**Title:** Malignant liver cancers have distinct myeloid-derived suppressor cell signatures

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**Abbreviations**

| | |
|---|---|
| AFP | Alpha-fetoprotein |
| AST | Aspartate aminotransferase |
| ALT | Alanine transaminase |
| APC | Antigen-presenting cells |

| | |
|---|---|
| CCA | Cholangiocarcinoma |
| CEA | Carcinoembryonic antigen |
| CRLM | Colorectal liver metastases |
| DC | Dendritic cell |
| gMDSC | Granulocytic myeloid-derived suppressor cells |
| HCC | Hepatocellular carcinoma |
| MDSC | Myeloid-derived suppressor cells |
| mMDSC | Monocytic myeloid-derived suppressor cells |
| NET | Neuroendocrine tumor |
| PBMC | Peripheral blood mononuclear cells |
| TCGA | The Cancer Genome Atlas |
| Treg | T regulatory cells |
| WBC | White blood cells |

**Abstract**

Myeloid-derived suppressor cells (MDSCs) are a heterogenous population of immunosuppressive cells that limit immune response in patients with cancer. It has been reported that circulating MDSC frequencies are increased in patients with viral-derived hepatocellular carcinoma (HCC). In this study, we compared the MDSC frequency of 100 patients with primary hepatobiliary malignancies or metastatic liver lesions. Non-viral HCC patients and patients with neuroendocrine tumors (NET) had the highest level of MDSCs ($CD33^{+}CD11b^{+}HLA\text{-}DR^{low/-}$), followed by colorectal carcinoma patients with liver metastases (CRLM). In contrast, MDSC levels were not significantly elevated in cholangiocarcinoma (CCA) cases. Another immunosuppressive cell population, regulatory T cells were not augmented in peripheral blood of patients with hepatobiliary malignancies or liver metastasis. Investigation of myeloid cell infiltration in HCC, NET and intrahepatic CCA tumors showed that the frequency of $CD33^{+}$ and $HLA\text{-}DR^{+}$ cells marking antigen-presenting cells were limited compared to benign lesions. Bioinformatic analysis of the Cancer Genome Atlas demonstrated that a high MDSC score in HCC patients predicted poor disease outcome. ***Conclusion:*** These data suggest that primary and metastatic hepatobiliary cancers have distinct immune activation status and MDSC signatures. Given our observation that MDSCs are increased in non-CCA malignant liver cancers, they may comprise suitable targets for effective immunotherapy approaches.

## Introduction

Primary hepatocellular carcinoma (HCC) is among the leading cause of cancer-related deaths in the U.S., with 40,000 new cases and 30,000 mortalities in 2018(1). Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, obesity, and excess alcohol consumption are risk factors associated with HCC(2). HCC resulting from chronic viral infections constitute 75% of all cases, while the non-viral causes account for the remaining 25%. Importantly, both viral and non-viral HCC involve inflammation of the liver and progress from cirrhosis(3). Secondary liver cancers, which originate by the metastatic spread of a primary tumor from a distant site, are more frequent than HCC(4). Although current treatment strategies including surgical resection, radiation, ablation, embolization, systemic/local chemotherapy infusion and liver transplantation have improved the outcome of patients with HCC and cholangiocarcinoma (CCA), liver cancers are diagnosed at advanced stages with poor clinical presentation and have high recurrence rates(5). Thus, there is an urgent need to gain mechanistic insight into the pathobiology of primary and metastatic liver cancers and develop more effective therapeutic opportunities.

Tumors employ multiple mechanisms to evade immune recognition and to establish an immunosuppressive microenvironment promoting their growth. Myeloid-derived suppressor cells (MDSCs) are a heterogenous population of immature myeloid cells that expand in patients with malignancies and infiltrate tumors, where they confine anti-tumor immune response by suppressing cytotoxic T cell and natural killer (NK) cell activity(6). Previous work demonstrated that high MDSC frequency in melanoma not only correlates with poor patient outcome but also associates with therapeutic irresponsiveness(6-9). Targeting MDSCs in multiple preclinical models including colorectal and liver cancers activated an anti-tumor immune response and reduced tumor growth, suggesting that modulating these cells is a promising strategy for cancer immunotherapy(10, 11). Several studies have reported that MDSCs are augmented in peripheral blood of HCC patients(12-15). However, the relevance of this observation in terms of other

hepatobiliary malignancies, including non-viral HCC, and patient clinical presentation has yet to be determined. Considering the range and pathobiological variation in primary and metastatic liver cancers, we hypothesized that MDSCs might have distinct associations with different types of hepatobiliary tumors. By screening peripheral blood samples and liver surgical specimens from patients with HCC, CCA, colorectal carcinoma with liver metastasis (CRLM) and neuroendocrine tumors (NET), we identified that MDSCs are elevated in most but not all liver cancers. Our findings demonstrate that MDSC augmentation is impacted by the type of liver tumors, and support testing of MDSC targeting strategies in patients with HCC, CRLM and NET.

## Materials and Methods

### Reagents

Fluorophore-conjugated anti-human CD3 (clone UCHT1), CD4 (clone SK3), CD8 (clone RPA-T8), CD14 (clone MφP9), CD15 (clone HI98), CD25 (clone M-A251), CD33 (clone WM53), CD107a (H4A3), CD127 (clone HIL-7R-M21) and HLA-DR (clone G46-6) antibodies were purchased from BD Biosciences. CD11b (clone CD11B29) antibody and LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit, for UV excitation was obtained from ThermoFisher Scientific.

### Patients

Peripheral blood samples were collected perioperatively from 100 patients with primary liver lesions or liver metastases in the Cleveland Clinic. All patients provided written informed consent under IRB 10-347 and the protocol was approved by the Cleveland Clinic Institutional Review Board. Only non-viral HCC specimens were included to the study and all the diagnoses were confirmed by the Cleveland Clinic Pathology Department. Patient information is provided in **Table 1**.

### Quantification of MDSC in peripheral blood

Peripheral blood samples were processed using a Ficoll-Paque PLUS gradient in a SepMateTM (Stem Cell Technologies) and frozen in 10% dimethyl sulfoxide (Fisher Scientific) and 90% fetal bovine serum (Gibco) until further analysis. Samples were stained with live/dead UV stain (Invitrogen) for 10 minutes on ice and blocked in FACS buffer (PBS, 2% BSA) containing an FcR blocking reagent at 1:50 (Miltenyi Biotec) on ice for 15 minutes. Samples were stained with fluorophore-conjugated antibody cocktails for 20 minutes on ice, washed with FACS buffer and analyzed using LSRFortessa (BD Biosciences). Analysis were performed using FlowJo software (Tree Star Inc.).

## Immunohistochemistry (IHC)

Liver biopsy specimens were available in 29 patients (6 HCC, 9 benign, 10 CCA and 4 NET) for IHC analysis. Formalin fixed paraffin-embedded blocks and H&E stained slides were retrieved and reviewed in these 29 cases. Additional IHC stains were performed on a selected representative block from each case. IHC was performed using the mouse monoclonal CD33 antibody (clone PWS44, Leica Biosystems) diluted 1:100 using SignalStain Diluent (Cell Signaling Technology) and incubated for 40 minutes at 37$^{o}$C. Automated staining used the VENTANA BenchMark Ultra platform, U iView DAB software, and was detected using iView DAB with the Endogenous Biotin Blocking Kit (Ventana Medical Systems Inc.). Antigen Retrieval used Cell Conditioning#1 selected for the Standard Time.

The mouse monoclonal HLA-DR/DP/DQ antibody (clone CR3/43, ThermoFisher) used with the VENTANA UltraView DAB detection kit, U ultraView DAB software, with the BenchMark Ultra automated stainer. Dilution was 1:100 using SignalStain diluent, and incubated for 40 minutes at 37$^{o}$C. Antigen Retrieval used Cell Conditioning#1 selected for the Standard Time. Mouse antibody amplification was selected. Normal lymph node tissue was used for positive run controls. For CD68 staining, samples were incubated with pre-diluted CD68 antibody (clone KP-1, Ventana) for 16 minutes at 37$^{o}$C and analyzed with OptiView DAB detection kit. IHC staining conditions are summarized in **Supplemental Figure 1**.

HLA-DR positive cells were assessed quantitatively within the lesional tissue, at a 200X magnification on an Olympus B41 microscope. Similarly, CD33 and CD68 positive cells were counted at the periphery of the lesion (tumor-normal interface) and intralesionally at 200x. For all three markers, the peak count was considered.

## Bioinformatic Analysis of Immune Populations

The Cancer Genome Atlas (TCGA) Liver Cancer (LIHC) database was accessed via University of California Santa Cruz (UCSC) Xena Browser [https://xenabrowser.net/heatmap/] on March 21, 2018 and December 13, 2018. CD33, CD11b (ITGAM), HLA-DRA, CD4, CD25 (IL-2RA), FoxP3 and CD8a expression levels, patient demographic information, viral status and overall survival data were extracted for further analysis. To generate MDSC signature, only samples with CD33 and CD11b expression above the median values were used. From this set, samples with low HLA-DRA expression were categorized into "high MDSC signature" group. $CD33^{high}CD11b^{high}HLA\text{-}DRA^{high}$ samples were used for the control population. Treg signature was generated based on initial CD4 positive gating and subsequent high CD25 and FoxP3 expression levels. Samples with CD25 and FoxP3 levels below the median served as controls. Finally, $CD8a^{high}CD107a^{high}$ samples were considered to have high levels of activated CD8 T cells, while samples with $CD8a^{high}CD107a^{low}$ expression levels were classified as controls.

Statistical Analysis

Two-way ANOVA with Tukey correction, Student's t-test and long-rank test were used for analysis, and p-value less than 0.05 was considered statistically significant (GraphPad Software Inc. and JMP Pro and Version 14. SAS Institute Inc.).

## Results

### MDSCs are increased in peripheral blood of primary and metastatic liver tumor patients

MDSCs are bone marrow-derived immature immunosuppressive cells that accumulate in patients with malignancies(8, 16). In human, MDSCs are broadly categorized into monocytic (mMDSCs, $CD33^{+}CD11b^{+}HLA\text{-}DR^{-/low}CD14^{+}$) and granulocytic (gMDSC, $CD33^{+}CD11b^{+}HLA\text{-}DR^{-/low}CD14^{-}$) subsets, which differ in their suppressive capacity(6, 17). Previous studies demonstrated that the monocytic subset of MDSCs are increased in circulation of HCC patients and their levels are impacted by radiation therapy(12-15). To gain a more comprehensive understanding of the link between MDSCs and liver cancers, we analyzed MDSC frequency from peripheral blood mononuclear cells (PBMC) of 100 patients with diagnosis ranging from benign lesions, HCC, CRLM, NET to CCA. Characteristics of these patients are summarized in **Table 1**. HCC patients compared to patients with benign tumors were older (median age 71.5 vs 38, $p<0.0001$) and more likely to be male ($p<0.001$). These patients also presented elevated levels of liver enzymes (ALT 41.4 vs 25.1, $p=0.2$ and AST 46.2 vs 27.1, $p<0.05$). A similar trend in age and liver enzyme levels were observed in HCC patients compared to CRLM patients (median age 71.5 vs 55 $p<0.001$, ALT 41.4 vs 27.5, $p<0.05$ and AST 46.2 vs 28.6, $p<0.0001$). In contrast, there were no significant differences in clinical markers between patients diagnosed with HCC vs CCA or NET.

As previously reported(12-15), $CD33^{+}CD11b^{+}HLA\text{-}DR^{-/low}$ MDSC frequency was increased HCC patients compared to patient with benign liver lesions (**Fig 1B**). Although patients with NET and CRLM also had MDSC expansion in their circulation, CCA patients had significantly lower rates of peripheral MDSCs in comparison to HCC patients (**Fig 1B**). MDSC percentages in CCA patients were indistinguishable from those with benign liver lesions (**Fig 1B**). Prior studies suggest that mMDSCs are the main population in patients with liver cancers(13-15). Consistently, our findings demonstrated that $CD14^{+}CD15^{-}$ mMDSC subset constitute the majority of MDSCs in peripheral circulation, and that these cells are present at higher rates in patients with HCC

compared to those with CCA or benign lesions (**Fig 1C-D**). Regulatory T cell (Tregs) constitute another major immunosuppressive cell population frequently increases in patients with cancer that suppresses activity of anti-tumoral $CD8^+$ T cells in the tumor microenvironment(18). It has been established that MDSCs and Tregs cross-talk to induce the generation and recruitment of one another(19). We analyzed the frequency of Tregs and activated $CD8^+$ T cells to investigate whether these populations correlate with disease status. Neither Tregs nor activated $CD8^+$ T cells were significantly different among various types of primary and metastatic liver malignancies (**Fig 1E-F**). Together, these results indicate that MDSCs are linked to malignancy but CCA represent an exception with lower MDSC frequency.

To investigate whether MDSC frequency correlates with clinical parameters, our patient cohort was split into a MDSC high (n=23) and MDSC low (n=76) group based on the mean peripheral MDSC percentage (4.8%) in the HCC subgroup. There was no significant difference between AST (34.3 vs 35.6, p=0.81) and ALT (35 vs 32, p=0.69) between MDSC high versus low groups (**Supplemental Fig 1A**). However, within the HCC group, there was a significant correlation between MDSC percentages and ALT levels ($p<0.001$), indicating that MDSCs could be impacted by the severity of liver damage (**Supplemental Fig 1B**). Carcinoembryonic antigen (CEA) is a marker elevated in peripheral blood of patients with colorectal cancer and an indicator of liver metastases(20). To examine whether MDSC frequency is linked to disease severity we separately investigated CEA levels in CRLM patients with high (>4.8%) versus low (<4.8%) MDSC score. CRLM patients with more MDSCs also exhibited increased levels of CEA ($p<0.01$, **Supplemental Fig 1C**).

**Benign liver lesions are infiltrated by antigen-presenting cells (APCs)**

Under normal physiological conditions, mMDSCs are capable of differentiating in to antigen-presenting dendritic cells (DCs) and macrophages(6). This pathway has been hypothesized to be halted under pathophysiological conditions such as cancer, leading to accumulation of these cells

in host. Thus, we investigated whether enhanced MDSC frequency associated with APC infiltration of liver lesions by performing immunohistochemistry staining of pan-myeloid marker CD33, MHC Class II HLA-DR and macrophage marker CD68 in a subset of HCC, CCA, NET and benign cases. Malignant tumors exhibited a similar immune profile with no significant differences in the number of CD33, HLA-DR and CD68 positive cells in peak areas counted within the tumors (**Fig 2A-B**). Compared to HCC and CCA, benign lesions had higher numbers of intralesional HLA-DR or CD33 positive cells ($HLA\text{-}DR^+$ cells in HCC-CCA 36.5 vs 54.9 in benign lesions, $p<0.05$; $CD33^+$ cells in HCC-CCA 16.7 vs 39.2 in benign lesions, $p<0.05$; **Fig 2C**), representing APCs. $CD68^+$ cells corresponding to macrophages, including resident Kupffer cells, were not different between malignant and benign liver lesions (**Fig 2B-C**), suggesting that DCs constitute the majority of infiltrating APCs in benign cases. In addition, we analyzed whether the immunosuppressive tumor microenvironment prevents infiltration of myeloid cells leading to the accumulation of these cells at the periphery (tumor-normal interface). Intralesional versus peripheral specimens from the matching tumors had similar numbers of CD33 or CD68 positive cells, CCA cases being the exception (**Fig 2D**). These results suggest that HCC and NETs result in reduced myeloid cell accumulation in the tumor as well as the adjacent tissue.

**MDSC frequency correlates with HCC outcome**

Although HCC patients had significantly higher levels of MDSCs compared to patients with CCA or benign lesions, this patient subpopulation could further be clustered into MDSC high (>4.8%) versus MDSC low (<4.8%) group based on flow cytometry analysis (**Fig 1B**). Based on this variation at the level of individual patient immune response, we investigated whether MDSC frequency correlates with disease outcome using the TCGA dataset containing gene expression information from 440 HCC patients. We generated a high versus low MDSC score by focusing on high CD33 and CD11b expression and further creating subgroups based on the median levels of HLA-DR expression. Patients with high MDSC score ($CD33^{high}CD11b^{high}HLA\text{-}DR^{low}$) had

significantly reduced overall survival duration, compared to those with high CD33, CD11b and HLA-DRA expression levels (**Fig 3A**). Importantly, these survival differences were not dependent on tumor grade, viral status or biological sex as no significant difference for these parameters was detected between cohorts (*data not shown*). Using the same strategy, we generated signatures for immunosuppressive Tregs ($CD4^{high}CD25^{high}FoxP3^{high}$) and activated $CD8^+$ T cells ($CD8^{high}CD107a^{high}$). There was no survival difference in patients with high Treg signature compared to those with low Treg ($CD4^{high}$) score (**Fig 3B**). Similarly, activated $CD8^+$ T cell signature did not predict survival advantage over having more naïve $CD107a^{low}$ T cells in the tumor microenvironment (**Fig 3C**). Together, these observations suggest that MDSCs but not Tregs or activated $CD8^+$ T cells associate with HCC outcome.

## Discussion

MDSCs have been linked to suppression of anti-tumor immune response and are emerging targets of cancer immunotherapy. Previous studies have demonstrated that the frequency of MDSCs is increased in various malignancies, including HCC(12-16), and can impair therapeutic response(8, 9). Here, we demonstrated that different types of primary and metastatic liver malignancies have distinct MDSC profiles. Despite increased MDSCs in blood of patients with HCC, CRLM and NET compared to benign lesions, MDSC levels were lower in CCA. CCA can arise at any portion of the biliary epithelium and depending on the location the disease is broadly categorized as intrahepatic or extrahepatic. Although symptoms, prognosis and treatment opportunities of the individual subtypes exhibit variation(21); intrahepatic versus extrahepatic CCA had no difference in MDSC frequency (*data not shown)*. Thus, our results suggest that low MDSC rates in this cancer type was related to cellular origin and pathogenesis of the disease, rather than the location of the tumor.

MDSCs subsets differ in their suppressive activity, ability to infiltrate tumors and disease associations. A recent study with a breast cancer model demonstrated that mMDSCs can modulate the local tumor microenvironment by interacting with cancer stem cells, while gMDSCs promote distant lung metastasis(17). Thus, it is important to determine the individual contribution of MDSC subsets in the context of malignancies. Consistent with the previous observations(13-15), our results demonstrated that $CD14^{+}/CD15^{-}/HLA\text{-}DR^{-}$ cells representing the mMDSC subset constitute the majority of MDSCs detected in peripheral circulation of patients with primary or metastatic liver tumors. However, it is noteworthy that the frequency of gMDSCs could be affected by sample storage conditions(22) and further analysis is required to determine whether the frequency of this population is augmented in hepatobiliary malignancies.

One of the mechanisms that MDSCs can support immunosuppression is induction of Tregs(19). Kalathil et al. demonstrated that compared to healthy controls the frequency of $FoxP3^{+}CTLA4^{+}$

Tregs was increased in HCC patients(12). However, we did not observe a difference in the peripheral blood levels of $CD25^{+}CD127^{-}$ Tregs in patients with malignant liver tumors as opposed to those with benign lesions. While FoxP3 is widely used as a biomarker of Tregs, lack of CD127 expression more reliably marks activated Tregs(23). Although the observed difference between the two studies might be related to the choice of Treg staining, it is also probable that variations in data sets impacted the outcome. Notably, our patient cohort excludes any viral HCC cases. Both HBV and HCV infection has shown to recruit Tregs to restrain anti-viral immunity and inflammation-related liver damage(24, 25). Therefore, it is possible that viral HCC patients have more Tregs than non-viral HCC patients. While 75% of the HCC cases result from chronic HBV/HCV infection, non-viral HCC patients are diagnosed with higher tumor burden, pointing out the importance of considering HCC etiology(26). Furthermore, it is predicted that with the improvements in HBV vaccine and HCV drugs, non-viral HCC will constitute the majority of cases in the future(5). Importantly, both viral and non-viral HCC progression involves inflammation, which could be the common driver of MDSCs(3). Identifying variations in the immunological signature of viral versus non-viral HCC for efficient targeting of HCC subtypes represents a priority for future studies. MDSCs also support tumorigenesis by attenuating cytotoxic T lymphocyte activity. By using CD107a (LAMP-1) expression as a marker of effector activity(27), we investigated potential changes in activated T cells. As expected, the percentage of activated $CD8^{+}$ T cells were relatively low in circulation but with no apparent difference between disease types. However, the lack of inverse correlation between MDSCs and activated T cells levels in circulation may not predict the degree of anti-tumor immunity as studies have shown that tumor-infiltrating but not peripheral MDSCs actively suppress T cell function(7).

Studies in breast cancer and glioblastoma have demonstrated that circulating MDSC frequency is elevated with tumor grade(28, 29). In our HCC cohort, two patients with the highest MDSC frequency had grade 3 tumors but due to limited sample size further analysis could not be

performed (*data not shown*). Moreover, there was no correlation between MDSC percentages and other clinical parameters such as AST and AFP levels. This indicated that MDSC frequency is an independent predictor of malignancy and not confounded by clinical variables. However, the majority of the patients in the entire cohort had AST and ALT levels <40 IU/ml suggesting that they had minimum liver damage(30). Still, we identified a positive correlation between serum ALT levels and MDSC frequency in non-viral HCC patients, which implies that chronic inflammation and accompanying liver damage may be linked to MDSC augmentation. Furthermore, CEA levels were significantly higher in CRLM patients with above average MDSC percentages. Despite lack of strong association between CEA levels and tumor burden, patients with Grade III and Grade IV colon cancers have higher serum CEA concentrations(31).

Unlike gMDSCs, which are hypothesized to be terminally differentiated, mMDSCs can polarize into DCs and macrophages. However, this maturation pathway is impaired under pathophysiological conditions(32, 33). Since mMDSCs were the prevalent cell type in patients with liver cancer, we speculated that mMDSC-APC differentiation axis was hindered in malignant tumors leading to accumulation of the former population in tissues. Lower number of intralesional CD33 positive cells in malignant tumors pointed out to a potential reduction in myeloid infiltration. It is also possible that the CD33 staining intensity is biasing the results, as APCs express higher levels of CD33 compared to immature myeloid cells(34). In contrast, the decreased number of positive intralesional cells observed in HLA-DR stain in CCA and HCC with respect to the benign lesions may suggest a reduced ability to elicit an immune response in these tumors.

In line with this observation, HCC patients with high tissue APC score, which was generated based on high CD33, high CD11b and high HLA-DR expression in TCGA dataset, had survival advantage. One possible explanation of decreased CD33 and HLA-DR positive cells in malignant tumors is that ENTPD2/CD39L expression by tumor cells hinders MDSC maturation in liver tumors(35). Although this is yet to be confirmed in HCC patients, low MDSC frequencies in CCA

patients suggest that there might be alternative mechanisms impairing trafficking or maturation of APCs in this tumor type.

Collectively, our results identify a distinct immune signature presented as high circulating MDSC levels and low tumor-infiltrating APCs in non-viral HCC as opposed to low peripheral MDSCs and more intrahepatic APCs in benign lesions (**Fig 4**). Our findings make the case for including blood MDSC measurement as a diagnostic test for primary and metastatic hepatobiliary malignancies. The use of metronomic chemotherapy for depletion of MDSCs(10, 36-38) is currently under clinical evaluation for glioblastoma and non-small cell lung cancer [NCT02669173, NCT03302247]. This approach compromises a promising therapeutic opportunity for HCC, CRLM and NETs as well.

## Figure Legends

Figure 1. Frequency of MDSCs is increased in HCC, CRLM and NET patients. A) Representative dot plots depicting the gating strategy of different immune populations. Frequency of B) $CD33^+CD11b^+HLA\text{-}DR^{-/low}$ MDSC, C) $CD33^+CD11b^+HLA\text{-}DR^{-/low}CD14^+$ mMDSC, D) $CD33^+CD11b^+HLA\text{-}DR^{-/low}CD14^-$ gMDSC, E) $CD3^+CD4^+CD25^+CD127^-$ Treg, and F) $CD3^+CD8^+CD107a^+$ activated T cells in PBMC of CRLM (n=41), HCC (n=18), CCA (n=18), NET (n=5) and benign (n=18) cases were analyzed with flow cytometry. * p<.05; ** p<0.01; *** p<0.001 as determined by 2-way ANOVA.

Figure 2. HCC, NET and CCA tumors have similar myeloid cell infiltration pattern. A) Representative immunohistochemistry images demonstrating infiltration $HLA\text{-}DR^+$ APCs, $CD33^+$ myeloid cells and $CD68^+$ macrophages. Quantification of peak area count of intralesional HLA-DR, CD33 and CD68 staining B) from HCC (n=6), CCA (n=10) and NET (n=4) cases C) from malignant liver cancers (n=20) versus benign lesions (n=9). D) Comparison of intralesional versus peripheral CD33 and CD68 peak area count. Data represented as min-to-max and *<p=0.05 as determined individually for each marker by two-tailed t-test with unequal variance; **<p=0.01 as by paired two-tailed t-test.

Figure 3. High MDSC score predicts poor HCC prognosis. Kaplan-Meier curved depicting overall survival of HCC patients with high A) MDSC ($CD33^+CD11b^+HLA\text{-}DR^{-/low}$), B) Treg ($CD4^+CD25^+FoxP3^+$) and C) Activated T cell ($CD8a^+CD107a^+$) score. Data are from TCGA and were obtained from UCSC Xena Browser. p <0.05 was determined by Log-rank (Mantel-Cox) test.

Figure 4. Malignant liver cancers have more circulating MDSCs and less tumor-infiltrating APCs.

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## Author Contributions

DB, JMB, DSA, JDL and FNA conceptualized the study and provided financial support; DB, ES, GAR, CL, MO and DSA designed and performed the experiments, DB, AL, LAA-M, DJS and DSA analyzed the data; DB, AL and JDL wrote the manuscript; and all authors provided final approval of the manuscript.

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## References

1. National Cancer Institute. Cancer Stat Facts: Liver and Intrahepatic Bile Duct Cancer. In; 2018.
2. Gomaa AI, Khan SA, Toledano MB, Waked I, Taylor-Robinson SD. Hepatocellular carcinoma: epidemiology, risk factors and pathogenesis. World J Gastroenterol 2008;14:4300-4308.
3. Yu LX, Ling Y, Wang HY. Role of nonresolving inflammation in hepatocellular carcinoma development and progression. NPJ Precis Oncol 2018;2:6.
4. Ananthakrishnan A, Gogineni V, Saeian K. Epidemiology of primary and secondary liver cancers. Semin Intervent Radiol 2006;23:47-63.
5. Karin M. New insights into the pathogenesis and treatment of non-viral hepatocellular carcinoma: a balancing act between immunosuppression and immunosurveillance. Precis Clin Med 2018;1:21-28.
6. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9:162-174.
7. Kumar V, Patel S, Tcyganov E, Gabrilovich DI. The Nature of Myeloid-Derived Suppressor Cells in the Tumor Microenvironment. Trends Immunol 2016;37:208-220.
8. Montero AJ, Diaz-Montero CM, Kyriakopoulos CE, Bronte V, Mandruzzato S. Myeloid-derived suppressor cells in cancer patients: a clinical perspective. J Immunother 2012;35:107-115.

9. Ramachandran IR, Condamine T, Lin C, Herlihy SE, Garfall A, Vogl DT, Gabrilovich DI, et al. Bone marrow PMN-MDSCs and neutrophils are functionally similar in protection of multiple myeloma from chemotherapy. Cancer Lett 2016;371:117-124.
10. Draghiciu O, Lubbers J, Nijman HW, Daemen T. Myeloid derived suppressor cells-An overview of combat strategies to increase immunotherapy efficacy. Oncoimmunology 2015;4:e954829.
11. Fleming V, Hu X, Weber R, Nagibin V, Groth C, Altevogt P, Utikal J, et al. Targeting Myeloid-Derived Suppressor Cells to Bypass Tumor-Induced Immunosuppression. Front Immunol 2018;9:398.
12. Kalathil S, Lugade AA, Miller A, Iyer R, Thanavala Y. Higher frequencies of GARP(+)CTLA-4(+)Foxp3(+) T regulatory cells and myeloid-derived suppressor cells in hepatocellular carcinoma patients are associated with impaired T-cell functionality. Cancer Res 2013;73:2435-2444.
13. Arihara F, Mizukoshi E, Kitahara M, Takata Y, Arai K, Yamashita T, Nakamoto Y, et al. Increase in CD14+HLA-DR -/low myeloid-derived suppressor cells in hepatocellular carcinoma patients and its impact on prognosis. Cancer Immunol Immunother 2013;62:1421-1430.
14. Hoechst B, Voigtlaender T, Ormandy L, Gamrekelashvili J, Zhao F, Wedemeyer H, Lehner F, et al. Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor. Hepatology 2009;50:799-807.
15. Wang D, An G, Xie S, Yao Y, Feng G. The clinical and prognostic significance of CD14(+)HLA-DR(-/low) myeloid-derived suppressor cells in hepatocellular carcinoma patients receiving radiotherapy. Tumour Biol 2016;37:10427-10433.
16. Greten TF, Manns MP, Korangy F. Myeloid derived suppressor cells in human diseases. Int Immunopharmacol 2011;11:802-807.
17. Ouzounova M, Lee E, Piranlioglu R, El Andaloussi A, Kolhe R, Demirci MF, Marasco D, et al. Monocytic and granulocytic myeloid derived suppressor cells differentially regulate spatiotemporal tumour plasticity during metastatic cascade. Nat Commun 2017;8:14979.
18. Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. Regulatory T cells in cancer. Adv Cancer Res 2010;107:57-117.
19. Lindau D, Gielen P, Kroesen M, Wesseling P, Adema GJ. The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. Immunology 2013;138:105-115.
20. Duffy MJ. Carcinoembryonic antigen as a marker for colorectal cancer: is it clinically useful? Clin Chem 2001;47:624-630.
21. Blechacz B. Cholangiocarcinoma: Current Knowledge and New Developments. Gut Liver 2017;11:13-26.
22. Kotsakis A, Harasymczuk M, Schilling B, Georgoulias V, Argiris A, Whiteside TL. Myeloid-derived suppressor cell measurements in fresh and cryopreserved blood samples. J Immunol Methods 2012;381:14-22.
23. Yu N, Li X, Song W, Li D, Yu D, Zeng X, Li M, et al. CD4(+)CD25 (+)CD127 (low/-) T cells: a more specific Treg population in human peripheral blood. Inflammation 2012;35:1773-1780.
24. Xu D, Fu J, Jin L, Zhang H, Zhou C, Zou Z, Zhao JM, et al. Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. J Immunol 2006;177:739-747.
25. Hartling HJ, Gaardbo JC, Ronit A, Knudsen LS, Ullum H, Vainer B, Clausen MR, et al. CD4(+) and CD8(+) regulatory T cells (Tregs) are elevated and display an active phenotype in patients with chronic HCV mono-infection and HIV/HCV co-infection. Scand J Immunol 2012;76:294-305.
26. Mohsen W, Rodov M, Prakoso E, Charlton B, Bowen DG, Koorey DJ, Shackel NA, et al. Patients with non-viral liver disease have a greater tumor burden and less curative treatment

options when diagnosed with hepatocellular carcinoma. World J Gastroenterol 2017;23:2763-2770.
27. Aktas E, Kucuksezer UC, Bilgic S, Erten G, Deniz G. Relationship between CD107a expression and cytotoxic activity. Cell Immunol 2009;254:149-154.
28. Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother 2009;58:49-59.
29. Alban TJ, Alvarado AG, Sorensen MD, Bayik D, Volovetz J, Serbinowski E, Mulkearns-Hubert EE, et al. Global immune fingerprinting in glioblastoma patient peripheral blood reveals immune-suppression signatures associated with prognosis. JCI Insight 2018;3.
30. Giannini EG, Testa R, Savarino V. Liver enzyme alteration: a guide for clinicians. CMAJ 2005;172:367-379.
31. Fletcher RH. Carcinoembryonic antigen. Ann Intern Med 1986;104:66-73.
32. Youn JI, Collazo M, Shalova IN, Biswas SK, Gabrilovich DI. Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. J Leukoc Biol 2012;91:167-181.
33. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol 2008;181:5791-5802.
34. Thomas R, Lipsky PE. Human peripheral blood dendritic cell subsets. Isolation and characterization of precursor and mature antigen-presenting cells. J Immunol 1994;153:4016-4028.
35. Chiu DK, Tse AP, Xu IM, Di Cui J, Lai RK, Li LL, Koh HY, et al. Hypoxia inducible factor HIF-1 promotes myeloid-derived suppressor cells accumulation through ENTPD2/CD39L1 in hepatocellular carcinoma. Nat Commun 2017;8:517.
36. Vincent J, Mignot G, Chalmin F, Ladoire S, Bruchard M, Chevriaux A, Martin F, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. Cancer Res 2010;70:3052-3061.
37. Otvos B, Silver DJ, Mulkearns-Hubert EE, Alvarado AG, Turaga SM, Sorensen MD, Rayman P, et al. Cancer Stem Cell-Secreted Macrophage Migration Inhibitory Factor Stimulates Myeloid Derived Suppressor Cell Function and Facilitates Glioblastoma Immune Evasion. Stem Cells 2016;34:2026-2039.
38. Wang Z, Till B, Gao Q. Chemotherapeutic agent-mediated elimination of myeloid-derived suppressor cells. Oncoimmunology 2017;6:e1331807.

**Table 1:** Patient demographic information

| | Benign (n=18) | HCC (n=18) | CRLM (n=41) | CCA (n=18) | NET (n=5) |
|---|---|---|---|---|---|
| **Age** | 38 (19-80) | 71.5 (54-87) | 55 (26-84) | 67 (60-86) | 60 (46-67) |
| **Sex (% male)** | 12% | 50% | 43% | 47% | 40% |
| **ALT** | 25.1 (±38.9) | 41.4 (±33.5) | 27.5 (±17) | 42.9 (±40.7) | 39.5 (± 20.4) |
| **AST** | 27.1 (±25.1) | 46.2 (±20.1) | 28.6 (±13.2) | 43.2 (±26.8) | 51.5 (± 51.3) |
| **Albumin** | 4.2 (± .3) | 4.0 (±.5) | 4.1 (±.4) | 3.9 (±.4) | 4.2 (± .3) |
| **WBC** | 6.9 (± 2.3) | 7.9 (±2.4) | 6.6 (±2.0) | 6.6 (±1.9) | 8.2 (± 1.2) |
| **Platelet** | 245 (±83) | 209 (±107.4) | 192.3 (±74.8) | 234.9 (±120.4) | 300.2 (± 102.1) |

**Figure 1**

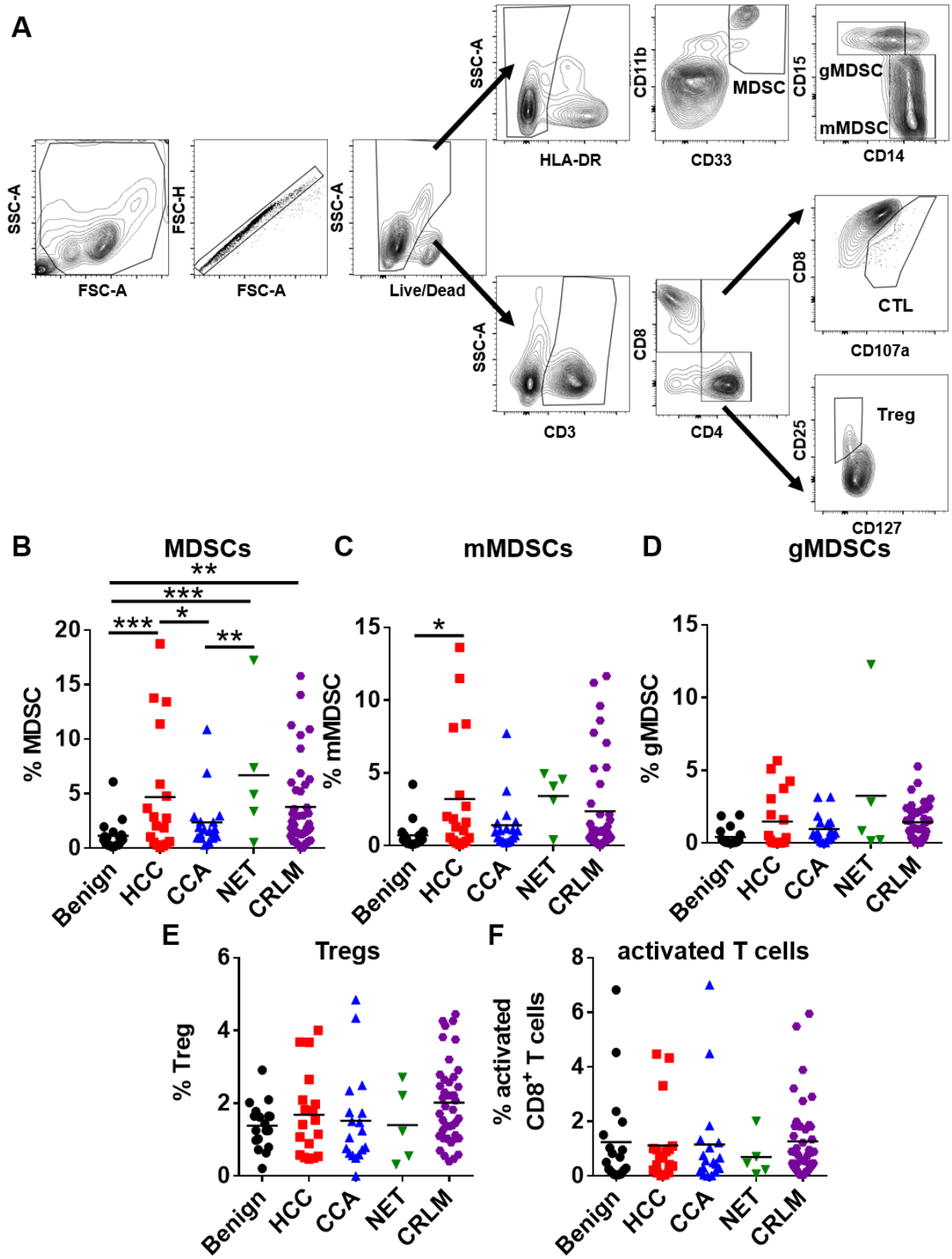

**Figure 2**

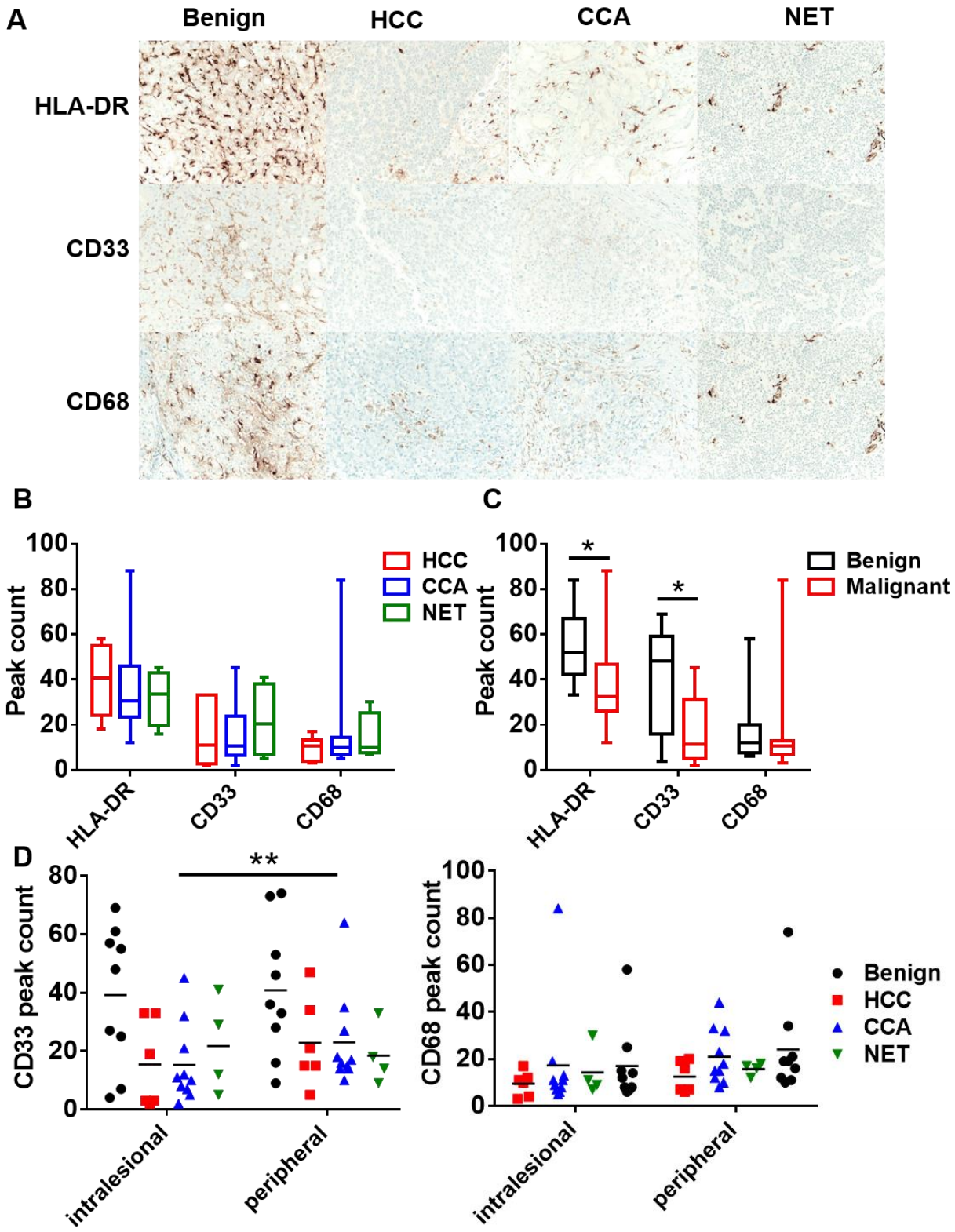

**Figure 3**

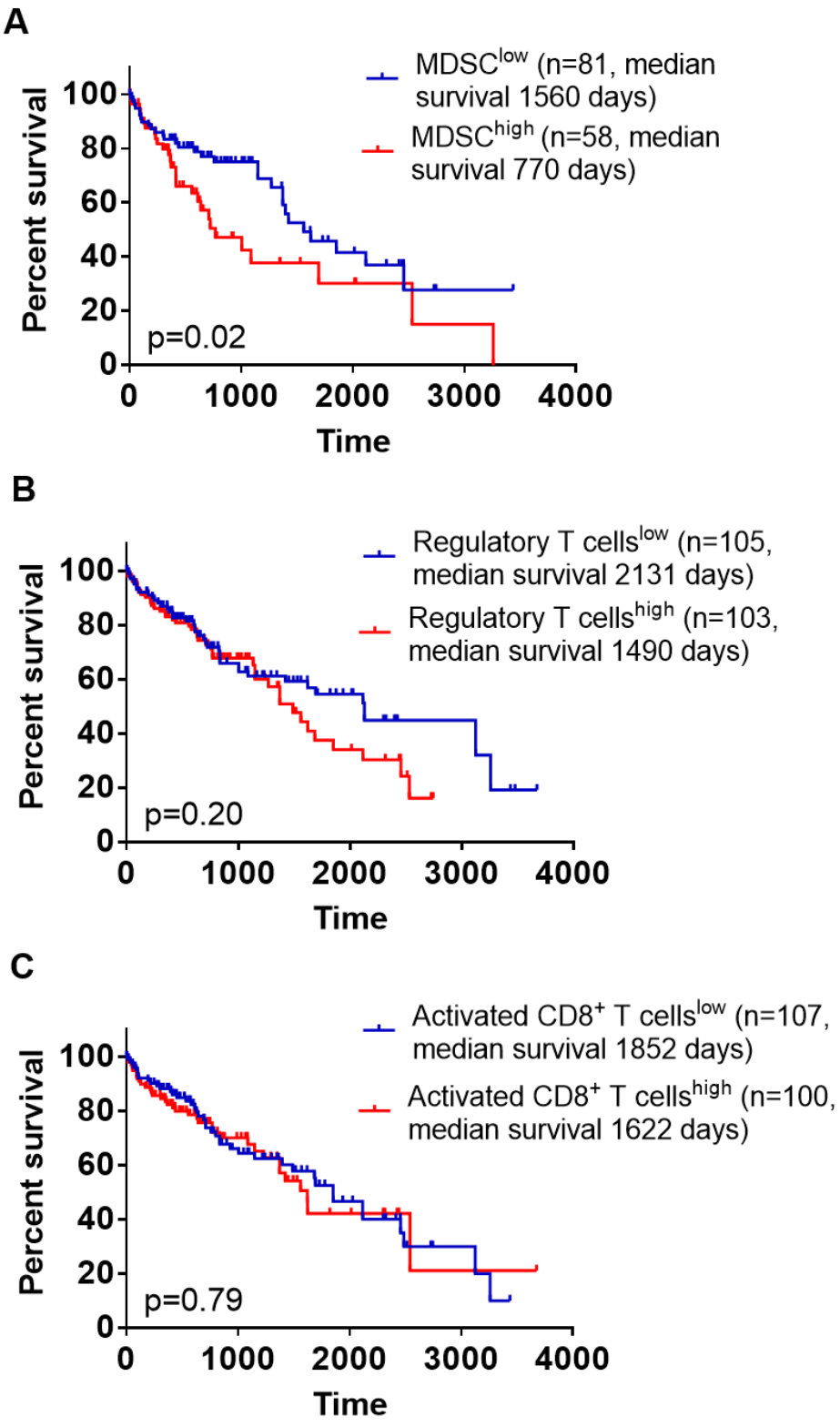

**Figure 4**