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Expansion of interferon-gamma-producing multifunctional CD4+ T-cells and dysfunctional CD8+ T-cells by glypican-3 peptide library in hepatocellular carcinoma patients☆

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Abstract

Glypican-3 is a promising target for immunotherapy for hepatocellular carcinoma, but limited data exist regarding its immunogenicity in patients with diverse HLA types, immunogenicity for CD4$^+$ T-cells, and the impact of inhibitory co-stimulation on glypican-3-specific T-cells. Using a 15mer overlapping peptide library for glypican-3, PBMC from patients with HCC were assessed *ex vivo* and after short-term *in vitro* expansion for tumor antigen-specific T-cell responses with and without blockade of PD-1/PD-L1 and CTLA-4 signaling. Glypican-3-specific T-cells were undetectable *ex vivo*, but primarily IFNγ$^+$ TNFα$^+$ CD4$^+$ T-cells expanded with short-term *in vitro* stimulation in 10/19 (52%) patients. Glypican-3-specific CD8$^+$ T-cells predominantly produced TNFα, but did not secrete IFNγ nor degranulate. CTLA-4 and PD-1 blockade minimally impacted the cytokine secretion and proliferation of glypican-3-specific T-cells. These data suggest that CD8$^+$ T-cell-directed tumor vaccines in HCC may have limited potential for efficacy unless optimal co-stimulation conditions can be identified but CD4$^+$-directed vaccines merit consideration.

Keywords

Hepatocellular carcinoma; T-cells; Interferon-gamma; PD-1; CTLA-4

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and third-leading cause of cancer death worldwide [1]. Hepatocellular carcinoma most frequently develops in

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patients with cirrhosis related to chronic viral hepatitis [2] at an approximate rate of 4% per year, often prior to the development of other complications of cirrhosis or portal hypertension. In these individuals, HCC primarily causes morbidity and mortality due to acceleration of hepatic failure and portal hypertension rather than via metastatic spread. Usually asymptomatic until late stages, HCC most often presents at an advanced stage at which time treatment options are quite limited [3]. For this reason, novel approaches including immunotherapy have been considered as a potential adjunctive therapy in advanced HCC.

There are several theoretical and empiric reasons to expect that hepatocellular carcinoma might be poorly immunogenic and thus a challenging target for tumor vaccines. First, the liver microenvironment exerts a toleragenic effect due to various properties of liver sinusoidal endothelial cells and Kupffer cells [4,5]. Second, HCC arises primarily in a cirrhotic liver, an immunosuppressive environment associated with phagocyte and antigen-presenting cell dysfunction [6,7]. Third, chronic viral infections, such as hepatitis B and C, which underlie the antecedent cirrhosis, induce intrahepatic antigen-specific effector T-cell exhaustion as well as regulatory T-cell generation [8,9]. Furthermore, there is mounting evidence that hepatocellular carcinomas may directly recruit regulatory cell populations, such as M2 macrophages, myeloid-derived suppressor cells, and regulatory T-cells, to suppress tumor-directed T-cell effector responses [10,11]. Not surprisingly, tumor-antigen specific T-cells in patients with HCC, when detectable, have generally exhibited restricted effector functions [12]. Nonetheless, some studies suggest that the presence of T-cell responses against panels of tumor antigens in HCC may be associated with improved prognosis [13]. Furthermore, T-cell activation by cytokines [14] and/or by therapeutic embolization of tumor-lysate-pulsed dendritic cells directly into HCCs has resulted in partial tumor control [15], suggesting that tumor-specific T-cells in HCC could play a role in retarding tumor growth.

Glypican-3 (GPC3), a glycosylphosphatidylinositol-linked heparan-sulfate proteoglycan, has been identified as a highly specific, membrane-associated, tumor antigen found in 66–100% of HCC [16,17] with little or no expression in non-tumorous cirrhotic liver tissue or other normal adult tissues [18,19]. GPC3 fosters HCC growth by altering Wnt signaling [20], modulating growth factors such as IGF-2, BMP-7 and FGF-2 [21], and possibly by playing a role in M2 macrophage recruitment [11]. GPC3 may be cleaved from the surface of expressing hepatocytes, thereby entering the circulation to allow serological detection [22]. While several groups have reported the ability to expand glypican-3-specific T-cells in mice [23–25] and from a small number of human subjects [23,26], this work has focused primarily on expanding $CD8^+$ T-cell using specific HLA-types and highly immunodominant epitopes [23,24,26] limiting the applicability of findings to more heterogeneous populations. Such approaches have also precluded the examination of the potential important role of tumor antigen-reactive $CD4^+$ T-cells [27,28]. Furthermore, cytolytic capacity of expanded GPC3-specific cells remained fairly weak even after long-term *in vitro* expansion under optimized conditions [23] suggesting that antigen-specific T-cells expanded from HCC patients remain dysfunctional.

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In this study, we sought to quantify the *in vivo* frequency and capacity for expansion of polyfunctional CD8+ and CD4+ antigen-specific T-cell response against glypican-3 in a cohort of patients with heterogeneous HLA types and to define the role of inhibitory co-stimulatory molecule expression on suppressing tumor antigen-specific peripheral T-cells. Our studies indicate that glypican-3-specific T-cells are functionally suppressed *ex vivo* in patients with HCC. However, IFNγ-producing CD4+ T-cells are expanded with short term *in vitro* stimulation in approximately half of HCC-bearing individuals. Glypican-3-reactive CD8+ T-cells are also expanded in approximately half of the HCC patients, but these CD8+ T-cells are functionally constrained to produce TNFα alone. CTLA-4 and PD-1 inhibitory co-stimulation pathways, which are quite important in suppressing hepatitis virus-specific T-cells, only modestly impact the cytokine secretion and proliferation of peripheral glypican-3-specific T-cells in HCC patients. Thus, peripheral glypican-3-specific T-cells may be poor targets for effective vaccine-induced augmentation or for expansion in adoptive immunotherapy protocols unless mechanisms to reverse tolerance are identified.

2. Materials and methods

2.1. Patients

Subjects and controls were recruited from the Gastroenterology Clinics at the Philadelphia Veterans Affairs Medical Center following informed consent on an institutional review board-approved protocol. All patients were assessed for baseline demographics, hepatitis viral serologies, alcohol use history, and prior therapy for hepatocellular carcinoma. HCC patients were diagnosed histologically or via standard radiological and serological criteria [29]. Controls included patients with hepatitis C-induced cirrhosis with no evidence of HCC by serial imaging and alpha-fetoprotein screening (cirrhotic group, CIR), hepatitis C patients with F1-2 fibrosis by biopsy within the preceding 5 years (early-stage viral hepatitis, EVH), and healthy donors (HD) with no evidence of chronic liver disease.

2.2. HLA Typing

In all patients, HLA-A2.1 expression was determined using flow cytometry with anti-HLA-A2.1 (clone BB7.2, BD Biosciences, Franklin Lakes NJ). Results were confirmed with Terasaki HLA Class I Typing Tray (One Lambda, Canoga Park CA).

2.3. Peptides and proteins

Libraries of 15mer peptides offset by 6 and overlapping by 9 amino acids for the human (580aa, NP_004475) and survivin (142aa, NP_001159) were commercially synthesized (Proimmune, Oxford UK). Selected individual 9–10mer peptides predicted to bind to human HLA-A2.1 based on online algorithms (BIMAS [30], SYPEITHI [31], and RankPep [32]) were also synthesized. Recombinant human glypican-3 (Gln 25–His 559) and survivin were obtained commercially (R&D Systems, Minneapolis MN and Genemega Inc., San Diego CA respectively). In previous studies using 15mer peptide libraries for the hepatitis C virus, 15mer peptides were shown to be immunogenic for both CD4+ and CD8+ T-cells [33]. A mixture of CMV, EBV, and Influenza (CEF) 9–10mer control peptides (Cellular Technology Ltd., Cleveland, OH) were used as positive controls for CD8+ effector T-cell responses.

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2.4. Isolation of PBMC

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Histopaque (Sigma, St. Louis MO) density centrifugation. Cells were resuspended in RPMI 1640 with L-glutamine (Invitrogen) with 10% human AB serum (Sigma Inc., St. Louis, MO), 1.5% HEPES (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

2.5. In vitro T-cell expansion

4×10^6 PBMC were stimulated with media control, pooled glypican-3 15mer peptides (95 peptides, 1 µg/ml each), or CEF peptide pool (1 µg/ml) for 8 days at 37 °C 5% CO_2 with rhIL-2 100U/ml (Novartis/ Chiron, Emeryville, CA) added on day 2 and day 5. Additional antigen-presenting cells (APC) were not utilized due to the presence of sufficient number of APCs in whole PBMC. For proliferation and blocking experiment, PBMC were labeled with 5 uM CFSE-DA (Molecular Probes, Eugene OR) per manufacturer's instructions, then incubated with survivin-, glypican-3- or control peptide pools in the presence of NA/LE control Ig (Biolegend, San Diego CA), anti-PD-L1 (clone 29A.2E3, Biolegend) and/or anti-CTLA-4 (clone BNI3, BD Biosciences, San Jose CA) with rhIL-2 added on day 2 and day 5.

2.6. Flow cytometry

All antibodies were purchased from Becton Dickinson (Becton Dickinson, Franklin Lakes, NJ) except where specified. The cutoff for each marker was based on the isotype antibody. Data were acquired on a modified FACSCanto (BD), and ana\lyzed using Flow Jo (Tree Star Inc., Ashland OR).

2.7. Intracellular cytokine staining

In vitro expanded cells were restimulated for 6 h with survivin- or glypican-3-derived 15mer peptide pools and controls in the presence of anti-CD107a-PE and monensin, fixed and permeabilized using BD Cytoperm/Cytofix (BD), then stained intracellularly for IFNγ PE-Cy7 and TNFα APC (BD).

2.8. IFNγ Elispot

Antigen-specific T cell IFNγ responses were examined *ex vivo* and after *in vitro* expansion in cytokine Elispot assay as previously described [34]. In *ex vivo* assays, PBMC (2×10^5/well) were plated in 96-well Elispot plates pre-coated with anti-IFNγ (5 µg/ml, Pierce, Rockford IL) and stimulated with peptide matrices for glypican-3 (95 peptides, 7×7×2 matrix) and human survivin (22 peptides, 5×5 matrix), pooled glypican-3 peptides (95 peptides), pooled surviving peptides (22 peptides)(1µg/ml), recombinant glypican-3, and recombinant survivin proteins (10 mg/ml) in duplicates. Negative control wells without peptides (6 replicates) and positive control wells with 2 µg/ml phytohemagglutinin, 1 µg/ml CEF and 20 µg/ml *C. albicans*. After 8 days *in vitro* expansion, 5×10^4 cells/ well were stimulated with each peptide pool (1 µg/ml) in duplicates with positive (PHA) and negative controls (media, irrelevant peptide pool). Plates were analyzed for spot forming units (SFU), excluding assays with high background (average > 10 SFU/well in negative control wells) or no response to positive control stimulation on an AID Elispot ELF04 (AID Diagnostika, Strassberg, Germany).

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2.9. HLA-A2.1 pentamer staining

In HLA-A2+ patients, HCC-specific T-cells were detected *ex vivo* and after *in vitro* expansion using peptide-loaded HLA-A2.1 pentamers (Proimmune, Oxford UK) loaded with following 9–10mer epitopes: Survivin$_{96–104}$ (LMLGEFLKL)(Sur1M2), Glypican-3$_{44–52}$ (RLQPGLKWV) [23], Glypican-3$_{136–145}$ (SLTPQAFEFV), Glypican-3$_{144–152}$ (FVGEFFTDV)[23], Glypican-3$_{169–177}$ (ELFDSLFPV)[23], Glypican-3$_{563–572}$ (KLLTSMAISVV), HTLV-I tax$_{11–19}$ (LLFGYPVYV), HCV NS3$_{1073–1081}$ (CINGVCWTV) and Influenza Matrix$_{58–66}$ (GILGFTFVL). 1×10^6 PBMC were stained with APC-labeled pentamer and the following antibodies (from BD, except as indicated): a dump channel containing anti-CD4 PerCP (RPA-T4), anti-CD14 PerCP (MDP9), anti-CD19 PerCP (SJ25C1) and Viaprobe; anti-CD8 APC-H7 (SK1); anti-PD-1 FITC (MIH4); and biotinylated anti-LAG-3 (BAF2319, R&D Systems) detected with streptavidin-Alexa700 (Invitrogen). Cells were permeabilized and fixed using BD Cytoperm/Cytofix and stained intracellularly for anti-CD152 PE (CTLA-4, BNI3).

2.10. Statistical analysis

The median values for clinical and immunologic parameters were compared using ANOVA, the nonparametric Kruskal–Wallis ANOVA, Wilcoxon Rank Sum or Mann–Whitney *U* test. The frequency of positive responses was compared using $\chi 2$ or Fisher's exact test based on sample size. Spearman rank correlation was used for bivariate correlation of variables. Multivariate regression was performed using JMP 7 (SAS Institute Inc, Cary NC). A p-value < 0.05 was considered significant.

3. Results

3.1. Patient characteristics

Thirty-three patients with hepatocellular carcinoma provided samples (Table 1). Median age was 57 years (range 49–67), 21 (63%) patients were black, and 12 (36%) were HLA-A2.1 positive. Except for one patient each with hepatitis B and non-alcoholic steatohepatitis, all patients were infected with hepatitis C virus and 25 had prior histories of alcohol dependence. Solitary tumors were present in 19/33 (58%, range 1–3 tumors) with a median size of the largest tumor being 3.8 cm (range 1.5–11.5 cm). 8/33 cases were confirmed histologically, the remainder confirmed using standard noninvasive criteria [29]. The distribution of BCLC tumor stages were as follows: A 16; B 13; C 3; D 1. Five patients received transarterial chemoembolization prior to recruitment, but none within 3 months of enrollment. Serum AFP levels were >20 mg/dl in 22 patients (67%) and >200 mg/dl in 12/33 (36%). Serum glypican-3 was detectable in 22/33 (66.7%). Controls included 10 patients with HCV cirrhosis with no evidence of HCC, 6 HCV patients with F1–2, and 15 healthy donors with no evidence of chronic liver disease (Table 2). HCC patients were all male and modestly older than healthy donors, a group that included 5 women, but were well matched with respect to age, gender and ethnicity with cirrhotic and non-cirrhotic hepatitis C controls.

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3.2. IFNγ$^+$ T-cell responses against glypican-3 cannot be detected ex vivo in HCC patients or controls

Utilizing 15mer overlapping peptides in IFNγ Elispot, we quantified total T-cell responses against glypican-3 in patients with HCC and relevant controls. Using an arbitrary cutoff of 50 SFU/10^6 PBMC (10 SFU/well above background) to define positive responses, we found no positive IFNγ responses in HCC patients against pooled glypican-3 15mer peptides (Fig. 1A) nor against pooled survivin 15mer peptides (Fig. 1B). Positive responses occurred in fewer than 10% of non-HCC controls. Consistent with results for pooled peptides, peptide matrices revealed no convincing detectable T-cell reactivity to any individual glypican-3 15mer peptide in any patient (data not shown). The lack of detection of T-cell IFNγ$^+$ response to the peptide libraries did not reflect global T-cell suppression as CD8$^+$ T-cell reactivity to CEF viral peptides and CD4$^+$ T-cell reactivity against *C. albicans* lysate remained detectable in the majority of cirrhotic patients (Figs. 1C and F). A significant minority of healthy donor patients (5/ 15) but no EVH, CIR and only a small minority of HCC (4/30, only 1 of whom had detectable serum glypican-3 levels) had detectable CD4$^+$ IFNγ$^+$ responses against recombinant human glypican-3 (Fig. 1D), suggesting that endogenously expanded T_h1 effector T-cells against glypican-3 are relatively frequently detectable in health but become suppressed during the course of HCV disease. By contrast, CD4$^+$ T-cell responses against human survivin were readily detected in most HCC patients as well as controls (Fig. 1E) indicating both the absence of a global CD4$^+$ T-cell defect as well as differential regulation of Th1 responses against various tumor antigens. Overall however, these data indicate that CD4$^+$ and CD8$^+$ T-cells reactive to glypican-3 either circulate at extremely low frequencies or are highly suppressed *in vivo* in HCC patients.

3.3. Glypican-3-specific IFNγ$^+$ cells can be expanded in vitro from nearly half of HCC patients

Where adequate lymphocytes were available we performed short-term *in vitro* expansion of freshly isolated PBMC with pooled 15mer peptides for glypican-3 in the presence of rhIL-2 and then re-examined peptide-specific cytokine secretion by IFNγ Elispot and intracellular cytokine staining. Unstimulated DMSO, survivin and CEF controls were used in all experiments. In all but 1 HCC patient, CEF control peptide stimulation resulted in expansion of antigen-specific IFNγ-secreting T-cells to greater than 1000 SFU/10^6 PBMC (data not shown) reflecting the absence of a global CD8$^+$ T-cell defect. Glypican-3-specific IFNγ-secreting T-cells could not be detected from PBMC expanded by rhIL-2 alone without peptides in any case (data not shown). As shown in Figs. 2A and B, detection of either glypican-3-or survivin-specific IFNγ$^+$ T-cells in non-HCC patients to levels greater than 500 SFU/10^6 PBMC (0.005%) with short-term *in vitro* expansion was a rare event, occurring in only one healthy control subject. By contrast, expansion of glypican-3-specific T-cells to greater than 500 SFU/10^6 PBMC (0.005%) occurred in 10/19 (52%) of HCC patients (Fig. 2C). Pre- and post-expansion glypican-3-specific IFNγ SFU were markedly increased in HCC patients (mean 9 vs. 815 SFU/10^6 PBMC, $p = 0.0038$) but were not statistically increased for either the non-cirrhotic or cirrhotic groups or combination thereof ($p = 0.11$). Interestingly, expansion of glypi-can-3-specific T-cells to greater than 500 SFU/10^6 PBMC within the HCC group was associated with greater antigen burden, using serum glypican-3

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levels as a surrogate marker of antigen expression (Fig. 2D, $p = 0.03$), similar to recent findings correlating AFP-specific $CD4^+$ T-cell IFNγ responses and serum AFP levels [27,28]. Thus, among HCC patients, presence of tumor antigen was associated with *in vivo* expansion of antigen-specific T-cells at a precursor frequency significantly greater than present in healthy donors, non-cirrhotic liver disease and cirrhotic controls.

3.4. Despite expansion of peripheral glypican-3-specific Th1 cells, glypican-3-specific CD8+ T-cells exhibit restricted functional responses

Peptide-expanded T-cells were restimulated on day 8 to measure CD107a uptake, a measure of degranulation, and production of IFNγ and TNFα by intracellular cytokine staining. On a per subset level, the median cytokine response (IFNγ, TNFα, and/or CD107a) was 0.29% of $CD4^+$ and 0.30% of $CD8^+$ T-cells among HCC patients. Expansion of glypican-3-specific IFNγ-secreting $CD8^+$ T-cells to greater than 0.25%/ $CD8^+$ T-cell was only seen in one normal donor (data not shown) and three of 18 HCC patients (median $IFN\gamma^+/CD8^+$ 0.03%, range 0–0.94%, Fig. 3A black plus grey bars). The predominant cytokine profile among $CD8^+$ T-cells was a $TNF\alpha^+IFN\gamma^-$ pattern (median of 0.13%/CD8+ T-cells) with 11/ 18 subjects having greater than 0.25% of $CD8^+$ T-cell responding only by TNFα secretion (Fig. 3A). $CD8^+$ T-cell cytokine secretion did not correlate with IFNγ Elispot results. While on a per subtype analysis, median $IFN\gamma^+$ secretion per $CD4^+$ T-cell was similar to $CD8^+$ T-cells (median $IFN\gamma^+$/ CD4+ 0.03%, range 0–0.38%), there was a strong correlation between $CD4+IFN\gamma^+$ frequency and IFNγ Elispot response (Fig. 3B). Reanalyzing these responses relative to all lymphoid cells (compensating for CD4:CD8 ratios), as shown in Fig. 3C, Elispot “responders” had significantly higher frequency of $CD4+IFN\gamma^+$ (black bars, 0.069 vs. 0.003%, $p = 0.0035$) but not CD8+IFNγ (white bars, 0.004 vs. 0.004%, $p = 0.92$). $CD4+TNF\alpha^+$ responses were similarly more frequent in Elispot responders than nonresponders (median 0.167% vs. 0.046%, $p = 0.004$) while no significant difference was seen for $CD8^+TNF\alpha^+$ response (Fig. 3D). Glypican-3-specific $CD8^+$ T-cells exhibited a strong polarization to TNFα secretion as a solitary response (72%) with modest fractions secreting TNFα with degranulation (16%) or degranulation alone (10%) (Figs. 3E white bars and G) with nearly no IFNγ secretion. By contrast, glypican-3-specific CD4+ T-cells a significantly broader array of response patterns with 45% manifesting more than 1 cytokine and/or degranulation function % (Figs. 3E black bars and G). The polarization of $CD8^+$ T-cell responses was antigen-specific and not global as CEF-expanded $CD8^+$ T-cells exhibited a predominant IFNγ response (Fig. 3F) that was most often multicytokine and associated with degranulation (CD107a data not shown).

3.5. Proliferation of glypican-3-specific $CD8^+$ T-cells in vitro was not associated with restoration of cytokine responses in HCC patients

In a subset of patients in whom CFSE-labeling was utilized, as shown with 4 representative patients in Fig. 4A, glypican-3 peptide expansion in the presence of IL-2 led to modest T-cell proliferation (dilution of CFSE), but proliferating CD8+ T-cells did not develop the capacity to secrete cytokine or degranulate; by contrast, peptide-specific responses were found exclusively in mono-functional TNFα-producing non-proliferating cells. While these 15mer peptides efficiently bind to class I MHC molecules (Supplemental Fig. 1), to exclude the possibility that $CD8^+$ T-cell polarization was solely attributable to the use of 15mer

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rather than 9–10mer optimal peptides, we utilized 9–10mer optimal peptides to expand glypican-3-specific CD8+ T-cells in HLA-A2$^+$ patients and tracked responder cell function by pentamer staining. As shown in Fig. 4B for one representative patient, despite readily detectable expansion of $GPC3_{44-52}$ CD8+ T-cells after 10 days of *in vitro* expansion, IFNγ secretion by expanded cells restimulated with cognate antigens was minimal-to-absent, particularly when compared to influenza-specific controls. Furthermore, these glypican-3-specific CD8$^+$ T-cells did not produce detectable amounts of IFNγ or TNFα by intracellular staining (data not shown). In total, while modest expansion of glypican-3-specific CD8$^+$ T-cells may occur with stimulation by 15mer peptides in approximately half of HCC patients, despite the activation of CD4$^+$ T-cell type 1 response, expanded CD8+ T-cells do not gain multifunctional effector capacity. CD8$^+$ T-cell cytokine responses that are detected are produced by non-dividing CD8$^+$ cells constrained to produce TNFα.

3.6. PD-L1 and CTLA-4 blockade do not significantly enhance the expansion or cytokine production of peripheral glypican-3-specific T-cells

Inhibitory co-stimulation via the PD-1 and CTLA-4 receptors have been previously been shown to be important in suppressing hepat it is virus-specific T-cell effector functions, and PD-1 expression by tumor antigen-specific T-cells may be important in limiting T-cell reactivity to HCC *in situ* [35,36]. Utilizing glypican-3-specific HLA-A2-restricted pentamers, we first confirmed the expression of PD-1 on peripheral glypican-3-specific CD8$^+$ T-cells, which was detectable *ex vivo* in 7/9 HCC patients tested ranging in frequency from 0.03 to 1.6% of CD8$^+$ T-cells (Table 3). As shown in Supplemental Fig. 2, PD-1 expression was significantly greater in pentamer-positive versus pentamer-negative CD8$^+$ T-cells for the $GPC3_{44-52}$ ($p = 0.013$) and $GPC3_{563-572}$ (strong trend $p<0.07$) epitopes. By contrast, CTLA-4 expression for GPC3-specific or control CD8+ T-cells was not strongly upregulated (Supplemental Fig. 3). Based on these data, we expanded CFSE-labeled T-cells from HCC patients for 7 days *in vitro* in the presence glypican-3 peptides, rhIL-2, with either control Ig, anti-PD-L1 mAb, anti-CTLA-4 mAb, or combined PD-L1/CTLA-4 blockade to determine the effect of inhibitory co-stimulation blockade on glypican-3-specific T-cell responses. As shown in Figs. 5A–B, PD-L1 and CTLA-4 had no effect on glypican-3-specific IFNγ$^+$ T-cell responses by Elispot. By contrast, surviving-specific and HCV NS3 responses were augmented by PD-L1. Dual blockade in 12 patients showed no suggestion of effect (data not shown). With PD-L1 and CTLA-4 blockade, 2/7 and 1/7 patients respectively showed significant increases in CD8$^+$ T-cell proliferation (Fig. 6A). However, when assessing mean peptide-induced proliferative and cytokine responses there was no significant difference in CD8$^+$ T-cell proliferation, TNFα production, or IFNγ production under blockade conditions. By contrast, CTLA-4 blockade produced a trend towards a small median increase in CD4$^+$ T-cell IFNγ and TNFα production while PD-L1 blockade also led to trend in CD4$^+$ TNFα production (Fig. 6B). In one patient in which inhibitory receptor blockade could be combined with pentamer analysis, PD-L1 blockade doubled the frequency of $GPC3_{44-52}$- and $GPC3_{136-145}$-specific CD8$^+$ T-cells after 7 days of expansion; however cytokine production appeared only minimally affected similar to findings with other HCC antigens [12]. In aggregate, these data suggest that PD-1 and CTLA-4 blockade might modestly increase the proliferation of a subset of tumor antigen-

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specific T-cells in a minority of HCC patients but that co-stimulation blockade does not effectively restore effector function to peripheral tumor-specific $CD8^+$ T-cells.

4. Discussion

Among tumor antigens that have been considered as immunotherapeutic targets for hepatocellular carcinoma, glypican-3 has several attractive properties including its high tumor-to-background expression, relatively low extra-hepatic expression, and potential for serological detection. This study was performed to determine the frequency and functional capacity of circulating glypican-3-specific $CD4^+$ and $CD8^+$ T-cells in an HLA-diverse HCC population with underlying cirrhosis, the population most likely to be considered for a therapeutic vaccine. Not unexpectedly, we were unable to detect IFNγ+ T-cells specific for glypican-3 (as well as those specific for survivin, another tumor antigen relevant to HCC) *ex vivo* using 15mer overlapping peptide libraries and peptide matrices. Interestingly, a small minority of HCC patients did have T-cell responses against recombinant glypican-3, likely derived from $CD4^+$ T-cells. Consistent with previous studies that utilized optimal HCC-related HLA-restricted epitopes *in vitro* [23,37–39], we were able to expand glypican-3-specific T-cells from approximately half of HCC patients, particularly in patients with evidence of higher serum antigen levels suggesting significant tumor-induced priming. Expansion using the 15mer peptide library approach in an HLA-independent manner generated both $CD4^+$ and $CD8^+$ glypican-3-specific T-cells. Glypican-3-specific $CD4^+$ T-cells retained or gained multiple effector functions during expansion. By contrast, expanded glypican-3-specific $CD8^+$ T-cells failed to degranulate or to produce IFNγ when restimulated and were functionally limited to the production of TNFα. This $CD8^+$ T-cell dysfunction could not be overcome by inhibition of PD-1 and/or CTLA-4 signaling. Thus, despite the presence of Th1-like $CD4^+$ T-cell response, tumor-specific CD8+ T-cells in HCC patients manifest a deeply exhausted or anergized phenotype that could not be reversed simply with inhibitory co-stimulation blockade.

To date, clinical studies using class I peptide-based tumor vaccination for HCC have yet to show convincing, sustained clinical benefit [37,40] while non-specific cytokine-activated lymphocytes [14,41] and/or stimulation with cell lysate-loaded APCs [15] after resection or ablation of tumors has demonstrated some evidence of clinical efficacy. While the lack of benefit of peptide-based approaches could be due to selection of advanced patients unlikely to benefit from inclusion, our data, similar to findings identified utilizing NY-ESO-$1b_{157–165}$-specific $CD8^+$ T-cells [42] and panels of HLA-A2-restricted tumor antigens [12], suggest that peptide-expanded $CD8^+$ T-cells often remain functionally impaired. Such dysfunction could be due to aberrant liver-specific priming of $CD8^+$ T-cells [43] or tumor-induced upregulation of multiple inhibitory receptors on these T-cells to inhibit TCR-induced activation (reviewed in [44]).

Blockade of inhibitory co-stimulation pathways is an emerging approach to overcome tumor-specific $CD8^+$ T-cell dysfunction for the treatment of solid tumors [45,46]. Previous work from our and other institutions strongly implicates the role of inhibitory co-stimulation by PD-1 and CTLA-4 in the suppression of $CD8^+$ T-cells in chronic hepatitis C [8], the underlying etiology for HCC in 32/33 patients we studied. The expression of PD-L1, an

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inducible ligand for PD-1, has been to shown to impart a poor prognosis in HCC [35]. PD-L1 is selectively upregulated on malignant hepatocytes and/or tumor-associated Kupffer cells as a result of T-cell-derived IFNγ or tumor-related IL-10, and reversibly inhibits effector PD-1$^+$ CD8$^+$ T-cell proliferation in some studies [36,47]. By contrast, we detected only modest expression of PD-1 on tumor antigen-specific peripheral blood CD8$^+$ T-cells in HCC patients possibly reflecting compartmentalization of PD-1^{hi} activated T-cells to the target environment, a finding suggested by other investigators using different HCC-associated tumor antigen-derived epitopes [12]. While peripheral CD8$^+$ T-cells specific for a subset of glypican-3 epitopes do express elevated levels of PD-1 (but not CTLA-4 and not specifically LAG-3), functional rescue *in vitro* by PD-L1 blockade did not markedly improve T-cell proliferation, cytokine production or degranulation both in assays using specific HLA-A2 epitopes and the 15mer peptide library. These data contrast with HCV-specific responses which in this and previous work [8] were augmented by PD-L1 blockade. While survivin-specific T-cell IFNγ responses were modestly increased by PD-L1 blockade, overall these data suggest that inhibitory co-stimulation blockade is unlikely to potently augment peripheral T-cell vaccination responses in HCC. However, studies on PBMC cannot be assumed to wholly reflect the impact of interventions within a tumor. Indeed, recent data suggest that PD-L1 blockade *in vivo* may stimulate effector T-cells in an indirect manner via reduction of the generation of intratumoral Tregs [48]. Thus, our findings would support the investigation of both direct and indirect effects of PD-1 blockade when such therapies are tested in patients.

The expansion and characterization of glypican-3-specific CD4$^+$ T-cells are novel findings that, along with data relating to other tumor antigen-specific CD4$^+$ T-cells in HCC, further highlights a population of cells that might be an important target for immune augmentation. The potential role for CD4$^+$ T-cells to exert a cytolytic effect on HCC has been suggested in both human and murine studies [49,50]. The ability to detect CD4$^+$ T-cells specific for NY-ESO-1 *ex vivo* has been described in a small number of HCC patients in one study [51]. The detection of AFP-specific CD4+ T-cells has generally required short-term *in vitro* expansion [27,28,52], resulting in the expansion of either effector [27,28,52] or TGFβ+ regulatory T-cells [53]. We were able to detect responses to recombinant glypican-3 by Elispot *ex vivo* in a small number of HCC patients, but no early fibrosis or cirrhotic patients, suggesting tumor-induced priming. Half of normal donors in our study had detectable *ex vivo* CD4$^+$ T-cell responses yet these did not translate into *in vitro* expansion of glypican-3-specific CD4$^+$ T-cells, also supporting the concept that tumor-priming is critical for establishing an expandable population of CD4+ T-cells [27]. Further support for this concept comes from our finding that the presence of serum antigen correlated strongly with the expansion of glypican-3-specific CD4$^+$ T-cells, as has been suggested by some [28] but not all [52] studies with AFP. The recent study from Witkowski et al. [28] demonstrates that the infrequent detection of HCC-reactive CD4+ T-cells *ex vivo* is not likely the result of compartmentalization of these cells into the tumors, nor was it likely due to circulation of dysfunctional CD4$^+$ T-cells, but rather due to low circulating precursor frequencies of potential effector cells. While we did not specifically assess TGFβ secretion [53] or a regulatory phenotype in expanded glypican-3-specific CD4+ T-cells, it is unlikely that the peripheral CD4$^+$ T-cells were regulatory given the T_h1-like functional profiles. In

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unpublished observations, we have found that roughly one-third of intratumoral CD4+ T-cells expressed foxp3 and CD25, and that TIL can suppress of glypican-3-specific IFNγ production among TIL and LIL possibly mediated by antigen-specific IL-10 suggesting possible antigen-specific Treg accumulation within the tumors. While the expansion of tumor-reactive peripheral $CD4^+$ T-cells in tumor-bearing patients might support the use of whole-antigen $CD4^+$ T-cell-directed components for therapeutic vaccination, the lack of generation of multifunctional $CD8^+$ T-cells despite the presence of significant $CD4^+$ T-cell responses in our system argues that CD4-directed approaches to increase “help” may not sufficiently help highly dysfunction $CD8^+$ T-cells to regain multi-effector function.

5. Conclusion

We have established that 15mer peptide libraries can expand $CD4^+$ and $CD8^+$ T-cells specific for glypican-3 and survivin in patients with hepatocellular carcinoma in an HLA-nonspecific manner. The $CD4^+$ T-cells retained a broad array of cytokine-secreting capacity, but glypican-3-reactive $CD8^+$ T-cells were functionally constrained suggesting a differentiated or exhausted state. However, this exhausted state could not be reversed by inhibitory co-stimulation blockade *in vitro* unlike the exhausted state of virus-specific T-cells in chronic viral hepatitis. Whole protein or peptide library-based stimulation of tumor antigen-specific T-cells may be able to generate effector $CD4^+$ T-cell responses in a large proportion of hepatocellular carcinoma patients, but further studies will be necessary to define optimal conditions, including the need for concomitant ablation of the tumor microenvironment, for the concomitant expansion of multifunctional effector $CD8^+$ T-cells to increase the likelihood of clinical efficacy.

Abbreviations

CIR	cirrhotic group
CTLA-4	cytotoxic T-lymphocyte antigen-4
EVH	early viral hepatitis
GPC3	glypican-3
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HD	healthy donor
IFN	interferon
LAG-3	lymphocyte-activation gene 3
PBMC	peripheral blood mononuclear cells
PD-1	programmed death-1
PD-L1	programmed death ligand-1
PHA	phytohemagglutinin

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SD	standard deviation
SFU	spot-forming unit
Th1	type 1 helper T-cell response
Tc1	type 1 effector T-cell response

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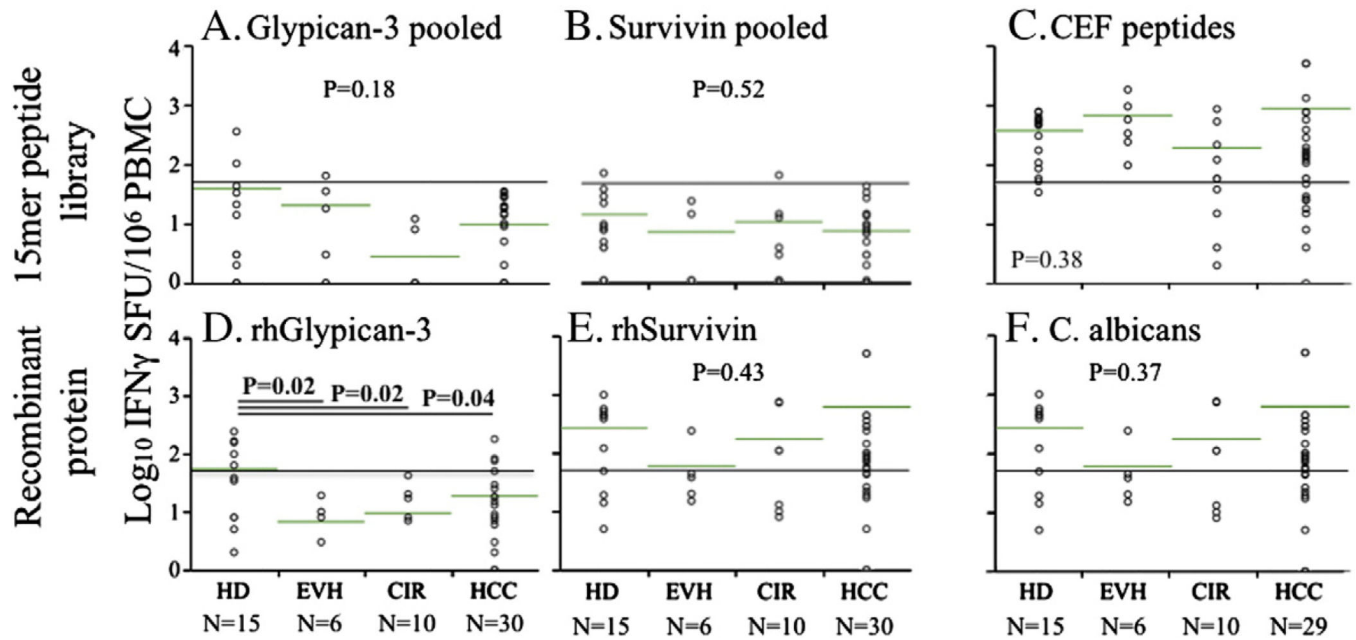

Figure 1.

T-cell reactivity to glypican-3 and control antigens *ex vivo* in HCC patients and controls. IFNγ SFU/10^6 PBMC in response to glypican-3 15mer peptides (A), survivin 15mer peptides (B), CEF 15mer peptides (C), recombinant human glypican-3 (D), recombinant human survivin (E), and *Candida albicans* (F) are shown for healthy donors, non-cirrhotic viral hepatitis, non-tumor-bearing cirrhotics, and HCC patients. Reference line indicates 50 SFU/10^6 PBMC and green bars show log-normalized mean values. P-values shown from ANOVA on log-normalized data.

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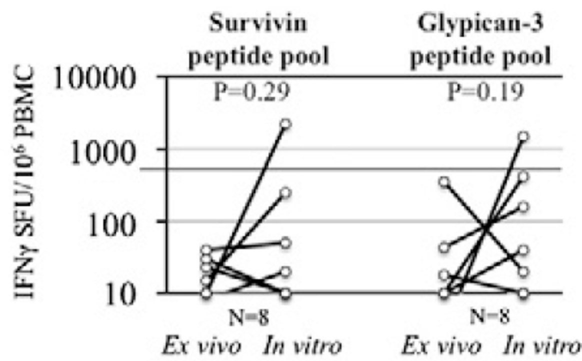

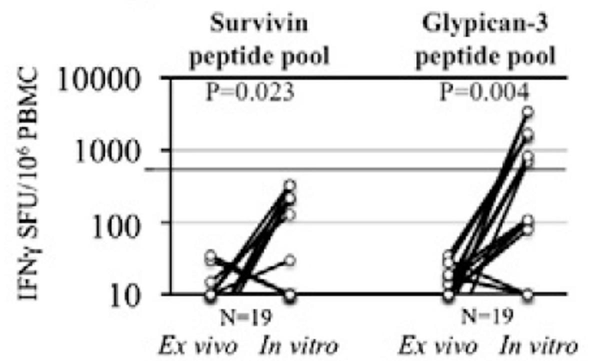

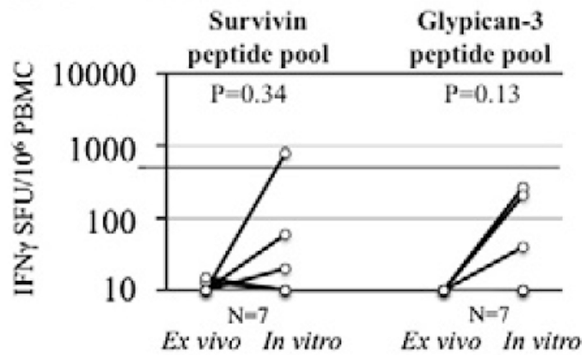

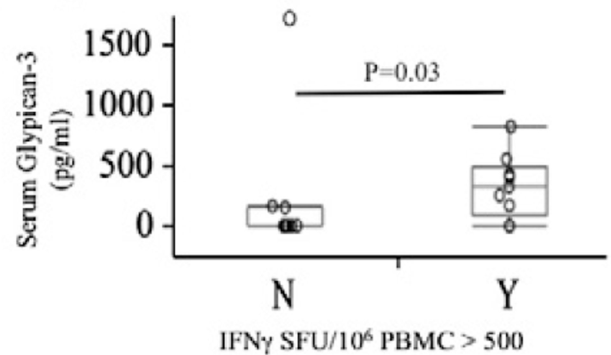

Figure 2.

Expansion of IFNγ^{+} T-cells with short-term *in vitro* expansion by Elispot. PBMC were stimulated with 1 mg/ml survivin or glypican-3 5mer peptide pools for 8 days with rhIL-2 100U/ml added on days 2 and 5. T-cell lines were re-stimulated with peptide pools in Elispot assays. Background was subtracted and results normalized to SFU per 10^6 PBMC. Comparison of *ex vivo* and *in vitro* 15mer peptide responses for non-cirrhotic patients (A), cirrhotic patients (B), and hepatocellular carcinoma patients (C). P-values were obtained by matched pair analyses. (D). Comparison of serum glypican-3 levels in patients with and without IFNγ^{+} glypican-3-specific T-cells greater than 500 SFU/10^6 PBMC after expansion.

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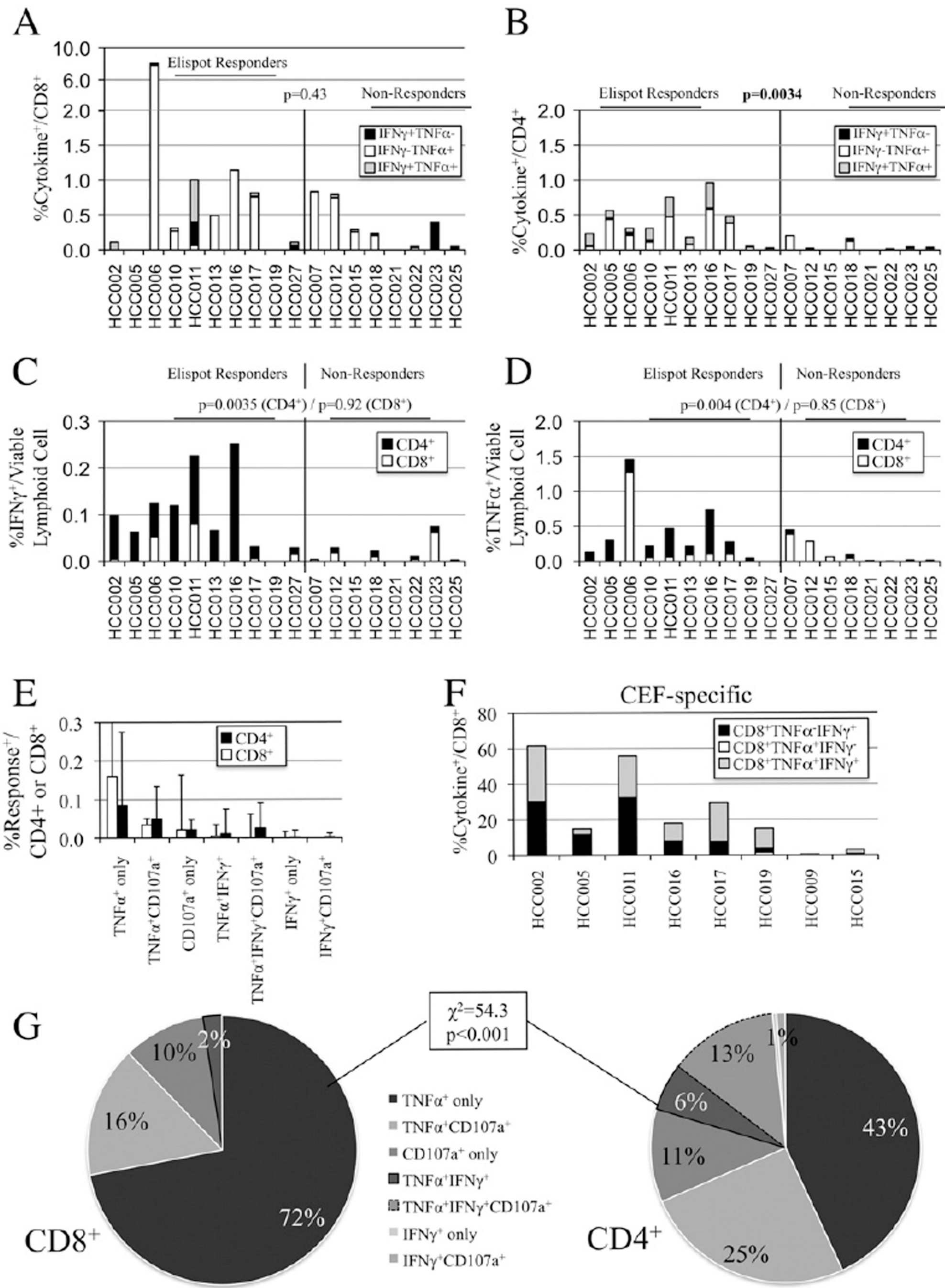

Figure 3.

Cytokine and degranulation profile of 15mer peptide short-term *in vitro*-expanded T-cells. A. Stacked column chart comparing median increase in glypican-3-specific TNFα- and/or IFNγ-secreting CD8+ T-cells in restimulated (subtracting derived from unrestimulated control wells) for patients with (left) and without (right) detectable IFNγ Elispot response. B. Stacked column chart comparing median increase in glypican-3-specific TNFα- and/or IFNγ-secreting CD4+ T-cells in restimulated for patients with (left) and without (right) detectable IFNγ Elispot response. C. Per lymphoid analysis demonstrating relative

contribution of CD4+ (black bars) and CD8+ (white bars) T-cells to IFNγ response. D. Per lymphoid analysis demonstrating relative contribution of CD4+ (black bars) and CD8+ (white bars) T-cells to TNFα response. E. Frequency per subset of CD4+ and CD8+ T-cell TNFα, IFNγ, and CD107a responses against glypican-3. F. Stacked column chart comparing median increase in CEF-specific TNFα- and/or IFNγ-secreting CD8+ T-cells under identical expansion conditions. G. Distribution of CD8+ (left) and CD4+ (right) T-cell TNFα, IFNγ, and CD107a responses against glypican-3.

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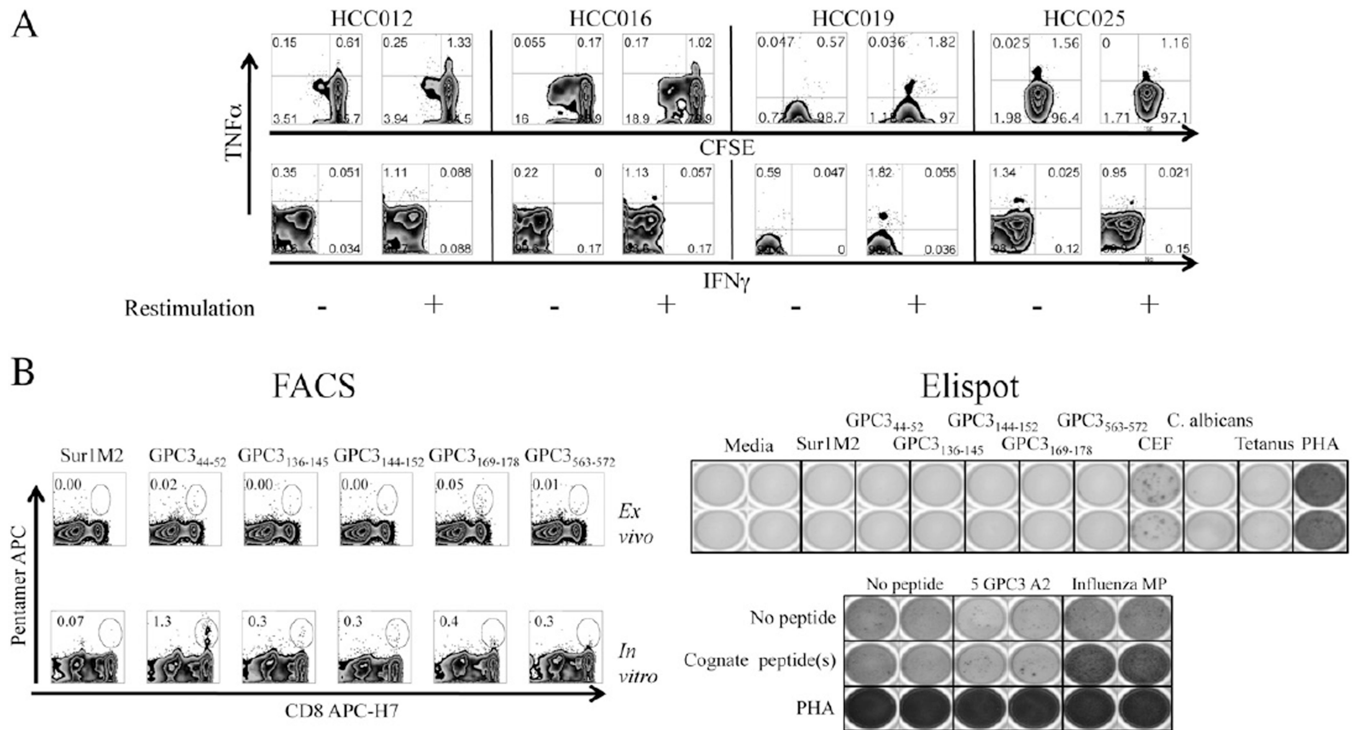

Figure 4.

Cytokine and degranulation profile of 15mer peptide short-term *in vitro*-expanded T-cells. A. Representative intracellular cytokine FACS plots showing CFSE dilution versus TNFα (upper) and TNFα vs IFNγ (lower) for four HCC patients with glypican-3-specific TNFα responses after 1 week of *in vitro* expansion using 15mer pooled peptides. B. Example in which PBMC from HLA-A2+ HCC patient were expanded with 9–10mer optimal peptides. Pentamer frequency *ex vivo* (top left) and after expansion *in vitro* (bottom left) for Sur1M2 and 5 GPC3 pentamers are shown. *Ex vivo* IFNγ Elispot (top right) shows no IFNγ^{+} responses against any glypican-3 peptide with lack of increase in IFNγ production by Elispot after restimulation with cognate antigen (bottom right).

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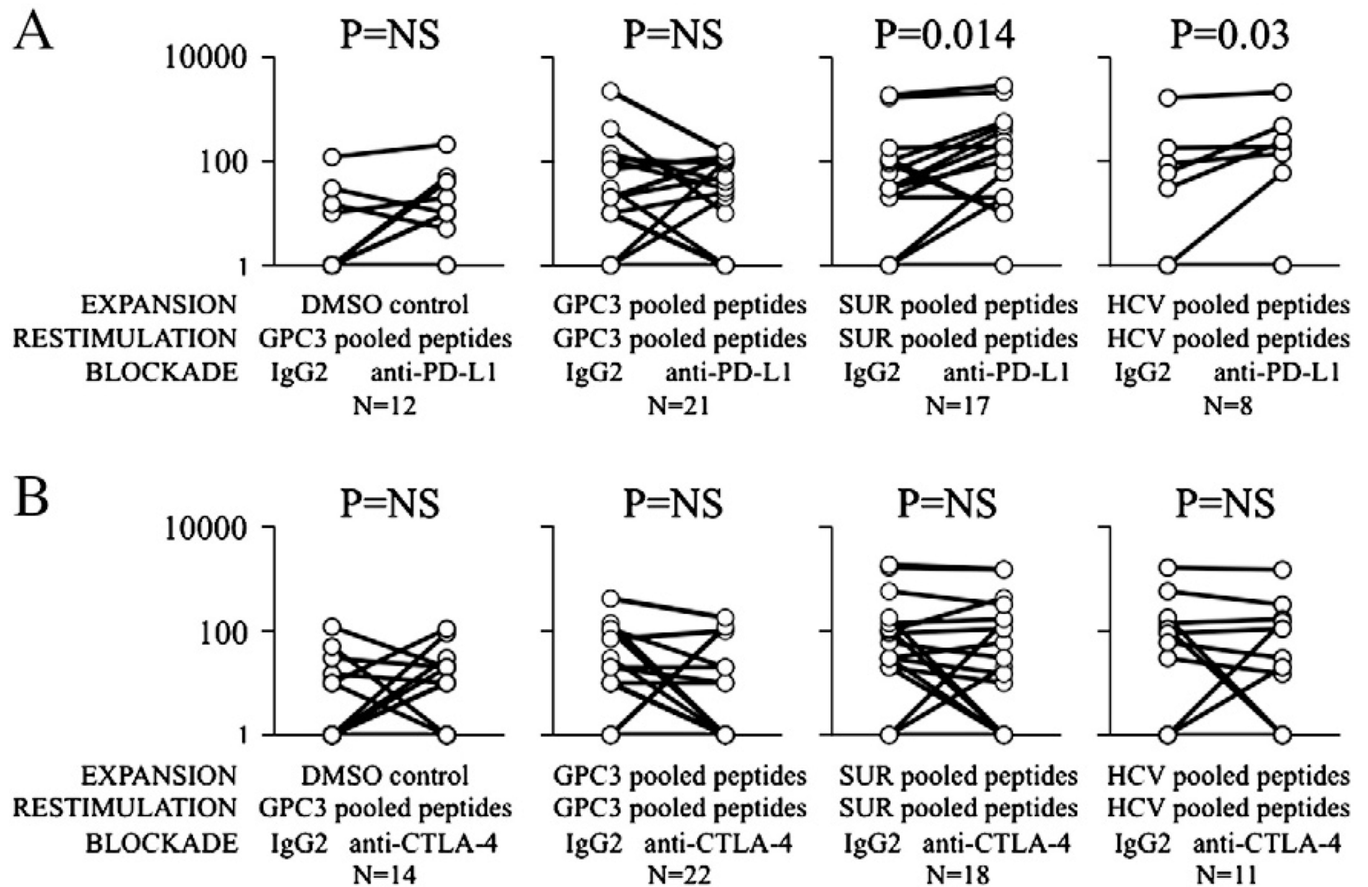

Figure 5.

Impact of PD-L1 and CTLA-4 blockade on total T-cell IFNγ production by Elispot. A. IFNγ response (SFU/10^6 PBMC) after 1 week of *in vitro* expansion with either DMSO control, glypican-3 15mer peptides, survivin 15mer peptides, or HCV NS3 15mer peptides in the presence of either controlIgor anti-PD-L1 mAb. P-value shown for matched pair analysis onlog-normalized data. B. IFNγ response after *in vitro* expansion with either DMSO control, glypican-3 15mer pooled peptides, survivin 15mer peptides, or HCV NS3 15mer pooled peptides in the presence of either control Ig or anti-CTLA-4 mAb.

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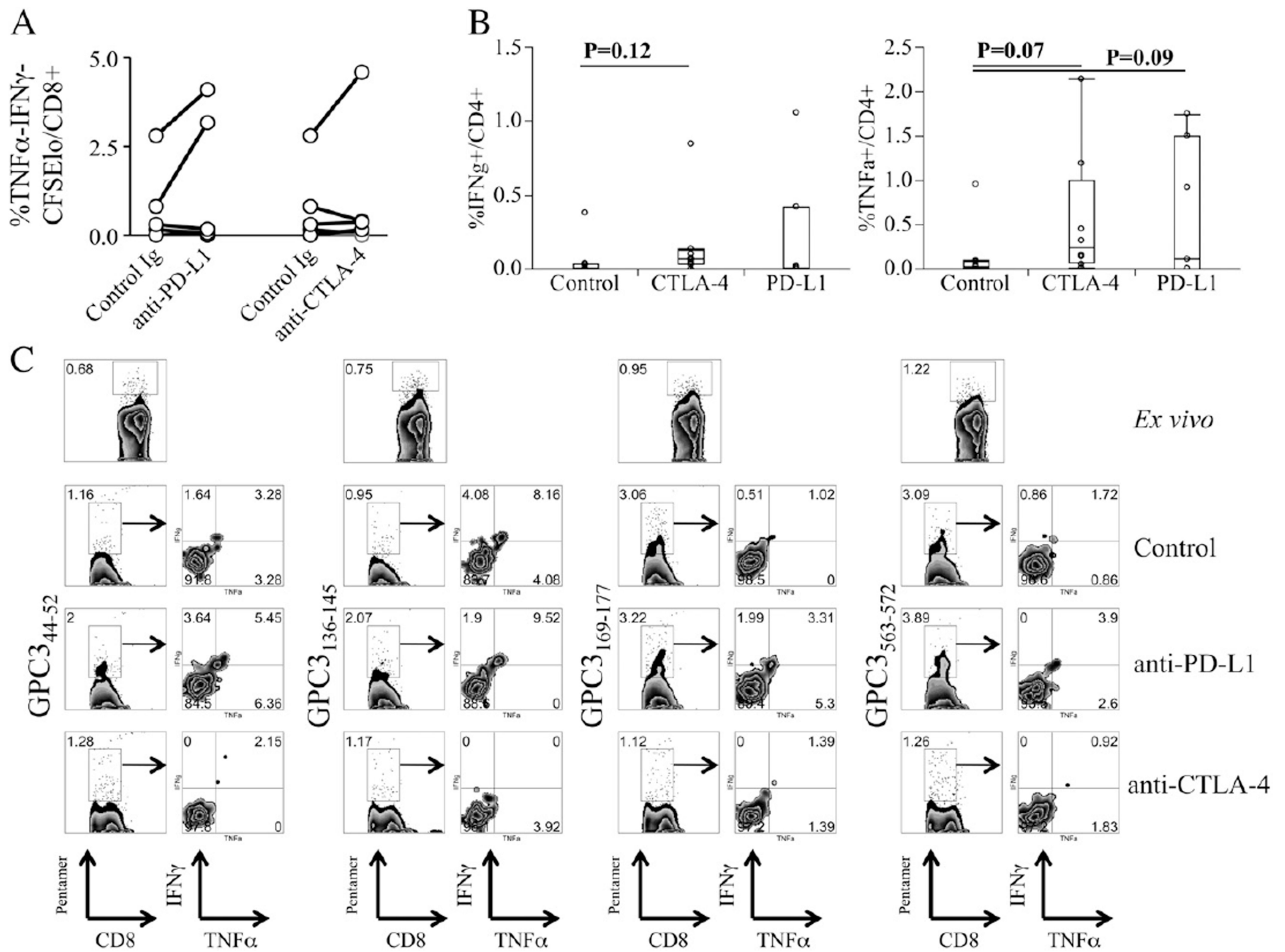

Figure 6.

Impact of PD-L1 and CTLA-4 blockade on CD8 T-cell proliferation and cytokine production by intracellular staining. A. The frequency of proliferated (CFSE^{lo}) TNFα^{-}IFNγ^{-} per CD8^{+} T-cell in presence of control Ig versus anti-PD-L1 and anti-CTLA-4 mAbs for 7 individual patients. B. Percentage of IFNγ^{+} (left) and TNFα^{+} (right) T-cells per CD4+ cells in presence of control Ig versus anti-PD-L1 and anti-CTLA-4 mAbs (p by matched pair analysis). C. Example of effect on expansion and cytokine production of pentamer-specific CD8^{+} T-cells in presence of control Ig, anti-PD-L1 and anti-CTLA-4 mAbs for four glypican-3 pentamers.

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Table 1

Baseline characteristics of hepatocellular carcinoma patients.

Patient ID	Age	Eth	Gnd	Underlying liver disease	Biopsy confirmed?	HLA-A02	Serum AFP mg/dI	Serum glypican-3 pg/mi	BCLC stage
HCCOO1	57	W	M	HCV/EtOH	+	+	58,700	219.2	B
HCCOO2	57	B	M	HCV/EtOH	+	+	13	<50	A
HCCOO3	54	B	M	HCV/EtOH	–	–	1720	<50	A
HCCOO4	60	W	M	HCV/EtOH	+	–	23,700	1724.4	B
HCCOO5	60	B	M	HCV/EtOH	–	+	12,600	820.7	B
HCCOO6	49	B	M	HCV/EtOH	–	–	58	406.0	A
HCCOO7	62	B	M	HCV/EtOH	–	–	421	1012.8	B
HCCOO8	58	B	M	HCV/EtOH	–	–	94	213.5	A
HCCOO9	61	W	M	HCV/EtOH	–	–	430	442.8	B
HCCO1O	53	B	M	HCV/EtOH	–	–	70	327.4	A
HCCO11	63	B	M	HCV/EtOH	+	+	5	169.8	B
HCCO12	61	W	M	HCV	–	–	3	<50	A
HCCO13	58	B	M	HCV/EtOH	–	–	17	<50	A
HCCO14	58	B	M	HCV	–	–	7230	253.2	B
HCCO15	57	B	M	HCV/EtOH	+	–	24	206.0	B
HCCO16	56	B	M	HCV/EtOH	–	–	4	310.3	A
HCCO17	55	B	M	HCV	–	+	9	<50	A
HCCO18	52	B	M	HCV	–	–	118	163.6	A
HCCO19	50	B	M	HCV/EtOH	–	+	1240	431.5	C
HCCO21	59	B	M	HCV/EtOH	+	+	21,000	<50	B
HCCO22	59	B	M	HCV	–	–	50	<50	B
HCCO23	57	W	M	HCV/EtOH	+	+	1130	152.9	B
HCCO24	67	W	M	HCV	–	+	12	<50	A
HCCO25	59	W	M	NAFLD	+	–	82	<50	A
HCCO26	56	B	M	HCV/EtOH	–	–	1150	<50	B
HCCO27	57	W	M	HCV	–	–	12	556.7	A
HCCO28	56	B	M	HCV/EtOH	–	+	46	115.4	A
HCCO29	58	B	M	HCV/EtOH	–	–	14	76.0	D

Patient ID	Age	Eth	Gnd	Underlying liver disease	Biopsy confirmed?	HLA-A02	Serum AFP mg/dI	Serum glypican-3 pg/mi	BCLC stage
HCCO3O	57	W	M	HCV/EtOH	–	–	9	<50	A
HCCO31	62	B	M	HCV/EtOH	–	–	2110	68.2	C
HCCO32	59	W	M	HCV/EtOH	–	–	53	850.1	B
HCCO33	54	W	M	HCV/EtOH	–	+	119	3040	C
HCCO34	56	W	M	HCV/EtOH	–	+	18	328.0	A

Ethnicity: W (White), B (Black).

Underlying liver diseases: HCV (hepatitis C). EtOH (alcoholic liver disease), NAFLD (non-alcoholic liver disease). HBV (hepatitis B).

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Table 2

Demographics of hepatocellular carcinoma patients and controls.

	HCC	CIR	EVH	HD	p
N	33	10	6	15	
Age (Median [Range])	57 [49–67]	55 [45–69]	56 [44–64]	49 [25–60]	0.0026[1]
Gender (M/F)	33/0	10/0	6/0	10/5	0.0011[2]
Ethnicity (W/B/H)	12/20/1	5/5/0	2/4/0	10/4/1	0.2
Hepatitis C infected (N [%])	31 [94%]	10 [100%]	6 (100%]	0 [0%]	

[1] HCC vs. HD p = 0.0001; HCC vs. CIR p = 0.049; and HCC vs. EVH p = 0.11.

[2] Difference entirely related HD group.

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Table 3

Frequency of $CD8^+$ pentamer T-cells in nine HLA-Ar hepatocellular carcinoma patients.

Pentamer	No. of patients	No. with pentamer+ cluster (%)	Median pentamer+/ CD8+ cells (range)
1 SurlM2	9	1 (11)	0.20 (0.20–0.20)
2 GPC3 44–52	9	3 (33)	0.20 (0.10–0.30)
3 GPC3 136–145	8	4 (50)	0.70 (0.05–1.30)
4 GPC3 144–152	9	2 (22)	0.47 (0.03–0.90)
5 GPC3 169–178	8	5 (63)	1.00 (0.20–1.60)
6 GPC3 563–572	9	4 (44)	0.45 (0.05–1.60)
7 HTLV-I	7	1 (14)	1.00 (0.10–1.00)
8 Flu matrix	9	5 (56)	0.40 (0.30–1.30)
9 NS3 1073	7	2 (29)	0.07 (0.03–0.10)