Tumor-Derived α-Fetoprotein Directly Drives Human Natural Killer–Cell Activation and Subsequent Cell Death

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Abstract

Hepatocellular carcinoma (HCC) patients with reduced natural killer (NK)–cell numbers and function have been shown to have a poor disease outcome. Mechanisms underlying NK-cell deficiency and dysfunction in HCC patients remain largely unresolved. α-Fetoprotein (AFP) is an oncofetal antigen produced by HCC. Previous studies demonstrated that tumor-derived AFP (tAFP) can indirectly impair NK-cell activity by suppressing dendritic cell function. However, a direct tAFP effect on NK cells remains unexplored. The purpose of this study was to examine the ability of cord blood-derived AFP (nAFP) and that of tAFP to directly modulate human NK-cell activity and longevity in vitro. Short-term exposure to tAFP and, especially, nAFP proteins induced a unique proinflammatory, IL2-hyperresponsive phenotype in NK cells as measured by IL1β, IL6, and TNF secretion, CD69 upregulation, and enhanced tumor cell killing. In contrast, extended coculture with tAFP, but not nAFP, negatively affected long-term NK-cell viability. NK-cell activation was directly mediated by the AFP protein itself, whereas their viability was affected by hydrophilic components within the low molecular mass cargo that copurified with tAFP. Identification of the distinct impact of circulating tAFP on NK-cell function and viability may be crucial to developing a strategy to ameliorate HCC patient NK-cell functional deficits. Cancer Immunol Res; 5(6); 493–502. ©2017 AACR.

Introduction

Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related deaths worldwide (1). It is an aggressive disease that has few viable treatment options, with the overall annual age-adjusted incidence rates increasing from 1.4 per 100,000 in 1975 to 1977 to 4.8 per 100,000 in 2005 to 2007 (2). It is believed to develop due to chronic liver inflammation, which causes multiple rounds of tissue damage, repair, and regeneration that ultimately leads to carcinogenesis (3, 4). The primary risk factors for developing HCC are cirrhosis (independent of its etiology; 80% of all HCC) and chronic infection with hepatitis B or C virus (HBV or HCV; refs. 5, 6).

Human α-fetoprotein (AFP) was the first oncofetal biomarker discovered, and its production has been associated with the development of HCC, as well as embryonic carcinoma, tumors of the yolk sac, teratocarcinoma, certain stromal tumors of the testis and ovary, hepatoblastoma, and flat-cellular liver cancer (7). It is a glycoprotein that is expressed by 50% to 80% of HCC (serum AFP concentrations are as high as 10 mg/mL). AFP belongs to the three-domain albuminoid protein family comprised of five members: albumin, vitamin D–binding protein, AFP, α-albumin, and the AFP-related gene (ARG) protein. It functions as a transporter of ligands, including copper, nickel, bilirubin, fatty acids, retinoids, steroids, phytoestrogens, and dioxins (8–11). AFP is the most abundant plasma protein during fetal development (with plasma concentrations in the fetus up to 3 mg/mL) and is thought to represent the fetal form of serum albumin. It is produced by the yolk sac and the fetal liver, and one of its suggested primary functions is to chaperone fatty acids into cells (11). The normal concentration of AFP in adult serum is 1 to 3 ng/mL, with increased concentrations found in individuals with liver disease. In cirrhotic patients, serum AFP is frequently elevated and, if >400 ng/mL, represents a biomarker for HCC (6). Cord blood “normal”– (nAFP) and tumor-derived AFP (tAFP) protein structures are identical, but they differ in their carbohydrate content. The fucosylated variant AFP-L3 is the predominant glycoform found in an HCC setting and is a poor prognosis marker. nAFP contains 5% of the fucosylated variant, whereas HCC patient serum contains >80% of the fucosylated variant (8, 11).

AFP has an immunosuppressive impact on natural killer (NK) cells, T cells, B cells, and dendritic cells (DC), which may play a role in HCC pathogenesis (11, 12). NK-cell function is inhibited in HCC patients compared with healthy donors (HD; ref. 13). HCC patient NK cells have impaired abilities to kill tumor targets and secrete cytokines (14, 15). In addition, overall frequencies of peripheral blood and liver NK cells are reduced in HCC patients.
and low infiltration of NK cells into tumors is associated with poor prognosis in patients with HCC (17, 18). NK cells are deficient in IFNγ production, a phenotype correlated with increased CD4+ CD25+ regulatory T cells and myeloid-derived suppressor cells in HCC patients (19).

APC can indirectly impair NK-cell activation by inhibiting IL12 production by DCs (20) and to inhibit NK-cell activity by enhancing the number and activity of bone marrow natural suppressor cells (7). However, the direct mechanism(s) underlying ACP-mediated NK-cell dysfunction remains unknown. The results of the current study suggest that nAFP and tAFP induce similar, yet distinct, changes in NK-cell function and viability, which may be influenced by their unique low molecular weight (LMW) cargos. We additionally show that nAFP and tAFP are both readily internalized by NK cells. These findings strongly suggest that future studies of AFP’s impact on immune function among HCC patients must specifically focus on tAFP.

Materials and Methods

Reagents

Human cord blood–derived nAFP (Cell Sciences), HCC cell line–derived tAFP (Bio-Rad; multiple lots of purified protein were tested, including numbers 0312612FP, 02A0913FP, and 64005863), and ovalbumin (OVA; Sigma) were used at 10 μg/mL in culture. NK-cell phenotype was examined using fluorochrome-conjugated antibodies against the following cell-surface markers: CD16-PE/ARC and CD3-PC7 (Beckman Coulter); CD56-FITC/PC7, CD69-PE, and BD Phosflow STAT3-PE (BD Biosciences); CD25/IL2 receptor (IL2R) e-PE, CD122/IL2R-Pe, and CD132/IL2R-PE (R&D Systems); and CD206/monosaccharide receptor (eBioscence); monoclonal mouse anti–human phospho-JAK1 and monoclonal rabbit anti–human phospho-JAK3 (Cell Signaling Technology). Alexa Fluor 647–conjugated polyclonal goat anti–mouse and goat anti–rabbit F(ab)2 fragments were used for secondary stainings (Cell Signaling Technology). For confocal microscopy analysis, Alexa Fluor 488–conjugated mouse anti–human AFP antibody (BD Biosciences) was used. Alexa Fluor 488 conjugation of nAFP, tAFP, and OVA, and OVA was performed using the Alexa Fluor 488 (Life Technologies) protein labeling Kit.

Cell lines

NK activity was tested against the human K562 erythroleukemia, whereas lymphokine-activated killer (LAK) activity was tested against the DAUDI lymphoma and HepG2 HCC cell lines. K562, DAUDI, and HepG2 cell lines were obtained from the ATCC in 2000, 2002, and 2016, respectively. K562 and DAUDI cells were passaged individually and tested monthly for cell-surface phenotype by flow cytometry. HepG2 was tested upon receipt from the ATCC by measuring AFP levels in cell-conditioned media. The cell lines were cultured in RPMI 1640 medium, supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% L-glutamine (Life Technologies), at 37 °C, in a humidified/5% CO2 atmosphere. K562 and DAUDI were passaged biweekly for >100 passages, when in culture. Cell lines were negative for mycoplasma contamination (monthly for K562 and Daudi, upon receipt from the ATCC for HepG2) by GEN-PROBE Mycoplasma Tissue Culture Non-Isotopic Rapid Detection System (Gen-Probe, Inc.).

Isolation of peripheral blood mononuclear cells

With informed consent, peripheral blood was obtained from HD and HCC patients (UPCI #04-001; UPCI #04-111). Donor and patient characteristics are described in Table 1. Peripheral blood mononuclear cells (PBMC) were separated from blood using Ficoll–Hypaque gradient centrifugation (Corning; ref. 21).

NK-cell isolation and culture

NK cells were purified from PBMCs by negative magnetic cell sorting selection using a human NK-cell isolation kit (Miltenyi Biotec). NK cells were cultured in AIM-V medium supplemented with 5% human AB serum (Life Technologies). NK cells were then left untreated (negative control), or they were cultured in the presence of 6,000 IU/mL recombinant human IL2 (i.e., 1,000 Cetus U/mL; PeproTech), nAFP, and/or tAFP for 1 to 6 days at 10^6 cells/mL (22, 23).

Flow cytometry

One-step staining of cell-surface antigens was performed using fluorochrome-conjugated primary antibodies as previously described (24–26). Cell viability was evaluated with the Annexin V–FITC Apoptosis Detection Kit II (BD Biosciences) according to the manufacturer’s protocol. Intracellular FACS staining using Alexa Fluor 488–conjugated anti–AFP or isotype IgG2a antibodies (BD Biosciences) was performed as per the manufacturer’s protocol. FACS analyses were performed using the Accuri C6 cytometer (BD Biosciences) and Gallios Flow Cytometer (Beckman Coulter), and analyzed using CFlow Plus and FlowJo v10 (FlowJo, LLC) software.

Intracellular cytokine staining

Healthy donor NK cells were stimulated with nAFP or tAFP for 2 hours at 37 °C. Cells cultured in media alone or with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) and ionomycin (Sigma; 2 μmol/L) were used as negative and positive controls, respectively. In order to induce NKP46 upregulation (an NK-cell–specific activation receptor), IL2 (6,000 IU/mL) was added to each stimulation except for negative control. Afterward, brefeldin A (Sigma) was added to cultures at 10 μg/mL, and cells were incubated for an additional 5 hours at 37 °C. Following the incubation, cells were analyzed for surface CD3–PC7, CD14–PE, CD56–FITC, and NKP46–BV510 (BD Biosciences) expression, and intracellular IL1β (APC; R&D Systems) or IL6 (APC; BD Biosciences) using the FoxP3 Staining Buffer Set (Miltenyi Biotec), and analyzed by flow cytometry.

NK-cell proliferation assays

Enriched NK cells were labeled using the Vybrant CFDA SE Cell Tracker Kit (CFSE; Life Technologies) according to the manufacturer’s protocol. Stained NK cells were cultured alone, with IL2 ± nAFP or tAFP. On day 6 of culture, cells were analyzed for CFSE dilution by flow cytometry using ModFit software (Verity Software House Inc.).

Antigen uptake and imaging flow cytometry

NK cells were cultured for 1 hour at 37 °C or on ice (negative control) with or without 10 μg/mL fluorescently labeled proteins in AIM-V media. For imaging flow cytometry, Alexa 488–conjugated antigen-loaded NK cells were costained with CD56–PC7, CD16–APC, Hoechst, and LysoTracker Red (LTR, used to label acidic vesicles; Life Technologies) per the manufacturers’ protocols. Cells were evaluated using the Amnis ImageStream X (EMD Millipore Sigma) imaging flow cytometer, and the data were analyzed with IDEAS software (EMD Millipore Sigma).
Microscopy
For live-cell imaging, freshly sorted NK cells, 10 μg/mL of Alexa Fluor 488–labeled protein, and Hoechst (nuclear stain; Life Technologies) were added to a 35-mm glass bottom microwell dish (MatTek Corp.) and placed on a live-cell environmental chamber stage (37°C/14°C, humidified, 5% CO2 atmosphere) for 1 hour to observe antigen uptake. Images were acquired using the Nikon LiveScan Swept Field Confocal Microscope (Nikon) with a 60x/1.4 NA oil immersion lens. The scan head was set to a 35 micron pinhole. Confocal was essential to exclude the green dye throughout the culture media. For confocal analysis of spontaneous serum AFP uptake by purified NK cells from HD and HCC patients with high concentrations of serum AFP, anti–AFP-Alexa 488 (BD Biosciences), Hoechst (nuclear stain), and rhodamine phalloidin (F-actin stain; Life Technologies) were used to label NK cells per the manufacturers’ protocols. After the staining, NK cells were cytospun onto glass slides and analyzed by a Nikon A1 confocal microscope (Nikon) with a 100X oil immersion lens. All image analysis was performed using the NIS-Elements (Nikon) software.

CellTracker Orange– and methylthiazolyldiphenyl-tetrazolium bromide–based cytotoxicity assays
CellTracker Orange–based flow cytometry assay was performed using K562 and DAUDI targets as previously described (26). Methylthiazolyldiphenyl-tetrazolium bromide–based cytotoxicity assay for adherent targets was performed as previously described using HepG2 adherent cells (27). The percentage of cytotoxic activity and lytic units (LU)10^7 effector cells were calculated as previously reported (28).

LMW and high molecular weight molecule separation and purification
nAFP and tAFP were first denatured in 10X PBS (Life Technologies) at 1:1 ratio for 30 minutes at room temperature. Protein solutions were diluted in ddH2O to 1X PBS and immediately separated into high molecular weight (HMW) and LMW fractions using Amicon Ultra 10 kDa Centrifugal Filters (Millipore). NK cells were cultured for 24 hours in the presence of 10 μg/mL of intact native proteins, individual LMW and HMW fractions extracted from 10 μg of protein, or HMW and LMW fractions together.

Reverse-phase high-performance liquid chromatography separation of hydrophilic molecules from tAFP
Note that 1 mg of tAFP (Bio-Rad) was separated on reverse-phase high-performance liquid chromatography (RP-HPLC) as previously described (29). Individual HPLC fractions were lyophilized to remove organic solvents (acetonitrile, trifluoroacetic acid). Samples were reconstituted in 500 μL of PBS, irradiated at 5,000 rads to sterilize, and stored at −80°C until use. Ten microliters of each test fraction was then used to assay for impact on NK-cell viability.

Measurement of secreted and cell-associated cytokines
Cell-free supernatants of NK-cell cultures were assessed for GM-CSF, IL1β, TNFa, IL6, and IFNy using the Cytokine Human 25-Plex Panel Luminex assay (Life Technologies).

Multiplex gene expression analysis
Total RNA samples isolated from NK cells (QIAGEN) were tested using the nCounter PanCancer Immune Profiling Panel
Statistical analysis

One-way ANOVA analyses were used to estimate statistical significance of differences of antigen upregulation, cytokine secretion, cell killing, and viability. Prior to analysis, the data were log-transformed. The calculations were performed using the GraphPad Prism v6 (GraphPad Software, Inc.). P values ≤ 0.05 were considered to be statistically significant.

Results

nAFP and tAFP were readily internalized by NK cells in vitro and in vivo

In order for nAFP or tAFP to directly affect NK-cell function, the proteins need to directly interact with NK cells. We evaluated the ability of AFP proteins to engage NK cells (Fig. 1). Fluorescently-

![Fluorescent images showing AFP internalization by NK cells](image_url)

**Figure 1.** Resting NK cells internalize AFP. Freshly-isolated HD NK cells were pulsed for 1 hour with Alexa Fluor 488-conjugated nAFP, tAFP, and OVA at 37°C or at 4°C. Unpulsed cells (Media) were used as the negative control. The ability of NK cells to internalize fluorescently-labeled proteins was measured by (A) flow cytometry and (B) confocal and live-cell microscopy, each method testing three HD in independent experiments. For flow cytometry, mean responses and SEMs are presented. For microscopy analyses, individual donor optical cross-sections showing protein localization within the cytoplasmic portion of the cells are shown. C, Imaging flow cytometry was also used to detect intracellular AFP and to evaluate whether internalized proteins were directed toward the endolysosomal compartments (details are described in Supplementary Fig. S1). Four internalization and colocalization patterns for AFP and LTR were observed (Supplementary Fig. S1B): AFP non-internalized and noncolocalized with LTR (Non-int. non-co-loc.), AFP internalized and non-co-localized with LTR (Int. non-co-loc.), AFP internalized and colocalized with LTR (Int. co-loc.), and AFP noninternalized and colocalized with LTR (Non-int. co-loc.). D, NK cells were sorted from 2 frozen HD and 2 HCC patient PBMCs (HCC1 and HCC2) and were analyzed for spontaneous in vivo AFP uptake by labeling cells with Alexa Fluor 488-conjugated anti-AFP antibody. Twenty images were captured for each of the donors/patients tested. Representative data from donors HD12 and HCC1 are shown. *, P < 0.05.
labeled nAFP, tAFP, and OVA were used to evaluate protein binding and uptake by NK cells by multiple approaches. FACS analyses showed that nAFP and OVA could each effectively label NK cells, even at 4°C (Fig. 1A). Confocal and live-cell microscopy showed intracellular punctate staining for nAFP, tAFP, and OVA and no cell surface labeling, suggesting that these proteins were internalized by HD NK cells (Fig. 1B). Imaging cytometry confirmed protein uptake and showed internalized pools of nAFP and OVA.

Figure 2.
Stimulation with nAFP and tAFP induces a proinflammatory, IL2-hyperresponsive cytokine profile in NK cells. HD (A) and HCC (B) NK cells were cultured overnight in the absence or presence of high-dose IL2, nAFP, tAFP and OVA as described in Materials and Methods. Cell activation status was subsequently determined by measuring secretion of IFNγ, GM-CSF, IL1β, IL6, and TNF in individual cell culture supernatants by Luminex. Data from 8 HD and 8 HCC patients independently tested are presented using Tukey box plot analysis. Triangles represent data outliers. *, P < 0.05.
nAFP and tAFP induced a unique proinflammatory, exaggerated IL2 response in NK cells

Earlier studies have suggested that nAFP desensitizes NK cells to subsequent IL2-mediated activation (30, 31). Here, by measuring cytokine secretion, CD69 and CD25/IL2Rα upregulation, and cytotoxic properties, we examined whether nAFP and tAFP were capable of inhibiting the activation and IL2 responsiveness of the three major NK-cell subsets: CD56lo CD16+, CD56+ CD16−, and CD56− CD16− (26, 32, 33). NK-cell supernatants were tested for the presence of a number of secreted/soluble factors. AFP proteins alone induced secretion of proinflammatory IL6. This was the only analyte that was readily induced from NK cells by AFP proteins (Fig. 2A). Low concentrations of IL1β and TNF were also induced in some HD. OVA did not promote cytokine secretion from NK cells. In contrast, IL2 modulated production of most of the assessed soluble factors except IL6. Production of these cytokines (e.g., IFNγ, GM-CSF, and TNF) was further enhanced when nAFP and tAFP, but not OVA, were combined with IL2 as a stimulus. In the case of IL1β and TNF, IL2 unexpectedly sensitized NK cells from the majority of HD to AFP, yielding enhanced cytokine production, demonstrating IL2/AFP synergism. These effects were AFP dose-dependent based on enhanced cytokine secretion as readout (Supplementary Fig. S2).

To confirm that IL6 and IL1β are derived from NK cells, we performed multi-color intracellular cytokine staining experiments. CD56+ NKp46+ CD3− CD14− NK-cell populations that were 96% to 99% pure were obtained according to FACS gating, and Annexin V and propidium iodide staining (Supplementary Fig. S3). Residual CD3+/CD14+ cells (<0.1% of total cell content) produced neither IL6 nor IL1β, whereas CD14+ cells (<0.03% of total cell content) did produce IL1β and IL6 in response to nAFP and tAFP (Supplementary Fig. S4). Considering the extremely low frequencies of monocytes in these cultures, it was unlikely that they contributed to the IL6 and IL1β amounts measured.

AFP-mediated NK-cell activation was further confirmed by testing CD69 and CD25 expression, which was increased in all three NK-cell subsets in response to nAFP and tAFP, suggesting activation (Supplementary Fig. S5). All NK-cell subsets in most donors evaluated their upregulated expression of CD69 and CD25 in response to nAFP and tAFP. On average, nAFP induced a greater degree of CD69 upregulation in comparison with tAFP. IL2 and AFP proteins (especially nAFP) synergized in their ability to activate NK cells. All NK-cell subsets in all donors showed elevated CD69 and CD25 expression when IL2 + nAFP and IL2-only treatments were compared. Again, nAFP was superior to tAFP at inducing CD69 upregulation. We further confirmed these observations with an exploratory multiplex gene expression analysis examining nAFP and tAFP effects on IFNγ, GM-CSF, IL1β, TNF, and CD25 (Supplementary Fig. S6). AFP/IL2 cooperation was not due to enhanced IL2 receptor signaling, because we observed modestly decreased Jak1 and Jak3 following IL2 activation (Supplementary Fig. S7).

NK- and LAK-cell activities were also enhanced by coculture with AFP. K562 targets were used to evaluate NK, whereas DAUDI and HepG2 cells, which are refractory to lysis mediated by resting NK cells, were used to assess alterations in LAK activity. nAFP and tAFP stimulation resulted in enhanced NK and LAK activities as measured by enhanced K562 and DAUDI killing, respectively (Supplementary Fig. S8A). In contrast to results related to CD69 upregulation and cytokine production, tAFP was more effective than nAFP at enhancing NK and, especially, LAK activities as seen by mean calculated lytic units (K562: 9575 vs. 6394; DAUDI: 12629 vs. 1264). Combined treatment with AFP + IL2 significantly enhanced NK-mediated killing of K562 cells in all donors tested. IL2 did not have a statistically additive effect with nAFP or tAFP on LAK activity as measured by DAUDI killing. Activated NK cells did lyse tAFP secreting HepG2 hepatoblastoma cells. Although nAFP and tAFP exposure did not enhance the ability of NK cells to lyse these cells, nAFP + IL2 stimulation did yield somewhat superior killing compared with IL2 alone, although
the difference was not statistically significant (Supplementary Fig. S7B).

NK cells isolated from HCC patients contained high levels of internalized AFP, regardless of their serum AFP concentrations (Fig. 1D; Supplementary Fig. S9; Table 1). It was important to examine whether exposing HCC patient NK cells to IL2 would induce a similar cytokine secretion profile to that of HD NK cells costimulated with nAFP/tAFP and IL2. After IL2 stimulation, only median concentrations of secreted TNF were within the range of HD NK cells costimulated with tAFP and IL2 (35 vs. 41 ng/mL for HD and HCC, respectively; Fig. 2B; Supplementary Table S1). Generally, HCC NK cells exhibited reduced or no secretion of IFN-γ, GM-CSF, IL1β, or IL6 when compared with HD NK cells.

Long-term exposure to tAFP, but not nAFP, induces NK-cell death

Given that AFP+ HCC patients are chronically exposed to tAFP, we next evaluated the effects of extended exposure to AFP on NK-cell frequency and function. tAFP negatively affected NK-cell viability (Fig. 3; Supplementary Fig. S10). We observed a significant decrease in the frequency of viable NK cells among all donors evaluated. Mean NK-cell viability was also moderately affected by nAFP and OVA (19.9% and 18.1% death, respectively), but not as drastically as tAFP (85.5% cell loss). A very limited effect on NK-cell proliferation by tAFP was seen (Supplementary Fig. S11A and S11B). Cytotoxicity assays revealed that nAFP and tAFP only promoted transient enhancement of NK and LAK activities, which was not sustained in long-term cultures (Supplementary Fig. S11C).

nAFP and tAFP glycoproteins and not their cargo directly activate NK cells

AFP can bind to and transport LMW hydrophobic ligands (8, 11). We have shown that both native tAFP and its LMW binding partners (<3 kDa) are needed to inhibit monocyte-derived DC differentiation and function (12). Based on these studies, we tested whether both AFP and its LMW fraction are needed to modulate NK-cell function. Purified HMW (AFP) and LMW (cargo copurified with AFP) molecules were compared for their ability to activate NK-cell subsets in short-term cultures (Fig. 4). HMW AFP exclusively induced activation of all NK-cell subsets as measured by CD69 upregulation, and IL1β, IL6, and TNF secretion. These data suggest that nAFP and tAFP glycoproteins, and not their LMW cargos, directly activated NK cells.

tAFP-derived hydrophilic LMW cargo negatively affects NK-cell survival

We next determined whether the negative effect of tAFP on NK-cell viability in long-term cultures was mediated by the tAFP protein or its LMW cargo (tAFP LMW). We observed that viability...
was exclusively affected by tAFP LMW (Fig. 5A). Evaluation of affected NK-cell subsets showed that the CD56loCD16+ subset was the most sensitive to tAFP LMW-induced cell death. Consequently, CD56loCD16- and CD56hiCD16- subsets became more predominant in extended cultures containing tAFP LMW (Fig. 5B). For the CD56hiCD16- subset of NK cells, it appeared that both tAFP LMW and HMW played a role in the observed increase in cell frequency. When both fractions were recombined, we observed that CD56hiCD16- NK-cell frequency modulation was equal to culture with unfraccionated tAFP.

To examine whether hydrophilic or hydrophobic components of tAFP LMW preferentially promote NK-cell death, RP-HPLC was used to separate these species (Supplementary Fig. S12). Consistent with the dominant inhibitory role of hydrophilic components on NK-cell viability, only early RP-HPLC fractions (i.e., fractions 3 and 4 that were eluted in low concentrations of organic solvent) induced NK-cell death in vitro (Fig. 5C).

**Discussion**

Reduced NK-cell numbers, function, and infiltration into tumors are poor prognostic indicators for patients with HCC (13–18). Indeed, preoperative NK-cell activity has been identified as an independent prognostic factor for HCC patients after hepatectomy (13). Mechanisms underlying NK-cell deficiency or dysfunction in HCC patients remain unresolved. Previous studies suggested that AFP can indirectly impair systemic NK-cell activation by preventing IL12 (a.k.a. NK-stimulating factor, NKSF) production by DCs, and by enhancing the number and activity of bone marrow natural suppressor cells (7, 20). However, no direct effect of AFP on NK-cell biology has been demonstrated to date.

Although human in vitro and murine studies suggested that AFP desensitizes NK cells to activation by IFNγ, poly I:C, or IL2 (30, 31), clinical data appear to suggest otherwise. Administration of rIL2 or rIFNα significantly increases LAK activity in HCC patients, albeit with limited clinical benefit (34, 35). Reduced NK-cell cytotoxicity has been directly correlated with tumor burden and not with HCC patient serum concentrations of AFP, suggesting that other factors play a central role in NK-cell dysfunction in these patients (35–37). In this regard, immunosuppressive cytokines such as IL10 and TGFβ have been implicated in HCC-associated NK-cell suppression (38).

The data reported in the current study may help explain the observed clinical impact of IL2 therapies on HCC patient NK-cell cytotoxicity. We show that nAFP and tAFP were not immunosuppressive in the short-term. Instead, both tAFP and nAFP induced a proinflammatory, IL2 hyperresponsive phenotype in HD NK cells. Functional AFP/IL2 synergy was not mediated by enhanced IL2 receptor signaling in NK cells, suggesting that an alternate signaling pathway was likely activated by AFP.

Our findings also showed that resting NK cells can readily take up AFP. We showed that nAFP and tAFP internalized by CD56loCD16+ and CD56hiCD16- subsets were localized in nonacidic vesicles, whereas at least half of CD56hiCD16- cells also contained internalized proteins in acidic vesicles. As intracellular AFP receptors have been reported to play a role in malignant tumor biology (39), it is feasible that nAFP and tAFP internalized by NK cells can also engage intracellular receptor(s), which could mediate NK-cell biology.

The cytokine profile of AFP-activated HD NK cells is of particular importance. Few reports support the ability of NK cells to produce proinflammatory IL1β and IL6 (40, 41). Sustained high production of IL6, IL1β, and TNF promotes liver inflammation, fibrosis, and HCC development (42, 43). Our data indicate that AFP may play a role in HCC oncogenesis by promoting chronic inflammation via sustained secretion of proinflammatory cytokines by NK cells. In contrast, the cytokine profile of HCC patient NK cells may suggest that during late-stage disease, TNF may represent the dominant proinflammatory cytokine involved in disease progression.

NK cells have also been implicated in inducing liver damage in patients with chronic hepatitis virus infections. Elevated serum
AFP is detected in HBV and HCV patients with chronic active hepatitis, cirrhosis, and in patients with acute, but not chronic/persistent hepatitis (44, 45). Furthermore, elevated concentrations of fucosylated AFP (i.e., higher amount of tAFP secretion) have been detected in HBV and HCV patients with cirrhosis (46). During these stages of HBV and HCV infection, similar aberrations in NK-cell function, frequency, and subset distribution have been observed as in HCC (47–50). These studies also show that in immune-activated patients with liver injury, NK cells do not produce IFNγ and are skewed toward cytolytic activity when compared with immune-tolerant carriers and HD. Here, we report that AFP-activated NK cells mediate superior cytoxicity, but do not produce IFNγ. It will be of future importance to examine whether NK cells in patients with HBV- or HCV-induced liver damage produce proinflammatory IL6, IL1β, and TNF and, if so, whether such production can be correlated to elevated serum AFP in these patients.

We also show that tAFP has dichotomous short- and long-term impact on NK cells based on the composite effects of its native glycoprotein and its LMW cargo, respectively. Although tAFP promotes NK-cell activation in short-term cultures, its hydrophilic LMW cargo inhibits NK-cell viability. CD56loCD16+ promotes NK-cell activation in short-term cultures, its hydrophilic glycoprotein and its LMW cargo, respectively. Although tAFP in these patients. AFP has been reported to bind to a broad array of endpoints can be correlated with serum tAFP concentrations in ultimately be of interest to determine whether these biological endpoints can be correlated with serum tAFP concentrations in these patients. AFP has been reported to bind to a broad array of partially characterized molecules. We are currently pursuing the molecular identity of the NK-suppressive LMW cargo of tAFP, in order to develop translational targeted interventional strategies.

Disclosure of Potential Conflicts of Interest

L.H. Butterfield is co-inventor on a patent regarding AFP-specific T cell activation that has not been licensed. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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