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REVIEW ARTICLE

Tumor-infiltrating lymphocytes and hepatocellular carcinoma: pathology and clinical management

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Abstract The presence of tumor-infiltrating lymphocytes (TILs) in hepatocellular carcinoma (HCC) is relatively rare. The prognosis of patients with HCC and marked TILs is better than that of patients with HCC without TILs. TILs in HCC tissues are mainly T cells, and previous reports suggested that TILs might be important antitumor effector cells. TILs have been extensively analyzed, and subpopulations of $CD3^+$, $CD4^+$, and $CD8^+$ T cells are often present in HCC. Some studies have reported that the percentage of $CD8^+$ T cells, which might have cytotoxic activity, is decreased in tumors with TILs, as compared with noncancerous tissues. Although the antitumor effects of TILs seem to be impaired in HCCs, the underlying mechanism has remained unclear until quite recently. Pathological and in vitro studies have now shown that regulatory T cells play important roles in the deterioration of the antitumor effects of TILs. The aim of this review is to introduce recent pathological findings for TILs in HCC and to evaluate new therapeutic strategies in this field.

Keywords Hepatocellular carcinoma · Tumor-infiltrating lymphocytes · Regulatory T cells

Abbreviations

HCC	Hepatocellular carcinoma
TIL	Tumor-infiltrating lymphocytes
T_{regs}	Regulatory T cells

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide [1]. In Japan, HCC accounts for 95% of all primary liver cancers. Although treatment options such as hepatic resection, liver transplantation, chemoembolization, ethanol injection, thermal ablation, and tyrosine kinase inhibitors (e.g., sorafenib) are widely used for HCC, HCC is still characterized by frequent recurrence [2], even after liver transplantation [3].

Clearly, new therapeutic strategies are needed to improve the survival of patients with HCC. Immunotherapy seems to offer one of the realistic new therapeutic modalities. Patients undergoing hepatic resection for HCC with prominent lymphocyte infiltration show reduced recurrence and better prognosis as compared with those without prominent lymphocyte infiltration [4, 5]. A randomized clinical by Takayama et al. [6] showed an improved recurrence-free rate after hepatic resection for HCC with adoptive immunotherapy using autologous lymphocytes activated in vitro with interleukin 2 (IL-2). Recurrence after liver transplantation for HCC is related to immunosuppression [7], and the presence of subpopulations of tumor-infiltrating lymphocytes (TILs) has been reported to predict recurrence [8].

Historically, TILs were categorized as being present or absent, based on morphologic analysis. With the development of immunohistochemistry and flow cytometry, many studies have shown that specific types of immune cells regulate the host defense against cancer, based on the subpopulations of TILs.

In this review, we discuss recent advances in basic research and clinical management of HCC, focusing on the roles of TILs.

Histological examination of TILs

Most of the previous papers regarding the presence of TILs in HCC were case reports [4, 9]. Thus, the incidence of TILs in HCC has remained unknown. Wada et al. [9] reported that, of 163 patients with HCC < 3 cm in diameter and who underwent hepatic resection, only 11 (7%) had HCC with marked TILs. The histological findings of HCC with TILs included piecemeal necrosis of the cancer nests. There was no difference in lymphocytes in noncancerous tissue between patients with and those without marked TILs. Furthermore, patients with HCC and the presence of TILs had better survival and disease-free survival than those with HCC without TILs. They also reported that, immunohistochemically, the main subpopulation of TILs was T cells. These findings strongly suggested that TILs might act as important antitumor effector cells.

However, the incidence of TILs in HCC is very low, suggesting the presence of a mechanism that suppresses the immune response of TILs against cancer in most cases.

TIL subpopulations

The subpopulations of TILs have been examined in several studies [10, 11] (Table 1). Immunohistochemical studies showed that the predominant subtype of T cell was $CD8^+$ T cells, followed by $CD4^+$ and $CD3^+$ T cells. Similar results were reported by Gao et al. [12]. Pang et al. [10] showed that, in the $CD3^+$ population, the percentage of $CD4^+$ cells was significantly higher in TILs than in lymphocytes infiltrating noncancerous tissue. Concurrently, the percentage of $CD8^+$ cells was decreased in TILs. In terms of the other lymphoid subsets, significant reductions were also observed for $CD3^-CD56^+$ natural killer (NK) cells, $CD3^+CD56^+$ NKT cells, and $\gamma\delta$ cells. Previous studies have reported an increased frequency of $CD4^+CD25^+$ T cells, which are thought to be regulatory T cells in HCC [10, 12–19].

Regulatory T cells [$CD4^+CD25^+$ cells (T_{regs})] in TILs

The concept of T_{regs} as suppressive T cells was first proposed in 1971 by Gershon and Kondo [20]. However, subsequent studies could not confirm this subpopulation because of the lack of specific molecular markers and difficulties in isolating and culturing of these cells [21]. Significant advances in the research of T_{regs} occurred quite recently, following the cloning of FOXP3, a member of the forkhead family of DNA transcription factors [22]. There is now considerable evidence that FOXP3 is a key molecule involved in the function of T_{regs}, and it is now widely accepted as an excellent marker for T_{regs} [14–18, 22].

Naturally occurring $CD4^+CD25^+$ FOXP3 regulatory T cells represent 5–10% of the peripheral $CD4^+$ T cells and are important regulators of immune tolerance [23]. In HCC, Unitt et al. [14] reported that 8.9% of $CD4^+CD25^+$ T_{regs} were TILs, and this percentage exceeded the percentage of cells in distant noncancerous tissue (2.4%).

In antitumor immunity, the T_{regs} may disturb the beneficial tumor-specific responses. T_{regs} have numerous immune targets, including $CD8^+$ T cells, $CD4^+CD25^-$ T cells, B cells, NK cells, and dendritic cells [24, 25]. The mechanisms involved in immunosuppression are not fully understood. The possible mechanisms include (a) T_{regs} secrete immunosuppressive cytokines, such as tumor growth factor (TGF)-β, interleukin (IL)-10, and IL-35 [26, 27]; (b) T_{regs} inhibit T-cell activation-dependent receptor (TCR) production of IL-2 and interferon (IFN)-γ [28, 29]; and (c) T_{regs} suppress antigen-presenting cells (APCs) through cell–cell contact via cell surface-bound TGF-β [30] and CTLA-4 [31].

These findings, particularly regarding cytokine expression, were confirmed in HCC patients. T_{regs} isolated from surgically resected HCC suppressed the proliferation of $CD4^+CD25^-$ cells and low activated CTL activity [17]. Furthermore, TGF-β may be related to the presence of $CD4^+CD25^+$ T cells. Pang et al. [10] reported that intratumoral $CD4^+$ T cells produced more IL-10 and less IFN-γ. This finding was also confirmed by Shen et al. [18], who detected high levels of IL-10 and TGF-β, and low levels of IFN-γ, in HCC. Ikeguchi et al. [32] reported that $CD8^+$ T cells expressing IL-2 were significantly suppressed in HCC and that patients with IL-2-positive tumors had a significant more favorable prognosis compared with those with IL-2-negative HCC. These results suggest the presence of T_{regs} among TILs in patients with IL-2-negative HCC.

Table 1 Possible cellular markers for immunohistochemistry

Subpopulation of TILs	Cellular markers	References
Memory T cells	CD45RO	[13]
Natural killer cells	Perforin	[14, 15]
	Granzyme B	[12, 14, 15]
Regulatory T cells	FOXP3	[8, 13, 17]
	$CD4^+CD25^+$	[19]

TIL, tumor-infiltrating lymphocytes; $CD4^+CD25^+$, double staining

Immunohistochemical markers of TILs

The recent progress in research of TILs in HCC has been aided by the development of immunohistochemical markers. In addition to the basic subpopulation of TILs, the function of T cells can be detected using monoclonal

antibody. The immunohistochemical markers of memory T cells, NK cells, and regulatory T cells are shown in Table 1.

Factors regulating the recruitment of T_{regs}

Previous studies showed that $CD4^+CD25^-$ T cells can be induced into suppressing $CD4^+CD25^+$ T cells by peripheral expansion [33]. Cao et al. [34] reported that the culture supernatants of HCC cells could promote $CD4^+CD25^+$ T-cell proliferation and inhibit $CD4^+CD25^-$ T-cell proliferation from peripheral blood mononuclear cells. Thus, HCC-derived soluble factors exhibit immunosuppressive activities.

The mechanisms that regulate the recruitment of tumor-associated T_{regs}, as well as those that trigger activation, remain unclear (Fig. 1). However, several hypotheses have been proposed: (a) programmed death 1 ligand (PD-L1) expression on dendritic cells (DCs) and monocytes [35, 36]; (b) induction of chemokines produced by tumor-derived macrophages, such as CCL22 [37]; and (c) recognition of tumor-specific antigens, such as NY-ESO-1 [38].

Tumor cells are reported to stimulate immature myeloid dendritic cells to secrete TGF-β and contribute to the conversion of naïve T cells to $FOXP3^+CD25^+CD4^+$ T_{regs} [39], and Wang et al. [40] subsequently reported that the expression of PD-1 on immature dendritic cells was necessary for this conversion. In HCC, Shi et al. [39] demonstrated that the increase in intratumoral PD-1-expressing $CD8^+$ T cells in HCC was associated with poor prognosis and that immunohistochemical expression of PD ligand 1 (PD-L1) in HCC was significantly associated with apoptotic infiltrating $CD8^+$ T cells in tumors. Furthermore, blocking the interaction between B7-H1 (PD L-1) with PD-1 CD8 T cells using neutralizing antibodies recovered the effector T-cell function [42]. Cao et al. [34] also reported that patients with HCC overexpressing PD-L1 had a poor prognosis, and they found a significant correlation between PD-L expression and $FOXP3^+$ lymphocyte infiltration. Considering these studies, PD-1/PD-L1 may constitute another pathway that hinders the antitumor immune response by T cells in HCC.

Zhou et al. [17] showed that the intratumoral prevalence of $FOXP3^+$ T_{regs} in HCC was associated with a high density of macrophages. Macrophages exposed to tumor culture supernatants form hepatoma-derived cell lines with increased frequency of $FOXP3^+$ T_{regs} in vitro, and this increase was partially blocked by anti-IL-10 antibodies.

Possible factors recruiting TILs: (1) tumor-associated antigens (TAA)

As previously shown, the antitumor immune responses can occur naturally in HCC through the activity of TILs and, in this situation, TILs may recognize cancer antigens. The so-called cancer-testis antigens are expressed in various solid tumors. The genes encoding cancer-testis antigens are not expressed in normal tissues with the exception of testis germline cells, which are major histocompatibility complex class I negative and are not recognized by T cells. Members of the MAGE A gene family were found to be expressed in up to 70% of HCCs [40]. For example, Riener et al. [41] showed that MAGE-C2 was expressed in 34% of HCCs.

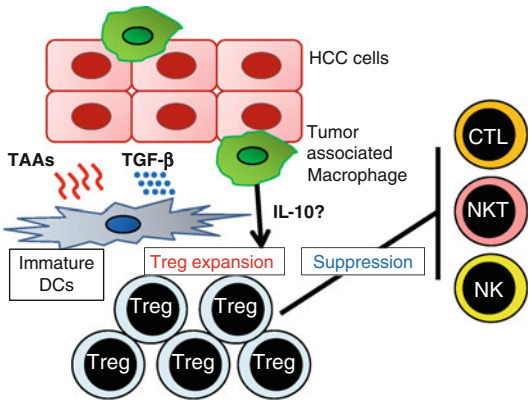

Fig. 1 Proposed functions in tumor immunology. Tumor-associated antigens (TAAs) and tumor growth factor-beta (TGF-β) induce regulatory T-cell (T_{reg}) expansion in combination with immature dendritic cells (DCs). Tumor-infiltrating macrophages may secrete interleukin 10 (IL-10), and IL-10 also may induce T_{reg} expansion. Expanded T_{regs} suppress the functions of CTL, NK, and NKT. *TAA*, tumor-associated antigen; *DC*, dendritic cell; *NK*, natural killer cells; *NKT*, natural killer T cell; *CTL*, $CD8^+$ cytotoxic T lymphocytes

Some patients with HCC show spontaneous humoral and cellular immune responses against a number of TAAs, including the cancer-testis antigens NY-ESO-1 and SSX-2; the MAGE antigens such as MAGE-1, A2, A3, A10, C2, and G2; telomerase reverse transcriptase (hTERT); and α-fetoprotein [42, 43].

Nishikawa et al. [44] reported that the cancer-testis antigen NY-ESO-1 is a tumor-specific antigen for which tumor-specific immunity is controlled by T_{regs}. Riener et al. [41] also demonstrated that the number of intratumoral $FOXP3^+$ regulatory T cells was increased in cancer-testis antigen-positive HCCs. Furthermore, recent studies have shown that $CD4^+$ T cells react with TAAs and can be expanded from peripheral blood T_{reg}-depleted $CD4^+$ T cells, but not from nondepleted $CD4^+$ cells [45].

Possible factors recruiting TILs: (2) humoral factors and chemokines

TILs and other cells in the tumor microenvironment produce a variety of cytokines, chemokines, and growth factors. These proteins participate in multiple biological

processes, including cellular differentiation, receptor activation, and cell survival. The interaction between these proteins and their receptors forms a comprehensive signaling network in tumors [46, 47].

The cytokine TGF-β has been reported to have antiinflammatory properties. The serum levels of TGF-β1 are elevated in patients with HCC [11, 14]. The size of the HCC was reported to be inversely proportional to serum TGF-β1 levels, which were inversely correlated with Fas expression in $CD4^+$ peripheral blood lymphocytes [11]. These findings may explain the phenomenon that tumors promote tolerance to tumor antigens by secreting downregulatory cytokines such as TGF-β1.

To date, more than 40 chemokines have been identified and grouped into four groups: XC, CC, CXC, and XC3C chemokines. Among these chemokines, the CXCL12–CXCR4, CCL20–CCR6, and fractalkine–CX3CR1 axes have been reported to be involved in HCC progression [48]. Chemokines have been reported to regulate cancer cell proliferation and progression, and antitumor immunity. In terms of antitumor immunity, Tang et al. [49] reported that CX3CR2 could upregulate tumor-specific cytotoxic T cells and increase the production of IL-2 and IFN-γ, which can inhibit tumor growth. Matsubara et al. [50] found that rate of recurrence was low in HCC with a high expression of fractalkine and its receptor CX3CR1, which suggests that these factors may be related to the prognosis of HCC patients because of their putative involvement in tumor immunity by killing tumor cells [51].

In contrast, the expression of chemokines such as monokines induced by IFN-γ (Mig: CXCL9) and IFN-inducible protein-10 (IP-10: CXCL10) in HCC have been reported to be important factors in TILs. For example, Yoong et al. [52] showed that Mig is expressed in HCC, particularly the epithelium, and that MIG is important in lymphocyte recruitment to HCC. Similarly, Hirano et al. [53] showed that some cytokines induced by IFNγ, such as Mig and IP-10, may promote lymphocyte recruitment to HCC.

In terms of T_{regs}, tumor cells and tumor-infiltrating macrophages produce the chemokine CCL22, which recruits $CD25^+CD4^+$ T_{regs} expressing CCR4 [37]. However, the role of CCL22 in TILs in HCC is still unclear.

Clinicopathological factors and the prognostic role of the TIL subpopulation

Several clinicopathological factors of HCC are related to the subpopulations of high density T_{regs} (Table 2). The prognostic value of the TIL subpopulation is shown in Table 3. Pang et al. [10] showed that a high density of $CD3^+CD56^+$ NKT cells was a significant predictor for favorable prognosis after hepatic resection. Similarly, Cai et al. [54] showed that a high density of dendritic cells, memory T cells, and $CD8^+$ T cells was also a good prognostic factor. Li et al. [13] reported that high density of tumor-associated macrophages and memory T cells was a good prognostic factor for both overall survival and disease-free survival. Recent studies have shown poor prognostic value of TILs, including $CD4^+CD25^+$ $FOXP3^+$ T_{regs}, in patients with HCC, undergoing hepatic resection. Recent reports [15–17] have shown that a high density of $FOXP3^+$ T_{regs} was a poor prognostic factor. Gao et al. [35] reported that the presence of low intratumoral T_{regs} in combination with high intratumoral activated $CD8^+$ cytotoxic cells was an independent prognostic factor for overall survival and disease-free survival.

A pathological study by Unitt et al. [14] showed that the CD4:CD8 ratio of intratumoral lymphocytes and reduced lymphocyte infiltration were significant prognostic factors for HCC after liver transplantation. The presence of $FOXP3^+$ lymphocytes was not a prognostic factor but was associated with vascular invasion. Still, it remains unclear

Table 2 High regulatory T cell (T_{reg}) density in hepatocellular carcinoma (HCC) and related clinicopathological factors

Variables	References
Clinical factors	
Advanced TNM stage	[18, 35]
High α-fetoprotein levels	[35]
Pathological factors	
Absence of encapsulation	[12]
Vascular invasion	[8, 35]
Poorly differentiation	[15, 35]
Multiple tumors	[35]

Table 3 Prognostic value of TILs for overall survival and disease-free survival of HCC

Variables	References
Hepatic resection	
High density of $CD3^+CD56^+$ NKT cells	[10]
High density of DCs, memory T cells, and $CD8^+$ T cells	[54]
High density of TAM and memory T cells	[13]
High density of $CD4^+CD25^+$ $FOXP3^+$ T_{regs}	[15, 16]
Low T_{regs} + high activated $CD8^+$ cytotoxic cells	[12]
High density of PD-1^+CD8^+ T cells	[39]
Liver transplantation	
CD4:CD8 ratio, reduced lymphocyte infiltration	[8]

NKT cells, natural killer T cells; TAM, tumor-associated macrophages

whether the increase in T_{regs} in TIL is the result of the tumor invasion or causes tumor invasion.

Collectively, these data suggest that the balance between antitumor effector T cells and T_{regs} may be a critical determinant of the immune response within a tumor. The net immune response within the tumor determines tumor aggressiveness and the outcome after treatment for HCC.

Clinical management

Yu et al. [46] suggested that some TILs may be friends while others may be foes. An increase in TILs may not always be associated with a better prognosis. Accumulation of T_{regs} inside a tumor suggests that aberrant immune responses can occur inside the tumors. Strategies to effectively reverse the immunologically suppressive environment may offer a new approach to enhance the efficacy of the immune response against tumors at local and distal sites.

We recently showed that intratumoral neoadjuvant immunotherapy using IL-12 [55] and IL-12 plus dendritic cells [56] offers an effective strategy to control murine HCC in immunosuppressive mice. Cao et al. [57] reported that IL-12 treatment blocked T_{reg} expansion. Meanwhile, Zabala et al. [47] demonstrated antitumor effects of IL-12 in a transgenic mouse model of liver cancer. This treatment approach shows transient efficacy during long-term observation and appears to be limited by the activation of immunosuppression, including recruitment of $FOXP3^+$ T_{regs} and elevated expression of immunosuppressive molecules such as PD-1, PD-L1, vascular endothelial growth factor (VEGF), CTLA-4, IDO, and IL-10. Thus, enhanced expression of effector cells in combination with suppression of T_{regs} seems to be important. Unfortunately, the effects of IL-12 on T_{regs} are still unknown.

Nishikawa et al. [58] suggested that combination therapy targeting several key molecules expressed in APCs, effector T cells, and T_{regs} offers a promising anticancer immunotherapy. Previous studies showed that HCCs frequently express TAAs, and spontaneous humoral and cellular immune responses can be detected in some patients with HCC. Nevertheless, T-cell exhaustion seems to occur in patients with HCC. Therefore, recovery of the T-cell population is an important target of immunotherapy in HCC.

There is currently great interest in determining the therapeutic implications of manipulating the T_{reg} pathway for cancer immunotherapy. Murine studies have revealed that selective elimination of T_{regs} using antibodies against CD25 enhances the antitumor T-cell responses and induces the regression of experimental tumors. However, nonselective depletion of T_{regs} may significantly increase the susceptibility to autoimmune diseases. Furthermore, anti-CD25-depleting antibodies may reduce $CD4^+CD25^+$ T_{regs}, as well as $CD25^+$-activated $CD4^+$ helper and $CD8^+$ effector cells [59]. Therefore, current strategies directed toward more selective depletion or inhibition of T_{regs} include targeting the local tumor cells versus peripheral blood cells; developing monoclonal antibodies for T_{reg} cell-surface molecules such as CD25, CTLA-4, and glucocorticoid-induced tumor necrosis factor-α receptor; and combination of T_{reg} elimination with CTL expansion.

Use of the correct stimulation regimen, such as using alternative anti-PD-L1 antibodies, may boost the frequency and recover the functionality of dendritic cells, and thus inhibit T_{regs}.

The quantification of $FOXP3^+$ T_{regs} and CTLs in HCC are immunohistochemical techniques that can be performed on formalin-fixed paraffin-embedded tissues. Determining the ratio of these two cell populations may not only help to predict the recurrence of HCC, but may also help us to identify patients who may benefit most from future immunotherapies targeting this pathway. In the near future, pathologists are expected to have a critical role in HCC therapy by assessing the type of TIL infiltration, thereby providing prognostic information as well as guidance in selecting the appropriate immunotherapeutic strategy for each patient.

Conflict of interest No author has any conflict of interest.

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