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## A new cross-talk between the Aryl hydrocarbon receptor and RelB, a member of the NF- $\kappa$ B family

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### Abstract

The discovery of the new crosstalk between the aryl hydrocarbon receptor (AhR) and the NF- $\kappa$ B subunit RelB may extend our understanding of the biological functions of the AhR and at the same time raises a number of questions, which will be addressed in this review. The characteristics of this interaction differ from that of AhR with RelA in that the latter appears to be mostly negative unlike the collaborative interactions of AhR/RelB. The AhR/RelB dimer is capable of binding to DNA response elements including the dioxin response element (DRE) as well as NF- $\kappa$ B binding sites supporting the activation of target genes of the AhR as well as NF- $\kappa$ B pathway. Further studies show that AhR/RelB complexes can be found not only in lymphoid cells but also in a human hepatoma cell line (HepG2) or breast cancer cell line (MDA-MB-231). RelB has been implicated in carcinogenesis of breast cancer for instance and RelB is known to be a critical factor for the function and differentiation of dendritic cells; interestingly the participation of AhR in both processes has been suggested recently, which offers the great potential to expand the scope of the physiological roles of the AhR. There is evidence indicating that RelB may serve as a pro-survival factor, including its ability to promote “inflammation resolution” besides the association of RelB with inflammatory disorders. Based on such information, a hypothesis has been proposed in this review that AhR together with RelB functions as a coordinator of inflammatory responses.

### Keywords

AhR; IDO; IL-8; NF-kappaB RelB; TCDD

### 1. Introduction

The AhR is a member of basic helix-loop-helix (bHLH-PAS) transcription factors including Period (Per), AhR nuclear translocator (ARNT), and single minded (SIM) regulating hypoxia, circadian rhythm, and cellular processes like differentiation and apoptosis [1]. The AhR is generally considered as a ligand-dependent transcription factor which dimerizes with ARNT to activate gene transcription through DRE located on the promoter of these target genes.

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Numerous exogenous compounds (e.g. polycyclic aromatic hydrocarbons, benzimidazoles and flavonoids) with various binding affinities have been shown to bind to and activate the AhR [2]. A number of endogenous ligands with varying binding affinities to the AhR have also been proposed. Especially derivatives from the essential amino acid tryptophan have been identified as high-affinity ligands to the AhR and are considered as potential endogenous AhR ligands [3,4]. The non-activated form of the AhR is complexed with heat shock protein 90 (HSP90) and X-associated protein 2 (XAP2) in the cytosol, but depending on cell type and physiological conditions the AhR is also located in the nucleus even in absence of exogenous ligand [5]. XAP2 may enhance the rate of nuclear translocation of the ligand-bound human AhR complex and modulates the sub-cellular localization of the mouse AhR [6,7]. The mechanism of classical AhR/Arnt signaling pathway will be discussed in detail elsewhere.

Although AhR agonists are known to induce immunosuppression, there is increasing evidence that one of the significant toxic actions of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is elicitation of chronic active inflammation in different organs and sites of the body [8-11]. TCDD is a prototype of polycyclic aromatic hydrocarbons (PAH) and exogenous compounds which binds with high affinity to the AhR. One of the routes of propagation of inflammatory signaling may be induction of proinflammatory genes including Tumor necrosis factor (TNF)  $\alpha$ , Interleukin (IL)-1 $\beta$ , Cyclooxygenase 2 (COX-2), and others by TCDD, which has been confirmed in numerous studies [12-15]. Recently we found that TCDD induces also IL-8 in human macrophages as well as in liver of C57BL/6 mice in an AhR-dependent manner [16-19]. IL-8 is a member of the CXC chemokine family. One of its functions is to control the process of leukocyte invasion into injured tissue which is a hallmark of inflammation [20]. The important point of showing all these examples of TCDD-induced inflammation and immunosuppression occurring in various tissues and cells is that these are the type of events, which cannot be readily explained on the basis of the existing model of the classical action mechanism of TCDD that is based on binding of AhR/ARNT to DRE.

Although tissue inflammation stimulates adaptive immunity, certain types of inflammation, particularly excessive or chronic inflammation, paradoxically suppresses adaptive immunity as well. In general, inflammation induces compensatory anti-inflammatory mechanisms that are essential to limit potentially dangerous immune responses and reduce tissue damage. Therefore it should be considered that inflammation enhances immunogenicity, but in some contexts, inflammation may also stimulate active suppression. The mechanism underlying TCDD's immunosuppressive effect is not well understood and it is still an open question if the AhR-mediated inflammatory responses might be the first event which eventually leads to immune suppression.

This review summarizes existing and original data of the new crosstalk between the AhR and NF- $\kappa$ B RelB and provides a new perspective of how AhR together with RelB may participate in immunoregulatory functions.

## 2. TCDD and the AhR

### 2.1. General mechanisms of action of TCDD

Following the original elucidation of the structure of the AhR by Chris Bradfield and his colleagues [21], there has been tremendous progress made in the field of AhR biology in terms of its basic structure [22], its cytosolic association with XAP2 [23] along with HSP90 proteins, its nuclear translocation and formation of its gene transactivation complex with its dimerization partner ARNT and several nuclear coactivators [24]. This line of investigation has been very fruitful and constructive in establishing the identity of this important receptor, particularly in mediating toxic signaling of TCDD and related dioxin-type pollutants with special emphasis on induction of a number of xenobiotic metabolizing enzymes of the AhR gene battery. Besides

dimerization with its well known partner ARNT, the AhR may also bind to other transcription factors to regulate gene expression [25-30]. Despite the above accomplishments, some questions remain unanswered in this field of science. The most pressing questions are: how does activation of AhR leads to toxic consequences especially inflammation as well as immunosuppression and how are developmental processes and physiological functions regulated through AhR?

## 2.2. Physiological roles of AhR

In this regard, the most illustrative findings indicating the role of AhR have been provided by several groups of scientists, who created strains of the AhR null mice. Among those, AhR null mice from one strain created by Frank Gonzalez and his colleagues [31] showed liver abnormality during the neonatal development which is associated with high rates of apoptosis. More recently, Chris Bradfield's group has reported that their strain of AhR null mice develop liver abnormality which is characterized with a patent developmental structure known as the ductus venosus (DV) during the first 48 h of their postnatal life. This abnormality is caused by the failure of the DV to close (decrease the diameter of the portal and the umbilical veins [32]. These observations clearly support the physiological role of AhR in the normal development of embryos and neonates. In addition, AhR null mice exhibit high production of TGF $\beta$ <sub>3</sub> in liver, which is accompanied with excess vitamin A [33]. Furthermore, Rodriguez-Sosa et al. [34] have reported that spleen of AhR null mice over-produces Interferon (IFN) $\gamma$  and IL-12 when challenged with ovalbumin, which is in line with previous findings [35] that AhR plays an important role in the normal development and function of the immune system. AhR null mice show decreased accumulation of lymphocytes in the spleen and lymph nodes [35]. This suggests the AhR is also located in the nucleus to regulate these physiological processes in the absence of exogenous ligands. Nuclear localization and activity of the AhR during embryonic development has also been reported [36].

Interestingly, from recent studies it becomes clear now that the AhR can regulate the generation of regulatory T cells (T<sub>reg</sub>) or pro-inflammatory T cells producing IL-17 (T<sub>H</sub>17) depending on the nature of its ligand. Activation of the AhR by TCDD induces differentiation of T-cell progenitor cells into T<sub>reg</sub> whereas ligand binding of AhR by 6-formylindolo[3,2-b]carbazole (FICZ) leads to differentiation of those progenitor cells into T<sub>H</sub>17 cell which produces interleukin (IL)-22 [37,38]. FICZ is a tryptophan-derived photoproduct that is thought to be an endogenous ligand with high affinity for the AhR [4]. It has long been recognized that derivatives of tryptophan can activate AhR [3]. In contrast to FICZ, the tryptophan-derived AhR endogenous ligand 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) suppressed autoimmune encephalomyelitis (EAE) in mice as demonstrated for TCDD [37]. Dioxin (= TCDD) is a prototype of a class of environmental contaminants with high affinity to the AhR. AhR activation by TCDD has broad effects on the immune system in humans [39] and the AhR-dependent induction of a T-cell population with characteristics of T<sub>reg</sub> has been described earlier [40,41]. The concept emerging from recent studies on T cell differentiation regulated by AhR proposes that activation of AhR dependent on its activating ligand can induce immunity or tolerance. Our most recent findings show, that activation of AhR by TCDD induces indoleamine-2,3-dioxygenase 1 (IDO1) and indoleamine-2,3-dioxygenase-like protein (IDO2) [42]. Expression of IDO1 and IDO2 is known to generate T<sub>reg</sub> which suggest a critical role of IDO to mediate immunosuppressive effects after activation of AhR by TCDD.

Future studies are necessary to show, if these ligand-specific effects on T cell differentiation are due to the capability of the AhR to interact with different transcription factors leading to distinct different outcomes or to the different pharmacological features of the AhR activating compounds.

It must be added that, while the connection between such a function of AhR to regulate the balancing of  $T_{reg}$  versus  $T_H17$  and that of RelB is not apparent so far, the possibility of the involvement of RelB will be elaborated in a later section.

### 3. The NF- $\kappa$ B subunit RelB

#### 3.1 Physiological roles of the NF- $\kappa$ B subunit RelB

The NF- $\kappa$ B or Rel proteins comprise a family of structurally related eukaryotic transcription factors that play critical roles in diverse cellular processes including adaptive and innate immunity, inflammatory responses, cell differentiation, proliferation, and apoptosis. The functions of Rel/NF- $\kappa$ B transcription factors are conserved from the fruit fly *Drosophila melanogaster* to humans; more recently, Rel/NF- $\kappa$ B homologs have also been found to occur even in organisms as simple as Cnidarians (e.g., sea anemones and corals) and Porifera (sponges). Transcriptionally active NF- $\kappa$ B dimers are formed by combinatorial association of five subunits: p50, RelA (p65), p52, c-Rel, and RelB [43]. Nuclear translocation of the NF- $\kappa$ B subunits is controlled by two main signaling pathways. The well known classical (canonical) NF- $\kappa$ B pathway consists majorly of the classic inducible NF- $\kappa$ B heterodimer p50 and RelA (=p65) subunits, each contacting one half of the DNA binding site. All Rel members can form heterodimers or homodimers, except for RelB, which can only form heterodimers. The individual dimers have specific DNA-binding sites and slight variations in the 10 base pair NF- $\kappa$ B consensus sequence (5'-GGGGYNNCCY-3') which is critical for the preference of selected Rel combinations [44] and regulation of a distinct set of genes. The activity of Rel dimers is controlled by a family of inhibitor proteins, known as I $\kappa$ Bs, whose degradation can be induced by a variety of stimuli and involves signal responsive activation of I $\kappa$ B kinase (IKK).

The activation of the IKK complex in the classical NF- $\kappa$ B pathway is generally regulated by IKK $\beta$ . In contrast, the alternative (non-canonical) NF- $\kappa$ B pathway is activated by phosphorylation of p100 through an IKK $\alpha$  homodimer lacking the regulatory subunit IKK $\gamma$ . IKK $\gamma$ , however, is absolutely required for activation of the classical pathway through ligation of members of the tumor necrosis factor receptor (TNFR) superfamily or initiation of pathogen sensing receptors the toll like receptors (TLR). Activation of the non-classical or alternative NF- $\kappa$ B pathway has been described to be mediated by several developmental stimuli such as Lymphotoxin (LT)- $\alpha_1\beta_2$  or TNFR ligands like B cell activating factor (BAFF), CD40 ligand (CD40L), or receptor activator of NF- $\kappa$ B ligand (RANKL). Upon binding of these ligands NF- $\kappa$ B-inducing kinase (NIK) and IKK $\alpha$  will be activated and p100 will be processed to mature p52 followed by nuclear translocation of RelB/p52 NF- $\kappa$ B dimers. Activation of the alternative NF- $\kappa$ B pathway through ligation of Lymphotoxin  $\beta$  receptor (LT $\beta$ R) for instance regulates expression of organogenic chemokines such as SLC (CCL12), BLC (CXCL13), ELC (CCL19), or SDF-1 (CXCL12) which is needed for organization of secondary lymphoid organs like maturation of spleen and lymph nodes [45-47]. NF- $\kappa$ B activation through the alternative pathway in response to developmental signals is weaker and delayed compared to inflammatory signals mediated via the classical pathway, which is usually a transient signal [48].

Genetic studies revealed that lymph node development requires the activity of both NF- $\kappa$ B pathways the classical (RelA and IKK $\beta$ ) and the alternative (RelB and IKK $\alpha$ ) pathway. For instance, signals mediated via CD40R or BAFFR which control B cell maturation were also shown to utilize the NIK pathway for RelA activation. Thus, the NF- $\kappa$ B RelA/p50 dimer does not only control a critical role in immune activation but plays also a role in immune development. For instance, development and maintenance of lymph nodes requires both RelA and RelB mediated NF- $\kappa$ B pathways. Their results further indicate that constitutive nuclear RelA/p50 activity is required to ensure expression of RelB and thus LT $\beta$ R mediated activation of the alternative RelB/p52 dimer. Recent studies performed by Alexander Hoffmann's group

support the crosstalk of the classical and alternative NF- $\kappa$ B pathway showing that inflammatory and developmental signaling of NF- $\kappa$ B dimers are not separate but highly interconnected pathways [49].

The cross-regulation of both NF- $\kappa$ B systems has been shown in RelA deficient mice which are defective in RelB activation. Evidence came from recent reports, showing that stimulation of the alternative pathway via LT $\beta$ R implicates a first phase of RelA activity which is important for expression and de novo synthesis of RelB [50]. RelB deficient mice showed increased inflammatory infiltration in multiple organs as well as severe deficits in adaptive immunity [51]. The most impressive findings regarding the function of RelB have been reported by Burkly et al. [52]. In RelB deficient mice, they could demonstrate that the expression of RelB is required for the development of thymic medulla and dendritic cells (DC). More recent studies have shown that the expression of RelB is upregulated early during differentiation of DC and is required for proper development and function of DC [53]. DCs are considered as key regulators of immunity or tolerance and are also an important source of IDO1 to induce T<sub>reg</sub> [54]. It has been shown that the alternative NF- $\kappa$ B pathway is critical for the induction of IDO1 in DC through ligation of CD40 for instance [55]. Thus, it is not unlikely that the TCDD-mediated induction of IDO1 does not only require AhR as shown recently [42], but also involves RelB as in the case of IL-8 [18].

### 3.2 The alternative NF- $\kappa$ B pathway in pathophysiology and the etiology of diseases

NF- $\kappa$ B transcription factors are persistently active in a number of disease states, including arthritis, chronic inflammation, asthma, neurodegenerative diseases, cardiovascular disease, and cancer. Inflammation caused by deregulation of NF- $\kappa$ B activity has been well identified as a tumor promoter. Both the classical and the alternative NF- $\kappa$ B pathways were found to be activated by viral oncoproteins, in particular the HTLV1-encoded Tax protein in adult T-cell leukemia and the EBV-encoded LMP1 protein in B-cell lymphoma [56].

Several reports indicate that the alternative NF- $\kappa$ B pathway is activated in specific subtypes of lymphoid leukemia and lymphoma. Chromosomal translocations disrupting the *p100* gene that generate truncated p100 proteins and constitutive processing of p100 to p52 were identified in cutaneous T-cell lymphoma, and, more rarely, in B-cell non-Hodgkin lymphoma, chronic lymphocytic leukemia, and multiple myeloma [45,56,57]. Transgenic expression of a truncated p100 protein led to the development of B-cell lymphomas in mice, thus demonstrating the oncogenic potential of *p100* mutations [58]. Recently, we demonstrated the AhR-mediated development of lymphoma in vivo which was associated with increased expression of inflammatory markers such as COX-2. In addition, the expression of Bcl-x1 an anti-apoptotic gene of the Bcl-2 family was significantly increased in clearly enlarged superficial lymph nodes of TCDD treated animals [59]. Interestingly, RelB/p52 complexes have been shown to induce Bcl-2 promoter activity [60] and future studies are needed to verify the possible involvement of AhR/RelB in lymphoproliferative disorder promoted by the TCDD-activated AhR.

In other cancer types such as breast cancer NF- $\kappa$ B has been shown to be constitutively active and located in the nucleus in over 90% of the breast cancers [61]. This could be due to chronic stimulation of the IKK pathway or defective I $\kappa$ B genes. Both NF- $\kappa$ B pathways can affect tumor growth. More recently the overexpression of RelB has been demonstrated in mammary tumorigenesis. Therefore the alternative signaling pathway has been considered to play a critical role in controlling the activity of NF- $\kappa$ B which is associated with cancer cells. Beside breast cancers (ER-negative, inflammatory type) [62], RelB has also been found to be overexpressed in some of prostate cancers [63]. It has been observed that in the above types of cancers as those cancer cells progress toward more advanced states of malignancy the nuclear titer of RelA protein decreases and that of RelB protein increases. The important point is that these are the type of breast cancer cells, which also exhibit overexpression of AhR and require



RelB, not RelA to survive in the hostile environment (Vogel and Matsumura, unpublished data, 2008). Such an observation of RelB contributing to cancer cell survival should include their acquisition of immune tolerance. Studies investigating the epithelial-mesenchymal transition (EMT) in mammary tumorigenesis showed overexpression and nuclear translocation of RelB contributes to the phenotype of inflammatory breast cancer which is the most aggressive breast cancer with high metastatic potential [64,65]. Especially in estrogen receptor (ER) $\alpha$  negative breast tumor cell types the expression of RelB is increased which is obviously based on a negative control of RelB de novo synthesis by ER $\alpha$  [60]. From their studies the authors concluded an inverse relationship exists between the expression of ER $\alpha$  and RelB in MCF-7, transformed mammary epithelial cells. Other data suggest that estrogenic status is also important in AhR regulation and can influence the effects of xenobiotics-mediated carcinogenicity [66]. A strong link between mammary tumorigenesis and AhR overexpression derived from studies performed by David Sherr's group [67]. Recent reports by G. Lazennec show that development of this type of breast tumor is associated with inflammation and an inappropriate production of chemokines especially IL-8 which is a critical phase in breast tumor metastasis [68-71]. Besides these recent findings little is known about the role of AhR together with RelB in human breast disease. Since we know that AhR/RelB complexes are involved in the regulation of IL-8 and that IL-8, AhR, and RelB are overexpressed and active in many breast cancer cells, we consider that it is not a mere coincidence that formation of the AhR/RelB dimer is found in those ER $\alpha$ -negative, transformed mammary epithelial cells. Rather the AhR/RelB dimer likely contributes to mammary tumorigenesis. This hypothesis is supported by the association of elevated AhR activity in breast tumor cells with formation of the AhR/RelB complex as found by co-immunoprecipitation in MDA-MB-231 breast cancer cells (personal communication, D. Sherr and G. Sonenshein, Boston University). Our own data confirm a distinct increase (10-fold) of AhR/RelB binding activity in EMSA with nuclear extracts of the breast epithelial cell line MCF10A after long-term treatment with estradiol for 20 weeks (P. Wong, C.F. Vogel and F. Matsumura, unpublished data). A better understanding of the overexpression of inflammatory markers such as IL-8 in breast cancer is crucial to get a better insight into the role of the cytokines/chemokines in mammary tumorigenesis.

## 4. AhR and RelB

### 4.1. Implication for the interaction of AhR and RelB

Recently it was reported from this laboratory [18] that in U937 macrophages the NF- $\kappa$ B subunit RelB is functionally associated with the AhR and mediates transcription of chemokines such as IL-8 via activation of AhR that is assisted by protein kinase A (PKA). RelB was found to physically interact with AhR, as attested to by their co-immunoprecipitation and their association as detected in electromobility shift assays (EMSA). We have shown that this dimer binds to a previously unrecognized RelB/AhR response element (RelBAhRE) of the IL-8 promoter linking two signaling pathways to activate gene transcription. We found a time-dependent recruitment of AhR to the RelBAhRE site of IL-8 mediated by the AhR ligand, TCDD and via activation of PKA. Furthermore, NF- $\kappa$ B-binding sites that are preferentially recognized by RelB/p52 are a target for RelB/AhR complexes without addition of any exogenous ligands, implicating the endogenous function of the AhR. RelB/AhR complexes are also found to bind on DRE as well as NF- $\kappa$ B consensus elements, and RelB drastically increases the TCDD-induced DRE-Luc reporter activity.

Since this discovery of the interaction of AhR and RelB is so recent, not many data are available from the literature showing the crosstalk of the AhR and RelB. However, some recent reports from other groups support the interaction between both transcription factors AhR and RelB [72] and may give us some more insight into the physiological and toxicological relevance of this novel heterodimer.

In a previous report we showed that TCDD dose-dependently induces the expression of IL-8 in human U937 macrophages [19]. Mechanistic studies on IL-8 activation showed that TCDD or forskolin (FSK) mediated nuclear entry of the AhR which represents a new alternative mechanism of AhR regulation which dependent at least partly on activation of PKA and the association of AhR with RelB [18]. Previous studies from other groups have shown that FSK induces IL-8 [73] and that FSK or cAMP may activate AhR through a PKA-dependent mechanism [74]. Interestingly, the PKA-dependent activation of AhR was associated with nuclear translocation, but without dimerization with ARNT. The potential dimerization partner of AhR remained unknown at that time. Recently, we found that the FSK- and TCDD-induced activation of the IL-8 involves an unidentified RelB/AhR binding element (RelBAhRE) close to the TATA box of the IL-8 gene. This RelBAhRE sequence is distinctly different from DRE or NF- $\kappa$ B consensus elements since it does not bind to ARNT nor to the NF- $\kappa$ B subunit p50, which are well known dimerization partners of AhR and RelB, respectively. Further studies in our laboratory have revealed that NF- $\kappa$ B binding sites, which preferentially bind RelB/p52 complex, are also bound by the AhR/RelB dimer. Besides IL-8, such RelB/AhR responsive elements have been found on genes like B-cell activating factor of the tumor necrosis factor family (BAFF), B-lymphocyte chemoattractant (BLC), CC-chemokine ligand 1 (CCL1), and the transcription factor interferon  $\gamma$  responsive factor (IFR3), which are known targets of the alternative NF- $\kappa$ B pathway [29]. Results from these studies also showed that the RelB/AhR heterodimer binds to DRE as well as NF- $\kappa$ B consensus elements suggesting that AhR together with RelB may also regulate classical NF- $\kappa$ B and AhR signaling pathways. Binding of AhR/RelB on DRE elements is not limited to hematopoietic cell lines like U937 and was also found in the human hepatoma cell line HepG2 (Fig. 1) and other cell lines like mouse Hepa1c1c7 or 3T3 L1 fibroblasts (Data not shown). This is an important point since Chris Bradfield's group has found that DNA binding of the AhR is required to mediate liver development as well as toxic endpoints elicited by TCDD [75]. Based on these data we proposed a model of an alternative AhR/RelB pathway for the action of the non-liganded AhR in which the crosstalk between AhR and RelB regulates the expression of inflammatory genes like IL-8 [18].

The unexpected finding that the AhR also binds on NF- $\kappa$ B consensus sites suggests that the AhR may affect the classical NF- $\kappa$ B pathway as well. Previous studies have shown that TCDD-mediated activation of the AhR may suppress the effects elicited by LPS or TNF $\alpha$  for instance [26]. The antagonistic action of AhR with the classical NF- $\kappa$ B pathway will be discussed in more detail in a different chapter of this issue. However, there is no doubt that TCDD together with LPS or TNF $\alpha$  may also act synergistically on target genes such IL-8 or COX-2 [76]. In collaboration with Nancy Kerkvliet we asked the question if the AhR may play a role in the induction mechanism of the classical NF- $\kappa$ B pathway mediated by inducers like LPS, for instance. For this purpose AhR null mice were challenged in Nancy Kerkvliet's lab with 50  $\mu$ g LPS for 3 and 6 hrs. Then we analyzed the expression of NF- $\kappa$ B target genes like KC (=IL-8 equivalent in mice) and COX-2 in liver and lung of LPS treated AhR null mice and compared the response towards LPS in wild type C57BL/6 mice. Surprisingly, we found that the LPS-induced up-regulation of KC mRNA expression in liver as well as that of COX-2 mRNA in lung was significantly lower in AhR null mice compared to wild type C57BL/6 mice (Fig. 2A and D). In contrast, LPS-mediated induction of KC in lung and COX-2 in liver was not significantly different in AhR null mice from that what was found in the matched wild type mice (Fig. 2B and C). As expected, TCDD had no effect on the expression of KC or COX-2 in AhR null mice (Fig. 2A-D). Previous studies have shown that both KC and COX-2 are targets of the AhR activated by TCDD [17,77], and therefore, these data indicate that even in the absence of exogenously added ligand the AhR must be helping those cells in liver and lung to maintain their responsiveness to LPS. The main implication of this finding may be that the AhR functions not only in AhR signaling but also NF- $\kappa$ B signaling that is activated by classical inducers like LPS. Results from another study by Thatcher et al. [72] showing the accelerated degradation of RelB in AhR null mice after LPS challenge, support the critical role of AhR

and RelB in response to LPS. Thatcher et al. [72] investigated the inflammatory response in lung of LPS and cigarette smoke exposed mice. They found that mice which inhaled cigarette smoke or LPS express higher levels of TNF $\alpha$  and IL-6 in the bronchoalveolar lavage of AhR null mice compared to wild type mice. In contrast to our findings (Fig. 2) and a previous report [17], these authors found that both constitutive as well as LPS-induced expression of KC was expressed at a higher level in AhR null mice compared to wild type BL/6 mice. The authors concluded that this phenomenon might be due to an elevated NF- $\kappa$ B activity in control animals of AhR null mice which is not the case in wild type mice under normal and healthy conditions [72]. Interestingly, the increased inflammatory response in cigarette smoke or LPS exposed AhR deficient mice was associated with a rapid loss of RelB protein. In a more recent study the same group reported that the heightened inflammatory response observed in fibroblasts from AhR null mice is not the result of NF- $\kappa$ B (p50/RelA) activation [78] as speculated previously [72]. The authors concluded that AhR helps to maintain the expression of RelB and thus limits pro-inflammatory COX-2 and prostaglandin (PG) production induced by cigarette smoke [78]. This report is in contrast to a previous study performed by the same group showing that the AhR is required and plays an important role in cigarette smoke-mediated COX-2 and PG production in human lung fibroblasts and may contribute to tobacco-associated inflammation [79]. One possibility is that specific components of cigarette smoke such as PAHs require AhR to mediate their effects whereas other components of cigarette smoke rather activate the classical NF- $\kappa$ B pathway through oxidative stress and may benefit from the absence of the AhR and RelB to induce an inflammatory response.

Nevertheless, these studies confirm our previous findings [17] on the critical role of the AhR in regulation of the inflammatory response. More importantly these studies support our reports of the interaction of AhR complexed with RelB [18], if we interpret their data to mean that the presence of AhR helps stabilization of RelB in normal mice by extending their observation that in the absence of AhR, RelB protein is more susceptible for degradation.

Lee et al. [80] investigated the effect of TCDD on the differentiation of DCs. Activation of the AhR through environmental pollutants like dioxin is well known to induce profound suppression of immune responses, especially T cell dependent responses, which is the primary toxicity associated with the exposure to dioxin [81]. Emerging from recent studies it becomes clear that the AhR can regulate the generation of regulatory T cells (T<sub>reg</sub>) which could explain TCDD's immune suppressive effects. Interestingly, induction of T<sub>reg</sub> after activation of AhR by an anti-inflammatory compound (VAF347) has been found to be mediated at least in part through DCs [82]. Therefore, DCs should be considered as a potential target for TCDD. DCs are the most potent antigen-presenting cells (APC) and are key regulators of the choice between immunity and tolerance [83]. One of the most potent ways in which DCs can create immunosuppression is by activating T<sub>reg</sub> cells [84]. Lee et al. [80] showed that treatment of mouse DC derived from bone marrow with TCDD significantly affected the differentiation and maturation of DCs. TCDD-treated DCs expressed significantly higher level of DC differentiation markers such as MHC class II and the co-stimulatory molecule CD86, whereas the expression of CD11c and the production of IL-10 was reduced by TCDD. These results agree with studies performed in Nancy Kerkvliet's lab [85,86] showing an increased expression of the DC surface markers CD86, MHC class II, and ICAM-1, whereas LFA-1 was found to be decreased in splenic DC of TCDD treated mice. In summary, the authors described an activation-like stimulus to DC after treatment with TCDD in an AhR-dependent manner which is supported by our most recent report [42]. In that we could show that activation of AhR by TCDD or the tryptophan derived photoproduct FICZ increases the expression of surface markers especially CD86 in human U937 monocyte-derived DCs, which was associated with morphological changes of DCs into more elongated and stellate cells typical for dendritic cells. In addition, we found that activation of AhR by TCDD leads to indolamine-2,3,-dioxygenase (IDO) expressing DCs and induction of IDO1 and IDO2 in spleen of BL/6 mice. The induction



of IDO in spleen was associated with the increase of the  $T_{reg}$  marker Foxp3 which was suppressed by treatment with a pharmacological inhibitor of IDO. These data indicate that induction of IDO by TCDD may play an important role in AhR-dependent regulation of T cell differentiation and immune suppression.

Lee et al. [80] reported that DCs from TCDD-treated mice showed enhanced ability to stimulate allogeneic T cell proliferation in a mixed lymphocyte reaction. Furthermore, the authors found that TCDD as well as Benzo(a)pyren downregulate the expression of RelB upon stimulation of DC maturation with LPS in an AhR-dependent manner. As mentioned earlier one critical physiological function of RelB is regulation of differentiation of DCs. Thus the TCDD-mediated and AhR-dependent effects on DC differentiation seem to implicate the function of RelB. With respect to our findings describing the role of the AhR/RelB complex in the regulation of cytokines and chemokines it is likely that the interference on DC differentiation caused by TCDD is mediated through an activated AhR complexed with RelB.

In another study Ruby et al. [87] investigated whether TCDD would alter the activation of NF- $\kappa$ B/Rel in DCs. The dendritic cell line DC2.4 was exposed to TCDD before treatment with TNF- $\alpha$  or anti-CD40, to activate both the classical and alternative NF- $\kappa$ B/Rel pathway. TCDD suppressed the binding of NF- $\kappa$ B/Rel to its cognate response element in TNF- $\alpha$ - and anti-CD40-treated cells and blocked translocation to the nucleus. The AhR was shown to associate with RelA, after coimmunoprecipitation, and seemed to block its binding to DNA. In contrast to the studies of Lee et al. [80] and our studies in U937 macrophages, this interaction was limited to RelA, but RelB did not seem to interact with AhR in DCs transfected with AhR. Ruby et al. [87] concluded from their results that the defects in the DCs and suppression of the immune response mediated by TCDD may be due to a shifted balance between NF- $\kappa$ B/Rel heterodimers and transcriptional inhibitory p50 homodimers in DCs. In contrast to TCDD, Lawrence et al. [88] reported recently that an anti-inflammatory low molecular weight compound (VAF347) activates the AhR signaling pathway resulting in anti-inflammatory effects on DC by inhibiting expression of the DC markers CD86 and MHC class II. Thus, the role of the AhR mediating activation-like or anti-inflammatory responses in DC might be due to its capacity to interact with different transcription factors in a ligand-specific manner as suggested for the differentiation of T cells into  $T_{reg}$  or  $T_H17$ . Another explanation could be that VAF347 affects additional signaling pathways independent of the AhR. The knowledge of how activation of AhR by specific ligands results in biologically different endpoints might improve our understanding of the toxicity of various AhR ligands and more importantly the physiological role of the AhR.

## 5. Concluding Remark

Evidence has been accumulating to indicate that there are close interactions between AhR and NF- $\kappa$ B family members such as RelA and RelB. Interestingly interactions appear to occur between RelB with the form of AhR either bound with its ligand or without. The consequence of RelA/AhR interactions appears to be mostly negative (i.e. antagonistic) as in the case of CYP1A1 [26] or IL-6 [89] gene activity by forming complexes with AhR which have obviously no DNA-binding capacity or transcriptional activity with the exception of the *c-myc* gene (Fig. 3). Kim et al. [90] reported that AhR and RelA may functionally cooperate to bind to NF- $\kappa$ B elements and induce *c-myc* gene expression. So far as we have found, the interaction of RelB/AhR appears to be rather positive by increasing DRE-reporter activity of CYP1A1 and transcription of NF- $\kappa$ B target genes such as IL-8 and other chemokines [17,18] through binding on specific RelBAhRE sequences (Fig. 4). However, we have to consider that the interaction of the AhR with NF- $\kappa$ B signaling pathways (classical or alternative) seems to be much more complex and should not exclude the possibility that enhanced formation of AhR/RelB dimers could also have suppressive action on the activity of certain target genes under certain

conditions. It must be added that in both cases (i.e. RelA/AhR and RelB/AhR interactions), their joint effects are exacerbated when AhR is activated by its prototypical ligand TCDD. The main question we must raise is then why does such an opposite consequence of their interactions exist between RelA/AhR versus RelB/AhR? This difference between RelA and RelB cannot be coincidental; after all, if RelA and RelB serve for the same purpose, there would be no need for two elaborate although interacting NF- $\kappa$ B signaling pathways (i.e. the classical pathway for RelA and the alternative pathway for RelB). One of the possibilities is that AhR assists the function of RelB not only to mediate chronic inflammation but also to promote RelB's function in resolution of inflammation via negative feedback mechanisms for instance [91,92], whereas AhR antagonizes the action of RelA to moderate acute cellular inflammation, particularly after stimulation by LPS or other potent inflammation inducing agents and/or protect cells from unwanted side-effects of full activation of inflammatory effects of RelA. Viewed in this way, it makes it easier to understand the reason why RelB/AhR interactions tend to produce mostly synergistic consequences of inflammation, while that of RelA/AhR could act antagonistically.

Exacerbated activation of the alternative NF- $\kappa$ B RelB pathway is associated with various inflammatory disorders including cancer. Recent reports indicate that activation of RelB through IKK $\alpha$  (as opposed to activation of RelA, which largely depends on activation of IKK $\beta$ ) may also lead to resolution (a term used to express the effect of anti-inflammatory agents such as aspirin or the negative feedback of cells to reduce the effects of inflammation) of RelA-induced inflammation [93]. Indeed, Matos and Jordan [94] consider that RelB acts essentially as the negative feedback to the inflammation pathway mediated by RelA. Furthermore, there is now enough evidence to indicate that RelB acts as a major cell survival factor such as conferring radiation resistance [95] and anti-apoptotic properties of cancer cells [96]. Since the finding of the RelB/AhR interaction is so recent, the possible assisting role of AhR in the above cases has not been addressed adequately yet. If such a view is confirmed, it would provide the important clue to one of the major functions of AhR to act as the factor promoting inflammation in cells and alternately it can act as a factor promoting resolution of inflammation to protect cells that are experiencing intense inflammation.

Certainly studies on the interaction of NF- $\kappa$ B proteins and signaling pathways are evolving rather fast, and we must closely keep up with future changes occurring in this fascinating research area. Nevertheless, the realization that AhR participates in NF- $\kappa$ B signaling pathways in regulating inflammation, immune responses, resolution, survival, and probably other functions, this aspect of the biological roles of the AhR merits close attention in the future. Furthermore, this finding also fits the model of AhR being capable of directly forming dimers with individual members of other types of nuclear transcription factors, even without its ligand, e.g. with ER $\alpha$  as in the case of BRCA-1 gene activation [97]. With the additional evidence of direct interactions of AhR with other transcription factors presented in this special issue, such a mode of AhR action to coordinate its activities with other major cellular regulatory pathways through direct AhR protein interactions in the nucleus should be considered as one of the major paradigm of AhR operations. Finally studies on the interaction between AhR and RelB support our previous findings that one of the cellular physiological roles of AhR includes regulation of the cellular inflammatory state.

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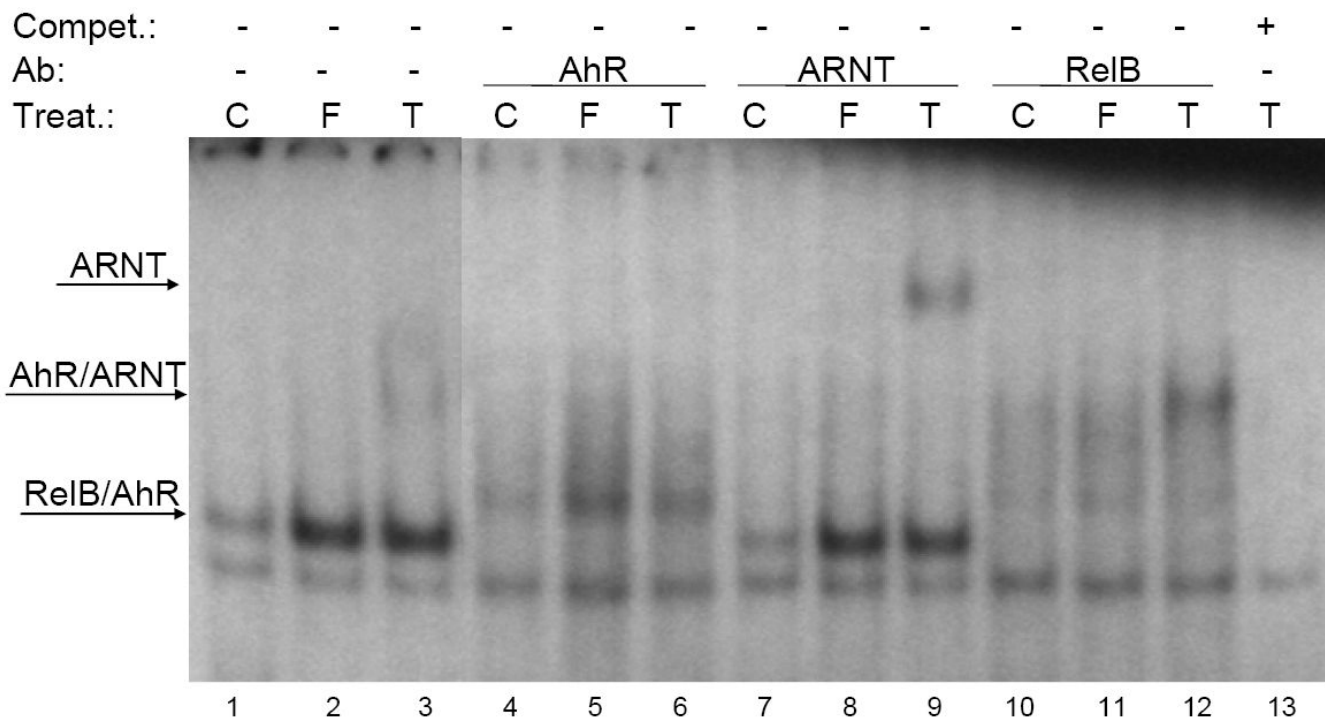
## Abbreviations

<b>AhR</b>	aryl hydrocarbon receptor
<b>ARNT</b>	AhR nuclear translocator
<b>COX-2</b>	Cyclooxygenase 2
<b>DC</b>	dendritic cell
<b>DRE</b>	dioxin response element
<b>FICZ</b>	6-formylindolo[3,2-b]carbazole
<b>IDO</b>	indoleamine-2,3-dioxygenase
<b>IL-8</b>	Interleukin 8
<b>IFN</b>	Interferon
<b>RelBAhRE</b>	RelB/AhR response element
<b>PKA</b>	protein kinase A
<b>TCDD</b>	2,3,7,8-tetrachlorodibenzo-p-dioxin
<b>T<sub>reg</sub></b>	

regulatory T cells

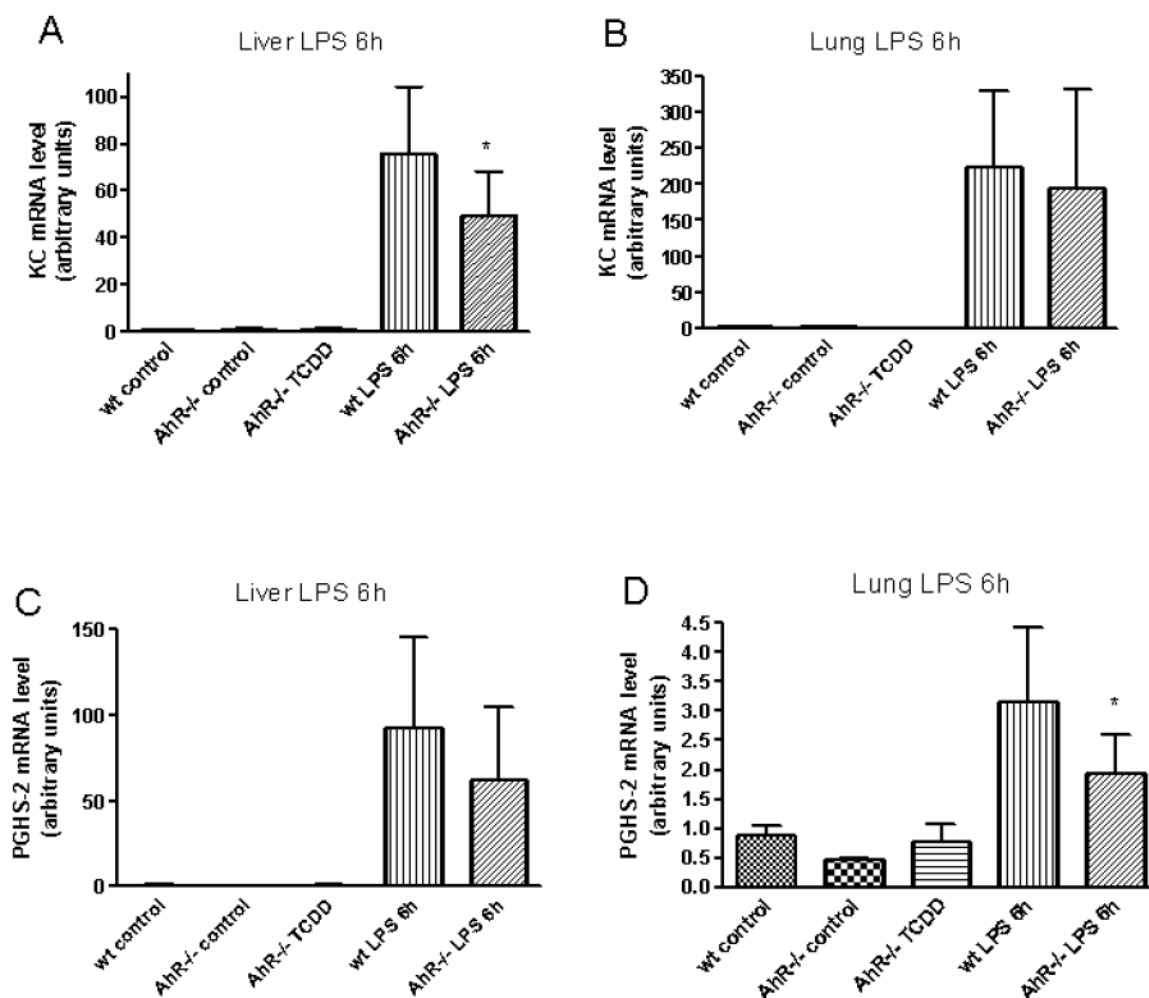
**XAP2**

X-associated protein 2



**Figure 1.** Nuclear protein extracts from control (C), FSK- (F), or TCDD-stimulated (T) HepG2 cells were incubated with <sup>32</sup>P-labeled oligonucleotide containing a DRE consensus element of the CYP1A1 promoter. Nuclear proteins were extracted after treatment (Treat.) of HepG2 cells (ATCC, Manassas, VA) with 10 nM TCDD or 5 μM FSK for 2h. TCDD (>99% purity) was originally obtained from Dow Chemical Co. (Midland, MI) and FSK was purchased from Sigma (St. Louis, MO). A possible binding of AhR, ARNT, and RelB was identified by supershift analyses using AhR-, ARNT-, or RelB-specific antibodies (Ab). Monoclonal ARNT (Santa Cruz Biotech Inc, Santa Cruz, CA), RelB (Active Motif, Carlsbad, CA) and polyclonal AhR (Novus Biologicals, Littleton, CO) antibodies were used for supershift analysis. To confirm specificity a 100-fold excess of unlabeled DRE consensus oligonucleotide was added. A 100-fold molar excess of unlabeled oligonucleotides was added as competitor (Compet.).

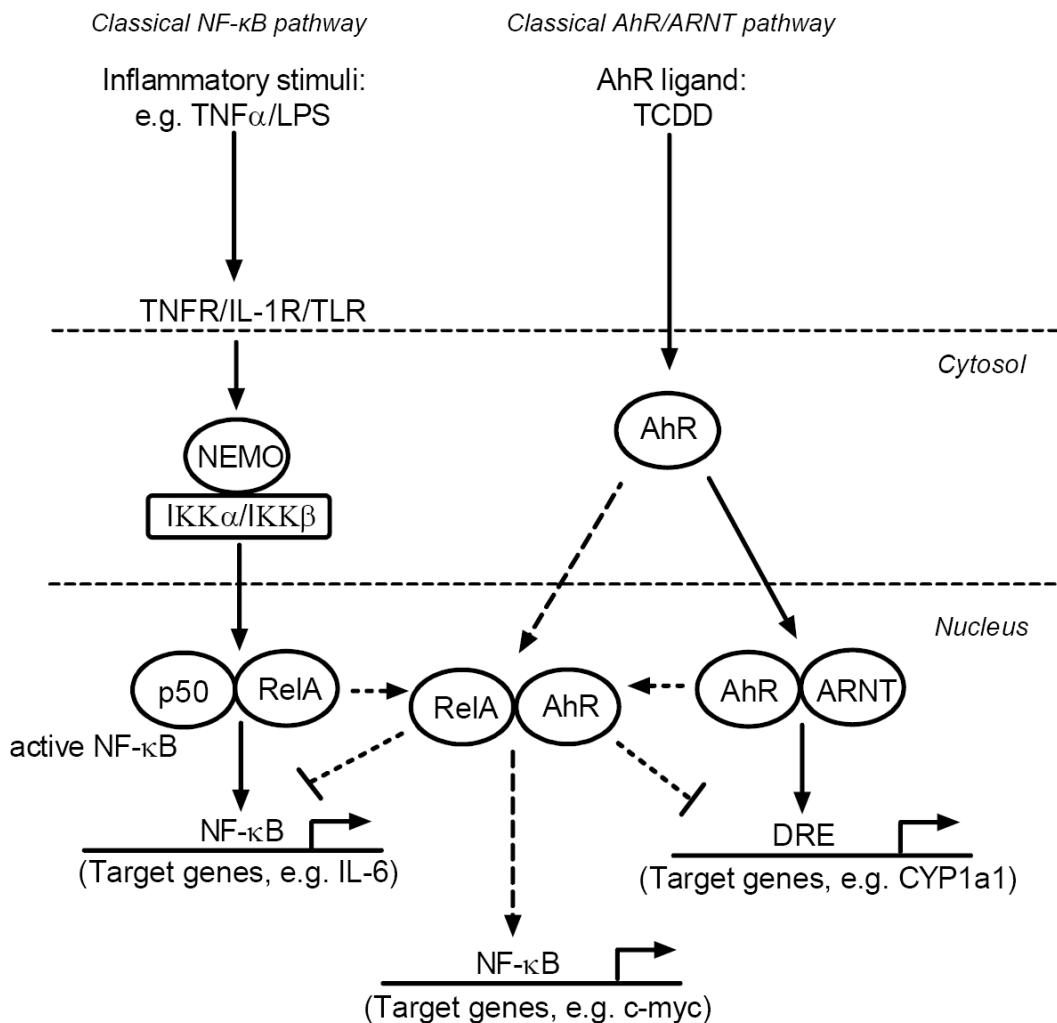




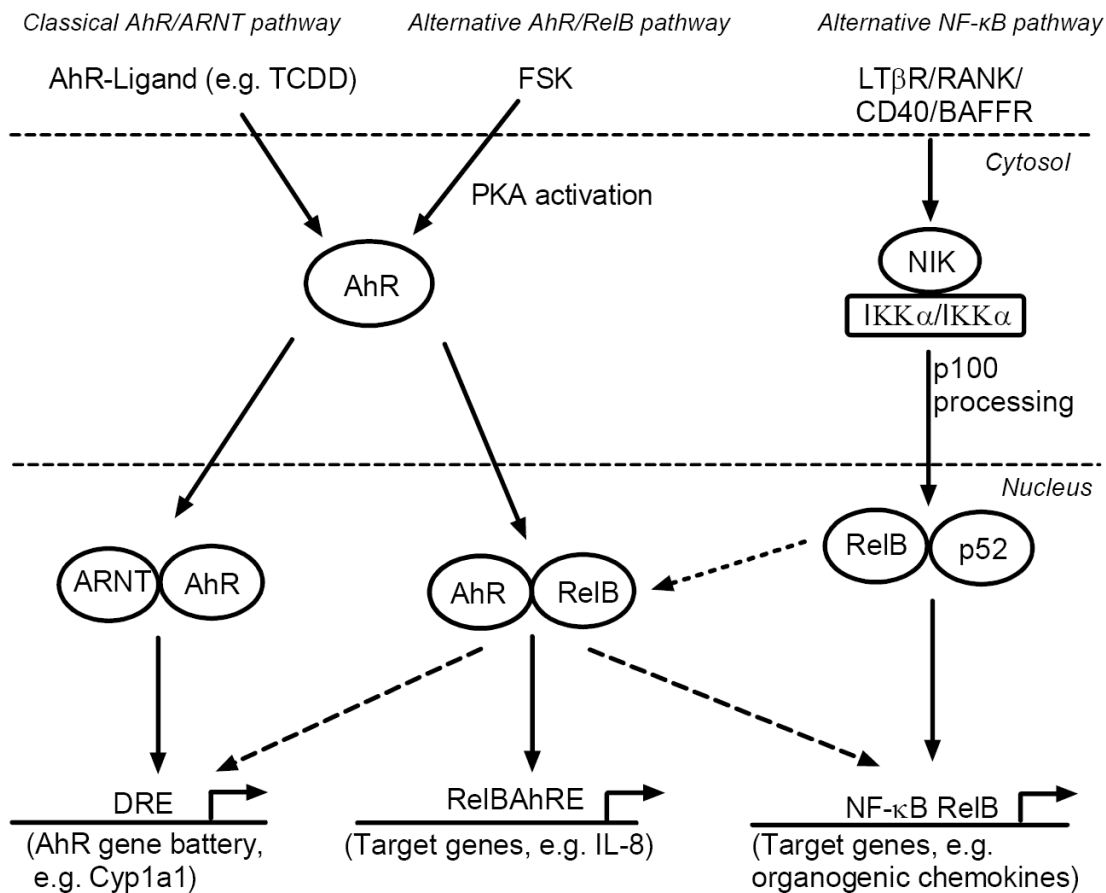
**Figure 2.**

C57BL/6 wild type (wt) and AhR homozygous null mice ( $AhR^{-/-}$ ) were treated with 50  $\mu$ g LPS *E. coli* 055:B5 (Sigma L4005, St. Louis, MO). In addition  $AhR^{-/-}$  mice were treated with 15  $\mu$ g/kg TCDD. After 6 hrs animals were sacrificed and tissues were prepared for RNA analysis by real time PCR. Values of KC and prostaglandin endoperoxide H synthase-2 (PGHS-2 or COX-2) mRNA are normalized to the expression of  $\beta$ -actin. C57BL/6J (BL6) and AhR null ( $AhR^{-/-}$ ) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME).

\* significantly different from LPS-treated wild type mice  $p \leq 0.05$



**Figure 3.** Putative crosstalk between the classical pathway of NF-κB and AhR activation. Ligand engagement of specific membrane receptors (TNFR, IL-1R or TLR) triggers phosphorylation and activation of IKK $\beta$ . Free NF-κB translocates to the nucleus and activates target genes via p50/RelA dimers. Activated free RelA may also bind to ligand-activated AhR generating transcriptionally inactive complexes that could suppress the classical activation pathways of NF-κB [89] and AhR [26] or positively regulate gene expression as shown for c-myc [90]. IL-1R, IL-1 receptor; NEMO, NF-κB essential modulator; TLR, Toll like receptor; TNFR, TNF receptor.



**Figure 4.** New mechanism of cross talk between AhR and RelB. Ligand-activated or unliganded AhR activated by PKA translocates into the nucleus and interacts with RelB and occupy RelB/AhR-response elements (RelBAhRE) of promoters as in the case of IL-8 (alternative AhR/RelB pathway). AhR agonists induce the recruitment of AhR/ARNT complexes to DRE-responsive promoters such as CYP1a1 (classical AhR/ARNT pathway). Alternative pathway of NF-κB activation through the LTβR, RANK, CD40, or BAFFR leads to the release of active NIK which phosphorylates IKK. After processing of p100, RelB/p52 heterodimers are generated and activate target genes. BAFFR, B-cell activating factor receptor; FSK, forskolin; IKK, IκB kinase; LTβR, Lymphotoxin β receptor; NIK, NF-κB-inducing kinase; NF-κB, nuclear factor-κB; PKA, Protein kinase A; RANK, receptor activator of NF-κB. From Vogel et al. [18] reproduced with permission from *Molecular Endocrinology* 2008.