Exogenous Stimuli Maintain Intraepithelial Lymphocytes via Aryl Hydrocarbon Receptor Activation

Ying Li, Silvia Innocentin, David R. Withers, Natalie A. Roberts, Alec R. Gallagher, Elena F. Grigorieva, Christoph Wilhelm, and Marc Veldhoen

1Division of Molecular Immunology
2Division of Biological Services
3Division of Developmental Neurobiology
MRC National Institute for Medical Research, Mill Hill, London NW7 1AA, UK
4Laboratory of Lymphocyte Signalling and Development, Babraham Institute, Cambridge CB22 3AT, UK
5MRC Centre for Immune Regulation, Institute for Biomedical Research, Medical School, University of Birmingham, Birmingham B15 2TT, UK
6Present address: Department of Molecular, Cell and Developmental Biology, Yale University, New Haven, CT 06520, USA
*Correspondence: marc.veldhoen@babraham.ac.uk
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SUMMARY

The body’s surfaces form the interface with the external environment, protecting the host. These epithelial barriers are also colonized by a controlled diversity of microorganisms, disturbances of which can give rise to disease. Specialized intraepithelial lymphocytes (IELs), which reside at these sites, are important as a first line of defense as well as in epithelial barrier organization and wound repair. We show here that the aryl hydrocarbon receptor (AhR) is a crucial regulator in maintaining IEL numbers in both the skin and the intestine. In the intestine, AhR deficiency or the lack of AhR ligands compromises the maintenance of IELs and the control of the microbial load and composition, resulting in heightened immune activation and increased vulnerability to epithelial damage. AhR activity can be regulated by dietary components, such as those present in cruciferous vegetables, providing a mechanistic link between dietary compounds, the intestinal immune system, and the microbiota.

INTRODUCTION

Our surfaces are colonized with a large variety of microorganisms. In the intestine, many of these aid in nutrient processing, immunity, tissue development, and provision of metabolic compounds. Although many microorganisms are beneficial, the tissues need protection against assault from these and others. A first line of defense is the physical obstruction provided by a single- or multi-cell layer, the epithelial barrier. In the skin this is a tight, although not impregnable, seal. In contrast, the epithelial cells of the intestine form leaky barriers required for supporting the exchange of nutrients and fluids. Underneath the epithelial barrier there is a network of immune cells, predominantly consisting of specialized intraepithelial lymphocytes (IELs). IELs are comprised of a distinct population of T cell receptor (TCR)γδ cells in the skin and TCRγδ and TCRαβCD8εε+ cells in the gut. They populate these sites before birth in preparation for subsequent colonization with microorganisms (Carding et al., 1990).

We recently reported the induced expression of the transcription factor aryl hydrocarbon receptor (AhR) in differentiated TCRαβ+CD4+ T helper (Th) 17 cells (Veldhoen et al., 2008), which is mirrored in populations of CD44hiCCR6+ TCRγδ+ T cells found in the skin and CD44hiCCR6+ TCRγδ+ T cells found in secondary lymphoid organs (Martin et al., 2009). However, specialized TCRγδ+ T cells found at epithelial sites, which predominantly display an alert phenotype without full activation (CD44intCD25−/C0CD69+/C0CCR6+), were not analyzed. In this study, we sought to determine whether these IELs express Th17-related factors that may play a role in barrier immunity. We report here that IELs express high levels of the AhR. Although the absence of AhR has no effect on the number or composition of general lymphoid cell populations, IELs have an intrinsic requirement for AhR activity. In the absence of AhR, IELs are no longer maintained at the epithelial sites, in both the skin and the intestine. We reveal that intestinal AhR ligands are predominantly diet derived, with high levels present in cruciferous vegetables. The absence of AhR activity reduces epithelial cell turnover and the ability to control the microbial load and composition. As a consequence of low AhR activity, the intestine is in a heightened state of immune activation, dominated by a type 1 response, and more susceptible to immunopathology.

RESULTS

High Basal AhR Expression in IELs
IELs expressing an invariant TCR consisting of TCRVγ3 and TCRVδ1 receptor chains (see Garman et al., 1986 for
nomenclature) are among the first wave of T lymphocytes to mature in the embryonic thymus and populate the epidermis during keratinocyte stratification. By contrast, intestinal TCRγδ+ cells develop in the thymus as well as extrathympically and mainly express TCRVγδ5 determinants that can pair with multiple TCRVδ chains (Rocha et al., 1994). We compared the gene expression profiles of fluorescence-activated cell sorting (FACS)-sorted skin-derived TCRVγδ3, intestinal TCRVγδ5, and lymph node-sourced populations of TCRVγδ1.1 and TCRVγδ2 cells from nonimmune mice housed under specific pathogen-free conditions. We note a high basal ex vivo expression of AhR in both IEL subsets without additional cell activation. AhR expression significantly exceeded that found in TCRVγδ2 and TCRVγδ1.1 cell populations (which contain both naive and previously activated cells) (Figure 1A), differentiated Th17 cells, and hepatocytes (Veldhoen et al., 2008). This is in agreement with an earlier study that noted differential expression of AhR in intestinal IELs compared with lymph node-sourced TCRVδ+ CD8δ+ T cells as well as intestinal epithelial cells (Fahrer et al., 2001). We confirmed that expression of the Th17 cell transcriptional regulator, the orphan nuclear receptor RORγt, was enriched in TCRVγδ2+ cells (Martin et al., 2009), whereas all TCRγδ subsets expressed the closely related factor RORA (Figure 1A). Other mRNA levels for relevant Th transcription factors are tabulated (Figure 1C; Figure S1A available online).

In confirmation of their state of “alertness” but not full activation, ex vivo interferon (IFN)-γ transcripts were very low in TCRVγδ3+ cells (Figures 1B and S1B), and only low amounts could be detected in the TCRVγδ5+ intestinal IEL fraction (Figures 1B and S1C). Consistent with the absence of RORγt (Figure 1A), IL-17 transcripts and protein were absent in IELs (Figures 1B, S1B, and S1C). This was in stark contrast with TCRγδ cells found in the dermis, which displayed an activated (CD44hiCD25+ CCR6+) phenotype and of which up to 50% readily produced IL-17 (Figure S1B). Despite high basal levels of AhR transcripts, no IL-22 transcripts or protein could be detected in ex vivo TCRVγδ3+ and TCRVγδ5+ cells (Figures 1B, S1B, and S1C), concordant with our earlier finding that AhR may be required but alone is not sufficient for IL-22 production (Veldhoen et al., 2009).

Figure 1. Gene Expression Profiles of Ex Vivo TCRγδ Subpopulations
(A and B) Average relative mRNA transcript levels to Hprt of indicated transcription factors (A) and cytokines (B) in FACS-purified populations of TCRγδ T cells (n = 5) identified by their TCRVγδ usage.
(C) Overview table of TCRγδ subsets and their ex vivo gene profiles.
Data represent averages ± standard deviation (SD). See also Figure S1.

AhR Deficiency Results in the Specific Loss of IELs

The basal expression of AhR in IEL populations led us to explore the consequences of the loss of AhR. Mice deficient in one or both AhR alleles showed no significant changes in the proportions or numbers of TCRγδ T cell subsets in lymph nodes, spleen, or thymus (Figures 2A, 2C, and S1D). However, there was a striking loss of over 95% of TCRγδ cells in the small intestine in the absence of AhR compared with controls, without bias toward Thy1 expression levels (Figures 2A, 2C, and 2F). Furthermore, skin isolates from AhR-deficient mice, containing both dermis and epidermis, showed a distinctly different TCRγδ profile with the absence of TCRγδ35 cells (see arrow, Figure 2A). TCRVγ3-specific staining positively identified these cells, highlighting the absence of this epidermis-specific subset in AhR-deficient mice (Figures S2A and S2B), as previously reported (Schmidt et al., 1996). However, TCRβ3+CD8+ T cells, the other major population of intestinal IELs with elevated AhR expression (Figure S2C), are also significantly reduced in AhR-deficient mice (Figures 2E and S2D). By contrast, the proportions (and numbers) of regulatory T cells (Tregs), CD4+ lymphoid tissue inducer (LTi)-like cells, or natural killer (NK) p46+ NK-like cells in the lamina propria (LP) of the intestine or spleen were not significantly altered in AhR-deficient mice compared with controls (Figures S2E–S2G). Although the absence of TCRVγ3+ cells in AhR-deficient mice dramatically changed the proportional representation of TCRγδ T cells in the skin, in the intestine the relative distribution of TCRVγ3 chains followed that of control mice, dominated by TCRVγ5+ IELs (Figure S1D). In situ staining for TCRγδ confirmed these findings and illustrates the close spatial relationship of IELs with epithelial cells in the intestine and the dense network of cells in the epidermis (Figures 2F and 2G).

IELs Develop in the Absence of AhR

AhR deficiency has been linked with altered development of peripheral lymphocytes in one of the three differently gene-targeted AhR-deficient mouse strains, albeit not in the other two (Lahvis and Bradfield, 1998; Schmidt et al., 1996). Furthermore, exposure to the nonbiodegradable AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) may contribute to thymic involution (Knutson and Poland, 1982). This raises the possibility that the selective reduction in IELs observed in AhR-deficient hosts could have a developmental origin. However, we did not observe proportional or numerical differences of TCRVγ3 cells in the embryonic thymus between AhR-sufficient and -deficient mice (Figure S3A). In addition, even if TCRVγ3 cells are reduced to 10% of fetal thymocytes, they still predominate in the adult epidermis (Xiong et al., 2002). Furthermore, TCRVγ3+ T cells did home to the skin in the absence of AhR, as they were detectable in neonates and 5-week-old mice, but they were lost over time (Figures S3B, S3C, 2A, and 2G). We did not encounter TCRVγ3+ cells at other anatomical sites examined, including the dermis and peritoneum, excluding aberrant homing properties in the absence of AhR. TCRVγ5+ T cells, which can be generated in the adult thymus, could also be detected in the adult thymus as well as in peripheral lymphoid organs of AhR-deficient mice, in ratios and absolute numbers similar to those of control mice (Figure S1D and data not shown). This is consistent with the hypothesis that the generation and migration of epithelial TCRγδ T cell subsets proceeds similarly in AhR-deficient hosts compared to controls, but that their maintenance at mucosal sites is compromised in the absence of AhR-dependent signals.

Loss of IELs in the Absence of AhR Is Cell Intrinsic

As AhR is expressed in many tissues, we determined whether IEL-intrinsic or -extrinsic mechanisms were responsible for the specific reduction in IELs. Adoptive transfers of FACS-purified AhR-sufficient TCRγδ T cells, from secondary lymphoid organs or intestine, only effectively reconstituted in the small intestinal epithelia of AhR-deficient but not control mice (data not shown). This is consistent with the absence of an endogenous IEL population in AhR-deficient mice, providing a niche for the transferred cells. Chimeras generated by transfer of bone marrow cells from control mice reconstituted the intestinal IEL populations in recombinase-activated gene (Rag) 1-deficient hosts even when the hosts were Rag1 and AhR double-deficient (Figures S3D and S3E). Although AhR-deficient bone marrow successfully reconstituted secondary lymphoid organs as well as TCRβ3+ cells in organs such as liver and intestine (data not shown), it failed to reconstitute intestinal IELs (Figure S3D).

To distinguish between potential AhR-dependent activity in bone marrow-derived lymphoid or accessory cells, we made use of mice with a floxed AhR allele (Walisser et al., 2005) and introduced Cre recombinase under the promoter of the Rag1 gene, allowing lymphocyte-specific deletion of AhR. In these mice, the defect in intestinal IELs (Figure 3A) and TCRVγ3 (TCRγδ35CCR6+) cells in the skin was recapitulated (Figure 3B) and indistinguishable from that in germline AhR-deficient mice (Figure 2). The collective data obtained support the view that the maintenance of IELs depends critically on T cell-intrinsic AhR activity.

IELs Proliferate in the Absence of AhR

AhR-derived signals have been suggested to modulate a broad array of genes, including factors that play a role in cell-cycle regulation and cell death (Boitano et al., 2010; Marlowe and Puga, 2005). To investigate the contribution of AhR-dependent proliferative and cell survival signals in IELs, we assessed their proliferative capacity by incorporation of 5-ethynyl-2′-deoxyuridine (EdU) in vivo. Two days of EdU administration showed a significantly increased proliferative capacity of AhR-deficient IELs compared with controls (Figures 3C and S3F). However, 4 days of administration resulted in a similar proportion of IELs that incorporated EdU in both groups of mice. Furthermore, termination of EdU administration resulted in a rapid contraction of EdU+ IELs in AhR-deficient mice compared with controls (Figures 3C and S3F). These results indicate that the proliferative capacity of IELs is not affected by the absence of AhR, but that their reduced survival potential is the likely cause of their diminished numeric presence.

Intestinal AhR Ligands Are Diet Derived

AhR contains two highly conserved, period clock-AhR nuclear transporter (Amtl)-single-minded (PAS) domains. Phylogenetic analysis indicates that PAS domain-containing proteins have
Figure 2. Specific Reduction of IELs in the Absence of AhR

(A–D) Analysis of TCR-V<sub>gδ</sub> T cell subpopulations, identified by their TCR-V<sub>γ</sub> usage, present in indicated tissues in control (upper panels) and AhR-deficient (lower panels) mice analyzed by FACS (n > 10) (A and B) and absolute numbers (n = 8) (C and D).

(E) TCR-V<sub>γδ</sub><sup>+</sup> cells in skin (ears) from Ahr<sup>−/−</sup>, Ahr<sup>+/−</sup>, and Ahr<sup>+/+</sup> mice.

(F) Small intestine

(G) Skin
primarily evolved to respond to environmental changes in energy (Taylor and Zhulin, 1999). The PAS domains in AhR/Arnt closely resemble those present in photoactive yellow protein (PYP) (Pellequer et al., 1998). Analogous to PYP, AhR is responsive to the tryptophan photoproducts 6-formylindolo [3,2-b] carbazole (FICZ) and 6,12-diformylindolo[3,2-b] carbazole (dFICZ) (Oberg et al., 2005), and AhR-dependent Cyp1a1 expression can be found in the skin in the absence of exogenous xenobiotics (Sadek and Allen-Hoffmann, 1994). However, the equivalent to mouse TCRV \( \gamma^3 \) IELs are not present in humans due to the absence of skinT1 (Boyden et al., 2008), and further studies were concentrated on intestinal IELs.

Expression of the AhR target gene Cyp1a1 in the intestine has been shown to depend directly on dietary AhR ligands (Ito et al., 2007). Indeed, we observed AhR-dependent Cyp1a1 expression (Figure 4A). The chemical complexity of the diet makes it difficult to determine the exact nature of all potential AhR ligands. However, the tryptophan-derived phytochemical I3C, found in cruciferous vegetables, can be converted into the high-affinity AhR ligands indolo[3,2-b]carbazole (ICZ) and 3,3-diindolylmethane (DIM) (Bjeldanes et al., 1991). In order to evaluate a dietary source of AhR ligands, we fed control C57BL/6 mice either standard diet (5021-3 Autoclavable Rodent Lab Diet) or a synthetic purified diet (AIN-76A Purified Rodent Diet). Exposure of control mice for 3 weeks to the purified synthetic diet significantly reduced Cyp1a1 expression in the ileum compared to standard diet (Figure 4A). Supplementation of AIN-76A with 200 ppm I3C was able to induce a higher level of Cyp1a1 transcripts than our standard diet (Figure 4A). In line with our hypothesis that AhR expression is required for maintaining IELs, we found a significant decrease in both TCR\( \gamma^\delta \) and TCR\( \alpha^\beta \)/CD8\( \alpha^\alpha \) IELs in the small intestine of mice fed with synthetic diet compared with standard diet (Figures 4B and S4A). Supplementing the same synthetic diet with only the AhR ligand precursor I3C restored both TCR\( \gamma^\delta \) and TCR\( \alpha^\beta \)/CD8\( \alpha^\alpha \) IELs to levels comparable to those with the standard diet (Figures 4B and S4A).

### AhR Deficiency Results in an Increased Bacterial Burden

IELs share many properties with conventional TCR\( \alpha^\beta \) T cells (Fahrer et al., 2001), which suggests a degree of overlap in their functional properties. Two crucial differences are the capacity of IELs to respond swiftly, without the need for clonal expansion or priming, and their crosstalk with cells that make up the epithelial barriers. Consistent with the reduction of IELs in AhR-deficient mice, intestinal epithelial turnover was reduced compared with control mice (Figure S4B) (Boismenu and Havran, 1994; Komano et al., 1995). In line with this, adoptive transfer of

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**Figure 3. IEL Maintenance Depends on Intrinsic AhR Activity**

(A and B) Representative FACS plots (n > 6) of control mice or mice with targeted AhR deficiency in Rag1-expressing cells in the intestine, staining for TCR\( \gamma^\delta \) (upper panels) or pregated TCR\( \alpha^\beta \) (bottom panel) cells (A), and skin, revealing TCRV\( \gamma^3 \) cells via staining for TCR\( \gamma^\delta \) and CCR6 (B).

(C) Percentage of proliferating cells detected by EdU incorporation at indicated time points and after (+) termination of EdU administration in control (■) and AhR-deficient (○) mice (n = 4).

Data represent averages ± SD. See also Figure S3.

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(E) FACS analysis of TCR\( \beta^\gamma \) lymphocytes in the IEL small intestinal fraction of control mice and those deficient for AhR at one or both alleles, showing relative contributions of CD8\( \alpha^\alpha \) and CD8\( \alpha^\alpha \) populations.

(F and G) Tissue samples from control (upper panels) or AhR-deficient (lower panels) mice were stained with antibodies against EpCam (green) and TCR\( \gamma^\delta \) (red) in the small intestine (F) or TCRV\( \gamma^3 \) (green) in the skin (mice aged 8 weeks) (G).

Figures are representative of at least four experiments. Data represent averages ± SD. See also Figure S2.
AhR-sufficient intestinal TCRγδ+ lymphocytes into AhR-deficient recipients restored epithelial cell proliferation to a level seen in control mice. TCRγδ deficiency can result in more robust development of host immunity, often accompanied by increased immune pathology, implicating IELs in tolerance (Girardi et al., 2003). In
our colony of AhR-deficient mice bred under SPF conditions, we see no overt signs of ill health, but transfer of such mice to a less controlled environment increases the occurrence of rectal prolapse and signs of colitis in aging mice and of deaths in young mice due to severe ileitis. Other AhR-deficient colonies, experiencing different environments, were reported to suffer from skin abnormalities, rectal prolapse, and premature deaths (Fernandez-Salgueiro et al., 1995; Kimura et al., 2009). We hypothesized that the reduction in IELs could result in bacterial translocation into the sterile tissues due to increased barrier permeability, but we did not detect any bacteria in the colon-draining mesenteric lymph node, nor did we observe an increase in serum levels of LPS-binding protein (LBP) or increased intestinal permeability in healthy, AhR-deficient mice compared with controls after dextran-FITC feeding (Figure S4C and data not shown). Histological analyses of the small intestine did not reveal intestinal damage but did highlight increased villus length, epithelial cell size, and apical cytoplasmic mucin distention in AhR-deficient mice compared with controls (Figure S4D). This observation may be the result of decreased epithelial cell turnover in AhR-deficient mice and may contribute toward maintaining barrier integrity in the absence of IELs.

IELs are directly involved in immune surveillance, inducing apoptosis in infected cells via expression of high levels of granzymes (Fahrer et al., 2001). In line with the significant reduction of IELs in mice with reduced AhR stimulation, we detected significantly lower levels of granzyme A and B expression in the small intestine compared with control mice and those on a synthetic diet supplemented with I3C (Figure 4C and data not shown). This suggested that in the absence of AhR, there might be a reduction in the control of microorganisms, prompting the analysis of bactericidal gene expression in the gut. Although the small intestine did not show differential expression of the antimicrobial cryptidins tested (Figure S4E), the expression of matrix metalloproteinase (MMP)-7, which is required for wound healing and the bactericidal activity of cryptidins (Wilson et al., 1999), was significantly reduced in mice with reduced AhR activity compared with controls (Figure 4C). In addition, significant differences were found between controls and mice deficient in AhR or those maintained on a diet with low AhR ligands in the expression levels of the antimicrobial C-type lectins regenerating islet-derived protein 3 (RegIII)β and RegIIIγ (Figure 4C). These lectins are secreted into the intestinal lumen and enhance the clearance of Gram-positive bacteria (Abreu, 2010). The reduced expression of granzymes, C-type lectins, and MMP-7 suggests a diminished control of the intestinal microbial load in mice with reduced or absent AhR activity.

In line with this expectation, qPCR analysis of microbial-specific ribosomal 16S DNA revealed a 4-fold increase in bacterial load in the small intestine, but not the colons, of AhR-deficient mice compared with controls (Figure 4E). A reduced intake of dietary AhR ligands for 3 weeks increased the bacterial load, whereas supplementation of this diet with I3C reduced the microbial load compared with controls, especially in the colon. The contribution to the microbial make-up by the phyla Firmicutes, Actinomycetes, or Proteobacteria was not significantly altered between the experimental groups (Figures S4G and S4H). The heightened bacterial load in the small intestine could be attributed to the increase in the phylum Bacteroidetes (Figure 4F), which contains members of the genus Bacteroides. Although largely mutualistic, Bacteroides contain many known opportunistic pathogens and have been implicated in a mouse model of inflammatory bowel diseases (IBD) (Bloom et al., 2011). Although the bacterial load in the colon was similar in all experimental groups, a proportional increase in the contribution of Bacteroidetes was also observed here in the context of reduced AhR activity, compared with controls (Figure 4F).

Expression of bactericidal C-type lectins, MMP-7, or granzymes was low in the colon compared with the small intestine in all groups of mice (Figure S4F). Cryptdin transcripts, however, although absent in the colons of control mice and of those on synthetic diet supplemented with I3C, could readily be detected at highly significant levels in the colons of AhR-deficient mice and of control mice on a synthetic AhR ligand-low diet (Figure 4D). In addition to this indication of a heightened state of immune activation, we found increased expression levels of IFN-γ, but not TGF-β1, in mice with no or low AhR activity compared with controls (Figure S5A). The synthetic diet, irrespective of the presence of I3C, induced significantly higher levels of IL-10 transcripts in the colon compared with our standard diet.

AhR Deficiency Increases Epithelial Immunopathology

The increased immune cell activation status prompted further analysis of the adaptive immune response in the colonic LP and IEL compartments. Lymphocyte analysis revealed an increased proportion and number of TCRαβCD4+ and CD8+ cells in the colonic IEL fraction (Figure 5A), with those in both the IEL and LP compartments producing high levels of IFN-γ but little IL-17 (Figures 5A and S5C). Administration of the broad-spectrum antibiotic Enrofloxacin (Baytril, Bayer) was able to prevent both the numerical increase of T cells and their IFN-γ production (Figure 5A), illustrating the causative effect of the microbiota on immune activation in the absence of AhR activity.

In order to address the consequences of AhR deficiency for intestinal physiology, we employed dextran sodium sulfate (DSS)-induced colitis, a model of chemically induced epithelial damage and inflammation that is exacerbated by bacterial dissemination, especially in the absence of IELs (Small et al., 2009; Wirtz et al., 2007). Administration of 3% DSS resulted in rapid weight loss in control mice but full recovery after DSS withdrawal at day 6 (Figure 5B). However, in the absence of AhR, DSS caused accelerated weight loss, extreme shortening of the colon, and severe hemorrhaging, with 13 out of 16 mice reaching over 20% of body weight loss 1 day after DSS was withdrawn (Figures 5B and S5B). In agreement with the critical role of AhR, control mice fed with an AhR ligand-low diet for 4 weeks rapidly lost weight and showed a shortening of the colon similar to that in AhR-deficient mice, with 11 out of 16 mice losing over 20% of body weight (Figures 5C and S5B). However, mice fed the purified diet supplemented with I3C showed only mild signs of colitis, with reduced weight loss and shortening of the colon, followed by a rapid recovery post-DSS administration (Figures 5C and S5D). Reconstitution of AhR-deficient hosts with control IELs 8 weeks prior to 3% DSS administration did reduce the initial weight loss, but this was still increased compared with controls (Figure 5D). However, all of the AhR-deficient mice
reconstituted with control IELs (16 out of 16) made a full recovery, emphasizing the importance of IELs in reducing DSS-induced colitis.

The severity of the tissue damage in AhR-deficient colons was illustrated in histological sections, showing a diffuse LP, increased destruction of colonic epithelium, but reduced immune cell infiltration (Figures 5E and 5F). Importantly, in control mice, colonic crypt stem cells retain the ability to proliferate (indicated by arrows) and replace the damaged epithelial cells. By contrast, this epithelial repair mechanism was not observed in AhR-deficient colons (Figure 5G). These data suggest that AhR activation by dietary compounds is required to maintain IEL populations, thereby reducing the susceptibility to intestinal pathology via increased microbial control. This results in reduced recruitment of T lymphocytes. In addition, increased microbial control reduces the bacterial load and the proportion of potential

Figure 5. The Absence of AhR or Dietary AhR Ligands Increases the Severity of Colitis
(A) Representative FACS plots (n = 6–8) of cells harvested from the lamina propria (LP) or intraepithelial cell (IEL) fraction of the colon of control or AhR-deficient mice or the latter after antibiotic treatment, plotting CD4 against IFN-γ.
(B–D) Relative weights to starting weight of control (■) and AhR-deficient (□) mice after 3% DSS administration (n = 8) (B); or of control mice fed standard diet (■), AhR ligand-low diet (○), or AhR ligand-low diet supplemented with I3C (△) (C); or of control (■) and AhR-deficient mice 8 weeks after adoptive transfer with control IELs (□) fed standard diet (D). n = 8 per group, representative of at least two biological repeats.
(E, F, and G) H&E staining of colons of untreated or 3% DSS-treated controls or AhR-deficient mice. (E) Images are 250 x, insets are 65 x, and (F) and (G) are 400 x magnification. (G) Arrows indicate proliferating cells at the base of colonic crypts. All figures are representative of at least six individual mice. Data represent averages ± standard error of the mean (SEM). See also Figure S5.
pathogens thereby reducing bacterial dissemination upon epithelial damage. Furthermore, IELs enable rapid barrier repair with acute and local production of epithelial growth factors.

**DISCUSSION**

We provide experimental evidence that external environmental factors can have a direct and profound impact on the host’s immune defense capacity. Barrier-resident immune cells, the IELs, found in the murine skin and the small intestine have a cell-intrinsic requirement for the ligand activation of the AhR. The IEL populations TCR$\gamma\delta$CD8$^{+}$ and TCR$\gamma\delta$ lymphocytes express high levels of AhR. Its activation directly affects the maintenance, but not the development, homing, or proliferation, of IELs. In addition, we show that cruciferous vegetables are an important source of AhR ligand(s) in the intestine. These vegetables are enriched in the breakdown product of glucosinolate glucobrassicin, I3C, which under the influence of stomach acids can be converted to the high-affinity AhR ligands DIM and ICZ.

Importantly, the same epithelial barrier sites that harbor IELs are also host to a diverse but tightly managed composition and amount of microorganisms, harboring species that contribute positively to health but also pathogenic and opportunistic ones that form a potential threat. We show that a reduction in AhR activity in mice reduces their intestinal cytotoxic capacity with a profound reduction in perforin and granzymes and reduces their capacity to express or activate antimicrobial proteins. Although the microbicidal action of these peptides is often considered mild, concentrations in the crypts can reach 10 mg/ml after microbial stimulation, sufficient for strong bacterial lysis (Ayabe et al., 2000). As a result, the bacterial load in mice with reduced AhR activity is increased, particularly associated with an enhanced contribution of species of the Bacteroidetes phylum.

An altered bacterial composition, but not load, in the absence of AhR activity was not confined to the small intestine, where the IEL populations are reduced, but was also observed in the colon. This results in an accumulation of lymphocytes that show a predominant type 1 profile characterized by the production of IFN-$\gamma$. This is in line with other murine models of colitis that report a deleterious role of the intestinal microbiota, especially during the transfer of T cells, and are dominated by a type 1 profile (Cong et al., 1998). The direct importance of the microbiota in the activation of T cells was illustrated when AhR-deficient mice were treated with a broad-spectrum antibiotic, reducing both the number of T cells and their activation status. Our data are in also line with observations made in mice genetically engineered to lack TCR$\gamma\delta$ lymphocytes, contributing over half of the intestinal IELs, which are more susceptible to certain bacterial, protozoal, and viral infections and show increased morbidity (King et al., 1999; Mombaerts et al., 1993; Moore et al., 2000; Roberts et al., 1996; Selin et al., 2001), and they are in agreement with increased morbidity of AhR-deficient mice and their increased susceptibility to colitis and rectal prolapse (Fernandez-Salguero et al., 1997; Kimura et al., 2009).

We show that AhR deficiency or the reduced intake of dietary AhR ligands contributes to increased immunopathology in a model of DSS-induced colitis, causing injury to colonic epithelial cells with enhanced immunopathology due to bacterial dissemination (Wirtz et al., 2007). Although reduced control of the microbial load or composition in mice deficient in critical microorganism control mechanisms results in increased susceptibility to DSS-induced injury and reduced epithelial proliferation (Rakoff-Nahoum et al., 2004; Vijay-Kumar et al., 2007), mice treated with broad-spectrum antibiotics or bred under germ-free conditions are similarly susceptible to DSS-induced colitis (Maslowski et al., 2009, Rakoff-Nahoum et al., 2004). This indicates that both elements of the microbiota and detection mechanisms are required for maintaining a healthy epithelial barrier (Mazmanian et al., 2008). Although IELs were important in reducing excessive DSS-induced colitis, the kinetics of initial accelerated weight loss after adoptive transfer of control IELs (Figure 5D) could indicate that other cell types may play an additional role. In line with this, we show that in the absence of IELs, approximately 50% of AhR transcripts can still be detected in the small intestine of mice fed an AhR ligand-low diet compared with controls (Figure 4A). It is possible that AhR, although not essential for the survival, development, or migration of other cell types, is important for their function, for example, for the induced production of IL-22 in Th17 cells or in populations of innate lymphoid cells (Cua and Tato, 2010; Veldhoen et al., 2008) that may influence recovery from DSS.

Whether AhR and its ligands affect human intestinal immunobiology is currently unknown. However, there are some observations of interest. Increased CD4-mediated IFN-$\gamma$ production, epithelial damage, hyperplasia, apical cytoplasmic mucin desorientation, reduced $\alpha$-defensin expression, and an increased bacterial load have all been associated with IBD (Fuss et al., 1996; Wehkamp et al., 2005). A body of data correlate individual components or alterations in the intestinal microbial composition in driving the mucosal inflammatory response in susceptible individuals suffering from IBD (Frank et al., 2007; Harper et al., 1985; Sokol et al., 2006). It is of note that a number of studies have also shown an association between high faecal or mucosal levels of *Bacteroides*, the genus of Bacteroidetes predominantly found in mammals, and active IBD (Andoh et al., 2011). Furthermore, lesions in IBD occur in those areas with highest bacterial exposure, and broad-spectrum antibiotics can be successfully used as primary therapy in uncomplicated IBD (Shanahan, 2000). Isolated bacterial species involved are nonpathogenic in healthy hosts, suggesting that effective regulatory mechanisms are normally in place to maintain intestinal homeostasis preventing inflammation. Epidemiological studies indicate a strong environmental influence on IBD, with a low concordance rate between identical twins and a link with a Western lifestyle (Loftus, 2004), a diet low in fruit and vegetables being one of the major risk factors (Amre et al., 2007; D’Souza et al., 2008; Sousa Guerreiro et al., 2007). This is in line with the premise that IBD develops in genetically susceptible hosts but that environmental factors precipitate the onset or reactivation of disease. Determining whether AhR or its downstream elements can play an additional role in determining human disease onset or severity requires future studies.

Our data also make a genetic link between the IELs of the intestine and the skin, both depending on AhR-mediated signals for their maintenance. In mice, adoptive transfer of T lymphocytes
into immunodeficient animals induces both colitis and psoriasis-like phenotypes (Davenport et al., 2002; Leon et al., 2006). A view of an association between IBD and cutaneous disorders (Bernstein et al., 2005; Najarian and Gottlieb, 2003), of which the most frequent example is psoriasis (7%–11% of IBD population versus 1%–2% of general population) (Hoffmann and Kruis, 2004), in line with common susceptibility loci (Cargill et al., 2007; Duerr et al., 2006; Einarsdottir et al., 2009) has been proposed. However, the epidermal TCRγδ population is absent in human skin, and although an alternative lymphocyte population is present (Duhem et al., 2009), to our knowledge AhR has not been reported as a susceptibility locus to date.

How AhR-mediated signals maintain IELs at epithelial sites is currently unknown and is the subject of further investigation. AhR may regulate particular metabolic pathways within IELs. In line with this, orphan receptors such as AhR have been shown to have strong connections with lipid and hormone metabolic pathways (Fernandez-Salguero et al., 1997; Kliewer et al., 1999), components of which show differential expression in IELs when compared to lymph node-sourced TCRγδ+CD8+ T cells (Fahrer et al., 2001). Importantly, metabolic gene expression in IELs is often found to be complementary to that in epithelial cells (Fahrer et al., 2001). In turn, the maintenance of IELs is instrumental in the safeguarding and repair of epithelial-barrier sites, providing protection against damage and microbial invasion, and in controlling the intestinal microfloral load and composition.

In conclusion, our data highlight the evolutionarily highly conserved AhR system as a previously unknown link between external environmental stimuli and the maintenance of specialized immune cell populations (IELs), as well as the control of the microbiota. Our results provide a molecular basis for the importance of cruciferous vegetable-derived phytonutrients as the microbiota. Our results provide a molecular basis for the importance of cruciferous vegetable-derived phytonutrients as biotics, in the physiology and homeostasis of epithelial barrier sites.

**EXPERIMENTAL PROCEDURES**

**Mice**

CS7BL/6 (B6), Rag1-deficient (Rag1−/−), Rag1-Cre, floxed AhR (AhRflx), and AhR-deficient mice (AhR−/−), all on a B6 background, were bred and maintained under specific pathogen-free conditions at NIMR, and all animal experiments were done according to institutional guidelines approved by the local ethical panel and by a project license from the UK Home Office.

**T Cell Isolation and FACS Staining**

Intestinal IELs were isolated by longitudinal opening of the small intestine. Intestinal IELs were isolated by longitudinal opening of the small intestine. T Cell Isolation and FACS Staining

**Statistical Analysis**

For statistical analysis, unpaired Student’s t tests were used. Significance of p < 0.01 is indicated by *. Error bars represent standard deviations unless otherwise specified.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, five figures, and one table and can be found with this article online at doi:10.1016/j.cell.2011.09.025.

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