The Aryl Hydrocarbon receptor and intestinal immunity: an overview of ligands derived from microbial metabolism and food

Fleur de Mooij
3257843

Master thesis Toxicology and Environmental Health

Supervisors:
Dr. Linda Loonen, Wageningen University
Prof. Harry Wichers, Wageningen University
Dr. Raymond Pieters, IRAS, Utrecht University

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Layman's summary

Everybody knows that eating vegetables and overall healthy food is beneficial for your health. In recent years advertisers also started to tell us that products such as Yakult© that contain bacteria can improve the wellbeing of our intestines[1]. This is why a lot of research has been going towards finding the mechanisms behind these healthy bacteria and the beneficial effects of healthy food. It is now suggested that there could be a link between these two.

Our bodies are built of cells that obtain their energy from food. In our intestines we have a lot of bacteria that helps us digest our food. This may sound weird, but in contrast to bacteria in other parts of our body, bacteria in the intestine are not harmful as long as there is a state of symbiosis. The cells of our intestines are not able to use all the food that we eat because they lack the machinery to make little pieces of it that can get absorbed into the cells. That's where our resident bacteria come in. They have different machinery that help us process certain types of food into the small pieces we need. When these small pieces of food are taken up by the intestinal cells, they can start processes within the cells by binding to receptors. For instance, this could lead to the development and activation of the immune system. This can help to keep the balance between the intestine and the resident bacteria. Recent research has shown us that an important receptor in this process is the Aryl Hydrocarbon Receptor (AhR).

The AhR can assist in protecting our intestines from harm by activating part of our immune system. They do so by activating the cells to produce signaling molecules, one of the most important ones is interleukin 22 (IL-22). This IL-22 is on its own able to bind its own specific receptor on other cells thereby amplifying the signal and activating these cells to start secreting small proteins such as defensins. As the name suggests, these defensins are small proteins that can kill the bacteria. With the secretion of defensins the amount of bacteria can be controlled, hereby keeping the balance to provide symbiosis. Next to the activation of cells to produce defensins, the activation of the AhR also leads to the development of other cells from our immune system, thereby making sure that we are well protected in the long run.

As described, via activation of the AhR we can modulate the health of our intestines. It is therefore very interesting to know how we can activate this receptor. It is now suggested that the small molecules that bind the AhR can come from our food. For instance, tryptophan, which is an amino acid, gets cut by the gut bacteria into kynurenine which is then able to bind and activate the AhR. A particular group of bacteria, the lactobacilli, are very able to contribute to this process. Additionally, small molecules derived from food such as broccoli and cabbage have been shown to activate the AhR. One of these molecules is known as indole-3-carbinol.

In conclusion, activating the AhR in the intestines could improve the health of our intestines. Molecules from our food could trigger this process. This provides a mechanism to explain that consumption of certain types of food, or supplementing our food with certain bacteria strains, has the potential to increase our health.

Introduction

The Aryl Hydrocarbon receptor (AhR) is a cytosolic receptor that is expressed by many cells in the human body. It is known to bind 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which was the reason its biology is well researched. Next to dioxins, pharmaceuticals or metabolites from pharmaceuticals can act as AhR ligands[2]. Although the AHR is mainly known for its ability to bind dioxins, recent research has shown that the function of the AhR could be more extensive. Binding of various types of ligands to the AhR can lead to the activation of several components of the immune system. It is therefore thought that the AhR could be a main modulator in mucosal immunity. The nature of these ligands that bind to the AhR is now the main focus of many research groups. In this thesis an overview will portrayed of the AhR itself, the effects it has on the immune system and the possible ligands that bind to this receptor. The main focus will be on the possibility that these ligands originate from food degradation by microbiota.

The AhR

The Aryl Hydrocarbon Receptor (AhR) is a basic helix-loop-helix ligand-activated transcription factor. In its inactive state it resides in the cytosol as an inactive complex bound to the chaperones heat shock protein (HSP90), ARA9, (or AIP2), p23 and the c-SRC protein kinase[3].

The AhR can be activated through two types of pathways, the classical and nonclassical AhR signaling pathway. In the classical signaling pathway, agonist binding leads to the translocation of the AhR complex into the nucleus. Here the chaperone proteins dissociate and are replaced by the aryl hydrocarbon receptor nuclear transporter (ARNT). This complex of ligand-AhR-ARNT then binds to specific enhancer sequences in the promoter of the responsive genes namely the dioxin receptor element (DRE) and the xenobiotic receptor element (XRE) which then activate the transcription of target genes[4].

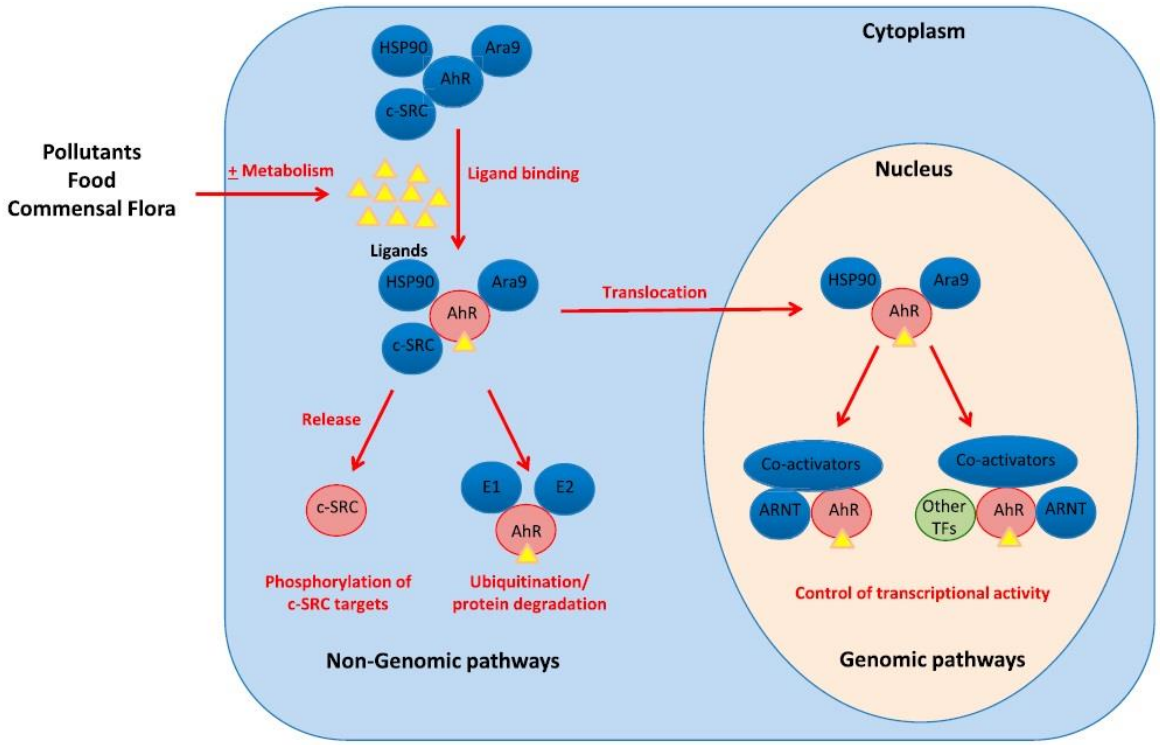

Figure 1: The AhR signaling pathways Binding of ligands derived from pollutant, food or the commensal flora leads to the dissociation of the inactive AhR complex. Translocation of the AhR to the nucleus leads to gene transcription. Dissociating proteins from the inactive AhR complex lead to activation of non-genomic pathways (Quintana *et al.*2013) *Colors are adjusted.*

It has recently been described that next to these known binding sites, the complex also recognizes other sequences, thereby possibly activating even more genes than currently described. Also, there may be more genes within these responsive elements than currently known, which would mean that binding leads to the activation of more processes[5].

The nonclassical AhR signaling pathway covers the pathways that is activated after the dissociation of the inactive complex. The disassociated proteins then activate pathways on their own. For example, the dissociation of c-SRC leads to the phosphorylation of c-SRC targets inducing the enzyme cyclooxygenase 2 (COX2) and other cell-migratory programs[5,6]. Also, the AhR has been linked to the activation of proteasome-dependent degradation of specific transcription factors leading to the degradation of AhR-interacting proteins (Figure 1).

Well known genes that respond in connection to AhR-activation are those of the cytochrome P450 family (CYP): 1A1, 1A2 and 1B1 which are xenobiotic metabolizing enzymes[3].

The AhR and intestinal immunity

To protect the body from pathogens, the outer layers of the body such as the intestine are lined with epithelial cells. Behind these epithelial cells a barrier of immune cells of the innate and adaptive immune system is present, guaranteeing an immediate response followed by a specific response upon danger. It has been shown that, for the development and activation of certain immune cells, activation of the AhR is necessary.

The gut microbiome

Our intestines are lined with mucosal surfaces that keep the delicate balance between tolerance and response to the huge number of microbes that reside in our intestines. A lot of microbes are commensal, but changes in the intestinal environment can make them switch to parasitism, thereby harming the host. In the healthy gut the anaerobes form the largest group of the microbiota consisting of Bacteroidetes and Firmimicutes from which the phyla Clostridiales and Lactobacillus are most abundant[7]. To keep this balance our intestines have a large number of residing immune cells that make sure that the microbiota stay commensal.

Innate lymphoid cells (ILC's)

ILC's are innate versions of T-cells of the adaptive immunity. They respond by excreting effector cytokines when there is non-specific danger, thereby forming the front line of protection. In recent years a lot of research has been performed on different ILC subsets and various names and numbers have been given to the subsets. Nowadays, the cells are divided into categories based on their phenotype, function and transcriptional regions. This has led to the definition of three categories of ILCs. ILC1 contains the Natural Killers (NK) cells, ILC2 are known as the nuocytes and excrete IL-5 and IL-13, but our main interest is in the subset of ILC3 which produce IL-17 and IL-22[8] (Figure 2). Cells that are also considered ILC3 are the ILC22 cells, known for the constitutive excretion of IL-22[9].

AhR activation leads to the development of Rorγt⁺ ILCs that produce IL-22

As figure 2 shows, the ILC3 cells develop in response to AhR-activation. The ILC3 originate from the common lymphoid progenitors (CLP) that differentiate into the common innate lymphoid progenitors (CILP) under the influence of the transcription factor Id2[8]. These CILP then differentiate into a retinoic acid related orphan receptor (ROR) γt-expressing ILC. RORγt⁺ ILCs are innate lymphocytes that rely on RORγt for their development.

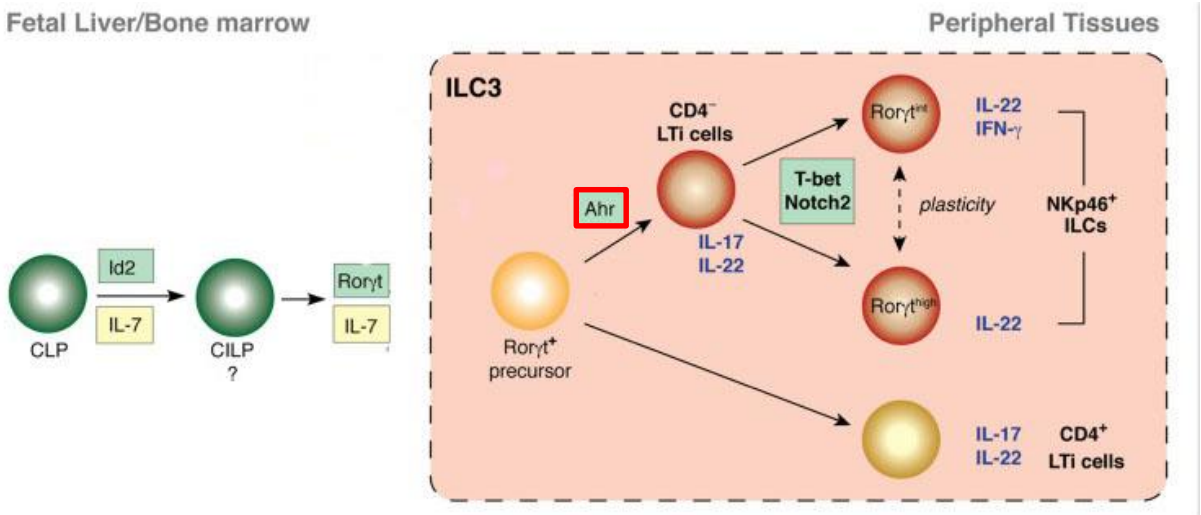

Figure 2: Development of the ILC3 subset cells. ILC3 cells rely on the activation of the AhR to develop into CD4⁺ LTi cells. These can then mature into Rorγt⁺ ILCs that express the Rorγt are an important source of IL-22 and IL-17. *Picture adapted from Rankin et al. (March 2013)* [8].

Activation of the AhR then causes differentiation of the precursor cells into $CD4^-$ LTi (lymphoid tissue inducer) cells that can express IL-17 and IL-22. The expression of T-bet and Notch2 in the $CD4^-$ LTi cells then leads to $NKp46^+$ ILCs that produce abundant IL-22 but no IL-17. Activation of the AhR induces Notch[9]. Without the AhR activation the $ROR\gamma t^+$ precursor cells turn into $CD4^+$ LTi cells that produce IL-17 and IL-22 (these cells are also known as $NKp46^- CD4^+$ LTi cells).

$ROR\gamma t^+$ ILCs have been known to have LTi function, meaning that they play a role in induction of lymphoid tissue. These $ROR\gamma t^+$ ILCs lie in cryopatches (CP) in the intestinal lamina propria and constitutively produce IL-22. CP are known to attract B cells and thereby form isolated lymphoid follicles (ILF). Here, the activation of the AhR leads to the adaptation of the organisms' response to natural ligands via DCs and correspondence with B cells. Even though the development of secondary lymphoid organs does not require the activation of the AhR, the postnatal development of CP requires AhR-expression by the $ROR\gamma t^+$ ILC's. Although $ROR\gamma t^+$ ILCs are already present at birth, they seem to need AhR-activation to maintain and expand[10].

All these cells have in common that they produce IL-22 which seems to be a key modulator in both innate and adaptive response to pathogens.

IL-22 as modulator for the microbial balance

The induction of IL-22 in the intestine leads to the production of antimicrobial peptides (AMPs) by ILCs. These peptides regulate the balance between commensal and parasitism of our microbiome. They do so by creating pores in the membranes of the bacteria leading to death[11]. A direct link between AhR-induced IL-22 excretion and AMPs can be found through the Paneth cells. They produce human α-defensin 5 and 6 in the intestine upon IL-22 detection[12]. Paneth cells constitutively excrete these defensins that target both Gram negative and Gram positive bacteria (Figure 3) [11].

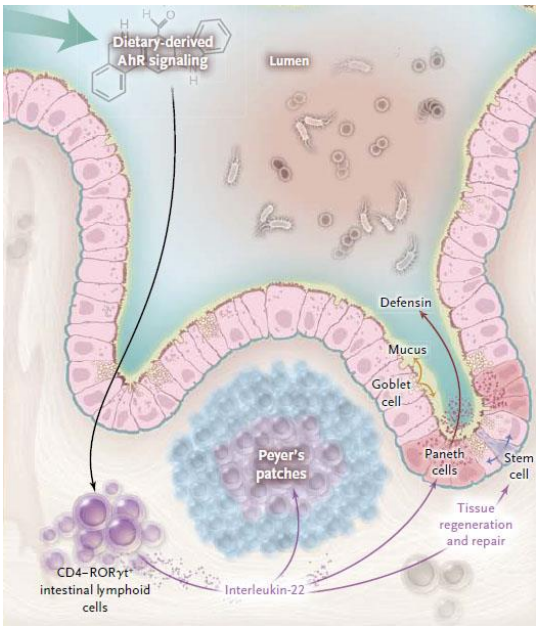

Figure 3: Excretion of defensin by Paneth cells upon IL-22 detection in the small intestine. Ahr-activation leads to the secretion of IL-22 by $CD4^-$-$ROR\gamma t^+$ cells. Upon detection of IL-22, Paneth cells excrete α-defensin 5 and 6 that cause death to bacteria[12]. Picture from Tilg *et al.* 2013

The AhR and the development of innate immune cells

As mentioned above, the AhR plays a role in the development and maintenance of the innate immune system, but it is also known for its role in the control of the adaptive immune response. The AhR controls the differentiation and activity of certain T-cell subsets, thereby having an effect on T-cells and antigen presenting cells (APCs). $CD4^+$ regulatory T-cells (Tregs) need the transcription factor Forkhead box P3 (FoxP3) to increase the accessibility to the transcription machinery leading to increased differentiation and functioning of Tregs[13]. FoxP3

is downstream of AhR indicating a direct link between the receptor and transcription factor[14]. Next to FoxP3, the AhR increases the expression of Mothers Against Decapentaplegic Homolog 1 (SMAD1) in human Tregs, which in its turn lead to the steady expression of FoxP3. The AhR can also inhibit the Signal Transducers and Activators of Transcription family (STAT1) transcription factor. STAT1 normally leads to a decrease in FOXP3 but the inhibition of STAT1 leads to abundant FOXp3. So the AhR has an effect on the expression of FoxP3 (either direct or via SMAD1) and inhibits STAT1, thereby controlling the Treg population[3].

Another subset of Treg cells, the Tr1 cells, are also activated via the AhR, but these do not express FoxP3. These Tr1 cells are activated via the AhR upon IL-27 signaling, which leads to the interaction of the AhR with c-maf. This protein complex can then activate the IL-10 promoter which also leads to the production of IL-21. This then shifts the Tr1 cells from inactive, to IL-10 and IL-21 excreting cells, which is a defense against colitis[3,15].

Next to Tregs, a link has been found between the AhR and the differentiation of T- helper 17 cells (Th17 cells). Activation of the AhR (*in vitro*) leads to the generation of IL-17$^+$ T cells and increases the production of IL-21 and IL-22[16]. A deficiency in AhR-expression was shown to abolish the IL-22 production of the TH17 cells in mice. The exact mechanism of the AhR on the differentiation of Th17 cells has not been verified yet, but recent data shows that there is a link between the inhibition of IL-2 by AhR activation and the differentiation of Th17 cells[3].

Lastly, the AhR has an effect on the Dendritic cells (DCs). DCs control T-cell activation and polarization. The AhR influences DC generation and activity, although these pathways have not yet been clarified. A set of transcription factors that control the response of DCs has been clarified but these included none of the direct transcriptional factors expressed via the AhR. Therefore it is thought that the AhR pathway produces proteins that are necessary for the protein-protein interaction that can activate the DCs. Upon infection or microbial dysbiosis the DC's are activated and this then leads to the excretion of IL-23. This IL-23 can then activate ILC22 (such as the IL3C) to start producing IL-22.

As shown in the figure 4, there are already some links made between the AhR and the regulation of the innate immune system, although it seems to be the tip of the iceberg.

To underpin the finding that IL-22 is a modulator in intestinal immunity, it was found that in IL-22$^{-/-}$ mice a higher rate of weight loss was measured upon infection with *C. rodentium* compared to the WT mice. In the second week of infection that caused a mortality rate of almost 100% of the IL-22$^{-/-}$ mice. In the colonic crypts higher counts of bacteria were observed in the IL-22$^{-/-}$ mice compared to the WT. This suggests that IL-22 plays a key role in controlling bacterial infections[17].

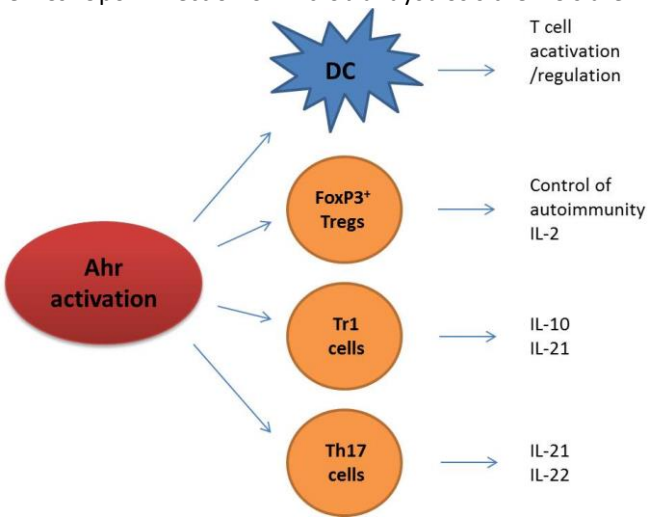

Figure 4: Overview of innate cells regulated by activation of AhR. Indicated processes in the left row are most relevant to mucosal immunity and maintenance of this barrier in the intestine. Therefore it could be that key regulators for other processes are not mentioned.

AhR activation leads to IL-22 (direct and indirect)

Concluding, the AhR plays a role in the activation of the innate and adaptive immune cells. It can activate a direct line of defense such as the ILC3s but can also activate the adaptive system. Abundant AhR ligands in the gut could thereby cause more activation of the immune system and lead to more protection of the intestine. The key mediator for this process seems to be IL-22, whether it is produced by the innate IL3C subset or the TH17 cells upon infection. This was also underlined by the finding that AhR$^{-/-}$ produce little to no IL-22 [9].

Tryptophan as source of ligands for the AhR

Our bodies host 10^{14} microbes in our gastrointestinal tract, varying from bacteria and fungi to archaea[18]. Our gutflora is very important in the digestion of our food as bacteria can ferment undigested carbohydrates and produce vitamins B and K, processes that our body cannot complete. The microbiome also possesses enzymes that the human cells lack enabling them to metabolize certain xenobiotics, bile acids, and sterols.

In the intestines bacteria don't harm us as long as there is a state of eubiosis. When a state of microbial dysbiosis is reached, this commensal occupation can shift to parasitism[7]. As discussed earlier, AhR ligands can induce the production of local IL-22 by ILCs from the ILC3 category. Among other factors, this IL-22 protects the intestine from damage by the commensal bacteria as is discussed in the chapter on immunity[9,19].

It is now suggested that the ligands that activate the AhR can also derive from the metabolism of food by the microbiome. Proposed ligands derive from tryptophan metabolism, resveratrol, eicosanoids, cytokines, dioxins, phytochemicals and flavonoids such as grapes. In this chapter the main focus will be on the ligands that originate from tryptophan (trp). Trp is an essential amino acid for the human body which means that our bodies cannot make it and therefore we have to obtain it by diet. These ligands can be divided into three categories: 1) already present as ligands in the food (cabbage and broccoli), 2) resulting from trp-metabolization by human enzymes, and 3) metabolized by the microbiome.

Indole-3-carbinol and 3,3'-di-indolylmethane

The first category of trp-derived ligands are direct ligands for the AhR. This means that there is no metabolizing step necessary in the intestine to form ligands. Vegetables from the Brassica family, such as broccoli and cabbage, contain high amounts of indole-3-carbinol (I3C). Oxidation of I3C occurs in the upper gastrointestinal tract by gastric acid. This gives rise to indole-3-carboxylic acid which is condensed into 3,3'-di-indolylmethane (DIM) in an acidic environment. DIM was shown to be an AhR-ligand thereby linking the consumption of food to the activation of the AhR[16].

To demonstrate the link between I3C and the AhR, mice were administered a phytochemical free diet. This led to the phenotype that was similar to a AhR-deficient mice characterized by the reduction in RORytcells at a young age (4 weeks). Supplementing the diet with indole-3-carbinol (I3C) led to a level of CPs and RORyt ILC cells as mice on a normal phytochemical rich diet[20]. This showed that I3C is a key modulator, derived from food, responsible for the maintenance of the RORyt ILC cell population. To show that this is via the AhR, $AhR^{-/-}$ mice were fed the I3C enriched diet. These mice were not able to recover the RORyt ILC population, showing that the I3C involves the AhR. This linked the modulation of intestinal immunity to activation of the AhR through ligands derived from food[10,20].

Trp metabolism routes via human enzymes

The second category are the ligands that are derived from trp by metabolism of human enzymes. When trp enters the intestine, it is taken up by the epithelial cells through the ACE2/B^0AT1 transporter into the cytosol[21] where it can be metabolized into AhR ligands. Various metabolizing routes have been suggested, primarily those involving human enzymes such as the indoleamine 2,3-dioxygenase 1 (IDO1) and IDO2. Trp is mainly used in protein synthesis but of all the excess Trp 99% is metabolized by IDO1[22]. Recently it has been discussed that our microbiome also holds a large group of Trp metabolizing enzymes capable of producing large quantities of AhR ligands derived from Trp[7].

The main route of Trp metabolism is the degradation by IDO1 into the metabolite kynurenine[23]. Kynurenine is a potent AhR ligand together with its downstream metabolites kynurenic acid and xanthurenic acid that are also

known to bind the AhR[24]. This pathway is known as the kynurenine pathway. The most potent kynurenine is ʟ-kynurenine, the first product of Trp-degradation by the IDO1 enzymes. Binding of ʟ-kynurenine to the AhR has been connected to the development of Tregs[25].

In the skin there is also a group of tryptophan metabolites known as photometabolites. UV light causes Trp to convert to 6-formylindolo[3,2-b] carbazole (FICZ). FICZ is known for its existence in the skin where it was involved in inflammatory responses by binding with high affinity to the AhR. Although FICZ was thought to only be available in the skin, metabolites were also detected in urine, suggesting a more systemic role for FICZ. Because FICZ is able to activate the AhR at nanomolar concentrations there could be a bigger role in AhR activation for FICZ than currently thought[26].

The activation of the AhR microbiome derived Trp ligands

Our microbiome produces enzymes that the human body does not, enabling it to process food in different ways giving rise to a different pool of possible ligands for the AhR. For example, the trp pathway by IDO1/2 gives rise to kynurenine, a known ligand for the AhR. But if trp follows the indole pathway it is transferred into an indole by tryptophanase. Tryptophanase is only expressed by a small subset of enteric bacteria such as the *Bifidobacterium* species[27]. This was demonstrated by measuring indoles in germ free (GF) mice which showed that GF mice had levels of indole in their plasma that were up to 60% lower than mice with a conventional gut flora[28]. These indoles can then be transferred further by the microbiome into indole-3-carbinol which is also a potent AhR ligand[28].

Trp can also be degraded by tryptophan hydroxylase and decarboxylase, both bacterial enzymes. The metabolite that derives from these pathways is tryptamine. Besides its own qualities to bind the AhR, tryptamine metabolites such as indole acetic acid, are also AhR ligands[27].

'Unknown' microbiome derived AhR ligands detection by IDO$^{-/-}$ knock-out mice

To analyze whether our gut microbiome is able to produce other, still 'unknown', ligands that are able to bind to the AhR, Zelante *et al.* experimented with IDO1$^{-/-}$ knock-out mice, lacking the enzyme to metabolize trp. One would expect that the intestinal immunity diminishes when the main production line for AhR ligands is knocked out, but this was not the case. This indicated that ligands can be derived from food sources through other pathways. With the term 'microbiota-AhR axis' Zelante *et al.* stated that our microbiome produces ligands for the AhR, mainly derived from trp, which can activate the AhR and influence the mucosal immunity of the intestine[7].

Using metabolomics, indole derivatives were detected ex vivo in a culture of *L. reuteri* and *L. Johnsonii* that were recovered from the gut of IDO$^{-/-}$ mice. Here it was shown that in a condition of carbohydrate starvation the *L. reuteri* bacteria produce large amounts of indole-3-aldehyde (IAld) which was shown to be a AhR ligand. This increase of IAld was not seen in cultures with bacteria that had tryptophanase activity, suggesting that the *L. reuteri* are the main trp metabolizing microbiota in a IDO$^{-/-}$ environment. This data was supported by the finding that in IDO$^{-/-}$ mice that were fed trp rich diets, *L. reuteri* quickly expanded. This finding proposed alternative trp-metabolizing pathways next to the conventional IDO1/2-catalyzed pathways[7].

Indole-3-aldehyde and the link to *L. reuteri*

Indole-3-aldehyde (IAld) is a well-known metabolite of trp and can arise via various degrading pathways. One of the main pathways is the indole-pyruvate-pathway that is catalyzed by the aromatic amino acid transferase (ArAT). ArAT is conserved in many bacterial species such as the Lactobacilli. In contrast to other bacterial species Lactobacilli showed to be the main bacterial species to metabolize Trp[7]. Further research with *L. reuteri* showed that there is a direct link between the metabolism of trp into IAld by *L. reuteri* and the increase in IL-22. This was not visible in the same tests with other intestinal bacteria strains such as the *L. Johnsonii* or *Clostridia* species[7].

These results seem promising as they show a direct link between the metabolism by *L. reuteri* and the induction of IL-22, but the circumstances under which this was found were not physiologically relevant. When the wild type (WT) mice were fed with either a trp rich or trp depleted diet a higher amount of IAld, IL-22, and IL-22 producing ILC3 cells were detected. To check whether this was due to *L. reuteri*, another group of WT mice were treated with ampicillin which decreases the amount of Lactobacillus species in the intestine. In these mice a decrease in IAld, IL-22, and ILC3 cells was measured. Upon feeding with a Trp+ diet, the expansion of *L. reuteri* was measured[7], showing the direct link between trp and *L. reuteri* .

IAld is an important microbiome produced metabolite of trp and the ability of IAld to bind the AhR and promote IL-22 secretion seems to be a good therapeutic target. In case of chronic inflammation, the administration of IAld even leads to the recovery of the antifungal resistance and an increased IL-22 production by $NKp46^{+}$ ILC3 cells. In $IDO^{-/-}$ mice the administration of IALd even lead to the recovery of the Treg and Th1 cellular immune response that was decreased to the $IDO^{-/-}$ knock-out[7].

All these data show that microbial metabolism of trp can lead to reactivation or improvement of the intestinal immunity. Especially the metabolite IAld is a very potent AhR agonist and could therefore be considered for therapeutic purposes[7].

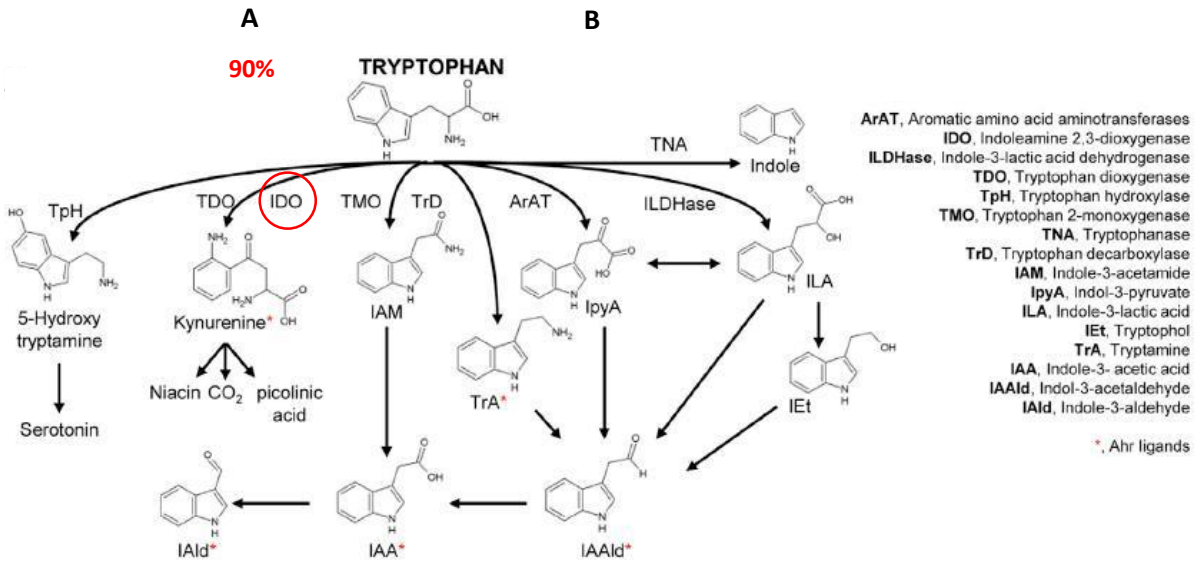

Figure 5: Tryptophan metabolites functioning as AhR ligands As described Trp can be metabolized into various ligands via various pathways. Above the main ones are described with the two most important ones for AhR activation marked A and B. (A) is the kynurenine pathway catalyzed by the IDO1/2 enzymes, giving rise to kynurenine which binds to the AhR. (B) is the indole (pyruvate) pathway in which ArAT metabolizes Trp into IAld which can bind to the AhR. The Indoles that are produced can be further metabolized into other AhR ligands such as indole-3-carbinol[27]. All the metabolites marked with * are AhR ligands. Picture modified form Zelante *et al.* 2013[7].

As can be seen in figure 5, the microbial metabolism of trp can give rise to many ligands that can bind the AhR[7]. The role for Trp derivatives as potent ligands that can increase the intestinal immunity was supported by recent research into the ACE2 enzyme in mice. Hashimoto *et al.* used a wild type mouse model that was fed with a trp-free diet. Their interest was the ACE2 enzyme, an enzyme that regulates the uptake of amino acids in the small intestine by binding to the transporter $B^{0}AT1$, resulting in the uptake of trp. The uptake of trp would then lead to the activation of the mTOR pathway which then regulates antimicrobial peptides. They found that in these

mice that missed trp in their diet a downregulation of antimicrobial peptides such as defensins were measured, linking the dietary starvation of trp to a reduction of intestinal immunity[21].

In conclusion it has been shown that a large pool of AhR ligands is produced by the degradation of trp by microbiota and that altering the composition of the microbiota also leads to less activation of the AhR. Food derived ligands such as I3C from the Brassica family have been a widely described phenomenon and have shown to activate the AhR. Still this seems to be the tip of the iceberg as promising results have been shown connecting the consumption of food with high amounts of isoflavones and flavones to AhR activation.

Probiotics

As mentioned in the introduction the addition of bacterial strains to food is already a used method to enrich our food. These are known as probiotics and are commensal bacteria that are beneficial. Drinks such as Yakult© contain live bacterial strains that expand the microbiome in our intestine as an increase in the specific added strains was detected in humans[1]. This would mean that if the microbiome contains high amounts of beneficial bacteria, this could have a positive effect on the intestinal health. A link with the AhR could be found in an increase of AhR ligands after administration of certain bacterial strains.

Indole-3-propionic acid (IPA)

To investigate a link between the addition of bacterial strains and the induction of the AhR, an AhR ligand was used as a target for selecting bacterial strains. Wikoff *et al.* found that, *in vitro*, *Clostridium sporogenes* produces indole-3-propionic acid (IPA) which is an AhR ligand and a product of tryptophanase. To see if the addition of C. Sporogenes really led to an increased amount of IPA, they administered the bacteria to germ free mice (method is not verified in article, it is suggested that the administration was via oral gavage). After the administration an increase in IPA is detected in the blood suggesting that adding live bacteria can indeed increase the amount of AhR ligands[28].

***L. bulgaricus* OLL1181**

Lactic acid bacterial strains have often been suggested as a probiotic but the connection between these bacteria and the AhR was not described yet. To identify possible candidates for the use as a probiotic, Takamura *et al.* did an *in vitro* screening of 62 heat killed lactic acid bacteria strains. They used a specific screening method to analyse if these bacteria were able to activate the AhR. After the screening, 42 candidates remained but only *L. bulgaricus* OLL1181, which was already used as an addition to yoghurts and other food, was chosen for the *in vivo* experiments. After oral administration of the *L. bulgaricus* OLL1181 an increase in CYP1A1 activity was measured suggesting that this strain was able to activate the AhR. To confirm specific CYP1A1 induction a negative control was also used which showed no increase in CYP1A1. To check whether it was indeed AhR activation, a test on prostaglandin 2 (PGE2) was performed because TCDD can induce PGE2 via the AhR. *In vitro* this induction of PGE2 due to *L. bulgaricus* OLL1181 was also detected suggesting that this lactobacilli can really activate the AhR. The exact mode of action has not been clarified yet, although it is suggested that these are also derivatives of trp[29].

Both these examples, as well as the study by Zelante *et al.*, show that there are bacteria able to produce ligands to activate the AhR. As there have not been many examples yet it is very probable that more bacteria strains have this property. Adding these probiotics to food could hence stimulate the AhR and induce intestinal health.

Discussion

The use of various ligand sources may seem to be a perfect solution but a lot of aspects concerning these ligands and the AhR itself have not been addressed. As a ubiquitously expressed receptor in almost all vertebrate cells, the AhR as a target could have serious side effects. A safer option would to further research the production of AhR ligands either by the microbiome or derived from food.

Kynurenine in brain can lead to suppression of the immune response

As described earlier the trp metabolite kynurenine is a ligand for the AhR. This ligand is mainly taken up from food but Opitz *et al.* recently described that tumor cells are also able to produce kynurenine and activate the AhR[25]. This activation then leads to the suppression of antitumor immune responses. Although this mechanism has mainly been described in the brain[25], this could also play a role in the intestine.

Polymorphisms alter the binding capacity of ligands to the AhR

The availability of abundant ligands would suggest an infinite binding and activation of the AhR but this does not seem to be true. Polymorphisms in the AhR showed to decrease the binding capacity of ligands. These tests are until now only performed for TCDD. With the tests it was shown that an analogous mutation at position 381 in humans, which changes a valine into an aspartic acid, highly weakened the affinity for TCDD[30]. Next to the effect of single polymorphisms that show a lower affinity, the translation of results from mice to men should be monitored closely. The same affinity tests with TCDD showed that there is a tenfold difference in the affinity for TCDD between mice and men ex vivo[31]. This could mean that possible ligands found in mice may not show the same affinity in men.

Activation of the AhR leads to depletion of its own ligands

The activation of the AhR often leads to the upregulation of enzymes that can degrade its own ligands again such as the CYP1A1 family of enzymes. This could mean that if there are tryptophan-derived ligands available that activate the AhR, a bigger response or effect on the immunity of the mucosal layer is not guaranteed. The AhR can activate a negative feedback loop that rapidly decreases its own ligands such as tryptamine which is a substrate for the CYP1A1 enzymes[32]. This finding was also supported by experiments where the downregulation of CYP1A1 increased the AhR transcriptional activity[33]. This negative feedback loop could diminish the beneficial effect on the AhR that would be expected in case of abundant ligands. These are represented by the red arrows in figure 6.

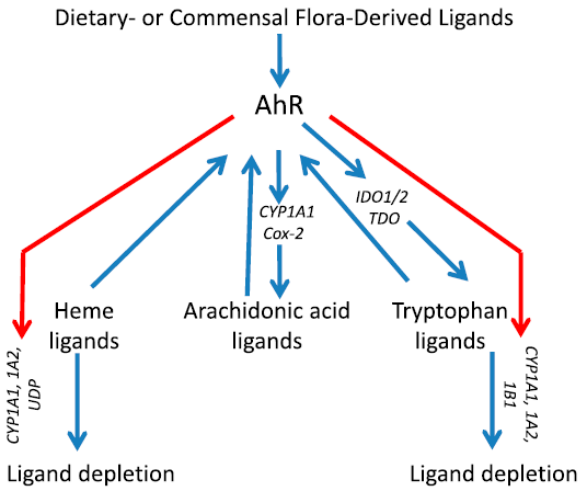

Figure 6: Upregulation of AhR dependent enzymes leads to degradation of its own ligands. The activation of the AhR leads to the upregulation of CYP1A1, 1A2 and 1B1, these enzymes are able to degrade possible ligands for the AhR. This creates a negative feedbackloop that could diminish the beneficial effects of abundant ligands[3].

There are also experiments that suggest that it is the other way around and that there are positive feedback loops especially in the case of arachidonic acid derived ligands. The AhR activation leads to the upregulation of CYP1A1 and Cox-2 which in their pathways produce arachidonic acid derived eicosanoids. These eicosanoids can bind to the AhR creating a positive feedback loop[32].

It is also known that the binding to the AhR of tryptophan metabolites can lead to increased amounts of IDO enzymes. These enzymes can then degrade tryptophan at a higher rate thereby creating a positive feedback loop[24].

New ligands for the AhR could be a misinterpretation due to the serotonin pathway

Serotonin is a derivative of tryptophan and abundant in the intestine. Serotonin itself is a very weak AhR activator but its downstream pathway can activate the CYP1A1, 1A2 and 1B1 enzymes. Serotonin metabolism leads to formation of melatonin and eventually 6-hydroxymelatonin, a process performed by the CYP enzymes. This pathway leads to a fast increase of CYP enzymes and consequently a fast decrease in tryptophan metabolites and depletion of AhR ligands, also named a metabolic sink. Because of the abundance of serotonin and the constant activation of this pathway, the effects of AhR ligands are placed into another perspective. Quintana *et al.* doubt if the effects that are seen after the administration of an AhR binding compound are in fact due to higher AhR activity or if this effect is seen due to the blocking of this metabolic sink[3]. How this would work is not yet known but certain drugs, such as serotonin antagonists against nausea and intestinal irritation, could have this effect[34]. The blockage of the metabolic sink would lead to a decrease in CYP enzymes and thereby abundant tryptophan metabolites, causing a higher AhR activity. This would mean that the positive effects after a ligand candidate administration could lead to falsely naming a compound an AhR ligand[3].

AhR as therapy difficult to modulate due to cross talk between pathways

As described earlier there is a large subset of exogenous ligands that can activate the AhR. This would suggest that in the case of a therapeutic approach these ligands would make perfect candidates. Still, after the point of activation of the AhR a large part of the knowledge about the mechanism is missing. The AhR can activate different XRE and DRE elements of which most are not even verified yet[5], but the AhR can also crosstalk with other pathways such as the NF-kB pathway and activate various processes in the cell[35]. Besides the fact that we do not know which processes are exactly active when ligands bind, it is now suggested that binding of different ligands leads to the activation of different processes although it is the same receptor[3]. This would mean that the binding of a trp-derived ligand activates other pathways than when a flavonoid type ligand binds. Knowing this you could conclude that the AhR as a therapeutic target is not a safe option yet. We may now know which ligands bind the AhR and we can see if this is beneficial or not, but as long as we cannot completely verify which ligands activates which pathway or process, AhR therapy could not have the projected effects. This is also backed by the appearance of feedback loops described above.

Does our food contain 'enough' ligands

It has been shown that food can cause the activation of the AhR. This link has mainly been found *in vitro*, *in vivo* it is hard to prove whether the beneficial effects for the animal were due to the diet they were given together with the activation of the AhR. Also, in most of these experiments the diet contained a very high amount of a certain ligand source, for instance broccoli supplements. It is doubtable that humans can get the same beneficial effects from eating the proposed amount of healthy foods[36]. If the link between food and the AhR can be confirmed then maybe supplements for human use could be a good therapeutic agent.

It is also suggested that the activation of the AhR by exogenous ligands can kickstart the production of endogenous ligands thereby starting a signaling cascade[3]. This would mean that a high concentration of ligands in the food is not necessary to create a positive effect.

Conclusion

The microbiome and intestine have always had a delicate balance in which the bacteria that live in our intestines do not harm our bodies. Many species such as *Candida* and *Lactobacillus* normally are commensal in the human gut, however, weak mucosal immunity can give room for these bacteria and fungi to shift to parasitism. Through AhR activation this mucosal immunity against the microbiome can be maintained as the microbiota-AhR axis has shown. Upon activation of the AhR, cells of the innate immune system secrete IL-22, activating Paneth cells to secrete defensins. But the activation of the AhR can also maintain the cells of the adaptive immune system and send certain subsets of immune cells into further development, hereby increasing the mucosal immunity and protecting our bodies from harmful infections.

To activate the AhR, a pool of ligands has been described of which the main ones, the kynurenines from the IDO pathway, were the most well-known. To demonstrate that there are other AhR ligands besides Trp derivates from IDO metabolism, Zelante *et al.* used $Ido^{-/-}$ mice that lack the enzyme to transfer Trp into kynurenine. They expected to find a decrease in IL-22 secretion, meaning that the mucosal immunity would be impaired. The result was opposite showing a steady excretion of IL-22. This meant that the AhR can be activated by other ligands, for instance from food, maintaining the mucosal immunity especially against fungi such as *Candida.*

Besides the increase of AhR ligands through the administration of food, adjusting the microbiome could also lead to more AhR ligands and thereby activation of the AhR. Adding certain bacterial strains as a supplement in food could lead to an adjustment in the microbiome, leading to an increase in 'good bacteria', indirectly leading to more AhR ligands.

Both addition of food that is rich in AhR ligands or AhR ligand producing bacterial strains could lead to a higher AhR activity suggesting an improvement in intestinal immunity. Still the direct effects of higher amounts of AhR ligands or bacteria has not been shown. As the underlying pathways still remain a mystery and it is suggested that there could be cross talk between different pathways, therapy would be too hard to modulate as the outcomes cannot be predicted yet.

There seems to be a strong link between the increase of AhR ligands and the increase in intestinal immunity. Fine tuning of the activation of the AhR should be the next step, together with defining the pathways that get activated and checking if activation can be modulated due to abundant ligand administration.

Concluding, the AhR could be a good target for therapy to increase intestinal immunity. However, to be able to use it as a therapy a lot of blanks will need to be filled in.

Suggestions for future research

In my opinion a lot of ground work still has to be done concerning the underlying pathways after AhR activation. The literature suggests activation of certain different pathways but also mentions that cross talk between pathways should not be forgotten.

The three pathways that are mentioned the most are the NF-κB pathway, the mTOR pathway, and AhR's own AhR-ligand-Arnt pathway. Next to the fact that there could be activation of other still unknown pathways, it is also suggested that different ligands activate different pathways. It would be interesting to see which ligand activates which pathway.

Therefore, I would start with an *in vitro* set-up that contains the most cited ligands for the AhR, for instance ʟ-kynurenine, indole-3-carbinol, FICZ en indole-3-aldehyde. As a positive control I would use TCDD. Although it is debated often for not being specific enough, I would detect CYP1A1 as a control for AhR activation. The NF-κB pathway activation can be detected by a specific luciferase assay (there are commercial kits available). mTOR activation can be detected in a specific ELISA (there are also kits available). I would not know which cell line to use but I do know that there is a Hepa1c1c7 cell line that expresses the AhR and that a modified version can be used for luciferase assays. CYP1A1 can be detected by RT-PCR.

In exposure assays wherein the cells are exposed to different amounts of the ligands, I would research which pathways gets activated. This will not be easy but I think this is the beginning. To detect possible cross talk, an inhibitor for the individual pathways could be added to see if the outcome is still the same in these cells.

I would also like to look at FICZ although it is not discussed often as a potential candidate. Wikoff *et al.* showed that it can be detected in the blood stream which would suggest that it could be active at other sites than the skin. FICZ can bind at very low concentrations, so I think it is important to know what effect the binding of FICZ has.

If this set-up would work, you would get an overview of the effects that different ligands have and which pathways they activate. From that point you could start with *in vivo* studies to see if the addition of the proposed ligands improve intestinal health *in vivo*. This study could then be extended with the addition or depletion of certain bacterial strains to see if this has an effect.

The portrayed set-up is of course a simplified version of reality, but I feel this is the basis to start from. Still, if it would work, a lot more could be said about AhR activation through the different subsets of ligands and therapeutic outcomes could be better predicted.

Abbreviations

Abbreviation	Definition
ACE2	Angiotensin converting enzyme 2
AhR	Aryl hydrocarbon receptor
AMP	Antimicrobial peptide
APC	Antigen presenting cells
AraT	Aromatic amino acid transferase
ARNT	Aryl hydrocarbon receptor nuclear translocator
CILP	Common innate lymphoid progenitors
CLP	Common lymphoid progenitors
COX-2	Cyclo oxygenase 2
CP	Cryopatches
CYP (1A1, 1A2, 1B1)	Cytochrome
DIM	3,3'-di-indolylmethane
DRE	Dioxin responsive element
FICZ	6-formylindolo[3,2-b] carbazole
FoxP3	Forkhead box P3
HSP90	Heat shock protein 90
I3C	Indole-3-carbinol
IAld	Indole-3-aldehyde
IDO1/2	Indoleamine 2,3-dioxygenase 1/2
IL- (3,5,17,22,23)	Interleukin
ILC's	Innate lymphoid cells
ILF	Innate lymphoid follicle
LTi	Lymphoid tissue inducer cells
mTOR	mammalian target of rapamycin
NK	Natural Killer cells
PGE2	Prostaglandin 2
PP	Peyer's patches
SMAD1	Mothers Against Decapentaplegic Homolog 1
STAT1	Signal Transducers and Activators of Transcription family 1
TCDD	2,3,7,8-tetrachlorodibenzo-p¬-dioxin
TH17	T helper 17 cells
Tregs	T regulator cells
Trp	Tryptophan
XRE	Xenobiotic responsive element

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