Differential dependence on microbiota of IL-23/IL-22-dependent gene expression between the small- and large-intestinal epithelia

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Keywords: intestinal epithelial cells, IL-23/IL-22 axis, gut microbiota,

lipid metabolism, cell proliferation

Abstract

In the intestine, interleukin (IL)-23 and IL-22 from immune cells in the lamina propria contribute to maintenance of the gut epithelial barrier through the induction of antimicrobial production and the promotion of epithelial cell proliferation. Several previous studies suggested that some of the functions of the IL-23/IL-22 axis on intestinal epithelial cells are shared between the small and large intestines. However, the similarities and differences of the IL-23/IL-22 axis on epithelial cells between these two anatomical sites remain unclear. Here, we comprehensively analyzed the gene expression of intestinal epithelial cells in the ileum and colon of germ-free, *Il23*$^{-/-}$, and *Il22*$^{-/-}$ mice by RNA-sequencing. We found that while the IL-23/IL-22 axis is largely dependent on gut microbiota in the small intestine, it is much less dependent on it in the large intestine. In addition, the negative regulation of lipid metabolism in the epithelial cells by IL-23 and IL-22 in the small intestine was revealed, whereas the positive regulation of epithelial cell proliferation by IL-23 and IL-22 in the large intestine was highlighted. These findings shed light on the intestinal site-specific role of the IL-23/IL-22 axis in maintaining the physiological functions of intestinal epithelial cells.

Introduction

The intestine is a very complicated organ, in which commensal bacteria, intestinal epithelial cells, and immune cells interact with each other to maintain gut homeostasis (Kayama, Okumura, & Takeda, 2020). Various environmental factors, including diet, exercise, intake of antibiotics, and psychological stress, alter the microbial composition and metabolism in the gut (Gomaa, 2020). The host intestinal epithelial cells and immune cells sense these alterations of gut environment and, in cooperation, cope with them by communicating with each other through the production of cytokines and chemokines (Mahapatro, Erkert, & Becker, 2021; Zimmerman, Vongsa, Wendt et al., 2008).

Among various cytokines produced from intestinal immune cells in the lamina propria, interleukin (IL)-23 and IL-22 cooperatively play a crucial role in maintaining gut homeostasis and regulating immune responses in the intestine (Fatkhullina, Peshkova, Dzutsev et al., 2018; Keir, Yi, Lu et al., 2020; Sano, Huang, Hall et al., 2016). The production of IL-23 from myeloid cells such as $CD103^+$ $CD11b^+$ dendritic cells, activated by specific bacteria such as segmented filamentous bacteria, leads to the secretion of IL-22 from type 3 innate lymphoid cells (ILC3) and T helper (Th)17 cells (Kinnebrew, Buffie, Diehl et al., 2012; Sano et al., 2016). IL-22 reportedly promotes the production of antimicrobial peptides, tight junction proteins, and mucins in intestinal epithelial cells (Sugimoto, Ogawa, Mizoguchi et al., 2008; Tsai, Zhang, He et al., 2017; Zheng, Valdez, Danilenko et al., 2008), which prevent commensal bacteria and pathogens from invading the intestinal tissue, thereby contributing to the maintenance of gut homeostasis. In addition, a previous paper demonstrated that IL-22 is required for epithelial repair and regeneration in the gut by promoting the

proliferation of epithelial cells in the case of epithelial injury (Lindemans, Calafiore, Mertelsmann et al., 2015). Moreover, a recent paper showed that IL-22 protects intestinal stem cells against DNA damage (Gronke, Hernandez, Zimmermann et al., 2019). With respect to the similarities and differences of IL-22 functions on intestinal epithelial cells between the small and large intestines, IL-22 exerts several similar functions at these two anatomical sites (Keir et al., 2020). However, to the best of our knowledge, no comprehensive studies have evaluated the differences of IL-22 functions on epithelial cells between the small and large intestines.

In this study, we comprehensively analyzed the gene expression of intestinal epithelial cells in the ileum and colon from wild-type mice under specific pathogen-free (SPF) or germ-free (GF) conditions, and *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice by RNA-sequencing (seq). We then compared the gene expression profiles among these groups. Our comprehensive gene expression analysis revealed that the functions of the IL-23/IL-22 axis on intestinal epithelial cells are less dependent on gut microbiota in the large intestine, unlike the case in the small intestine. The similarities and differences in the intestinal site-specific roles of the IL-23/IL-22 axis between the small and large intestines were clearly demonstrated by the current transcriptomic analysis.

Results

The IL-23/IL-22-mediated gene expression of epithelia differentially depends on microbiota between small and large intestines

To compare the functions of IL-23 and IL-22 on epithelial cells between the small and large intestines, we performed RNA-seq of intestinal epithelial cells from the ileum and colon of *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice. Because previous studies demonstrated that gut microbiota upregulates the expression of IL-23 and IL-22 from immune cells in the lamina propria of the small intestine (Kinnebrew et al., 2012; Sano et al., 2016), the intestinal epithelial cells of wild-type (WT) mice under SPF and GF were also analyzed. The RNA-seq data were analyzed using integrated Differential Expression and Pathway analysis (iDEP) (http://bioinformatics.sdstate.edu/idep96/) (Ge, Son, & Yao, 2018). We performed hierarchical clustering and principal component analysis (PCA) of the top 2000 variable genes to gain insights into the similarity or dissimilarity of gene expression patterns among the groups (Fig. 1). In the hierarchical clustering analysis of the ileal data, the gene expression patterns are primarily divided into two clusters: WT (SPF) and the other group (Fig. 1A – [1]), consisting of WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice (Fig. 1A – [2]). This suggests that WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice exhibit similar gene expression patterns that are distinct from those of WT (SPF) mice (Fig. 1A). Furthermore, within the same cluster (Fig. 1A – [2]), the gene expression patterns are further separated into two subclusters: WT (GF) and *Il22*$^{-/-}$/*Il23*$^{-/-}$ mice. This indicates minor differences in gene expression in the epithelial cells between germ-free mice and mice lacking IL-22 or IL-23 in the small intestine (Fig. 1A). Moving on to the large intestine, the gene expression patterns are primarily divided into two clusters: WT (GF)/WT (SPF) mice and *Il22*$^{-/-}$/*Il23*$^{-/-}$ mice. This suggests significant differences in

gene expression between germ-free mice and mice lacking IL-22 or IL-23 in the large intestine, in contrast to the findings in the small intestine (Fig. 1B). In the PCA of the ileal data, the gene expression of WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice shows a positive correlation with PC1, accounting for 40% of the variance, while the expression of WT (SPF) mice shows a negative correlation (Fig. 1C). This suggests that WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice exhibit relatively similar gene expression patterns in the small intestine. Additionally, *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice show higher expression levels of genes positively associated with PC2, accounting for 20% of the variance, unlike WT (GF). This indicates some differences in gene expression between germ-free mice and mice deficient in IL-22 or IL-23. Conversely, in the large intestine, the gene expression of *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice shows a positive correlation with PC1, accounting for 46% of the variance, while both WT (SPF) and WT (GF) mice show a negative correlation (Fig. 1D). This suggests that the gene expression patterns of *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice in the large intestine are different from those of WT mice regardless of the presence of gut microbiota, and the IL-23/IL-22 axis regulates the gene expression of intestinal epithelial cells through the microbiota-independent pathway in the large intestine. These findings from hierarchical clustering and PCA demonstrate that the IL-23/IL-22-mediated gene expression of small intestinal epithelia largely depends on microbiota in the small intestine. However, in the large intestine, the IL-23/IL-22 axis and gut microbiota act independently on the epithelial cells.

We next selected differentially expressed genes (DEGs) between groups by DESeq2 using iDEP [false discovery rate (FDR) < 0.1, fold change (FC) >2] (Fig. 2).

The numbers of DEGs in ileal epithelial cells of WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice in comparison with the WT (SPF) levels were higher than those in colonic epithelial cells, suggesting that the deficiency of either gut microbiota or the IL-23/IL-22 axis has a greater impact on epithelial cells in the small intestine than in the large intestine (Fig. 2A–D). The downregulated genes in ileal epithelial cells in common among WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice included *Reg3b*, *Reg3g*, and *Saa1*, all of which are reportedly upregulated by the commensal bacteria/IL-23/IL-22 axis in the small intestine (Sano et al., 2016; Zheng et al., 2008) (Fig. 2E–G). By contrast, in the large intestine, the expression of *Saa1* was elevated only in WT (GF), unlike in the small intestine, whereas the expression of *Reg3b* and *Reg3g* was downregulated in the three groups, like in the small intestine (Fig. 2H–J).

Differential dependence on microbiota of IL-23/IL-22-mediated immune response between small- and large-intestinal epithelia

We next performed K-means clustering (k = 4) to identify functionally related sets of genes upregulated or downregulated in WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice compared with the levels in WT (SPF) mice (Fig. 3A and B). The K-means clustering based on the ileal data revealed that a set of genes related to immune responses, including defense response to other organisms and response to bacteria, were downregulated in WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice (Fig. 3A), indicating that the microbiota/IL-23/IL-22 axis is critical for host defense in the small intestine, as previously reported (Kinnebrew et al., 2012; Sano et al., 2016; Zheng et al., 2008). By contrast, the K-means clustering based on the colonic data showed that immune response-related genes are downregulated only in WT (GF), but not in *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice (Fig. 3B), suggesting that microbial

signals enhance the host defense through some pathways other than the IL-23/IL-22 axis in the large intestine, where this axis is not so important for epithelial barrier function. In terms of the expression of each gene related to the mucosal barrier, the expression of *Reg3b* and *Reg3b*, both of which encode bactericidal proteins against Gram-negative and Gram-positive bacteria, respectively (Miki, Holst, & Hardt, 2012; Vaishnava, Yamamoto, Severson et al., 2011), was strongly downregulated by the deficiency of IL-23 and IL-22 in the small intestine as well as the large intestine (Fig. 4A and B). By contrast, some mucosal barrier protein-encoding genes such as *Dmbt1* (Renner, Bergmann, Krebs et al., 2007) and *Lypd8* (Okumura, Kurakawa, Nakano et al., 2016) are downregulated in the small intestine of WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice, but they are constitutively expressed in the large intestine of every mouse. With respect to the chemokine expression, most chemokine-coding genes including *Ccl5*, *Ccl9*, and *Ccl20* were downregulated in the ileum of WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice (Fig. 4C), which was not clearly seen in the colon (Fig. 4D). Thus, the IL-23/IL-22-dependent expression of immune response genes differs somewhat between the small and large intestines.

Microbiota/IL-23/IL-22 axis regulates lipid absorption in the small intestine

The small intestine is responsible for nutrient absorption. Several recent studies demonstrated that intestinal IL-22 regulates systemic lipid metabolism, and thereby contributes to the prevention of metabolic disorder including diabetes and arteriosclerosis (Fatkhullina et al., 2018; Wang, Ota, Manzanillo et al., 2014). However, the molecular mechanism behind this remains unclear. In this regard, our RNA-seq data showed that a set of genes involved in lipid metabolism were upregulated in the ileal

epithelial cells of WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice (Fig. 3A). A previous study demonstrated that high IL-22 production from activated ILC3 in *Rag1*$^{-/-}$ mice substantially inhibits the expression of genes related to lipid absorption, such as *Cd36*, *Fabp1*, and *Fabp2*, in the ileum (Mao, Baptista, Tamoutounour et al., 2018). Fatty acid and cholesterol are taken up through various transporters such as CD36 on the apical side of the cell membrane [CM (AP)]. They are then transported to the endoplasmic reticulum (ER) by fatty acid-binding proteins and converted to triacylglycerol (TG) or cholesterol esters (CE) in the ER, where chylomicrons composed of TG and CE are formed by apolipoproteins. These chylomicrons mature in the Golgi apparatus, and are then discharged by exocytosis from the basal side of the cell membrane [CM (BS)] to the lymph circulation (Fig. 4E). In the case of deficiency in the microbiota/IL-23/IL-22 axis, most of the lipid absorption-related genes including *Cd36* in CM(AP), *Fabp1*, *Fabp2* in cytoplasm (CP), *Apoa1* and *Apoa4* in Golgi, and *Abca1* in CM(BS) were upregulated in the ileal epithelial cells (Fig. 4F), which is compatible with a previous paper (Mao et al., 2018). These results clearly indicate that certain commensal bacteria regulate lipid absorption in the small intestine through the induction of IL-23 and IL-22 production from immune cells in the lamina propria.

IL-23/IL-22 axis promotes proliferation of epithelial cells in the large intestine

Several previous studies demonstrated that IL-22 promotes the proliferation of intestinal epithelial cells by *in vitro* experiments using organoids and *in vivo* experiments, such as via treatment with recombinant IL-22, mucosal injury models, and cancer models (Aparicio-Domingo, Romera-Hernandez, Karrich et al., 2015; Kirchberger, Royston, Boulard et al., 2013; Lindemans et al., 2015). However, the molecular mechanism by

which IL-22 accelerates cell proliferation remains unclear. K-means clustering showed that genes related to cell proliferation such as cell division and the cell cycle were downregulated in the colonic epithelia of *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice, but not in those of WT(GF) mice (Fig. 3B). This suggests that the IL-23/IL-22 axis promotes epithelial cell proliferation particularly in the large intestine in a microbiota-independent manner, even in a steady state. The cell cycle is regulated by various molecules including cyclins (Cyc) and cyclin-dependent kinase (CDK), both of which play a crucial role in driving cell cycle progression (Suski, Braun, Strmiska et al., 2021) (Fig. 5A). The expression of *Ccna1* (coding Cyclin A1), *Ccna2* (coding Cyclin A2), *Ccnb1* (coding Cyclin B1), *Ccnb2* (coding Cyclin B2), and *Cdk1* was downregulated in *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice (Fig. 5B), which may contribute to the brake of the cell cycle in the intestinal epithelial cells of *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice. In addition, among the genes coding molecules controlling the activation of Cyc–CDK complexes, the expression of *Sfn* coding Stratifin/14-3-3σ, which reportedly negatively regulates activation of the CycB–CDK1 complex (Gardino, & Yaffe, 2011), was substantially upregulated in *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice (Fig. 5B). Moreover, *Cdc25b* and *Cdc25c* coding Cdc25b and Cdc25c, respectively, which are reported to activate the CycB–CDK1 complex (Liu, Zheng, Lu et al., 2020), were downregulated in *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice (Fig. 5B). With regard to genes related to the establishment of sister chromatid cohesion in the S phase, the expression of *Ecos1/2*, *Stag1/2*, *Smc3*, *Rad21*, and *Pds5a/b* was reduced by the deficiency of IL-23 and IL-22 (Fig. 5C and D). These findings suggest that the IL-23/IL-22 axis enhances the cell cycle progression of colonic epithelial cells by upregulating the expression of Cyc and CDK and cohesion establishment-related

molecules, and by downregulating the expression of Stratifin/14-3-3σ, a suppressor of the cell cycle.

Discussion

In the intestine, a variety of immune cells in the lamina propria orchestrate the physiological functions of intestinal epithelial cells through the production of cytokines. Among various cytokines, IL-23 and IL-22 are key for maintaining the physiological functions of intestinal epithelial cells. To precisely understand the function of these two cytokines on intestinal epithelial cells in the small and large intestines, we comprehensively analyzed the gene expression of intestinal epithelial cells in the ileum and colon of *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice by RNA-seq.

Several recent studies demonstrated that the microbiota/IL-23/IL-22 axis plays an important role in maintaining epithelial barrier functions to regulate gut microbiota in the small intestine (Kinnebrew et al., 2012; Sano et al., 2016; Zheng et al., 2008). However, whether this is also true in the large intestine has remained unclear. In this context, the gene expression patterns in the colonic epithelia of *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice were found to differ from that of GF mice, unlike in the ileal epithelia, suggesting that the IL-23/IL-22 axis is much less dependent on commensal bacteria in the large intestine. In addition, the expression of several barrier molecule-encoding genes including *Dmbt1* (Renner et al., 2007) and *Lypd8* (Okumura et al., 2016) was downregulated by deficiency of IL-23 and IL-22 only in the small intestine, but not in the large intestine, suggesting that the effect of the IL-23/IL-22 axis on regulation of the epithelial barrier is greater in the small intestine than in the large intestine.

We found the differential dependence of the IL-23/IL-22 axis on epithelial cells in the small and large intestines. In this regard, several possible mechanisms can be considered: differential expression of the IL-22 receptor on epithelial cells / the IL-23 receptor on ILC3/Th17 cells in the small and large intestines, differential

composition of epithelial cell subsets showing different IL-22 responses in the small and large intestines. Regarding the divergent impacts of gut microbiota on gene expression of epithelial cells in the small and large intestines, it is noteworthy that the number of DEGs in the ileal epithelial cells between SPF and GF mice was greater than that in the colonic epithelial cells, although the large intestine harbors a higher number and diversity of gut microbes than the small intestine. This could be due to the fact that some bacteria, such as segmented filamentous bacteria, directly attach or adhere the epithelial cell in the small intestine (Atarashi, Tanoue, Ando et al., 2015; Ivanov, Atarashi, Manel et al., 2009), whereas bacteria are segregated from epithelial cells by the presence of thick mucus in the large intestine (Okumura, & Takeda, 2017). It is also possible that the response to bacteria is higher in the small intestinal epithelial cells because of the higher expression of pattern recognition receptors. To gain a comprehensive understanding of the underlying mechanisms, further analyses, such as single-cell (sc) RNA-seq of epithelial cells and immune cells, as well as network analysis based on sc RNA-seq data, should be performed in the future.

Interestingly, the current RNA-seq analysis revealed that the commensal bacteria/IL-23/IL-22 axis suppresses the expression of genes involved in lipid absorption, such as *Cd36*, *Fabp1*, *Fabp2*, *Apoa1*, and *Apoa4*, in the ileal epithelia, indicating that certain kinds of commensal bacteria capable of inducing IL-23 and IL-22 production from immune cells can downregulate lipid absorption in the small intestine. This result is compatible with a previous report showing that activated ILC3 in *Rag1*$^{-/-}$ mice inhibits the expression of genes related to lipid absorption through the production of IL-22 (Mao et al., 2018). Meanwhile, another paper showed that *in vivo* treatment with recombinant IL-22 reduces the serum concentrations of triglyceride and cholesterol

in mice fed a high-fat diet (HFD) (Wang et al., 2014). Therefore, future work should examine the concentrations of triglyceride, cholesterol, and free fatty acids in the portal and peripheral blood of germ-free, *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice fed an HFD.

In comparison with the levels in ileal epithelia, a set of genes involved in cell proliferation were downregulated in the colonic epithelia by deficiency of either IL-23 or IL-22, which is almost independent of commensal bacteria. Although several studies have demonstrated that IL-22 promotes the proliferation of intestinal epithelial cells, particularly in the case of mucosal damage and cancer (Hanash, Dudakov, Hua et al., 2012; Kryczek, Lin, Nagarsheth et al., 2014; Lindemans et al., 2015), the molecular mechanism involved has remained unclear. Our gene expression analysis showed that the expression of Cyclin genes and *Cdk1* gene was downregulated in colonic epithelia of *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice, which may be a cause of the delayed cell cycle progression of intestinal epithelial cells in such mice. In addition, the expression of *Sfn* coding Stratifin/14-3-3σ, which reportedly suppresses the cell cycle (Gardino, & Yaffe, 2011), was significantly higher in the colonic epithelia of *Il22*$^{-/-}$ and *Il23*$^{-/-}$ mice, but not in the ileal epithelia, indicating that the IL-23/IL-22 axis inhibits Stratifin/14-3-3σ expression particularly in the large intestine. IL-22 is reported to promote the progression of colon cancer (McCuaig, Barras, Mann et al., 2020; Perez, Kempski, McGee et al., 2020; Wang, Gong, Sheh et al., 2017). Meanwhile, several recent reports have suggested that Stratifin/14-3-3σ functions as a tumor suppressor in colorectal cancer (Winter, Rokavec, & Hermeking, 2021; Young, Radhakrishnan, Centuori et al., 2015). These previous and current findings suggest that the downregulation of Stratifin/14-3-3σ by the IL-23/IL-22 axis may be one of the causes of colon cancer progression.

IL-23 and IL-22 from immune cells are well known to have an important function in tissue repair and defense against pathogens in both the small and large intestines (Keir et al., 2020). The current study clearly highlights that the IL-23/IL-22 axis is predominantly dependent on gut microbiota in the small intestine, but it is much less dependent on gut microbiota in the large intestine. Besides the above well-known functions, this study shows the intestinal site-specific functions of the IL-23/IL-22 axis on epithelial cells: the regulation of lipid absorption in the small intestine and the promotion of epithelial cell proliferation in the large intestine. Since intestinal IL-23 and IL-22 are known to be involved in the pathogenesis of various diseases such as inflammatory bowel diseases, colorectal cancer, and metabolic disorders, further analyses to elucidate the molecular mechanism underlying the intestinal site-specific functions of IL-23 and IL-22 should be conducted in the future.

Experimental procedures

Mice

C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). Pseudopregnant ICR female mice were purchased from CLEA Japan Inc. (Tokyo, Japan). *IL23*$^{-/-}$ mice were generated as described previously (Kitada, Kayama, Okuzaki et al., 2017). *Il22*$^{-/-}$ mice were generated using the CRISPR/Cas9 system. Age- and sex-matched mice were used for the experiments. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Osaka University.

Generation of *Il22*$^{-/-}$ mice using CRISPR/Cas9 system

To generate *Il22*$^{-/-}$ mice, single guide RNA (sgRNA) target sequences for the upstream of *Il22* exon 1 (5′-GACGAACATGCTCCCCTGAT-3′) or within that exon (5′-ATGCGCTGCCCGTCAACACC-3′) were designed using the CRISPRdirect suite (https://crispr.dbcls.jp/), selecting for minimal 12-mer and 8-mer scores to reduce off-target risk (Fig. S1). Purified crRNAs containing the 20-mer target sequences along with the tracrRNA (Merck, Darmstadt, Germany) were assembled into a ribonucleoprotein (RNP) complex with Cas9 protein (Thermo Fisher Scientific, Waltham, MA, USA). The RNPs were electroporated into embryos of C57BL/6J mice using a NEPA21 Super Electroporator (Nepa Gene, Chiba, Japan). The embryos were cultured overnight to the two-cell stage before being transferred into the oviducts of pseudopregnant ICR female mice. Founder mutations in pups born were identified by Sanger sequencing. Mice were genotyped by PCR using the following primer set:

forward primer 5′-GGATCCCCCGATGGCTATAAAAGCAGC -3′ and reverse primer 5′-GAATTCTTCTAAATGCGCCTCTGTAGA -3′ for *Il22*$^{-/-}$.

RNA-seq analysis of intestinal epithelial cells

To isolate small- and large-intestinal epithelial cells from mice, each part of the intestines was opened longitudinally and washed with PBS to remove feces. The washed intestine was shaken in Hanks' Balanced Salt Solution (HBSS) containing 5 mM ethylenediaminetetraacetic acid (EDTA) at 37°C for 20 min to detach epithelial cells. After removing the intestinal tissues, the suspended epithelial cells were centrifuged at 780 × *g* and 4°C for 5 min and then washed with PBS. For the extraction of total RNA, the isolated epithelial cells were resuspended with TRI reagent (Merck). Total RNA was extracted from cells with a miRNeasy Micro kit (Qiagen) following the manufacturer's instruction. Library preparation was performed using the TruSeq Stranded mRNA sample prep kit (Illumina, San Diego, CA), according to the manufacturer's instructions. Sequencing was performed on an Illumina NovaSeq 6000 sequencer (Illumina) in 101-base single-end mode. Sequenced reads were mapped to the mouse reference genome sequence (mm10) with TopHat version 2.1.1 in combination with Bowtie2 ver. 2.2.8 and SAMtools ver. 0.1.18. Fragments per kilobase of exons per million mapped fragments (FPKMs) were calculated using Cufflinks version 2.2.1. The present RNA-seq data (GSE #236445) and our previous RNA-seq data (GSE #160379), which are the gene expression profiles of intestinal epithelial cells from SPF and germ-free mice [15], are collocated in this study. The read count data were analyzed using iDEP.96 (http://bioinformatics.sdstate.edu/idep96/) (Ge et al., 2018; Ge, 2021),

hierarchical clustering and principal component analysis were performed, and differentially expressed (DE) genes (FDR<0.1) were selected by DESeq2 (Love, Huber, & Anders, 2014). Heatmaps of the gene expression levels were generated based on the FPKM values using GraphPad Prism version 9.4.0 (GraphPad Software, San Diego, CA, USA). Volcano plots showing the change of gene expression based on DESeq2 analysis were generated using EnhancedVolcano R package (version1.18.0).

Data availability

The present RNA-seq data (GSE #236445) are deposited in the Gene Expression Omnibus (GEO). These data and our previous RNA-seq data (GSE #160379), which are the gene expression profiles of intestinal epithelial cells from SPF and germ-free mice (Tani, Li, Kusu et al., 2021), are collocated in this study.

Author contributions

A.N. analyzed the data and wrote the manuscript. R.O. planned and directed the research, analyzed the data, and wrote the manuscript. A.I., S.O., K.S., and Y.I. isolated intestinal epithelial cells from mice. D.O. purified RNA from intestinal epithelial cells and performed next-generation sequencing. K.T. planned and directed the research, and reviewed and edited the manuscript.

Acknowledgments

We thank Y. Magota for technical assistance, Edanz Group for editing a draft of this manuscript, and C. Hidaka for secretarial assistance. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan [JP18K15187 (RO), JP21H050430 (KT)], the Japan Agency for Medical Research and Development [JP21gm1010004 (KT)], and the Uehara Memorial Foundation (RO).

Declaration of interests

The authors declare no competing interests.

Figure legends

Figure 1.

(A and B) Heatmaps of most variable genes (top 2000) in the ileal epithelia (A) and colonic epithelia (B) by hierarchical clustering among wild-type (WT) mice under specific pathogen-free (SPF) and germ-free (GF) conditions, and *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice (n = 2: 1 male and 1 female per each group). **(C and D)** Principal component analysis of transcriptomes in the ileal epithelia (C) and colonic epithelia (D) among WT (SPF), WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice (n = 2: 1 male and 1 female per each group).

Figure 2.

(A and B) Venn diagrams indicating the numbers of upregulated (A) and downregulated (B) differentially expressed genes (DEGs) in the ileal epithelia of WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice (n = 2: 1 male and 1 female per each group). **(C and D)** Venn diagrams indicating the numbers of upregulated (C) and downregulated (D) DEGs in the colonic epithelia of WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice. **(E–G)** Volcano plots of DEGs in the ileal epithelia of WT (GF) (E), *Il22*$^{-/-}$, (F) and *Il23*$^{-/-}$ (G) mice in comparison with WT (SPF) mice. **(H–J)** Volcano plots of DEGs in the colonic epithelia of WT (GF) (H), *Il22*$^{-/-}$, (I) and *Il23*$^{-/-}$ (J) mice in comparison with WT (SPF) mice.

Figure 3.

(A and B) Heatmaps of most variable genes (top 2000) in the ileal epithelia (A) and colonic epithelia (B) with K-means clustering (k=4) among WT (SPF), WT (GF),

Il22$^{-/-}$, and *Il23*$^{-/-}$ mice (n = 2: 1 male and 1 female per each group). Gene ontology terms enriched in each cluster and false discovery rate for each term are shown.

Figure 4.

(A and B) Heatmaps of the expression levels of epithelial barrier-related genes (FPKM value > 2.0 in all groups) in the ileal (A) and colonic (B) epithelia among WT (SPF), WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice (n = 2: 1 male and 1 female per each group). FC: fold change. **(C and D)** Heatmaps of the expression levels of chemokine genes (FPKM value > 2.0 in all groups) in the ileal (C) and colonic (D) epithelia among WT (SPF), WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice (n = 2: 1 male and 1 female per each group). **(E)** Schematic diagram showing the lipid absorption in the intestinal epithelial cells. Abca1: ATP-binding 4 cassette, sub-family A (ABC1), member 1, Abcg5/8: ATP-binding cassette sub-family G member 5/8, Acat: acetyl-Coenzyme A acetyltransferase, Apgat: 1-acylglycerol-3-phosphate O-acyltransferase, Apoa1: apolipoprotein A-I, Apoa4: apolipoprotein A-IV, Apob: apolipoprotein B, CE: cholesterol esters, CL: cholesterol, Dgat: diacylglycerol O-acyltransferase, ER: endoplasmic reticulum, FA: fatty acid, Fabp: fatty acid binding protein, HDL: high-density lipoprotein, Mgat: mannosyl (alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase, Mttp: microsomal triglyceride transfer protein, Npc1l1: NPC1-like intracellular cholesterol transporter 1, and Slc27a4: solute carrier family 27, member 4. **(F)** Heatmap of the expression levels of lipid absorption-related genes in the ileal epithelia among WT (SPF), WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice (n = 2: 1 male and 1 female per each group). CM (AP): cell membrane (apical), CP: cytoplasm, and CM (BS): cell membrane (basal).

Figure 5.

(A) Schematic diagram showing the regulation of cell cycle and cell division. Cdc25B/C: cell division cycle 25B/C, CDK1: cyclin-dependent kinase 1, ESCO1/2: establishment of sister chromatid cohesion N-acetyltransferase 1/2, P: phosphoric acid, PDS5A/B: PDS5 cohesin associated factor A/B, RAD21: RAD21 cohesin complex component, Sfn: stratifin, SMC3: structural maintenance of chromosomes 3, STAG1/2: stromal antigen 1/2

(B–D) Heatmap of the expression levels of cell cycle-related genes in the G_2–M (B) and G_1–S phases (C and D) in the colonic epithelia among WT (SPF), WT (GF), *Il22*$^{-/-}$, and *Il23*$^{-/-}$ mice (n = 2: 1 male and 1 female per each group).

Supplementary Material

Figure S1. Generation of *Il22*$^{-/-}$ mice using the CRISPR/Cas9 system.

Schematic diagram of the *Il22* locus with the gRNA target sequences for the generation of IL-22-deficient mice, and the results of PCR genotyping of wild-type (+/+), *Il22*$^{+/-}$ (+/–), and *Il22*$^{-/-}$ (–/–) mice are shown.

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