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Tumor-Derived α -Fetoprotein Impairs the Differentiation and T Cell Stimulatory Activity of Human Dendritic Cells

Angela D. Pardee,* Jian Shi,* and Lisa H. Butterfield*,†,‡,§

Several tumor-derived factors have been implicated in dendritic cell (DC) dysfunction in cancer patients. α -fetoprotein (AFP) is an oncofetal Ag that is highly expressed in abnormalities of prenatal development and several epithelial cancers, including hepatocellular carcinoma (HCC). In HCC patients exhibiting high levels of serum AFP, we observed a lower ratio of myeloid/plasmacytoid circulating DCs compared with patients with low serum AFP levels and healthy donors. To test the effect of AFP on DC differentiation in vitro, peripheral blood monocytes from healthy donors were cultured in the presence of cord blood–derived normal AFP (nAFP) or HCC tumor-derived AFP (tAFP), and DC phenotype and function were assessed. Although the nAFP and tAFP isoforms only differ at one carbohydrate group, low (physiological) levels of tAFP, but not nAFP, significantly inhibited DC differentiation. tAFP-conditioned DCs expressed diminished levels of DC maturation markers, retained a monocyte-like morphology, exhibited limited production of inflammatory mediators, and failed to induce robust T cell proliferative responses. Mechanistic studies revealed that the suppressive activity of tAFP is dependent on the presence of low molecular mass (LMM) species that copurify with tAFP and function equivalently to the LMM fractions of both tumor and nontumor cell lysates. These data reveal the unique ability of tAFP to serve as a chaperone protein for LMM molecules, both endogenous and ubiquitous in nature, which function cooperatively to impair DC differentiation and function. Therefore, novel therapeutic approaches that antagonize the regulatory properties of tAFP will be critical to enhance immunity and improve clinical outcomes. *The Journal of Immunology*, 2014, 193: 5723–5732.

An oncofetal protein synthesized in the yolk sac and fetal liver, α -fetoprotein (AFP) is the most abundant serum protein in the fetus, reaching a maximum level of 3 mg/ml in the third month of gestation (1). Neural tube defects, chromosomal abnormalities, and other fetal abnormalities can be identified by elevated levels of AFP in the amniotic fluid. AFP is transcriptionally repressed shortly after birth, and normal adult levels typically range between 1 and 40 ng/ml. Reappearance of AFP in the circulation of adults is associated with liver regeneration, hepatitis, chronic liver diseases, and malignant growth, particularly hepatomas, teratomas, and gastrointestinal cancers of endodermal origin (2). Various glycoforms of AFP have been identified in the serum of hepatocellular carcinoma (HCC) patients (3, 4). The fucosylated variant AFP-L3 is

the major glycoform found in individuals with HCC and is associated with poor prognosis. Although cord serum–derived AFP contains <5% of the fucosylated variant, >80% of the fucosylated variant has been observed in HCC patient serum.

Several functions of AFP have been described. With its structural similarity to albumin, it was hypothesized to play a role in the transport of serum components, including fatty acids, steroids, and heavy metals, and it may serve as an embryonic analog of albumin (2). There also were reports of AFP interfering with intracellular signaling, including both the caspase-3 and PI3K/AKT pathways (5, 6). An immunoregulatory role for AFP also was proposed. Early studies revealed an inhibitory effect of cord blood–derived human AFP on lymphocyte function, whereas more recent reports suggest that AFP exerts its immunosuppressive activity through the induction of dendritic cell (DC) dysfunction (7–12). However, there is little consensus about which cell subsets and/or signaling pathways are the primary targets of AFP-mediated immunosuppression. Furthermore, because the majority of these studies solely used cord blood AFP preparations, the immunoregulatory nature of tumor-derived AFP remains unknown.

Given its specificity to HCC tumors, AFP represents an attractive target for immunotherapy. Several MHC class I and II restricted epitopes have been identified (13). Our group observed that CD8⁺ T cells from healthy donors (HDs) can recognize 4 dominant and 10 subdominant HLA-A*0201–restricted peptides in vitro (14). Two clinical trials were initiated to test four immunodominant AFP peptides emulsified in Montanide adjuvant and AFP peptide-pulsed autologous DCs (15, 16). Although no objective clinical responses were observed in the small numbers of vaccinated patients, AFP-specific T cell responses were either generated or enhanced in the majority of patients, providing proof-of-principle for AFP-targeted immunotherapeutic approaches.

To more fully characterize the immunosuppressive activity of AFP, as well as to inform the rational design of AFP protein-based vaccines, we examined the effects of cord blood–derived normal AFP (nAFP) or HCC tumor-derived AFP (tAFP) on DC differentiation

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; AFP, α -fetoprotein; DC, dendritic cell; HCC, hepatocellular carcinoma; HD, healthy donor; HMM, high molecular mass; iDC, immature DC; Lin1, lineage mixture 1; LMM, low molecular mass; mDC, mature DC; MDSC, myeloid-derived suppressor cell; MMCO, molecular mass cutoff; myDC, myeloid DC; nAFP, cord blood–derived normal AFP; pDC, plasmacytoid DC; PNGaseF, peptide-N-glycosidase F; tAFP, tumor-derived AFP.

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and function. In this study, we show that tAFP has profound negative effects on the generation, maturation, and function of monocyte-derived DCs and that this activity is dependent on the presence of low molecular mass (LMM) molecules that copurify with tAFP. Moreover, we report that elevated serum levels of AFP are associated with alterations in the frequency of DC subsets in HCC patient peripheral blood.

Materials and Methods

Abs and reagents

Purified OVA (Fisher Scientific), human cord serum AFP (Cell Sciences; purity > 95% by SDS-PAGE), and HCC cell line culture-derived AFP (Bio-Rad; purity > 95% by SDS-PAGE) were added to cultures at 10 µg/ml, unless otherwise indicated. AFP concentrations were routinely confirmed by clinical laboratory tests (University of Pittsburgh Medical Center), and the degree of fucosylation (AFP-L3 percentage) was determined by Quest Diagnostics (both Clinical Laboratory Improvement Amendments–certified assays). As expected, the tAFP preparations used in our experiments exhibited a high degree of fucosylation (61.7–84.6%) compared with nAFP (3.6–5.2%). Lot-to-lot variability in the preparations' inhibitory activity was negligible (data not shown). AFP proteins were examined by stained SDS-PAGE gels and Western blot to confirm identity (Santa Cruz anti-AFP SC-8399) (Supplemental Fig. 1A). AFP and OVA preparations were divided into high molecular mass (HMM) and LMM fractions using Amicon Ultra 3kDa MWCO Centrifugal Filters (Millipore). DC, T cell, and myeloid-derived suppressor cell (MDSC) phenotypes were examined using fluorochrome-conjugated Abs against the following cell surface markers: HLA-ABC (BioLegend), CD206 (mannose receptor), CD40, CD80, CD83, IL-2, IFN-γ, lineage mixture 1 (Lin1; CD3, CD14, CD16, CD19, CD20, CD56), CD33 (BD Biosciences), CD11c/BDCA-1, CD303a/BDCA-2 (eBioscience), CD4, CD8, TNF-α, HLA-DR, CD14, and CD11b (Beckman Coulter). DC apoptosis was detected using annexin V-FITC and 7-aminoactinomycin D (7-AAD) staining (BD Pharmingen).

Cell lines

T2 cells (HLA-A2⁺; American Type Culture Collection), T2-DR4 cells (HLA-DR4⁺; kindly provided by Dr. Janice Blum, Indiana University School of Medicine, Indianapolis, IN), and the HepG2 hepatoma cell line (American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine (all reagents from Life Technologies). Cultures were maintained in a humidified 37°C incubator under 5% CO₂ tension.

Isolation of PBMCs

PBMCs were obtained from HDs and from HCC patients enrolled in an AFP DNA plasmid prime/adenovirus boost vaccine or a peptide-pulsed DC vaccine clinical trial (UPCI #04-001; UPCI #04-101, IND BB13653; UCLA IRB #00-01-026, IND BB9395; informed consent was obtained from all patients and donors). Limited patient data are listed in Supplemental Table I. PBMCs were separated from blood using gradient centrifugation (Ficoll-Paque; GE Healthcare).

DC preparation

CD14⁺ monocytes were isolated from PBMCs using MACS (Miltenyi Biotec) and cultured for 5 d in 800 IU/ml rGM-CSF (sargramostim; Genzyme) and 500 IU/ml rIL-4 (R&D Systems). In some cases, DCs were matured with IFN-γ (1000 IU/ml; PeproTech) and LPS (250 ng/ml; Sigma-Aldrich) for an additional 24 h prior to collection. DC culture supernatants were tested by multiplex Luminex assay (Life Technologies) for 30 analytes (cytokines, chemokines, and growth factors), as previously described (17).

Confocal immunofluorescence staining and imaging

DCs cultured in eight-well chamber slides (Nunc) were fixed (with 4% paraformaldehyde), permeabilized (0.1% Triton X-100), and stained with rhodamine phalloidin (Life Technologies) and DRAQ5 (eBioscience) to label F-actin and nuclei, respectively. Images were acquired using a Leica TCS-SL confocal microscope.

T cell proliferation, multimer staining, and cytokine production assays

For MLR assays, 2×10^4 mature DCs were cocultured with 2×10^5 allogeneic CFSE-labeled CD8⁺ or CD4⁺ T cells in T cell media (RPMI 1640 medium supplemented with 10% human AB serum in the presence of

recombinant human IL-2 [30 IU/ml; PeproTech]). Cell proliferation was tested by measuring CFSE dilution on day 6. For intracellular cytokine staining, T cells were stimulated with PMA (0.2 ng/ml) and ionomycin (0.2 µM) in the presence of brefeldin A. Six hours later, T cells were stained for surface markers, fixed, permeabilized, and stained for intracellular cytokines (IL-2, TNF-α, and IFN-γ).

For flu peptide-specific assays, immature DCs from HLA-A2⁺ or HLA-DR4⁺ donors were cultured overnight with 10 µg/ml FluM1_{58–66} (University of Pittsburgh Peptide Synthesis Facility) or HA_{307–319} (Mimotopes), respectively, in the presence of IFN-γ and LPS. The next day, 2×10^5 FluM1_{58–66} or HA_{307–319} loaded mature DCs were cocultured with 1×10^6 autologous CD8⁺ or CD4⁺ T cells, respectively, in T cell media plus 30 IU/ml IL-2. Cultures were supplemented with IL-2 (30 U/ml) every 3–4 d. After 11 d, CD8⁺ T cells were collected and stained with PE-labeled FluM1_{58–66} pentamer (ProImmune) plus CD8-allophycocyanin. For intracellular cytokine staining, CD8⁺ or CD4⁺ T cells were restimulated with FluM1_{58–66}-pulsed T2 cells or HA_{307–319}-pulsed T2-DR4 cells in the presence of brefeldin A. Six hours later, T cells were stained for surface markers, fixed, permeabilized, and stained for intracellular cytokines (IL-2, TNF-α, and IFN-γ). Data were acquired with an Accuri C6 cytometer (BD Biosciences) and analyzed using Accuri C6 software.

Enzymatic treatment of AFP

For all enzymatic treatments, AFP and OVA preparations were first heat denatured at 95°C for 15 min. Proteinase K (Ambion; 0.5 µg enzyme/1 µg AFP) or OVA control was added at 56°C for 4 h. AFP degradation was confirmed by a clinical laboratory test, which detected 0 ng/ml AFP. Peptide-N-glycosidase F (PNGase F) (New England BioLabs) was added to AFP and OVA preparations, according to the manufacturer's instructions, and incubated for 2 h at 37°C. α-L-Fucosidase (Sigma-Aldrich) was added to AFP and OVA preparations at 0.5 U/ml and incubated overnight at 37°C. Enzymes were used at concentrations recommended by the manufacturer based on functional testing by lot. After all treatments, enzymes were heat inactivated at 95°C for 15 min before AFP and OVA preparations were added to culture.

Preparation of cell lysates

HepG2 hepatoma cells and HD PBMCs were resuspended in 0.9% saline and lysed by three freeze (on methanol and dry ice)–thaw (37°C) cycles. Larger particles were removed by centrifugation (13,000 rpm, 20 min, 4°C), and supernatants were either left whole or separated into HMM and LMM fractions using Amicon Ultra 3kDa MWCO Centrifugal Filters (Millipore). Lysates were added to cultures on day 0 at a lysed cell/monocyte ratio of 1:1, and DC were collected and analyzed on day 5.

Gene regulation and signaling analysis

DCs cultured with nAFP, tAFP, or OVA, as described above, were lysed, and total mRNA was isolated and quantified. Transcriptome analysis was performed by Affymetrix HG-U133A array and analyzed by Significance Analysis of Microarrays and Ingenuity software. Selected immune-related gene regulation by nAFP and tAFP was confirmed by real-time PCR using MicroFast 96-well quantitative PCR plates and primers (Life Technologies) and qPCR SuperMix (VWR), with HPRT1 used as a housekeeping gene for normalization. To confirm NF-κB pathway changes, total NF-κB p65, phospho-NF-κB, total IκBα, and phospho-IκBα were examined by Western blot (Cell Signaling NF-κB sampler), with β-actin used as a housekeeping protein for densitometric calculations. Expression of selected NF-κB pathway immune-related genes were tested further by real-time PCR using TaqMan Gene Expression MasterMix and MicroFast 96-well plates (Life Technologies), with HPRT1 as the housekeeping gene, according to the manufacturer's instructions.

Statistical analysis

All comparisons of intergroup means were performed using a Student *t* test, with *p* values < 0.05 considered significant.

Results

Elevated serum AFP levels are associated with a lower myeloid DC/plasmacytoid DC ratio in the peripheral blood of HCC patients

Two independent studies showed that the frequency of circulating myeloid DCs (myDC) is decreased in HCC patients (18), and the frequency of circulating plasmacytoid DCs (pDCs) is significantly increased in the context of advanced liver disease (19). We tested

banked PBMC samples from HCC patients (Supplemental Table I) for levels of myDCs (Lin1[−]CD1c⁺) and pDCs (Lin1[−]CD303a⁺) (Fig. 1A). When patients were stratified into AFP^{low} (serum AFP < 1 μg/ml) and AFP^{high} (serum AFP > 1 μg/ml) groups, the mean myDC/pDC ratio decreased significantly for AFP^{high} HCC patients compared with AFP^{low} HCC patients and HDs (Fig. 1B).

DC differentiation is inhibited by physiologic levels of tAFP

To investigate the effect of AFP on DC differentiation *in vitro*, monocytes were isolated from the PBMCs of HDs and cultured for 5 d in the presence of GM-CSF + IL-4 and either nAFP or tAFP. Because median AFP serum levels of 9 μg/ml have been observed in HCC patients (2), nAFP and tAFP were added to the culture at a biologically relevant dose range (1–20 μg/ml), with OVA (a member of the albuminoid gene superfamily) used as a control. Both nAFP- and tAFP-treated DCs expressed diminished levels of the DC markers HLA-ABC, CD206 (mannose receptor), CD40, CD80, and CD83, whereas elevated levels of the monocyte marker CD14 were observed (Fig. 2A). Expression levels of HLA-DR, CCR7, and CD86 were not significantly altered (data not shown). However, a high dose of nAFP (10–20 μg/ml) was necessary to suppress DC differentiation, consistent with a previous report (9). Conversely, tAFP demonstrated suppressive activity at lower doses (5–10 μg/ml), suggesting that DCs are particularly sensitive to the tumor-derived isoform of AFP. As depicted in Fig. 2B, when cells were treated at a concentration of 10 μg/ml (used for all subsequent experiments), only CD83 expression was significantly

diminished in nAFP-conditioned DCs, whereas expression levels of all five DC markers were significantly reduced on tAFP-conditioned DCs compared with both nAFP- and OVA-treated cells. CD14 expression was significantly elevated by nAFP treatment ($p < 0.05$ versus OVA) and was enhanced to an even greater extent by tAFP treatment ($p < 0.01$ versus OVA), suggesting that AFP (and tAFP in particular) blocks the differentiation of DCs from monocyte precursors.

It was documented that the differentiation program of DC precursors can be skewed toward MDSC generation by various tumor-associated factors (20). Therefore, we assessed our AFP-treated, GM-CSF- and IL-4-cultured DCs for an MDSC phenotype (Lin1[−]HLA-DR^{low}CD11b⁺CD33⁺), but no evidence of MDSC skewing was observed (Supplemental Fig. 1B).

Consistent with their phenotype, tAFP-conditioned DCs also displayed a poorly differentiated morphology, as determined by fluorescent microscopy (Fig. 3A). tAFP-treated DCs lacked prominent dendrites, which were present in their nAFP- and OVA-treated counterparts and, in general, retained a monocyte-like morphology. Along with the surface marker analysis above, these data indicate that both DC phenotype and morphology are negatively impacted by physiologic levels of tAFP. Although cell recovery was modestly reduced in the tAFP-treated group (Fig. 3B), the percentage of apoptotic cells after 5 d of treatment did not exceed 14% (Fig. 3C), suggesting that tAFP does not impair DC differentiation primarily through the induction of cell death.

The production of inflammatory cytokines and chemokines is compromised in tAFP-conditioned DCs

To further characterize the effect of AFP on DC generation and function, cell-free supernatants from the previous cultures (Fig. 2B) were assessed by a 30-plex Luminex assay. Production of several cytokines, including IL-1β, IL-6, TNF-α, IL-7, and IL-15, were not significantly changed by nAFP or tAFP treatment, and IL-12 was undetectable in these samples (data not shown). Although not statistically significant, there was a trend toward lower levels of IFN-α secreted by tAFP-conditioned DCs (Fig. 3D). However, we observed a highly significant ($p < 0.01$) decrease in IL-8 production by tAFP-conditioned DCs compared with both nAFP- and OVA-treated cells. We also observed elevated levels of IL-10 in the culture supernatant of both nAFP- and tAFP-treated DCs. The chemokines MCP-1/CCL2, MIP-1α/CCL3, and MIP-1β/CCL4, which are known to recruit innate cells (neutrophils, monocytes, NK cells) to sites of infection/inflammation and enhance the cytolytic activity of NK cells, were produced at markedly lower levels in tAFP-conditioned DCs compared with nAFP- and OVA-treated cells (21, 22).

To examine the molecular mechanism involved in the phenotypic and secreted molecule changes induced by tAFP and nAFP, DC from five different donors were tested for gene expression changes by gene array. Several immune-related genes were significantly downregulated in tAFP-treated DCs, including CCR7, CXCL1, and MIP-1α/CCL3, whereas IL-6 and CD14 mRNAs were upregulated (Supplemental Fig. 2A). Because the NF-κB pathway is known to be critical for DC maturation and inflammatory cytokine regulation (23), and several of the specific gene products we identified by microarray, we examined whether this signaling pathway was modulated by tAFP. We tested this first by examining gene products specifically regulated by the NF-κB pathway by real-time PCR. In immature DCs (iDC), tAFP increased IL-6 mRNA levels and decreased expression of CD40, IL-12A, IL-12B, and CD83 (Supplemental Fig. 2B). In mature DCs (mDC), tAFP increased IL-10 mRNA levels and decreased IL-12A, IL-12B, and CD83 (Supplemental Fig. 2B). To further confirm the modulation

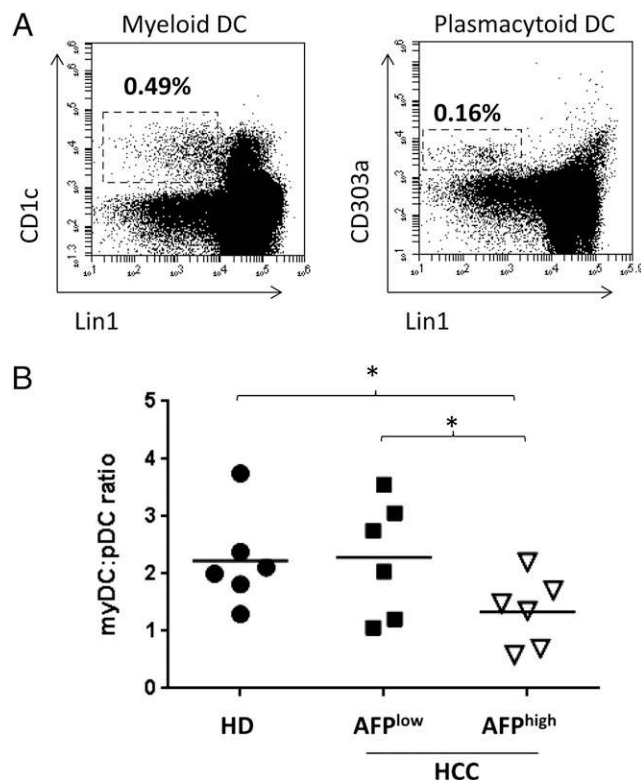


FIGURE 1. Alteration of circulating DC subsets in the peripheral blood of AFP^{high} HCC patients. **(A)** Circulating DC gating strategy. Percentages of myDCs (Lin1[−]CD1c⁺) and pDCs (Lin1[−]CD303a⁺) among total PBMCs. Shown is representative data from HCC patient C3. **(B)** Myeloid DC/pDC ratio in the peripheral blood of HDs ($n = 6$) or HCC patients ($n = 12$) was determined by flow cytometry. HCC patients were divided into AFP^{low} (serum AFP < 1 μg/ml) and AFP^{high} (serum AFP > 1 μg/ml) groups. * $p < 0.05$.

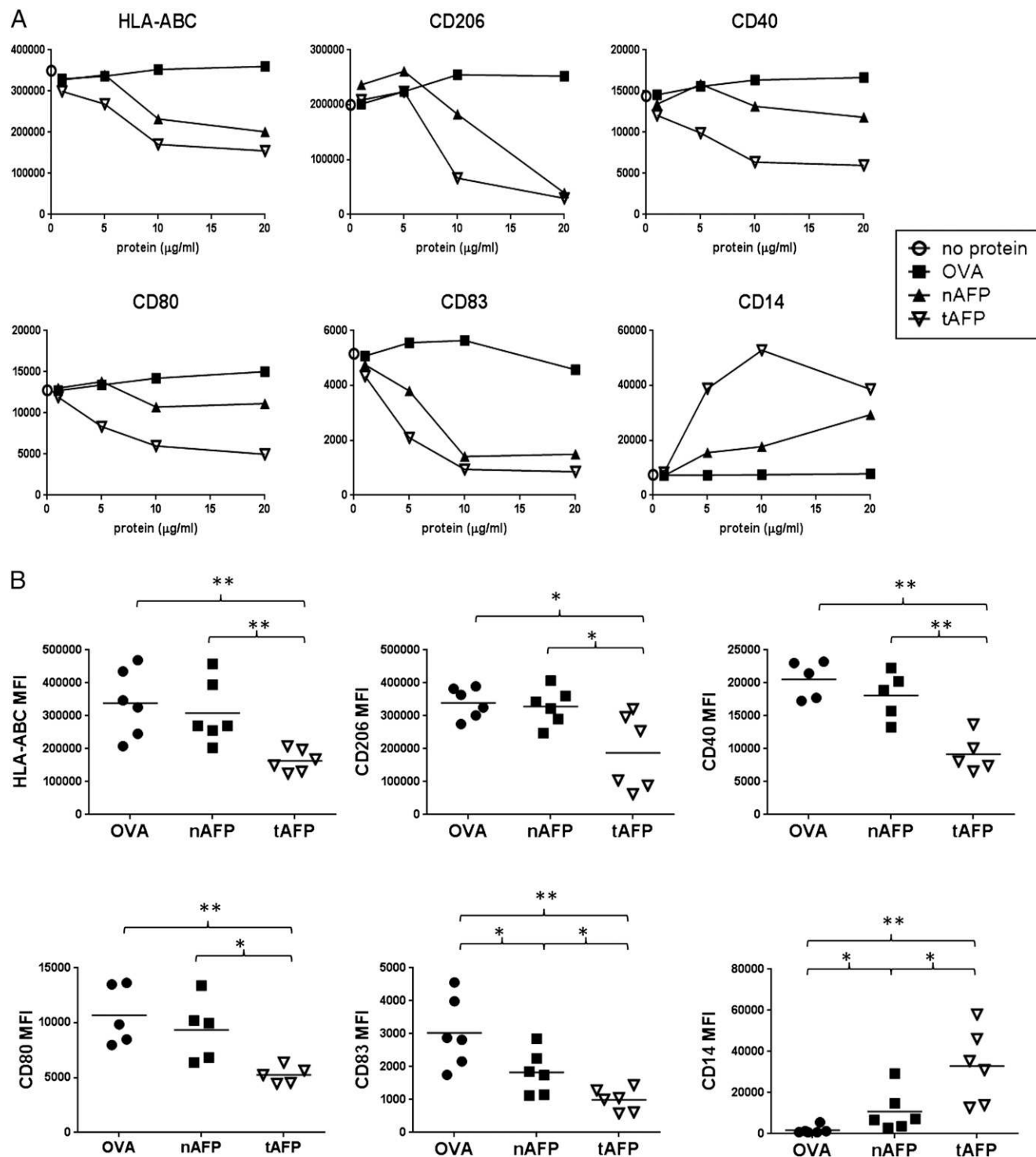


FIGURE 2. DCs fail to acquire a fully differentiated phenotype when cultured in the presence of tAFP. **(A)** CD14⁺ monocytes isolated from the peripheral blood of HDs were cultured with GM-CSF and IL-4 for 5 d either alone (“no protein”) or in the presence of increasing doses of OVA, nAFP, or tAFP (1, 5, 10, 20 μg/ml). Mean fluorescence intensity (MFI) of HLA-ABC, CD206 (mannose receptor), CD40, CD80, CD83, and CD14 is indicated. Data from one representative HD (of three HDs tested) are shown. **(B)** Monocytes from HDs ($n = 5-6$) were differentiated into DCs as in **(A)** in the presence of OVA, nAFP, or tAFP (all at 10 μg/ml). The horizontal lines represent the mean values. * $p < 0.05$, ** $p < 0.01$.

of the NF- κ B pathway by tAFP, Western blotting showed that, in both iDCs and mDCs, tAFP-treated DC have less total NF- κ B protein. As expected, maturation with IFN- γ and LPS increases phospho-NF- κ B in both OVA-conditioned and tAFP-conditioned DCs. I κ B α phosphorylation was reduced by 51–63% in both iDCs and mDCs exposed to tAFP (Supplemental Fig. 2C). Together, these tAFP-induced changes in gene expression, protein expres-

sion, and phosphorylation support tAFP modulation of the NF- κ B pathway.

Alloreactive T cell proliferation, but not cytokine production, is impaired by tAFP-conditioned DCs

To enhance their allostimulatory activity, OVA-, nAFP-, and tAFP-conditioned DCs were cultured overnight with LPS and IFN- γ to

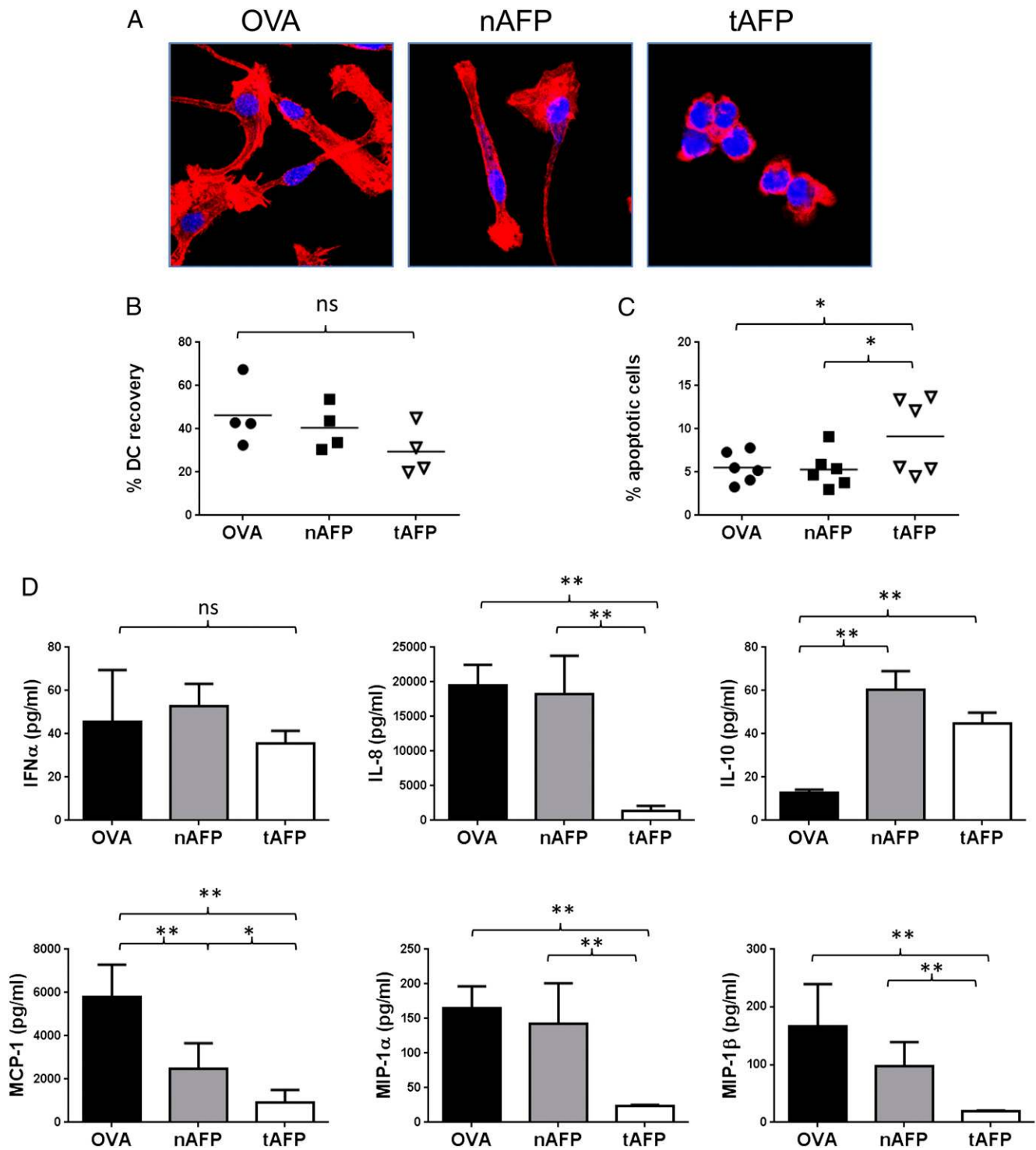


FIGURE 3. tAFP impairs DC morphology, viability, and the production of inflammatory cytokines and chemokines. Purified monocytes from HDs ($n = 4$) were differentiated into DCs for 5 d in the presence of OVA, nAFP, or tAFP (all at 10 $\mu\text{g/ml}$). **(A)** Representative confocal analysis of OVA-, nAFP-, and tAFP-conditioned DC. Actin (red), nuclei (blue). Images are representative of three independent experiments performed and were taken using a 63 \times objective. **(B)** Percentage of DC recovery (from total plated monocytes) on day 5 of culture is shown for four HDs. **(C)** DC apoptosis was assessed by annexin V and 7-AAD staining on day 5 of cultures, with the percentage of apoptotic cells calculated as the sum of the percentage of annexin V⁺/7AAD⁻ and annexin V⁺/7AAD⁺ cells. Data from six HDs are shown. **(D)** Cell-free supernatants were collected from the above cultures and tested for IFN- α , IL-8, IL-10, MCP-1/CCL2, MIP-1 α /CCL3, and MIP-1 β /CCL4 by Luminex assay. * $p < 0.05$, ** $p < 0.01$. ns, $p \geq 0.05$.

generate mDCs. Although this maturation step enhanced the expression of HLA-ABC, CD206, and CD40 by tAFP-conditioned DCs to levels similar to those expressed by OVA-DCs, CD80 and CD83 expression remained significantly lower (and CD14 significantly higher) in tAFP-treated mDCs compared with their

nAFP- and OVA-treated counterparts (Supplemental Fig. 3A). The production of inflammatory cytokines and chemokines (TNF- α , IL-12, MCP-1, and MIP-1 α) also was significantly reduced in tAFP-treated mDCs (Supplemental Fig. 3B). Similar to immature DCs, the recovery and viability of tAFP-treated mDCs were

slightly impaired, although the percentage of apoptotic cells after 5 d of treatment did not exceed 10% (Supplemental Fig. 3C, 3D).

When these OVA-, nAFP-, and tAFP-conditioned mDCs were used to stimulate allogeneic T cells in an MLR, considerable variability between donor-pair responses was observed, with OVA-conditioned mDC-induced proliferation at day 6 ranging from 34 to 72%. To standardize the donor-pair responses, nAFP-conditioned mDC-induced and tAFP-conditioned mDC-induced proliferation were normalized to control OVA-conditioned mDC-induced proliferation (set at 100%). This showed that both nAFP-conditioned mDCs and tAFP-conditioned mDCs, but particularly tAFP-conditioned mDCs, fail to induce a robust allogeneic T cell proliferative response (Fig. 4A). CD8⁺ T cell proliferation was significantly impaired by both nAFP-conditioned mDCs and tAFP-conditioned mDCs compared with OVA-conditioned mDC control ($p < 0.01$ for both). Meanwhile, proliferation of the CD4⁺ T cell subset was only inhibited by tAFP-conditioned mDCs in comparison with nAFP-conditioned mDCs ($p < 0.05$) and OVA-conditioned mDC control ($p < 0.01$).

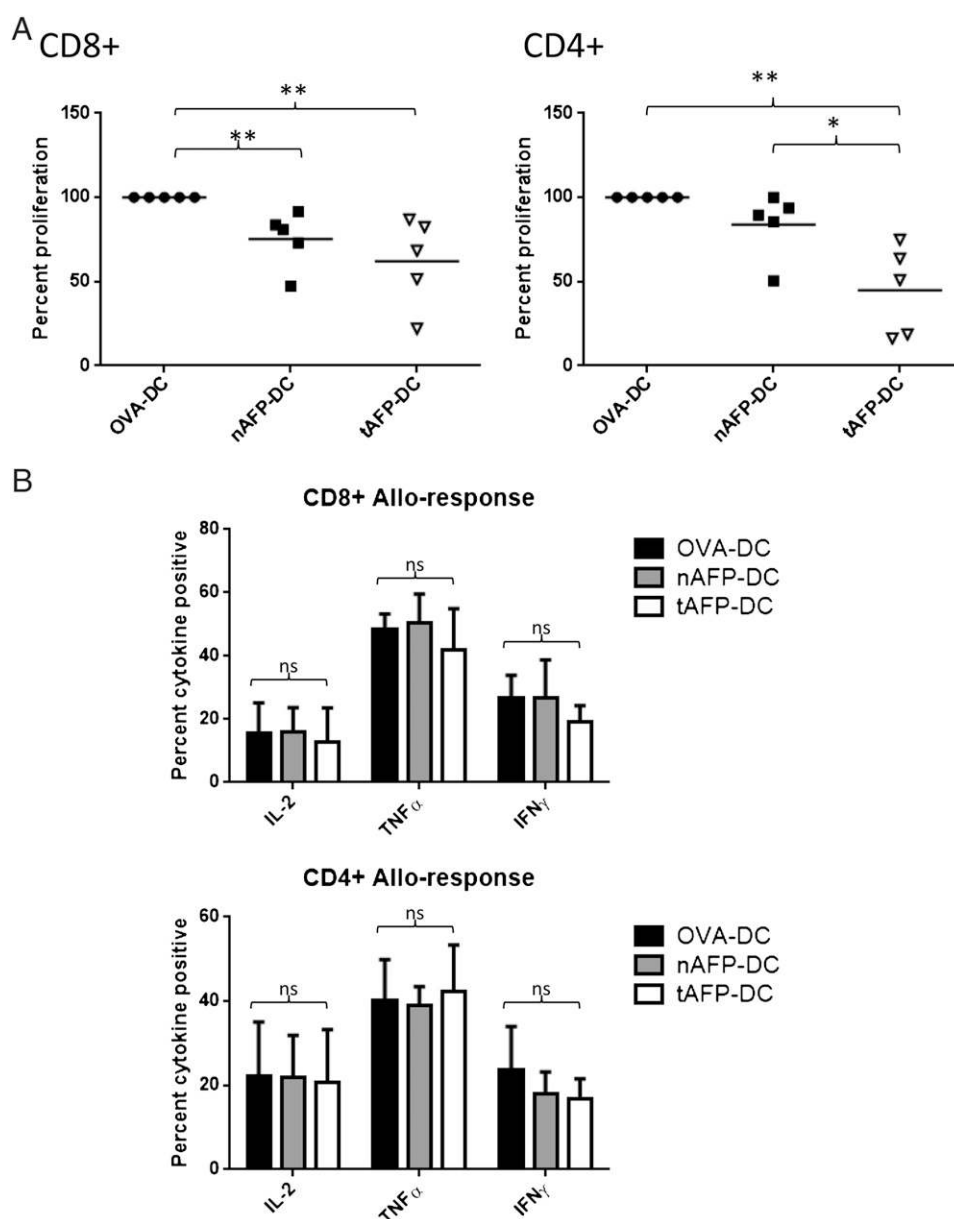
Alloreactive T cells also were analyzed for the ability to produce inflammatory cytokines. After a brief restimulation, the percent-

ages of IL-2⁺, TNF- α ⁺, and IFN- γ ⁺ T cells were unaltered among the OVA-conditioned, nAFP-conditioned, and tAFP-conditioned mDC groups (Fig. 4B). This was true for both CD8⁺ and CD4⁺ T cell subsets.

tAFP-conditioned DCs are poor stimulators of Ag-specific T cell expansion

We next tested a more biologically relevant model by using autologous AFP-conditioned DCs to stimulate flu peptide-specific T cell activation. Autologous CD8⁺ T cells were stimulated for 11 d by FluM1₅₈₋₆₆ peptide-pulsed OVA-conditioned, nAFP-conditioned, or tAFP-conditioned mDCs. Multimer staining revealed that the expansion of FluM1₅₈₋₆₆ peptide-specific CD8⁺ T cells was impaired in both the nAFP-conditioned and tAFP-conditioned mDC groups (Fig. 5A). To standardize the variability that was observed among donors (multimer⁺ cells in the OVA-conditioned mDC group ranged from 2.4 to 7.5%), the nAFP-conditioned mDC and tAFP-conditioned mDC groups were normalized to control OVA-conditioned mDCs (set at 1.0% for each individual donor). As depicted in Fig. 5A (right panel), expansion of FluM1₅₈₋₆₆ peptide-specific CD8⁺ T cells from three HDs was

FIGURE 4. tAFP-conditioned DCs fail to induce robust allogeneic T cell proliferation. Monocytes from HDs ($n = 5$) were differentiated into DCs for 5 d in the presence of OVA, nAFP, or tAFP (all at 10 $\mu\text{g/ml}$). DCs were then matured for an additional 24 h prior to collection. (A) Conditioned mDCs were cultured with allogeneic CFSE-labeled CD8⁺ or CD4⁺ T cells for 6 d. T cell proliferation was assessed by measuring CFSE dilution. The horizontal line represents the mean value. For each allogeneic donor pair, nAFP-conditioned mDC-induced and tAFP-conditioned mDC-induced proliferation was normalized to control OVA-conditioned mDC-induced proliferation (set at 100%). (B) Conditioned mDCs were cultured with allogeneic CD8⁺ or CD4⁺ T cells for 7 d. After stimulation with PMA and ionomycin, the cells were surface stained for CD8 or CD4 expression, and intracellular cytokine production was assayed by flow cytometry. Data are mean \pm SD. * $p < 0.05$, ** $p < 0.01$. ns, $p \geq 0.05$.



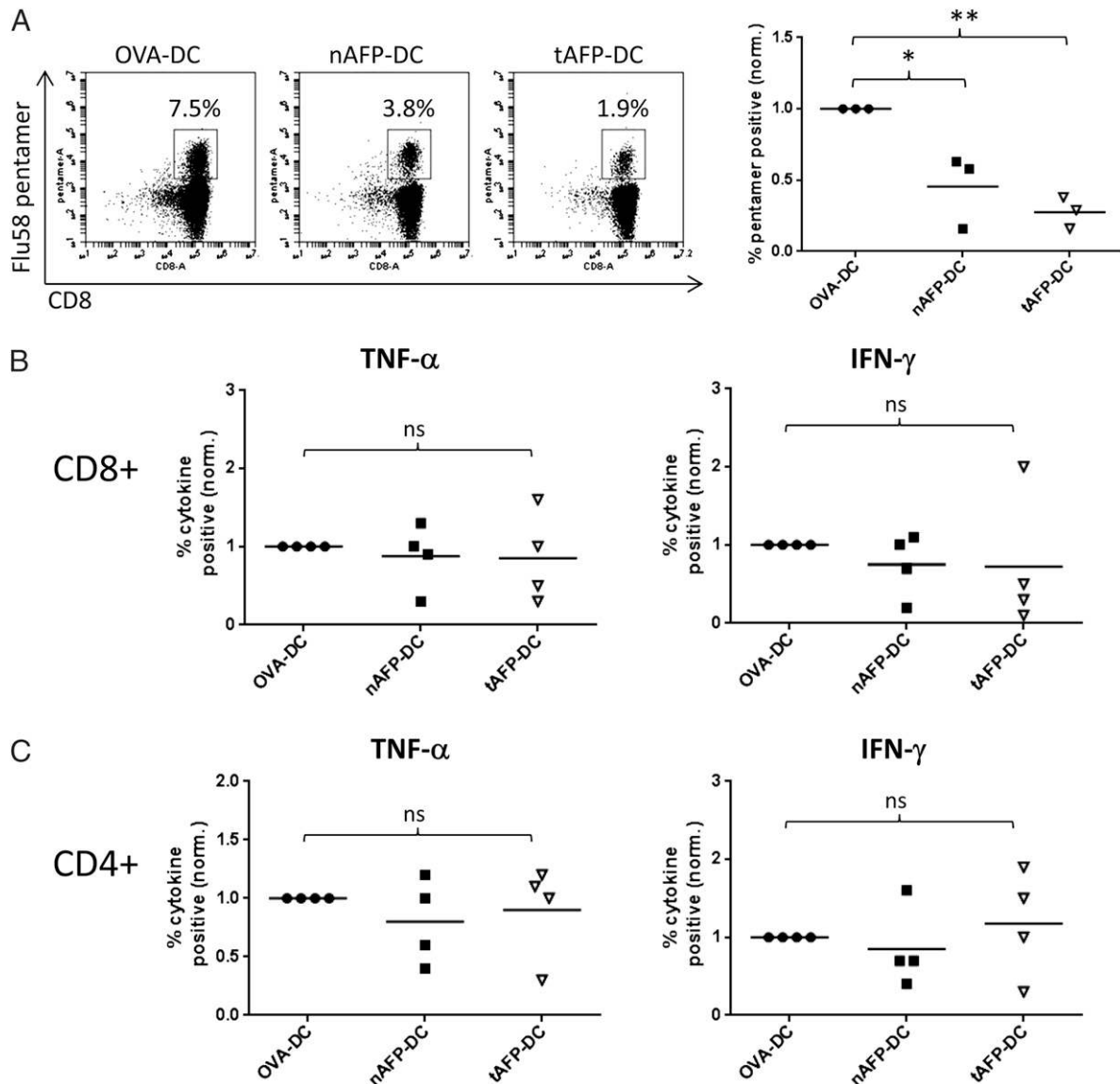


FIGURE 5. Ag-specific T cell expansion, but not effector function, is blunted by tAFP-conditioned DCs. (**A** and **B**) Monocytes from healthy HLA-A2⁺ donors were differentiated into DCs in the presence of OVA, nAFP, or tAFP and then matured overnight prior to collection. Conditioned mDCs were cultured with autologous CD8⁺ T cells in the presence of peptide FluM1_{58–66} for 11 d. (**A**) Representative FluM1_{58–66} pentamer staining (left panels) and from all donors (right panel) is shown. For each donor ($n = 3$), nAFP-conditioned mDCs and tAFP-conditioned mDCs were normalized to the control OVA-mDC group, which was set at 1.0%. (**B**) After a 6-h stimulation of collected CD8⁺ T cells with FluM1_{58–66}, intracellular cytokine production was assayed by flow cytometry. (**C**) Monocytes from healthy HLA-DR4⁺ donors were differentiated into DCs in the presence of OVA, nAFP, or tAFP and then matured overnight prior to collection. Conditioned mDCs were cultured with autologous CD8⁺ T cells in the presence of peptide HA₃₀₇ for 11 d. After a 6-h stimulation of collected CD4⁺ T cells with HA_{307–319}, intracellular cytokine production was assayed by flow cytometry. (**B** and **C**) For each donor ($n = 4$), nAFP-conditioned mDC-induced and tAFP-conditioned mDC-induced cytokine production was normalized to control OVA-conditioned mDC-induced cytokine production, which was set at 1.0%. * $p < 0.05$, ** $p < 0.01$, ns, $p \geq 0.05$.

significantly diminished by nAFP-conditioned mDCs ($p < 0.05$) and reduced even more by tAFP-conditioned mDCs ($p < 0.01$) compared with OVA-conditioned mDC control.

CD8⁺ T cells from this FluM1_{58–66} in vitro stimulation were studied further for cytokine production following a brief peptide-specific restimulation. Again, there was notable donor-to-donor variability. In the OVA-conditioned mDC control group, TNF- α ⁺ cells ranged from 0.2 to 1.1%, and IFN- γ ⁺ cells ranged from 0.2 to 2.1%. The nAFP-conditioned mDC and tAFP-conditioned mDC groups were normalized to control OVA-conditioned mDCs (set at 1.0%). However, no alterations in TNF- α or IFN- γ production were observed among the different DC treatment groups (Fig. 5B).

Next, autologous CD4⁺ T cells were stimulated with HA_{307–319} peptide-pulsed OVA-conditioned, nAFP-conditioned, and tAFP-conditioned mDCs. After 11 d, cells were briefly restimulated, and intracellular cytokine production was determined by flow cytometry. Donor variability (OVA-conditioned mDC control, TNF- α : 0.5–2.3%; IFN- γ : 0.3–1.2%) was standardized by setting OVA-conditioned mDC-induced cytokine production at 1.0%. Again, HA_{307–319} peptide-specific cytokine production by CD4⁺ T cells was not impaired by nAFP-conditioned or tAFP-conditioned mDCs (Fig. 5C). Along with the results from the MLR, these data demonstrate that, although effector function is unaltered, the ability of T cells to proliferate in response to both alloantigen and cognate Ag was significantly impaired by AFP-conditioned DCs.

An LMM AFP copurifying ligand is required for tAFP-induced DC dysfunction

Previous reports suggested that protein conformation (24), the degree of glycosylation (25), and fucosylation status (26) play important roles in the function of AFP. However, when OVA, nAFP, and tAFP preparations were heat denatured or treated with the enzymes PNGase F or α -L-fucosidase (to remove *N*-linked glycan or fucose residues, respectively), tAFP retained suppressive activity, as evidenced by diminished levels of the surface markers CD206 (Fig. 6A), as well as HLA-ABC, CD40, CD80, and CD83 (data not shown), on DCs. To evaluate whether the inhibitory activity of tAFP is dependent on full-length protein, we next pretreated OVA, nAFP, and tAFP with a serine protease (proteinase K). As shown in Fig. 6A, CD206 expression in DCs treated with digested tAFP was restored to levels observed in digested OVA-treated or nAFP-treated cells, indicating that an intact primary structure is necessary for tAFP activity. Similarly, expression of HLA-ABC, CD40, CD80, and CD83 (data not shown) was almost completely restored to control levels with protease-digested tAFP.

Because one of the main functions of AFP (69 kDa) is to bind and transport LMM hydrophobic ligands (4), we divided our OVA, nAFP, and tAFP preparations into HMM and LMM fractions (>3 and <3 kDa, respectively). Compared with the native tAFP preparation, neither the isolated HMM nor LMM fraction of

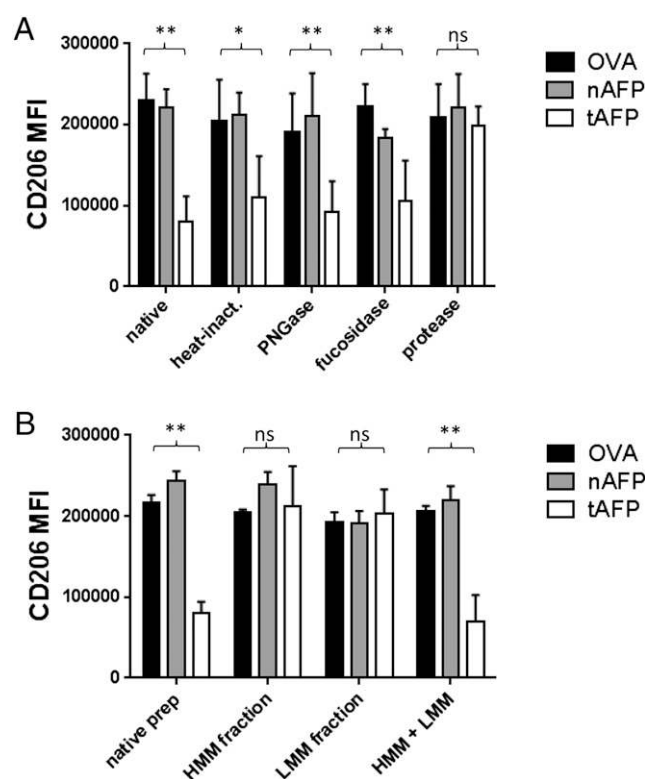


FIGURE 6. The inhibitory activity of tAFP requires intact whole protein and LMM copurification products. **(A)** DCs were differentiated for 5 d in the presence of native OVA, nAFP, or tAFP (all at 10 μ g/ml) or the same preparations prepared with heat denaturation or enzymatic treatment with PNGase F, fucosidase, or protease (as described in *Materials and Methods*). **(B)** OVA, nAFP, and tAFP (all at 10 μ g/ml) were separated into HMM and LMM fractions with a 3-kDa MMCO. DCs were differentiated for 5 d in the presence of intact native preparations, HMM fraction, LMM fraction, or HMM and LMM fractions together. MFI of CD206 is shown. Similar patterns were observed for HLA-ABC, CD40, CD80, and CD83. Data are mean \pm SD ($n = 4$ HD). * $p < 0.05$, ** $p < 0.01$. ns, $p \geq 0.05$.

tAFP inhibited DC expression of CD206 (Fig. 6B), HLA-ABC, CD40, CD80, and CD83 (data not shown). However, when HMM and LMM fractions of tAFP were added to cultures together, we observed suppressive activity to the extent of the native tAFP preparation. We next dialyzed tAFP against PBS using membranes of various molecular mass cutoffs (MMCOs). Even using an MMCO of 0.5 kDa, the HMM fraction of tAFP lacked suppressive activity (data not shown), indicating that the copurifying ligands in the LMM fraction of tAFP are <0.5 kDa. Extensive analysis of our native AFP preparations and isolated HMM and LMM fractions via mass spectrometry revealed no unexpected protein or peptide species (other than AFP itself) or glycosylation pattern that correlated with the immunosuppressive activity of AFP (data not shown), confirming the expected identity of these preparations and suggesting that the LMM ligands are not peptide-based molecules.

Finally, to further characterize the LMM species that function cooperatively with tAFP, cell lysates were prepared from HepG2 hepatoma cells and HD PBMCs (representing tumor and nontumor tissue, respectively); they were either left whole or separated into HMM and LMM fractions. DCs were then differentiated for 5 d in the presence of lysates, with or without the purified (HMM) fractions of OVA, nAFP, or tAFP. As illustrated in Fig. 7, CD206 expression was unaltered by HepG2 or PBMC lysates (both whole and fractionated) alone. Also, no change was observed when LMM lysates were added to cultures in combination with HMM OVA or nAFP. However, when the LMM lysates of either HepG2 cells or PBMCs were added to cultures together with HMM tAFP, expression levels of CD206 (Fig. 7), HLA-ABC, CD40, CD80, and CD83 (data not shown) were reduced significantly, effectively reproducing the tAFP-conditioned DC phenotype. These data suggest that the immunoregulatory LMM copurifying species in our tAFP preparations is a ubiquitous self molecule that is present in both tumor and healthy tissue.

Cumulatively, we observed that the ability of tAFP to impair DC differentiation and function is not dependent on native protein conformation or glycosylation status; rather, full-length AFP protein, along with the presence of LMM copurifying species, is absolutely essential for tAFP to exert its immunosuppressive activity.

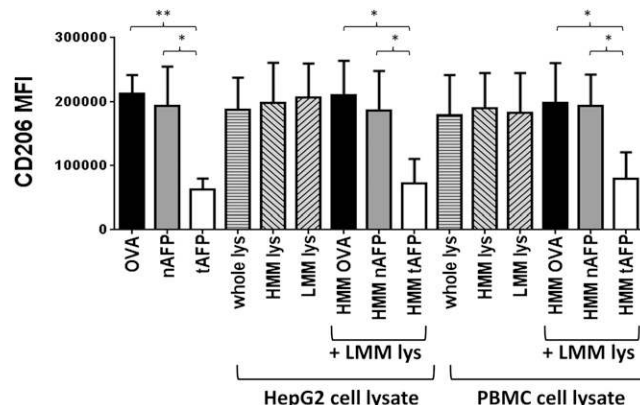


FIGURE 7. LMM cell lysates in combination with purified tAFP fully recapitulate the tAFP-conditioned DC phenotype. Lysates were prepared from HepG2 hepatoma cells and HD PBMCs and either left whole ("whole lys") or separated into HMM and LMM fractions ("HMM lys" and "LMM lys," respectively). OVA, nAFP, and tAFP (all at 10 μ g/ml) were either left intact or separated into HMM and LMM fractions. DCs were differentiated for 5 d in the presence of these preparations, as indicated. MFI of CD206 is shown. Similar patterns were observed for HLA-ABC, CD40, CD80, and CD83. Data are mean \pm SD ($n = 3$ HD). * $p < 0.05$, ** $p < 0.01$.

Discussion

Compared with HDs, reduced frequencies of circulating myeloid DCs are observed in the peripheral blood of HCC patients, whereas DCs generated in vitro from HCC patient PBMCs exhibit impaired IL-12 production and limited allostimulatory activity (18, 27). In this article, we show a significant relationship between AFP serum levels and the ratio of distinct DC subsets in the peripheral blood of HCC patients. Several tumor-derived factors have been implicated in DC dysfunction in cancer patients and tumor-bearing mice. These include VEGF, gangliosides, PGs, TGF- β , and IDO (28). Our current data suggest that AFP, which can be found at high levels in the serum of patients with gastrointestinal (i.e., HCC, stomach, pancreatic) and reproductive (i.e., yolk sac, teratoma) cancers, serves as a key regulator of DC differentiation.

Consistent with a previous study, nAFP exerted inhibitory activity in our DC cultures at higher concentrations ($>10 \mu\text{g/ml}$) (9). However, tumor-derived AFP preparations induced aberrant DC differentiation at much lower doses. These tAFP-conditioned DCs retain a monocyte-like phenotype and morphology, downregulate MHC and costimulatory molecule expression, and produce limited levels of inflammatory cytokines and chemokines, including IL-8, MCP-1, MIP-1 α , and MIP-1 β . This reduced stimulatory phenotype involves NF- κB pathway signaling. We recently identified DC-derived IL-8 as a crucial factor in NK cell chemotaxis (22). Moreover, the chemokines MCP-1/CCL2, MIP-1 α /CCL3, and MIP-1 β /CCL4 are known to recruit NK cells to sites of inflammation and enhance their IFN- γ production and cytolytic activity (21, 29). Notably, limited numbers of NK cells were reported in the peripheral blood and tumor lesions of HCC patients (30), and the density of intratumoral NK cells correlates with patient survival (31). Our data suggest that this paucity of NK cells in HCC patients, which serves as a significant barrier to anticancer immunity, may be a result of tAFP-mediated mechanisms that reduce inflammatory chemokine production. This is a new area that we are currently investigating.

Based on the poorly differentiated phenotype and limited cytokine/chemokine profile of tAFP-conditioned DCs, we hypothesized that these cells would be unable to induce a robust T cell response. Indeed, in both allogeneic and autologous T cell stimulation models, tAFP-conditioned DCs, and nAFP-conditioned DCs to a lesser extent, drove limited T cell proliferation compared with OVA-conditioned DC control. Conversely, cytokine production by activated T cells was not impaired by nAFP-conditioned and tAFP-conditioned DCs. This dichotomy may be explained by a recent study in which it was reported that, although a low density of TCR-CD3 complexes was sufficient to induce cytokine secretion, the threshold for T cell proliferation was significantly higher (32). Therefore, although tAFP-conditioned DCs may be capable of engaging a sufficient level of TCR-CD3 complexes to allow for cytokine production by T cells, they are unable to surpass the threshold necessary to drive proliferation.

To identify the molecular basis of tAFP's immune-suppressive activity, nAFP and tAFP preparations were tested under several conditions to identify the impact of glycosylation, structure, and conformation. Currently, the only reported variation between nAFP and tAFP isolates is altered glycosylation (i.e., the predominance of the fucosylated variant in tAFP preparations) (33). However, when we pretreated OVA, nAFP, and tAFP with fucosidase, the immunosuppressive activity of tAFP remained intact. Similarly, when these preparations were pretreated with the glycosidase PNGase F, the immunosuppressive activity of tAFP was unaltered. This is consistent with an earlier study in which it was shown that several AFP isolates exerted immunoregulatory activity, irrespective of their carbohydrate content (34).

An alternative hypothesis is that only specific conformational states of AFP protein possess immune-suppressive activity. AFP can assume three conformations: the natural compact conformation found during circulation in the blood; a slightly denatured state, termed the molten globule form, typically present when AFP is localized in the cytoplasmic compartment of cells; and a completely denatured state induced by extreme, nonphysiologic environments (4, 24). Heat denaturation of our AFP preparations failed to abrogate tAFP-mediated DC dysfunction, indicating that tAFP in both its compact (natural) and completely denatured states is immunosuppressive.

One of the main functions of AFP is to bind and transport LMM hydrophobic ligands, such as fatty acids (35), neopterin (G. Mizejewski, personal communication), and bilirubin (36). Immunologically, it was shown that high levels of these serum factors are capable of inducing immune dysfunction (37–39). Setiyono et al. (12) reported that domains 2 and 3, but not domain 1, of the AFP protein possess immunoregulatory activity. Notably, domain 2 contains the major fatty acid binding site of the protein, providing further evidence for a potential role for fatty acids in tAFP-based DC dysfunction (40). These observations, along with our current data, support a model in which LMM species that copurify with tAFP and are abundant in the LMM fractions of both tumor and nontumor cell lysates are efficiently chaperoned into monocytes/DCs by tAFP, where they interfere with molecular-programming pathways and inhibit full DC differentiation. We are currently using mass spectrometry and biochemical methods to uncover the identity of this molecule(s).

Purified AFP is being explored as a therapeutic in several clinical settings. Based on its immunosuppressive properties, recombinant AFP is under development for the treatment of autoimmune diseases (41). In the setting of AFP-expressing cancers (i.e., HCC), AFP serves as an attractive target for immunotherapy, and several groups investigated the anticancer potential of HCC tumor lysate- or AFP-based vaccines (16, 42, 43). Our data suggest that caution must be exercised in the formulation of these therapeutics. In the setting of autoimmune therapy, it will be essential to maintain AFP-copurifying, LMM-immunoregulatory species, whereas for AFP-based cancer vaccines, considerable effort must be taken to eliminate this component.

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Disclosures

L.H.B. is coinventor of patents covering aspects of AFP as a target for T cell-mediated anti-HCC immunity. The other authors have no financial conflicts of interest.

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