Title
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Journal
Trends in Immunology, 43(6)

ISSN
1471-4906

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Publication Date
2022-06-01

DOI
10.1016/j.it.2022.04.001

Peer reviewed

CellPress
OPEN ACCESS

Trends in
Immunology

Opinion

Immunomodulatory impact of α-fetoprotein

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α-Fetoprotein (AFP) is a fetal glycoprotein produced by most human hepatocellular carcinoma tumors. Research has focused on its immunosuppressive properties in pregnancy, autoimmunity, and cancer, and human AFP directly limits the viability and functionality of human natural killer (NK) cells, monocytes, and dendritic cells (DCs). AFP-altered DCs can promote the differentiation of naïve T cells into regulatory T cells. These properties may work to shield tumors from the immune system. Recent efforts to define the molecular characteristics of AFP identified key structural immunoregulatory domains and bioactive roles of AFP-bound ligands in immunomodulation. We propose that a key mechanism of AFP immunomodulation skews DC function through cellular metabolism. Delineating differences between fetal 'normal' AFP (nAFP) and tumor-derived AFP (tAFP) has uncovered a novel role for tAFP in altering metabolism via lipid-binding partners.

Highlights

Cord blood-derived alpha-fetoprotein (AFP) has an incompletely understood role in pregnancy, as well as in autoimmune diseases. AFP is currently being investigated as a potential therapeutic for multiple autoimmune diseases. These diverse biological roles are complicated by earlier approaches that did not account for AFP sources (where a tumor-derived AFP might be more potently immunosuppressive than normal AFP due to differential binding partner molecules.

Tumor-derived AFP has broad immunosuppressive effects on multiple cell types, including NK cells, NKT cells, and dendritic cells (DC). The mechanisms of action include AFP protein and non-protein binding partners. AFP can also have indirect effects on lymphocytes after uptake by DCs, as well as after direct uptake by lymphocytes.

A key mechanism of AFP-mediated immunosuppression involves the binding of lipid partners which can modulate cellular metabolism. Lipid-AFP complexes can impact DC phenotype, function, and metabolic pathway utilization. Some of these lipids have been identified as polyunsaturated fatty acids which can preferentially bind AFP.

AFP: more than a hepatocellular carcinoma (HCC) biomarker

AFP (see Glossary) is a 70-kDa immunoregulatory fetal glycoprotein [1], similar to albumin, produced by most **hepatocellular carcinoma (HCC)** tumors [2] and by the fetus. The human fetus produces nAFP [3]. In HCC, elevated AFP synthesis in the tumor correlates with larger tumor size, differentiation stage, and inferior survival in HCC patients [4]. The speculation that AFP might function to prevent antifetal or antitumor immune responses has paved the way for numerous studies focusing on the roles of both nAFP and tAFP and their effects on a variety of immune cell types (Figure 1) [5]. Indeed, previous work has examined the immunosuppressive properties of AFP in pregnancy (Box 1), autoimmunity (Box 2), and cancer. Here, we re-examine nAFP and tAFP biology, as well as newly identified mechanisms of how AFP might exert certain immunosuppressive effects. In this opinion article we note the differential effects of AFP molecules (nAFP vs. tAFP) on immune cells such as T cells, **DCs,** and **NK cells**, and we discuss potential strategies to overcome AFP-mediated immunosuppression by understanding its putative role in immunometabolism. The ability of AFP to bind molecules that modify its function results in pleiotropic immunomodulatory effects.

AFP binds fatty acids

AFP can bind a variety of molecules, including bilirubin [6], metals [7], and fatty acids [8]. When evaluating bound species for tAFP immunosuppressive effects, the source and purification method, as well as the immunological readout, are essential. For instance, rat AFP binds estrogen [9], which harbors immunological properties; however, human AFP cannot bind estrogen [10]. In 1978, the binding of fatty acids to AFP was quantified [11]; adult albumin and nAFP bound a similar amount of fatty acids at a molar ratio of approximately 2:1. However, adult albumin exhibited a higher affinity for saturated fatty acids, whereas nAFP preferentially bound unsaturated fatty acids. In particular, nAFP bound **polyunsaturated fatty acids (PUFAs)** more potently than human or fetal albumin. These nAFP-bound PUFAs were enriched in docosahexaenoic acid (22:6), arachidonic acid (20:4), and to a lesser extent linoleic acid (18:2) [10]. The binding of fatty acids to AFP is relevant for immunosuppression mechanisms and will be discussed further (Figure 2).

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Putative intrinsic and extrinsic mechanisms of AFP immunosuppression

Direct evidence that specifically evaluates AFP-mediated immune cell suppression came from murine studies; for instance, a report demonstrated that human AFP could inhibit antibody responses [12] and the proliferation of human blood isolated lymphocytes *in vitro* [13], and that nAFP was more effective than tAFP at inhibiting mitogen-induced mixed lymphocyte reactions (MLR) [13]. These data showed initial evidence of the biological differences between nAFP and tAFP in their presumed immunoregulatory properties. The early observation also suggested that AFP might potentially suppress immune responses via intrinsic factors (e.g., the abundance of different isoforms, glycosylation patterns, etc.), as well as extrinsic factors such as binding partners (e.g., various hormones, prostaglandins, fatty acids) (Figure 2).

In addition to AFP intrinsic factors, additional work explored whether noncovalently bound low molecular mass (LMM) binding partners of AFP (that could be eluted away from AFP) might play an immunosuppressive role in host responses. For example, indirect evidence has suggested that noncovalently bound species might be responsible for AFP-mediated immunosuppression, stemming from two studies that isolated rat AFP [14] and human AFP [15,16] by elution from immunosorbent columns. This purification method (with serum antibodies and size exclusion) removed noncovalently bound molecules and eliminated the inhibitory activities of AFP in both preparations; this suggested that these noncovalently bound LMM species might be important, contributing to immunosuppressive activities on allogeneic MLR proliferation to human AFP [16].

Evidence for AFP-mediated immunosuppressive effects on NK cell function and survival

Causal evidence for an impact of AFP on NK cells came from a study that evaluated the effects of AFP *in vitro* and demonstrated that murine NK cells pretreated with nAFP exhibited diminished cytolytic effector functions compared with nontreated cells [17]. Moreover, these effects seemed to be independent of any bound products of nAFP (e.g., prostaglandins) because the preparation biochemically removed any such species, suggesting a potential intrinsic inhibitory effect of nAFP [17]. Furthermore, another study evaluated the effects of nAFP on human NK cells [18] *in vitro*, and – in agreement with a previous report showing NK suppression *in vivo* in Japanese quail [19] – determined that the nAFP-mediated suppression of NK cells was due to an indirect mechanism involving the modulation of DC function. Specifically, nAFP-treated NK cells exhibited reduced target-cell killing because AFP treatment *in vitro* decreased DC production of interleukin 12 (IL-12) compared with albumin [20].

Our research group found that both nAFP and tAFP induced a proinflammatory secreted IL-2 response *in vitro* and could hyperactivate human NK cells independently from any AFP LMM ligands [20], similar to some of the findings listed earlier [17]. However, we found that tAFP, and not nAFP, induced NK cell death *in vitro* and depended on the presence of a hydrophobic LMM ligand (purified by size-exclusion column chromatography and examined by mass spectrometry) [20]. We further demonstrated that the NK cells internalized labeled AFP *in vitro*, and that NK cells derived from HCC patients harbored detectable AFP intracellularly [20]. Common apoptosis assays showing the direct induction of apoptosis by tAFP in NK cells [20] and DCs [21] *in vitro* suggested that cell death might be an essential feature of tAFP-mediated immunoregulation, at least in this context. We argue that both direct and indirect mechanisms might contribute to promoting NK-cell apoptosis as well as limiting IL-12 production by DCs in certain scenarios; however, further research is warranted to dissect these mechanisms [20,21].

Glossary

α-Fetoprotein (AFP): a fetal glycoprotein. 'Normal' (nAFP) is cord-blood-derived AFP. Tumor-derived AFP (tAFP) is differentiated from nAFP by its glycosylation (specifically, it is fucosylated at the single amino acid that is glycosylated in all forms of AFP); it is not reported to be mutated compared with nAFP. These molecules are closely related to albumin, but bind different molecules and have different biological properties.
CD1d: a cell surface molecule on antigen-presenting cells such as DCs; it presents lipid antigen to NKT cells.
CD303⁺ plasmacytoid DCs: these cells have some myeloid as well as lymphoid cell properties; they can produce large amounts of IFNs upon stimulation.
Chimeric antigen receptor engineered T cells (CAR-T cells): used as a strategy to change the natural T cell receptor-mediated peptide specificity of a T cell to instead recognize a cell-surface protein in a way analogous to that in which an antibody recognizes a protein shape. The CAR construct often incorporates an antibody variable region linked to intracellular signaling molecules, triggering the T cell to proliferate and be activated by a cell surface protein.
Cyclooxygenase (COX) subunit 1: the mitochondrially encoded gene for cytochrome C oxidase I, the main subunit of the cytochrome C oxidase complex in the electron transport chain.
Dendritic cells (DCs): the most potent antigen-presenting cells. They can be differentiated from monocytes *in vitro* or purified from *in vivo* blood and tissues as several phenotypic subtypes (including conventional CD1c⁺/CD141⁺). DCs are involved in the immune antigen presentation pathway of cross-presentation: exogenous antigens are taken up, processed, and presented via MHC classes I and II to CD4⁺ and CD8⁺ T cells, respectively. Immature DCs are specialized for environmental antigen capture and processing. Matured DCs (responding to a variety of environmental cues such as pathogen-derived signals) are more specialized in the presentation of processed antigens to T cells.
Epigenetic imprinting: heritable structural changes in the way in which nuclear DNA is remodeled (without affecting the DNA sequence), making it more or less accessible to transcription factors modulating gene expression.
Exhausted phenotypes: cells (e.g., T cells) that have repeatedly encountered

AFP can modulate monocyte and DC immune responses

While previous studies suggested that both nAFP and tAFP harbored immunoregulatory properties [21], the precise mechanism of a DC-mediated contribution was unclear. Of note, the suppressive effects of AFP on human DCs were shown to persist *in vitro* for days after its removal [22], suggesting that other downstream effects might occur in the DCs (e.g., long-term reprogramming via a mechanism such as **epigenetic imprinting**) or might involve the induction of immunosuppressive cells propagating immunosuppression over time, even in the absence of AFP [23]. This possibility is interesting, as long-term effects on DC biology might suggest more than a transient change in an AFP-mediated signal, but might also suggest an alteration to the epigenome, leading to more stable changes in DC biology; however, this possibility remains to be tested.

Subsequent studies in mouse models demonstrated that AFP induced T cells to suppress primary antibody responses *in vitro* [24]. These findings suggested that at least one mechanism of AFP-mediated immunosuppression might include the skewing of $CD4^+$ T-cell differentiation towards suppressive **regulatory T cells (Tregs)**. However, the factors and/or cells driving this skewing remained unknown until experiments *in vitro* demonstrated that such AFP-mediated induction of suppressor T cells depended on the effect of AFP on monocytes in mice [25]. Moreover, the results indicated that AFP was unlikely to directly impact T cells, but instead, based on T cell subset cocultures, altered monocyte functions [24,25]. The observed effects of AFP on monocytes led to the hypothesis that they might preferentially take up AFP through unique surface receptors (see Outstanding questions). To test this hypothesis, unique receptors were biochemically identified on the U937 monocyte cell line [26]; a 65-kD putative AFP receptor was isolated, although it remains uncharacterized. As discussed earlier, efficient routes of AFP uptaken by multiple immune cells have been reported. Furthermore, AFP treatment on phorbol 12-myristate-13-acetate (PMA)-, lipopolysaccharide (LPS)-, and interferon γ (IFN-γ)-stimulated human monocytes was assessed via the production of tumor necrosis factor α (TNFα) and IL-1β [27]. The study found that decreased TNFα and IL-1β production from murine monocytes following AFP treatment *in vitro* (and for all stimuli) depended on the synthesis of the potently immunoregulatory prostaglandin E2 (PGE_2). While these *in vitro* data were limited to cell lines,

an antigen, or which have encountered an antigen in a way that does not support cell activation and differentiation (which should occur upon activated DC priming); this can lead T cells to express multiple negative signaling molecules on their surface, including expression of inhibitory checkpoint receptors such as PD-1, CTLA-4, TIGIT, and LAG-3.

Hepatocellular carcinoma (HCC): primary liver tumor derived from hepatocytes.

Mammalian target of rapamycin complex 1 (mTORC1): the downstream metabolic regulators SREBP-1, FASN, and ACLY are a group of important enzymes in cellular metabolism.

Natural killer (NK) cells: innate cytolytic cells that play an essential role in clearing virally infected cells or tumor cells without needing antigen-specific priming.

Peroxisome proliferator-activated receptor-gamma coactivator-1-α (PGC1-α): a transcription factor which contributes to regulating mitochondrial biogenesis.

Polyunsaturated fatty acids (PUFAs): fatty acids containing two or more double bonds.

Regulatory T cells (Tregs): a subset of $CD4^+$ T cells expressing the FoxP3 nuclear transcription factor; they are capable of suppressing immune responses in antigen-specific and antigen-nonspecific ways.

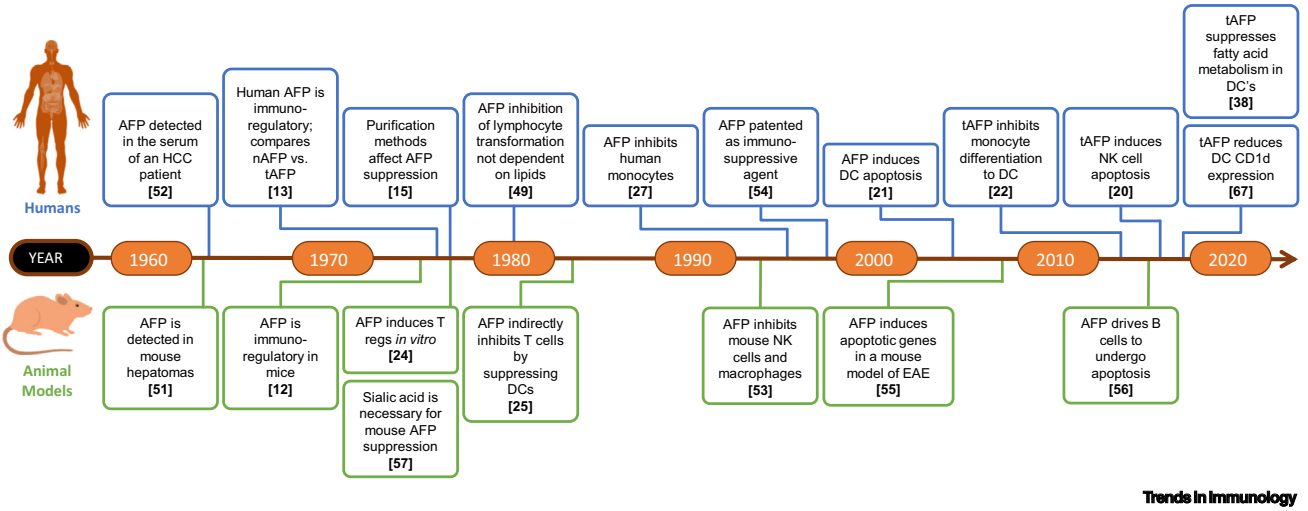

Figure 1. Timeline of α-fetoprotein (AFP) discoveries in animal models (e.g., rodents) and humans. AFP biology has been investigated for over 60 years, particularly as a biomarker for tumor growth in hepatocellular carcinoma (HCC). Studies on AFP as an immunomodulatory protein began 50 years ago. Illustrated are brief synopses of select papers from studies involving humans (blue, above the arrow) and animal models (green, below the arrow), along with the reference and year published (bold). Studies that focused on the immunoregulatory properties of AFP were prioritized. Abbreviations: DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; nAFP, 'normal' cord-blood-derived AFP; NK, natural killer; tAFP, tumor-derived AFP. See [12,13,15,20–22,24,25,27,38,49,51–57,67].

Box 1. The role of AFP during pregnancy

Initial *in vivo* evidence for the role of AFP in immunosuppression began in pregnant animal models and humans. The first indirect evidence that AFP has immunosuppressive properties *in vivo* was demonstrated by infusing anti-AFP antibodies into pregnant rabbits, which caused fetal rejection, potentially limiting suppressive maternal immune responses [58]. However, the authors could not rule out the possibility that the anti-AFP antibodies were directly harming the embryo. Another study provided further evidence that nAFP suppressed maternal immunity by demonstrating that pregnant mouse serum inhibited antibody synthesis *in vitro* in an nAFP-dependent manner [59]. Notably, the study showed that pregnant mice had a diminished immune response to vaccination. However, a variety of non-nAFP pregnancy-associated factors could have limited vaccine responsiveness. In addition to the role of nAFP in healthy fetal development [60] (e.g., promoting erythropoiesis [61]), these findings suggested that nAFP played a role in suppressing the maternal immune system to limit antifetal immune responses and prevent fetal rejection. In partial agreement with these findings from mice, nAFP played a role in remission of symptoms of myasthenia gravis in pregnant women [62]. Myasthenia gravis is an autoimmune disease caused by autoantibodies to the acetylcholine receptor (AcChoR), which results in muscle fatigue. In contrast to prior work, the mechanism was the inhibition of antibody and antigen binding rather than AFP indirectly impacting anti-AcChoR antibody production [62]. These findings contributed to explain the observation that newborns had a transient expression of myasthenia gravis symptoms because of the continued presence of maternal anti-AcChorR antibodies and the subsequent loss of endogenous nAFP [62].

they provide relevant mechanistic insight, indicating that monocytes take up AFP and subsequently produce PGE_2 which limits their functional capacity by limiting the secretion of such inflammatory cytokines [27].

Subsequent work evaluated AFP-mediated immunosuppression mechanisms using primary monocyte-derived human DCs. The effects of AFP on monocyte-derived DCs from healthy people and HCC patients were analyzed; two significant functional impacts of AFP exposure were shown: (i) relative to controls, reduced *in vitro* DC-derived production of TNFα and IL-12 was observed, and (ii) DC apoptosis ensued [21]. Moreover, because AFP can in general bind various compounds that might limit immune function (not specified here), a question that arose was whether AFP-mediated inhibition of DC function depended on AFP-bound LMM species, or whether it was intrinsic to the molecule itself. Thus, an approach was to understand which portion of the AFP molecule inhibited DC IL-12 production. Recombinant AFP was expressed in *Escherichia coli* as its separate domains (D1, D2, and D3), and full-length AFP (FL-AFP) was used as a control molecule [28]. D2 and D3, but not D1, potently suppressed DC-derived IL-12 production *in vitro*. Of note, D2 is the domain that can bind fatty acids, consistent with the earlier mention of AFP-bound fatty acids [29]. In addition, tAFP and nAFP naturally exist as glycoproteins; however, because this study used AFP synthesized in *E. coli* – which lacks eukaryotic glycosylation enzymes – these molecules lacked any attached sugar moieties. Therefore, it was concluded that the observed DC functional suppression was independent of AFP glycosylation, suggesting that molecules binding D2 and D3 might be functionally important; however, this finding does not rule out the possibility that glycans might contribute in some way to other immunoregulatory properties of AFP [28].

To better evaluate the differences between nAFP and tAFP, our group sought to examine more closely these differences in the context of human monocyte differentiation into DCs *in vitro* [22]. tAFP potently inhibited the differentiation of $CD14^+$ monocytes into immature or mature DCs

Box 2. AFP as a therapeutic agent against autoimmunity

AFP has been investigated as a possible therapeutic agent against different types of autoimmunity. One pharmaceutical company tested recombinant AFP purified from transgenic goats' milk [63]; it was tested in clinical trials in 2003. These trials were supported by preclinical *in vitro* and *in vivo* data, including observations of remission of rheumatoid arthritis and multiple sclerosis in pregnancy which correlated with fetal AFP concentrations. The tested form of recombinant AFP lacked the single glycosylation site. Recently, another company has been testing a proprietary form of AFP based on preclinical data in mice showing reversal of some effects of myasthenia gravis, and human trials are ongoing.

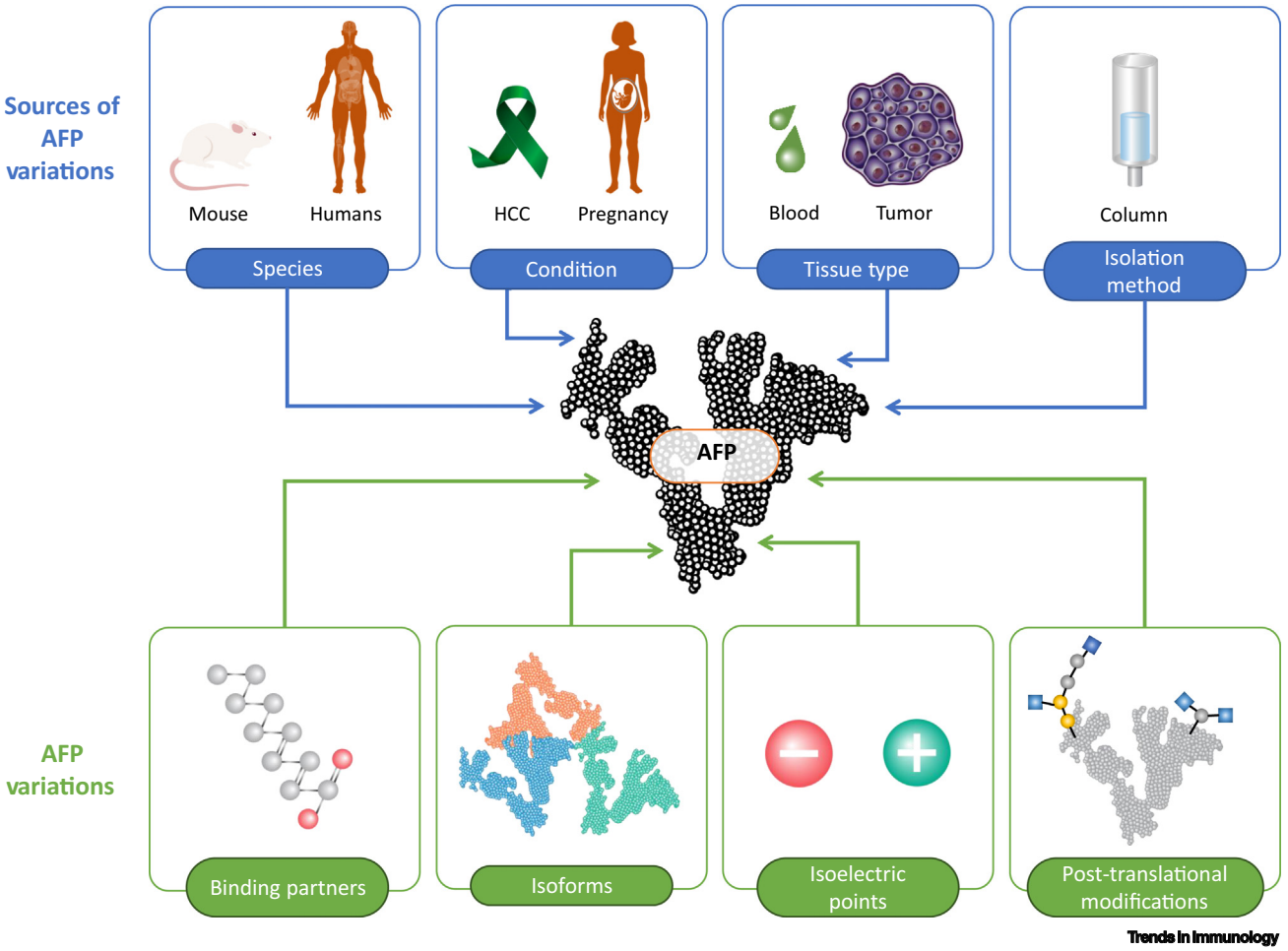

Figure 2. Sources of α-fetoprotein (AFP) biochemical diversity. AFP has biological effects in individual protein domains, as an intact protein, as well as in a complex with a variety of binding partners. Shown are sources of AFP variation (blue, top row) and biochemical variability among AFP (green, bottom row). The primary sources of AFP variability include the species, clinical condition, tissue, and isolation method. Microheterogeneity of tumor-derived AFP (tAFP) includes the ligands or binding partners, isoforms, isoelectric points, and post-translational modifications. Abbreviation: HCC, hepatocellular carcinoma.

[as evidenced by the decreased expression of MHC human leukocyte antigen – DR isotype (HLA-DR) and CD206] when compared to cord-blood-derived nAFP – which has the same amino acid sequence as tAFP, but is not fucosylated (altered glycosylation relative to tAFP) – or ovalbumin (OVA)-treated controls [22]. OVA is an evolutionarily related albuminoid protein with the same size and structure as human AFP and human albumin, and the regulation of MHC class II expression and CD206 expression were emblematic of at least eight DC phenotypic molecules. The observed tAFP immunoregulatory effects depended on the binding of eluted hydrophobic LMM partners; indeed, the tAFP high molar mass (HMM) or the LMM fractions alone could not inhibit monocyte-to-DC differentiation, unlike HMM–AFP protein complexed with the LMM fraction. These findings suggested that the binding of fatty acids might have inhibited monocyte differentiation into DCs *in vitro*, a possibility which remains to be rigorously assessed *in vivo*. Moreover, the molecular composition of such tAFP binding partners, as well as whether certain immunosuppressive effects are dependent on a particular fatty acid remain unknown, but certainly warrant further investigation.

The effects of AFP binding to fatty acids

One study testing human fetal tissue AFP characterized bound fatty acids and demonstrated that by removing fatty acids from nAFP, followed by the subsequent addition of the eluted arachidonic acid, restored the native protein isoelectric point; the arachidonic acid therefore constituted an important AFP binding partner [11]. Moreover, another study showed that PUFAs blocked DC activation [30]: specifically, arachidonic acid (20:4), which binds nAFP [11], robustly inhibited the expression of costimulatory molecules CD40 and CD80 on DCs. Notably, the inhibitory effects of arachidonic acid were observed even after potent LPS stimulation, as evidenced by the decreased IL-12 production by DCs compared with untreated controls. Of note, in mouse models, arachidonic and docosahexaenoic acids can program DCs to produce prostaglandins (e.g., PGE_2) which subsequently limit $CD4^+$ T cell proliferation and increase FoxP3 expression *in vitro* [31]. The findings using arachidonic acid mirror the effects of AFP to induce PGE_2 synthesis [27], and are consistent with the hypothesis that fatty acids may be essential for nAFP- or tAFP-mediated immunosuppression via Treg induction (see Outstanding questions).

Polyunsaturated fatty acids may also play a role in limiting NK cell function. Eicosapentaenoic acid (20:50), similar to arachidonic acid but with one additional double bond, limits NK cell cytotoxicity *in vitro* [32]. In support of these findings, a randomized, placebo-controlled, double-blind trial found that dietary supplementation of eicosapentaenoic acid – but not arachidonic acid or fish oil – led to reduced NK cell activity in healthy subjects aged 55–75 years [33]. However, there is limited evidence that nAFP can bind eicosapentaenoic acid, and it is unclear whether these results extend to human tAFP [8]. Nevertheless, some evidence suggests that NK cells, unlike monocytes and DCs, require arachidonic acid for cytolytic effector functions [34]. Thus, the potential role of PUFAs on NK cell activity, and whether PUFAs are necessary for tAFP-mediated NK cell cytotoxicity (and/or NK cell apoptosis) warrant further investigation.

tAFP can alter certain immunometabolic processes

Metabolic reprogramming associated with glycolytic and mitochondrial respiration is central to DC differentiation, migration, and antigen presentation [35]. DCs require fatty acid synthesis during maturation and activation in mice and humans [36]. Given prior work implicating lipids [22] or the decrease in **CD1d** lipid antigen presentation (Box 3) in AFP-mediated DC differentiation and phenotype, we assessed the effects of nAFP versus tAFP on DC metabolism [38]. Compared to OVA-treated DCs, tAFP significantly inhibited **mammalian target of rapamycin complex 1 (mTORC1)** downstream metabolic regulators sterol regulatory element-binding protein-1 (SREBP-1), fatty acid synthase (FASN), and ATP citrate lyase (ACLY) [38]. Both the mature and the sterol-mediated cleaved forms of SREBP-1 were predominantly downregulated in tAFP-exposed DCs compared with nAFP and OVA. Furthermore, reduced intracellular amounts of **peroxisome proliferator-activated receptor-gamma coactivator-1-α (PGC1-α)** due to inhibition of mTORC1 resulted in decreased mass and depolarization of mitochondria, and tAFP-treated DCs exhibited low **cyclooxygenase (COX) subunit 1** expression relative to controls; this resulted in suppressed oxygen-dependent respiratory capacity in human DCs, suggesting

Box 3. NKT cell dysregulation by tAFP

Unlike their T cell counterparts, NKT cells are innate-like cells that do not recognize MHC restricted amino acid sequences but rather CD1d-presented lipidic antigens [64]. NKT cells are also prominent in the microvasculature of the liver and play essential roles in normal physiology [65]. Given the prominence of NKTs in the liver and their emerging role in cancer immunotherapy [66], our group also sought to better understand how AFP might regulate CD1d molecules on DCs, thereby limiting NKT cell function *in vitro* [67]. We found that both nAFP and tAFP led to significant downregulation of CD1d on DCs; however, this did not diminish their potential to stimulate NKT cells [67]. Additional work is needed to fully understand the interplay between DCs and NKT cells, including the modulation of cytokines produced by NKT cells, focusing on a role for lipids in this cellular immune interaction.

that a key metabolic pathway (oxidative phosphorylation) was shut down. Moreover, in contrast to healthy donors (HDs), HCC patient-derived DC subsets – including conventional $CD1c^+/CD141^+$ and **$CD303^+$ plasmacytoid DCs** – expressed reduced amounts of PGC1-α and diminished mitochondrial potential. Also, HCC $CD1c^+$ DCs exhibited impaired cross-presentation capacity relative to HDs, as they induced $CD8^+$ T cells with reduced activation, as well as IFNγ, TNFα, and CD69 expression [38]. Therefore, AFP^+ HCC patients may have defective DCs which are less capable of activating autologous T cells than those in healthy individuals.

Towards AFP-based therapeutic approaches in HCC

NK cells account for a large proportion (25–50%) of human liver lymphocytes, and they play an essential role in liver immunity and homeostasis (reviewed in [37]). Given the ability of tAFP to dysregulate and kill NK cells directly *in vitro*, we propose that strategies aimed at diminishing tAFP production or shielding NK cells from the proapoptotic effects of tAFP might be necessary to ensure the full antitumor cytotoxic potential of NK cells. Additionally, we argue that AFP-induced Tregs might also pose a barrier to achieving a robust NK cell response. In support of this concept, Tregs from HCC patients were associated with diminished autologous NK cell cytotoxicity and IFN-γ production *in vitro* [38] relative to controls. Our group also demonstrated that an AFP-based DC vaccine administered subcutaneously to HCC patients both increased NK cell activation and decreased Treg frequencies [39]. While vaccination activated the HCC patient-derived NK cells, effector functions were less robust than those of NK cells from HD

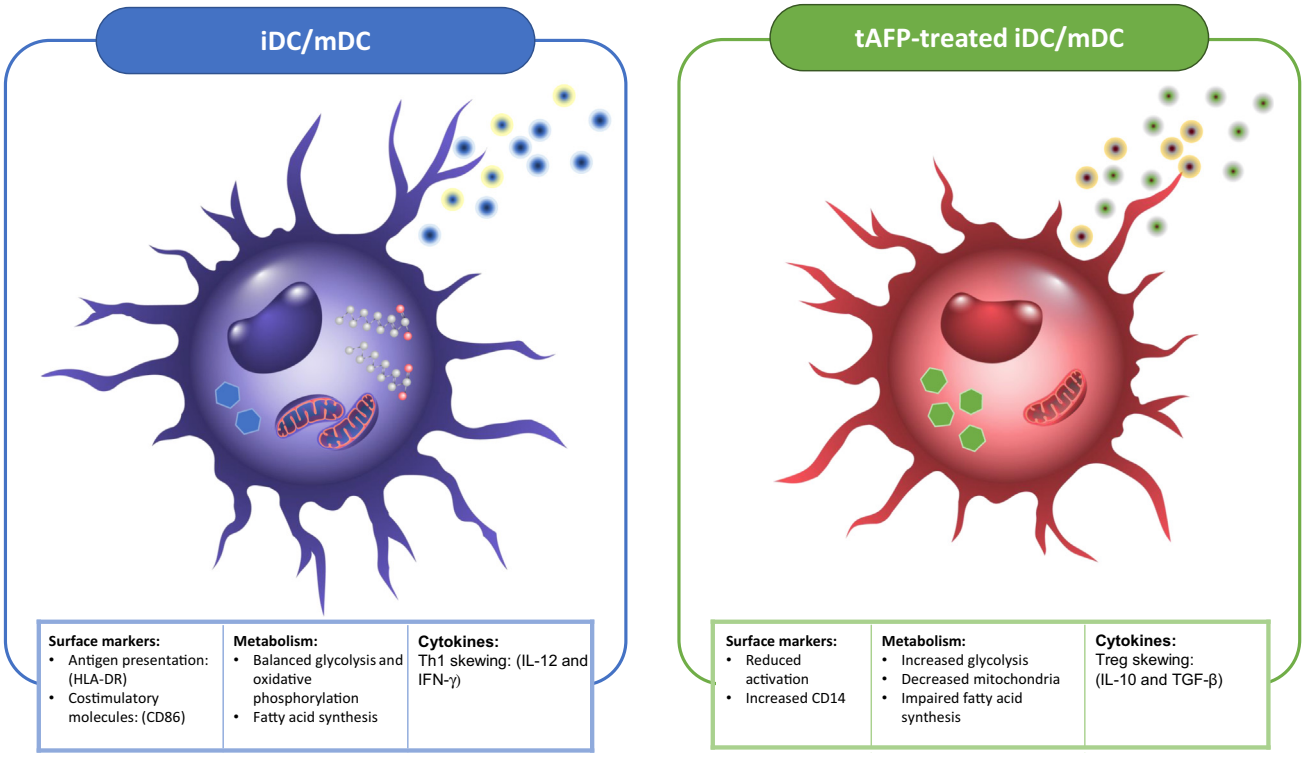

Figure 3. Effects of tumor-derived α-fetoprotein (tAFP) on immature, monocyte-derived dendritic cells (DCs). Typical characteristics of *in vitro* generated monocyte-derived (iDC/mDC) (left) versus tAFP-treated (right) are shown. Naturally, DCs will robustly express antigen presentation, costimulatory molecules, and cytokines to induce robust T cell responses. By contrast, tAFP-treated DCs exhibit reduced antigen presentation and costimulatory molecules and secrete immunoregulatory cytokines. These tAFP-treated DCs also exhibit an altered metabolic profile when compared to typical DCs. Abbreviations: HLA-DR, human leukocyte antigen – DR isotype; IL-12, interleukin 12; IFN-γ, interferon γ; TGFb, transforming growth factor β.

controls. These data suggest that combining agents that might neutralize tAFP and diminish Treg frequencies, administered in conjunction with an AFP-targeting vaccine to ideally promote immunity against tAFP-producing tumor cells, might be a candidate strategy to induce a potent response and restore maximal functionality of NK cells, although this remains conjectural.

Of note, we recently showed that tAFP requires PUFAs for potent immunosuppression of monocytes and DCs [20,22] (Munson *et al*., unpublished). Additionally, tAFP exposure dysregulated DC mitochondrial homeostasis and metabolism and this, in turn, limited DC antigen presentation and T cell stimulatory functions *in vitro*, as noted earlier (Figure 3) [38]. However, an important limitation to these findings is that it is unknown whether such tAFP effects can be reversed *in vivo*; moreover, *in vitro*, potent DC maturation signals only partially reversed the observed suppressive DC phenotype of defective antigen presentation and expression of costimulatory molecules [22]. If replicated *in vivo*, we argue that strategies that reverse AFP effects might not be effective, but by contrast, blocking AFP secretion together with promoting the *de novo* differentiation of DCs and other immune cells from progenitors might (and should) be tested.

In the setting of HCC, as mentioned, AFP can limit innate immunity by inducing tolerogenic DCs and promoting NK cell apoptosis [19–22] (Figure 4). We propose a model whereby such tolerogenic DCs might block the development of cancer-specific CD8$^+$ cytotoxic T cells (CTLs) and support the expansion of Tregs. Presumably, by blocking the anticipated regulatory effects

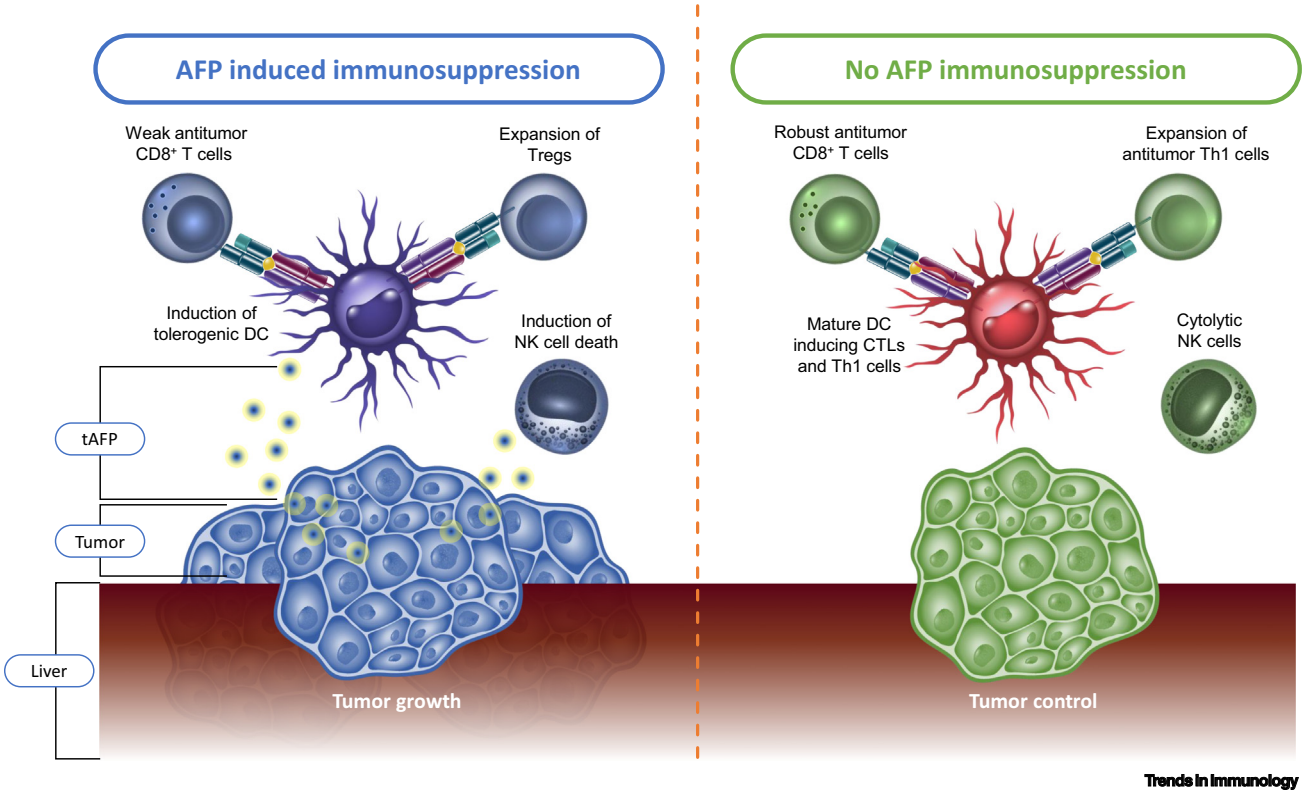

Figure 4. Model of potential effect of blocking α-fetoprotein (AFP)-mediated immunosuppression. The diagram depicts AFP-induced immunosuppression (left) and the theoretical outcome of blocking AFP-mediated suppression (right). In the setting of AFP$^+$ tumors, the model proposes that dendritic cells (DCs) are skewed towards a tolerogenic phenotype that weakens CD8$^+$ cytotoxic T cells (CTLs), induces Tregs, and limits natural killer (NK) cell activity. Blocking the effects of tumor-derived AFP (tAFP) might reinvigorate DC function and ideally potentiate a robust T cell response and reversal of NK cell dysfunction.

of AFP, a robust innate immune response might be induced. Cytolytic NK cells might then kill tumor cells and release cancer antigens into the environment to be internalized by DCs. Such DCs, capable of cross-presentation, might present these cancer antigens to naïve $CD8^+$ T cells and ideally elicit robust *de novo* CTL responses as well as potent Th1 responses. We speculate that, by working in concert, these immune responses might limit tumor growth and improve patient outcomes. Therefore, understanding how and under which contexts AFP can exert its suppressive effects might lead to rationally designed candidate AFP-based vaccines aimed at limiting the immunoregulatory effects of AFP while ideally preserving antigenicity. Because the D2 and D3 domains of AFP, but not the D1 domain, suppressed DC function *in vitro*, we propose that a D1-based AFP vaccine might limit the potential suppressive effects of AFP. Accordingly, several vaccine approaches have focused on minimal HLA-A*02-restricted CD8 epitopes that are unlikely to retain the suppressive properties of full-length AFP, and these vaccines are immunogenic. However, these vaccines harbor modest immunogenicity, which might in theory be due to insufficient $CD4^+$ T cell help deemed important for the induction and maintenance of AFP-specific $CD8^+$ T cells (based on IFNγ ELISPOT and MHC multimer assays) [40]. In support of this concept, AFP-specific $CD4^+$ T cells have been detected in the blood of HCC patients [41], and these responses have been enhanced *in vitro* with an HLA-DR-restricted peptide [42] as well as with DC vaccines expressing full-length AFP [43]. A recent study identified an immunodominant CD4 epitope in AFP, and a synthesized vaccine could induce AFP-specific $CD4^+$ T cells in HCC patients [44]. Therefore, we argue that an approach to develop a successful candidate DC-based AFP vaccine might be to use the full-length AFP, bearing precise point mutations that could specifically block the binding of ligands predicted to be immunosuppressive, while ideally inducing robust $CD4^+$ and $CD8^+$ T cell responses (see Outstanding questions).

Other immunotherapeutic approaches to treating HCC might include the use of AFP-specific *ex vivo* engineered T cells [45]. We propose that by using cells generated *ex vivo* , it might be possible to address some of the limitations of using AFP vaccine-generated T cells, including their low frequency within tumors and **exhausted phenotypes.** Indeed, to develop an engineered T cell receptor (TCR)-based approach, one group generated AFP-specific $CD8^+$ T cells *in vitro* from HDs [46]. AFP-specific $CD8^+$ T cell TCR α/β chain genes were inserted into a lentiviral vector to engineer such T cells. These AFP-specific engineered cells killed HepG2 cells *in vitro* and *in vivo* when implanted into immunodeficient mouse tumor models [46]. Of note, AFP-specific **chimeric antigen receptor engineered T cells (CAR-T cells)** are currently being developed towards MHC-restricted AFP peptides, initially showing that they are highly specific for the HLA-A*02:01-restricted $AFP_{158–166}$ peptide, and have led to a rapid and robust decrease in tumor growth of both HepG2 and SK-HEP-1 cells in mouse models [47]. Consequently, these studies encourage the further development of engineered T cells to target AFP^+ tumors.

Concluding remarks

Prior work has yielded significant insights, reporting certain immunosuppressive properties of AFP which can affect NK cell viability and functionality, and limit monocyte and DC function, as well as indirectly skewing $CD4^+$ T cells towards a Treg phenotype *in vitro*. The proposed mechanisms for these suppressive functions may include: (i) the transportation of immunosuppressive AFP-bound LMM species, and (ii) certain intrinsic properties of the AFP protein which may include immune cell functions. However, many apparently contradictory data from previous studies [9,10,13,17,48,49] might be the result of differences in host species, clinical conditions, tissue sources, and isolation methods (Figure 2). These differences may affect various biochemical properties, such as the removal of bound ligands, the enrichment of select isoforms, as well as the status of isoelectric points and post-translational modifications (i.e., sialylation). The variation in biochemical properties may in turn limit or enhance the immunosuppressive properties of AFP,

Outstanding questions

How are fatty acids that are bound to AFP protein metabolized intracellularly to impact cellular metabolism? AFP can bind multiple fatty acids, but the resulting impact on cellular metabolism may likely depend on how these molecules are metabolized into signaling mediators. Additional research is needed to determine if PUFAs mediate nAFP- or tAFP-induced DC suppression or the generation of tolerogenic DCs, which might potentially identify opportunities for intervention.

Do the pathways by which AFP is taken up by different cell types impact the functional outcomes? There is a putative AFP cell surface receptor, as well as AFP uptake by the mannose receptor and scavenger receptors. Does the mode of uptake lead to differential biological impacts? If so, blockade of some receptors might be an opportunity to inhibit AFP uptake.

What is the best approach to block immunosuppressive effects of AFP in cancer? Targeting AFP-positive tumor cells is complex. Is there another approach to blocking AFP secretion by tumors? The results of adoptive T cell therapy clinical trials may yield new perspectives.

How can AFP-mediated immunosuppression be harnessed to treat certain autoimmune diseases? Will it behave similarly for different autoimmune diseases? Will systemic infusion of AFP protein be sufficient to improve autoimmune disease pathology, or should AFP complexes and other molecules such as fatty acids be tested? AFP stably complexed with specific fatty acids might potentially yield more robust inhibitory effects compared to AFP alone.

and thus many questions remain (see Outstanding questions). Moreover, even when anticipating an ideally full reversal of tAFP-specific effects, there are clearly multiple other immunoregulatory barriers to developing successful immunotherapies against HCC [50]. This tolerance might likely be, at least in part, because the liver is exposed to blood which is rich in gut-derived microbial products, and food-derived antigens enter the liver via the portal vein. Immune cells in the liver can produce IL-10, among potently suppressive molecules. The liver's tolerogenic nature, including the production of immunosuppressive IL-10, might contribute to explaining why the 5-year HCC-specific survival of patients not receiving surgery has been reported as being 14.7% for AFP-negative patients versus 6.1% for AFP-positive patients, but this remains to be robustly investigated [4]. Nevertheless, we posit that strategies to reverse the suppressive effects of tAFP while also increasing tumor-specific immune responses might contribute to help improve HCC patient survival.

Acknowledgments

Funding was provided by the Parker Institute for Cancer Immunotherapy. Expert graphic design assistance with the figures was provided by Khriszha H. Quema-Yee (Parker Institute for Cancer Immunotherapy).

Declaration of interests

No interests are declared.

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