AHR and the transcriptional regulation of Type-17/22 ILC

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Mucosal innate lymphoid cells (ILCs) are an emerging population of diverse and heterogeneous immune cells, all with the unique ability to mount a rapid response against invading pathogens. They are further divided into subsets based on their differing cell surface markers as well as in their functional specialization. In this review, we summarize recent reports describing the importance of the transcription factor aryl hydrocarbon receptor (AHR) in regulating the development of one of these subsets, the Type-17/22 ILCs, as well as in the organization of postnatal lymphoid structures. We discuss the mechanisms behind the AHR dependence for development in Type-17/22 ILCs as well as reviewing the proposed physiological ligands that are mediating this effect.

Keywords: innate lymphoid cell, IL-22, AHR, isolated lymphoid follicle, kynurenine, NKp46

INTRODUCTION

INNATE LYMPHOCYTE HETEROGENEITY AND FUNCTIONAL SPECIALIZATION

Mucosal innate lymphoid cells (ILCs) are a population of immune cells that have broad roles in host immunity as well as maintaining homeostasis between the host and its environment. As their name suggests, ILCs are a family of lymphoid cells from the innate immune system, and develop from hematopoietic precursors (Sips and Di Santo, 2011). Murine ILCs are heterogeneous and they include two major subsets that we will define here as Type-17/22 ILCs and Type-2 ILCs. These major subsets can be further divided to include subsets that differ in the expression of cell surface markers as well as in their functional specialization. Type-17/22 ILCs comprise NKp46+ ILCs (also known as NK-22 in humans; Satoh-Takayama et al., 2008; Cella et al., 2009; Luci et al., 2009; Sanos et al., 2009), LTi-like ILCs (CD4+ CD44hi LTI-like cells; Takatori et al., 2009) and Thy1high Scat+ ILCs (Buonocore et al., 2010). These populations collectively known as Type-17/22 ILCs, specialize in the production of IL-22 and/or IL-17. Type-2 ILCs include “natural helper cells” (NH; Moro et al., 2010), “nuocytes” (Niehl et al., 2010), innate helper type-2 (Ih2) cells (Price et al., 2010), and multipotent progenitor type-2 cells (MPPtype2; Saenz et al., 2010) which specialize in the production of IL-5 and IL-13. Type-17/22 ILCs (Cella et al., 2009; Cupedo et al., 2009; Crellin et al., 2010) and Type-2 ILCs (Moro et al., 2010; Mjösberg et al., 2011; Monticelli et al., 2011) have also been described in humans. Most ILCs are ablated in Il2rc−/− mice (Satoh-Takayama et al., 2008; Koyasu and Moro, 2011), suggesting the requirement of a γc-associated cytokine receptor for their development and/or expansion. Interestingly, an IL-22 producing innate splenic population independent of Rag2 and the γc but resembling CD44hi LTI-like ILCs have been described (Dumoutier et al., 2011). Whether these cells represent a precursor of intestinal IL-22 producing ILCs or its central counterpart has yet to be determined. Type-17/22 ILC subsets are developmentally related in their requirement for Id2 and RORγt expression, as IId2 (Yokota et al., 1999; Satoh-Takayama et al., 2010) and RORγt (Satoh-Takayama et al., 2008; Luci et al., 2009; Sanos et al., 2009) deficient animals have greatly reduced numbers of these ILCs. The key transcription factors that drive development of Type-2 ILCs are not well defined. However, they seem to develop independently of RORγt (Moro et al., 2010). In addition, while Type-17/22 ILCs are largely confined to gut and lung mucosa and found in low numbers in secondary lymphoid organs, Type-2 ILCs have some distinct tissue distribution. Similar to Type-17/22 ILCs, nuocytes, MPPtype2, and Ih2 were found in spleen, lymph nodes, small intestine, gut-associated lymphoid tissue, and liver; however and in contrast to Type-17/22 ILCs, NH ILCs are uniquely localized in “fat associated lymphoid clusters” (Moro et al., 2010). Although the exact relationship among the Type-2 ILC subsets is currently not as clear as the relationship among the Type-17/22 ILC subsets, it is likely that Type-2 ILC subsets are strictly related and may represent the same cell type with different tissue localization although it cannot be excluded that they derive from a common lymphoid precursor and represent different cell lineages (Yang et al., 2011).

One of the main features that characterize Type-17/22 ILCs is their ability to promptly respond to the inflammatory cytokine IL-23 by secreting IL-22 and/or IL-17 (Sonnenberg et al., 2011). IL-18 alongside IL-23 has been shown promote IL-17 and IL-22 production in murine invariant Natural Killer T cells (Doisne et al., 2011) and γδ T cells (Sutton et al., 2009), NKp46+ ILCs (Reyniers et al., 2011), as well as sustaining IL-22 production in human ILCs cultured in vitro (Cella et al., 2010; Hughes et al., 2010). Interestingly, the lymphotoxin pathway has also been demonstrated to play an important role in controlling IL-22 production.

Mucosal lymphoid structures can be divided into lymph nodes and isolated lymphoid follicles (ILFs). ILFs are unique lymphoid structures found in the gut, liver, and skin and represent a site of immune cell accumulation, as well as a site of T cell differentiation. The ILF is a highly specialized lymphoid structure that acts as an immune checkpoint that maintains the balance between homeostasis and inflammation. The ILF is a site of immune cell accumulation, as well as a site of T cell differentiation. The ILF is a highly specialized lymphoid structure that acts as an immune checkpoint that maintains the balance between homeostasis and inflammation. The ILF is a site of immune cell accumulation, as well as a site of T cell differentiation. The ILF is a highly specialized lymphoid structure that acts as an immune checkpoint that maintains the balance between homeostasis and inflammation. The ILF is a site of immune cell accumulation, as well as a site of T cell differentiation. 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by Type-17/22 ILCs (Wang et al., 2010; Tumanov et al., 2011). Conversely, one of the primary characteristics of Type-2 ILCs is their capacity to quickly respond to the inflammatory cytokines IL-33 and/or IL-25 by producing IL-5 and IL-13. The unique ability to respond within hours to changing environmental cues has demonstrated increasing evidence that ILCs play a critical role in dictating appropriate tissue responses to incoming pathogens. ILCs are crucial for limiting tissue damage at mucosal sites and they provide the initial innate layer of protection while appropriate adaptive responses are developing. The intrinsic ability of ILCs to quickly respond to cytokines closely resembles the long-ago recognized capacity of conventional NK cells to respond to IL-12 and IL-18 by rapidly producing IFN-γ (Takeda et al., 1998). In light of this unique attribute, IL-12 and IL-18 responding cells, including conventional NK cells, should be viewed as Type-1 ILCs. The characteristics of the ILC subsets are summarized in (Table 1).

Considering the relevance of ILCs in disease and homeostasis, understanding the developmental pathways of ILCs is critical in their manipulation toward therapeutic exploitation. Therefore, increasing effort is ongoing into understanding how ILCs develop, expand, differentiate, and respond to a rapidly changing environment. One of the main focuses of recent research has been the attempt to elucidate transcriptional factors that are crucial for ILC differentiation. Here in this review, we will discuss recent findings on the role of the transcription factors aryl hydrocarbon receptor (AHR) and Notch in the development and function of Type-17/22 ILCs.

THE ARYL HYDROCARBON RECEPTOR PATHWAY

Numerous studies have shown that AHR is a master regulator of drug metabolism and that it mediates the biochemical and toxic effects of dioxins, polyaromatic hydrocarbons, and related compounds and has been reviewed (Nebert et al., 1993; Wilson and Safe, 1998; Mimura and Fujii-Kuriyama, 2003; Nguyen and Bradfield, 2008). While AHR was initially identified as a key regulator of xenobiotic metabolism, it has since been shown to modulate the expression of certain genes and the activity of other transcription factors to influence other cellular functions such as apoptosis, proliferation, cell growth, and differentiation. AHR contains a basic helix–loop–helix (bHLH) domain similar to that present in many DNA-binding proteins, and is a member of the Per–Arnt–Sim (PAS) superfamily of proteins which contain proteins involved in detection of intracellular or environmental changes, sensing light, oxygen, and circadian rhythm (Gu et al., 2000). In the absence of agonists, AHR resides in the cytosol as an inactive complex including the chaperones heat shock protein (Hsp90; Perdew, 1988), aryl hydrocarbon receptor interacting protein (AIP; Carver and Bradfield, 1997), and p23 (Kazlauskas et al., 1999). The PAS domain on AHR is an important site for agonist binding, which leads to a conformational change in AHR. This change results in a modified interaction with its chaperones and exposes a nuclear localization signal on AHR. After migrating into the nucleus, AHR heterodimerizes with another bHLH-PAS protein known as the aryl hydrocarbon receptor nuclear translocator (ARNT; Reyes et al., 1992) and this complex binds specific enhancer sequences adjacent to target promoters called dioxin responsive elements (DRE), inducing transcription of multiple target genes. These genes include xenobiotic metabolizing monoxygenases such as the members of the Cytochrome P450 family Cyp1a1, Cyp1a2, and Cyp1b1, but also encompass a large number of other genes involved in important developmental and physiological processes (Nguyen and Bradfield, 2008).

DEVELOPMENTAL DEFECTS IN AHR DEFICIENT MICE

The hypothesis that AHR has a role in development independent of binding xenobiotics was greatly augmented throughTable 1 | Characteristics of ILC subsets.

<table>
<thead>
<tr>
<th>ILC Subtype</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td><strong>TYPE-17/22 ILC: RESPONSIVE TO IL-23 AND IL-1β</strong></td>
<td></td>
</tr>
<tr>
<td>NKp46+ ILC (ILC22)</td>
<td>Secreting IL-22 (&lt;br&gt;CD4+, CD4&lt;sup&gt;-&lt;/sup&gt; subsets)</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;-&lt;/sup&gt; subsets</td>
<td>Secreting IL-22 and IL-13</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;-&lt;/sup&gt; subsets</td>
<td>Postnatal lymphoid tissues (CP and ILF)</td>
</tr>
<tr>
<td><strong>TYPE-2 ILC: RESPONSIVE TO IL-33 AND/OR IL-25&lt;sup&gt;*&lt;/sup&gt;</strong></td>
<td></td>
</tr>
<tr>
<td>NH</td>
<td>Secreting IL-5 and IL-13</td>
</tr>
<tr>
<td>Nuocyte</td>
<td>Secreting IL-5 and IL-13</td>
</tr>
<tr>
<td>Ih2</td>
<td>Secreting IL-5 and IL-13</td>
</tr>
<tr>
<td>Mpp&lt;sup&gt;type2&lt;/sup&gt;</td>
<td>Secreting IL-5 and IL-13</td>
</tr>
<tr>
<td><strong>TYPE-1 ILC: RESPONSIVE TO IL-12 AND IL-18</strong></td>
<td></td>
</tr>
<tr>
<td>NK cells</td>
<td>Secreting IFN-γ</td>
</tr>
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ILC, innate lymphoid cells; LTi, lymphoid tissue inducer; CP, cryptopatches; ILF, isolated lymphoid folicles; NH, natural helper; Ih2, innate helper type-2; Mpp<sup>type2</sup>, multipotent progenitor cell; FALC, fat associated lymphoid clusters; NK, natural killer.
the generation of three independent AHR deficient animals (Fernandez-Salguero et al., 1995; Schmidt et al., 1996; Shimizu et al., 2000). Examination of AHR null mice revealed common developmental anomalies (Lahvis et al., 2000) including increased neonatal lethality, portal vascular hypertrophy, and patent ductus venosus (DV; Esser, 2009). Lymphocytes from the lymph nodes and spleen were greatly reduced in the mouse generated by Fernandez-Salguero et al. but a similar phenotype was not observed in the other two knockout mice. While the explanation of these contrasting phenotypes is still unclear, the common developmental defects in AHR deficient mice provides evidence supporting the hypothesis that AHR plays an important role in normal development and that AHR is regulating normal vascular or hematopoietic development. This important impact on development would suggest that AHR is more than simply a xenobiotic sensor, and thus it is of importance to identify and characterize the vast diversity of AHR ligands as well as elucidating the mechanism behind AHR regulated development.

**AHR IN THE IMMUNE SYSTEM**

While AHR biology has mainly been focused on environmental toxicology studies and more recently into its importance during normal physiology and development, AHR has found its way into mainstream immunology research in the past few years. Several studies have identified the importance of AHR in both adaptive and innate immune systems and have recently been reviewed (Stevens et al., 2009; Stockinger et al., 2011). The focus of this review will be centered on the emerging evidence connecting AHR, its ligands, and intestinal immune function.

**AHR AND TYPE-17/22 ILC**

While the importance of AHR expression on the differentiation and function of Th17, Treg, and DC subsets has been described (Stockinger et al., 2011), recent reports have also demonstrated AHR expression in human and murine mucosal RORγt-dependent Type-17/22 ILCs (Kiss et al., 2011; Lee et al., 2011; Qiu et al., 2011; Figure 1). Driven by the hypothesis that similar to T H17 cells, AHR was necessary for the production of IL-22 in Type-17/22 ILCs, it was of great interest to test the impact of AHR deficiency on the function and/or development of these ILCs. Similar to the phenotype seen in AHR deficient T H17 cells, Peyer’s patches, and lamina propria cells isolated from the mucosal tissues of AHR deficient animals stimulated with IL-23 failed to produce IL-22. Further investigation demonstrated that surprisingly, AHR signaling in vivo was critical for the presence of Type-17/22 ILCs and not simply for their ability to secrete IL-22, as these cells were absent in Ahr−/− mice. AHR deficiency affected Nkp46+ ILCs as well as the CD4+ and CD4− LTi-like ILCs. Histological examination of gut-associated secondary lymphoid structures found an interesting and unique phenotype in AHR deficient animals. While embryonically implanted lymphoid structures, including the mesenteric lymph nodes, Peyer’s patches, and cecum-associated patches, remained largely intact in AHR deficient animals, the postnatally implanted lymphoid structures were severely diminished. These included small intestine lamina propria cryptopatches (CPs) and isolated lymphoid follicles (ILFs), as well as colon associated lymphoid patches.

**CPs AND ILFs**

Cryptopatches and ILFs are well-organized structures along the gastrointestinal tract that are thought to be functionally similar to Peyer’s patches in serving as important sites for the induction of protective mucosal immune responses (Lorenz and Newberry, 2004; Shikina et al., 2004). CPs are located in the lamina propria, and are composed of clusters of RORγt+ LTi-like ILCs scattered within the gastrointestinal tract. These structures are not present at birth, but start to develop about 2 weeks after birth. Their formation depends on factors necessary for the development and proper function of Type-17/22 ILCs such as IL-7 signaling (Adachi, 1998; Yoshida, 1999) and the expression of RORγt (Eberl et al., 2004) but seem to be independent of B and T cells (Hamada et al., 2002). Although original reports suggested that Type-17/22 ILCs required intestinal microbiota to develop (Sakoh-Takahaya et al., 2008; Sanos et al., 2009), subsequent investigation failed to confirm the need for microbiota (Lee et al., 2011; Reynders et al., 2011; Sawa et al., 2011). It is thought that inflammatory signals acting through Nod1 (Bouskra et al., 2008) as well as other TLRs during microbial colonization of the gut promote the transition of CPs into ILFs. Accordingly, germ-free animals have normal numbers of CPs (Kanamori et al., 1996) and medium-size ILFs (Hamada et al., 2002) but reduced number of large-size ILFs (Lorenz and Newberry, 2004). ILFs are thought to develop from CPs as CCR6+ CXCRC5+ B cells are recruited through the chemokines CCL20 and CXCL13 produced by epithelial cells and dendritic cells respectively (McDonald et al., 2010; van de Pauw and Mebius, 2010). These structures are quite dynamic and plastic in nature, representing a spectrum of varying maturity states.
possibly influenced by the constant interaction between the host and its luminal contents.

As mentioned earlier, the development of CPs and ILFs requires factors involved in the proper functioning of Type-17/22 ILCs, with AHR now added to the growing list. It is important to clarify that AHR has broad expression patterns within different cell types including immune cells. Therefore it was important to determine whether AHR signaling was critical in Type-17/22 ILCs or was required in other subsets that orchestrate Type-17/22 ILC recruitment to CPs and ILFs or to gut lamina propria. Using various conditional deletion mutants for AHR, it was found that the requirement for AHR in the development of Type-17/22 ILCs (Kiss et al., 2011; Lee et al., 2011) as well as the organization of CPs and ILFs was indeed intrinsic to RORγ expressing cells. CPs and ILFs from AHR conditional deletion mutants driven by RORγ-Cre recombinase had the same phenotype as AHR null animals while these structures from AHR conditional deletion mutants driven by CD11c or Villin – Cre were unaffected (Kiss et al., 2011). These studies seem to indicate that while AHR is expressed in intestinal epithelial cells or other mucosal-associated immune populations, AHR signaling within RORγ expressing ILCs is critical for their development as well as the formation of CPs and ILFs.

PROPOSED MECHANISMS FOR AHR REQUIREMENT IN TYPE-17/22 ILC DEVELOPMENT

C-KIT

What is the mechanism behind the AHR-mediated development of Type-17/22 ILCs? Interestingly using similar approaches, two different mechanisms have been proposed. AHR has been shown to induce the transcription of many target genes, and it was important to determine which of these target genes were important for Type-17/22 ILC development. One study focused on the receptor tyrosine kinase c-kit as a downstream target of AHR signaling (Kiss et al., 2011). Type-17/22 ILCs are identified by their expression of c-kit, and c-kit was shown to regulate intestinal ILC numbers and its luminal contents.

Along the same lines, a second study has also demonstrated normal frequencies of intestinal Type-17/22 ILCs in WT or AHR deficient mice at embryonic day 17.5 (Qiu et al., 2011). This study suggests that AHR is important for the survival and maintenance of Type-17/22 ILCs. Intestinal Type-17/22 ILCs had higher Annexin V staining as well as decreased expression of the anti-apoptotic genes Bcl2 and Bcl2l1, perhaps through the reduction of IL-7 in the large intestines of AHR deficient mice. However the exact mechanisms behind the regulation of AHR, IL-7, and antiapoptotic genes need to be addressed further.

NOTCH

A third study has proposed the induction of Notch as the mechanism behind AHR-driven development of at least small intestine lamina propria Nkp46+ ILCs (Lee et al., 2011). Notch signaling affects tissue renewal, maintenance, and cell differentiation decisions across a broad range of cell types and at different steps during cell lineage progression. Notch proteins are cell surface receptors, four of which have been described in mammals: Notch1–Notch4 (Kopan and Ilagan, 2009). The requirement for Notch signaling during T cell differentiation as well as activation is well characterized and has been reviewed (Osborne and Minter, 2007). Notch has also been implicated in the development of adult but not fetal RORγ+ ILCs in vitro (Possot et al., 2011) as well as in promoting the differentiation of TγH17 cells (Mukherjee et al., 2009; Keerthivasan et al., 2011). Another interesting report suggested a potential Notch–AHR axis for IL-22 production through the Notch induced generation of as of yet unknown endogenous AHR ligands in CD4+ T cells (Alam et al., 2010). However a connection between AHR signaling and the upregulation of Notch have not been clearly demonstrated. AHR agonists administered in vivo resulted in the rapid induction of Notch genes in both the liver and in the lamina propria cells isolated from the small intestine. NK cell lines transfected with a constitutively active form of AHR also upregulated Notch2, demonstrating that AHR signaling in part, regulates the expression of Notch. Animals lacking RBP-J in hematopoietic cells partially resembled the phenotype of AHR deficient animals. RBP-J is the binding partner of all Notch proteins and necessary for Notch signaling (Tanigaki and Honjo, 2010). These animals showed a severe reduction of Nkp46+ ILCs in the small intestine lamina propria. However, in contrast to AHR deficient animals, the CD4+ T-like ILCs were to some extent preserved. Subsequent histological examination of diffuse postnatal lymphoid follicles in the RBP-J deficient animals revealed intact CPs and ILFs, confirming the presence of functional T-like ILCs. It is possible that additional signals downstream of AHR partially compensate for the lack of Notch signaling in T-like ILCs. However, this dichotomy in the requirement for AHR by all Type-17/22 ILCs but the specific requirement of Notch signaling by Nkp46+ ILCs, open new and interesting questions.

Fate mapping experiments of Type-17/22 ILCs have shown that the frequencies of RORγ+ Nkp46+ ILCs increase with time after birth, reaching a plateau 4–5 weeks after birth (Sawa et al., 2010). However, RORγ+ CD4+ T-like ILCs have an opposite behavior, as their frequencies steadily decrease over time after birth, reaching a lower set point 2–3 weeks after birth. This evidence raises the
possibility that indeed NKp46+ ILCs only originate from adult, most likely bone marrow derived, precursor cells through a pathway that is both AHR and Notch-dependent. Although some of these cells may localize into CPs and ILFs, they are mostly dispersed throughout the lamina propria and are irrelevant for CP and ILF formation. On the contrary, CD4− LTI-like ILCs detectable during the first 2 weeks of age may still be of fetal origin, possibly from the fetal spleen or fetal liver hematopoietic stem cells (Mebius et al., 1997; Kim et al., 2008). These cells are AHR dependent but, as suggested by in vitro experiments, Notch-independent (Posset et al., 2011) for their development, and they may represent the crucial clue in the generation of CP and ILF structures.

EXOGENOUS AND ENDOGENOUS SOURCES OF AHR LIGANDS; WHICH MATTERS THE MOST?

What are the AHR ligands responsible for development and/or maintenance of Type-17/22 ILCs? Currently, known environmental ligands of AHR include halogenated aromatic hydrocarbons, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs; Nguyen and Bradfield, 2008). Endogenous AHR ligands isolated from mammalian tissues have also been identified. Kynurenine, a tryptophan metabolite generated from the indoleamine-2,3 dioxygenase (IDO) pathway, has recently been demonstrated to induce Cyp1a1 in bone marrow derived dendritic cells (Mezrich et al., 2010; Nguyen et al., 2010), as well as IDO expression as well as its induction has been shown to be dependent upon AHR signaling (Mezrich et al., 2010; Nguyen et al., 2010), and IDO enzymatic dependent and independent mechanisms of immunomodulation have been described (Pallotta et al., 2011). While the physiologically relevant endogenous agonist has yet to be concretely established, some attractive candidates have been identified. Kynurenine, a tryptophan metabolite generated from the indoleamine-2,3 dioxygenase (IDO) pathway, has recently been demonstrated to induce Cyp1a1 in bone marrow derived dendritic cells (Mezrich et al., 2010; Nguyen et al., 2010), as well as IDO expression as well as its induction has been shown to be dependent upon AHR signaling (Mezrich et al., 2010; Nguyen et al., 2010), and IDO enzymatic dependent and independent mechanisms of immunomodulation have been described (Pallotta et al., 2011). Many studies have suggested that AHR expression in DCs is critical to expand Treg through an IDO-dependent production of the tryptophan catabolite kynurenine, that acts as an endogenous

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AHR ligand (Veldhoen et al., 2009; Mezrich et al., 2010; Nguyen et al., 2010; Wu et al., 2011). This mechanism of Treg induction is most likely highly active in tumors, since it has been recently shown that kynurenine is abundantly expressed in many tumors (Opitz et al., 2011). In tumors, however, kynurenine is not produced by the tryptophan metabolizing enzymes IDO1 and/or IDO2, as in dendritic cells, but is uniquely generated by the enzyme tryptophan-2,3 dioxygenase (TDO; Opitz et al., 2011). Given the abundance of studies demonstrating the important role of tryptophan metabolism in modulating immune responses, further investigation into the AHR/kynurenine pathway in Type-17/22 ILCs is quite promising. While synthetic highly purified diets low in AHR agonists may reduce the environmental contribution of AHR ligands, it may be more difficult to distinguish among other factors such as nutrients, preservatives, as well as housing and bedding variances. New ways to partially block formation or deplete endogenous AHR ligands may be a promising avenue to dissect the contribution of natural endogenous versus exogenous compounds that drive the differentiation of Type-17/22 ILCs.

CONCLUSION

A recent string of publications connecting the classical dioxin sensor AHR with the development and maintenance of mucosal immune cell populations demonstrate an unexplored frontier in mucosal immunology. While teasing out the source of these exogenous and/or endogenous ligands is not clearly understood at this moment, the hypothesis that dietary derived compounds are intricately involved in the development of mucosal immune structures as well as the maintenance of immune cells in the gut is quite intriguing. Further studies will need to carefully characterize the exact mechanism by which AHR signaling seems to be exerting its effects, as well as identifying the natural ligands responsible; either from endogenous compounds or from exogenous dietary sources.

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Requirement of AHR for type-17/22 ILC development


