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AHR in the intestinal microenvironment: safeguarding barrier function

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Abstract

Mammalian aryl hydrocarbon receptor (AHR) is a ligand-dependent transcription factor that belongs to the basic helix-loop-helix (bHLH)-PAS family of transcription factors, which are evolutionarily conserved environmental sensors. In the absence of ligands, AHR resides in the cytoplasm in a complex with molecular chaperones such as HSP90, XAP2 and p23. Upon ligand binding, AHR translocates into the nuclear compartment, where it dimerizes with its partner protein, AHR nuclear translocator (ARNT), an obligatory partner for the DNA-binding and functional activity. Historically, AHR had mostly been considered as a key intermediary for the detrimental effects of environmental pollutants on the body. However, following the discovery of AHR-mediated functions in various immune cells, as well as the emergence of non-toxic 'natural' AHR ligands, this view slowly began to change, and the study of AHR-deficient mice revealed a plethora of important beneficial functions linked to AHR activation. This Review focuses on regulation of the AHR pathway and the barrier-protective roles AHR has in haematopoietic, as well as non-haematopoietic, cells within the intestinal microenvironment. It covers the nature of AHR ligands and feedback regulation of the AHR pathway, outlining the currently known physiological functions in immune, epithelial, endothelial and neuronal cells of the intestine.

The existence of a receptor responsible for binding to dioxin was postulated in the 1980s and was finally proven with the cloning of mammalian *Ahr* (which encodes aryl hydrocarbon receptor (AHR)) in 1992 (REF[1]). This spurred a large number of molecular studies on the structure and mode of action of AHR[2] across different species but they remained firmly in the domain of toxicology. The discovery of AHR expression and its consequences in mouse T helper 17 (T_H17) cells[3] and regulatory T (T_{reg}) cells[4] finally propelled AHR into the field

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of immunology and beyond, with an ever-increasing number of publications on the functional implications of AHR activation. The recognition of AHR as a key regulator of homeostatic processes at barrier sites such as the skin, lung and gut[5], in addition to its role in detoxification, led to intensified searches for ‘physiological’ (in contrast to xenobiotic) ligands[6,7]. It became clear that the gastrointestinal tract is a rich source of such ligands, derived from dietary and microbial metabolites (see the section The nature of AHR ligands). Another concept that emerged was the importance of negative-feedback regulation of AHR signalling[8,9]. The existence of negative-feedback mechanisms suggests that curtailment of AHR signalling is physiologically important (see the section Feedback regulation of AHR activity).

This topic brings to the forefront a pervasive problem in the AHR field. The toxicological view of AHR, which tends to emphasize a pathological role in physiology, is at odds with accumulating evidence for the beneficial roles of AHR activation in many cell types and tissues, especially in barrier organs. Notably, many of the paradigms in the field of AHR biology were established in cell lines and might need to be revisited in appropriate animal models that have been generated in the last 5 years, and translation to humans is urgently needed (Box 1).

Although it is generally accepted that AHR activation by xenobiotics can interfere with its physiological functions, the mechanisms underlying this distinction remain unclear[10]. One possibility is that differential binding of xenobiotic versus physiological ligands triggers different downstream signalling cascades. Nonetheless, although differences in ligand binding have been demonstrated in a combination of computational and experimental analyses with purified compounds in vitro[11], it remains to be seen whether this truly translates into differential AHR signalling in vivo. Another possibility is that inadequate metabolic clearance of xenobiotics by cytochrome P450 family 1 (CYP1) enzymes might lead to artificially prolonged AHR activation, and that this consequently interferes with physiological short-term signalling, thereby dysregulating AHR function. As AHR is expressed in a wide range of cell types, with a multitude of potential consequences for various physiological functions, it stands to reason that its activation and regulation need to be tightly controlled to ensure optimal functionality. This is exemplified in particular in the gastrointestinal environment, where AHR expression is prominent in immune cell types, as well as in epithelial, endothelial and neuronal cells. AHR deficiency or inadequate AHR activation has fundamental effects on the survival and function of intestinal immune cells and predisposes to inflammatory disorders as well as inflammation-associated intestinal malignancy. In this Review, we discuss advances in understanding the nature of AHR ligands as well as the important principles underlying regulation of the AHR pathway, and provide an overview of the range of physiological functions involving AHR in different intestinal cell types.

The nature of AHR ligands

The recognition of AHR as a key regulator of homeostatic processes has led to a number of studies focused on identification of ‘physiological’ or ‘natural’ AHR ligands (in contrast to xenobiotic ligands)[6,7,12]. Nevertheless, there remains some controversy in the field as to

whether the identified ligands are true, bona fide AHR ligands. Strictly speaking, only direct binding studies can confidently identify ligands, whereas the induction of an activation signal in reporter cell lines or the expression of target genes in any biological system could be due to indirect or alternative mechanisms[13].

Ligands

Molecules or compounds exhibiting specific binding to a receptor.

'Natural' AHR ligands

Ligands derived from the diet or the gut microbiota or formed endogenously.

Efficacy

Biological effectiveness of the ligand; that is, its ability to induce an active form of the receptor and the degree to which it produces a biological response.

Potency

The concentration or amount of the ligand needed to produce a specific biological effect; that is, the sum of a ligand's affinity and efficacy.

Affinity

The strength with which a ligand binds to its receptor; that is, how well it fits into the ligand-binding pocket.

Studies have shown that molecular characteristics such as size, planarity, polarity and electrostatic properties seem to have large effects on AHR–ligand binding[14]. To date, a large repertoire of chemicals with strikingly divergent structures has been proposed to interact with and activate AHR. In addition to the long list of synthetic xenobiotics, this repertoire includes various naturally occurring compounds derived from the diet, formed endogenously or by microbial action in the gut (reviewed elsewhere[6,15,16]). However, bearing in mind the structural and physicochemical characteristics required for fitting in the ligand-binding pocket, it is more than reasonable to assume that not all of the huge numbers of postulated AHR activators are in fact bona fide ligands for the receptor, as mentioned in the previous section. The definition of a ligand is a molecule that exhibits specific binding to a receptor. However, a capacity to bind to a receptor does not predict the outcome of the binding. That is, whether it generates an agonistic response, rendering the receptor to its active form and thereby causing a biological response, or an antagonistic response, rendering the receptor inactive. Furthermore, the role a ligand might have in AHR signalling and subsequent biological functions is determined by its efficacy and potency[17]; that is, the amount required to produce a defined biological effect. Many compounds postulated as AHR ligands have not been properly evaluated as true agonists, creating confusion in the field. In vitro studies in cell lines have shown that AHR can also be indirectly activated by compounds inhibiting the CYP1-mediated negative regulation of AHR, thereby causing an enhanced receptor signalling, which is wrongly interpreted as an activation[9,18,19]. Furthermore, some postulated ligands are in fact 'pro-ligands' — that is, precursors that are chemically transformed to high-affinity ligands, often via non-enzymatic and spontaneous condensation reactions. An example is kynurenine, a tryptophan-derived metabolite generated by indoleamine 2,3-dioxygenase (IDO) activity, which has commonly been cited as an AHR

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ligand, although kynurenine itself does not have AHR-binding activity. Instead, it is a kynurenine transformation product, a so-called trace-extended aromatic condensation product, that functions as a high-affinity ligand for AHR in reporter luciferase assays[20].

In the gastrointestinal tract, proligands are continuously formed from dietary constituents by microbial metabolism or non-enzymatic condensation reactions[21]. The best-described example of this is the high-affinity ligand indolo[3,2-ĥ]carbazole, which is formed through an acid-catalysed condensation reaction of the diet-derived proligand indole-3-carbinol (I3C) in the stomach[22]. In addition to indolo[3,2-*b*]carbazole, indole and several tryptophan-derived compounds formed by microbial metabolism have been shown to possess AHR-activating properties in vitro, such as indole-3-acetaldehyde, indole-3-pyruvate, indole-3-aldehyde, 2-oxoindole, 3-methylindole, indole-3-ethanol, indole-3-pyruvate and tryptamine[23–26]. Although some have been shown to act as weak-to-medium-affinity AHR ligands, many of these might be transformed into more-potent ligands, such as 6-formylindolo [3,2-*b*]carbazole[27].

With AHR research extending beyond the field of toxicology, the pursuit of endogenous ligands has intensified. To date, there are several suggested ligands, of which the most potent contain an indole structure. Interestingly, AHR activation by some indole compounds, such as indirubin and indigo, shows strong species specificity favouring human AHR[28], whereas the affinity of other indoles, such as 6-formylindolo[3,2-b] carbazole, seems to be evolutionarily conserved[27]. From the findings taken together, there are multiple sources of proligands that might be transformed into AHR agonists. However, it is important to consider not only the potency of a ligand but also its kinetic properties in vivo, such as its uptake, distribution to target organs, metabolism and final excretion. Although there are many studies describing these parameters for xenobiotic ligands, very few of the suggested natural ligands have been studied in such detail, obstructing understanding of their importance and potential physiological functions. These issues as well as potential differences between human and mouse ligand binding, which remain as unresolved, are of importance in consideration of AHR agonists as therapeutic agents. To date, a number of studies have tested either natural or synthetic AHR-activating substances in humans with inflammatory bowel disease (IBD) or in mouse models of colitis. For instance, a randomized, placebo-controlled 8-week trial with indigo naturalis, a traditional Chinese medicine that contains ligands for AHR, showed benefits in patients with ulcerative colitis[29], and synthetic AHR ligands induced IL-22 in intestinal immune cells from patients with IBD in vitro as well as amelioration of colitis symptoms in mice[30,31]. Nevertheless, we predict that concerns about potential toxicity due to aberrant AHR stimulation will hamper the widespread application of AHR agonist therapy.

Feedback regulation of AHR activity

Cellular outcomes of AHR activation are often context dependent, resulting in seemingly contradictory outcomes such as tumour promotion and suppression, induction of proliferation and differentiation, and stimulation and suppression of immunity[32,33]. Although this observation is sometimes ascribed to the timing and dose of ligand exposure or ligand-specific effects[34], it might also reflect differences in the regulation of AHR action.

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To date, three independent mechanisms for negative-feedback regulation of AHR activity have been described: ligand degradation by CYP1 enzymes[8,9,19], repression of AHR transactivation by AHRR[35] and degradation of the receptor protein through induced ribosylation[36] mediated by TIPARP[37] (FIG. 1).

It is generally thought that endogenous ligands are metabolized rapidly and efficiently by AHR-induced CYP1 enzymes, leading to short-duration AHR signalling, whereas many xenobiotics are poor substrates for CYP1 enzymes[38,39], leading to prolonged or even sustained signalling. On the other hand, a paradigm in the toxicology field centres on the CYP1A1-mediated metabolism of xenobiotics such as benzo[*a*]pyrene (BaP) to carcinogenic metabolites, forming DNA adducts in a process termed 'metabolic potentiation'[40,41]. This and other, similar phenomena resulted in the reluctance of the pharmaceutical industry to pursue compounds that trigger the expression of CYP1 enzymes for therapeutic purposes[42].

Interestingly, it appears that inhibition of CYP1 enzymes in a human keratinocyte cell line by a wide variety of substrates can indirectly cause prolonged or enhanced AHR activation to endogenous ligands[9]. This phenomenon might explain why AHR can be activated by a seemingly vast number of compounds that do not have typical ligand structures. The wide availability of AHR ligand sources makes it less probable that AHR activation is limited by ligand availability; tryptophan, a major precursor of AHR ligands, is an essential amino acid, and as such it is required for survival. Instead, metabolism by the three AHR-controlled CYP1 enzymes, CYP1A1, CYP1A2 and CYP1B1, might determine the extent and duration of AHR signalling.

Negative-feedback circuits can coexist within a single cell type, but it is not clear whether all of them are active in all cells where they are expressed. Their inter-connectivity and relative contribution to AHR signalling strength and duration is currently unknown, and it likewise remains unclear whether and why certain modes of inhibition might be more important for one cell type than for another.

Altered function of these regulatory circuits causes adverse effects. For example, knockout of *Cyp1a1* expression in mice causes increased toxicity upon exposure to BaP[41], suggesting that the CYP-mediated generation of secondary toxic metabolites is more of an in vitro phenomenon. Likewise, constitutive expression of *Cyp1a1* has negative consequences, resulting in a phenocopy of AHR deficiency with inability to resolve enteric infection by *Citrobacter rodentium* and impaired intestinal barrier integrity[19,43]. Specific overexpression of *Cyp1a1* in mouse intestinal epithelial cells (IECs) is sufficient to cause these effects, indicating that CYP1 enzymes are central to physiological AHR functions in the gut by regulating local and systemic ligand availability.

Regulation of AHR signalling mediated by AHRR has been described in several in vitro studies with human or animal cell lines[44,45]. AHR suppression by AHRR might result from AHRR dimerizing with AHR nuclear translocator (ARNT) and competing with the AHR–ARNT complex for binding to the AHR response element[45] or by influencing the transcriptional activity at the promoter regions of *Ahr*-responsive genes such as *Cyp1a1* (REF.[44]). Its repressive function in vivo is not well understood but seems to be less

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effective[35]. *Ahrr* is predominantly expressed in immune cells of the skin and intestine of mice, unlike *Cyp1a1*, which is expressed in epithelial cells of these tissues as well as in AHR-expressing immune cells[46–48]. Cells in the intestinal tract are exposed to potentially high concentrations of AHR ligands from the diet and microbial metabolism. A study published in 2020 indicates that induction of *Ahrr* in mouse intestinal immune cells, particularly myeloid cells, is more prominent in response to dietary rather than microbial ligands in vivo[49]. *Ahrr* basal expression in mice seems to be negatively correlated with chemical inducibility of *Cyp1a1* (REF.[50]). This observation was confirmed also in *Ahrr* reporter mice, in which cells with high expression of the reporter had low *Cyp1a1* expression[46]. *Ahrr* depletion causes induction of *Cyp1a1* expression in a tissue-specific manner in *Ahrr*-knockout mice[51], although mRNA expression of *Cyp1a1* does not necessarily coincide with enzymatic activity[52]. This observation suggests that perhaps only one of these negative-feedback systems is active in any one type of cell. AHRR prevents excessive proinflammatory signalling in the intestine of mice in homeostatic conditions, whereas it enhances production of proinflammatory interferon-γ (IFNγ) in the inflamed gut[46], indicating context-specific proinflammatory and anti-inflammatory functions.

Of the three regulatory pathways, *Tiparp* is the latest to be described and consequently the least studied. Ligand-dependent activation of AHR sets in motion the proteolytic degradation of the receptor protein[36] involving TIPARP-mediated ribosylation. This process was initially demonstrated in vitro[37], but later studies in vivo showed that Tiparp-knockout mice have increased sensitivity to dioxin-induced wasting syndrome, steatohepatitis and lethality[53,54] and impaired AHR-regulated type I interferon-mediated antiviral innate defence[55]. Its role in the intestine is, however, still unknown.

Altogether, the cell specificity and differences in the inducibility of these regulatory circuits strongly indicate that each mode of feedback control can differentially affect AHR signalling in response to ligands.

AHR in the intestinal microenvironment

Since the discovery of *Ahr* expression in T_H17 cells and T_{reg} cells, there has been continued focus on the role of AHR in immune cells throughout the body, including the intestine. In this section, we summarize findings in the immune compartment from the past 3 years, but otherwise focus specifically on less well-known or emerging roles of AHR in non-haematopoietic cell types (FIG. 2).

Immune cells

The intestinal immune compartment is specialized in immunosurveillance of the intestinal epithelial barrier and the prevention of systemic microbial transgression. Simultaneously, it also has a critical role in preserving tissue homeostasis through the formation of immunoregulatory networks that function to prevent pathophysiological interactions between the host and the microbiota[56]. Various immune populations in the intestine depend on AHR signalling for their postnatal maintenance and function.

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One example is intraepithelial lymphocytes[57]. A rare subset of intraepithelial lymphocytes termed 'doublepositive $CD4^{+}CD8\alpha\alpha^{+}$ intraepithelial lymphocytes', which are found only in mice colonized with *Lactobacillus reuteri*, require *Ahr* not only for their survival but also for their generation. *L. reuteri* expresses high levels of aromatic aminotransferase, which converts L-tryptophan into AHR activators and in conjunction with transforming growth factor-β (TGFβ) drives reprogramming of CD4+ T cells into double-positive intraepithelial lymphocytes in the gut[58]. There is also evidence that AHR ligands can influence intraepithelial lymphocyte activity by promoting the upregulation of IL-10 and downregulation of IFNγ in colitic mice, suggesting that *Ahr* is important for balancing the immunoregulatory and defence roles of intraepithelial lymphocytes[59].

Innate lymphoid cells (ILCs) resident in the lamina propria of barrier sites are key early responders in the initiation of inflammatory responses, and their established role in preservation of intestinal homeostasis has made ILCs (particularly group 3 ILCs (ILC3s)) a significant subject of interest in the field of AHR immunity[60,61]. AHR activation is required for the maintenance of IL-22+ ILC3s in the first 2–3 weeks after birth, which is likely acquired from maternal AHR ligands derived from microbial metabolism of tryptophan[62]. They are transferred to offspring either during development, partially aided by maternal antibodies via the maternal gut–placenta–fetus axis, or postnatally through milk[62]. After weaning, ILC3s are largely sustained by AHR ligands derived from dietary and microbial proligands, as shown in mouse studies with different diets[19,63]. Decreased AHR signalling in ILC3s can cause alterations in the balance between ILC3 and ILC1 populations and promote the development or persistence of colitis. For example, patients with active Crohn's disease exhibit conversion of ILC3 to ILC1 due to decreased AHR signalling in ILC3s, resulting in a phenotypic shift to an ILC1-like profile, thereby exacerbating inflammation and pathology[64,65]. The reduction of AHR signalling could be due to reduced availability of AHR ligands, but another explanation could be the high expression of miR-124, a microRNA known to inhibit *Ahr* in colonic tissues of patients with active Crohn's disease[66].

A 2018 study highlighted that ILC2s also express high levels of *Ahr*. *Ahr* expression by ILC2s is intestine specific and is mediated by cooperative action between AHR and GFI1, as ILC2s from GFI1-deficient mice exhibit reduced AHR transcription[67]. In contrast to ILC3s, the levels of ILC2s are increased in AHR-deficient mice, suggesting that AHR mediates the balance between ILC subsets through negative regulation of key ILC2 genes such as *ST2*, which encodes a receptor for IL-33 that is required for ILC2 expansion and production of the type 2 cytokines IL-5 and IL-13 (REF[67]). Consequently, loss of *Ahr* in ILC2s resulted in an increased capacity to mount an antihelminth response.

The hallmark cytokine for ILC3s, IL-22, is crucial for the maintenance of IECs and defence against pathogens via IL-22-induced secretion of antimicrobial peptides by IECs (for example, RegIIIβ and RegIII*γ*)[68]. This cytokine is also produced by the T_H17 cell subset of CD4+ T cells. *Ahr* expression was first demonstrated in the T_H17 cell subset[3] and in T_{reg} cells[4] of mice. *Ahr* drives the expression of IL-22 in T_h17 cells but is not required for their differentiation[3]. Mice lacking *Ahr* expression in T cells are highly susceptible to infection with *C. rodentium* [19,69] as early IL-22 production by ILC3s is not sufficient to counteract the intestinal epithelial damage incurred over the course of this infection[69]. During infection

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with pathogens such as *Nippostrongylus brasiliensis* or *Staphylococcus aureus*, which trigger self-limiting inflammatory responses, intestinal T_H17 cells can be converted into anti-inflammatory, IL-10-producing T_{reg} type 1 (T_R1) cells that no longer express IL-17A in a process that depends on coordinated action between AHR and TGFβ signalling. This suggests that *Ahr* might be important in modulating T_H17 cell function by restraining its conversion towards a T_H1 cell-like profile and instead promoting the transition of T_H17 cells into a T_R1 cell-like state[70].

FOXP3$^+$ T_{reg} cells are critical for suppression of tissue inflammation through their release of canonical cytokines such as TGFβ and IL-10 (REF[71]). A large population of tissue-resident T_{reg} cells induced in response to dietary antigens and the microbiota resides in the intestine[72,73]. The role of *Ahr* in T_{reg} cell differentiation and function is controversial owing to conflicting data on the precise mechanism by which AHR influences these processes[4,74,75]. On the whole, *Ahr* expression in T_{reg} cells found in the spleen and lymph nodes is very low, whereas *Ahr* is highly expressed in intestinal T_{reg} cells[75], and cell-specific loss of *Ahr* in T_{reg} cells reduces their numbers in the intestine of mice. This observation is due to impaired intestinal homing as a result of reduced expression of key intestinal-homing genes, such as those encoding GPR15 (REF.[76]), CD103 and CCR6, rather than proliferation or survival defects[75]. Furthermore, AHR-deficient T_{reg} cells exhibit a T_H1 cell-like expression profile (for example, increased IFNγ and IL-17 expression) and consequently have a diminished capacity to suppress inflammation in a T cell transfer mouse model of colitis[75].

There are few studies on the roles of AHR in other immune cell types in the gut, such as B cells or myeloid cells. Various studies on the therapeutic value of AHR ligands in colitis report alterations in the recruitment and activation of myeloid cells, such as neutrophils and dendritic cells. For example, supplementation with the AHR proligand indole-3-pyruvic acid limited colonic inflammation in a T cell transfer mouse model of colitis as indole-3-pyruvic acid increased the number of CD103$^+$ dendritic cells at the cost of the number of CD103$^-$ dendritic cells, which are known to induce IFNγ production in CD4$^+$ T cells[59]. In vitro studies showed that AHR can influence monocyte fate, driving differentiation of monocyte-derived dendritic cells over monocyte-derived macrophages by regulating interferon regulatory factor 4 (IRF4; which drives dendritic cell commitment) in the presence of IL-4, suggesting that AHR is a key driver of macrophage–dendritic cell balance in inflamed tissues, with key consequences for disease outcomes[77]. Lastly, neutrophils recruited in the intestine in a mouse T cell-driven colitis model can respond to IL-23 signalling with upregulation of the gene encoding RORγt and *Ahr* and subsequent generation of IL-22 (REF[78]).

Intestinal epithelial cells

The intestinal epithelial barrier is the primary site of contact with AHR ligands derived from the diet or microbial metabolism. *Ahr* is widely expressed in the intestinal epithelium, with a gradient of expression from the proximal to the distal regions. The highest expression is found in the upper small intestine adjacent to the stomach of mice[79], where dietary AHR ligands are transformed from proligands into active ligands[22]. Ligand-induced AHR

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activation and induction of CYP1A1 and CYP1B1 results in metabolic clearance of ligands, which is a key regulatory step for ligand distribution throughout the body[19,80]. Constitutive expression of *Cyp1a1* in IECs of mice results in an AHR-deficiency phenotype[19]. This effect is reversible upon dietary supplementation of the AHR proligand I3C[19], indicating that the AHR-deficiency phenotype is caused by excessive ligand metabolism. Importantly, constitutive *Cyp1a1* expression in IECs also affects intestinal immune cells that rely on AHR ligands for their survival and function[19]. Thus, IECs can be considered ‘gatekeepers’ for the supply of ligands systemically. This notion is in keeping with an older study showing that IEC-specific deletion of the cofactor *Arnt* in mice resulted in widespread upregulation of *Cyp1a1* throughout the body except the gut, due to an overabundance of AHR ligands that were not metabolized in the absence of *Ahr*-mediated *Cyp1a1* upregulation[80].

AHR also has distinct, cell-intrinsic roles in IECs, primarily in stem cells. The intestinal epithelium undergoes constant renewal, sourced by stem cells that reside in the intestinal crypt[81]. The activity of crypt stem cells is largely dependent on the tightly regulated integration of signals emanating from signalling pathways such as WNT and NOTCH[82]. Dysregulation of these key pathways, as in the case of WNT signalling through mutations in the *APC* gene, is a hallmark of intestinal cancers[83]. Studies have demonstrated a role for AHR signalling in the regulation of stem cell differentiation in various tissues and contexts, such as haematopoietic stem cells, embryonic stem cells or stem cells in the upper respiratory tract[84–86]. Furthermore, AHR also functions as a tumour suppressor in cancer stem cells, such as leukaemic stem cells, in which AHR activation suppresses self-renewal and promotes differentiation in human leukaemic cells in vitro[87]. Early in vitro studies demonstrated an intersection between AHR and WNT signalling, providing evidence that AHR could function as part of an E3 ubiquitin ligase complex that initiates ubiquitylation of β-catenin and consequently its degradation[88,89]. More recently, AHR deficiency in IECs was shown to dysregulate the WNT–β-catenin pathway upstream of β-catenin degradation, as the lack of *Ahr* reduced transcription of the genes encoding two related E3 ubiquitin ligases (*Znrf3* and *Rnf43)* that target WNT–Frizzled receptors for degradation[90]. The loss of *Ahr* in mouse IECs resulted in unrestricted intestinal stem cell proliferation and impaired differentiation, rendering IECs vulnerable to malignant transformation[43]. In addition, regenerative differentiation following infection with *C. rodentium* was found to be compromised. *C. rodentium* infection causes a transient loss of mature IECs such as goblet cells in wild-type mice, but AHR deficiency in IECs results in prolonged and extensive failure of differentiation, which causes increased lethality in this infection[43]. Furthermore, *Ahr* is required for IDO1-mediated promotion of secretory cell differentiation in the mouse intestine through repression of NOTCH signalling[91]. *Ahr* is also involved in regulation of tight junctions, which are disrupted in chronic inflammatory intestinal diseases, leading to persistent activation of lamina propria immune cells by translocating microorganisms[92]. Administration of the AHR ligand 6-formylindolo[3,2-b]carbazole attenuated the loss of tight junction proteins in a dextran sulfate sodium (DSS) colitis mouse model[92]; however, whether this was an epithelial-intrinsic effect or was due to increased cytokine production by immune cells was not tested. The intersection of the *Ahr* and nuclear factor erythroid 2-related factor 2 gene (*Nrf2*) pathways was involved in upregulation of tight junction proteins such as claudin 4 and occludin by the antioxidant urolithin A[93].

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Thus, activation of the AHR pathway in IECs and its effect on the regulation of stem cell differentiation are crucial components of barrier integrity (FIG. 3).

Endothelial cells

One of the most prominent phenotypes in *Ahr*-null mice is patent ductus venosus, a condition characterized by portosystemic shunting in the liver resulting in decreased liver weight as well as high numbers of the predominant vessel of the fetal liver, anastomotic sinusoids, which persists to adulthood in *Ahr*-null mice due to defective maturation of the hepatic portal vein in the absence of *Ahr* expression in endothelial cells[94,95]. The persistence of fetal-like vascular structures is not restricted to the liver, as they are also retained in the eyes and kidneys of *Ahr*-null mice, suggesting that these findings might have relevance in other vascular organs, such as the intestine. Collectively, these early findings provide evidence for *Ahr* as an important sensor for factors that regulate the transition and maturation of fetal vasculature into adult vasculature. Furthermore, endothelial cell-specific loss of *Ahr* was shown to cause hypotension due to reduced responsiveness to angiotensin II, resulting in decreased ability to regulate vascular tone in a mouse model[96]. Given that approximately one third of cardiac output is redirected towards the gastrointestinal tract, and given that the mesenteric vasculature is particularly sensitive to angiotensin II, loss of angiotensin II responsiveness in the intestine could lead to hypoperfusion, resulting in deleterious consequences for intestinal barrier function[97]. Laminar flow has a profound effect on the vascular phenotype, with prolonged shear stress leading to cell-cycle arrest, stabilization of vessels and decreased inflammatory responsiveness, which are traits of quiescent endothelial cells[98,99]. Studies on human and mouse endothelial cells in vitro demonstrate the involvement of shear stress in the activation of AHR signalling in endothelial cells[100–102]. AHR regulates shear stress-responsive genes in response to physiological shear stress, independent of mechanosensing[100]. Collectively, these findings suggest that AHR regulates various facets of endothelial biology from development to function.

Although no studies have specifically examined the role of *Ahr* in the intestinal vasculature, endothelial cells in this intestinal tissue of mice express high levels of CYP1A1, similarly to adult lung endothelial cells, and are receptive to further activation upon addition of ligands (B.S. and K.S., unpublished observations). Thus, *Ahr* in endothelial cells could potentially influence intestinal repair and regeneration upon injury, in which proinflammatory and angiogenic cues released by cells such as epithelial and immune cells at the site of injury can trigger adult endothelial cells to exit quiescence and switch to an angiogenic state[103,104]. However, the functional significance of active AHR signalling for intestinal vascular homeostasis in general or, vascular remodelling and regeneration associated with the wound healing process, is unknown[105–107]. Excessive angiogenesis is associated with dysregulated repair responses, as seen in chronic inflammatory disorders such as psoriasis and colitis or IBD[107]. In psoriasis, angiogenesis is a major driver of pathology, and a mouse study has shown that loss of *Ahr* in cutaneous endothelial cells in this context leads to enhanced tissue inflammation with marked neutrophilia and epidermal acanthosis[108]. In IBD, microvascular endothelial dysfunction is proangiogenic and proinflammatory, but the role of *Ahr* in endothelial cells in this context has not been investigated. Given the link between *Ahr* and

IBD protection, and growing evidence for *Ahr* in regulating vascular homeostasis, the function of *Ahr in* endothelial cells warrants further study.

Enteric nervous system

The enteric nervous system (ENS) consists of different neuronal and glial subtypes in the intestine. Collectively, these fine-tuned neural circuits are critical for regulating various processes within the gastrointestinal tract, such as blood flow, fluid exchange and peristalsis. In addition, enteric glia and neurons also have critical roles in the activation of AHR-regulated immune cells, such as ILC3s and ILC2s[109,110].

Although many studies over the past decade have addressed the role of AHR in neuronal development of both invertebrates and vertebrates[111], *Ahr* expression and function in the ENS has been identified only in the past 2 years. A study identifying transcription factors involved in ENS development found that AHR was expressed in the colonic ENS of mice between embryonic day 15 and embryonic day 18 (REF[112]); however, the functional importance of its emergence in the late stages of embryonic development remained unclear. In adult mice, sustained expression of *Ahr* in the ENS was found to be microbiota dependent and restricted to colonic neurons[113].

The gut microbiota has been identified as a key driver of ENS maturation[114,115]. Compared with specificpathogen-free mice, germ-free mice have a larger proportion of nestin-postive neural precursors in the intestine, correlating with functional abnormalities within the ENS such as delayed intestinal motility, which reflects the immaturity of the ENS in axenic conditions[115]. Delayed transit time leads to adverse consequences for intestinal homeostasis, leading to complications such as bacterial overgrowth and chronic constipation in patients[116]. Microbial-derived metabolites promote serotonin (also known as 5-HT) biosynthesis from enterochromaffin cells in the colon, and studies demonstrated that peristaltic defects in germ-free mice were due to decreased 5-HT levels[117]. As 5-HT has been shown to influence AHR activity in human IEC lines by augmenting AHR induction of *Cyp1a1* and consequently the clearance of AHR ligands[118], it is possible that this synergistic interaction might similarly occur in colonic neurons. Loss of *Ahr* in ENS neurons of mice results in delayed colonic transit, suggesting that microbiota-driven regulation of peristaltic activity in the colon is driven to some extent by ligand-dependent AHR activation[113]. Promotion of peristalsis was attributed in part to *Ahr*-mediated transcriptional upregulation of KCNJ12, a lipid-gated ion channel, which encodes the primary subunit for IK1. IK1 is responsible for stabilizing the resting membrane potential and driving electro-physiological maturation of electroconductive cells such as neurons and cardiomyocytes[119].

Although *Ahr* has been identified as a regulator of peristatic activity under homeostatic conditions, its function in ENS regeneration upon injury has yet to be explored. Central nervous system regeneration is controversial in humans[120], but the ENS retains regenerative potential due to retention and renewal of neural precursors throughout adulthood as shown in rats[121]. Indeed, isolation and transplantation of these precursors is currently being explored as an option for use in spinal-cord regeneration and for ENS disorders such as Hirschsprung disease[122]. The role of AHR in regulating regenerative responses in the intestinal barrier,

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and its role in driving neurogenesis in the central nervous system[123], suggests that AHR might also have relevance in the regeneration of the ENS after injury.

Intestinal inflammation and CRC

The role of *Ahr* in tumorigenesis remains ambiguous, with conflicting reports of *Ahr* functioning as either a driver of tumorigenesis or a tumour suppressor[33,124]. *Ahr* is associated with tumorigenesis in the lung due to activation by substances such as BaP in tobacco smoke, which is then metabolized by CYP1A1 to DNA-damaging epoxides in human cell lines[125]. However, as outlined earlier, it is debatable whether this process constitutes a general carcinogenic mechanism of AHR activation. Later studies using mice with CYP1 gene deletions provided evidence that the generation of carcinogenic BaP metabolites might be more of an in vitro phenomenon, whereas detoxification by CYP1 enzymes is the overriding *Ahr*-dependent function in vivo[42]. Furthermore, transgenic overexpression of constitutively active Ahr[126] leads to development of stomach and liver cancers in mice[127,128]; however, transgenic whole-body *Ahr* overexpression is artefactual, and no mechanistic insight into the formation of tumours was presented. In addition, a detrimental role for *Ahr* is frequently extrapolated from findings of increased *Ahr* expression and/or activity in advanced tumours or from correlative data from cell lines without clear mechanistic insight[33].

The role of *Ahr* as a tumour suppressor in colorectal cancer (CRC) is gaining consensus[129–131]. The protective roles of *Ahr* in this context are in part due to its influence on the mucosal immune system, which it shifts towards a non-inflammatory state. There are clear connections between chronic inflammation and intestinal tumorigenesis, as well as pronounced lifestyle associations such as nutrition[132–136]. Although mice that lack *Ahr* systemically appear overall healthy if kept under strict specific-pathogen-free conditions, they have intestinal inflammation, often manifesting itself as rectal prolapse, more frequently than *Ahr* wild type littermates[137]. AHR-deficient mice show increased serum levels of IL-6 in advanced age, which is indicative of chronic inflammation[43], and are very susceptible to unresolved intestinal inflammation in models such as DSS colitis or infection with the intestinal pathogen *C.* rodentium[19,69,138]. Furthermore, intestinal inflammation associated with knockout of *Card9*, a susceptibility gene for IBD, alters the composition of the gut microbiota and reduces AHR ligand availability through depletion of tryptophan-metabolizing bacteria such as *Lactobacillus reuteri*, resulting in increased severity of disease in a mouse model of colitis[63]. AHR activation by tryptophan metabolites might also be involved in upregulation of IL-10R on IECs in a DSS mouse model of colitis[139].

The cytokine IL-22 has a complex role in CRC[140]. It is linked to maintenance of barrier integrity (as discussed earlier) and the prevention of CRC development[141] in a mouse model of inflammation-driven carcinogenesis (induced by azoxymethane–DSS), in which the absence of IL-22 signalling in IECs resulted in the continued survival of cells carrying mutations that initiated tumour growth. On the other hand, IL-22 activity in the colon was also associated with tumour progression as a mediator of the inflammatory trigger required for perpetuation of CRC[142]. Furthermore, a single-nucleotide polymorphism in the human *IL22* gene locus is associated with a 50% increase in colon cancer incidence in heterozygous

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carriers[143]. Although the increased vulnerability of AHR-deficient mice in the gut is often attributed to effects on IL-22, data from cell type-specific deletion of *Ahr* indicate that AHR has critical functions not just in intestinal immune cells but also in epithelial cells themselves[19,43]. *Ahr*-induced expression of *Cyp1a1* in IECs determines how much ligand is accessible to the intestinal immune system, and AHR deficiency restricted to IECs results in similar susceptibility to colitis and tumorigenesis as complete *Ahr* deletion, even though *Ahr* expression in the immune system (and the availability of IL-22) is not affected in this case[19]. A study on rationally designed heterocyclic AHR agonists that were characterized to show drug-like properties and favourable pharmacokinetics described beneficial effects in a mouse model of DSS colitis, with induction of IL-22 in mouse T cells and in T cells from patients with IBD[31]. Although the readout for AHR activity was restricted to IL-22 induction, the study nevertheless further emphasizes that the AHR pathway represents a promising therapeutic target in intestinal inflammatory disorders.

Thus, the intestinal barrier is a frontier for manifestation of symptoms due to AHR deficiency. As the state of the gut barrier has profound influences on the whole organism and AHR ligands are systemically distributed, it is likely that AHR deficiency in the gut affects other organs in the body (FIG. 4). Examples of this are activation of type I interferon signalling in astrocytes by AHR ligands derived from microbial metabolism of dietary tryptophan, resulting in reduced experimental autoimmune encephalomyelitis-driven central nervous system inflammation[144], or the limitation of systemic lupus erythematosus (SLE) pathology in SLE-prone mice by dietary supplementation of I3C, which resulted in TLR9-dependent AHR activation and acquisition of a tolerogenic phenotype in myeloid cells[145]. Furthermore, upholding barrier integrity in the intestine can limit development of atherosclerosis through limiting the growth of a proatherogenic microbiota[146], and the gut microbiota-derived short-chain fatty acid butyrate supports growth of microorganisms capable of tryptophan metabolism and production of 5-hydroxyindoleacetic acid, which drives AHR activity in B cells to promote regulatory B cell differentiation and suppression of inflammation in arthritis[147]. Impaired production of AHR ligands by the gut microbiota is an important factor in metabolic syndrome, and supplementation with AHR ligand-producing strains reduces both dietary-induced and genetically driven dysfunction in glucose metabolism and liver steatosis, as shown in faecal samples of humans with metabolic disorders as well as in mouse models[148]. It has become increasingly clear that the status of the gut microbiota and the availability of its metabolites, which are derived from dietary substrates, have fundamental roles in preserving the integrity of the mucosal environment and the prevention of local intestinal, as well as systemic, inflammation[149]. Among the many metabolites that contribute to intestinal health are those that stimulate AHR to provide its critical functions throughout the body.

Conclusions and future strategies

The AHR pathway has an important and beneficial role in the intestinal microenvironment.

Under homeostatic conditions, an adequate diet rich in indole components derived from vegetables, as well as a balanced gut microbiota containing tryptophan-metabolizing species, safeguards and promotes AHR function in a way that supports intestinal health on multiple

levels. However, it seems that AHR has an even more important role following injury and during regenerative processes. Although the effects of AHR on immune cells have received substantial attention in the past few years, in this Review we have highlighted that AHR plays important roles in other cell types, notably epithelial cells, endothelial cells and enteric neurons, which warrants further investigation. These findings are dependent on the use of genetically modified mouse strains with cell type-specific deletion of *Ahr*, as widespread expression of *Ahr* makes functional defects in fully AHR-deficient mice difficult to interpret. Moreover, the use of dietary interventions to increase availability of AHR ligands might be a useful tool to explore the physiological consequences of enhanced AHR activation. Indeed, dietary supplementation with AHR proligands such as I3C is very effective in mouse models of inflammation[19,43,150] and cancer[129,151,152]. On the other hand, many studies have also emphasized the role of the gut microbiota in the provision of AHR ligands[6,26,63]. As the gut microbiota and diet are interlinked, it remains to be determined whether a diet promoting physiological AHR activation influences the representation of tryptophan-metabolizing bacteria that can generate AHR ligands in the gut flora (Box 1).

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Key points

- Aryl hydrocarbon receptor (AHR) is widely expressed in the intestinal microenvironment, and its activation by natural ligands results in barrier-protective effects.
- In addition to well-documented functions in intestinal immune cells, AHR has important and less well-studied functions in epithelial and endothelial cells as well as in neurons of the enteric nervous system.
- AHR involvement can appear subtle under homeostatic conditions but becomes prominent in regeneration following tissue injury.
- AHR acts as a tumour suppressor for development of colonic malignancy.
- New models and tools enabling the dissection of cell type-specific and tissue type-specific AHR functions in vivo are needed to understand the effect of environmental signals on inflammation and tissue repair in the gastrointestinal barrier.

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Box 1

Open questions or research needs

- What is the molecular basis for differential outcomes of aryl hydrocarbon receptor (AHR) activation by xenobiotics versus natural ligands?
- What are the relative contributions of dietary versus gut microbiota-derived AHR ligands?
- Are natural ligands (as opposed to xenobiotic) safe or do they have a toxicity threshold?
- Integration of cell type-specific effects of AHR in vivo.
- Role of AHR in endothelial cells and the enteric nervous system in vivo.
- Generation of tools that enable functional analysis of AHR in whole-animal models rather than merely in cell lines.
- Translation of findings in animal models to humans.

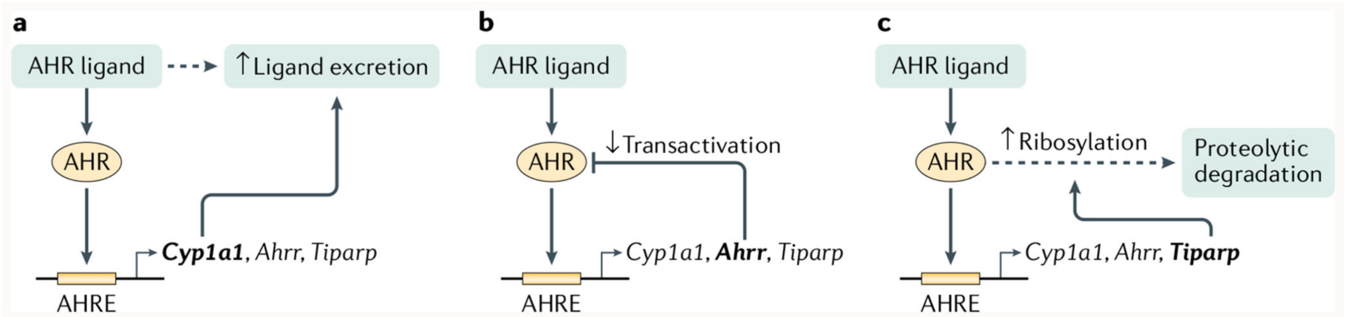

Fig. 1. Mechanisms for negative-feedback regulation of AHR activity.

a | Cytochrome P450 family 1 (CYP1)-catalysed degradation of aryl hydrocarbon receptor (AHR)-activating ligand. **b** | Repression of AHR transactivation by AHR repressor (AHRR). **c** | Induced degradation of AHR through 2,3,7,8-tetrachlorodibenzo-*p*-dioxin poly(ADP-ribose) polymerase (TIPARP)-mediated ribosylation. AHRE, AHR response element (DNA-binding site for AHR).

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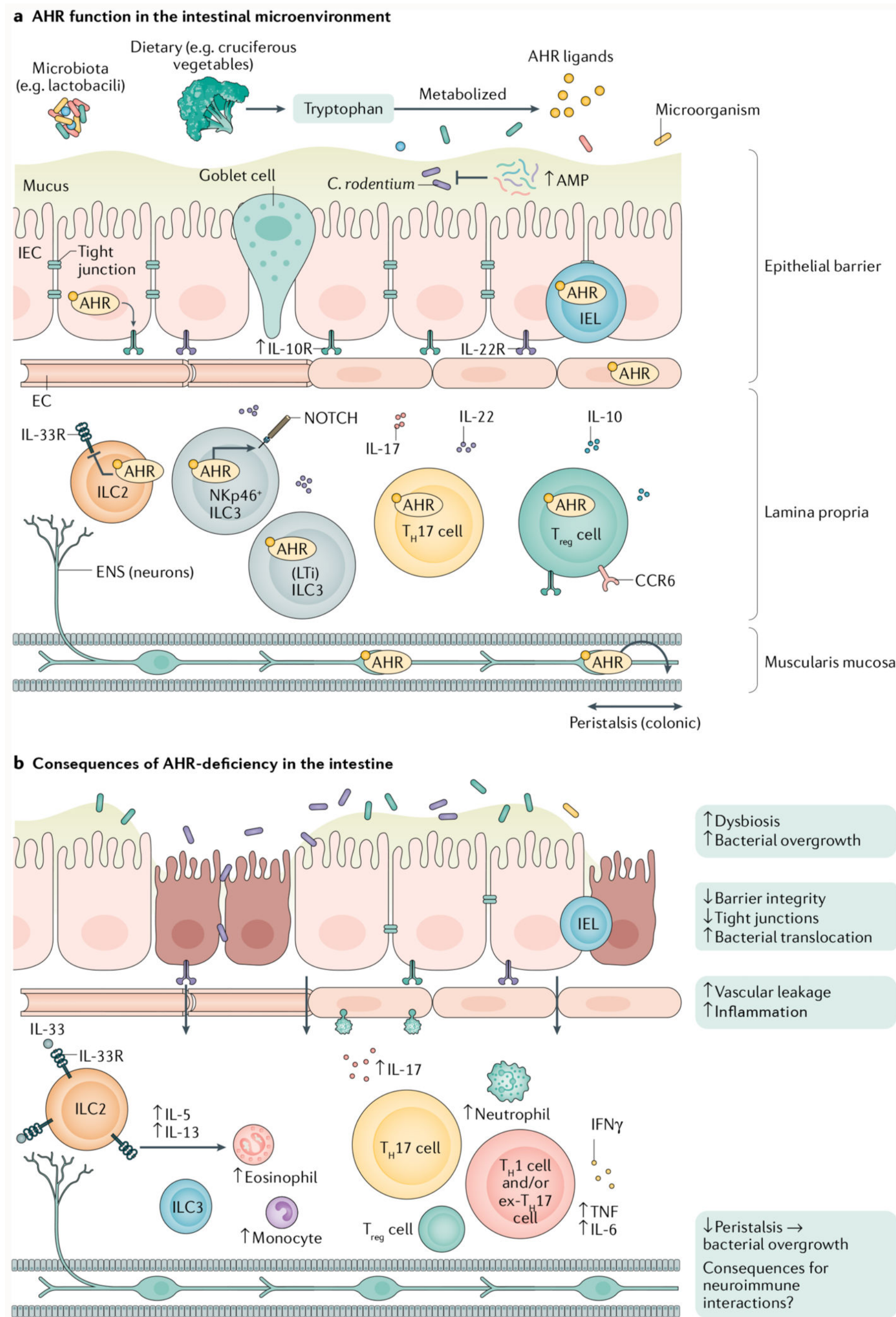

Fig. 2. AHR in the intestinal microenvironment.

a | Under homeostatic conditions, ligand-dependent aryl hydrocarbon receptor (AHR) signalling via dietary or microbiota-derived ligands in various immune and non-haematopoietic cells is critical for the preservation of intestinal barrier integrity and function via secretion of IL-22, induction of IL-10R, strengthening of tight junctions and effects on colonic neurons. Several cell types depend on AHR signalling either for their survival (intraepithelial lymphocytes (IELs) and group 3 innate lymphoid cells (ILC3s)) or for their functional activity (T helper 17 (T_H17) cells, regulatory T (T_{reg}) cells and ILC2s). IL-22 is

produced mainly by ILC3s under steady-state conditions and induces secretion of antimicrobial peptides by intestinal epithelial cells (IECs) for barrier protection. Following infection with pathogens such as *Citrobacter rodentium*, T_H17 cells take over from ILC3s as major producers of IL-22. T_{reg} cells will balance the inflammatory response via secretion of IL-10. Expression of AHR in colonic neurons supports motility, which increases gut microbiota density. **b** | AHR deficiency leads to perturbation of ILC balance, an increase in the numbers of eosinophils and neutrophils and a decrease in the numbers of ILC3s and IELs. This leads to a lack of IL-22, increased levels of proinflammatory cytokines (tumour necrosis factor (TNF), IL-6, IL-17 and interferon-γ (IFNγ)), vascular leakage, reduced mucus layer and disruption of the barrier (impairment of tight junctions). Lack of AHR in colonic neurons negatively affects gut motility (peristalsis) and leads to overgrowth of intestinal bacteria. EC, endothelial cell; ENS, enteric nervous system; ex-T_H17 cell, T_H17 cell secreting IFNγ due to plasticity; LTi, lymphoid tissue inducer.

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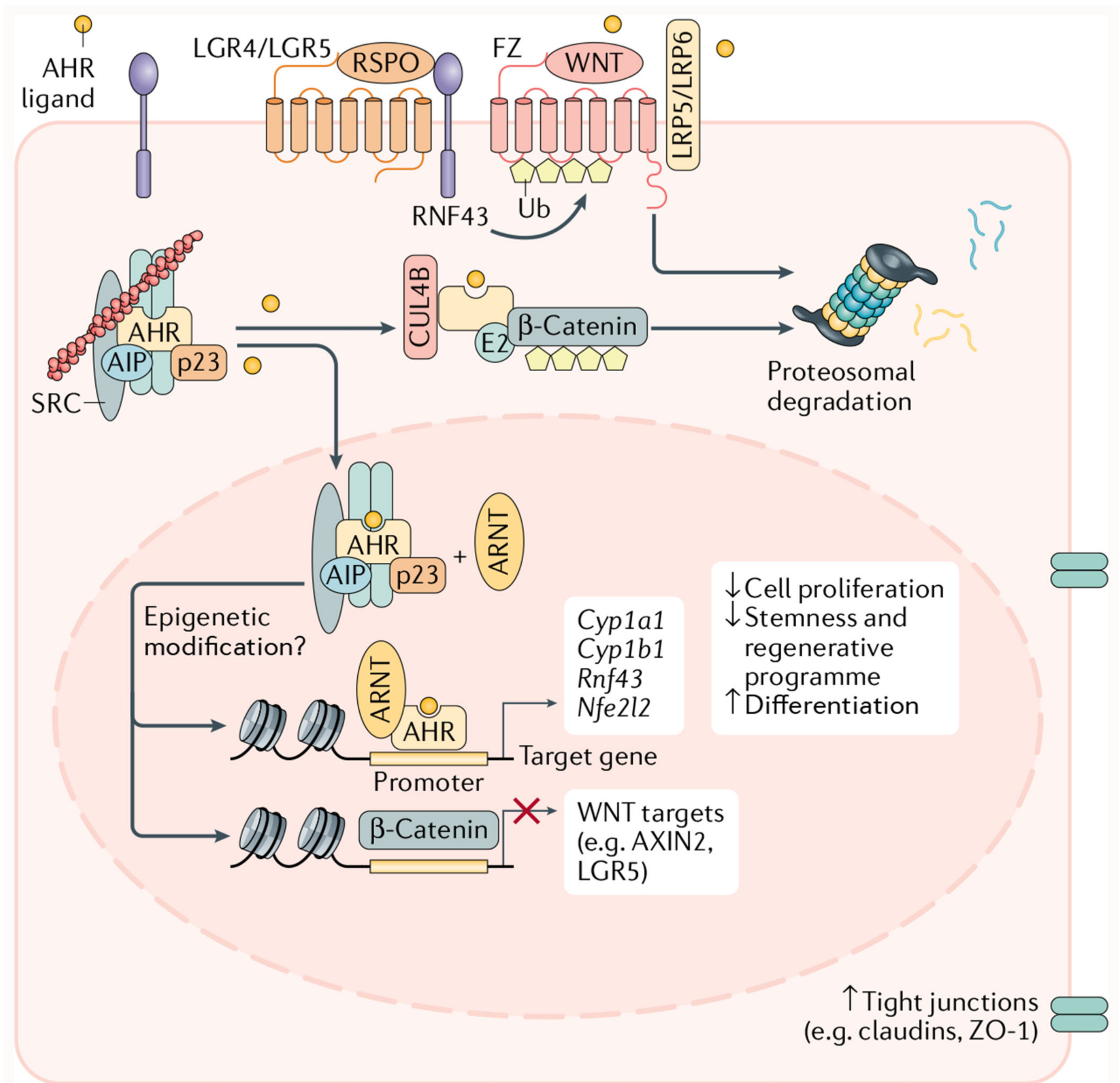

Fig. 3. AHR function in intestinal epithelial cells.

In the absence of ligands (yellow), epithelial aryl hydrocarbon receptor (AHR) is inactive and sequestered in an actin-bound complex containing AHR-interacting protein (AIP), p23, SRC and heat shock protein 90 (HSP90), which maintains AHR in a configuration conducive to ligand binding while preventing proteasomal degradation. Upon ligand binding the complex translocates into the nucleus and AHR dimerizes with its nuclear-localized cofactor AHR nuclear translocator (ARNT) to drive transcription of AHR target genes. AHR signalling restrains stemness programmes through transcriptional regulation of factors such as RNF43, an E3 ubiquitin ligase, which counteracts WNT-β-catenin by degrading WNT

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receptors (for example, Frizzled (FZ)). It might also act post-transcriptionally as part of an E3 ubiquitin ligase complex containing cullin 4B ubiquitin ligase (CUL4B), which causes the proteasomal degradation of β-catenin in the cytosol. Furthermore, ligand-bound AHR also functions to promote barrier integrity by upregulation of tight junction proteins in intestinal epithelial cells. RSPO, R-spondin; Ub, ubiquitin.

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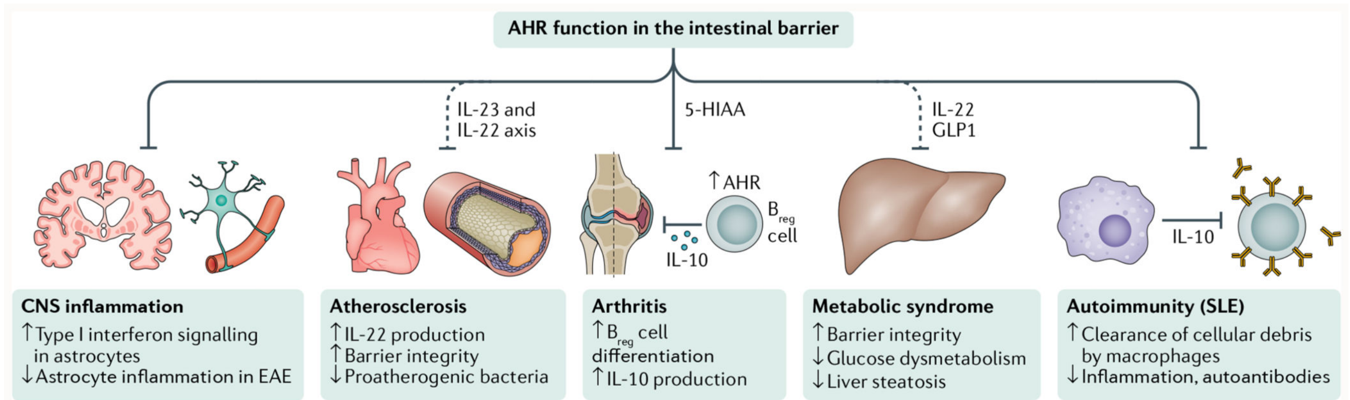

Fig. 4. From the gut and beyond: systemic implications of AHR signalling in the intestine.
Aryl hydrocarbon receptor (AHR) signalling in the gut can protect against the development of diseases in distal sites. AHR ligands from microbial metabolism of dietary tryptophan activate astrocytes to limit experimental autoimmune encephalomyelitis (EAE)-driven central nervous system (CNS) inflammation by facilitating type I interferon signalling. Phagocytosis of apoptotic cell debris triggers AHR activation in a ToLL-Like receptor 9 (TLR9)-dependent manner and is required for acquisition of a tolerant phenotype in myeloid cells to prevent systemic lupus erythematous (SLE)-like autoimmunity. IL-22 release by group 3 innate lymphoid cells and T helper 17 cells in the intestine can limit atherosclerosis development through upholding barrier integrity and limiting the growth of proatherogenic microbiota. The gut microbiota-derived short-chain fatty acid butyrate enhances production of the serotonin-derived metabolite 5-hydroxyindoleacetic acid (5-HIAA), selecting for growth of microbiota capable of tryptophan metabolism. 5-HIAA drives AHR activity in B cells, which promotes regulatory B (B_{reg}) cell differentiation and suppression of inflammation in arthritis. AHR signalling in intestinal epithelial cells stimulates production of intestinal-derived incretins (for example, glucagon-like peptide 1 (GLP1)), leading to improved glucose metabolism and protection from liver steatosis in mice receiving a high-fat diet. Black lines indicate studies with a direct AHR link, dotted lines refer to hypothesized links based on AHR function.

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