SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

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TITOLO DELLA RELAZIONE: Aryl hydrocarbon Receptor and RelB control long-term expression of indoleamine 2,3-dioxygenase 1 in dendritic cells favoring endotoxin tolerance

RELAZIONE:

The aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, acts as a heterodimeric transcriptional regulator via association with the AhR nuclear translocator (ARNT). Widely expressed in a variety of animal species as well as in humans and initially credited with a specific function – namely, the hepatic clearance of xenobiotics, including dioxin – AhR has physiological functions that are only now beginning to be appreciated [1]. Animal and human data indicate that AhR is involved in various signaling pathways critical to cell normal homeostasis and even neoplasia [2], including cell proliferation and differentiation, gene regulation, cell motility and migration, inflammation and others [3, 4]. Signal molecules function as ligands for AhR, and activated AhR binds promoter recognition sequences of the target genes. The AhR–ARNT complex may then require coactivators – including members of other families of transcription factors [5] – in order to initiate transcription and to unwind histone-bound DNA for exposing additional promoter recognition sites via their histone acetyltransferase function.

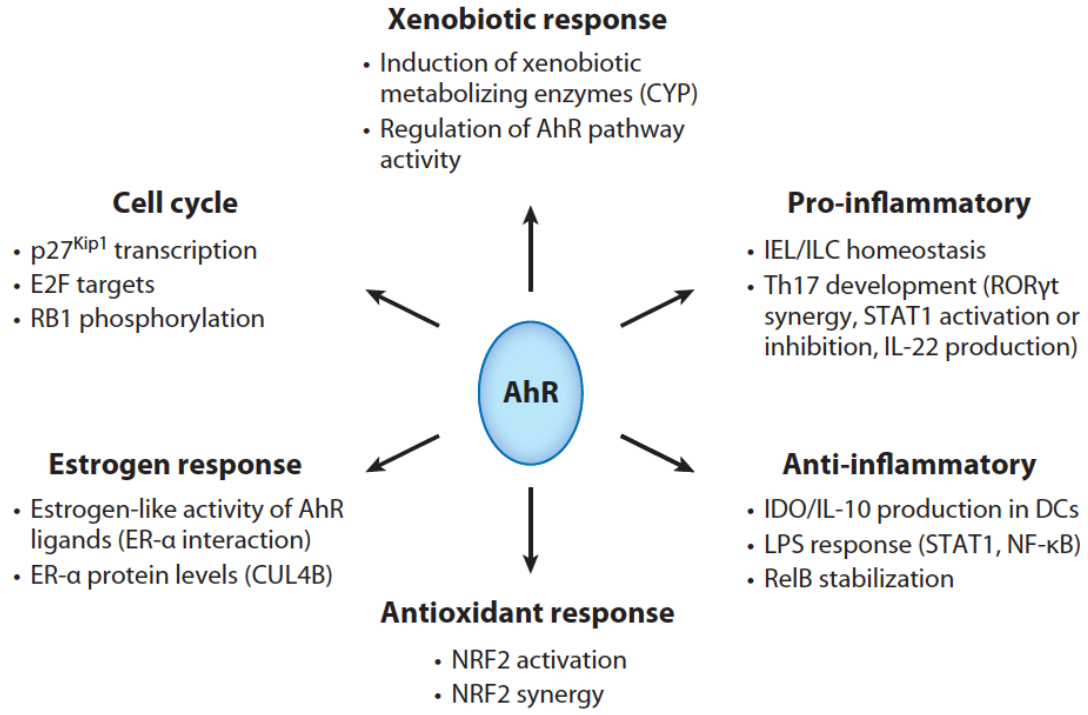

Figure 1. Multiple putative AhR functions in the immune system. AhR ligands affect several pathways and responses according to the cellular context. The exact molecular mechanisms through which many of these functions are achieved remain elusive, but several components have been described, pointing to a synergy or antagonism at the protein-protein interaction or the chromatin level. (Abbreviations: DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; IEL, intraepithelial lymphocyte; ILC, innate lymphoid cell; LPS, lipopolysaccharide; NRF2, nuclear factor erythroid 2–related factor 2)

Three major factors may affect to the outcome of gene transcriptional regulation by AhR, namely, the nature of the ligand, the local tissue microenvironment, and the presence of coactivators in the cell. Much interest in now centered on the nature of AhR's physiological ligands as well as on its mode of action in response to molecules that are different in nature as to their endogenous source and chemical structure [6]. AhR activation by physiologically relevant ligands occurs in gut innate lymphoid cells by microbiota-derived indole-3-aldehyde (IAld) [7], in skin keratinocytes by endogenous 6-formylindolo[3,2-b]carbazole (FICZ) [8], and in lymphoid tissue dendritic cells by a product of tryptophan catabolic enzymes, L-kynurenine [9]. This receptor features multiple conformations of the binding site endowed with diverse properties to accommodate structurally different ligands.

Recently, in our laboratory, we demonstrated the nature of kynurenine as a ligand of AhR. We obtained a functionally relevant, mutated form of AhR—we developed homology models of the ligand-binding domain of AhR by comparing L-kynurenine (a low-affinity ligand) with 2,3,7,8 - tetrachlorodibenzo-

p-dioxin (TCDD; a high-affinity ligand). As a result of docking experiments, TCDD and L-kynurenine appeared to bind different conformations of the receptor, which are endowed with rather opposite properties of the binding site that nicely fit the respective hydrophilic and hydrophobic properties of the ligands. In particular, Gln377 appeared to be crucial for binding L-kynurenine. A mutant receptor was engineered carrying a Gln377 to Ala mutation. AhR-deficient cDCs reconstituted with the mutated AhR bearing the Q377A mutation, followed by L-kynurenine treatment, were ineffective in inducing IDO1 both at the mRNA and protein levels [9]. Moreover, the production of both TGF-β and IL-10 was greatly impaired in AhR-deficient cDCs reconstituted with the mutant AhR form and then stimulated with L-kynurenine, as compared to WT counterparts. These results, underlining the different behaviors of L-kynurenine and TCDD as AhR ligands, showed a crucial requirement for the Gln377 residue of AhR for L-kynurenine interaction.

RESULTS

During of my PhD training, I contrubuted to clarifying the specific mechanisms that control hyperinflammatory responses to acute endotoxin challenge. These mechanisms operate in the complex phenomenon whereby an initial exposure to endotoxin induces a durable state of resistance to subsequent endotoxemia[10, 11, 12, 13]. Here, we demonstrated that an initial exposure to lipopolysaccharide (LPS) induces a rapid increase in endogenous kynurenine in wild-type as well as indoleamine 2,3 dioxygenases (IDO1 or IDO2*)*-deficient mice. In contrast, LPS fails to promote protective kynurenine/AhR-dependent responses in mice lacking hepatic tryptophan 2,3 dioxygenase (TDO2), which is an early component of the acute phase protein response in inflammation. These results suggest a critical role of TDO2 in the kynurenine-driven AhR repression of early, LPS-responsive inflammatory genes. Increased levels of proinflammatory cytokines were detected in sera from TDO2-deficient mice as compared to WT mice or mice lacking IDO1 or IDO2, with the recruitment of migratory neutrophils, responsible for multiorgan infiltration. In summary, our data suggest that, upon LPS exposure, TDO2-dependent production of L-kynurenine activates AhR to limit inflammation and systemic host damage (**Fig.2**).

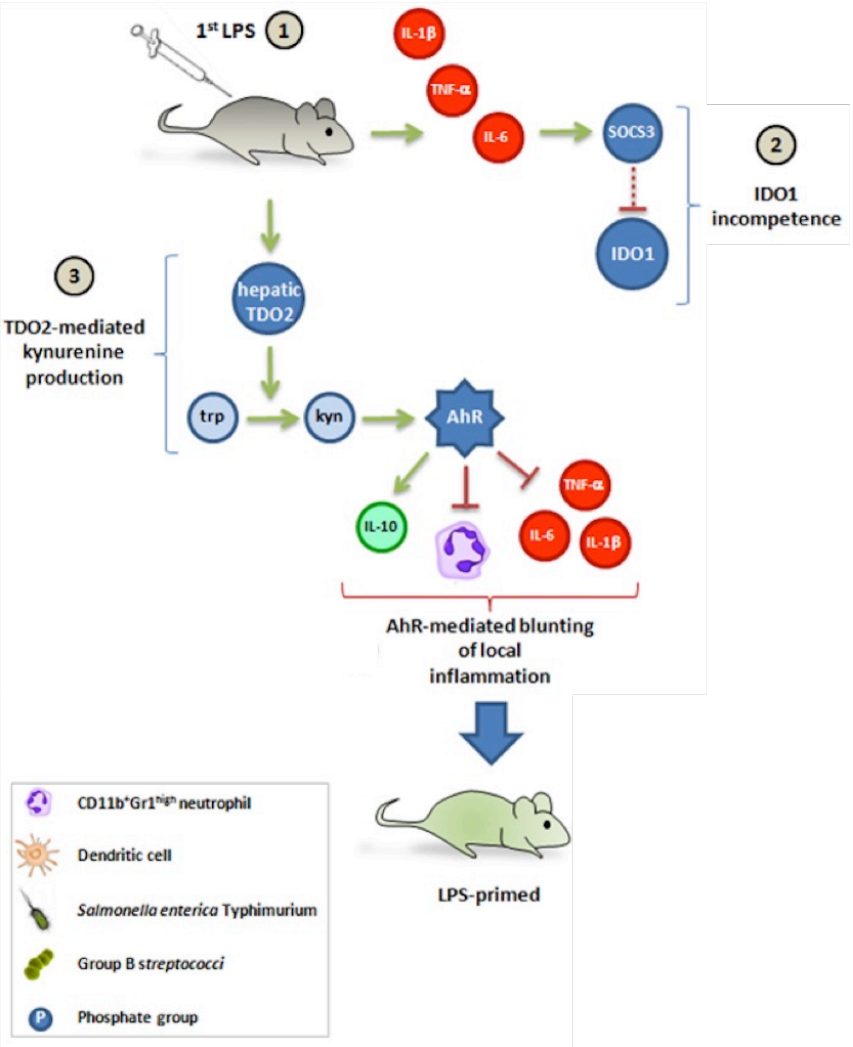

Figure 2. Schematic representation of specific mechanisms that control hyperinflammatory responses to acute endotoxin challenge. (1) Intraperitoneal administration of an LPS sublethal dose (10 mg/kg) promotes activation of hepatic TDO2 that mediates the cleavage of the 2,3-double bond of the indole ring of tryptophan (trp) leading to kynurenine (kyn) production **(2)**. **(3)** Kynurenine, by acting as an AhR ligand increases IL-10 and decreases IL-1β, TNF-α and IL-6 production, thus leading to reduced systemic inflammation.

Recently, it has been demostrate that AhR can cross-talk with many signalling pathways and interact with multiple transcription factors[14]. NF-κB and AhR interplay in various situations. Notably, AhR/RelB heterodimer has been reported to promote expression of both AhR and RelB/p52 target genes. Moreover, a specific RelB/AhR responsive element (RelBAhRE) has recently been identified as responsible of *Il8* transactivation in macrophages[15]. It is noteworthy that the heterodimer has been found in TCDD-treated bone marrow-derived dendritic cells (BMDCs) nuclear extracts binding to RelBAhRE *in vitro*, and IDO induction was observed under the same conditions [16].

For these reasons, in the Kenneth Murphy lab, we investigated what was the molecular mechanism activated by L-Kynurenine-AhR binding.

In this framework, our study aimed at elucidating two important points:

1. Can Kyn activate AhR in DCs to sustain *Ido1* transcription?
2. Do AhR and RelB actually interact to achieve IDO1 induction in the same setting?

L-kynurenine promotes IDO1 long-term expression in LPS-primed cDCs

It is known that dendritic cells express IDO1 upon inflammatory stimuli such as LPS treatment [17, 18]. Previous studies also show that IDO product L-kynurenine acts as an AhR ligand promoting Foxp3+ inducible T_{reg} (iT_{reg}) lymphocytes development in co-cultures of DCs and $CD4^+$ T cells [17, 19]. Based on these data, we set out to test if the first tryptophan metabolite, Kyn, might affect AhR-mediated IDO1 expression in DCs.

Splenic conventional DCs obtained from WT and $AhR^{-/-}$ mice were stimulated with LPS or with Kyn, this latter either alone or after LPS priming. IDO1 expression was investigated at protein level by western blotting. As shown in **Fig.3**, in WT DCs LPS promoted IDO1 synthesis, whereas Kyn alone raised non effect. However, combination of LPS and Kyn largely overtook LPS performance. By contrast, no trace of IDO1 protein could be detected in $AhR^{-/-}$ DCs under any condition. These data suggest that Kyn enhances induction of IDO1 expression elicited by LPS through AhR mediation.

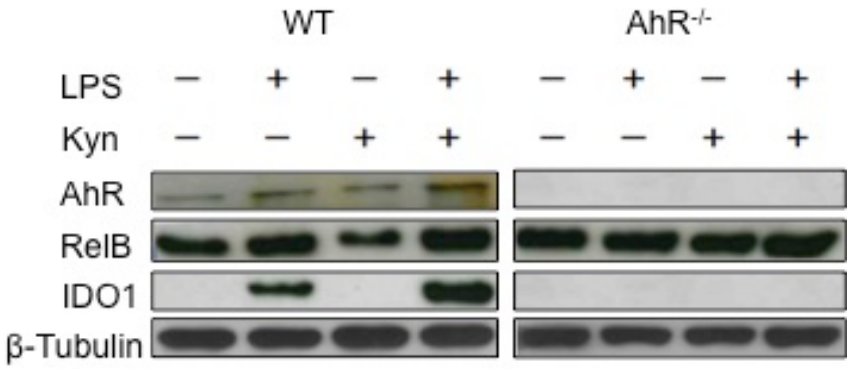

Figure 3. LPS-primed splenic cDCs, treated with L-kynurenine promote IDO1 expression. IDO1 expression was assessed by immunoblotting in WT or $AhR^{-/-}$ cDCs, treated with LPS *in vitro* (1 µg/ml overnight), and then treated with L-kynurenine (Kyn, 50 µM for 24 h). Results are from one experiment representative of three.

If L-kynurenine is an IDO product and IDO inducer at the same time, it is possible to hypothesize that it may be part of a feedforward loop amplifying IDO1 activity. To verify this hypothesis, we measured L-kynurenine to tryptophan ratios at different times in cell supernatants from WT cDCs under mentioned conditions. **Fig.4** attests that while enzymatic activity profile shows a single peak at 24 hours after treatment with LPS alone, L-kynurenine addition produces a different profile curve with heightened 12- and 24-h yields which are maintained up to 48 hours from challenge. Such results indicate that Kyn sustains enhanced and prolonged expression and activity of tolerogenic IDO1 in LPS-primed cDCs.

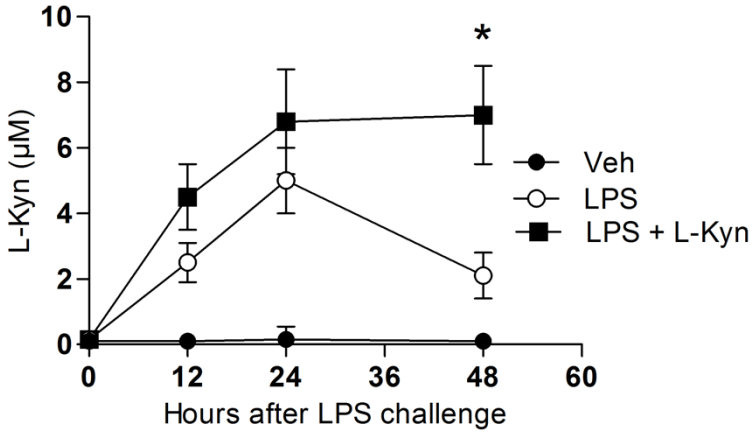

Figure 4. Treatment of cDCs with LPS plus L-kynurenine promotes long term IDO1 enzymic activity. L-kynurenine to tryptophan (Kyn/trp) ratios (means ± s.d. of three experiments) were determined at different times in cell supernatants from WT cDCs, either unprimed (medium) or treated with LPS alone or in combination with Kyn (LPS + Kyn). *$P < 0.001$ (LPS *vs.* combined treatment; Student's *t*-test).

AhR and RelB interact in LPS-primed cDCs treated with L-kynurenine

To investigate the possible mechanisms underlying our findings, we considered previous studies demonstrating that LPS prolonged stimulation promotes expression of RelB, which downregulates proinflammatory NF-κB canonical pathway [20-22]. RelB was also shown to induce IDO production following CD40 activation [23]. Furthermore, recent reports from Vogel *et al.* indicate that TCDD causes AhR and RelB to associate and bind a specific RelBAhRE consensus sequence, and that was even observed in the same setting leading to IDO mRNA heightened transcription in BMDCs [24-26]. Hence, provided that IDO expression was elicited in AhR-dependent manner upon combined treatment with LPS and Kyn, we assessed whether an interaction between AhR and RelB was implicated.

First, we found—as expected—that RelB is expressed in cDCs, but its protein levels were markedly increased in whole cell lysates from samples treated with LPS in combination with L-kynurenine compared

both to controls and LPS-stimulated samples (**Fig.5**). Co-immunoprecipitation experiments with anti-AhR antibody revealed the presence of RelB only in the lysates from cDCs co-treated with both stimuli (i.e. LPS and Kyn) but not in lysates from control untreated or LPS single-treated cDCs (**Fig.5**). These results indicate that in LPS-primed DCs, L-kynurenine stimulation promotes physical association between the two transcription factors AhR and RelB.

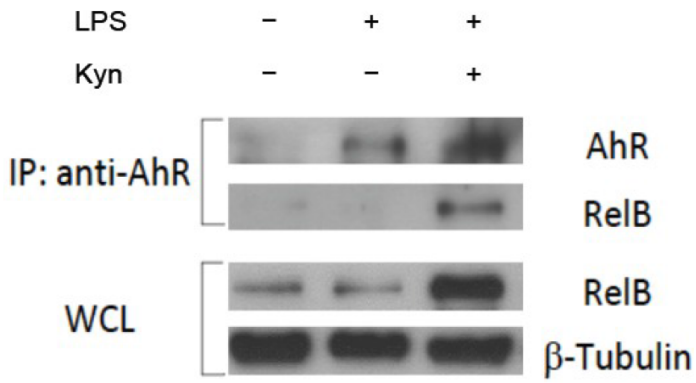

Figure 6. AhR and RelB interaction in LPS-primed cDCs after L-kynurenine treatment. RelB expression, by immunoblotting, in whole cell lysates (WCL) from WT cDCs treated with LPS *in vitro* (1 μg/ml overnight), and then treated with L-kynurenine (kyn, 50 μM for 6 h). Also shown are the results of AhR and RelB blotting after AhR immunoprecipitation (IP). IgG immunoprecipitates served as an antibody specificity control

AhR and RelB transactivate *Ido1* promoter

Next, we went on testing RelB contribution to *Ido1* transactivation. Murine *Ido1* gene promoter nucleotide sequence was searched for possible RelB-binding elements. Three possible mouse RelB responsive elements (mRelB) were identified at positions -3403, -3735, and -4473 relative to the start site of *Ido1* transcription, and designated mRelB-1 to 3, respectively (**Fig.6**). mRelB-1 and -3 core sequences correspond to that of canonical XRE, while mRelB-2 is the same found by Vogel in RelBAhRE (Table 1).

Target gene Responsive element	Sequence	Model	AhR ligand
CYP1A1 canonical XRE	TnGCGTG A/G G/C A	Hepatic cell lines, hepatocytes and others	Halogenated or polycyclic Ahs
CYP1A2 XREII	CATG(N)6CTTG	Hepatic cell lines, hepatocytes	3-MC, TCDD
Bax TCDD-unresponsive, BaP-responsive XRE	AcaagcctggGCGTGggc tatattg	Mice oocytes	BaP
PON1 TCDD-unresponsive, polyphenol-responsive XRE	TgccgacccgGCGGGgagg ggcggg	HuH7 cell line	3-Mc, BaP, resveratrol, quercetin
Tyrosine hydroxylase AhREIII	tgtcttcatgTCGTGtctagggc gg	Neuro2a cell line	TCDD
Cathepsin D, c-Fos, pS2 iXRE	CnG GCGTG A/G G/C C	MCF-7 cell line	TCDD
RelBAhRE	AGATGAGGGTGCATA AGTTC	U937 cell line	TCDD, forskolin

Table 1. Identified AhR-responsive elements, corresponding controlled-genes, the model used to characterize them and the type of ligand that leads to recruitment of the AhR. Core nucleotidic elements determining affinity to AhR are underlined.

To verify the ability of these sequences to bind RelB, we performed chromatin immunoprecipitation studies (ChIP) in WT cDCs treated with LPS alone or in combination with Kyn. Results, shown in **Fig.6**, indicate that RelB is able to interact with the three putative consensus sequences; this interaction mainly occurred in mRelb-3 and mRelb-1 and was significantly augmented by cDCs co-treatment with the two stimuli. When anti-AhR antibody was used, ChIP analysis gave similar outcomes. These data show that both AhR and RelB recognise and bind the three novel responsive elements on *Ido1* promoter.

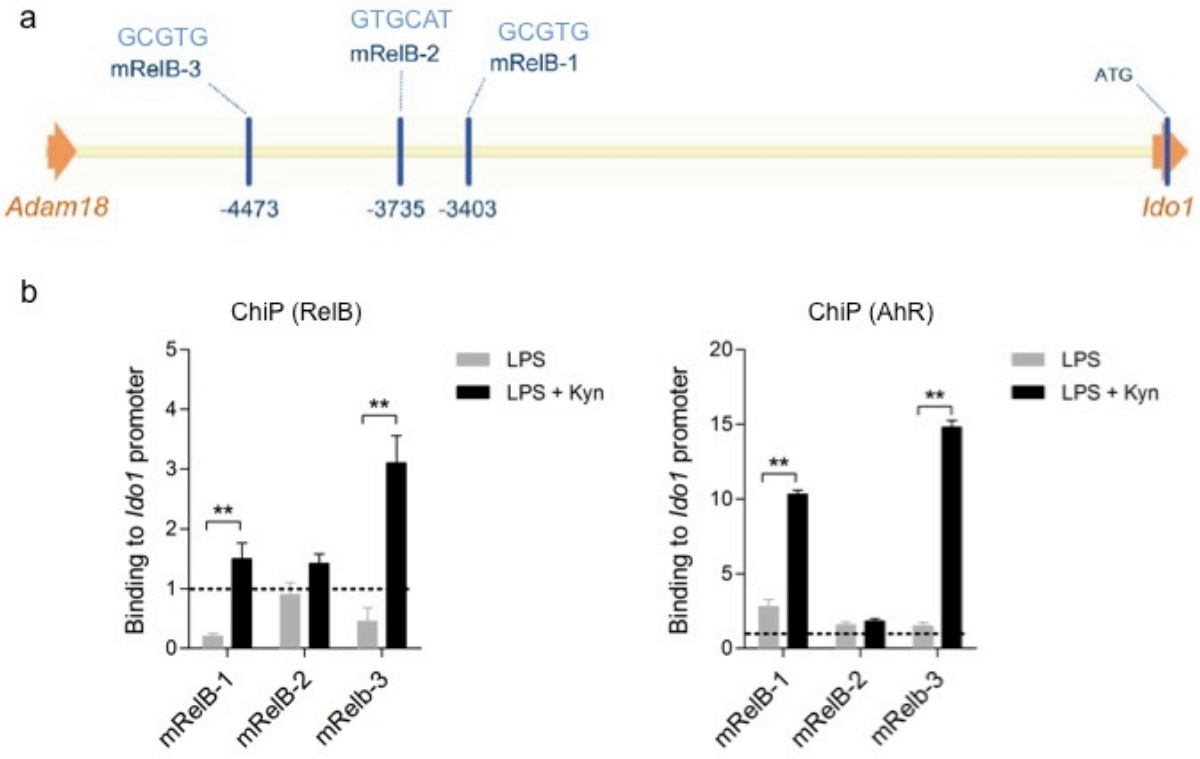

Figure 6. ***Ido1*** **promoter contains three mouse Relb responsive elements (mRelB).** **a)** Schematic design of the mouse Ido1 promoter. Shown are the known positions of the putative mRelB-AhR and noncanonical NF-κB response elements (RE) along the *Ido1* promoter, relative to the start of *Ido1* transcription. **b)** Chromatin immuno-precipitation assay with anti-RelB antibody or anti-AhR antibody on the potentially responsive elements (RelB-1 to 3) in the *Ido1* promoter using cell extracts from WT cDCs, either unprimed or treated with LPS alone or in combination with L-kynurenine (LPS + kyn). Data (mean ± s.d. of three experiments) are presented as normalized binding level in the samples relative to normalized binding in control cultures (that is, unprimed cells cultured *ab initio* in medium alone, in which fold change = 1; dotted line). **P* < 0.05 and ***P* < 0.001, Shapiro test.

In order to confirm the requirement of the RelB and AhR on IDO1 expression, we measured enzyme mRNA levels by RT-PCR after RelB or AhR specific silencing through small interfering RNA technology (siRNA) in cDCs (**Fig.7**). Lack of RelB definitely abolished IDO1 induction elicited in cDCs by either LPS alone or LPS priming and L-kynurenine treatment. As expected, AhR-siRNA elicited similar effects. Collectively these results indicate that both AhR and RelB are strictly needed to transactivate *Ido1* gene under examined conditions. Moreover RelB and AhR heterodimers are able to bind three novel putative consensus elements identified on *Ido1* gene promoter to induce its synthesis.

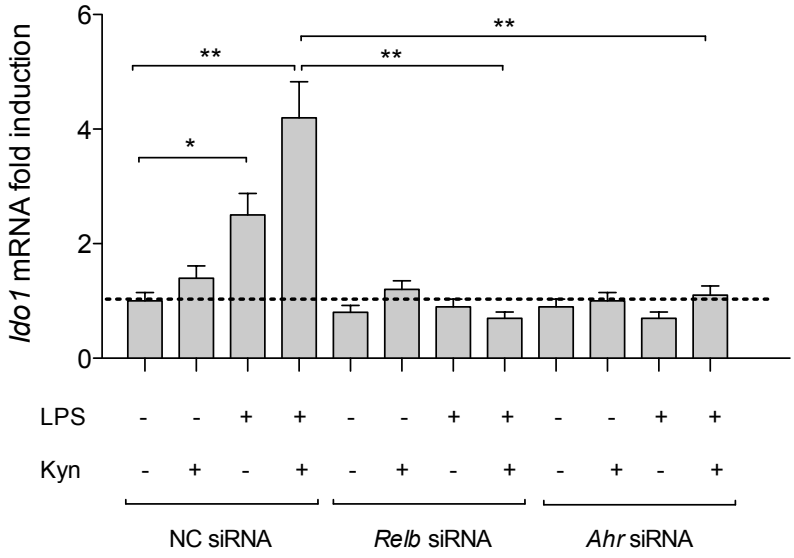

Figure 7. Relb and Ahr silencing impairs IDO1 expression in cDCs treated with LPS and L-kynurenine Quantitative RT-PCR expression of *Ido1* in WT or *Relb* and *AhR*-silenced cDCs, treated with LPS in vitro (1 µg/ml; overnight), and then stimulated with L-kynurenine (kyn, 50 µM for 6 h). Data (mean ± s.d. of three experiments) are presented as normalized transcript expression in the samples relative to normalized transcript expression in control cultures (that is, unprimed cells cultured *ab initio* in medium alone, in which fold change = 1; dotted line). *P < 0.05 and **P < 0.001 (Shapiro test).

LPS-primed, L-kynurenine–conditioned IDO1-competent cDCs confer resistence to LPS challange in vivo

The IDO1^{high} phenotype LPS-primed, L-kynurenine–conditioned IDO1-competent cDCs prompted us to investigate whether those cells could exert protective effects in an in vivo model of endotoxemia and whether IDO1 could be involved in this effect. Unstimulated, LPS primed, or LPS-primed L-kynurenine-conditioned cDCs were transferred i.v. into naïve mice to be challenged at 48 h with a lethal i.p. dose of LPS. A group of sham-tolerized controls received medium alone. As expected, all control animals died within 48 h of LPS challenge (**Fig. 8a**). No changes in survival were observed in mice transferred with unstimulated or LPS primed cDCs. In contrast, as many as 60% of mice receiving LPS-primed cDCs survived LPS challenge (**Fig. 8b**). However, the protective effect of the adoptive transfer was lost when Ido1$^{-/-}$ cDCs were transferred into recipient hosts (**Fig. 8b**). Thus successful adoptive transfer of tolerance by cDCs required that the transferred LPS-primed cells be competent for IDO1.

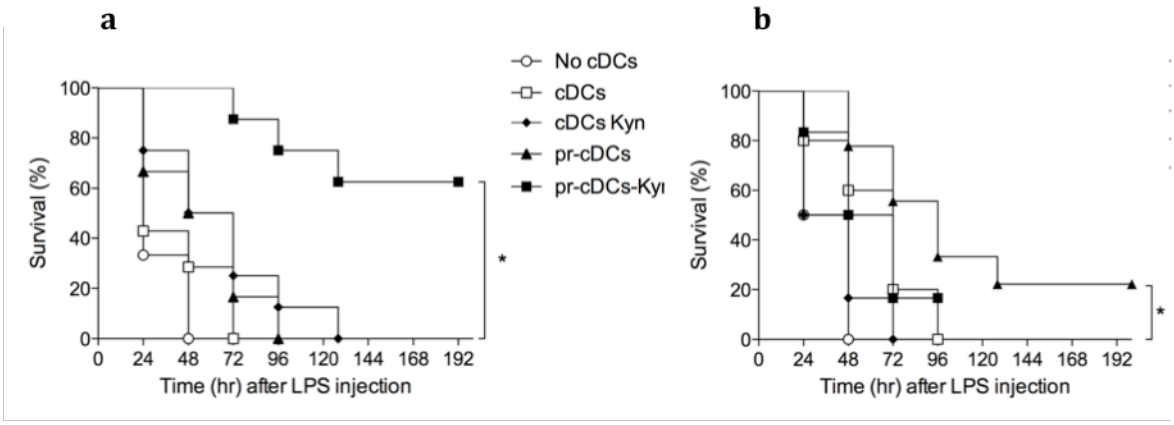

Figure 8. Adoptive transfer with LPS-primed, L-kynurenine-conditioned cDCs protects from lethal endotoxemia in an IDO1-dependent manner. a) Survival rates of WT mice transferred with unstimulated, LPS primed, or LPS-primed L-kynurenine-conditioned cDCs (1×10^6/mouse) and challenged i.p. with 30 mg/kg LPS after 48 h. Vehicle-injected mice were used as control. Survival was monitored every 24 h through day eight of LPS challenge, n = 10 per group,in one of three experiments; *p < 0.001. **b)** Survival rates of WT mice transferred with unstimulated, LPS primed, or LPS-primed L-kynurenine-conditioned Ido$^{-/-}$cDCs (1×10^6/mouse) and challenged i.p. with 30 mg/kg LPS after 48 h. Vehicle-injected mice were used as control. Survival was monitored every 24 h through day eight of LPS challenge, n = 10 per group,in one of three experiments; *p < 0.001.

CONCLUSION

AhR is a transcription factor that, existing as a dormant complex with chaperone proteins, is usually activated by ligand binding and plays a large variety of roles. Initially studied in toxicology as a mediator of xenobiotic metabolism, it was later implicated in cell cycle and immune system regulation. Many AHR-mediated immunoregulatory mechanisms have been discovered, and this knowledge may enhance our understanding of the molecular pathogenesis of autoimmune inflammatory syndromes such as collagen-induced arthritis, experimental autoimmune encephalomyelitis, and experimental colitis.

AhR is able to recognise a wide range of both exogenous and endogenous small molecules, and can also be activated in the absence of obvious ligands. Beyond the canonical XRE, it interacts with alternative DNA consensus sequences, thus enlarging the target gene spectrum. Finally, it interplays with a variety of signalling pathways and physically interacts with other transcription factors, acquiring further functions.

Recent findings have elucidated the critical link between AHR and indoleamine 2,3- dioxigenase 1 (IDO1) in the development of regulatory T (T_{reg}) cells and Th17 cells, which are key factors in a variety of human autoimmune diseases. Induction of IDO1 and IDO1-mediated tryptophan catabolism, together with its downstream products such as kynurenine, is an important immunoregulatory mechanism underlying immunosuppression, tolerance, and immunity. Recent studies revealed that induction of IDO depends on AHR expression.

The present report discussion encloses all these elements. We started from known facts: both AhR and NF-κB subunit RelB promote a tolerogenic phenotype in dendritic cells through IDO expression; IDO product L-kynurenine is an active mediator of tolerance and an endogenous AhR agonist; AhR and RelB can physically interact to mutually enhance transactivation of respective target genes and to induce further genes expression. Then, we performed *in vitro* experiments in DCs and found a novel pattern involved in immune modulation. We propose that in LPS-primed splenic conventional DCs, treatment with L-kynurenine stimulates AhR nuclear translocation and heterodimerization with RelB; the AhR/RelB complex binds three consensus sequences on *Ido1* promoter, which consist in two XREs and one recently identified specific AhR/RelB responsive element; the final outcome is long-term expression and activity of IDO1, which produces more Kyn and thus finances a feedforward circuit involved in immunological tolerance *in vitro* (**Fig. 9**). In our experiments of successful adoptive transfer of endotoxin tolerance by LPS-primed cDCs this is clearly demonstrated by the requirement for IDO1 competence in those cells. Thus our current experiments not only provide proof-of-principle for the protective activity of IDO1 in septic shock, but they also provide further mechanistic insight into how inflammation in general, and susceptibility to endotoxin in particular, are self-regulating processes, which could be exploited therapeutically in inflammatory settings

This model highlights some as yet poorly defined aspects in the literature. Notably, it provides a

mechanistic explanation of IDO induction in DCs under physiologically occurring conditions during inflammation, and it delineates a role for Kyn in AhR-mediated signalling in DCs. Along with that, it supports recent findings on AhR interplay with RelB, and corroborates the importance of endogenous activators in AhR function.

On the other hand, our study suggests new questions, including:

1. Does each ligand induces a specific conformation of the receptor that interacts with a specific transcription factor?
2. Does each ligand induce the recognition of specific target gene responsive elements?
3. Do LPS-primed, L-kynurenine–conditioned cDCs induce a memory state in innate immune cells?

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