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Abstract

The amino-acid catabolizing enzyme Interleukin-4 induced gene 1 (IL4I1) remains poorly characterized despite it is emerging as a pertinent therapeutic target for cancer. IL4I1 is secreted in the synaptic cleft by antigen-presenting cells. It inhibits TCR signaling, modulates naïve T cell differentiation and limits effector T cell proliferation. IL4I1 expression in tumors shapes the tumor microenvironment and impairs the antitumor cytotoxic T cell response, thereby facilitating cancer immune escape. Several mechanisms participate in these effects. Recent data suggest a role of new IL4I1 metabolites in activation of the aryl-hydrocarbon receptor (AHR). Here, we observe that expression of IL4I1 is poorly correlated with that of validated targets of AHR in human cancers. Moreover, dendritic cells do not upregulate AHR target genes in relation with IL4I1 expression *in vivo*. Finally, IL4I1 activity towards tryptophan leading to production of AHR-activating products is very low, and should be negligible when tryptophan-degrading enzymes of higher affinity compete for the substrate. We recently showed that IL4I1 expression by dendritic cells directly regulates immune synapse formation and modulates the repertoire and memory differentiation of responding CD8 T cells after viral infection. Thus, IL4I1 may restrain tumor control through regulating the priming of tumor-specific CD8 T cells, independently of AHR activation.

Introduction

Amino-acid catabolizing enzymes, responsible for the diminution of T-cell activation, proliferation, and/or function, have gained growing importance in immunology over the last 20 years (*1*). Several are now clearly perceived to be central actors of tumor escape from immunosurveillance. Illustrating this interest, indoleamine 2,3-dioxigenase (IDO1) has been proposed as a possible target for the treatment of cancer and small-molecule inhibitors have been tested in preclinical models and Phase I/II trials, with promising results. However, in a Phase III clinical trial in melanoma patients, the absence of any additional clinical benefit of the most advanced molecule, epacadostat, in combination with pembrolizumab has prompted rethinking of this strategy (*2*). Other immunosuppressive enzymes represent possible candidates for chemical inhibition.

Since the first study to identify human IL4I1 as a phenylalanine (Phe) oxidase secreted by antigen-presenting cells (dendritic cells [DC], monocytes, macrophages, and B-cells) in 2007, it has also been demonstrated to inhibit human T-cell proliferation *in vitro* (*3*, *4*). Both $CD8^+$ and $CD4^+$ T cells were equally inhibited, but $CD45RO^+$ (memory) cells were more sensitive than $CD45RA^+$ (naïve) cells. IL4I1 also facilitated the differentiation of $FoxP3^+$ regulatory T cells from naïve $CD4^+$ T cells (*5*). IL4I1 is produced by the macrophages that infiltrate most human solid tumors and by cancerous cells in the case of certain B-cell lymphoma types (*6*). Recent single cell analyses have also identified IL4I1 expression in tolerogenic DC subpopulations of the tumor microenvironment, which can express *IDO1* (*7*, *8*).

The role of IL4I1 has been characterized in mouse models of transplanted and spontaneous melanoma, both in WT and IL4I1 deficient backgrounds, clearly showing that it facilitates tumor growth by inhibiting the antitumor cytotoxic T-cell response and remodeling the tumor immune microenvironment (*9*, *10*). These observations have been recently extended to a model of chronic lymphocytic leukemia (*11*). In line with this, clinical correlations have been reported between IL4I1 expression by stromal cells and invasion of the sentinel lymph nodes, a higher melanoma stage, and rapid relapse in human primary cutaneous melanomas, in which IL4I1 expression was analyzed by immunohistochemistry (*12*). Most interestingly, zones in which IL4I1 expression was concentrated were depleted of cytotoxic $CD8^+$ T cells and enriched with regulatory $FoxP3^+$ T cells. Moreover, *IL4I1* was overexpressed in melanoma patients with progressive disease under treatment with the anti-PD-1 antibody nivolumab, suggesting a relation between IL4I1 expression and resistance to immune-checkpoint blockade (*11*).

We initially proposed IL4I1 as a prognostic biomarker and therapeutic target in cancer in 2009 (*6*). Transcriptomic data from several groups also indicated that IL4I1 is associated with poor outcome in certain human carcinomas (breast, renal, and colon carcinoma and glioma) (*13* and reviewed in *14*). Sadik *et al* recently showed IL4I1 expression in tumor cells of low-grade gliomas and glioblastomas and observed correlation with diminished patient survival (*11*). Glioblastoma is particular in that it is suspected to be derived from neural stem cells (*15*) and isoform 2 of IL4I1 is expressed in several neural cell types (*16*), with a still undefined function that may involve control of the level of neurotransmitter amines (*17*, *18*). In the same work, they demonstrate for the first time that IL4I1 promotes glioblastoma motility *in vitro*, an effect that may be highly relevant for metastatic processes. Finally, their work brings new insights by proposing that the immunosuppressive effect of IL4I1 in tumors is mediated by activation of the ligand-activated transcription factor aryl hydrocarbon receptor (AHR). AHR is widely distributed in tissues and displays very diverse activities, depending on the cellular and molecular context. Its role in cancer is complex and incompletely deciphered, involving both pro and anti-tumorigenic effects that affect directly tumor cells or the tumor microenvironment (*19*). Here, we examine the hypothesis of IL4I1-mediated AHR activation and propose a model of IL4I1 action during cancer development.

Material and methods

Transcriptomic analysis of human primary tumors and mouse DCs

Data from human primary tumors: breast, colon, and ovarian cancer, melanoma, mesothelioma, and glioblastoma were retrieved from The Cancer Genome Atlas (TCGA) using the Xena platform. Among genes used in the Sadik *et al.* study (*11*), we defined 45 experimentally-confirmed AHR target genes according to the Harmonizome platform (*20*), which report genes identified by Lo and Mathews (*21*). Spearman's rho were calculated using GraphPad Prism 8.

Genes modulated by 2,3,7,8-tétrachlorodibenzo-p-dioxine (TCDD) in human cells (hepatocytes, $CD34^+$ hematopoietic stem cells, MCF7 and HL60 cell lines) were retrieved from GSE7765, GSE14553, GSE16160, GSE24193, GSE46874, GSE122518 (NCBI GEO site), based on metaanalysis by Oshchepkova et al (*22*).

DCs were obtained by negative cell sorting from spleens of WT and $IL4I1^{-/-}$ mice at steady state and at 24h of infection with lymphocytic choriomeningitis virus (LMCV) strain WE2.2, as described in Puiffe et al. (*23*). Mouse experiments were approved by the local Ethical Committee for Animal Experimentation (Cometh Anses/ENVA/UPEC) and the French Research ministry under the number 05338.03.

Total RNA was isolated from purified DCs using RLT buffer and RNeasy columns (Qiagen). Libraries of polyA mRNA were generated using the TruSeq® Stranded mRNA Library Prep kit (Illumina) with double indexing using TruSeq RNA UD Illumina Indexes. RNA was reverse transcribed using SuperScript™ II (Invitrogen). Next-generation sequencing was performed by 75 bp single reading with the NextSeq 500/550 High Output Kit v2.5 (75 Cycles) on a NextSeq 500 analyzer (all from Illumina).

The twelve samples (40,675,782 ± 2,630,770 reads/sample) were quality-checked using the software FastQC (version 0.11.8). We checked that rRNA depletion had the expected quality (less than 2.32 ± 0.88 of total RNA, no prokaryotic contamination) and that more than 93% of the reads mapped to the mouse genome (GRCm38) using SortMeRNA (version 2.1b), FastQScreen (version 0.13) and Kraken2 (version 2.0.9 / default database). Trimmomatic (version 0.39) was used to filter reads using a quality 20 (sliding window of 5 reads) and minimal length of 50pb, which led to more than 96% of surviving reads. Filtered reads (39,151,723 ± 2,545,395) were aligned to the mouse genome (GRCm38) using STAR (version 2.6.1d). The mapping of the reads for the different regions of the genome and the level of gene expression was calculated using RSEM (version 1.3.2). The level of gene expression was normalized in CPM (counts per million). A heatmap was generated using GraphPad Prism 8 for the selected AHR-dependent genes and IL4I1, showing the mean CPM from three mice per condition.

IL4I1 enzymatic activity

Phe and Trp were dissolved in 0.1 N HCl and a 100 ng/ml solution of human recombinant IL4I1 (R&D) was used. Hydrogen peroxide production from IL4I1 activity was measured at 30 minutes according to Carbonnelle-Puscian et al. (*6*) with serial dilutions (1250 to 78 µM) of each amino acid. The Km and Vmax were calculated using GraphPad Prism 8. IL4I1 activity was also measured on the inducible recombinant HEK cell line expressing IL4I1 (described in Boulland et al (*3*). Lysates in Phosphate Buffer Saline containing protease inhibitors (Complete mini, Roche, Meylan, France) were obtained by 3 freeze-thaw cycles. The enzymatic test was performed as described above, on 40 µL cell lysates corresponding to $5x10^5$ cells. Activity was calculated as pmol H_2O_2 produced per min and per mL.

Results and discussion

Relation between IL4I1 expression and activation of the transcription factor AHR.

T-cell inhibition by IL4I1 has been mainly attributed to its enzymatic activity via the metabolic consequences of Phe consumption and the effect of hydrogen peroxide (*3*, *4*), although inhibition of early TCR signaling by IL4I1 does not involve amino acid deficiency, nor the presence of Phe catabolites (*24*). Sadik *et al.* establish a correlation between the expression of IL4I1 and of the pan-tissue AHR signature they generated. We examined the list of genes used for this AHR signature (*11*) and were puzzled by the fact that it contains several genes, of which the expression may reflect the immune infiltrate of the tumor, in particular, *IFNG*, *CD3E*, and *CD8A* (Table I). Indeed, the expression of *IL4I1* in primary tumors from TCGA correlates very strongly with that of T-cell related genes, as well as others, such as *IDO1*, which is indicative of the activation of a Th1 and cytotoxic T-cell response in the tumor bed (CD3E = 0.57±0.11, CD8A = 0.49±0.13, IFNG = 0.47±0.13, IDO1 = 0.55±0.15; mean rho ± SD on the six tumor types analyzed**; Fig. 1A).** This is in accordance with previous results in human and mouse melanomas (*10*, *12*) and with the known regulation of IL4I1 expression by interferons (*4*). This is also in line with the reanalysis by Sadik *et al* of data from Riaz et al (*25*), indicating concomitant induction of *IL4I1* and *IDO1* in patients with advanced melanoma treated with anti-PD-1. This induction may result from restored local production of IFNγ by reinvigorated T cells. As IL4I1 is produced by tumor-associated macrophages in most solid tumors (*6*), we also observed a strong correlation between IL4I1 and various genes attesting the presence of macrophages in the tumor bed (*CD68* = 0.57±0.15; *CD14* = 0.63 ± 0.15; *CD163* = 0.51 ± 0.17; mean rho ± SD on the six tumor types analyzed).

ABCC4	C21orf33	CYP3A4	FOXQ1	IL1B	**MSI2**	PCK2	SERPINE1	TNFSF9
ABCG2	CARD11	DKK3	FPR2	**IL1R2**	MYC	PDE2A	SESN2	TXNRD1
ACTA2	CAV1	DLX3	GATA3	IL2	NANOG	PDS5B	SH3KBP1	UGT1A6
ADM	CCL5	DUOX2	GFI1	IL6	**NCOA2**	PER1	SLC10A1	VAV3
AHR	**CCND1**	**EBF1**	GHR	**INSIG1**	NCOR2	PHGDH	SLC3A2	XDH
AHRR	CD36	**EDN1**	GNA13	IRF8	NDRG1	PIWIL1	**SLC7A5**	ZIC3
ALDH3A1	CD3E	**EGFR**	GSTA2	JAG1	**NEDD9**	PIWIL2	SMAD3	
AMIGO2	CD8A	EGR1	GSTM1	JUP	**NFE2L2**	PLA2G4A	SMAD7	
AQP3	CDK4	EPGN	**HES1**	KDM1A	NOS1	**PNPLA7**	**SOCS2**	
AREG	CDKN1A	EREG	HIF1A	KIAA1549	NOS3	POLK	**SORL1**	
ARG2	CFTR	**ESR1**	HLA-DRB4	KIT	NPTX1	PPARGC1A	**SOS1**	
ATP12A	CRH	F3	HMOX1	KMO	NQO1	**PRDM1**	SPRR2D	
ATP5IF1	CXCL2	FAS	HSD17B4	**LEPR**	NR1H3	PTGS2	**STC2**	
ATP5ME	CYBB	FAT1	HSPB2	LHCGR	NR1H4	**RARA**	**TFF1**	
ATP6AP2	CYP19A1	FBXO32	ID1	LIFR	**NR3C1**	REL	TGFBI	
BCL11B	**CYP1A1**	**FGFR2**	ID2	LPL	**NRIP1**	**RFC3**	TGM1	
BCL2	**CYP1A2**	FIG4	IFNG	LTBP1	**NSDHL**	RSPO3	TH	
BCL6	**CYP1B1**	FLG	IGF2	LYN	OVOL1	SCARB1	THBS1	
BLNK	CYP2B6	**FOS**	IGFBP1	MID1	**PAX5**	SCIN	**TIPARP**	
BRCA1	CYP2E1	FOXA1	**IKZF3**	MMP1	PCK1	SERPINB2	TJP1	

Table I. Genes of the AHR signature in Sadik et al. (n = 166). Forty-five genes identified as AHR targets by CHIP-Seq are in bold (*21*). Blue and red boxes indicate genes that are upregulated and dowregulated, respectively, by TCDD in GSE7765, GSE14553, GSE16160, GSE24193, GSE46874, GSE122518. HLA-DRB4 is upregulated in $CD34^+$ cells, but downregulated in hepatocytes. The most robustly AHR-dependent genes in this list are *AHRR*, *CYP1A1*, *CYP1B1*, *NFE2L2*, *SLC7A5*, *TIPARP* (all upregulated) according to the metaanalysis of Oshchepkova et a l(*22*) .

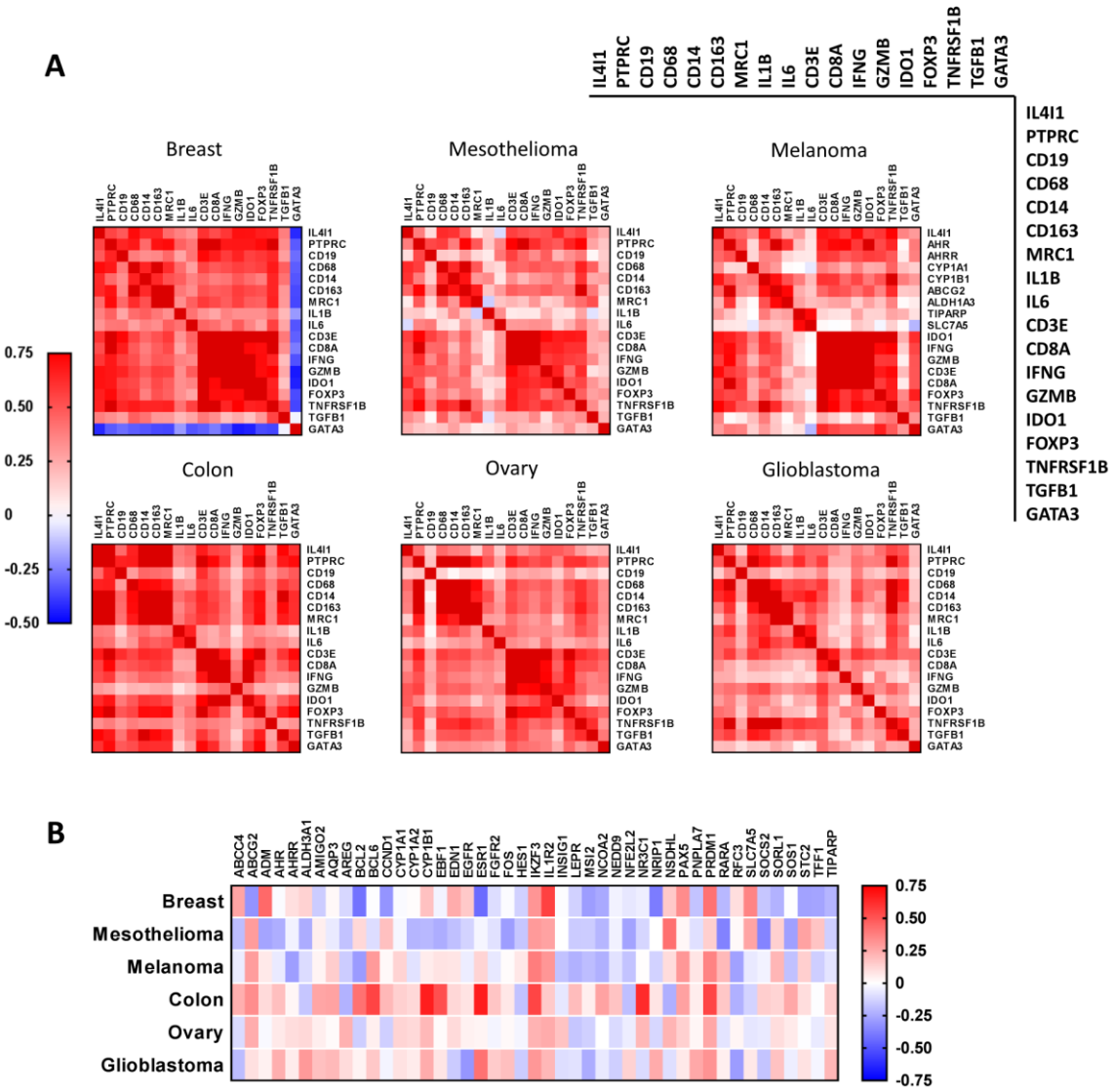

Fig.1. Correlation between the expression of IL4I1, immune-response genes and AHR-regulated genes in primary tumors. (**A**) Correlation between *IL4I1* and *PTPRC* (coding the panleukocyte marker CD45), *CD19* (B cells), *CD68*, *CD14*, *CD163*, *MRC1*, *IL1B*, *IL6* (macrophages and inflammation), *CD3E*, *CD8A*, *IFNG*, *GZMB*, *IDO1*, *FOXP3*, *TGFB1*, *TNFRSF1B and GATA3* (T-cell infiltration, Th1 response, regulatory T-cells, Th2 cells). (**B**) Correlation between *IL4I1* and AHR-dependent genes. Each line shows results for one of the six primary cancer types analyzed. Values above 0.5 are considered highly significant correlations.

A comparison of the AHR signature reported by Sadik *et al.* to validated AHR targets identified by CHIP-seq in the study of Lo and Matthews (*21*), retrieved 45 genes common to both lists, which did not include IFNG, CD3E, or CD8A. We analyzed these 45 genes for correlations with IL4I1 expression in primary tumors from the TCGA database using the Xena platform (*26*) (**Fig. 1B**). With few exceptions, Spearman's rho was consistently inferior to 0.25; depending on tumor type, 1 to 15 genes had rho > 0.25 and 0 to 9 had rho <-0.25 (total, 1 to 15 genes modulated). Colon carcinoma displayed the highest correlation between *IL4I1* and AHR-regulated genes (15 genes upregulated with rho>0.25, 7 with rho>0.5, none downregulated with rho <-0.25). Three of these genes (*CYP1B1*, *ESR1, ALDH3A1*) are known to be upregulated by TCDD *in vitro* (Table I). However, in colon cancer, the diet and/or the microbiota can provide numerous AHR ligands.

To confirm these observations on murine cells, we infected WT and IL4I1-deficient ($IL4I1^{-/-}$) mice with LCMV and sorted splenic DCs both at steady state and after infection (**Fig. 2**). Transcriptomic analysis

did not show any relationship between IL4I1 expression and activation of the 45 AHR target genes. Despite vigorous induction of IL4I1 expression in response to LCMV, AHR-related genes were not significantly upregulated compared to the steady state. In addition, these genes were similarly expressed in DCs from both mouse strains. Thus, the relationship between IL4I1 expression and induction of the AHR pathway may be only indirect, implying that targeting of the AHR pathway and IL4I1 for cancer treatment may be independent, and, potentially synergistic.

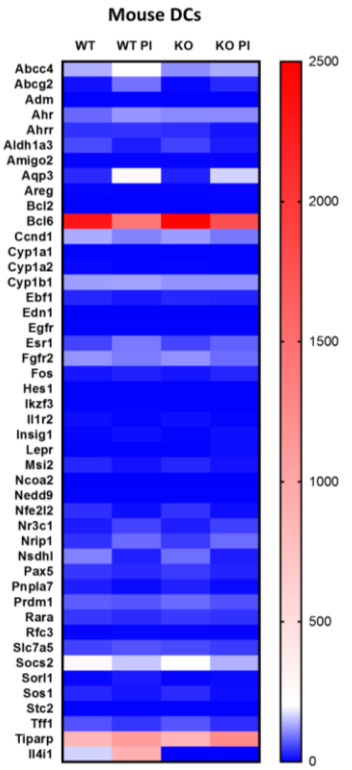

Fig. 2. Transcriptomic analysis of purified DCs from WT and IL4I1$^{-/-}$ mice at steady state and after 24 h of LCMV infection. The heat map shows counts per million for the selected AHR-dependent genes and *IL4I1* (mean from three mice per group). A previous analysis of the cDNA library at steady state has been published in Puiffe et al. (*23*). PI: post-infection; KO: IL4I1$^{-/-}$.

IL4I1 degradation of tryptophan (Trp)

Sadik *et al.* demonstrate that IL4I1 degrades the essential amino acid Trp into indole-3-pyruvate (I3P), which is immediately converted *in vitro* to kynurenic acid (KynA) and other derivatives. I3P, KynA, and indole-3-aldehyde all activate AHR (*11*). A study contemporaneously published by Zhang *et al.* also provides arguments for I3P production by IL4I1 activity (*27*). However, IL4I1 has a strong preference towards Phe. A greater than five-fold higher Phe-degrading than Trp-degrading activity was initially observed using a relatively insensitive colorimetric test (*3*). Indeed, although measurement of the Km using an optimized technique shows similar values for the two amino acids, the Vmax of IL4I1 towards Phe is almost 10-fold higher than that towards Trp (**Fig. 3A**). We validated these data on human cell lysates obtained from recombinant HEK cells expressing IL4I1 (**Fig. 3B**). The difference between Phe and Trp catabolism was even greater than with the purified enzyme. This might be due to different glycosylation, as commercial IL4I1 is produced in Chinese hamster ovary cells. Moreover, in the human body, the concentration of Phe is equal to or slightly higher than that of Trp and the concentration of Phe is substantially higher in classical culture media than that of Trp (**Fig. 3C**). Thus, the *in vitro* and *in vivo* catabolism of Trp by IL4I1 should be less than that of Phe and the production of phenylpyruvate should overtake that of I3P. This also suggests that IDO1, whose affinity for the Trp substrate is around 50 folds higher than IL4I1 (Trp Km~20 µM for IDO (*28*) versus 974 µM for IL4I1) and which is often expressed concomitantly with IL4I1, as stated above, may degrade Trp more efficiently than IL4I1. In accordance, a metabolomic study demonstrates a significant increase in phenylpyruvate and phenyllactacte in ovarian cancer in comparison to normal

ovary, but does not detect variations in indole compounds (*29*). Another indirect argument may come from a study in an orthotopic model of mouse pancreatic cancer. In this model, tumor regression after treatment with metformin and torin 2 (mTORC1 and mTORC2 inhibitors) was associated with a 4-fold increase of plasmatic phenylalanine (*30*). Finally, the use of a specific IL4I1 inhibitor in mouse with B16 melanoma led to significant decrease of phenylpyruvate levels in the tumors (*31*).

The I3P produced by IL4I1 in the presence of H_2O_2 (which is a byproduct of the enzymatic reaction) would spontaneously be degraded to KynA the main mediator of AHR activation (*11*). However, KynA could be produced independently of IL4I1 activity and experiments showing AHR activation *in vitro* required largely supraphysiological concentrations of the products (3.13 to 40 µM I3P) and Trp consumption required five days of culture to be measured.

Overall, these observations raise questions about the *in vivo* relevance of Trp degradation by IL4I1, in particular, in comparison to the effect of IDO1 on the same substrate. In any case, treatment with IL4I1 inhibitors should block the enzyme, regardless of the amino-acid substrate, Phe, Trp, or even arginine, which has also been demonstrated to be a minor IL4I1 substrate (*14*).

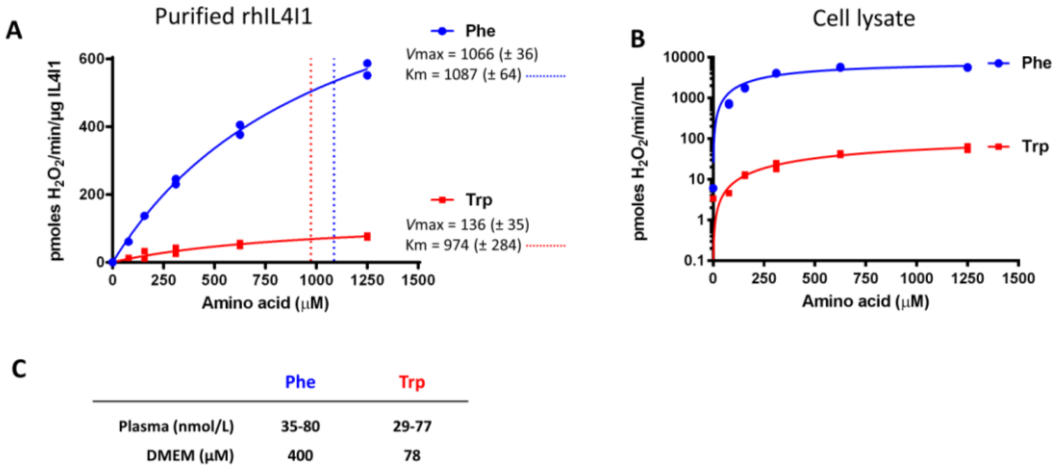

Fig. 3. IL4I1 activity towards phenylalanine (Phe) and tryptophan (Trp). (**A**) Hydrogen peroxide production from the activity of recombinant human IL4I1 was measured in two independent experiments performed in duplicate. Values for Km and Vmax (± SD) were determined. The blue and red dotted lines indicate Km for Phe and Trp, respectively. (**B**) IL4I1 activity was measured as in A on cell lysates from recombinant HEK cells expressing human IL4I1 (one experiment performed in duplicate). (**C**) Concentration of Phe and Trp in human plasma (reference values from the Mayo clinic) and in DMEM.

Implications for cancer of IL4I1 modulation of CD8 T cell priming

We recently dissected the effect of IL4I1 during the priming of naïve CD8 T cells using a model of acute LCMV infection in WT and IL4I1$^{-/-}$ mice (*23*). This work revealed unsuspected aspects of IL4I1 action. Somewhat counterintuitively, the effector CD8 T cell response was stronger in WT (IL4I1^{+}) mice than in IL4I1$^{-/-}$ mice. Indeed, the genetic inactivation of *IL4I1* was associated with delayed and slightly diminished expansion of functional short lived-effector CD8 T cells, but enhanced memory T cell differentiation. These observations were not related to intrinsic differences of CD8 T cells between WT and IL4I1$^{-/-}$ mice, but resulted from modulation of immune synapse formation and early activation events by IL4I1-expressing DCs. Indeed, IL4I1 enhanced the T cell activation threshold, thereby favoring the priming of high-affinity clones, restriction of the response to the most immunodominant peptides and rapid acquisition of effector differentiation.

These effects, which are unrelated to AHR activation (**Fig. 2**), are relevant for the T-cell response against cancer. Indeed, IL4I1 expression not only inhibits the proliferation and function of anti-tumor effector T cells in the tumor microenvironment, as previously shown, but its presence in secondary lymphoid organs may restrain the repertoire of primed anti-tumor CD8 T-cells, limiting control of mutant tumor subclones. Moreover, as IL4I1 is associated with diminished differentiation of memory CD8 T cells, it may limit the capacity to durably regenerate anti-tumor effector cells. Thus, inhibition of IL4I1 should both improve the antitumor effector response and allow long-lasting revitalized immunity against cancer.

Overall, it has been clearly demonstrated in the literature that IL4I1 alters the antitumor CD8 T-cell response and that IL4I1-mediated immunoregulation facilitates cancer growth, affecting patient survival and potentially fostering resistance to immune checkpoint inhibitors. Despite these new advances, there are still uncertainties concerning the mechanism of the action of IL4I1 on the T-cell response at the molecular and cellular levels. Recent data suggest that IL4I1 affects directly both CD8 T cells and tumor cells (**Fig. 4**). Indeed, IL4I1-dependent modulation of the affinity and functional properties of the CD8 T-cell repertoire elicited at priming may interfere with its capacity to combat tumor cells (*23*). In this case, IL4I1 effects on early T cell signaling and immune synapse formation are too rapid to involve gene regulation. In particular contexts where sufficient Trp catabolites are generated, IL4I1-mediated AHR activation may play a role in enhancing tumor-cell migration and metastasis (*11*), or tumor cell survival, as I3P has also been recently proposed to elicit a cell protective gene expression program (*32*). Pharmacological blockade of IL4I1 is therefore of high interest.

In contrast to other immunoregulatory enzymes, IL4I1 is a secreted protein and is therefore easily accessible to pharmacological blockade (*33*). An orally-available compound with potent IL4I1 inhibitory activity *in vitro* has been recently developed (*31*). This compound limits the growth of several tumors in mouse models, without significant toxicity, opening the way to IL4I1 targeting in human cancer. Thus, a complete understanding of the actions of IL4I1 will be fundamental to facilitating clinical development and avoiding the failure that occurred with epacadostat (*2*).

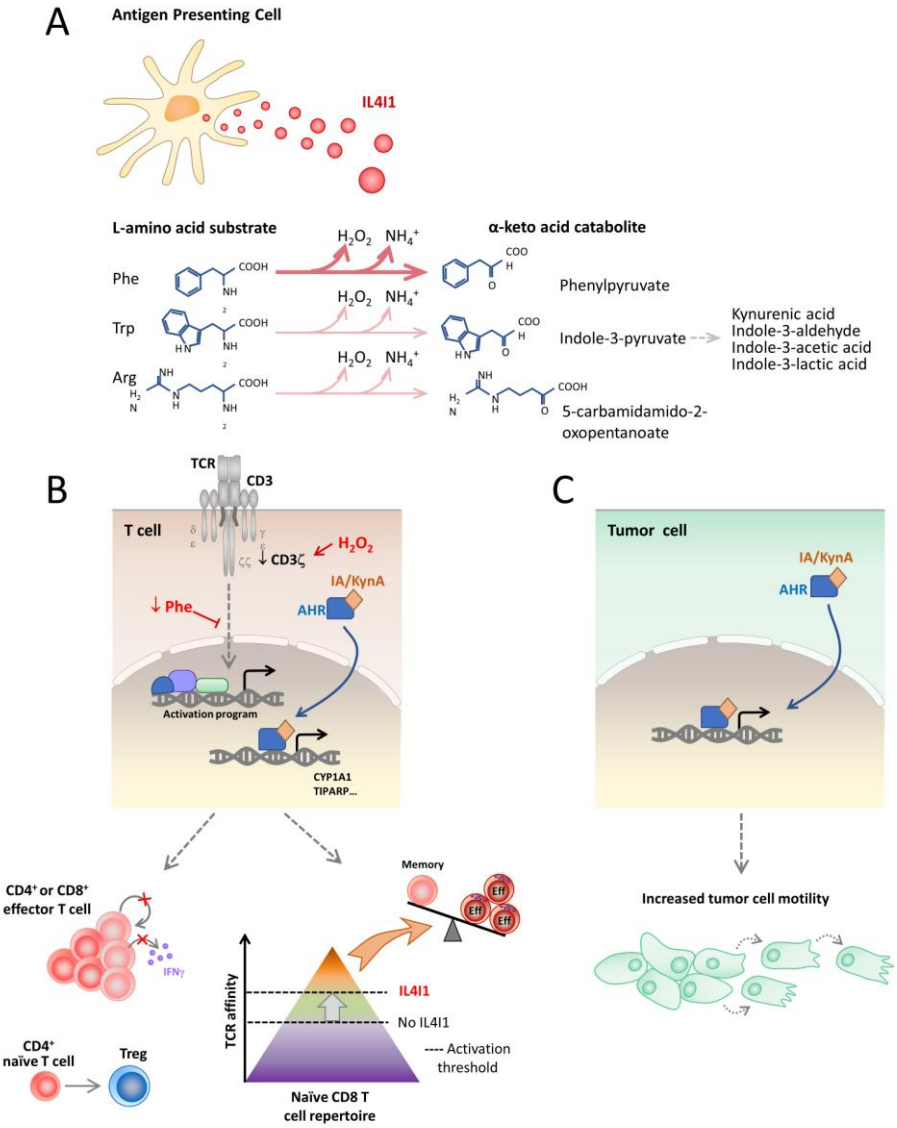

Fig. 4. Mechanism of action of IL4I1 in the context of cancer. (**A**) IL4I1 is secreted into the synaptic cleft between an antigen-presenting cell and a T cell. L-amino acid oxidation by IL4I1 produces an α-keto acid, while liberating hydrogen peroxide (H_2O_2) and ammonia (NH_4^+). Phe is the most rapidly degraded amino acid but Trp and arginine (Arg) are also IL4I1 substrates. Sadik *et al.* showed that indole-3-pyruvate produced by Trp degradation is not detectable, as it is rapidly converted into non AHR-stimulating indole derivatives and kynurenic acid (KynA) and indole-3-aldehyde (IA), which are AHR agonists. (**B**) IL4I1 enzymatic activity is responsible for the consumption of Phe, which deprives T cells of this essential amino acid, and potentially participates in limiting the availability of Trp and Arg. This leads to decreased activation of the mTORC1 pathway downstream of the TCR and costimulatory receptors. T-cell signaling is also sensitive to hydrogen peroxide, which reduces membrane exposure of the CD3 ζ chain. The mechanism of inhibition of early TCR signaling (ZAP70 activation) is still unresolved. Phe depletion and IL4I1-produced second messengers limit effector T-cell proliferation and favor regulatory T-cell differentiation from naïve CD4 T cells, individually or in combination. As shown by Puiffe *et al.*, IL4I1 also increases the threshold of CD8 T-cell activation, thus restraining the primed repertoire to high-affinity clones and favoring the differentiation of effector cells at the expense of memory cells. Cancer development is favored by the diminution of the quality and intensity of the T-cell response. (**C**) Metastasis may also be promoted by increased tumor-cell motility induced by KynA and IA stimulation of the AHR pathway in tumor cells.

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Bibliography

1. P. J. Murray, Amino acid auxotrophy as a system of immunological control nodes. *Nat. Immunol.* **17**, 132–139 (2016).
2. B. J. Van den Eynde, N. van Baren, J.-F. Baurain, Is There a Clinical Future for IDO1 Inhibitors After the Failure of Epacadostat in Melanoma? *Annu. Rev. Cancer Biol.* **4**, 241–256 (2020).
3. M.-L. Boulland, J. Marquet, V. Molinier-Frenkel, P. Möller, C. Guiter, F. Lasoudris, C. Copie-Bergman, M. Baia, P. Gaulard, K. Leroy, F. Castellano, Human IL4I1 is a secreted L-phenylalanine oxidase expressed by mature dendritic cells that inhibits T-lymphocyte proliferation. *Blood*. **110**, 220–227 (2007).
4. J. Marquet, F. Lasoudris, C. Cousin, M.-L. Puiffe, N. Martin-Garcia, V. Baud, F. Chereau, J.-P. Farcet, V. Molinier-Frenkel, F. Castellano, Dichotomy between factors inducing the immunosuppressive enzyme IL-4-induced gene 1 (IL4I1) in B lymphocytes and mononuclear phagocytes. *Eur. J. Immunol.* **40**, 2557–2568 (2010).
5. C. Cousin, A. Aubatin, S. Le Gouvello, L. Apetoh, F. Castellano, V. Molinier-Frenkel, The immunosuppressive enzyme IL4I1 promotes FoxP3(+) regulatory T lymphocyte differentiation. *Eur. J. Immunol.* **45**, 1772–1782 (2015).
6. A. Carbonnelle-Puscian, C. Copie-Bergman, M. Baia, N. Martin-Garcia, Y. Allory, C. Haioun, A. Crémades, I. Abd-Alsamad, J.-P. Farcet, P. Gaulard, F. Castellano, V. Molinier-Frenkel, The novel immunosuppressive enzyme IL4I1 is expressed by neoplastic cells of several B-cell lymphomas and by tumor-associated macrophages. *Leukemia*. **23**, 952–960 (2009).
7. B. Maier, A. M. Leader, S. T. Chen, N. Tung, C. Chang, J. LeBerichel, A. Chudnovskiy, S. Maskey, L. Walker, J. P. Finnigan, M. E. Kirkling, B. Reizis, S. Ghosh, N. R. D'Amore, N. Bhardwaj, C. V. Rothlin, A. Wolf, R. Flores, T. Marron, A. H. Rahman, E. Kenigsberg, B. D. Brown, M. Merad, A conserved dendritic-cell regulatory program limits antitumour immunity. *Nature*. **580**, 257–262 (2020).
8. Y. Liu, S. He, X.-L. Wang, W. Peng, Q.-Y. Chen, D.-M. Chi, J.-R. Chen, B.-W. Han, G.-W. Lin, Y.-Q. Li, Q.-Y. Wang, R.-J. Peng, P.-P. Wei, X. Guo, B. Li, X. Xia, H.-Q. Mai, X.-D. Hu, Z. Zhang, Y.-X. Zeng, J.-X. Bei, Tumour heterogeneity and intercellular networks of nasopharyngeal carcinoma at single cell resolution. *Nat. Commun.* **12**, 741 (2021).
9. F. Lasoudris, C. Cousin, A. Prevost-Blondel, N. Martin-Garcia, I. Abd-Alsamad, N. Ortonne, J.-P. Farcet, F. Castellano, V. Molinier-Frenkel, IL4I1: an inhibitor of the CD8+ antitumor T-cell response in vivo. *Eur. J. Immunol.* **41**, 1629–1638 (2011).
10. L. Bod, R. Lengagne, L. Wrobel, J. P. Ramspott, M. Kato, M.-F. Avril, F. Castellano, V. Molinier-Frenkel, A. Prévost-Blondel, IL4-induced gene 1 promotes tumor growth by shaping the immune microenvironment in melanoma. *Oncoimmunology*. **6**, e1278331 (2017).
11. A. Sadik, L. F. Somarribas Patterson, S. Öztürk, S. R. Mohapatra, V. Panitz, P. F. Secker, P. Pfänder, S. Loth, H. Salem, M. T. Prentzell, B. Berdel, M. Iskar, E. Faessler, F. Reuter, I. Kirst, V. Kalter, K. I. Foerster, E. Jäger, C. R. Guevara, M. Sobeh, T. Hielscher, G. Poschet, A. Reinhardt, J. C. Hassel, M. Zapatka, U. Hahn, A. von Deimling, C. Hopf, R. Schlichting, B. I. Escher, J. Burhenne, W. E. Haefeli, N. Ishaque, A. Böhme, S. Schäuble, K. Thedieck, S. Trump, M. Seiffert, C. A. Opitz, IL4I1 Is a Metabolic Immune Checkpoint that Activates the AHR and Promotes Tumor Progression. *Cell*, S0092867420309466 (2020).
12. J. P. Ramspott, F. Bekkat, L. Bod, M. Favier, B. Terris, A. Salomon, L. Djerroudi, K. S. Zaenker, Y. Richard, V. Molinier-Frenkel, F. Castellano, M.-F. Avril, A. Prévost-Blondel, Emerging Role of IL-4-Induced Gene 1 as a Prognostic Biomarker Affecting the Local T-Cell Response in Human Cutaneous Melanoma. *J. Invest. Dermatol.* **138**, 2625–2634 (2018).
13. M. Liu, Q. Pan, R. Xiao, Y. Yu, W. Lu, L. Wang, A cluster of metabolism-related genes predict prognosis and progression of clear cell renal cell carcinoma. *Sci. Rep.* **10**, 12949 (2020).
14. V. Molinier-Frenkel, A. Prévost-Blondel, F. Castellano, The IL4I1 Enzyme: A New Player in the Immunosuppressive Tumor Microenvironment. *Cells*. **8** (2019), doi:10.3390/cells8070757.
15. J. H. Lee, J. E. Lee, J. Y. Kahng, S. H. Kim, J. S. Park, S. J. Yoon, J.-Y. Um, W. K. Kim, J.-K. Lee, J. Park, E. H.

Kim, J.-H. Lee, J.-H. Lee, W.-S. Chung, Y. S. Ju, S.-H. Park, J. H. Chang, S.-G. Kang, J. H. Lee, Human glioblastoma arises from subventricular zone cells with low-level driver mutations. *Nature*. **560**, 243–247 (2018).
16. S. Wiemann, A. Kolb-Kokocinski, A. Poustka, Alternative pre-mRNA processing regulates cell-type specific expression of the IL4I1 and NUP62 genes. *BMC Biol.* **3**, 16 (2005).
17. Y. Gao, J. Mu, T. Xu, T. Linghu, H. Zhao, J. Tian, X. Qin, Metabolomic analysis of the hippocampus in a rat model of chronic mild unpredictable stress-induced depression based on a pathway crosstalk and network module approach. *J. Pharm. Biomed. Anal.* **193**, 113755 (2021).
18. F. Castellano, V. Molinier-Frenkel, Control of T-cell activation and signaling by amino-acid catabolizing enzymes. *Front. Cell Dev. Biol.*
19. A. Paris, N. Tardif, M.-D. Galibert, S. Corre, AhR and Cancer: From Gene Profiling to Targeted Therapy. *Int. J. Mol. Sci.* **22**, 752 (2021).
20. A. D. Rouillard, G. W. Gundersen, N. F. Fernandez, Z. Wang, C. D. Monteiro, M. G. McDermott, A. Ma'ayan, The harmonizome: a collection of processed datasets gathered to serve and mine knowledge about genes and proteins. *Database*. **2016**, baw100 (2016).
21. R. Lo, J. Matthews, High-resolution genome-wide mapping of AHR and ARNT binding sites by ChIP-Seq. *Toxicol. Sci. Off. J. Soc. Toxicol.* **130**, 349–361 (2012).
22. E. Oshchepkova, Y. Sizentsova, D. Wiebe, V. Mironova, N. Kolchanov, Meta-Analysis of Transcriptome Data Detected New Potential Players in Response to Dioxin Exposure in Humans. *Int. J. Mol. Sci.* **21** (2020), doi:10.3390/ijms21217858.
23. M.-L. Puiffe, A. Dupont, N. Sako, J. Gatineau, J. L. Cohen, D. Mestivier, A. Lebon, A. Prévost-Blondel, F. Castellano, V. Molinier-Frenkel, IL4I1 Accelerates the Expansion of Effector CD8+ T Cells at the Expense of Memory Precursors by Increasing the Threshold of T-Cell Activation. *Front. Immunol.* **11**, 600012 (2020).
24. A. Aubatin, N. Sako, X. Decrouy, E. Donnadieu, V. Molinier-Frenkel, F. Castellano, IL4-induced gene 1 is secreted at the immune synapse and modulates TCR activation independently of its enzymatic activity. *Eur. J. Immunol.* **48**, 106–119 (2018).
25. N. Riaz, J. J. Havel, V. Makarov, A. Desrichard, W. J. Urba, J. S. Sims, F. S. Hodi, S. Martín-Algarra, R. Mandal, W. H. Sharfman, S. Bhatia, W.-J. Hwu, T. F. Gajewski, C. L. Slingluff, D. Chowell, S. M. Kendall, H. Chang, R. Shah, F. Kuo, L. G. T. Morris, J.-W. Sidhom, J. P. Schneck, C. E. Horak, N. Weinhold, T. A. Chan, Tumor and Microenvironment Evolution during Immunotherapy with Nivolumab. *Cell*. **171**, 934-949.e16 (2017).
26. M. J. Goldman, B. Craft, M. Hastie, K. Repečka, F. McDade, A. Kamath, A. Banerjee, Y. Luo, D. Rogers, A. N. Brooks, J. Zhu, D. Haussler, Visualizing and interpreting cancer genomics data via the Xena platform. *Nat. Biotechnol.* **38**, 675–678 (2020).
27. X. Zhang, M. Gan, J. Li, H. Li, M. Su, D. Tan, S. Wang, M. Jia, L. Zhang, G. Chen, *J. Agric. Food Chem.*, in press, doi:10.1021/acs.jafc.0c03735.
28. L. Zhai, E. Ladomersky, A. Lenzen, B. Nguyen, R. Patel, K. L. Lauing, M. Wu, D. A. Wainwright, IDO1 in cancer: a Gemini of immune checkpoints. *Cell. Mol. Immunol.* **15**, 447–457 (2018).
29. M. Y. Fong, J. McDunn, S. S. Kakar, Identification of metabolites in the normal ovary and their transformation in primary and metastatic ovarian cancer. *PloS One*. **6**, e19963 (2011).
30. G. A. Soliman, S. M. Steenson, A. H. Etekpo, Effects of Metformin and a Mammalian Target of Rapamycin (mTOR) ATP-Competitive Inhibitor on Targeted Metabolomics in Pancreatic Cancer Cell Line. *Metabolomics Open Access*. **6** (2016), doi:10.4172/2153-0769.1000183.
31. A. MacKinnon, D. Bhupathi, J. Chen, T. Huang, W. Li, Y. Ma, N. Sotirovska, S. Steggerda, W. Zhang, F. Parlati, 705 Anti-tumor activity of CB-668, a potent, selective and orally bioavailable small-molecule inhibitor of the immuno-suppressive enzyme Interleukin 4 (IL-4)-Induced Gene 1 (IL4I1). *J. Immunother. Cancer*. **8**, A747–A747 (2020).
32. L. Zeitler, A. Fiore, C. Meyer, M. Russier, G. Zanella, S. Suppmann, M. Gargaro, S. S. Sidhu, S. Seshagiri, C. Ohnmacht, T. Köcher, F. Fallarino, A. Linkermann, P. J. Murray, Anti-ferroptotic mechanism of IL4i1-mediated amino acid metabolism. *eLife*. **10** (2021), doi:10.7554/eLife.64806.
33. M. Presset, D. Djordjevic, A. Dupont, E. Le Gall, V. Molinier-Frenkel, F. Castellano, Identification of inhibitors of the immunosuppressive enzyme IL4I1. *Bioorganic Chem.* **94**, 103463 (2020).