing the *ex vivo* functional assays. We found that the fold increase of CD4$^+$ TIL proliferation in the presence of nivolumab was negatively correlated with the percentage of PD1$^+$ Tregs in tumors (Fig. S4). Whereas in the presence of ipilimumab, the fold increase of CD8$^+$ TIL proliferation was positively correlated with the percentage of granzyme B$^+$ cells in CD8$^+$ TIL (Fig. S5). In the presence of GITRL, the fold

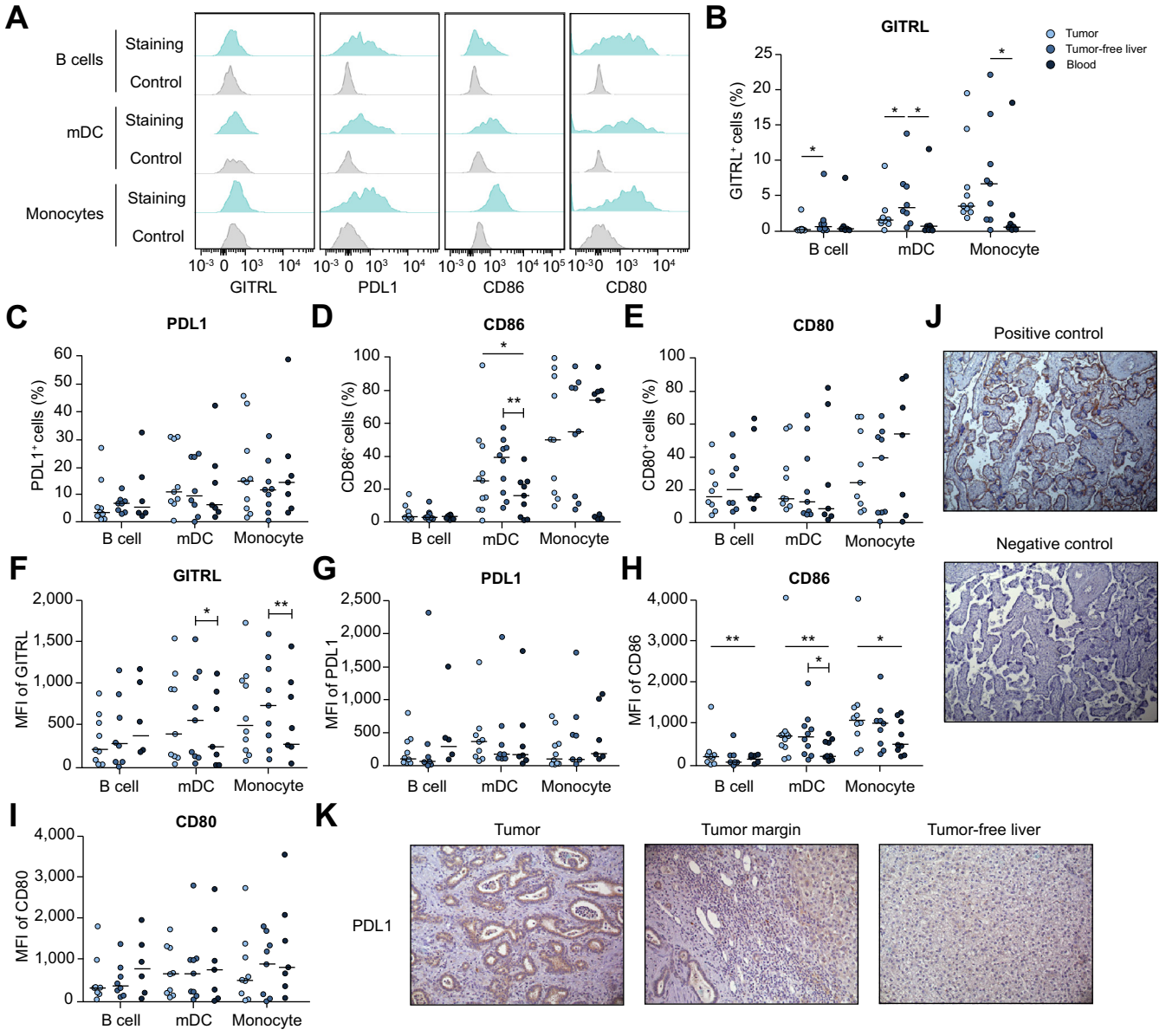

Fig. 5. Expression of co-stimulatory and co-inhibitory ligands on antigen-presenting cells in matched tumors, TFL and blood. (A) Representative histograms of ligand staining and fluorescence minus one control on B cells, mDCs and monocytes/macrophages isolated from tumors. (B–E) The frequencies of ligand positive cells within B cells, mDCs and monocytes/macrophages in tumors, TFL and blood of patients with CCA. (F–I) MFI of ligands. Values of individual patients are shown, lines depict medians. Differences were analyzed by paired *t* test (|—|) or Wilcoxon matched pairs test (—); **p* <0.05, ***p* <0.01. Representative pictures show (J) the positive and negative control (omission of anti-PDL1 antibody) stains of PDL1 in placenta, and (K) PDL1 staining in CCA tumor, tumor margin and TFL areas (100× magnification). CCA, cholangiocarcinoma; mDCs, myeloid dendritic cells; MFI, median fluorescence intensity; TFL, tumor-free liver.

increase of TNF-α production was positively correlated with the percentage of GITR$^+$ cells in CD8$^+$ TILs (Fig. S6). These findings suggest that some of the inter-individual variations in TIL responses to targeting immune checkpoints are related to differences in the immune phenotype of TILs. These parameters might become useful as biomarkers to predict clinical responsiveness of individual patients with CCA to immune checkpoint treatment.

Discussion

Our immunohistochemistry findings confirm previous reports[12,23] showing that CD8$^+$ and CD4$^+$ cells in CCA are predominantly sequestered at the tumor margin, while Foxp3$^+$ cells are present both inside the tumor and at the tumor margin (Fig. 2). Since Foxp3$^+$ cells represent CD4$^+$Foxp3$^+$ Tregs (flow cytometry demonstrated that all Foxp3$^+$ TIL are CD4$^+$, data not shown), we believe that most CD4$^+$ cells accumulating at the tumor margin are Th cells that are unable to enter the tumor. Because several CD56 antibodies failed to specifically stain NK cells in tumors and TFL by immunohistochemistry, we used an NKp46 antibody validated by others for detection of NK cells in liver tissue,[24,25] and found that both tumors and tumor margins contained fewer NKp46$^+$ cells compared with TFL. These data suggest that CCA belongs to the type of tumors with relatively low numbers of tumor-infiltrating cytotoxic immune cells. The density of CD8$^+$ cells in CCA tumors is even lower than that in primary colorectal cancer, liver metastasis of colorectal cancer and HCC,[26–31] while the density of NKp46$^+$ cells in CCA is lower than that in primary colorectal cancer but similar to

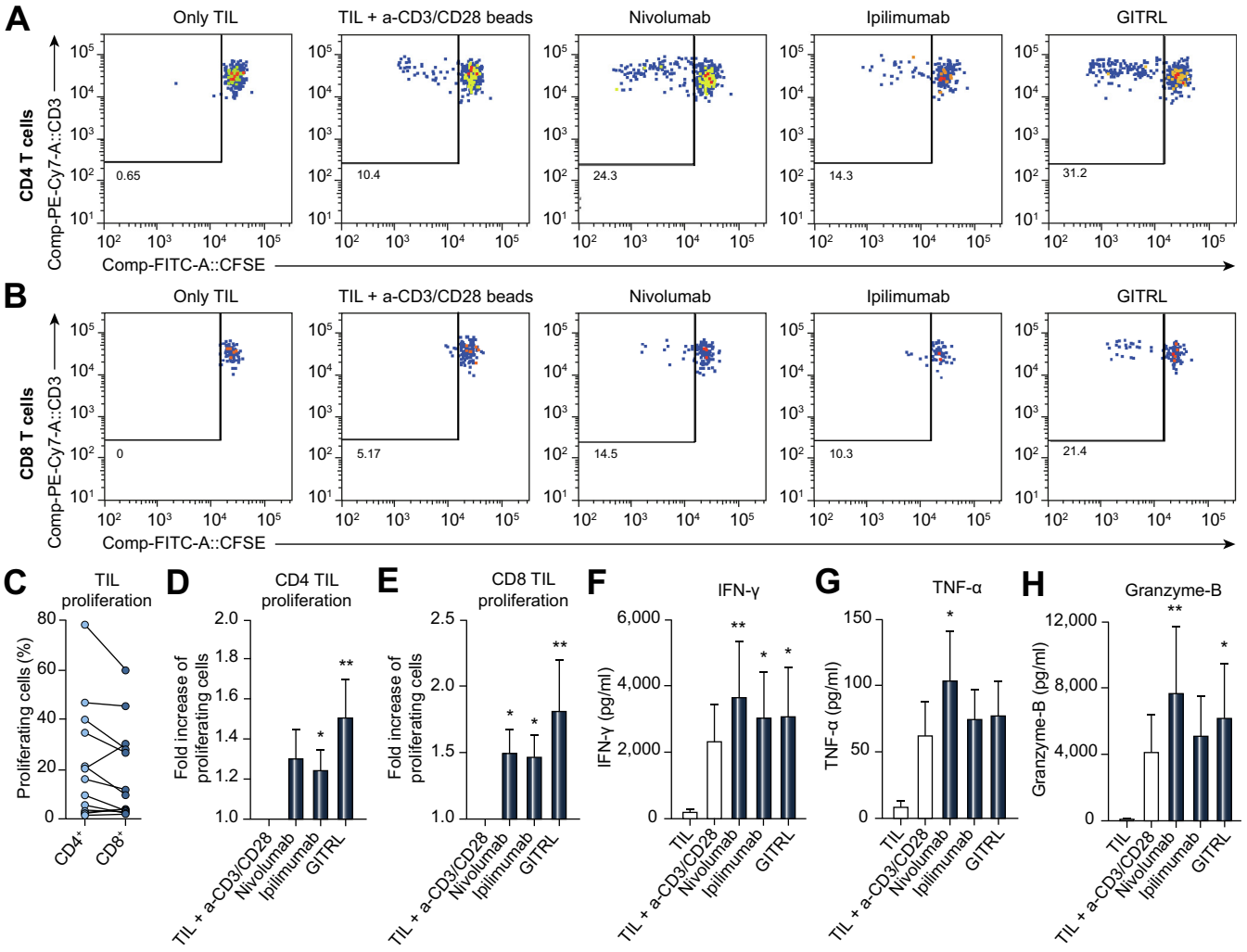

Fig. 6. Tumor-infiltrating T cell functions are enhanced by targeting GITR, PD1 or CTLA4. (A, B) Representative FACS dot plots of $CD3^+CD4^+$ and $CD3^+CD8^+$ TIL proliferation in response to CD3/CD28 stimulations. (C) The baseline percentages (in the condition with only anti-CD3/CD28 beads) and (D, E) the fold increase (in the presence of nivolumab, ipilimumab or GITRL) of proliferating cells (CFSE-low) within $CD4^+$ TIL and $CD8^+$ TIL are shown. The results of proliferative responses to antibodies are reported as fold increase calculated by dividing the percentage of proliferating T cells in the presence of the antibody, by the percentage in the baseline condition. (C) Individual data are shown, each line represents 1 patient. (D, E) Values are means with standard error of the mean (n = 12 different patients with CCA, with detectable suboptimal TIL proliferations). (F-H) Accumulation of IFN-γ, TNF-α and granzyme B in culture supernatants was quantified. Values are means with standard error of the mean (n = 14–17 patients, with detectable cytokine concentrations). Differences were analyzed by paired *t* test or Wilcoxon matched pairs test compared to the baseline condition with only anti-CD3/CD28 beads; $^*p$ <0.05, $^{**}p$ <0.01. (This figure appears in colour on the web.)

that in liver metastasis of colorectal cancer,[30,32] The density of $Foxp3^+$ cells in CCA is lower than that in HCC, but comparable to that in primary colorectal cancer and distant metastases from colorectal cancer.[28,29,31]

Using flow cytometry measuring isolated immune cells, we corroborated that the proportion of $CD8^+$ T cells within the isolated $CD3^+$ TILs was decreased while the proportion of $CD4^+Foxp3^+$ Tregs in $CD3^+$ TILs was increased compared with TFL (Fig. 1I, K). The frequency of $CD8^+$ T cells within $CD3^+$ T cells in CCA is also lower than that in healthy liver biopsies and donor liver perfusates.[33] In addition, using the combination of CD3 and CD56 markers, we observed that percentages of NKT cells and NK cells within $CD45^+$ cells were lower in tumors than in TFL. The observed reductions of the 3 main types of cytotoxic immune cells and the increase of suppressive Tregs in the tumors, are similar to our previous data in patients with HCC[34,35] and reconfirm that the CCA tumor microenvironment prevents penetration of cytotoxic immune cells but not of Tregs into the tumors. Therefore, CCA tumors belong to the category of immune-excluded tumors in which most effector T cells are sequestered at the tumor margin.[17]

We are the first to isolate TILs from CCA tumors and analyze their characteristics. $CD8^+$ T cells in CCA tumors displayed a slightly more activated and proliferative status than those in TFL according to their enhanced expression of activation marker and proliferative status (Fig. 3A, D). This is corroborated by the observed over-expression of co-signaling receptors (Fig. 4), which is also a sign of T cell activation.[36] Nevertheless, compared with TFL, fewer $CD8^+$ T cells in CCA tumors expressed the cytolytic proteins perforin and granzyme B, similar to what we observed in HCC tumors.[34] In addition, less NK cells and NKT cells expressed perforin in tumors compared with TFL (Fig. 3E-F). Tregs in tumors also displayed a slightly more activated status than those in TFL as demonstrated by elevated expression of co-signaling molecules (Fig. 4B, D-E, J, M). This may implicate that CCA tumor-infiltrating Tregs can strongly suppress effector

T cells in the tumor microenvironment, as we previously demonstrated that increased activation status of Tregs in HCC tumors coincides with enhanced suppressive capacity.[34]

There is immunohistochemical evidence that co-inhibitory molecules PD1, CTLA4 and PDL1 are expressed in CCA tumor tissues,[13,37] and that PDL1 can be expressed by both tumor cells and tumor-infiltrating immune cells.[9,38] However, which types of immune cells in CCA tumors express these co-inhibitory molecules was unknown, and data on expression of other immune checkpoint molecules in CCA were lacking. Flow cytometric analysis of the expression of multiple co-stimulatory and co-inhibitory molecules on isolated immune cells displayed that Tregs, Th and $CD8^+$ T cells in CCA tumors over-expressed GITR, PD1 and CTLA4 in comparison to their counterparts in TFL and blood (Fig. 4B, D-E, J, L-M). Previously, we had similar observations in HCC and liver metastasis of colorectal cancer.[5,6,34,35] However, in contrast to HCC and liver metastasis of colorectal cancer[5,6] LAG3 was not upregulated on tumor-infiltrating T cells in CCA tumors (Fig. 4F, N), showing that expression of co-signaling molecules can differ between different types of tumors in the liver. Like in liver metastasis of colorectal cancer,[6] tumor-infiltrating Tregs displayed the highest expression of the checkpoint receptors compared with Th and $CD8^+$ T cells. The frequency of $PD1^+$ cells within $CD8^+$ T cells is also higher in CCA than in healthy donor blood.[39] The ligands of co-inhibitory receptors were variably expressed on B cells, mDCs and monocytes/macrophages (Fig. 5B-I), and we confirmed that PDL1 can be expressed on CCA tumor cells (Fig. 5K), like in HCC tumors.[5,40] The over-expression of PD1 and CTLA4 on tumor-infiltrating T cells and their respective ligands in the tumors suggest that these co-inhibitory pathways can potentially be targeted to invigorate intratumoral immunity in patients with CCA. In addition, the over-expression of the co-stimulatory receptor GITR on tumor-infiltrating T cells compared to the virtually non-existent expression of GITRL in tumors, suggests that agonistic targeting of GITR may offer another possibility to revitalize intratumoral immunity in CCA.

Functional effects of targeting co-stimulatory or co-inhibitory receptors on tumor-infiltrating T cells of patients with CCA have not been studied before. Our previous data have demonstrated that soluble GITRL as well as CTLA4 blockade partially abrogate suppression mediated by tumor-infiltrating Tregs derived from HCC,[34,35] while PDL1 blockade enhances functional responses of $CD8^+$ and $CD4^+$ TILs, taken from patients with HCC, in *ex vivo* assays.[5] Here we used low doses of soluble ligand GITRL in *ex vivo* stimulations of CCA-derived TILs to stimulate T cells via their co-stimulatory receptor GITR, and low doses of therapeutic human antibodies nivolumab and ipilimumab to block co-inhibitory receptors PD1 and CTLA4 on T cells, respectively. We are the first to reveal that the secretion of the cytotoxic mediator granzyme and effector cytokines as well as T cell proliferation of TILs were increased by the 3 reagents (Fig. 6, Figs. S7-8). Therefore, these immune checkpoint molecules are promising targets to evaluate for immunotherapy of patients with CCA. However, similar to other immune-excluded tumors,[17] benefit of immune checkpoint therapy in patients with CCA may require additional therapy to enable effector T cell infiltration into the tumors, such as immunogenic chemotherapy.[41,42]

Our study has some limitations: i) due to limited numbers of isolated TILs, not all types of experiments could be performed for every patient with CCA; ii) the patient cohort was recent and small, so we were unable to associate immunological data with patient survival.

In summary, decreased numbers of cytotoxic immune cells and increased numbers of Tregs, together with the expression of 2 co-inhibitory receptors PD1 and CTLA4 on tumor-infiltrating T cells and their respective ligands in the tumors, indicate that the tumor microenvironment in CCA is immunosuppressive. Blockade of PD1 or CTLA4, as well as stimulation of GITR, enhances the *ex vivo* effector functions of tumor-infiltrating T cells from patients with CCA, suggesting that these molecules may be potential targets for immunotherapy of patients with CCA. However, clinically effective immunotherapy may require combination with another treatment aiming to facilitate effector T cell penetration from the tumor margin into the tumor bed.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

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Supplementary data

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