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Liver specific gene immunotherapies resolve immune suppressive ectopic lymphoid structures of liver metastases and prolong survival

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ABSTRACT

The ability to generate potent immunotherapies locally and transiently for the treatment of cancers is a promising strategy to improve efficacy and decrease off-target toxicities. Here, we explored an alternative approach for the delivery of immunotherapeutic agents, in which we deliver the pDNA of an engineered PD-L1 trap and/or CXCL12 trap to the nucleus of liver hepatocytes via a lipid calcium phosphate nanoparticle. This strategy greatly increased the concentrations of immunotherapeutic agents in the local tissue, allowing the therapy to inhibit the accumulation of immune suppressive cells and liver metastasis. Furthermore, we find that the lipid calcium phosphate nanoparticles containing the pCXCL12 trap resolved the formation of immune suppressive ectopic lymphoid structures, while the pPD-L1 trap promoted T-cell survival and migration into the liver following vaccination against tumor antigens (>180% increase in survival). This approach showed superior efficacy in the treatment of the liver metastasis compared to free protein immunotherapies. This strategy should be considered as an approach to support liver metastasis therapies as well as for future research interested in manipulating the chemokine/cytokine immune factors within the liver.

Significance: Our approach results in transient liver specific expression of gene immunotherapies with improved efficacy and reduced off-target toxicities over traditional systemically administered immunotherapies. This approach would allow clinicians to manipulate the liver and immune microenvironment to resist cancer invasion, improve organ health, and prolong patient survival.

1. Introduction

In the United States alone approximately 381,150 patients were diagnosed with colorectal cancer, resulting in over 89,640 deaths in 2015. These numbers make colorectal cancer the third most common type of cancer worldwide, and is the second most deadly [1]. The cause of death is rarely due to the primary cancer burden, as local resection is quite efficient. The establishment of metastasis is the leading cause of death, in which at early stages of cancer detection, before metastases form, the five-year survival rate is approximately 90%. Once liver metastasis has been established, this rate drops drastically to less than 12% [1]. In the United States and Europe, liver metastasis is much more common than primary liver cancers such as hepatocellular carcinoma (HCC). Although resection and varying chemotherapeutic treatment regimens yield improved efficacy over other traditional treatments, the off-target toxicities along with a minimal increase in median survival, 12–20 months, presents a less than desirable outcome [2]. Therefore, researchers aim to improve the treatment of cancers through boosting the patient's own immune system against cancer specific antigens. Numerous anti-cancer vaccines for colorectal liver metastasis are being investigated in clinical trials. However, no anti-cancer vaccine for colorectal liver

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metastasis has reached the market due to lack of efficacy or systemic toxicities. The liver and tumors immune suppressive microenvironment is one major obstacle which must be overcome before these vaccines will have a significant impact on cancer patients. Recently, a study by Finkin et al. reported that the formation of immune suppressive ectopic lymphoid structures (ELS) in patients correlated with reduced survival and increased recurrence of liver cancer [3]. Therefore, targeting central factors that are critical for the formation and establishment of the immune suppressive tumor microenvironment and ELS is one such strategy to decrease tumor burden.

A crucial factor, chemokine CXCL12, has been shown to increase tumor progression through promoting the immune suppressive environment that suppresses $CD8^+$ T-cell activation. The hepatic stellate cells are resident perisinusoidal cells that have been shown to produce high levels of endogenous CXCL12 for recruitment of immunosuppressive lymphocytes (i.e. regulatory T cells) and myeloid derived suppressor cells (MDSC) to areas of inflammation. In the presence of high levels of CXCL12, CXCR4+ cancer and immune cells accumulate to establish an immune suppressive tumor niche [4,5]. Further clinical analysis on human colorectal cancer biopsies have found that increased liver metastasis correlates with increasing CXCR4 expression [6,7].

Additionally, major progress has been made in recent years aimed at increasing $CD8^+$ T-cell activation through blocking the programmed cell death receptor 1/programmed cell death ligand 1 (PD-1/PD-L1) receptor/ligand pair. It has been well documented that the immune suppressive cell populations such as T-regulatory (T_{reg}), MDSC, and numerous other immune cell types express high levels of PD-L1, which directly deactivates PD-1+ T-cells. Therefore, this PD-L1/PD-1 has become a promising target to increase tumor specific $CD8^+$ T-cell activation and killing. However, while PD-1/PD-L1 targeting in cancer has shown beneficial responses in some cancer types such as melanoma, it has not shown great efficacy in immune suppressive cancers such as liver metastasis or solid tumors. Furthermore, a variety of side effects termed immune-related adverse events (irAEs) have been observed following systemic delivery of PD-1/PD-L1 antibodies, which has limited their therapeutic applications in clinical trials [8].

Therefore, we developed a new anti-cancer strategy centered on the liver-specific delivery of genes encoding small affinity proteins to entrap such factors as CXCL12 and/or PD-L1. We recently demonstrated this strategy by trapping the CXCL12 in the liver [9]. However, through these studies we found that trapping CXCL12 can only decrease the incidence of liver metastasis for a limited time due to low $CD8^+$ T-cell recruitment and activation in the metastatic niche. Therefore, we hypothesize that it is necessary to utilize a combination of therapies aimed at reducing the immune suppressive environment while simultaneously increasing $CD8^+$ T-cell recruitment and activation.

In the current study, we utilize the LCP platform to deliver two engineered plasmid DNAs (pCXCL12 trap/pPD-L1 trap) encoding proteins trapping both CXCL12 and PD-L1 to treat colorectal liver metastasis. The small CXCL12 protein trap, CXCL12 trap, was designed based on an anti-CXCL12 antibody sequences, by fusing a V_H and a V_L domain. The PD-L1 protein trap, PD-L1 trap, was developed based on the ability to convert the extracellular PD-L1 binding domain from endogenous PD-1 into a trivalent ligand through genetic fusion with a trimerization domain. The resulting trivalent PD-L1 trap achieved approximately 219 pM binding affinity to mouse PD-L1, 82,000 times higher affinity compared to endogenous PD-1 and PD-L1 (Miao et al., publication pending). Furthermore, to increase the $CD8^+$ T-cell activation and recruitment to the metastatic niche, we delivered a previously reported LCP vaccine formulation loaded with a RIG-1 ligand, 5'pppdsRNA, and a phosphorylated model cancer specific antigen, p-AH1-A5.

The establishment of an aggressive murine colorectal liver metastasis via hemi-splenic inoculation of CT-26FL3 (RFP/Luc) allows us to investigate our therapies ability to alter the liver microenvironment and the tumor/immune cell profile. Furthermore, this model has minimal primary tumor growth, due to surgical resection, which mirrors the clinical scenario of colorectal liver metastasis patients in which the primary tumors are resected. The use of this syngeneic model also mirrors the clinical cases of the liver metastasis immune environment in which numerous immune cell ELS aggregates are established. Therefore, investigating strategies to resolve the establishment of these aggregates as well as promoting $CD8^+$ T-cell recruitment to the metastatic niche will be further elucidated.

2. Results

2.1. Formulation and characterization of galactose-LCP pDNA-mc-CR8C nanoparticles

Hu et al. first reported the formulation and delivery of the galactose-LCP with pDNA-mc-CR8C cargo to the liver hepatocytes of mice [10]. The formulation of the pCXCL12 trap LCP was reported by Goodwin et al. and the methods used in the cited paper were used for all pTrap LCP formulations used in this study [9]. Furthermore, the 5'pppdsRNA p-AH1-A5 LCP vaccine formulation was first reported by Goodwin et al. [11] which is based on the phosphorylated peptide LCP vaccine formulation first reported by Xu et al. [12] The core structure can be visualized under transmission electron microscopy (TEM) (Fig. 1B). The DOPA monolayer surrounding the $Ca_3(PO_4)_2$ core allows for the addition of the cationic outer leaflet lipids (1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), helper lipid cholesterol, and galactose or mannose conjugated to 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-*N*-succinyl(polyethylene glycol)-2000 (DSPE-PEG2000) to assist in RES evasion and dendritic cell (by mannose) or hepatocyte (by galactose) uptake. In this report, the hydrodynamic diameter and the surface charge of the LCP particles were found via dynamic light scattering zetasizer analysis to be approximately 45 nm and 10 mV, respectively (Fig. 1B). The pDNA encapsulation efficiency was found to be approximately 50–60%. The p-AH1-A5 and 5'pppdsRNA encapsulation efficiency was found to be approximately 70%, which corresponds to the reported efficiency by Goodwin et al. [11].

2.2. In vivo expression profile of pDNA (pPD-L1 and pCXCL12 trap) via LCP formulation

Expression of the pCXCL12 trap and pPD-L1 trap was assessed through ELISA analysis via the His(6×)-tag engineered at the C-terminus of the protein traps (Fig. 1A). Mice were treated via IV injection of pCXCL12 or pPD-L1 trap LCPs and compared to the free CXCL12 or PD-L1 protein traps (1 mg/kg QOD x 3). Studies demonstrated the transient expression time profile following analysis of the livers, serum, spleen, and kidneys on days 1, 2, 4, and 8 following final IV administration is shown in Fig. 1C. The results indicate that the pDNA LCP vectors elicits transient liver specific expression of the CXCL12 or PD-L1 trap, yielding high expression for 4 days post final injection with minimal trap expression found on day 8 (Fig. 1C). These results demonstrate that the galactose-LCP vector allows for local and transient expression in the liver hepatocytes, with minimal expression in other organs or serum compared to the IV administration of free CXCL12 or PD-L1 traps (Fig. 1C).