Tumor-derived α-fetoprotein suppresses fatty acid metabolism and oxidative phosphorylation in dendritic cells

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ABSTRACT

Cellular metabolism supports immune cell function. Here, we identify a reduction in fatty acid synthesis and mitochondrial metabolism in dendritic cells (DCs) due to α-fetoprotein (AFP), a protein secreted by hepatocellular cancer (HCC). DCs cultured in the presence of AFP show reduced expression of the metabolic regulatory molecules SREBP-1 and PGC1-α. The negative effect of AFP on mitochondrial metabolism and ATP production was confirmed with observation of reduction in basal oxygen consumption rate (OCR) in DCs exposed to AFP derived from cord blood. More severe reduction in basal OCR was observed in tumor-derived DCs exposed to AFP due to downregulation of cytochrome c oxidase. We also showed reduced expression of PGC1-α in circulating myeloid DCs of HCC patients and impaired capacity to stimulate antigen-specific effector functions. These data show the negative effects of AFP on DC metabolism. These findings elucidate a mechanism of immune suppression in HCC and may help generate therapeutic approaches to reverse such immunosuppression.

INTRODUCTION

Hepatocellular carcinoma (HCC) remains a serious global health problem, with approximately 781,600 deaths with 841,100 new cases diagnosed worldwide in 2018 (American Cancer Society: https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2018.html). In the US, about 42,000 new cases were diagnosed in 2018. Incidence and mortality rates are expected to increase each year (1). Major risk factors for HCC include chronic infections with HBV and HCV, cirrhosis, heavy alcohol consumption and smoking. Other risk factors include metabolic disorders such as diabetes, obesity and non-alcoholic fatty liver disease (2,3). Current treatments, which include surgery, transplantation, ablation, radiation,
embolization and chemotherapy, are largely palliative especially for advanced cases (4). Overall prognosis of the disease once diagnosed remains dismal with a 5 year survival rate of 17% (5). Phase III HCC clinical trials for five different small molecule inhibitors have all been unsuccessful (4). Although there are some encouraging data for PD-1 blockade in HCC (6), most current therapies are inadequate. A better understanding of treatment resistance mechanisms and improved treatment options is needed.

The liver sustains a tolerogenic environment to prevent immune responses to innocuous antigens encountered as part of normal liver function (7). In addition to this normal tolerogenic environment in the liver, HCC patients exhibit systemic immune suppression. HCC patients have reduced NK cell frequencies (8), reduced frequencies of myeloid DCs with impaired IL-12 production (9), and higher frequencies of circulating myeloid-derived suppressor cells (MDSCs) (10) and regulatory T cells (11). HCC tumors can also overproduce VEGF, COX2, IL-10, and TGF-β, all of which contribute to the immune suppressive environment (12-14).

α-Fetoprotein (AFP), a single-chain glycoprotein, is the most abundant serum protein present in the fetus, reaching serum concentrations of up to 3 mg/mL. AFP may, similar to albumin, bind and transport various serum components such as fatty acids, heavy metals and steroids (15). Transcription of AFP is repressed immediately after birth such that serum concentrations drop to a range of 1-40 ng/mL in healthy adults. However, 50-80% of HCC tumors secrete AFP. Serum concentrations of AFP at (>200ng/ml) are diagnostic of disease; reductions in AFP concentrations are used to monitor treatment responsiveness (15). Tumor-derived AFP (tAFP) carries a different glycosylation profile than does AFP derived from normal cord blood (nAFP). nAFP contains <5% of the fucosylated variant AFP-L3, whereas AFP in HCC patient serum is
>80% AFP-L3. High AFP-L3 content in HCC patient serum is associated with poor disease prognosis (16).

AFP is taken up by epithelial cells, tumor cells, activated lymphocytes, NK cells and dendritic cells (DCs). A study from our group showed that DCs take up both nAFP and tAFP efficiently through the mannose receptor and can also utilize scavenger receptors (17). AFP has a suppressive effect on immune cells including NK cells, T cells and DCs (18-21). We have shown that tAFP, and to a lesser extent nAFP, inhibited monocyte differentiation into dendritic cells. DCs exposed to tAFP expressed less of DC maturation markers and are poor stimulators of antigen-specific T cell activation and proliferation (22).

Studies have characterized the connection between metabolism and immune cell function (reviewed in (23)). The protein complex mTORC1 senses nutrients and integrates signals from upstream signaling pathways activated via growth factors as well as signals from receptors and sensors that convey changes in intracellular nutrients and energy. Integrating extracellular and intracellular stimuli, mTORC1 regulates downstream cellular processes including protein synthesis, nucleotide synthesis, lipid biosynthesis, mitochondrial biogenesis and glycolysis in immune cells (24). Metabolism produces energy in the form of ATP. Naive T cells use mitochondrial fatty acid β-oxidation and oxidative phosphorylation (OXPHOS) to generate ATP. Upon T cell activation, these cells shift to using aerobic glycolysis to generate most of the ATP needed, rapidly proliferate, and become T effector cells (25). Likewise, resting bone marrow-derived murine DCs rely on fatty acid β-oxidation and OXPHOS to produce ATP (26). However, DC maturation and activation triggered by TLR agonists drives the metabolic transition from
OXPHOS to aerobic glycolysis (27). In addition to ATP production, fatty acid synthesis, another essential cellular process, produces lipids used for energy storage and membrane construction.

In this study, we examined the effect of AFP on SREBP-1 regulated fatty acid synthesis and mitochondrial metabolism of monocyte-derived DCs. Our findings show that AFP decreases SREBP-1 activity through inhibition of mTORC1. We show that the mitochondrial regulator PGC1-α is decreased upon AFP exposure. In addition to inhibiting DC maturation, nAFP, and especially tAFP, inhibits mitochondrial oxidative phosphorylation. The effect of AFP on DC metabolism occurs within 24 hours of AFP exposure and the defects in OXPHOS are due to AFP-induced decreases in mitochondrial protein cytochrome c oxidase amounts. We also show that different circulating DC subsets in HCC patients have decreased expression of the mitochondrial biosynthesis transactivator PGC1-α.
MATERIALS AND METHODS

Isolation of PBMCs and DC culture

PBMC were obtained from healthy donor (HD) blood or HCC patients (Supplementary Table S1) with informed consent (Pitt IRB #UPCI 04-001 and UPCI 04-111) conducted in accordance with the Declaration of Helsinki using density gradient centrifugation (Ficoll-Paque, GE Healthcare). To generate DCs, CD14 monocytes from 3-5 different healthy donors per experiment, were isolated from PBMC using magnetic separation with CD14 microbeads (Miltenyi Biotec) per manufacturer’s instructions and cultured for 5 days in 800 IU/mL rGM-CSF (Miltenyi Biotech; Sanofi) and 500 IU/mL rIL-4 (R&D Systems; Gemini Bio-Products). Purified chicken egg ovalbumin (OVA) (Fisher Scientific), human cord serum nAFP (Cell Sciences, lot numbers 4111706-4111708, 4111710-4111713) and HCC cell line-derived tAFP (Bio-Rad, lot numbers 0312612FP, 02A0913FP, 64005863, 64024255) were added to cultures at 10 μg/mL for 5 days.

Flow cytometry analysis

After 5 days in culture, DCs were collected and counted. For mitochondrial staining, cells were stained with MitoTracker Deep Red (ThermoFisher), Mitotracker Green (ThermoFisher) and TMRE (ThermoFisher). For PGC1-α staining, cells were fixed and permeabilized using eBiosciences FOXP3 buffer kit per manufacturer’s instructions. Cells were stained with PGC1-α (H-300, Santa Cruz Biotechnology), washed and stained with secondary antibody anti-rabbit IgG conjugated to Alexa 647 (Jackson Immunolabs or Cell Signaling Technology). For pS6 and p4E-BP1 staining, cells were fixed with 1.5% PFA in 1x Permeabilization Buffer (eBiosciences) and subsequently permeabilized and stained with pS6 (Ser240/244), p4E-BP1 (Thr37/46), S6
(54D2), 4E-BP1 (53H11) or isotype control (DA1E) from Cell Signaling Technology. For analysis of myeloid DC subsets, PBMC were stained with viability dye Zombie Aqua (Biolegend) and then surface stained with CD3 (UCHT1), CD19 (HIB19), CD56 (B159), CD11c (B-ly6), CD14 (MφP-9), CD303(V24-785), HLA-DR (G46-6) (BD Biosciences) and CD1c (L161) and CD141 (M80) (Biolegend) prior to intracellular staining for PGC1-α. For cell sorting, PBMC were thawed and stained with the same surface markers in addition to Mitotracker DR and TMRE. Lin (CD3/CD19/CD56)^− HLA-DR^+ CD1c^+ myeloid DCs were sorted in addition to Lin (CD3/CD19/CD56)^− HLA-DR^− cells for autologous T cells using MoFlo Astrios (Beckman Coulter). DCs and T cells were cultured in 1:5 ratio for 8 days in the presence of 1 μg/mL CEF-MHC Class I peptides (Immunospot), 10 ng/mL IL7 and 10 U/mL IL2. Collected T cells were then stimulated with autologous CD3-depleted PBMC with 1 μg/mL CEF-MHC class I and 10 μg/mL brefeldin A (Sigma). After 5 hours, cells were stained with CD3 (SK7), CD8 (RPA-T8) and CD107a (H4A3) (BD Biosciences) and were fixed and permeabilized as described previously prior to intracellular staining with CD69 (FN50), IFN-γ (B27) and TNFα (Mab11) (BD Biosciences). Data were acquired with an Accuri C6 or LSR II Fortessa cytometer (BD Biosciences) and analyzed using FlowJo software version 10.0.8 (TreeStar).

**Metabolic Assays and ATP Measurement**

After 5 days in culture, DCs were collected and counted and plated at 200,000 cells/well on Seahorse culture plates in assay media consisting of minimal, unbuffered DMEM supplemented with 1% BSA and 25 mM glucose, 1 mM pyruvate, and 2 mM glutamine and analyzed using a Seahorse XFe96 (Agilent). Basal oxygen consumption and extracellular acidification rates were taken for 30 minutes. Cells were stimulated with oligomycin (2 μM), FCCP (0.5 μM), 2-
deoxyglucose (10 mM) and rotenone/antimycin A (0.5 μM) to obtain maximal respiratory and control values. For ATP measurement, cells were collected and resuspended at 1x10^4 cells/μL and in PBS (Gibco) and 10 μL cell suspension was plated per well in a 96 well luminometer plate (Perkin Elmer). ATP measurement was performed in triplicates per manufacturer’s instructions using ATP Assay kit (Millipore Sigma).

**Gene and protein expression analysis**

DCs cultured with OVA, nAFP or tAFP as described above were used for mRNA and protein isolation and quantification. Transcriptome analysis was performed with Affymetrix HG-U133A array as described in (22). The data discussed in this publication is accessible through GEO Series accession number GSE62005. For protein expression analyses, dendritic cell pellets were lysed in cold RIPA lysis buffer (ThermoFisher Scientific) containing protease inhibitor cocktail (ThermoFisher Scientific). The protein concentration of cell lysates was measured using BCA Protein Assay kit (ThermoFisher Scientific) and DU-520 UV-visible Scanning Spectrophotometer (Beckman Coulter). Samples were mixed with 2X or 4X loading buffer (Bio-Rad) containing SDS, and 20-50 μg of protein were loaded in a 6%, 8% or 12% SDS-PAGE gel. After electrophoresis, the gel was transferred to a PVDF membrane (Bio-Rad) and blocked with 5% non-fat milk in 1x TBST for 1 hour at room temperature. Western blot was performed by incubating with primary antibodies from the Total OXPHOS Rodent WB Antibody kit (ab110413, AbCam), or antibodies SREBP-1 (A-4, sc-365513) FASN (A-5, sc-55580), or ACLY (5F8D11, sc-517627) from Santa Cruz Biotechnology, overnight at 4°C and using β-actin antibody (A1978, Sigma) as a loading control. After primary antibody incubation, the membrane was washed with 1x TBST three times, and then incubated with secondary antibodies: goat anti-
mouse IgG-HRP (sc-2005, Santa Cruz Biotechnology) or mouse IgG kappa binding protein conjugated to Horseradish Peroxidase (sc-56102, Santa Cruz Biotechnology), for 1 hour at room temperature. The Western blot was developed with Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate (Perkin Elmer). Densitometry data was collected from scanned film images using Image J.

Statistics

Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparisons test (Graphpad Prism 6.0), with p values < 0.05 considered as significant.
RESULTS

Expression of enzymes involved in fatty acid synthesis is downregulated upon exposure to AFP

We have previously shown that AFP-exposed monocyte-derived DCs have defects in maturation and reduced antigen presentation function (22). Examination of gene array data for differentially expressed genes due to AFP exposure of DCs cultured for 5 days identified several metabolism-related genes that were downregulated after exposure. Gene expression was more reduced in tAFP DCs compared to nAFP DCs. Most of the genes that were differentially expressed are involved in lipid metabolism, such as enzymes involved in fatty acid and cholesterol synthesis (Supplementary Table S2). The bHLH transcription factor SREBP-1 regulates the expression of enzymes such as FASN and ACLY which are key in fatty acid synthesis. SREBP-1 is a 128 KDa protein that is localized in membranes of the ER and nucleus, and can be cleaved into a 68 KDa form that can translocate to the nucleus (28). Based on gene array findings, we hypothesized that SREBP-1 expression will be reduced in DCs upon 5 days of AFP exposure. Analysis of expression of both precursor and mature forms of SREBP-1 in DCs shows that precursor SREBP-1 is significantly reduced by 35% and 70% in nAFP DCs and tAFP DCs, respectively compared to control OVA DCs (Fig. 1A). The amount of mature SREBP-1, which has been shown to be regulated by sterol mediated proteolysis (28), is reduced in tAFP DCs only. We examined the expression of SREBP-1 gene targets to determine if SREBP-1 transcriptional activity is also inhibited by AFP. Results show that expression of FASN, an enzyme in fatty acid biosynthesis that catalyzes the production of palmitate from acetyl-CoA and malonyl-CoA, was also downregulated by 35% and 70% in nAFP DCs and tAFP DCs respectively compared to OVA DCs (Fig. 1B). Likewise, we also saw a reduction in expression of ACLY, an enzyme responsible for synthesis of cytosolic acetyl-coA, in both nAFP and tAFP exposed DCs (Fig.
Therefore, DC exposure to AFP results in inhibition of expression of SREBP-1 and downstream target proteins. Exposure of DCs to tAFP further inhibits sterol-mediated cleavage of SREBP-1.

SREBP-1 expression and activity is controlled by the nutrient sensor mTORC1, a regulator of cell growth, proliferation and metabolism (29). mTORC1 is a serine/threonine kinase that phosphorylates downstream targets, such as cap-dependent translation inhibitor 4E-BP1 and ribosomal protein S6 kinase (S6K1), to promote protein synthesis (30). We hypothesized that mTORC1 activity in DCs will be inhibited by AFP exposure (as reflected in the changes observed in SREBP-1 expression). We examined the expression of these two targets of the mTORC1 pathway. As expected, the frequency of cells expressing p4E-BP1 was reduced by 18% in nAFP DCs and by 60% in tAFP DCs (Fig. 1D). Expression of pS6 in immature DCs immediately after exposure to either nAFP or tAFP was also examined (Fig. 1E). DCs exposed to nAFP and OVA had increased expression of pS6 within an hour, peaking at around 2 hours after protein exposure. This correlates with our previous finding on DC uptake of AFP into the perinuclear space within 30 minutes of exposure (17). In contrast, DCs exposed to tAFP for as long as 6 hours did not result in increased pS6 expression. We did not observe any changes in either total 4E-BP1 or total S6 expression (Supplementary Fig. S1). Together, these data show that AFP inhibits mTORC1 activity.

*DCs exposed to AFP show downregulation of PGC1-α and exhibit mitochondrial defects.*

In addition to SREBP-1, mTORC1 has also been shown to regulate PGC1-α expression (31), therefore we determined whether PGC1-α would also be downregulated in nAFP and tAFP DCs,
reflecting the reduced activation of mTORC1. nAFP and tAFP DCs have reduced intracellular PGC1-α expression compared to OVA DCs (reduced by 26% and 51% respectively, Fig. 2A). PGC1-α is a transcriptional coactivator that promotes mitochondrial biogenesis and respiration. Furthermore, mitochondrial biogenesis and function can be enhanced by the activation of the PGC1-α pathway (32). To investigate the impact of AFP on mitochondrial metabolism, we utilized the probes Mitotracker Deep Red and Mitotracker Green to measure total mitochondrial content. Both nAFP and tAFP DCs exposed to AFP showed significant reductions in mitochondrial content (Fig. 2B and 2C). The mitochondrial mass of nAFP DCs was reduced by an average of 23% whereas that of tAFP DCs was reduced by 35%. Similar findings were observed when using Mitotracker Green. We then examined differences in the mitochondrial membrane potential in nAFP- and tAFP-exposed DCs. Results showed reductions of 30% and 45% in nAFP and tAFP DCs respectively compared to OVA DCs (Fig. 2D). These results demonstrate that DCs exposed to AFP have decreased PGC1-α, reduced mitochondrial mass and more depolarized mitochondria, suggesting defects in mitochondrial function.

**nAFP and tAFP DCs have less oxygen consumption activity but unaffected glycolysis**

Previous studies have shown that resting DC use OXPHOS as the primary metabolic pathway and, upon TLR-dependent activation, undergo a metabolic switch to glycolysis that is PI3K/AKT-dependent (27,33). We hypothesized that reductions in PGC1-α and depolarized mitochondria in DCs exposed to AFP would lead to defects in mitochondrial metabolism. To test DC mitochondrial function, we performed a metabolic assay stress test on DCs cultured for 5 days in the presence of OVA, nAFP or tAFP. The oxygen consumption rate (OCR) was used to determine mitochondrial OXPHOS activity. Results show that nAFP DCs had lower basal
oxygen consumption rates than OVA DCs (reduced by 55%, Fig. 3A-3B). In particular, tAFP DCs had little oxygen consumption (reduced by 98% vs OVA-DC, Fig. 3A). Use of the uncoupling agent FCCP to stimulate maximal oxygen consumption revealed that tAFP DCs have suppressed respiratory capacity. In contrast, glycolysis, as shown by ECAR rates, did not significantly differ among OVA DCs, nAFP DCs and tAFP DCs (Fig. 3C, D). There were no changes in the frequency of viable cells among the different treatment groups throughout the 5-day culture (Supplementary Fig. S2). nAFP DCs showed a 60% reduction and tAFP DCs an 80% reduction in ATP content compared to OVA DCs (Fig. 3E). There were no increases in oxidative phosphorylation after 24 hours of treatment with LPS in AFP-exposed DCs, in contrast to that observed in OVA DCs (Supplementary Fig. S3). Together, these data indicate that tAFP, and to a lesser extent nAFP, inhibits DC mitochondrial metabolism in a manner that cannot be rescued by TLR stimulation. Thus, ATP production is diminished even though AFP does not affect DC capacity to utilize glycolysis.

Changes in DC metabolism occur within 24 hours

Monocytes were used to model DC precursors exposed to AFP derived from a fetus or an HCC tumor. Therefore, we determined the time point at which AFP exposure begins to affect DC metabolism. Each protein (OVA, nAFP, tAFP) was added at day 0, day 2, day 3 or day 4 prior to testing metabolic function. Consistent with our earlier results, DCs that were exposed to AFP for 5 days had significantly reduced oxidative phosphorylation (Fig. 4A-F, Supplementary Fig. S4). Changes in OXPHOS activity were observable in DCs after 1 day exposure to nAFP and tAFP, compared to OVA (reduced by 23% and 54% respectively, Fig. 4A, C). Changes in OXPHOS become more pronounced after 2 days of nAFP and tAFP exposure (reduced by 37% and 82%
respectively, Fig. 4B-4C). Similar to what we found in DCs cultured for 5 days with nAFP and tAFP, the reductions in basal OCR were more severe in DCs that were cultured with tAFP than with nAFP in all the time points measured. This early AFP effect is consistent with our previous finding examining the time course of changes in DC phenotype with AFP exposure (22).

Because loss of mitochondrial activity can promote a dependence on aerobic glycolysis for generation of ATP and regeneration of NAD$^+$, glycolysis was also measured. A transient change in glycolysis was observed as early as 1 day after protein exposure, but only in tAFP DCs (Fig. 4D and 4F). In particular, DC glycolysis was increased by 30% and 32% compared to OVA DCs and nAFP DCs respectively, after 1 day of culture with tAFP. Glycolysis was also increased in tAFP DCs after 2 days in culture by 37% and 29% compared to OVA DCs and nAFP DCs respectively (Fig. 4E and 4F). Glycolysis in nAFP DCs and OVA DCs was similar after both 1 and 2 days in culture (Fig. 4D-4E). After 3 days in culture, glycolysis was comparable for all three conditions, tAFP DCs, OVA DCs, and nAFP DCs (Fig. 4F). Thus, there is a transient increase in glycolysis in DCs exposed to tAFP that is not sustained.

**Oxidative phosphorylation defects correlate with loss of cytochrome c oxidase**

To determine the mechanism behind the reduced mitochondrial metabolism in nAFP and tAFP DCs, we examined expression of mitochondrial electron transport proteins responsible for oxidative phosphorylation by Western blot (Fig. 5A). A significant increase in expression of complex III subunit UQCRC2 in tAFP DCs was identified. We did not observe any AFP-specific differences in the expression of enzymatic components of complex I, II and ATP synthase (Fig. 5). However, the protein expression of cytochrome c oxidase subunit 1 (MTCO1/COX1), a
mitochondrially encoded subunit, is significantly decreased (14% and 39%) by both nAFP and tAFP respectively (Fig. 5B). Direct comparison of both AFP DC groups shows that tAFP DCs had less MTCO1 expression than nAFP DCs. This is in agreement with the altered bioenergetics observed in nAFP and tAFP DCs, as indicated by membrane depolarization and reductions in basal OCR and PGC1α. MTCO1 serves as a catalytic subunit of cytochrome c oxidase (COX), the terminal enzyme of the electron transport chain that catalyzes transfer of electrons to molecular oxygen (34). Thus our results suggest that exposure to AFP, especially tAFP, leads to reduced cytochrome c oxidase expression, which disrupts reduction of oxygen to water and generation of a proton gradient across the inner mitochondrial membrane. The disrupted proton gradient causes membrane depolarization. These findings demonstrate that AFP induces a decrease in PGC1-α expression in monocyte-derived DCs which correlates to observed reductions in COX expression and OXPHOS activity.

Overall, these data indicate that treatment with nAFP affected both SREBP-1 and PGC1-α downregulation. However, exposure to tAFP had a more profound effect on mTORC1 activity. As a consequence, SREBP-1 expression and activation in addition to PGC1-α and mitochondrial function is compromised in DCs, revealing a mechanism by which tAFP causes immune suppression.

*Myeloid DCs in HCC carcinoma patients show reduced expression of PGC1-α*

Because tAFP can be secreted into the serum by over 50% of hepatocellular carcinomas, we wanted to determine whether mitochondrial metabolism in circulating DC will also be suppressed similar to previous studies indicating that tAFP have suppressive effects on DC
function [22-24, 48]. The rarity of circulating DCs in peripheral blood precluded obtaining sufficient cells for a functional metabolic assay, hence, we tested whether PGC1-α expression in circulating DCs in HCC patients was different compared to healthy donors instead. We obtained peripheral blood lymphocytes from HCC patients and healthy donors and identified different DC subsets via staining with surface markers as previously described (35). We examined PGC1-α expression in three DC subsets including conventional myeloid CD1c+ DCs, a CD141+ expressing myeloid DC subset that is specialized for cross-presentation, CD303+ plasmacytoid DCs, and HLA-DR+ Lin− (CD3/CD19/CD56) CD14+ monocytes from HCC patients and HD. We observed significant reductions in PGC1-α expression in all three DC subsets examined (Fig. 6A and 6B), indicating that the tumor microenvironment in HCC patients not only affects DC function, but might also negatively regulate mitochondrial metabolism in these cells. To investigate the potential differences in mitochondrial integrity, we examined Mitotracker DR and TMRE fluorescence in the conventional myeloid CD1c+ DC subset in HD or HCC patients. There were no differences observed in Mitotracker DR signal (Fig. 6C), however CD1c+ DCs from HCC patients had less TMRE fluorescence compared to HD DCs (Fig. 6D) indicating a defect in mitochondrial membrane potential in these patients. To determine whether DC function is compromised in HCC patients, sorted CD1c+ DCs were used to stimulate autologous T cells using a CEF I peptide pool that contains 32 ubiquitous HLA class I-restricted T cell epitopes as antigens. Analysis of antigen-specific CD8+ T cell effector function after in vitro co-culture showed that T cells stimulated by HCC DCs had less robust effector function as well as reduced TNFα production and CD69 expression (Fig. 6E). Together, these data show that mitochondrial integrity and DC function are impaired in HCC CD1c+ DCs.
DISCUSSION

Tumor-derived AFP is used as a serum biomarker to diagnose HCC. In addition, poor prognosis and low overall survival is correlated with AFP+ HCC tumors and high serum AFP concentrations (36). Several functions of AFP have been described. Given its structural similarity to albumin, AFP may play a role in the transport of serum components, including fatty acids, steroids, and heavy metals (15). There have been reports of AFP interfering with intracellular signaling, including both caspase-3 and PI3K/AKT pathways (37). An immunoregulatory role for AFP has also been proposed. Studies show an inhibitory effect of nAFP on lymphocyte function and a protective role in autoimmunity models wherein autoimmunity can go into remission during the third trimester of pregnancy (38-40). Based on these and related findings, AFP has been clinically tested for immune suppressive activity in MS patients. Reports suggest that AFP exerts its immunosuppressive activity through the inhibition of DC function (19-21,41). However, there is little consensus about which cell subsets and/or signaling pathways are the primary targets of AFP-mediated immunosuppression. Furthermore, because most of these studies used cord blood nAFP, less is known about the immunoregulatory nature of tAFP.

In this study, we have identified an AFP-specific inhibitory effect on DC metabolism. This is characterized by DCs with decreased expression of SREBP-1 and PGC1-α, likely due to AFP mediated inhibition of mTORC1 signaling. Studies have shown that DC differentiation driven by Flt3L in murine DCs or GM-CSF and IL-4 in human DCs is dependent on constitutive mTOR activation (42,43). Furthermore, DCs differentiated in the presence of rapamycin, an mTORC1 inhibitor, show reduced expression of MHC and co-stimulatory molecules and are poor stimulators of T cells (44). In agreement with our previous findings, the inhibitory effect of AFP
on DC metabolism differed in severity when comparing cord serum derived AFP versus tumor derived AFP. Here, we show that tAFP has an immediate inhibitory effect on mTORC1 signaling compared to nAFP in immature DCs. In addition, differences in protein expression and mitochondrial activity are also observed between nAFP and tAFP, which may be indicative of the microenvironment in which these two forms of AFP can be normally found.

DCs require fatty acid synthesis during development and differentiation as well as during maturation and activation (26,45). Blocking fatty acid synthesis inhibits DC development in both humans and mice (45). Here, we show that DCs exposed to AFP downregulate expression of SREBP-1-mediated transcription of enzymes involved in fatty acid synthesis, indicating changes in fatty acid content. The synthesis of fatty acids is an essential cellular process used to produce lipids that can be utilized for membrane synthesis or ATP production. Previous studies have shown differences in the effects of lipid content on DC function. For instance, high lipid content of liver resident DCs positively correlates with immunogenicity (46), whereas in the tumor microenvironment, DCs with high fatty acid content are associated with impaired immune priming (47). However, based on our current and previous work, the effect of AFP exposure on DCs and its subsequent effect on SREBP-1 mediated fatty acid synthesis is one route through which DC development and differentiation is negatively affected by AFP, resulting in poor DC function.

A study by Ma et. al, in a murine model of HCC induced by non-alcoholic fatty acid liver disease highlights the role of mitochondrial dysfunction in promoting HCC via loss of hepatic CD4+ T cells due to ROS accumulation (48). Here, we show reduced expression of PGC1-α, a positive regulator of mitochondrial biogenesis and function, in DCs upon AFP exposure. Different
subsets of circulating DCs from HCC patients also show reduced PGC1-α expression. These findings indicate that the tumor microenvironment of HCC patients can promote mitochondrial and immune dysfunction in immune cells to favor HCC establishment and/or progression. Expression and activity of PGC1-α, which is involved in metabolism and mitochondrial function, is controlled by several mechanisms. For instance, signaling pathways involving cAMP, p38MAPK, AMP-activated protein kinase (AMPK), mTOR and FOXO1 have all been shown to activate PGC1-α transcription (49,50). PGC1-α activity can be regulated by protein phosphorylation, acetylation and methylation (49). Although we show that DC exposure to tAFP results in mTORC1 repression, it is possible that other factors in addition to mTORC1 inhibition are also involved in the downregulation of PGC1-α following AFP exposure because DCs exposed to nAFP also have reduced PGC1-α expression. Thus, further studies are needed to determine the mechanism(s) by which PGC1-α expression is repressed following AFP exposure.

Studies show that resting DCs utilize fatty acid oxidation to generate ATP by oxidative phosphorylation (26,27). In contrast, DC activation by TLR agonists results in increased glucose utilization by glycolysis for ATP production despite the presence of oxygen (27). This process of aerobic glycolysis (known as Warburg effect) also occurs in tumor cells as well as T cells (23,26). Furthermore, blocking glycolysis with 2-deoxyglucose (2-DG) inhibited DC activation indicating that the switch to glycolysis is a part of DC activation (27). It has been reported that the shift from oxidative phosphorylation to glycolysis in TLR-activated DC is due to direct inhibition of oxidative phosphorylation by nitric oxide (NO) generated as a consequence of inducible nitric oxide synthase (iNOS) expression (33). Similar to what has been shown by Everts et al, upon DC stimulation with TLR agonists (33), the inhibitory effect of tAFP on
oxidative phosphorylation and concomitant increase in glycolysis can be seen as early as 24 hours after protein exposure. Furthermore, although basal OXPHOS activity is inhibited by tAFP, glycolysis in DCs likely increases within 1-2 days after protein exposure to compensate for the decreased OXPHOS activity. However, this compensatory increase is not sustained after more than 2 days of protein exposure despite low mitochondrial oxygen consumption. In addition, DCs exposed to tAFP during differentiation become poorly immunogenic and exhibit a monocytic and less mature DC phenotype (22). This is in contrast to the activated mature phenotype that DC acquire after TLR stimulation. Therefore, the inhibition of OXPHOS activity in tAFP DCs is probably not due to the inhibitory presence of NO, but rather due to decreased PGC1-α expression resulting in mitochondria dysfunction.

AFP has been a blood biomarker for HCC tumor size and recurrence for decades. Understanding the function of AFP in immunity and metabolism will lead to new approaches to reverse its metabolic effects. Targeting AFP expressing tumor cells (e.g. with adoptive transfer of T cells) may reverse the negative immune and metabolic effects of this tumor secreted molecule.
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FIGURE LEGENDS

Figure 1. AFP inhibits downstream targets of mTORC1 pathway

(A-C). Western blot was performed to analyze protein expression by loading 20-50 µg of cell lysate from dendritic cells cultured with 10 µg/mL of OVA, nAFP or tAFP for 5 days. Representative Western blots from 2 different donors are shown for (A) SREBP-1, (B) FASN and (C) ACLY along with densitometric quantification for each protein normalized to β-actin amounts, shown as fold change in expression relative to OVA (n=4). (D) Expression of p4E-BP1 in AFP-exposed DCs was analyzed by flow cytometry. Two representative experiments along with summary of p4E-BP1 expression are shown (n=6). (E) Immature DCs were grown from purified monocytes cultured for 5 days with GM-CSF and IL4. On day 5, OVA, nAFP, tAFP (at 10 µg/mL), or no protein were added to the cells for the indicated exposure times. Cells were harvested and amount of pS6 was measured by flow cytometry. Summary of n=3 to 4 experiments per time point showing the mean MFI of pS6 are shown. * p<0.05; ns not significant.

Figure 2. AFP inhibits expression of PGC1-α and causes mitochondrial dysfunction.

Human monocytes from peripheral blood were differentiated into DCs for 5 days in the presence of OVA, nAFP or tAFP at 10 µg/mL (n=5-6). (A) Intracellular amounts of PGC1-α were measured by flow cytometry. Representative histograms from 2 different healthy donors along with summary graphs of PGC1-α MFI normalized to OVA are shown. Cells were collected and stained with (B) MitoTracker Deep Red, or (C) Mitotracker Green and (D) TMRE to examine mitochondrial mass and mitochondrial activity respectively. MFI values were normalized to OVA MFI. * p<0.05; ns not significant.
**Figure 3. AFP-exposed DCs have reduced mitochondrial metabolism resulting in less cellular ATP.**

Purified monocytes from HD (n=3) were differentiated into DCs in the presence of OVA, nAFP or tAFP at 10 μg/mL for 5 days and then subjected to metabolic stress test using Seahorse extracellular flux analyzer. (A) Summary graph of measured basal OCR of all replicates from n=3 independent experiments. Representative OCR traces from (B) healthy donor 1 and healthy donor 2 are shown. (C) Summary graph of measured basal ECAR of all replicates from n=3 independent experiments. Representative ECAR traces from (D) healthy donor 1 and healthy donor 2 are shown. (E) DCs were collected after 5 days of AFP exposure and cellular concentration of ATP was measured (n=6). ***p<0.001; ns not significant.

**Figure 4. Alterations in DC bioenergetics occur within 24 hours of AFP exposure.**

Human monocytes from peripheral blood were differentiated into DCs for 5 days as previously described. OVA, nAFP or tAFP were added in the cultures as indicated for a total of 5, 3, 2 and 1 day(s) exposure. Cells were collected, counted and subjected to metabolic stress test. Graph of basal OCR of all replicates from n=4 experiments after (A) 1 day or (B) 2 days of protein exposure. (C) Summary graph of measured basal OCR showing all the time points tested. Summary graph of basal ECAR of all replicates from n=4 experiments after (D) 1 day or (E) 2 days of protein exposure. (F) Summary graph of measured basal ECAR showing all the time points tested. * p<0.05; ** p<0.01; ns not significant.

**Figure 5. Decreased expression of cytochrome c oxidase in DCs treated with AFP.**
Western blot was performed to analyze the expression of mitochondrial complexes by loading 20 μg of cell lysate from dendritic cells cultured with 10 μg/mL of OVA, nAFP or tAFP for 5 days (n=4). Rat heart mitochondria lysate was used as a positive control and was loaded at 6 μg. (A) representative Western blots from 2 different donors are shown for complex I, II, III, IV and ATP synthase. (B) Densitometric quantification for each protein complex was normalized to β-actin levels. Results are shown as fold change in expression relative to OVA. * p<0.05; ns not significant.

**Figure 6. Circulating CD1c⁺ conventional myeloid DCs from HCC patients show reduction in PGC1-α and TMRE and weak antigen-specific effector function compared to healthy donors.**

PBMC from healthy donors or HCC patients were thawed and stained with surface markers to identify different DC subsets prior to fixation and intracellular staining with PGC1-α. (A) Gating strategy used to identify CD1c⁺ conventional myeloid DCs, CD141⁺ cross-presenting DCs, CD303⁺ plasmacytoid DCs and HLA-DR⁺ CD14⁺ monocytes. (B) Comparison of PGC1-α expression in healthy donors or HCC patients in the different DC subsets tested (n=6-8 per group). Representative histograms showing PGC1-α expression is also shown. PBMC from healthy donors or HCC patients were thawed and stained with surface markers to identify CD1c⁺ DCs as well as analyze (C) Mitotracker DR and (D) TMRE expression prior to cell sorting. (E) Sorted CD1c⁺ DCs from HD or HCC patients were cultured with autologous CD8⁺ T cells in the presence of CEF I peptide pool for 8 days. After a 5-hour stimulation of collected CD8⁺ T cells with CEF I peptide pool, expression of CD107a, CD69 and intracellular production of IFN-γ and
TNFα was assayed by flow cytometry (n=4-5 per group). Each symbol represents one HD or HCC sample, * p<0.05; ns not significant.
Figure 1

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Figure 2

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Healthy Donor 1
Healthy Donor 2

PGC-1a

MFI normalized to OVA

0 50 100 150

OVA nAFP tAFP

B

Mitotracker DR

Mitotracker DR MFI normalized

0 50 100 150

OVA nAFP tAFP

C

Mitotracker Green

Mitotracker Green MFI normalized

0 50 100 150

OVA nAFP tAFP

D

TMRE

TMRE MFI normalized

0 50 100 150

OVA nAFP tAFP
Figure 3

A. Basal OCR

B. Healthy Donor 1 and Healthy Donor 2

C. Basal ECAR

D. Healthy Donor 1 and Healthy Donor 2

E. [ATP]
Figure 4

A

OCR 1d

basal OCR (pmol/min)

OVA nAFP tAFP

B

OCR 2d

basal OCR (pmol/min)

OVA nAFP tAFP

C

basal OCR (pmol/min)

days with protein

D

ECAR 1d

basal ECAR (pH/min)

OVA nAFP tAFP

E

ECAR 2d

basal ECAR (pH/min)

OVA nAFP tAFP

F

basal ECAR (mV/min)

days with protein

- OVA
- nAFP
- tAFP
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Tumor-derived α-fetoprotein suppresses fatty acid metabolism and oxidative phosphorylation in dendritic cells

Patricia M Santos, Ashley V Menk, Jian Shi, et al.

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