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REVIEW

Immunosuppression in liver tumors: opening the portal to effective immunotherapy

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We have recently witnessed substantial progress with immunotherapy for selected diseases. Checkpoint inhibitors and chimeric antigen receptor T (CAR-T) cells are among the most promising agents. Whereas much of the early success with CAR-T cells has been demonstrated with hematological malignancies, important barriers remain for the application of CAR-T cell therapies for the management of metastatic solid tumors. The challenges are particularly apparent when considering primary and metastatic tumors in the liver. At baseline, the intrahepatic space is immunosuppressive and this feature is exploited by malignant cells. Fortunately, our evolving understanding of liver immune cell biology is allowing the development of novel immunotherapeutic strategies for the treatment of liver tumors. Furthermore, the unique anatomic features of the liver make possible highly selective immunotherapeutic delivery approaches that may maximize antitumor efficacy while limiting off-target damage to healthy tissues. This review summarizes the immunobiology of the intrahepatic space and how this knowledge enables identification of hurdles and potential solutions to the barriers facing immunotherapy for liver tumors.

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INTRODUCTION

The liver is a unique immunological organ, with an abundance of immune cells demonstrating a strong tendency toward promotion of tolerance or immune suppression. The propensity toward immune tolerance in the liver is advantageous for maintenance of normal biological function, as the liver is constantly bathed in foreign antigens and bacterial byproducts found in the portal blood. Unfortunately, the capacity of intrahepatic immune cells to suppress immunity and inflammation create fertile ground for primary and metastatic liver tumors to develop and progress. Suppressor cells in the liver are impediments to the development of effective antitumor immunotherapy strategies. A deeper understanding of liver immune cell biology will be essential for the development of novel immunotherapeutic approaches for liver tumors.

LIVER IMMUNOLOGY

A perfectly balanced immune system protects our bodies from external pathogens and endogenous threats such as malignant cells, while avoiding damage to healthy tissue. Two main arms of the immune system work in concert toward this ideal—innate and adaptive immunity (Figure 1). The innate immune system provides a broad primary response that is not dependent on the exquisite antigen specificity of antibodies or T-cell receptors. The innate response is mediated by natural killer (NK) cells, macrophages and dendritic cells (DCs). Innate immune cells have the capacity to process and present antigen to cells capable of more specific and long-lasting adaptive responses. T and B cells mediate highly specific responses to particular antigens, in addition to rapid recall or memory responses. Although immune cells offer important protective functions, regulatory mechanisms to control immune system activation are essential for prevention of damage to normal tissues. This is particularly true in the liver.

The liver contains an abundance of innate and adaptive immune cells, which are continuously exposed to ingested foreign antigens and bacterial products derived from gut flora. The liver is an immunologically rich and active organ, with a large number of DCs, Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), T cells, NK cells and B cells. Vigorous responses by liver immune cells to the steady stream of portal blood antigens would lead to a precarious situation with respect to liver damage and systemic inflammation.[1] As such, intrahepatic immune cells are skewed toward tolerance, with overtly suppressive functions or quiescence at baseline.

The tolerogenic properties of liver immune cells protect us from overexuberant responses to portal venous antigens but limit the ability of our immune systems to fight intrahepatic neoplasia. The propensity of primary and metastatic tumors to thrive in the liver is in part reflective of the tolerogenic nature of the intrahepatic milieu.[2,3] Intrahepatic tolerance to specific antigens is initiated upon uptake, processing and presentation of soluble antigens by antigen-presenting cells (APCs). LSECs capture and process antigen but are unable to activate T cells on their own.[4] DCs are also thought to promote tolerance in the liver, as studies have shown that these cells are less reactive than their counterparts elsewhere in the body.[5] Thorn *et al.*[6] have also shown that B cells can be suppressed in the liver that may also promote immune suppression, and liver T cells demonstrate tolerogenic properties.[5]

TUMOR-INFILTRATING LYMPHOCYTES (TILS)

Studies of TILs have provided important insight into the nature and limitations of the endogenous response to intrahepatic

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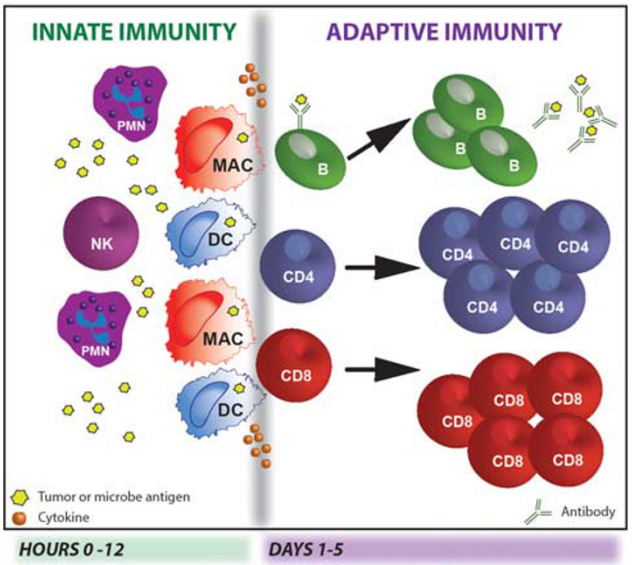

Figure 1. Innate and adaptive immunity. (**a**) Innate immunity includes the body's initial defences against infection. It includes certain complement proteins, epithelial barriers, natural killer (NK) cells, neutrophils (PMN), phagocytes such as macrophages (MAC) and antigen-presenting cells such as dendritic cells (DCs). Innate immune cells may directly kill tumor or infected cells and then present antigen to adaptive immune cells. (**b**), Adaptive immunity *i*ncludes B-cell-mediated humoral (dissolved) and T-cell-mediated cellular components. The innate and adaptive immune systems communicate by direct cellular contact or cytokine secretion. We obtained permission from Elsevier to use this figure, which is appearing in an upcoming new edition of *Jarnagin: Blumgart's Surgery of the Liver, Biliary Tract, and Pancreas*, 6th edn., Chapter 10: Liver immunology.

neoplasms. TILs may be used for therapeutic or prognostic purposes.[7] They are believed to represent an immune response to tumor antigens, although their presence does not imply effective antitumor immunity. Liver tumors, both primary and metastatic, have been evaluated extensively with regards to the presence of TILs in resected specimens and their prognostic value. Several types of TILs have been detected in liver malignancies, including T cells, NK cells, macrophages and B cells. $CD8^+$ and $CD4^+$ T cells are the most extensively studied liver TIL subsets as they are critical mediators of antitumor cellular immune responses. Regulatory T cells (Tregs), identified as $FOXP3^+$, are often present in liver tumors and represent mediators of intrahepatic immunosuppression.[8–10]

TILs have been evaluated in an attempt to predict clinical outcomes for primary liver tumors, including hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma. Li *et al.*[11] showed that elevated tumor-associated macrophage and memory T-cell infiltration were a prognostic factor for disease-free survival and overall survival in resectable HCC. Mathai *et al.*[10] reported that higher ratio of $FOXP3^+/CD8^+$ cells was associated with poorer differentiated tumors, recurrence and decreased overall survival. Interleukin 17 positive ($IL17^+$) T cells and $FOXP3^+$ cells are also believed to promote the progression and affect the prognosis of HCC by decreasing the level of $CD8^+$ T cells within the tumor.[12,13] Takagi *et al.*[14] evaluated $CD4^+$ and $CD8^+$ levels in resected intrahepatic cholangiocarcinoma and found that they correlated with DC density and outcome.

The liver is also a common site of metastatic disease, particularly from intra-abdominal cancers. Colorectal cancer liver metastasis (LM) patients who present with resectable disease have a 10-year survival of 17%, and fewer than 20% are surgical candidates at presentation.[15] Studies evaluating TILs in primary colorectal cancer have shown the type, location and density of TILs to be associated with outcomes.[16] TILs are also present in colorectal cancer LM specimens, with $CD8^+$ T-cell density being predictive of long-term survival.[17] Major histocompatibility complex (MHC) class I expression and its association with increased $CD8^+$ T-cell density was correlated with increased overall survival and time to recurrence.[18] Increased FOXP3 to $CD8^+$ or $CD4^+$ T-cell ratios were predictors of poor outcome in resected colorectal cancer LM patients, indicating the importance of suppressor–effector cell interactions.[8] Tregs have also been correlated with outcome following resection of neuroendocrine tumor LM.[19] Study of TILs from primary hepatic tumors and hepatic metastases have provided significant prognostic information regarding outcomes. Although the prognostic utility of TILs has been well demonstrated, a robust TIL response is generally insufficient to protect most patients from disease progression and death. As noted earlier, the suppressive tendencies of liver immune cells may have a large role in rendering TIL responses ineffective in addition to thwarting immunotherapeutic efforts.

BIOLOGY OF LIVER IMMUNOSUPPRESSIVE CELLS

As noted earlier, the liver has unique population of immune cells that promote immune tolerance as evidenced by liver allografts requiring less immunosuppressive therapy as compared with other solid organ transplants.[20] Although the tolerogenic nature of liver immune cells facilitate allograft acceptance, it is an impediment to cancer immunotherapy. Cancer immunotherapies include chimeric antigen receptor T (CAR-T) cell, antibodies that block immunoinhibitory or checkpoint molecules, and vaccines that attempt to induce a natural immune response against cancer. CAR-T cells are genetically engineered to express receptors that recognize an antigen on tumor cells.[21] However, hepatic immunosuppressive cells limit CAR-T cell performance within the intrahepatic space.[22] Detailed studies of intrahepatic immune cells have shed light on the mechanisms promoting liver tolerance and opportunities for enhancing immunotherapy for liver tumors.

LYMPHOCYTES

The lymphocyte pool in the liver is comprised of conventional T cells, γδ T cells, NK and NK T cells (NKT) and B cells.[5,6,23,24,25] Conventional liver $CD4^+$ and $CD8^+$ T cells have unique functional properties, while NKT and γδ T cells are particularly abundant. Most human liver $CD4^+$ and $CD8^+$ T cells are in an activated state and express CD25 and CD69.[26] In mice, liver $CD4^+$ T cells secrete both interferon γ (IFN γ) and IL4, indicative of T helper type 1 (Th1) and Th2 cell types.[27] Among $CD4^+$ T cells, Th1 programming is suppressed in the liver, resulting in a functional bias toward Th2 functionality.[28] Liver Th2 cells produce high levels of IL4 and IL10, which thwart antitumor immune responses.

Intrahepatic $CD4^+$ T cells are also reprogrammed into Tregs under neoplastic and inflammatory conditions.[8,29] Tregs with high expression levels of CD25 and Foxp3 are responsible for peripheral tolerance and protect the liver from immune-mediated damage.[30] In HCC specimens, Tregs downregulate costimulatory molecules and decrease the secretion of IL12 and tumor necrosis factor α (TNFα) and promote tolerance.[31] The liver also contains Th17 cells which secrete inflammatory cytokines that promotes hepatic inflammation and fibrosis. Liver Th17 cells may expand in response to programmed death-1 (PD-1, CD279) signaling and contribute to immunosuppression.[12,32] Intrahepatic T-cell subsets cooperate to create a tolerogenic milieu, which limits antitumor immunity.

NK cells may promote hepatic tolerance by producing suppressive factors such as transforming factor-beta (TGFβ) and

IL10, which inhibit DC function. This in turn induces the expansion of immunosuppressive Treg cells.[33,34] NKT cells have properties of both T cells and NK cells and share several of their surface markers. Activated NKT cells secrete IFNγ and IL4 similar to Th1 and Th2 cells, respectively.[35] NKT cells are known to clear hepatic infections and have a role in the developing inflammatory diseases. NKT cells are known to perform tumor surveillance and mediate tumor rejection by secreting IFNγ.[36] Also, NKT cells can suppress T-cell proliferation and thereby cause immunosuppression in the liver.[5] As such, liver NK and NKT function is highly contextual.

LIVER SINUSOID ENDOTHELIAL CELLS

LSECs line the hepatic sinusoids and are thus well positioned to take up and process the bulk of portal venous antigens.[37] LSECs are efficient APC and process antigens at levels similar to DCs.[4,38] LSECs are also capable of recruiting hepatic leukocytes via CD54 (intercellular adhesion molecule-1), CD106 (vascular cell adhesion molecule-1), vascular adhesion protein-1, CD44 and hyaluronan.[39,40] LSECs express low levels of MHCII and co-stimulatory molecules[41] and can induce CD4 T-cell reprogramming into suppressive IL10- and IL4-producing cells.[42] The tolerogenic effect of LSECs are also mediated by IL10 secretion and PD-1/programmed death-ligand 1 (PD-L1) signaling.[43]

DENDRITIC CELLS

DCs are a rare population in the liver, have poor immunostimulatory capability and contribute to intrahepatic tolerance.[44] Hepatic DCs are less immunostimulatory compared with splenic DCs.[45] There are four distinct DC subtypes ($CD8\alpha^{+}CD11b^{-}$, $CD8\alpha^{-}CD11b^{+}$, $CD8\alpha^{+}CD11b^{low/-}$, $CD8\alpha^{low/-}CD11b^{low/-}$) with specific functions. $CD8\alpha^{+}CD11b^{-}$ and $CD8\alpha^{-}CD11b^{+}$, which account for only 20% of total DC population in the liver, activate T cells causing an immunostimulatory effect. However, the other more predominant subtypes of hepatic DCs, $CD8\alpha^{+}CD11b^{low/-}$ and $CD8\alpha^{low/-}CD11b^{low/-}$, are poor T-cell stimulators. Thus, these two populations may contribute to tolerance. Direct physical interaction between Tregs and DCs inhibit DC maturation even in the presence of granulocyte macrophages colony-stimulating factor (GM-CSF), TNFα or IFNγ.[46] Human hepatic DCs are also tolerogenic in comparison to autologous blood DCs.[2]

KUPFFER CELLS

KCs are found within the liver sinusoids and constitute 80–90% of tissue macrophages in the body.[47] Depletion of KCs causes loss of oral tolerance and KC are less immunogenic than macrophages in other organs.[2,5] At baseline, they are more skewed toward tolerance. KCs can secrete anti-inflammatory cytokines or immunosuppressive factors (IL10, nitric oxide, TGFβ) in addition to pro-inflammatory cytokines (TNFα, IL6).[48–51] They can inhibit T-cell proliferation and the secretion of IL10 can induce the activation of Tregs thereby causing tolerance.[52] KCs can also express both PD-1 and PD-L1, which are known immunomodulatory molecules. PD-L1–PD-1 interactions between KCs, effector T cells and LSECs can modulate disease activity.[53]

MYELOID-DERIVED SUPPRESSOR CELLS (MDSCS)

MDSCs are a heterogeneous cell population of myeloid origin that have been reported in association with in liver tumors and in several inflammatory conditions, including sepsis, hepatitis and viral infections. T-cell suppression caused by MDSCs is mediated in part by L-arginine depletion by arginase 1 or by reactive oxygen species.[54] Liver MDSCs expand in response to GM-CSF secreted by tumor cells and GM-CSF enhances their capacity to suppress immune responses[22,55] (Figure 2) through exploitation of STAT3, indoleamine 2,3-dioxygenase (IDO) and PD-L1.[22,56]

HEPATIC STELLATE CELLS (HSCS)

HSCs are located in the luminal sinusoidal space of Disse. HSCs are derived from bone marrow precursors in mice and store vitamin A.[57] Once activated, HSCs metabolize vitamin A and produce extracellular matrix that induces hepatic fibrosis and cirrhosis.[58,59] HSCs express MHCI/MHCII and CD86 but are poor APCs. HSCs are capable of producing retinoic acid and TGFβ that induce Tregs.[60] On contact with activated T cells, HSCs express PD-L1, which attenuates T-cell response by increasing apoptosis.[61]

IMMUNOINHIBITORY SIGNALING

Immune checkpoint molecules prevent the development of unregulated immune response and autoimmune tissue damage. Some of the well-known immnuoinhibitory receptors include cytotoxic T-lymphocyte antigen-4 (CTLA-4), PD-1, T-cell immunoglobulin domain and mucin domain-3 (TIM3) and lymphocyte-activation gene (LAG3).[62–64] CTLA-4 counteracts CD28 costimulatory receptor activity on T cells. CTLA-4 and CD28 have common ligands in CD80 and CD86. However, the affinity of CTLA-4 for CD80 and CD86 is much higher than CD28 and thus prevents engagement of CD28. CTLA-4 blockade is known to enhance CD4 helper T cells and limit Treg suppressive activity.[65–68]

PD-1 causes immune tolerance in the tumor microenvironment.[69–72] Unlike CTLA-4, PD-1 limits T-cell activity in the peripheral tissue during inflammation and autoimmunity. PD-1 gets overexpressed during T-cell activation and is associated with proliferation of Tregs in the presence of its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC).[70] Recently, it was reported that there was a molecular interaction between PD-L1 expressed on APCs and CD80 expressed on T cells thereby eliciting inhibitory signals.[73,74] PD-1 is also expressed on other cell types such as B cells and NK cells, which limit their lytic activities.[75,76] However, PD-1 predominantly regulates effector T-cell proliferation and cytokine production, while CTLA-4 regulates early T-cell activation.

Other immune checkpoint markers of interest are LAG3 and TIM3, which are implicated in inhibiting lymphocyte activity and in anergy. These receptors are upregulated during T-cell activation. LAG3 signaling enhances immunosuppressive activity of Treg cells and inhibits the effector activity of CD8 T cells.[77,78] LAG3–MHCII interaction enhances T-cell proliferation and effector T-cell function *in vivo* and *in vitro*. PD-1 and LAG3 are co-expressed on anergic or exhausted T cells.[79,80] TIM3 co-expresses with PD-1 on tumor-specific CD8 T cells and their dual blockade enhance T-cell proliferation and cytokine secretion.[81–83]

OVERCOMING IMMUNE SUPPRESSION IN THE LIVER FOR ANTITUMOR IMMUNOTHERAPY

Few attempts have been made to specifically target intrahepatic neoplasms with immunotherapy and augment therapy through modulation of liver immune cells. Effective immunotherapy for liver tumors will require an effective tumor killing strategy, efficient intrahepatic delivery and agents capable of reversing suppressive function of liver immune cells. Intrahepatic tolerance can be reversed by depleting suppressor cells, activating hepatic immune cells or blocking the immune checkpoints. CAR-T cells are among the more promising immunotherapy technologies to emerge in recent years, given that highly specific immune responses can be manufactured as opposed to induced.

CAR-T cells have demonstrated success in hematological malignancies and have shown activity in solid tumors. CAR-T cells are autologous T cells that are bioengineered to express an immune receptor that is activated upon tumor antigen binding.

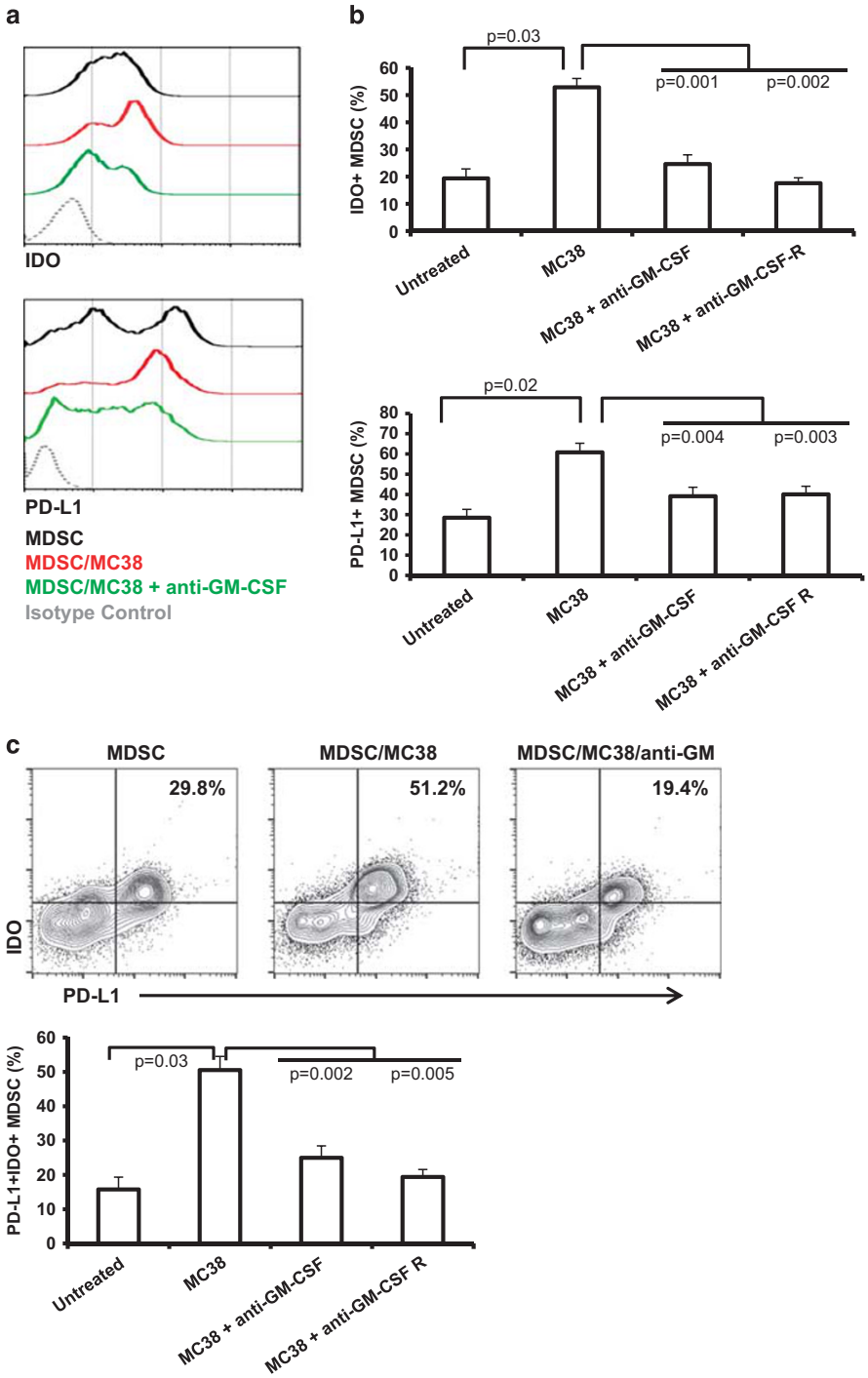

Figure 2. Tumor-derived granulocyte macrophages colony-stimulating factor (GM-CSF) drives indoleamine 2,3-dioxygenase (IDO) and programmed death-ligand 1 (PD-L1) expression in liver myeloid-derived suppressor cells (L-MDSCs). In all, 2.5×10^5 L-MDSCs were cultured with or without 5×10^4 MC38 tumor cells. Anti-GM-CSF and anti-GM-CSF receptor antibodies were added at the start or after 1 day of culture. After 48 h, L-MDSCs were purified for IDO and PD-L1 expression measurement (**a** and **b**). Isotype controls were used to define background staining and to set the threshold for positive IDO and PD-L1 expression. IDO/PD-L1 co-expression was visualized in L-MDSCs/MC38 cultures with or without anti-GM-CSF and anti-GM-CSF receptor (**c**). Bars show the expression averages of L-MDSCs isolated from four tumor-bearing livers and are representative of three experiments. Error bars are based on s.e.m. values. *P*-values were calculated using a two-tailed *t*-test. Previously published in Thorn *et al.*[89]

We have reported the biological activity and safety of anti-carcinoembryonic antigen (anti-CEA) CAR-T cell hepatic artery infusions (HAIs) in patients with heavily pretreated large tumor burdens.[84,85] CAR-T cell therapies for LM will likely be enhanced by strategies to inhibit the function of intrahepatic-suppressor cells, among which MDSC are particularly problematic. Liver MDSCs may be susceptible to inhibition of PD-1/PD-L1 interactions or IDO.[22] MDSCs express IDO and PD-L1, which mediate T-cell suppression.[56] Combinatorial immunotherapeutic approaches will enhance the antitumor activity of CAR-T cells.

ENHANCING THE EFFICACY AND SAFETY OF LIVER IMMUNOTHERAPY THROUGH TARGETED DELIVERY

In addition to addressing intrahepatic immunosuppression, regional delivery approaches have shown promise as a strategy to enhance biological activity and reduce off-target toxicity when targeting liver tumors with cellular immunotherapeutics.[84,85] The rationale for regional, intrahepatic infusion of CAR-T cell stems from the experiences with regional chemotherapy and immune cell treatments. Delivery of chemotherapy directly into the liver for metastasis treatment permits maximal exposure of the tumors to the agent, while minimizing the effects on healthy tissues elsewhere. This principle has been well demonstrated in patients receiving HAI of chemotherapy for LM. Response rates are consistently higher with HAI and systemic effects are minimized.[86] The regional infusion of lymphocytes into the liver has been demonstrated to be feasible and safe, with up to 80% of radiolabeled lymphocytes infused via the hepatic artery persist in the liver for up to 120 h.[87]

We recently completed a phase I trial to test CAR-T cell HAI to determine whether direct regional delivery of CAR-T cell to LM is safe and associated with signals of clinical efficacy.[84,85] Six patients completed the protocol, and three received anti-CEA CAR-T cell HAIs alone in dose-escalation manner (10^8, 10^9 and 10^{10} cells). We treated three additional patients with the maximum planned CAR-T cell HAI dose (10^{10} cells $\times 3$) along with IL2 infusional support. Four patients had >10 LMs, and patients were heavily pretreated with conventional systemic therapy.

No patient suffered a grade 3 or 4 adverse events related to the CAR-T cell HAIs and there were no deaths related to the study intervention. Importantly, regional infusion seemed to avoid severe cytokine release syndrome and significant off-target effects from direct destruction of normal CEA^+ tissue by CAR-T cells. Febrile adverse events were observed in four patients. A single patient experienced a marked increase in the peripheral eosinophil count. Given the reported association between IL2 infusion and cardiac thrombosis with other features of Loeffler's syndrome,[88] we obtained an echocardiogram and electrocardiogram, which were normal

Normal liver parenchyma and biliary structures were well preserved following CAR-T cell HAIs. Biopsies from normal liver did not demonstrate increased levels of inflammation or fibrosis following CAR-T cell HAI whether or not systemic IL2 was administered. Although all patients experienced transient elevations of alkaline phosphatase, total bilirubin and aspartate aminotransferase levels, the majority of values did not deviate significantly from baseline levels. Portal pressures and liver synthetic function were not adversely affected by the CAR-T cell HAIs, as reflected by no patient becoming thrombocytopenic or coagulopathic.

One patient remains alive with stable disease at 44 months following CAR-T cell HAI and five patients died of progressive disease. Among the patients in the cohort who received systemic IL2 support, CEA levels decreased 37% (range 19–48%) from baseline. Biopsies demonstrated an increase in LM necrosis or fibrosis in four of the six patients. Elevated serum IFNγ levels correlated with serum CEA responses. As CAR-T cell HAIs were well tolerated and associated with evidence of tumor cell killing in our subjects, further clinical testing of this approach alone and in combinatorial manner is underway. HAI of CAR-T cells for the treatment of liver tumor is a promising approach for enhancing clinical activity while avoiding some of the more problematic systemic effects of CAR-T cell infusions, including severe cytokine release syndrome.

SUMMARY

The liver contains an abundance of diverse immune cells that work in concert to create a highly tolerogenic milieu. Though hepatic tolerance is beneficial in liver transplantation and maintenance of normal physiological homeostasis, intrahepatic immunosuppression limits the effectiveness of endogenous and therapeutic antitumor immunity. Hepatic immunosuppression is mediated by a variety of cell types, cytokines and immunoinhibitory molecules. Reversal of intrahepatic immunosuppression will be an important element in combinatorial immunotherapy approaches designed to target intrahepatic tumors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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