The Role of Estrogen Related Receptor alpha (ERRα) in Intestinal Homeostasis and Inflammatory Bowel Diseases

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ASTRACT

Inflammatory bowel diseases (IBD) are a group of chronic idiopathic inflammatory disorders of the gastrointestinal tract characterized by alternating phases of relapse and remission. The two main forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). While the etiology of IBD remains elusive, it is thought to arise from an inappropriate immune response to the intestinal microbiota in genetically susceptible individuals. The estrogen-related receptors (ERRs) ERRα, ERRβ and ERRγ are founding members of the orphan nuclear receptors subgroup of the nuclear receptors (NR) superfamily. ERRα is constitutively expressed in metabolic tissues including the intestine. It acts at the intersection of cellular metabolism, oncogenesis, and immunity. Among its transcriptional targets are genes involved in mitochondrial activity and biogenesis, fatty acid metabolism, brown adipose tissue thermogenesis, glucose and fatty acid transport, autophagy, mitophagy, and inflammatory signaling. The transcriptional activity of ERRs is dependent on the co-activators PGC-1α and PGC-1β (Peroxisome proliferator activator receptor [PPAR]γ coactivator-1 (PGC-1). A previous study showed that PGC1α$^{\Delta IEC}$ mice, which lack PGC-1α specifically in intestinal epithelial cells (IECs), displayed increased susceptibility to experimental colitis induced by dextran sulfate sodium (DSS). In this project, we aimed at exploring the role of ERRα in intestinal homeostasis and IBD. Our hypothesis was that, akin to PPARγ, a therapeutic target in IBD (with the PPARγ agonist Rosiglitazone demonstrating some clinical efficacy in mild to moderate UC in a phase 2 clinical trial), ERRα might act as an important effector of intestinal homeostasis. In this thesis, we demonstrated that ERRα deficiency in mice resulted in increased susceptibility to experimental colitis induced with the dextran sulfate sodium (DSS) salt. We hope that our results will lead to exploring synthetic ERRα agonists as potential treatment for intestinal inflammation and IBD.

RÉSUMÉ

Les maladies inflammatoires chroniques de l'intestin (MICI) sont un groupe de troubles inflammatoires idiopathiques chroniques du tractus gastro-intestinal caractérisés par une alternance de phases de rechute et de rémission. Les principales formes de MICI sont la maladie de Crohn (MC) et la colite ulcéreuse (CU). Bien que l'étiologie des MICI reste incertaine, on pense qu'elles sont dues à une réponse immunitaire inappropriée au microbiote intestinal chez des individus génétiquement sensibles. Les récepteurs liés aux œstrogènes (ERR) ERRα, ERRβ et ERRγ sont les membres fondateurs du sous-groupe des récepteurs nucléaires orphelins de la superfamille des récepteurs nucléaires (NR). ERRα est exprimé de manière constitutive dans les tissus métaboliques, y compris l'intestin. Il agit à l'intersection du métabolisme cellulaire, de l'oncogenèse et de l'immunité. Parmi ses cibles de transcription figurent des gènes impliqués dans l'activité et la biogenèse mitochondriales, le métabolisme des acides gras, la thermogenèse du tissu adipeux brun, le transport du glucose et des acides gras, l'autophagie, la mitophagie et la régulation de l'inflammation. L'activité transcriptionnelle des ERR dépend des co-activateurs PGC-1α et PGC-1β (Peroxisome proliferator activator receptor [PPAR]γ coactivator-1 (PGC-1). Une étude précédente a montré que les souris PGC1α$^{\Delta IEC}$, qui ne possèdent pas de PGC-1α spécifiquement dans les cellules épithéliales intestinales (IEC), présentaient une sensibilité accrue à la colite expérimentale induite par le sulfate de dextrane-sodium (DSS). Dans ce projet, nous avons proposé d'explorer le rôle de ERRα dans l'homéostasie intestinale et les MICI. Notre hypothèse fut qu'à l'instar de PPARγ, une cible thérapeutique dans les MICI (l'agoniste de PPARγ, la rosiglitazone, ayant démontré une certaine efficacité clinique dans la rectocolite hémorragique (RCH) légère à modérée lors d'un essai clinique de phase 2), ERRα pourrait agir comme un important effecteur de l'homéostasie intestinale. Dans cette thèse, nous démontrons que le déficit en ERRα chez la souris entraîne une susceptibilité accrue à la colite induite par le DSS. On espère que ces résultats conduiront à l'utilisation d'agonistes synthétiques de ERRα pour le traitement de l'inflammation intestinale et des MICI.

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Contribution of Authors

Maya Saleh: Primary supervisor; provided guidance and direction for my project; edited the thesis and manuscript

Mario Songane: Conducted some preliminary data of the project

Claudia Champagne: Assisted with the immunofluorescence experiments, and RNA isolation from IECs

Luc Galarneau: Conducted bioinformatic analysis for RNA-sequencing, and microbiome DNA-sequencing

Nassima Fodil: Assisted with the bone-marrow chimera experiment

Vincent Giguère: Member of my advisory committee and provided input on the work

John White: Member of my advisory committee

Carlo Ouellet: *Esrra*$^{-/-}$ mice husbandry and genotyping

ABBREBIATIONS

ACTR	Activator of thyroid and retinoic acid receptor
ATG	Autophagy-related gene
AF	Activating function
BAT	Brown adipose tissue
CD	Crohn's disease
CTE	COOH-terminal extension
DAX	X chromosome gene-1
DBD	DNA binding domain
NTD	N-terminal domain
ER	Endoplasmic reticulum
ERR	Estrogen-related receptor
ERRE	Estrogen-related response element
FUNDC	FUN14 domain-containing protein
eQTL	Expression quantitative trait loci
GRIP	Glucocorticoid receptor interacting protein
GSDMD	Gasdermin D
GWAS	Genome-wide association studies
HRE	Hormone response element

IBD	Inflammatory bowel disease
IRGM	Immunity related GTPase M
LBD	Ligand binding domain
LD	Linkage disequilibrium
LDF	Lineage defining factor
LRH	Liver receptor homolog
NCoR	Nuclear receptor co-repressor
NLS	Nuclear localization sequence
NOD	Nucleotide-binding oligomerization domain-containing protein
NR	Nuclear receptor
NRF	Nuclear respiratory factor
NTD	N-terminal domain
OR	Odds ratio
OPTN	Optineurin
PPAR	Peroxisome proliferator activator receptor
PF	Pioneer factors
PGC	Peroxisome proliferator-activated receptor coactivator
PINK	PTEN-induced kinase
PTM	Post-translational modification

PTEN	Phosphatase and tensin homolog
RAR	Retinoic acid receptor
RIP	Receptor-interacting protein
RXR	Retinoid X receptor
SF1	Steroidogenic factor
SHP	Small heterodimer partner
SNP	Single nucleotide polymorphisms
SRC	Steroid hormone receptor
TBK	TANK-binding kinase
TF	Transcription factor
THR	Thyroid hormone receptor
UC	Ulcerative colitis
ULK	Unc-51-kinase
VDR	Vitamin D receptor
WGS	Whole-genome sequencing
WES	Whole-exome sequencing
XBP	X-box binding protein

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CHAPTER I – LITERATURE REVIEW

1.1 Nuclear receptor superfamily

The nuclear receptor (NR) superfamily is group of transcription factors (TFs) comprising of 48 human members (1) involved in diverse biological processes including cell cycle, development, metabolism, inflammation and reproduction (2-5). Typically, activation of NRs is mediated through binding to their cognate lipid-soluble hormones and signals such as steroids, retinoids and phospholipids (6). However, ligands for several NRs remain to be identified classified as 'orphan receptors' but some NRs can act without a bound ligand (7). The lipid-solubility of NR-associated ligands allows them to diffuse through cellular lipid bilayers, thus, these ligands can interact with their respective NRs within the cytoplasm and/or nucleus as reviewed in (8). When ligand-bound, NRs undergo an active conformational change as monomers, homodimers, or heterodimers and recognize DNA sequences termed hormone response elements (HREs) with consensus (5'-RGGTCA-3'; R is a purine) (9, 10). Subsequently, the NR complex recruits co-regulator proteins, chromatin remodelers, and the general transcriptional machinery at the promoter site to drive transcription of target genes (11). In contrast, NRs bound to antagonists induces a receptor conformational change that facilitates corepressor binding to repress transcriptional activity (11, 12). Since aberrant activity of NRs is reported in numerous diseases including cancer, diabetes, and chronic inflammation, development of synthetic ligands to modulate NR activity has been a focus of the pharmaceutical industry (13). Although NRs are ubiquitously expressed, their ability to regulate different genes in a cell-specific manner provides distinct functions within those cells (14, 15). The nature of selective gene regulation is defined by cell-specific enhancer selection conferred by lineage-determining factors (LDF) also known as pioneer factors (PFs). Mechanistically, PFs function to open up chromatin to allow for binding of TFs, thereby, augmenting the rate of transcription (16-18). NRs are classified into 7 subfamilies (Type 0 – Type 6) through sequence alignment and phylogenetic relationships (Table 1.1) (19).

1.1.1 Nuclear receptors mechanisms of action

Nuclear receptors can also be divided into four subtypes (I-IV) based on their mechanism of action and subcellular localization in the absence of their cognate ligand presented in **(Figure 1.1)** (2). Type I receptors comprise steroid receptors (SRs) that bind cholesterol-derived steroidal hormones and that are confined in the cytoplasm by chaperone proteins. They include the androgen receptor, glucocorticoid receptor, progesterone receptor, and estrogen receptors (20). Ligand binding of receptors leads to the dissociation of chaperones and exposes the nuclear localization sequence (NLS), thus, allowing for translocation to the nucleus. Once in the nucleus, type I receptors typically act as homodimers and associate with co-activator proteins to bind to DNA HRE consisting of two inverted repeats **(Figure 1.1)** (21, 22). In contrast, Type II receptors including retinoic acid receptor (RAR), retinoid X receptor (RXR), and thyroid hormone receptor (THR) (20, 23) are sequestered in the nucleus, and heterodimerize with retinoid X receptor (RXR) regardless of ligand binding state (24). In the absence of a ligand, they cooperate with NCoR and SMRT corepressor proteins and repress transcriptional activity of their target genes (25). Upon ligand binding, type II receptors dissociate from the corepressor complex in exchange for co-activators and transcriptional machinery to bind to DNA HREs of direct repeats **(Figure 1. B)** (2). Similarly, Type III receptors such as vitamin D receptor (VDR) function in the same manner as Type II receptors except that they bind to DNA HRE of direct repeat sequences as homodimers (26). Finally, Type IV receptors comprising but not limited to liver receptor homolog-1 (LRH-1), and steroidogenic factor 1 (SF1) also function similarly to Type II receptors but bind as monomers on DNA HREs of extended half sites (27, 28).

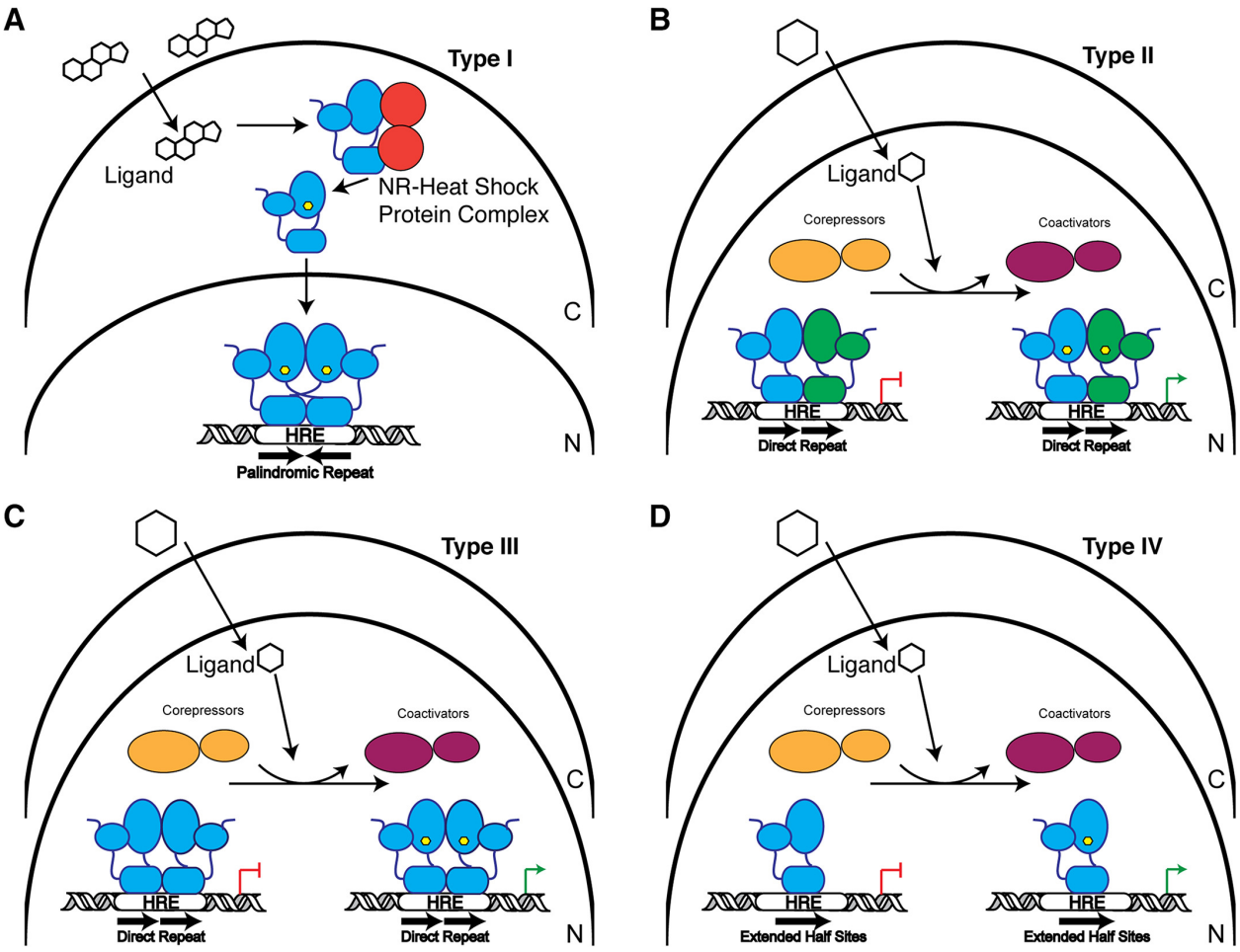


Figure 1.1 NR subtypes based on their signaling mechanisms. Type I receptors anchored in the cytoplasm by heat shock proteins. Ligand binding dissociates NR-heat shock protein complex mediating nuclear translocation and binding to palindromic repeats as homodimers **(A)**. Type II-V receptors reside in the nucleus and coupled to corepressors. Ligand binding results in the exchange of corepressors by coactivators and subsequently binding to direct repeats as heterodimers in Type II receptors **(B)** and homodimers in Type III receptors **(C)**. Type IV receptors bind as monomers on extended half sites **(D)**. Schematic adapted from (29).

1.1.2 Nuclear receptor co-regulators

The regulation of NR to mediate transcription of specific signaling pathways is controlled by associated coregulator complexes (30). These coregulators when complexed with NRs modulate

transcriptional activity by upregulating or downregulating the rate of target gene expression (30). To date, over 350 nuclear coregulator proteins exist (http://www.NURSA.org) that are categorized into coactivators or corepressors. These coregulators interact with AF-1 and AF-2 interfaces of NRs. However, ligand-dependent recruitment of coregulators only occurs via AF-2, while AF-1 relies on PTMs (31). Furthermore, the presence (holo-state) or absence (apo-state) of a bound ligand will determine the conformation of the AF-2, therefore, defining the specificity of NR recruitment (31). In general, ligand binding causes a conformational change in AF-2 by stabilizing the C-terminal helix (H12) into a fixed position, which forms the hydrophobic groove for coactivator interactions (32). This AF-2 structure allows for the interaction of coactivators through a conserved LxxLL motif, also known as the NR-box (33). Coactivator recruitment leads to the scaffolding of large multiprotein complexes containing chromatin remodeling and histone modifying enzymes (34). These factors facilitate the opening of chromatin, and sequentially or in a combinatorial manner, recruit further coregulators, transcriptional machinery, and RNA polymerase II to the accessible sites (35). Furthermore, coactivators that directly interact with AF-1 and AF-2 interfaces of NRs, are also known as 'primary' coactivators, which cooperate with 'secondary' coactivators to mediate transcriptional activity at the promoter regions (36). The most common coactivators for NRs are p160 family members, SRC-1, GRIP1/TIF/SRC-2, and pCIP/AIB1/SRC-3 (37). Conversely, unliganded or antagonist-bound receptors comprise an AF-2 with a flexible H12 conformation (38). This structure, in opposition to ligand-bound NRs, favours corepressor interaction via the motif (LxxH/IIxxxI/L), also termed as the CoRNR-box (30). Co-repressor interactions promote the recruitment of modifying enzymes that limit the chromatin opening, and access of transcriptional scaffolds, and thereby, mediating the repression of transcriptional activity (39). This is mediated by the recruitment of histone deacetylase enzymes such as HDAC1-HDAC7 and SIRT1 (40-42). This concept of ligand-bound, or unliganded/ antagonist-bound recruitment of coactivators and corepressors, respectively, was eventually challenged by the discovery of RIP140, which acts as a ligand-bound corepressor. Eventually, more ligand-dependent corepressors were found as reviewed in (43). Apart from ligand-dependent transcriptional regulation, NR activity can be controlled by its binding to 'positive' or 'negative' response elements, to either recruit coactivators, or corepressors, respectively (11, 44).

1.2 Orphan nuclear receptors

Apart from classical ligand-dependent NRs including steroids hormones, thyroid hormones and retinoic acid receptors, NR superfamily includes a subfamily of orphan receptors for which no ligand has been found since the time of their discovery. However, once the ligand(s) is identified, such NRs are referred to as an "adopted orphans".

1.2.1 Estrogen-related Receptor alpha (ERRα)

In 1988, the estrogen-related receptor alpha (ERRα; NR3B1) was the first to be identified within the orphan NR classification, and along with ERRβ within the ERR subfamily (45). Along with ERRβ, ERRα was cloned by screening recombinant cDNA libraries using the DNA-binding domain (DBD) of estrogen-receptor-alpha (ERα) as a hybridization probe (45). A decade later, a third ERR isoform was identified and referred to as ERRγ, thus, completing the ERR subgroup (46). Expression profiles show that ERRα is ubiquitously expressed but enriched in adult tissues with high oxidative capacity such as brown adipose tissue, heart, and intestine (15). Based on sequence homology, ERRs cluster into the NR subfamily group 3 of steroid hormone receptors. In relation to ERα/β, ERRα share 70%, and 36-37% amino acid sequence homology in their DBD and LBD, respectively (47). Despite their relatedness, ERRα does not bind to the ligand(s) of estrogen receptors (ERs) and E2-like molecules, nor engages in classic ER signaling pathways and biological processes (48). In fact, the natural endogenous ligand(s) of ERRα remains to be identified (48). While most steroid receptors are activated by the presence of a ligand, ERRα transcriptional activity is modulated by interaction with coregulators. The constitutive activity of ERRα depends on the presence of coactivators, notably, peroxisome-proliferator activated receptor g coactivator-1a (PGC-1α) and peroxisome-proliferator activated receptor g coactivator-1b (PGC-1β) (49-51).

Functionally, ERRα regulates transcriptional target genes as monomers, homodimers or heterodimers consisting of two different isoforms by binding to the consensus extended half-site (TNAAGGTCA), originally identified for steroidogenic factor 1, and currently known as the estrogen-related response element (ERRE) (52-55). ERRα monomer or dimer binding is

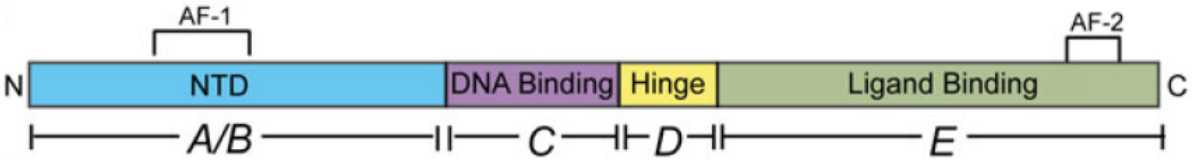
determined by the nucleotide base at N position, where C or G favours monomer binding, and A or T dimer binding (56). Although ERRα and ERα vastly regulate distinct genes, a small subset of target genes are shared by both NRs (52, 57). This is provided by the ability of ERRα to bind as a monomer or dimer to estrogen-response elements (ERE) containing the inverted repeat recognition motif (AGGTCAnnnTGACCT) (52, 58), with an ERRE embedded within the sequence (59, 60). As such, ERRα and ERα display transcriptional crosstalk in regulating such overlapping genes (57, 61-63). Moreover, ERRα and ERα can act in synergy by forming heterodimers, or as competitors for shared target genes (62, 64). The ERRα-PGC-1 interaction acts regulates genes involved in mitochondrial activity and biogenesis, fatty acid metabolism, brown adipose tissue thermogenesis, glucose and fatty acid transport, autophagy, mitophagy, and transcriptional induction of anti-inflammatory genes (65-67). ERRα bound to DNA can exerts both stimulatory or repressive activity of target genes depending on the cell-type (presence of coregulators, corepressors, and other TFs), response element, the promoter-context, and post-translational modifications of the receptor (47, 68).

1.2.2 Architecture of Nuclear Receptors and of ERRα

NRs share a common structure except for the atypical group small heterodimer partner (SHP) and X chromosome gene-1 (DAX) (29). Their fundamental architecture consists of five to six domains (A to F) as follows: beginning from the N-terminus is the highly variable N-terminal domain (NTD) (A/B domain; also referred to as activation function 1 (AF-1)), followed by a conserved DNA-binding domain (DNA binding domain; the C domain), a flexible hinge region (D domain) for subdomain linkage, and at the C-terminus ending with a ligand binding domain (LBD; E domain) shown in **(Figure 1.2. A)** (29). Activation function (AF) sites bind to co-regulators to control transcriptional activity of NRs. Therefore, transcriptional activation of NRs is dependent on the status and conformation of these binding sites for co-activators (69). Firstly, AF-1 contained within the NTD confers weak ligand-independent transcriptional activation, and its activity is constitutive via post-translational modifications (PTMs) (69). In contrast, the AF-2 resides in the LBD and possesses ligand-dependent function. When bound by a ligand, AF-2 undergoes a conformational change to facilitate the interaction with co-activators (69). Additionally, AF-1

synergizes with AF-2 to produce robust upregulation of gene expression (70). Some receptors also consist of a highly variable C-terminal F-region (E domain), with an unknown function (29). ERRα follows the prototypic structure (A-F domains) as depicted in **(Figure 1.2. B)**. The following sections describe the roles and functionalities of each domain in ERRα.

A.



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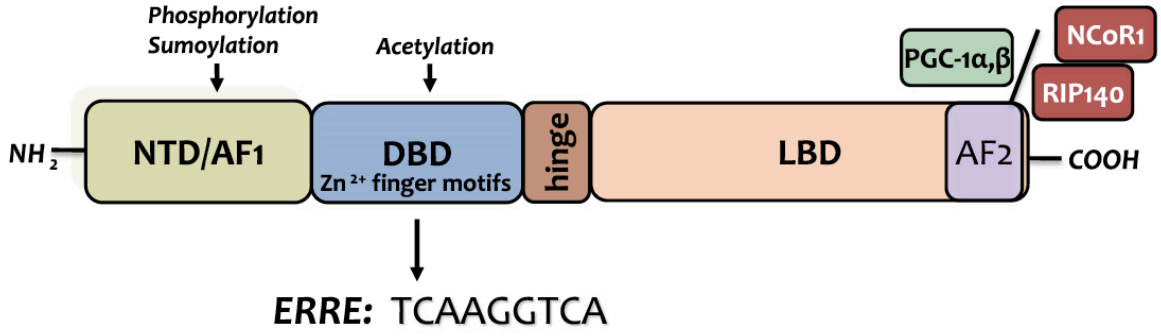

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Figure 1.2 Structure of Nuclear Receptor and subclass estrogen-related receptors. Representation of the general structure and its subdomains of NRs **(A)** and estrogen-related receptor **(B)**. NTD, N-terminal domain; DBD, DNA binding domain; LBD, ligand binding domain; AF, activating function; PGC-1, peroxisome proliferator-activated receptor coactivator-1; NCoR, nuclear receptor co-repressor, RIP140, receptor-interacting protein 140; ERRE, estrogen-related receptor response element. Schematic adapted from (29, 71).

1.2.1.1 N-terminal domain (NTD)

The NTD is a poorly conserved region across NRs as observed with ERRs shown by low sequence homology in all three isoforms **(Figure 1.2. B)** (69). However, AF-1 harboured by NTD are in fact conserved, and subject to post-translational modifications, specifically, phosphorylation and SUMOylating to regulate transcriptional activity (72).

1.2.1.2 DNA binding domain (DBD)

The DBD is the most conserved subdomain of NRs demonstrated by its high sequence homology among all ERR isoforms **(Figure 1.2. B)** (69). It binds DNA to activate gene transcription. Structurally, the DBD consists of three helices, two of which contain a zinc finger motif (N-terminal helix, and C-terminal helix), and COOH-terminal extension (CTE) helix. Although CTE belongs to the hinge region, it is considered to be an extension of the DBD due to its capacity to directly bind to DNA **(Figure 1.3. A)** (73). Therefore, the CTE is discussed in this section. Each zinc motif consists of 4 conserved cysteine residues, tetrahedrally arranged to chelate a zinc atom to provide structural stability of the DBD (74). The N-terminal helix and C-terminal helix each contain a zinc finger motif, also known as P box, and D box, respectively **(Figure 1.3. A)**. Generally, the N-terminal helix interacts at the major groove of DNA, and its P box determines the binding specificity of the NRs to DNA HREs (75-79). The C-terminal helix forms non-specific interactions with the DNA backbone, and its D box acts as a receptor dimerization interface (69, 78, 80, 81). Lastly, the CTE flanks the DBD and contains T Box and A box for receptor dimerization, and to make base-specific minor groove interactions to enhance receptor-DNA binding, respectively (27, 53, 77, 82-84). To date, of all three ERR isoforms, only ERRβ has a crystal structure of the receptor bound to DNA. In this report, the CTE of ERRβ acts as a motif to enhance DNA-binding stability providing the basis of ERRs' high affinity to monomerically bind to ERREs (53). In the context of ERRα, deacetylation of the four lysine residues of zinc motifs and the CTE by the deacetylases SIRT1 and HDAC8 increase its DNA binding affinity (85).

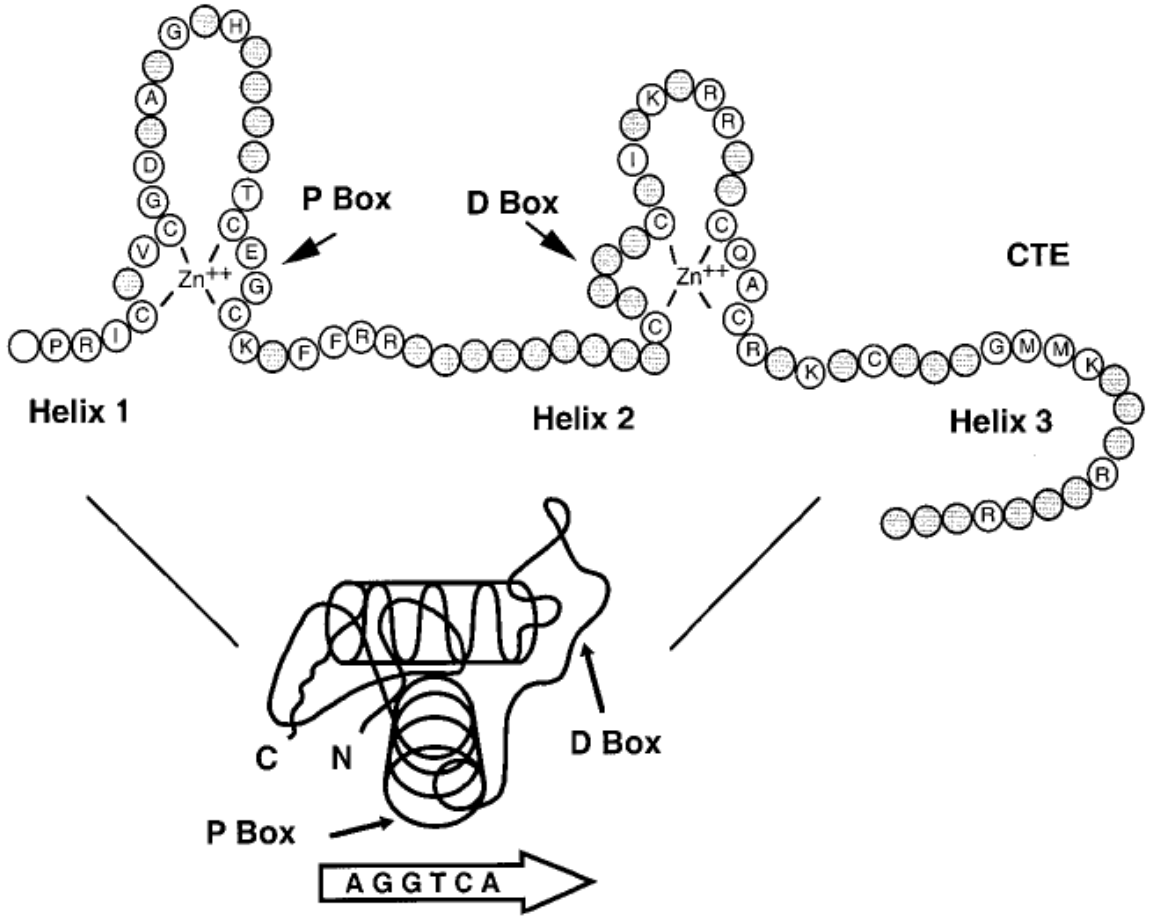

Figure 1.3 Diagram the DNA binding domain (DBD) and the COOH-terminal extension (CTE). This domain comprises three helices. Helix-1 (N-terminal helix) and helix-2 (C-terminal helix) each contain a zinc finger known as P box and D box, respectively. Generally, the P box is involved in basic-specific DNA interactions, while the D box acts as an interface for protein-protein dimerization. The CTE contains the T box and A box for dimerization, and further DNA contacts to enhance stability. The bottom panel illustrates the 3-D structure of the core DBD. Adapted from (86).

1.2.2.3 Hinge region

The hinge region (D domain) is the least conserved domain that acts as a flexible short protein bridge between the DBD and LBD (87). The flexibility of the hinge domain establishes and maintains the integrity of dimeric NR structures, and allows for the synergy between AF-1 and AF-2 (88, 89). Since NRs are found in both the cytoplasm and nucleus as discussed in **(Section**

1.1.1), the subcellular distribution is regulated by the nuclear localization signal contained in the hinge region (90-93). While nuclear localization sequence (NLS) of ERRα remains to be reported, its cellular localization is regulated by kinesin family member 19 (KIF17) (94). Binding of ERRα LBD to the KIF17 tail has been reported as essential for inhibiting nuclear translocation of endogenous ERRα in breast cancer cells (94). Furthermore, receptor-mediated transactivation and transrepression can be regulated by PTMs of the hinge domain, thereby, affecting DNA recognition, receptor dimerization, recruitment of coregulators, and ligand sensitivity (95-97). In the context of ERRα, it has been reported that the acetylation status of the hinge region modulates transcription by affecting its DNA-binding activity (85).

1.2.2.4 Ligand binding domain (LBD)

The C-terminal ligand binding domain (LBD) also known as the E-region is the most conserved domain among NRs. It is multifunctional site for ligand binding, an interface for receptor dimerization and interactions with heat-shock proteins and coregulators (38, 98). However, its primary function is ligand-dependent conformational change for the interaction with coregulators to mediate transcriptional activity (69). Structurally, the LBD is folded into three-layered anti-parallel sandwich comprised of 12 conserved alpha-helices (H1-H12), and beta-sheets (S1-S2) between H5 and H6 (69). Within the LBD is a hydrophobic ligand-binding pocket (LBP) with amino acids in helices 3, 5, and 10/11 involved in the interaction with cognate ligands (99). Moreover, the LBD contains a transactivation domain (AF-2) consisting of H3, H4, and H12. Upon ligand binding, H12 also known as the activation function helix undergoes a conformational change to complete the hydrophobic groove with H3, H4, and H5 (69, 98). This orientation of AF-2 facilitates the interaction with LXXLL signature motifs found in all primary NR coactivators (32). In contrast, NR transrepression mediated by the binding of an antagonist prevents the proper folding of H12 and favours the recruitment of corepressors (100-102). In the context of ERRα, the recruitment of coactivators does not necessitate ligand-binding to mediate transcriptional activation. This is due to amino acids with bulky side chains in the LBD that occupy the LBP, mimicing the ligand-bound form of classical NRs. This results in an active AF-2 conformation, facilitating the interaction with coactivators (51, 103, 104).

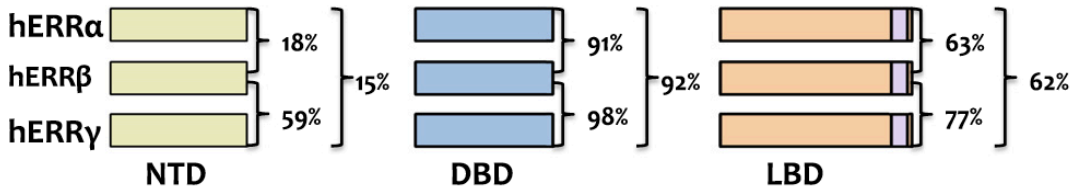

Figure 1.4 ERR sequence homology. Human ERR sequences and their degree of homology within each subdomain. NTD, N-terminal domain; DBD, DNA binding domain; LBD, ligand binding domain; hERR, human estrogen-related receptor. Illustration adapted from (71).

1.2.3 ERRα coregulators

As discussed above, the transcriptional regulation of NR is dependent on coregulator interactions mediated by PTMs in AF-1, and ligand-binding status of AF-2. To date, the natural ligand(s) of ERRs remain to be identified. Indeed, it is thought that ERRα is constitutively active with coactivators in the absence of ligands (103, 104). In fact, the agonist-independent mechanism of ERRα is described to function through the occupancy of bulky side chains of Phe^{328} in the ligand-binding pocket (LBP), thereby, inducing an active conformation of the LBD to interact with coactivator PGC-1a (104). Conversely, the crystal structure characterizing the ligand-absent state of the LBP demonstrated its capacity to accommodate a molecule with a size equivalent to four carbon atoms (104). More recently, it was discovered that cholesterol can act as a potential endogenous ERRα agonist by increasing its transcriptional activity, which is downregulated by inhibitors of cholesterol synthesis known as statins and bisphosphonates (105). The authors of this study claimed that cholesterol is the first identified endogenous ligand of ERRα, hence, de-orphanizing this NR. However, this conclusion has not been confirmed by additional studies. It is also noteworthy to mention that cholesterol has high binding affinity to many proteins. Therefore, it is generally accepted that cholesterol is not the putative ligand of ERRα, and to date, the NR remains within the orphan subclass. ERRα can be regulated by numerous families of coactivators namely by PGC-1α/β, among others including the p160 family such as activator of thyroid and retinoic acid receptors (ACTR), glucocorticoid receptor interacting protein 1 (GRIP1), and steroid receptor coactivator 1 (SRC-1) (51). Since PGC-1α/β act as key transcriptional coactivators of

ERRα transcriptional activation, they will be of focus in this report. Similar to ERRα, PGC-1α/β coactivators are widely expressed in tissues with a high oxidative capacity such as brown adipose tissue, skeletal muscle, heart, and kidney (106-108). By directly coactivating NR and non-NRs (109), they transduce upstream physiologic and nutritional stimuli to orchestrate the control of gene regulatory programs involved in cellular energy and mitochondrial oxidative metabolism (109-111). In particular, they can activate diverse signaling metabolic pathways such as mitochondrial biogenesis, beta oxidation, glucose uptake, and gluconeogenesis to fulfill energetic demands as an adaptive response to changing physiological conditions (112-115). However, the control of cellular metabolism by ERRα is dependent on the presence of PGC-1 coactivators (116). Indeed, its co-expression induces robust activation of ERRα target genes indicating the importance of the ERRα-PGC-1 functional complex in metabolic regulation (50, 117, 118). In addition to ERRα, PGC-1 exerts its metabolic control by coactivating other NRs involved in cellular energy metabolism including PPARs, and nuclear respiratory factors (NRFs) (119, 120). Interestingly, ERRα not only transcriptionally activates PPARs and NRFs, but can also cross-regulate target genes within these pathways (109, 117, 121). Moreover, crosstalk between ERRα and PGC1 was shown through ERRα transcriptional induction of PGC-1 (116, 122, 123). This is further supported by the fact that ERRα mRNA levels are highest when PGC-1 levels are expressed in tissues including heart, kidney, and muscle (116). This demonstrates that ERR-PGC1 signaling is not only limited to this single axis but involves a complex circuitry to augment activation of target genes implicated in energy metabolism.

The cofactor receptor interacting protein 140 (RIP140) functions as a coregulator and interacts with most NRs and several transcription factors (124). As a broadly expressed coregulator, it participates in diverse biological functions including female fertility, and lipid metabolism (125, 126). RIP140 functions as a ligand-dependent coactivator or a corepressor depending on the cell-type, context of the target-gene promoter, and interaction dynamics of other transcription factors (127-130). RIP140 was first identified to interact with ERs to repress their transcriptional activity, and subsequently discovered to restrict ERRs (127, 131). Although RIP140 was demonstrated to elicit repressive activity of ERRα specifically, on EREs and ERREs, it increases the transactivation of ERRα through Sp1 response elements, further supporting the dual coregulatory ability of this factor (127). As a corepressor, RIP140 functions via its LxxLL (L is

leucine, and x is any amino acid) interaction motif to cooperate with ERRα (32). RIP140 acts as a scaffold protein that recruits histone deacetylases (HDACs), DNA and histone methyltransferases, C-terminal binding protein (CtBP) to downregulate transcriptional activity (132-134).

Nuclear receptor corepressor 1 (NCoR1) is a broadly expressed coregulator that also interacts with ERRα to repress its transcriptional activity (135). In skeletal muscle, global gene expression analysis of NCoR1 deletion overlapped with that of PGC-1a overexpression. Further, the regulation of oxidative metabolism in muscle was controlled by NCoR1 antagonism of PGC-1a-mediated coactivation of ERRα (135). Similar to RIP140, NCoR scaffolds the recruitment of additional factors, specifically, HDAC3, and transducer beta-like proteins TBL1, and TBLR1 to mediate the repressive effect of chromatin (136).

1.3 Inflammatory Bowel Diseases

Inflammatory Bowel Disease (IBD) are a group of chronic idiopathic inflammatory conditions of the gastrointestinal (GI) tract comprising two main types, namely, Crohn's disease (CD), and ulcerative colitis (UC) (137). While the etiology of IBD remains elusive, the disease is thought to arise from the interplay between host genetics (138), and environmental factors (139) that induce an inappropriate immune response (140) to the microbiota (141) through a disrupted epithelial barrier (142). Both CD and UC are characterized by periods of relapse and remission associated with symptomatic features including abdominal pain, diarrhea, rectal bleeding, and weight loss (143). However, distinct clinical and pathophysiological characteristics distinguish the disease types as later discussed. Epidemiological studies of North America show that women had lower risk of CD until ages 10-14. However, the incidence of CD did not differ between sexes until 45 years of age, thereafter, men had higher incidences compared to women (144). Since the 20th century, IBD was considered as a disease of westernized-countries including North America, Europe, and Oceania (145, 146). Currently, North America has the highest incidence and prevalence rates with approximately 270,000 cases in Canada (147, 148). Although the incidence has been stable since 1990, the prevalence remains on the rise and is predicted to attain 404,000 cases in 2030 (147, 148). The incidence of IBD in newly industrialized countries has experienced

a dramatic increase, which could be explained by their westernization (146, 148, 149). Altogether, these trends address the global healthcare challenges posed by this complex disease, thus, effective public health measures, and efforts in medical research are deeply needed.

In CD, the inflammation can occur in any part of the GI tract, most commonly, in the ileum and colon, and affects all layers of the intestinal epithelium (150-152). While CD can appear at any age, it is most frequently diagnosed between ages 15-35 years (153). CD patients typically present with pain in the lower right abdomen and thickening of the bowel wall as a result of swelling (154). Since the small intestine is affected, weight loss is common due to malnutrition from impaired absorption (155). Moreover, perianal disease is more commonly observed in CD in cases where anal canal is affected, due to the transmural inflammation of the disease (156). Nonetheless, rectal bleeding is less frequent in CD. In contrast, the inflammation in UC is limited to the colon and rectum and affects the superficial mucosal layer (157). UC is associated with two peak onsets, beginning at ages 15-40 years, followed by 50-80 years of age (158). To clinically distinguish both disease types, UC presents symptoms of pain in the lower left abdomen, and is most often associated with rectal bleeding (159, 160). Another important clinical distinction between the two subtypes is that CD presents with continuous lesions, whereas UC presents with discontinuous lesions (161).

1.3.1 Genetics of IBD

Familial aggregation in IBD has been long observed in population studies with siblings or relatives of IBD patients having a higher risk of developing the disease compared to the general population (162). The concordance rate of CD and UC in monozygotic twins are 20-50% and 14-19%, respectively, and both <7% for dizygotic twins (163). Moreover, a positive family history of IBD is a major risk for the disease (164). By pooling of twin concordance studies, IBD inheritance has been calculated to be 75% for CD and 67% for UC for the progeny (165). However, IBD does not exhibit monogenic mendelian inheritance patterns, rather, it is the interplay of polygenic influences and environmental factors that cause the disease (138). Advancement in sequencing technologies has enabled the identification of common single nucleotide polymorphisms (SNPs) associated with

disease through genome wide-association studies (GWAS) (166). To date, pooling of large-scale GWAS and immunochip studies have identified a total of 242 susceptibility loci associated with IBD (167). The inheritance for CD and UC calculated from immunochip was 19% and 15%, and 26% and 19% from GWAS (165). The discrepancy in the inheritance from twin studies versus GWAS and immunochip studies is referred to as the "missing hereditability". To address this heritability gap, next-generation sequencing (NGS) methods such as whole-genome sequencing (WGS) and whole-exome sequencing (WES), and increasing the meta-analysis of GWAS sample size are some of the ways currently explored to uncover rare variants (minor allele frequency of <0.05) (168). GWAS have allowed for the identification of disease-associated variants, especially common genetic polymorphisms within populations, with minor allele frequencies (MAF) of >0.05 (169). These common variants, however, have modest effects sizes with (OR (odds ratio) of <1.2), and are often found in non-coding regions (167). In contrast, low-frequency variants (1-5%) and rare variants (<1%) can range from having moderate effect sizes to disease casual effects, and most often, are non-synonymous alleles (167). Since these variants are primarily in coding-regions, which only account for 1.5% of the whole genome (170), investigating the non-coding regions can further account for the missing heritability. Loci and alleles in close proximity are less subject to recombination due to linkage disequilibrium (LD) – the non-random association between alleles of different loci (171). Although LD allows for the identification of associated-SNPs within the same linkage block, it however, hinders the mapping resolution of casual versus neutral variants within the haplotype. To address this issue, fine-mapping of GWAS has been conducted to gain high-resolution mapping of casual variants (172). The authors found a total of 45 single casual variants with >50% certainty, found in protein-coding regions, transcription factor binding sites (TFBS), tissue-specific enhancers, cis-eQTLs, and the remaining residing in non-coding regions with no functional annotations (n=21). Indeed, most of the IBD associated loci are enriched in non-coding DNA, specifically in DNA regulatory elements (DREs) (172).

Nucleotide-binding and oligomerization domain containing 2 (*NOD2*) was the first susceptibility gene discovered for CD (173). Currently, there are three identified NOD2 susceptibility variants: L1007fs, R702W, and G908R (174). NOD2 is primarily expressed in myeloid cells such as macrophages and DCs and acts as a critical regulator of innate immunity by sensing muramyl dipeptide (MDP) to mediate NF-κB and MAPK pathway activation and the

induction of proinflammatory cytokines (175-177). Individuals homozygous for NOD2 mutated alleles are 20 to 40 times more at risk of developing CD relative to the general population (178, 179). The implication of NOD2 in IBD is hypothesized for its role in epithelial barrier, bacterial recognition, and immune defense. NOD2 mutations associated with CD have been reported to interfere with the recognition of MDP (180, 181). Moreover, protective immunity is also abolished as observed in NOD2-deficient mice, which have decreased expression of Paneth cell-derived anti-microbial peptides, as in CD patients (177). Paneth cells play a key role in regulating microbial composition (182), and innate immunity (183, 184), via the production of inflammatory cytokines (185-187), antimicrobial peptides, and other peptides (188). Therefore, it is not surprisingly that Paneth cells are linked to the pathogenesis of IBD (189).

NOD2 also regulates the activation of autophagy via its interaction with ATG16L1 (190). In this study, CD-associated NOD2 variants resulted in impaired NOD2-dependent autophagy-mediated clearance of *salmonella* (190). Indeed, several groups have further supported the link between NOD2 and ATG16L1 in mediating autophagy (190, 191).

Autophagy refers to an evolutionary conserved self-degradative process that directs cytosolic materials to lysosomes for the maintenance of intracellular homeostasis under conditions of stress or malnutrition (192). It is involved in recycling of misfolded proteins, protein aggregates or damaged organelles, engulfment of intracellular pathogens, immunity, nutrient and energy homeostasis, and resolution of endoplasmic reticulum (ER) stress (193, 194). Notably, GWAS have identified SNPs in the autophagy genes *ATG16L1* and *IRGM* to be associated with IBD (195, 196). Numerous studies have implicated defective autophagy in the onset and pathogenesis of IBD (197-201). For example, mice harbouring ATG16L1 susceptibility variants have reduced expression of this protein as it is more readily cleaved by caspase-3. As a result, these mice had defective autophagy-mediated clearance of intracellular pathogens, and inappropriate inflammatory responses (197). ATG16L1-deficient mice displayed abnormalities in Paneth cell granule exocytosis, similar to CD patients homozygous for the ATG16L1 risk allele (198). Moreover, mice with intestinal epithelial cell (IEC)- or myeloid-specific deletion of ATG16L1 were more susceptible to chronic colitis models (199, 200). Further, it has been well-established that ER-stress is implicated in intestinal inflammation and IBD. Adolph *et al.* showed that IEC-specific deletions of the unfolded protein response (UPR) protein Xbp1 and ATG16L1 resulted in

unresolved ER-stress and development of spontaneous ileitis (201). Altogether, these studies provided insights into the mechanisms by which NOD2 and ATG16L1 may contribute to IBD susceptibility and disease pathogenesis.

Additional genetic associations with IBD include SNPs in *IL18* that contribute to increased disease risk (202). Individuals with IBD have elevated levels of IL-18 relative to controls (203). IL-18 is a member of the IL-1 family of cytokines and is primarily expressed by myeloid cells and epithelial cells (204). It acts as a pro-inflammatory mediator upon processing of its pro-IL18 precursor to its mature, biologically active form, by caspase-1 in the inflammasome complex **(Section 1.4.1)**. IL-18 (interferon (IFN)-gamma inducing factor) has pleiotropic functions that stimulate various cell types such as T-lymphocytes, B-lymphocytes, NK-cells, mast cells, and basophils (205). It is mainly known for its role in facilitating Th1 responses (206). In support of this, neutralization of IL-18 in mice subjected to chemical-induced colitis ameliorated clinical disease severity by reducing colonic cytokine levels (207-209). On the other hand, our group and others have demonstrated that mice deficient in NLRP3, ASC, or caspase-1 were highly susceptible to dextran sodium sulfate (DSS)-colitis (210, 211). Importantly, these studies point to the critical role of inflammasome-dependent production of IL-18 in mediating intestinal epithelial regeneration and repair (210, 211). This discrepancy may be due to the differences in the duration of the colitis-models in mice. It is postulated that in response to acute colitis, IL-18 is necessary for IEC proliferation and repair in order to limit access of immunogenic contents that may otherwise promote further mucosal damage. In contrast, prolonged exposure of IL-18 in chronic colitis impairs the differentiation of goblet cells leading to a disrupted mucus layer, activates intestinal immune cells and possibly result in the overproduction of pro-inflammatory cytokines. Goblet cells produce and secrete mucin, which form the luminal mucus layer to protect the epithelium against invading pathogens and debris. Importantly, the depletion of goblet cells and the loss of the mucus layer have been associated with colitis in both humans and mouse models (212-215).

1.3.2 Environment, microbiota, and IBD

As discussed in **Section (1.3.1)**, genetics only explains a small fraction of IBD heritability; thus, environmental factors are thought to be a major component contributing to disease risk.

Monozygotic twin studies show concordance rate of 20-50% in CD, and 14-19% in UC, indicating that environmental factors play a critical role in pathogenesis of IBD (163). Environmental factors contributing to the risk of IBD could be explained by global epidemiological trends of the disease. For example, since the 1990s, North America and Europe have experienced a stable or decreasing incidence of IBD (148). In contrast, pronounced increases in incidence have occurred among industrialized countries and is associated with urbanization and westernization of society (148). Moreover, countries display gradients of IBD incidence along the north-south disease gradient in Europe, and east-west disease gradient in Canada (216, 217). These environmental factors could be broken down to regional scale (urbanization, air pollution, vitamin D, latitude of residence), or an individual-scale (cigarette smoking, appendectomy, diet, antibiotics, breast feeding, depression, stress, sleep disturbance, nonsteroidal anti-inflammatory drugs (NSAID) usage, and exercise) (139). Environmental factors can influence disease risk by impacting the microbiome, increasing gut permeability, and altering intestinal immunity (218). Early exposures to these environmental factors shape the intestinal microbiota in childhood which becomes stable thereafter (219). The interplay between environmental factors and the microbiome can result in dysbiosis, a characteristic hallmark of IBD patients (220). In fact, the gut microbiome of IBD patients has a lower diversity than that of a healthy gut, and is characterized by an increase in proteobacteria and a decrease in firmicutes and Bacteroides (220). Immuno-suppressive bacteria are decreased in IBD patients compared to heathy controls (220). To name a few, *F. Praunitzii*, a member of firmicutes, that produces the short-chain fatty acid (SCFA) butyrate is decreased in IBD patients (221). Consistently, F. *Praunitzii* reduction is associated with an increased risk of ileal CD recurrence after surgery (221). Furthermore, the immunomodulatory properties of F. *Praunitzii* have been evaluated; it stimulates higher secretion of IL-10 while attenuating the levels of pro-inflammatory cytokines (222). Also, adhesion-invasive *E.coli* (AIEC) is a pro-inflammatory bacteria that is characterized by its ability to adhere to the intestinal epithelium. Studies have reported a high prevalence of AIEC in patients with ileal CD compared to healthy controls (223). The increased colonization of pro-inflammatory invasive bacteria may lead to the induction of an inappropriate immune response, affecting gut permeability, and potentiation gut dysbiosis in a feed-forward cycle. Altogether, these studies demonstrated the potential influences of environmental factors on the gut microbiome.

1.3.3 Intestinal epithelium and IBD

The intestinal epithelium is a monolayer of columnar epithelial cells that line both the small intestine (SI) and the large intestine (colon) of the gastrointestinal (GI) tract (224). Fundamentally, these cells serve as a barrier to protect the host from entry of external factors. The IEC layer architecture and cellular composition differs between the SI and colon (225). The intestinal epithelial cells are tightly joined by tight junctions that form an impermeable barrier, and further supported by the underlaying mucosa, submucosa, muscular layer, and adventitia (226, 227). The SI is divided into three segments known as the duodenum, jejunum, and ileum, and is organized into crypts and villi to maximize surface area of nutrient absorption (228). In the colon, the regions are partitioned into 6 sections known as the cecum, transverse colon, descending colon, sigmoid colon, rectum, and anus (229). A wide array of differentiated intestinal epithelial cells with specialized functions are present in both the SI and colon. These include the most prevalent intestinal cell type, the enterocytes (230), responsible for nutrient absorption, Tuft cells a chemosensory cell type (231), secretory cells including mucin-secreting goblet cells (232), enteroendocrine cells that function to secrete hormones such as somatostatin, serotonin, and substance P (233), and intraepithelial lymphocytes (234). Other cells limited to the SI are Paneth cells responsible for secreting antimicrobial peptides (235), M cells involved in antigen uptake (236), and cup cells with no known function (237). The IEC layer is continuously renewed every 4-5 days through a process of renewal, maturation, and migration (230). This turnover process relies on proliferative stem cells that reside in the crypt base of the intestinal glands that produce undifferentiated progenitors known as transit amplifying (TA) cells (230). These TA cells undergo a limited number of cell divisions, and undergo upward migration where they are differentiated into their mature specialized cell type (238). In the SI, Paneth cells differentiate as they migrate downward to the base of the crypt. Matured intestinal cells age as they reach the top of the crypts and villi where they are shed off into the luminal space and die by apoptosis (230).

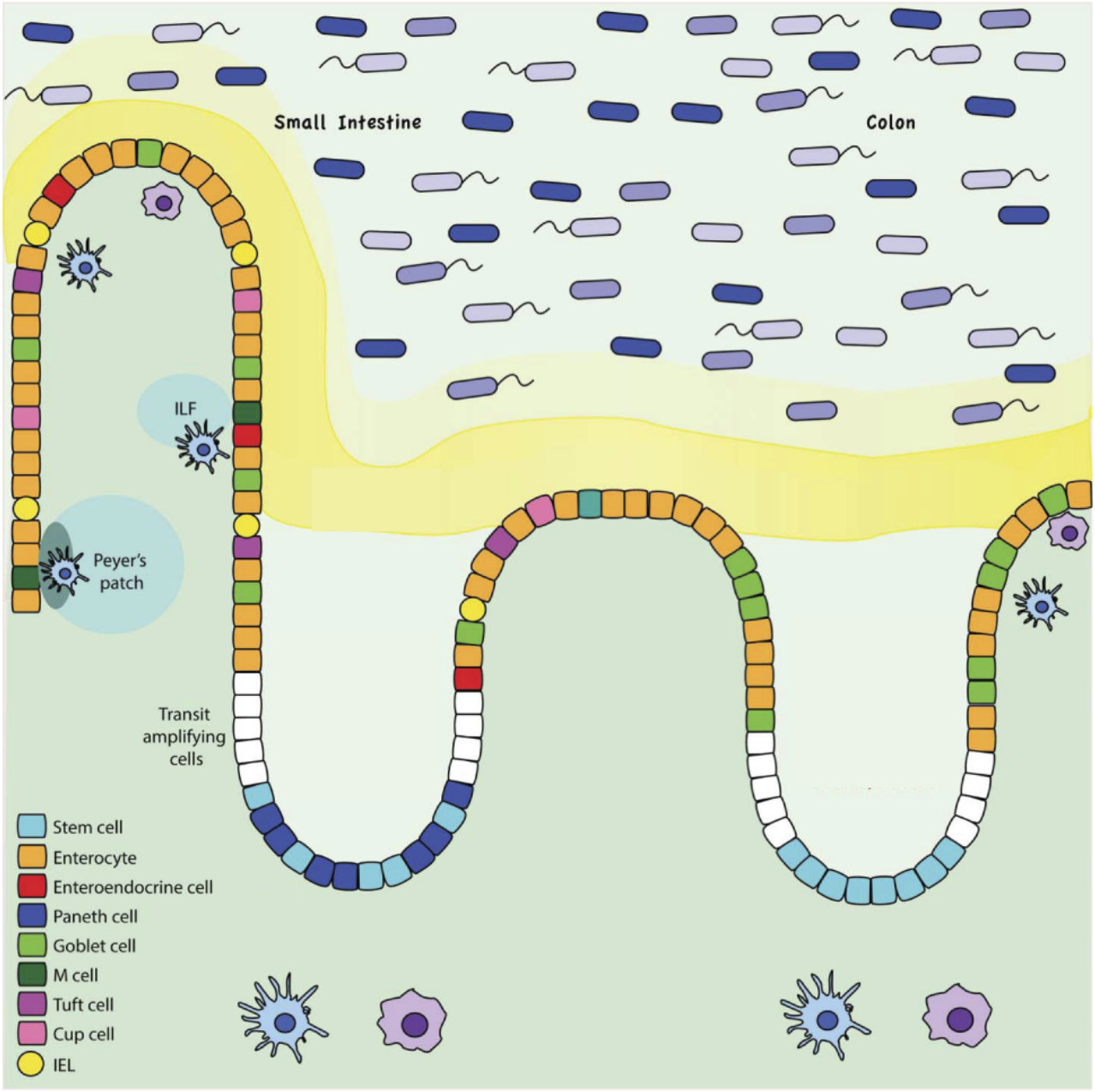

Figure 1.5 The Intestinal Epithelium. The small intestine (left) and colon (right) comprise of stem cells located at the bottom of the crypts that divide into transit amplifying cells, and subsequently proliferate and differentiate into 1 of 7 intestinal epithelial cell subtype (enterocytes, enteroendocrine cells, Paneth cells, goblet cells, M cells, tuft cells, cup cells, and intraepithelial lymphocytes) adjoined by cell junctions. Goblet cells produce a mucus layer comprising of an outer loose layer that harbours the gut microbiota, and a sterile inner dense layer that is devoid of

bacteria. The epithelial barrier alongside the mucus layer segregates the intraluminal contents from the innate immune system in the lamina propria. Illustration adapted and modified from (239).

1.3.4 Immunopathogenesis of IBD

The intestinal epithelial barrier and its mucous layer separate the intraluminal contents from the immune system in the subepithelial lamina propria (240). While the microbiota shapes the intestinal immune system, the proper functioning of intestinal immunity is indispensable for intestinal homeostasis (241). Microbiota dysbiosis, genetic, and environmental factors can impair epithelial barrier function, and increase intestinal permeability leading to an inappropriate immune activation (242). The dysregulated immune system associated with IBD is characterized by a decreased expression of retinoic acid, transforming growth factor (TGF)β, and thymic stromal lymphopoietin (TSLP) by intestinal epithelial cells (243-245). These factors are key mediators of tolerogenic IL-10 producing dendritic cells (DCs) (240, 243). In IBD, mucosal DCs and macrophages upregulate the expression of TLR2, TLR4, CD40, and chemokine receptor CCR7 and induce the pro-inflammatory cytokines IL-6, TNF, IL-1β and IL-18 (243, 246-248). The family of innate lymphoid cells (ILCs), in particular ILC1 and ILC3, accumulate in the intestinal mucosa of IBD patients (249-251). Cytokines secreted by antigen presentation cells (APCs) are critical to linking the innate and adaptive immune system (249). The cytokine secretion profile of DCs and macrophages promote the differentiation of naïve CD4+ T cells into effector T helper (Th) cells (Th1, Th2, Th17 and Th9) or into T regulatory cells (Tregs) (252). An increase in both Th1 and Th2 have been observed in the mucosa of IBD patients (253, 254). Likewise, Th17 cells produce the pro-inflammatory cytokines that promote IL-8 secretion by epithelial cells leading to the recruitment of neutrophils (255). More recently, IL-9 producing Th9 cells have been reported to be elevated in IBD patients, and in mouse models of colitis (256, 257). IL-9 has been shown to disrupt barrier integrity by inhibiting IEC proliferation and downregulating tight-junction proteins (257). Epithelial cell secretion of retinoic acid and TGFβ can induce the activation of CD103+ DCs to promote Treg differentiation (240, 243). Tregs exert immunosuppression by secreting anti-inflammatory IL-10 and TGFβ (248, 258). Studies have pointed to the importance of the Tregs/Th17 balance in the pathogenesis of IBD (259). Furthermore, increased numbers of B-lymphocytes and their associated immunoglobulin G (IgG) is found in the mucosa of IBD patients (260). Lastly, gut-homing markers that act to regulate lymphocyte trafficking have been implicated

in IBD. These markers include α4β7 integrin and CCR9 that bind to mucosal addressin cell-adhesion molecule 1 (MAdCAM1) and chemokine ligand CCL25, respectively, to migrate to the intestine (261, 262).

1.4 Inflammasomes

The inflammasomes are a group of cytosolic multiprotein protein complexes of the innate immune system that mediate protective responses against cellular stress and damage, and microbial invasion (263). Inflammasomes have been implicated in an array of diseases such as cancer, diabetes, myocardial infarction, and IBD (264-267). Since the mucosal intestinal barrier acts as a first line of defense, inflammasomes are widely expressed in this compartment with high levels in epithelial cells, macrophages, and DCs (268). Sensing of danger signals triggers the assembly of the inflammasome, generally, comprised of a sensor ((pattern recognition receptor (PRR)), an adaptor (apoptosis-associated speck-like protein containing caspase recruitment domain (ASC)), and an effector (caspase-1) (269). The formation of the inflammasome triggers the activation of caspase-1, resulting in the cleavage of pro-IL1β and pro-IL-18 into their mature and biologically forms (269). IL-1β is a proinflammatory cytokine predominantly expressed in blood mononuclear cells, macrophages, and DCs under TLR-stimulation or other cytokines (270-274). An early report demonstrated an increased production of IL-1β in IBD patients compared to healthy controls (275). In support of this, IL-10 deficiency triggers an increase in IL-1β that is associated with intestinal inflammation in both mice and humans (270). IL-1β enhances the differentiation of Th17 cells and stimulates naive and memory Th cell proliferation (276). A report demonstrated the pathological mechanism of IL-1β contributing to intestinal inflammation by promoting IL-17 responses from Th17 cells and ILCs (277). Among the PRRs, inflammasomes are formed by NLR-subsets (e.g. NLRP1, NLRP3, and NLRC4), AIM2, Pyrin, and IFI16 (278, 279).

1.4.1 NACHT, LRR, and PYD domain-containing protein 3 (NLRP3)

The Nod-like receptor NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome has been most extensively characterized and found to be implicated in IBD (264). NLRP3 is predominantly expressed in macrophages but also found in other immune cell types such as granulocytes and DCs (280, 281). Structurally, the NLRP3 inflammasome complex consists of three components including the sensor NLRP3, the adaptor ASC, and the cysteine protease caspase-1 (282). NLRP3 contains three domains known as N-terminal pyrin domain (PYD), a central NAIP, CITA, HET-E, and TP1 (NACHT) domain, and a C-terminal leucine rich repeats (LRRs) domain (283). First, the NACHT domain has ATPase activity to facilitate NLRP3 oligomerization, and the LRR domain folds in with the NACHT domain to inhibit its interaction with ASC at basal state (284). ASC contains an N-terminal PYD, and a C-terminal caspase recruitment domain (CARD) for homotypic interactions with NLRP3 on one end and caspase-1 on the other (285). Caspase-1 contains an N-terminal CARD, a central large catalytic domain (p20), and a C-terminal small catalytic domain (p10) (286). Upon exposure to pathogen/microbial-associated molecular patterns (PAMPs/MAMPs), danger-associated molecular patterns (DAMPs), or environmental irritants, NLRP3 undergoes a conformational change that exposes the PYD, enabling the interaction with ASC and pro-caspase-1 through their corresponding domains (287). The complete assembly of the NLRP3 molecular platform facilitates the autoproteolytic cleavage of pro-caspase-1 into mature caspase-1, resulting in the production and release of mature cytokines IL1β and IL-18 (288). Additionally, gasdermin-D (GSDMD) is cleaved by active caspase-1 to form pores in the cellular membrane and induce an inflammatory form of cell death termed pyroptosis (289).

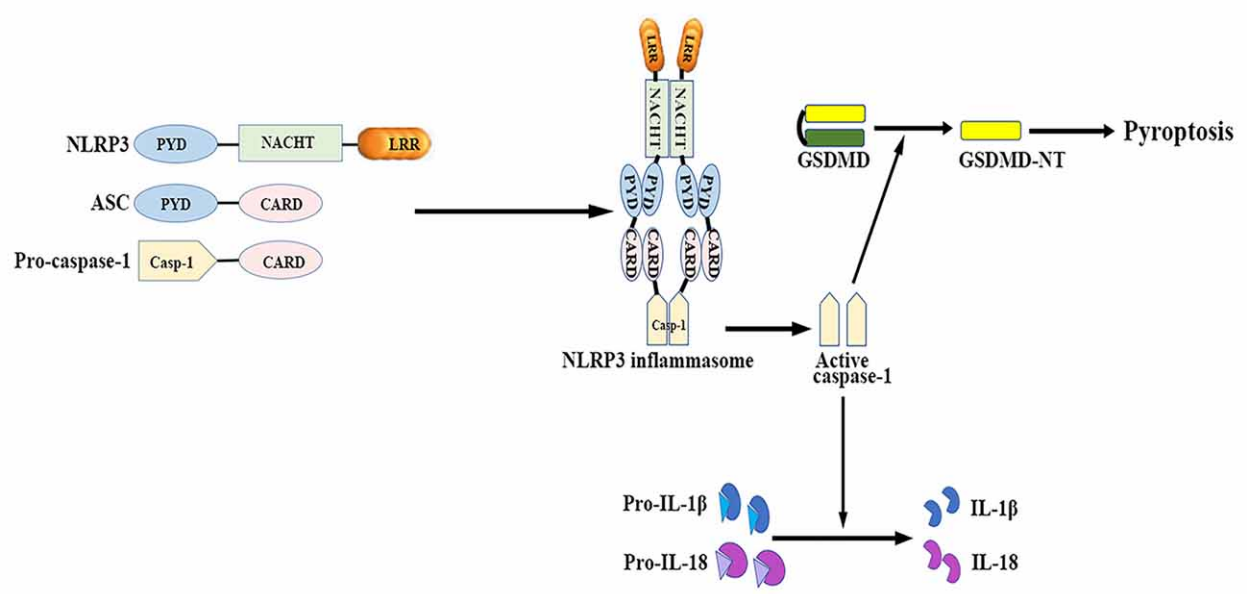

Figure 1.6 The NLRP3 Inflammasome structure and function. The NLRP3 inflammasome consists of 3 major components – the sensor molecule NLRP3, adaptor protein ASC, and the effector protein pro-caspase-1. Upon activation, the NLRP3 protein interacts with ASC via PYD, and the CARD domain of ASC binds to CARD of pro-caspase-1 to assemble NLRP-ASC-pro-caspase-1 complex termed the NLRP3 inflammasome. Activated NLRP3 inflammasome leads to activation of caspase-1, which subsequently mediates the cleavage of precursors pro-IL-1β and pro-IL-18 into mature IL-1β, and IL-18, respectively. Concurrently, activated caspase-1 cleaves gasdermin-D to mediate a form of inflammatory cell death, known as pyroptosis. NLRP3, nucleotide-binding oligomerization domain-, leucine-rich repeat- and pyrin domain-containing 3; CARD, caspase activation and recruitment domain; ASC, apoptosis-associated speck-like protein containing a CARD; PYD, pyrin-only domain; NACHT (NOD), nucleotide binding and oligomerization domain; LRR, leucine-rich repeat; GSDMD, gasdermin-D; GSDMD-NT, N-terminal fragment of GSDMD; IL, interleukin; Casp-1, caspase-1. Illustration adapted from (290).

Earlier studies discovered that activation of the NLRP3 inflammasome could be triggered by numerous stimuli including ATP (291). It was later proposed that its activation follows a two-signal model, first, a priming signal followed by an activation signal (292). The priming signal by toll-like receptors (TLRs), nucleotide-binding oligomerization domain-containing proteins 1 or 2 (NOD1/2) or cytokine receptors that trigger nuclear factor kappa B (NF-κB) activation and transcriptional upregulation of inflammasome components, including NLRP3 and pro-IL-1β (293-295). Other priming signals are mediated by post-translational modifications (PTMs) including ubiquitination, phosphorylation and sumoylation of NLRP3 (292). Next, the canonical activation of NLRP3 is triggered by ATP, crystals, or microbial products that converge on potassium ion (K^+) or chloride ion (Cl^-) efflux, calcium ion ($Ca^{2+)}$ influx and lysosomal disruption (292). Furthermore, the non-canonical activation of NLRP3 triggered by gram-negative bacteria can activate caspases-4 or -5 in humans, and caspase-11 in mice (287). Akin to caspase-1, caspase-11, triggers pyroptosis through the cleavage of GSDMD (287).

1.4.2 NLRP3 inflammasome and IBD

The NLRP3 inflammasome functions to activate an inflammatory response as a means to protect the host from microbial invasion and cellular damage (296). However, aberrant activation of this inflammasome is implicated in immune-mediated diseases including IBD, as reviewed in (297). Clinical studies report that NLRP3 activation in PBMCs stimulated by LPS is higher among IBD patients with long-standing disease (1.5> years) relative to healthy controls (298). GWAS have identified polymorphisms in the *NLRP3* gene associated with IBD in population of Chinese, Swedish, or European descent (299-301). In rodent models, mice deficient in NLRP3, ASC or caspase-1 have increased susceptibility to DSS-colitis compared to WT controls (210, 211).

1.5 Autophagy and mitophagy

Autophagy is a conserved, intracellular process of self-eating that delivers cytosolic cargo including organelles, misfolded proteins, protein aggregates, macromolecular complexes, and

foreign bodies to lysosomes where they are digested (192). This essential cellular process has been implicated in various diseases such as cancer, Parkinson's disease, osteoarthritis, and infectious diseases (302-305). Autophagy was initially considered as a non-selective bulk degradative process termed macroautophagy, induced as a survival mechanism under conditions of stress (306, 307). Macroautophagy is stimulated by nutrient deprivation, and subsequently catabolizes cytoplasmic contents to mediate recycling and overall turnover of cytoplasmic materials (308). However, it became evident that autophagy was also a highly selective process (193). Autophagy acts in response to other forms of stress including hypoxia, infection, growth factor depletion, ER stress, accumulation of ROS and DNA damage (194).

Stress signals induce autophagy by stimulating the formation of the Unc-51-kinase 1 (ULK1) complex consisting of ULK1, autophagy-related protein 13 (ATG13), RB1-inducible coiled-coil protein 1 (FIP200), and ATG101, which phosphorylates the class III PI3K (PI3KC3) complex I consisting of vacuolar protein sorting 34 (VPS34), ATG14, Beclin 1, activating molecule in Beclin 1-regulated autophagy protein 1 (AMBRA1), and general vesicular transport factor (p115) to mediate phagophore nucleation. Phosphorylated PI3KC3 complex I induces the production of phosphatidylinositol-3-phosphate (PI3P) at a subdomain of the endoplasmic reticulum (ER) membrane known as the omegasome (309). Next, PI3P recruits zinc-finger FYVE domain-containing protein 1 (DFCP1) (309), and WD repeat domain phosphoinositide-interacting proteins (WIPIs) (310, 311) to the omegasome, acting as a docking site for the ATG12-ATG5-ATG16L1 complex (312, 313). The latter couples ATG8 family proteins (ATG8s) such microtubule-associated protein light chain 3 (LC3) proteins (LC3A, LC3B, LC3C), and/or g-aminobutyric acid receptor-associate protein (GABARAP) subfamily (GABARAP, GABARAPL1, GATE-16/ GABARAPL2) to membrane-resident phospahatidylethanolamine (PE) and converts these ATG8s into their membrane-bound lipidated state (314, 315). Lipidated ATG8 is required for linking cargo to the autophagic membrane via their light chain 3 (LC3)-interacting region (LIR) and activates elongation and closure of the phagophore membrane (316). The complete closure of the double membrane phagophore matures into the autophagosome, which fuses with the lysosome to form the autolysosome (193).

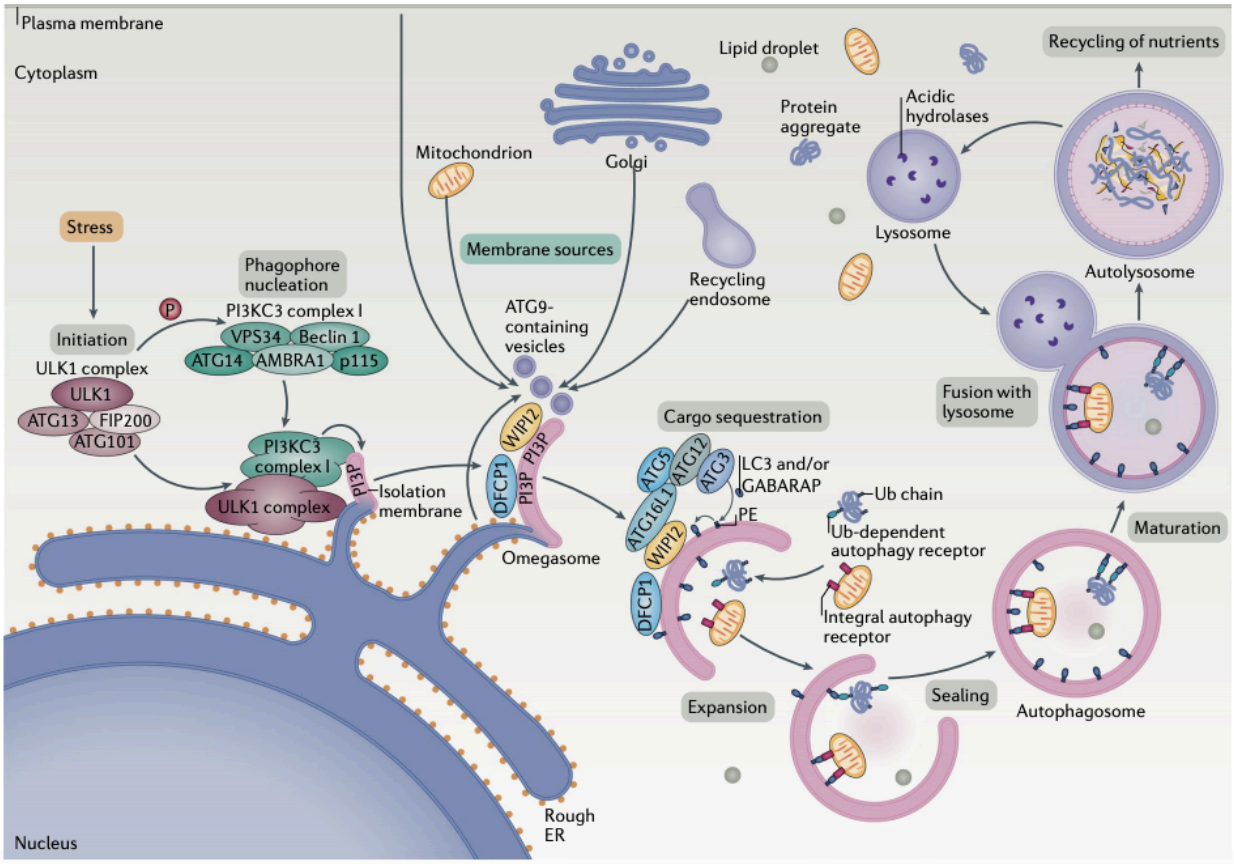

Figure 1.7 Summary of the autophagy process. Autophagosome formation is induced by stress signals and involves 3 main steps – initiation, expansion, and maturation. Activation of autophagy results in the formation of the ULK1 complex (ULK1, ATG13, FIP200, ATG101), which triggers the nucleation of the phagophore by the phosphorylation of PI3KC3 complex I (VPS34, Beclin 1, ATG14, AMBRA1, p115), then activates PI3P production at a subdomain of the endoplasmic reticulum, termed the omegasome. Next, the P3IP recruits DFCP1 and WIPI2, which binds to the ATG12~ATG5-ATG16L1 complex. This complex results in ATG3-mediated conjugation of ATG8s (LC3, GABARAPs) to the membrane-associated PE, and is converted to its lipidated forms. Thereafter, the autophagosome undergoes expansion by receiving donated membrane material from recycling endosomes, Golgi, mitochondrion, and plasma membrane via ATG9-containing vesicles. At the same time, ATG8s recruit cargo containing a LIR (LC3-interacting region)-containing receptors to be sequestered into the autophagosome. Subsequently, the autophagosome is sealed and matured into a double-membrane vesicle which is fused with acidic hydrolase-containing lysosomes. Finally, the formation of the autophagolysosome results in the

catabolism of autophagic cargo, and the recycled contents are reused by the cell. ULK, Unc-51-like kinase 1; ATG, autophagy-related protein; FIP200 (RB1-inducible coiled-coil protein 1), FAK family kinase-interacting protein of 200kDa; VPS34, vacuolar protein sorting 34, AMBRA1, activating molecule in Beclin-1-regulated autophagy protein 1; P115, general vesicular transport factor; PI3KC3, class III phosphoinositide 3-kinase; PI3P, phosphatidylinositol-3-phosphate; DFCP1, zinc-finger FYVE domain-containing protein 1; WIPI, WD repeat domain phosphoinositide-interacting proteins; LC3, microtubule-associated protein 1A/1B-light chain 3; GABARAP, gamma-aminobutyric acid receptor-associated protein; PE, phosphatidylethanolamine; Ub, ubiquitin; ER, endoplasmic reticulum; P, phosphorylation. Illustration adapted from (317).

1.5.1 Selective autophagy: mitophagy

Selective autophagy can target specific cellular components. When the mitochondria are targerted, the process is termed 'mitophagy' (318). There are three mechanisms by which the ATG8-conjugated phagophore can recognize damaged or dysregulated mitochondria to mediate cargo sequestration for degradation: 1) Ub-dependent receptors, 2) Ub-independent receptors, and 3) lipid-mediated cargo recognition. First, Ub-dependent receptor-mediated mitophagy is activated by damaged or depolarized mitochondria, where phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) recruits Ub E3 protein ligase Parkin (319, 320). PINK1 and Parkin complex attach phosphorylated Ub (pUB) chains to proteins of the outer mitochondrial membrane (OMM) leading to the recruitment of cargo receptors such as optineurin (OPTN) (321, 322). Moreover, TANK-binding kinase 1 (TBK1) functions to phosphorylate ubiquitin binding domains (UBDs) and LIRs of cargo receptors, thereby increasing the affinity to pUBs and ATG8s (323, 324). In Ub-independent mitophagy, mitochondrial proteins containing a LIR domain such as FUN14 domain-containing protein 1 (FUNDC1) act as cargo receptors that can bind to ATG8s conjugated to the phagophore (325). Lastly, lipid-mediated mitophagy depends on the exposure of phospholipids such as cardiolipin, and ceramide at the OMM surface, and their recognition by ATG8s of the phagophore (326).

1.6 Dextran sodium sulfate (DSS) model of colitis

Dextran sodium sulfate (DSS) is a model of colitis employed in mice that mimics intestinal inflammation as seen in UC (327). DSS is a water-soluble sulfated polysaccharide with a size range of 5-1400 kDa. It is administered to mice in sterile drinking water to induce intestinal inflammation (328). Of note, the penetrability of DSS in the intestinal monolayer is best at 40-50 kDa (329). This model is favourable due to its simplicity, reproducibility, and can be designed to be administered at varying concentrations, duration, and frequency (330). Other factors that could contribute to the effectiveness of DSS include manufacturer and batch, the genetics of mice strains, housing conditions, and microbial composition (330). Acute colitis is induced by administering DSS to mice for 5-10 days, which in our study was implemented for 5 days. In chronic colitis, mice are given DSS in three cycles of 5 days separated by 1 week of sterile water (327, 331). DSS exerts its chemical toxicity by directly damaging the intestinal epithelium (332). The clinical symptoms triggered by DSS are similar to those observed in IBD patients, including body weight loss, rectal bleeding, diarrhea, and hematochezia (333). DSS-induced colitis is characterized by altered expression of tight junctions, increased IEC apoptosis, depletion of mucin-secreting goblet cells, all of which contributes to a leaky gut (329). The entry of luminal microbes and antigens into the lamina propria leads to the infiltration of immune cells including granulocytes potentiating the erosion of the epithelial tissue (334). The inflammatory profiles of both acute and chronic DSS-induced colitis differ, whereby, acute inflammation is associated with a Th1 and Th17 response in contrast to a Th2 response in chronic inflammation (335). Altogether, the DSS-induced colitis model acts as a direct toxin to the intestinal epithelial barrier by altering the mucous properties, and direct cell injury, thus, inducing an inappropriate inflammatory response as seen in IBD.

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CHAPTER II – MANUSCRIPT

Estrogen-related Receptor alpha (ERRα) Protects against Experimental Colitis

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ABSTRACT

The estrogen-related receptor alpha (ERRα) is a primary regulator of mitochondrial energy metabolism, function, and dynamics, and has been implicated in autophagy and immune regulation. ERRα is abundantly expressed in the intestine and in cells of the immune system. However, its role inflammatory bowel disease (IBD) remains unknown. Here, we report a critical role of ERRα in intestinal homeostasis and IBD resistance. We found that mice deficient in ERRα were highly susceptible to experimental colitis exhibiting enhanced clinical signs of disease severity. This exacerbated colitis phenotype was mediated by impaired compensatory proliferation of intestinal epithelial cells (IEC) following injury, enhanced IEC apoptosis and necrosis, and increased colonic inflammation accompanied by reduced goblet cell count. Analysis of the bacterial community structures demonstrated reduced microbiome α-diversity in ERRα-deficient mice and depletion of healthy gut bacterial constituents. ERRα mediated its protective effects by acting within the radio-resistant compartment of the intestine, as revealed by bone marrow chimera experiments. Mechanistically, ERRα controlled the expression of key genes involved in tissue repair. These findings provide new insight on the role of ERRα in intestinal homeostasis and extends our current knowledge on nuclear receptors implicated in IBD.

Keywords: ERRα, intestine, cell death, metabolism, inflammation, colitis, autophagy, mitochondria

Highlights:

1. ERRα maintains intestinal homeostasis and protects against acute experimental colitis;
2. Deficiency of ERRα leads to enhanced colitis-associated pathology;
3. ERRα acts within the radio-resistant compartment to mediate its protective effects;
4. Colonic transcriptome and microbiome alterations between ERRα-/- and WT following DSS-colitis

INTRODUCTION

Inflammatory bowel diseases (IBD) are a group of chronic idiopathic inflammatory disorders of the gastrointestinal tract characterized by alternating phases of relapse and remission. The two main forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). While the etiology of IBD remains elusive, it is thought to arise from an inappropriate immune response to the intestinal microbiota in genetically susceptible individuals. The monolayer of the intestinal epithelium acts as a defensive barrier that ensures the segregation of luminal contents from the mucosal immune system. Therefore, perturbations of epithelial function and integrity can contribute to alterations in the intestinal microbiome, dysregulated mucosal immunity and disease pathogenesis. A fine-tuned regulation of the turnover of the gut epithelium and maintenance of immunological tolerance necessitate considerable energy supply, and the mitochondria are central organelles for meeting such high energy demands. In support of a crucial role of mitochondrial bioenergetics in intestinal homeostasis, mitochondrial abnormalities, such as a swollen structure and irregular cristae have been observed in the enterocytes of IBD patients (336). Consistently, IBD patients have lower ATP levels within the intestinal mucosa (337). Further, mice subjected to experimental colitis exhibited downregulation of mitochondrial biogenesis machinery, impaired mitochondrial function and structure, and oxidative stress (338).

Quality control of the mitochondria depends on mitochondrial biogenesis and dynamics, notably fission and fusion, and energy metabolism is contingent on the proper functioning of these homeostatic processes (339). In parallel to mitochondrial dynamics, the autophagy-dependent degradation of damaged mitochondria, known as mitophagy, contributes to organelle homeostasis (340). Defective autophagy has been implicated in IBD pathogenesis, as reported in genetic-association and functional studies (341). Indeed, defects in autophagy-dependent regulation of intestinal epithelial function, anti-microbial peptide secretion, bacterial handling and immune responses to the microbiota are hallmarks of IBD (167). Mitophagy in particular has been implicated in IEC maintenance in an experimental model of colitis (342). Consistently, defects in mitophagy and autophagy pathways led to the accumulation of damaged mitochondria and mitochondrial reactive oxygen species (ROS) (340, 343). Last, the mitochondria act as structural

and functional hubs for innate immune signaling (344). However, whether defective mitochondria are a cause or consequence of intestinal inflammation in IBD remains unclear.

The estrogen-related receptor alpha (ERRα; NR3B1) is a member of the orphan nuclear receptors family that is recognized as a key regulator of mitochondrial energy metabolism, mitochondrial biogenesis and dynamics (66, 118, 121, 345). Despite sharing high sequence homology with estrogen receptors (ERs), estrogen is not its natural ligand and its endogenous ligand(s) remains elusive, hence, termed as an 'orphan' (45). ERRα is enriched in tissues with high oxidative capacity including the intestine, and act at the intersection of cellular metabolism, oncogenesis, and immunity (67, 346, 347). In addition, ERRα regulates the expression of nuclearly-encoded mitochondrial proteins involved in all aspects of mitochondrial function (348). Therefore, it is not surprising that inhibition of ERRα results in impaired mitochondrial structure and function (349). Besides ERRα-mediated energy regulation, ERRα also regulates autophagy and mitophagy (65, 66). The transcriptional activity of ERRα is dependent on the co-activators PGC-1α and PGC-1β (Peroxisome proliferator activator receptor [PPAR]γ coactivator-1(PGC-1). Interestingly, a previous study demonstrated that IEC-specific deletion of PGC1α (PGC1αΔ^{IEC} mice) resulted in increased susceptibility to experimental colitis induced by dextran sodium sulfate (DSS) (338).

In the gut, several nuclear receptors (NRs) regulate intestinal physiology including barrier integrity and function, energy metabolism, nutrient uptake and elimination of toxic components or xenobiotics, and shaping the intestinal microbiota and immune tolerance (350). Furthermore, a number of NRs, e.g. members of the PPAR family, which are also co-activated by PGC-1α and PGC-1β, have been shown to prevent or counter intestinal inflammation, opening the window for pre-clinical and clinical trials of their respective ligands for the treatment of IBD (350). For instance, the PPARγ agonist, rosiglitazone, has shown some efficacy in the treatment of mild to moderate active ulcerative colitis (UC) in a phase 2 clinical trial (351). Here, we report that ERRα-deficient mice (*Esrra*$^{-/-}$) exhibited heightened susceptibility to DSS-induced experimental colitis, revealing a non-redundant role of this NR in intestinal homeostasis. Our results point to ERRα as a potential target to exploit for the treatment of IBD patients.

RESULTS

Esrra$^{-/-}$ mice exhibit heightened susceptibility to experimental colitis

To evaluate the role of ERRα in colitis, we subjected wild-type (WT) and *Esrra*$^{-/-}$ mice to 3% DSS in their drinking water for 5 days, followed by 3 days of regular drinking water (Figure 1A). *Esrra*$^{-/-}$ mice were markedly more susceptible to DSS colitis than WT mice, as evidenced by shortened colon length on day 8 following DSS at necropsy (Figure 1B, C). Histological analysis of colon tissue sections further confirmed the enhanced disease severity of *Esrra*$^{-/-}$ mice, as it revealed severe loss of colon crypt architecture and enhanced tissue injury in *Esrra*$^{-/-}$ mice relative to WT mice (Figure 1D). Quantitatively, ~80% of the colon tissue was damaged or eroded in *Esrra*$^{-/-}$ mice on day 8 post-DSS compared to ~30% in WT mice (Figure 1E). These results suggested that ERRα expression in the colon protects from colitis.

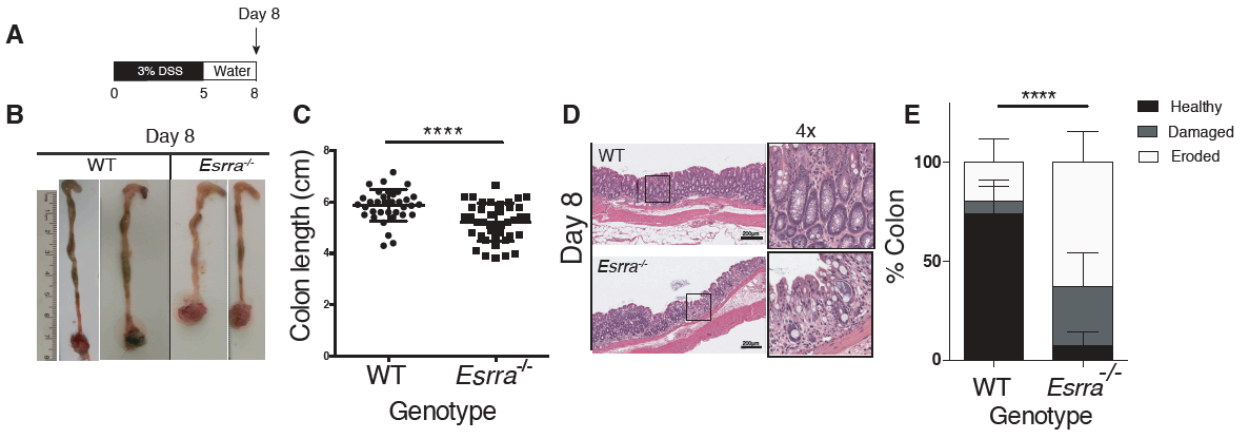


Figure 1. *Esrra*$^{-/-}$ mice are more susceptible to DSS-induced colitis

(A) Schematic representation of the experimental procedure for the DSS-colitis model. WT and *Esrra*$^{-/-}$ mice were subjected to 3% dextran sodium sulfate (DSS) in their drinking water for 5 days, followed by regular drinking water for an additional 3 days (days 5-8). The experiment was repeated 8 times. In each experiment, a group of 4 to 5 mice were used per genotype.

(B) Representative photographs of colon and cecum from WT or *Esrra*$^{-/-}$ mice on day 8 following DSS treatment.

(C) Colon length of WT or *Esrra*$^{-/-}$ mice on day 8 following DSS treatment. Each symbol represents 1 mouse; (day 8: WT [n=35], *Esrra*$^{-/-}$ [n=41]) the horizontal line represents the mean. Statistical analysis was preformed using Student's t-test (day 8, ****p<0.0001).
D) Representative Hematoxylin and eosin (H&E) staining of colon sections derived from WT or *Esrra*$^{-/-}$ mice on day 8 following DSS-treatment (magnification 100x, with 4x zoom).
E) Intestinal tissue damage and erosion were quantified by measuring the length of healthy, damaged, eroded sections, and each category expressed as a percentage of colon length. Data represent the mean ± SEM of 4 mice/ genotype. Statistical analysis was performed using two-way analysis of variance (ANOVA, ***p<0.001).

Impaired intestinal epithelial regeneration in *Esrra*$^{-/-}$ mice following injury

To investigate the mechanisms involved in the heightened susceptibility of *Esrra*$^{-/-}$ mice to colitis, we first evaluated the extent of apoptotic and necrotic cell death in the colon of DSS-treated animals. Immunofluorescence staining of colon tissue sections with anti-cleaved caspase-3 antibodies and TUNEL showed that loss of ERRα expression led to increased numbers of early apoptosis (cleaved caspase-3), late apoptotic (double positive), and necrotic (TUNEL) cells in *Esrra*$^{-/-}$ compared to WT mice (Figure 2A, B). Consistently, immunoblot analysis of total colon homogenates showed increased cleaved caspase-3 and phosphorylated RIPK1, markers of apoptosis and necroptosis, in the colon of in *Esrra*$^{-/-}$ mice relative to WT mice (Figure 2C). Second, we sought to determine the IEC proliferative capacity in the colon of *Esrra*$^{-/-}$ and WT mice. Immunofluorescence staining of colon tissue sections on day 8 post-DSS with anti-PCNA (proliferating cellular nuclear antigen) antibodies revealed markedly reduced IEC compensatory proliferation at the level of the stem cells and the transit amplifying cells of the crypts in *Esrra*$^{-/-}$ mice relative to WT mice, suggesting a role of ERRα in intestinal epithelial regeneration (Figure 2D, E). Since PCNA^{+} cells were confined to the bottom of the crypts, these cells were quantified by crypt column as opposed to the total area of the colonic epithelium. In addition, PCNA^{+} cells were quantified in both healthy and damaged regions of the colon. Last, we evaluated the number of goblet cells, a specialized IEC type responsible for mucus production and depleted in contexts of chronic inflammation, such as in IBD patients (352). Immunofluorescence staining of colon tissue sections on day 8 post-DSS with anti-mucin-2 (Muc-2) showed that DSS elicited a more

severe reduction in the number of Muc-2-producing goblet cells in *Esrra*$^{-/-}$ mice relative to WT mice (Figure 2F, G). Collectively, our data suggested that ERRα is required at multiple levels to maintain intestinal homeostasis and restore epithelial barrier integrity following injury, as its loss triggered cell death, impaired IEC proliferation and modulated goblet cell differentiation.

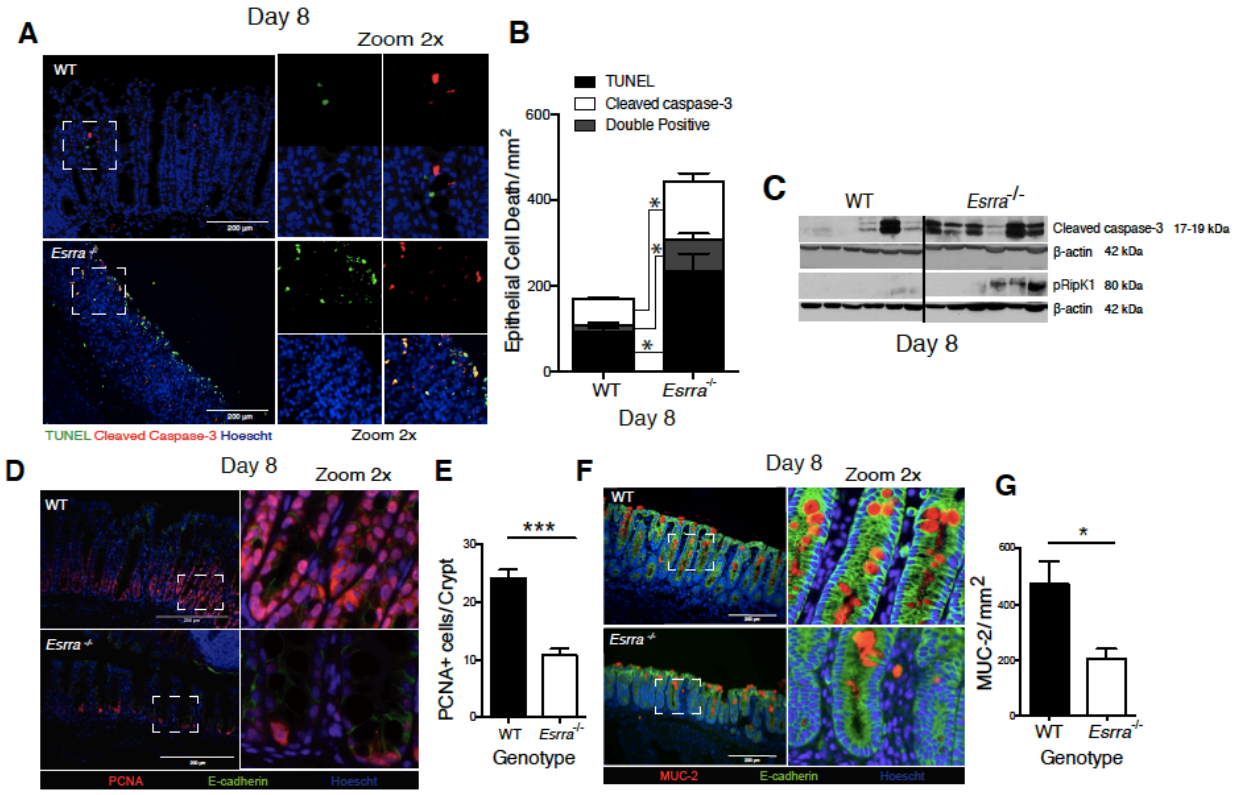


Figure 2. Impaired intestinal epithelial cell homeostasis in *Esrra*$^{-/-}$ mice after DSS injury

A) Immunofluorescence images of colon section from WT and *Esrra*$^{-/-}$ mice on day 8 after DSS treatment stained with antibodies against cleaved caspase-3 and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling). Zoomed images corresponding to boxed regions.

B) The quantification of cleaved caspase-3, TUNEL, and double positive, per area (mm^2) on day 8 following DSS treatment is shown (3 mice per genotype). Data represents the mean ± SEM of 3 mice/genotype. Statistical analysis was performed using Student's *t*-test, *p<0.05.

C) Western blots depicting cleaved caspase-3, phosphorylated RipK1, and beta-actin levels in colon homogenates from WT and *Esrra*$^{-/-}$ mice on day 8 after DSS treatment.

D) Immunofluorescence was performed on colon sections derived from WT and *Esrra*$^{-/-}$ mice on day 8 stained with antibodies against proliferating cellular nuclear antigen (PCNA) to mark cell replication, E-cadherin to mark intestinal epithelial cells (IECs), and Hoechst to label nuclei.
E) The quantification of PCNA^{+} cells per crypt on day 8 following DSS treatment is shown (5-10 crypts were scored; 3-4 mice per genotype were quantified. Data represent the mean ± SEM of n=3-4 mice/ genotype. Statistical analysis was performed using Student's *t*-test, ***p<0.001.
F) Immunofluorescence images of colon sections from WT and *Esrra*$^{-/-}$ mice on day 8 following DSS treatment stained with antibodies against MUC-2.
G) The quantification of MUC-2^{+} cells/mm^{2} on day 8 following DSS treatment is shown (3 mice per genotype were quantified; total number of cells per total area of colonic epithelium in each mouse were counted). Data represent the mean ± SEM of 3 mice/genotype. Statistical analysis was performed using Student's *t*-test, *p<0.05.

Loss of ERRα results in exacerbated colonic inflammation in experimental colitis

To further elucidate the mechanistic basis of *Esrra*$^{-/-}$ mice enhanced colitis pathology, we next examined markers of colonic inflammation. Colon sections on day 8 post-DSS were stained with antibodies to mark T lymphocytes (anti-CD3), macrophages (anti-F4/80), granulocytes (anti-Ly6G), and B cells (anti-CD19). We observed a marked increase in T cells and granulocytes in the colon of *Esrra*$^{-/-}$ mice compared to WT mice (Figure 3A – D), but no significant differences were seen for B cells or macrophages (not shown). To further characterize the inflammatory environment of the colon in both mouse genotypes, we used qRT-PCR to quantify mRNA levels of several inflammatory mediators in whole colon. We found significant increases in IL-6 and iNOS in *Esrra*$^{-/-}$ mice relative to WT mice (Figure 3E, F), and a trend of an increase in IL-22 and TNFα transcripts levels in *Esrra*$^{-/-}$ mice (not shown). Together, these results demonstrated that ERRα protected from colitis in part by attenuating inflammatory responses in the colon, although the exacerbated inflammation in *Esrra*$^{-/-}$ mice could be secondary to their marked epithelial tissue damage.

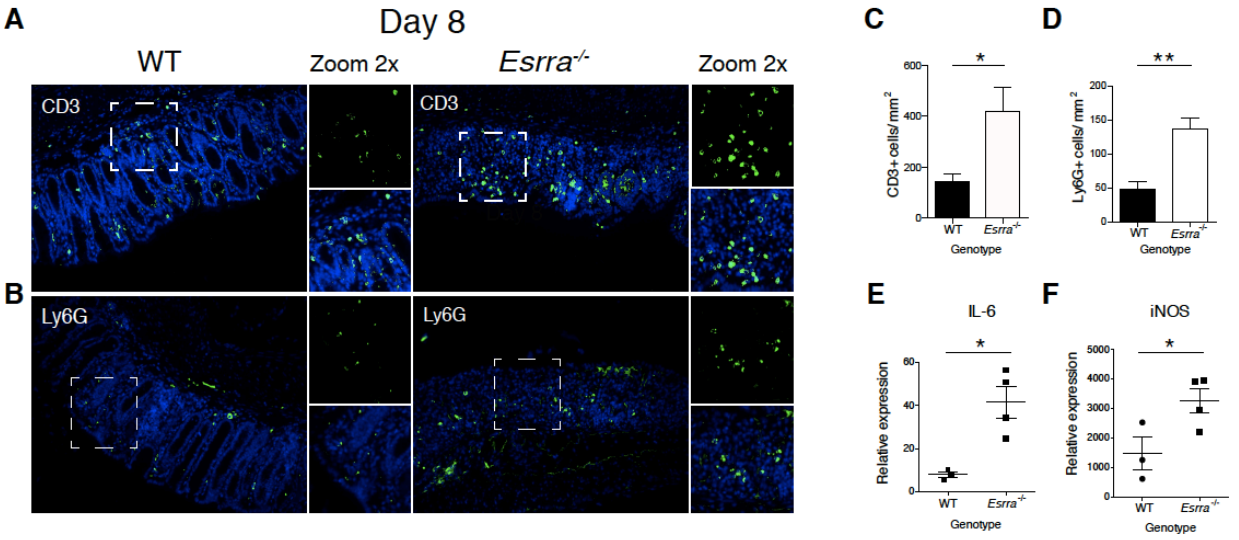


Figure 3. Increased Inflammation in the Colon of DSS-treated *Esrra*$^{-/-}$ mice

A and B) Immunofluorescence staining was performed on colon sections derived from WT and *Esrra*$^{-/-}$ mice on day 8 post-DSS stained with antibodies against CD3^{+}, Ly6G^{+} to mark for T cells, and neutrophils respectively, and Hoechst to label nuclei.

C and D) The quantification of CD3+, Ly6G+ cells/ area (mm^2) on day 8 following DSS-treatment is shown; 3-4 mice per genotype were quantified. Total number of cells per total area of colonic mucosa in each mouse were counted). Data represent the mean ± SEM of n=3-4 mice/ genotype. Statistical analysis was performed using Student's *t*-test, *p<0.05, **p<0.01.

E-F) mRNA transcripts derived from colon of WT or *Esrra*$^{-/-}$ on day 8 after DSS treatment and analyzed for expression of *NOS2* and *IL6* by qPCR. Data represents the mean ± SEM of n=4 mice/genotype. Statistical analysis was performed using Student's *t*-test, *p < 0.05.

The severe colitis of *Esrra*$^{-/-}$ mice is associated with alterations in microbiome diversity and composition

The gut microbiome is an essential factor in mediating the chronic intestinal inflammation in IBD. For this, we set on investigating the relationship between ERRα and the microbiota, at the intersection of the intestinal epithelial barrier defects and enhanced inflammation elicited by DSS. The bacterial communities of *Esrra*$^{-/-}$ and WT mice were characterized by 16S rRNA sequencing of fecal pellets collected at baseline (day 0) as well as longitudinally on days 2, 4, 6, and 7 post-DSS (Figure 4A). This analysis showed a quick decrease in microbial α-diversity as early as day

2 post-DSS treatment (signs of colitis pathology are not evident until day 8 post-DSS) in both genotypes. However, this loss of α-diversity was more severe in *Esrra*$^{-/-}$ mice (Figure 4B). At this early time point (day 2), *Esrra*$^{-/-}$ mice had increased levels of *Sutterella* (Figure 4C), a genus associated with human diseases including IBD (353) However, as colitis progressed, *Esrra*$^{-/-}$ mice had a marked decrease in read counts of bacteria associated with a healthy gut, including *Akkermansia* (Figure 4D) and *Verrucomicrobia* (Figure 4E). Overall, these results demonstrate the impact of ERRα on the gut microbiome but whether the observed alterations are a cause or a consequence of colitis requires further study.

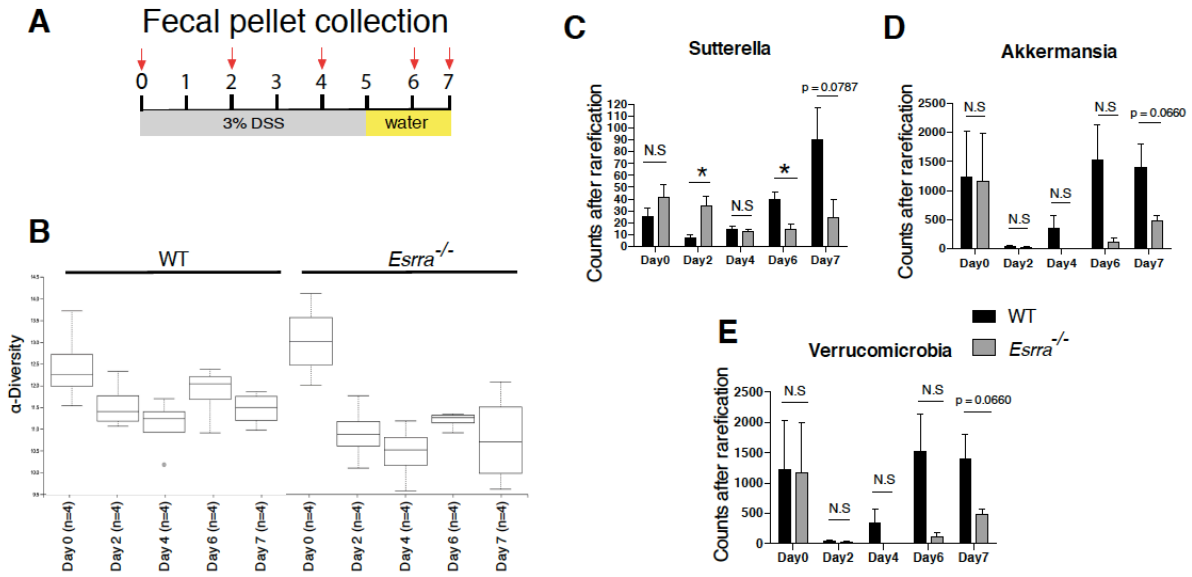


Figure 4. Changes in Microbial Composition in DSS-treated WT or *Esrra*$^{-/-}$ mice

A) Analysis of alpha diversity in *Esrra*$^{-/-}$ mice compared to WT mice post-DSS treatment. Differences in operation taxonomy units (OTUs) between WT and *Esrra*$^{-/-}$ mice in *Sutterella* (B), *Akkermansia* (C) and Verrucomicrobia (D). Data represent the ± SEM of n=4 mice/genotype. Statistical analysis was performed using Student's *t*-test; *p<0.05, **p<0.01, ***p<0.001.

Expression of ERRα in the radio-resistant compartment mediates resistance to DSS

To determine the cellular compartment in the intestine requiring ERRα expression to counter colitis, bone marrow chimeras were generated by the reconstitution of lethally irradiated

mice with bone marrow derived from donor mice of the same or different genotype. Twelve weeks after reconstitution, the four chimeric groups were treated with DSS. Our results showed that *Esrra*$^{-/-}$ mice remained highly susceptible to colitis regardless of their bone marrow transplant genotype (WT→ *Esrra*$^{-/-}$ or *Esrra*$^{-/-}$ → *Esrra*$^{-/-}$). In contrast, transplantation of bone marrow from *Esrra*$^{-/-}$ mice did not transfer the enhanced colitis susceptibility to WT mice (*Esrra*$^{-/-}$→WT), which were equivalent to WT controls (WT→WT). This was observed at the level of colon length (Figure 5A, B) and confirmed by histopathology (Figure 5C, D). These findings suggested that ERRα acts primarily within the radio-resistant compartment (IECs and stromal cells) of the colon to mediate its protective effects against DSS-induced colitis.

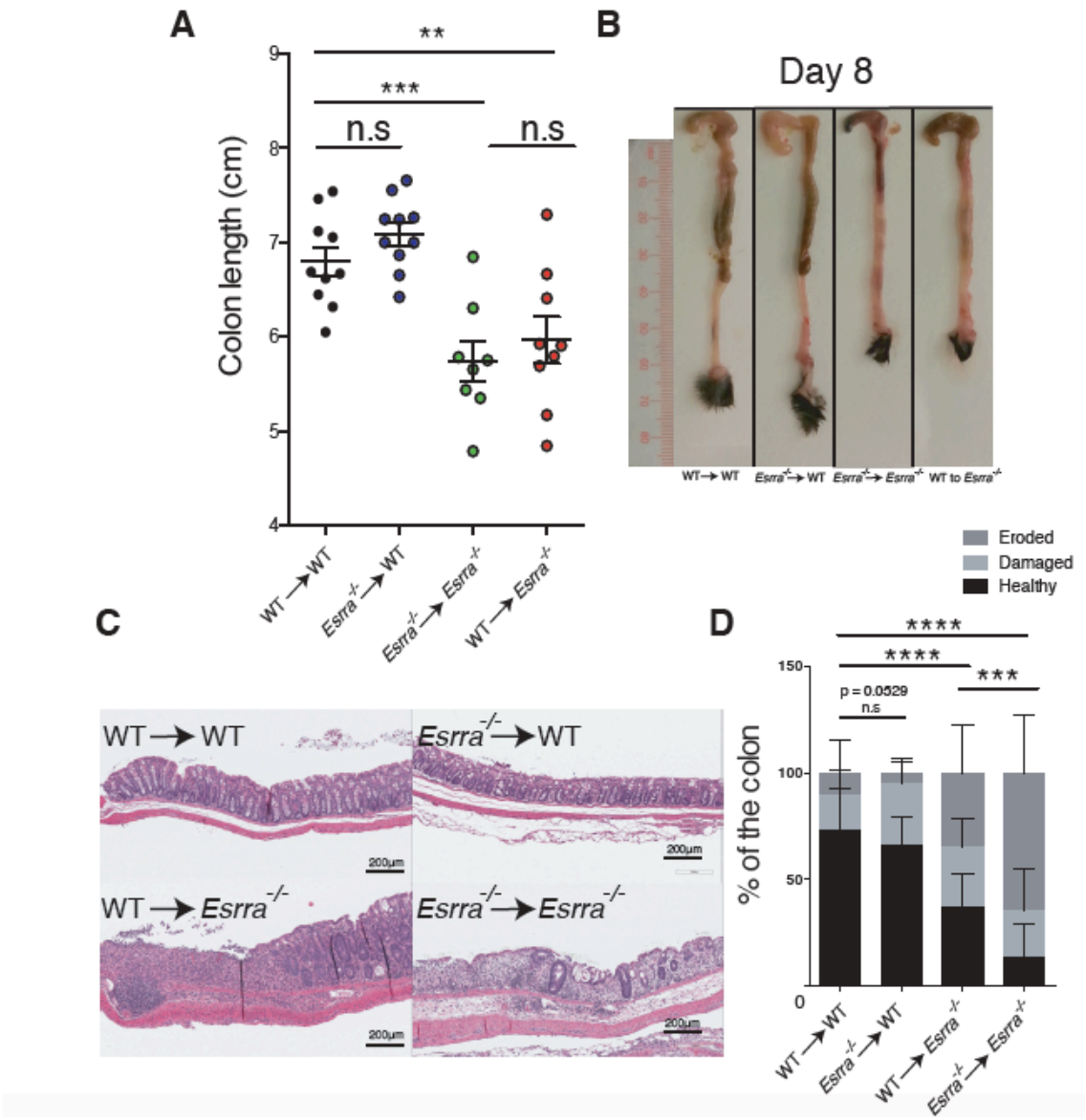


Figure 5. Expression of ERRα in the radio-resistant compartment mediates resistance to DSS colitis

A) Colon length of bone marrow chimera mice on day 8 following DSS treatment. Statistical analysis was performed with Student's *t*-test, *p<0.05, **p<0.01, ***p<0.001; *ns*, nonsignificant. Data represents the mean ± SEM of n=8-10 mice.

B) Representative photographs of colon and cecum on day 8 following DSS treatment.

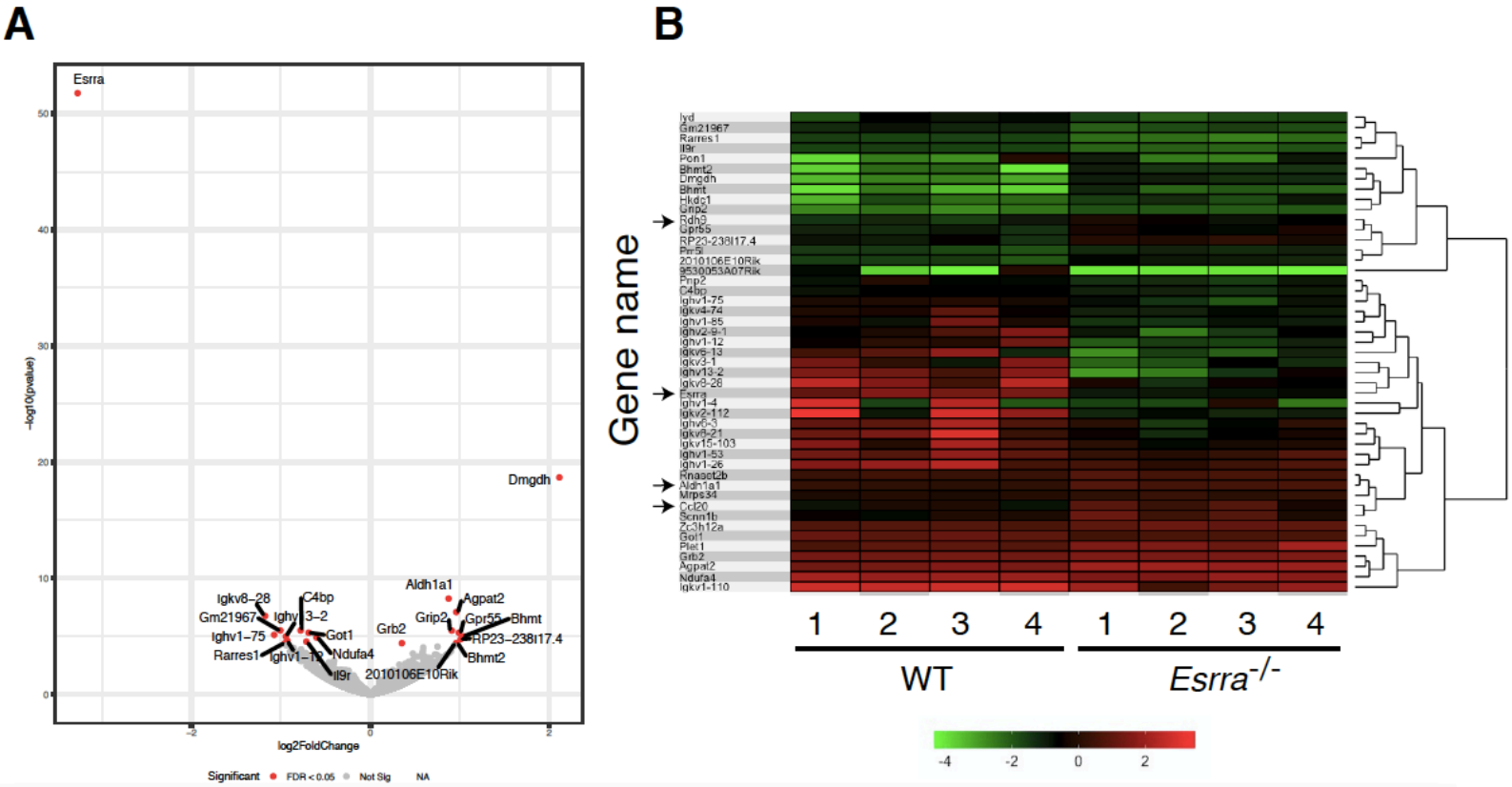
C) Representative Hematoxylin and eosin (H&E) staining of colon sections derived from WT/ *Esrra*$^{-/-}$ bone marrow chimera mice on day 8 following DSS treatment (magnification 100x, with 4x zoom).

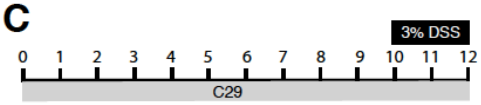
D) Intestinal tissue damage and erosion of bone marrow chimera mice on day 8 following DSS treatment were quantified by measuring the length of healthy, damaged, eroded sections, and each category expressed as a percentage of colon length. Data represent the ± SEM of 8-10 mice/ genotype. Statistical analysis was performed using two-way analysis of variance (ANOVA, ***p<0.001, ****p<0.0001; *ns*, nonsignificant).

The ERRα-dependent colon and IEC transcriptomes in colitis

To explore the ERRα transcriptional network in colitis, we first performed bulk RNA sequencing (RNAseq) on the colon of WT or *Esrra*$^{-/-}$ mice on day 8 post-DSS. The genes regulated by ERRα at endpoint are depicted in a volcano plot (Figure 6A) and a heatmap (Figure 6B). A total of 47 modestly differentially expressed genes were found between the two genotypes. Among these, 4 genes are direct ERRα transcriptional targets: *Rhdh9*, *Aldh1al, ccl20* and *esrra*. Since ERRα mediated its protective effects in colitis by acting in the radio-resistant compartment of the intestine, we next decided to explore the IEC transcriptome. We opted to analyze an earlier time point following DSS treatment (day 2) and upon acute inhibition of ERRα with an inverse agonist, named Compound 29 (C29) (354). WT mice were pre-treated with C29 for a total of 12 days prior to 2 days of DSS treatment (Figure 6C). The differentially expressed genes between C29 and vehicle-treated mice are shown in a volcano plot (Figure 6D) and a heatmap (Figure 6E). Interestingly, 3 genes known as direct ERRα transcriptional targets *Apoc3, Apoa1,* and *Scd1,* play a role in lipid metabolism and have been previously shown to be implicated in IBD (355, 356).

Colon bulk RNA-seq (day 8)





IEC RNA-seq

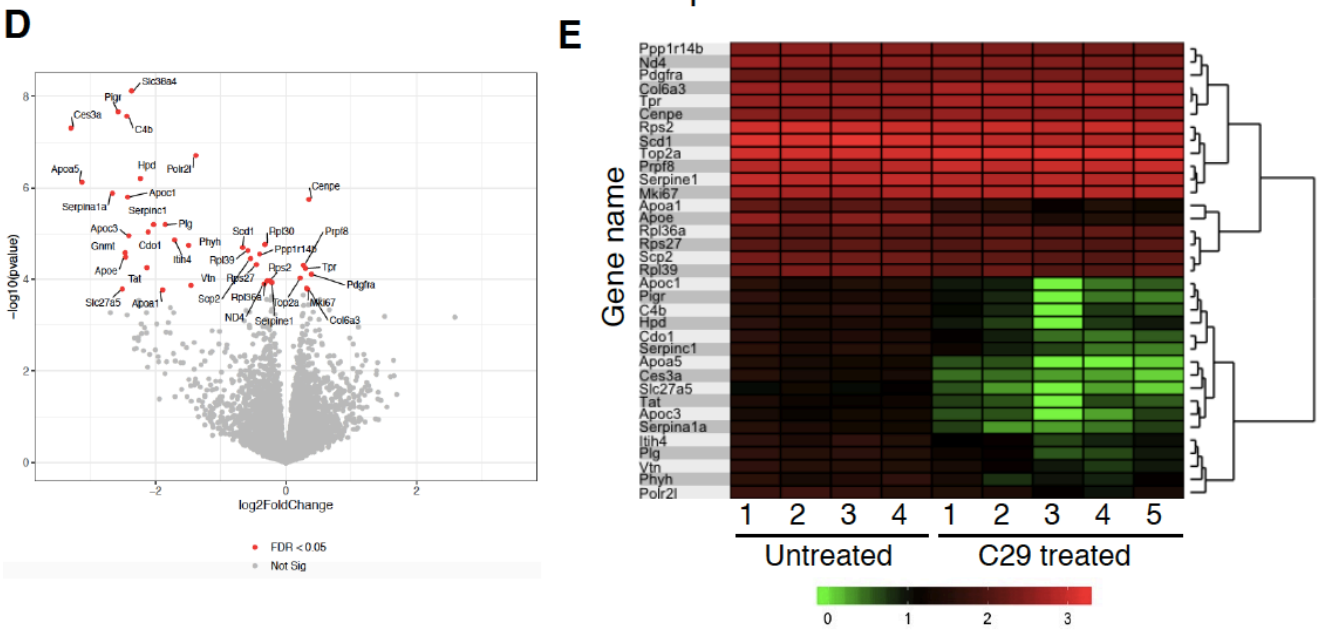


Figure 6. Differential Expression of ERRα-regulated genes in DSS Colitis

A) Volcano plot of RNA-sequencing data obtained from DEseq2 analysis of the colon of WT and *Esrra*$^{-/-}$ mice on day 8 post-DSS with FDR of <0.05 are highlighted in red. Data represents 4 mice/genotype.

B) Heatmap of enriched (red) or depleted (green) transcripts in the colon of WT and *Esrra*$^{-/-}$ mice on day 8 following DSS treatment.

C) Schematic representation of experimental procedure of DSS-colitis model following acute inhibition of ERRα. WT mice were either pre-treated with C29 or vehicle control for total of 12 days and subjected to 3% DSS in their drinking water on days 10-12. In this experiment, 3 mice were used per group, with 3-4 groups/ genotype.

D) Volcano plot of RNA transcripts from IECs of WT mice treated with C29 or vehicle control for 12 days followed by 2 days of DSS treatment. Significant differential expression of C29 injected mice with FDR of <0.05 are marked in red.

E) Heatmap of enriched (red) or depleted (green) transcripts in IECs isolated from mice injected with compound C29 or vehicle control for 12 days followed by 2 days of DSS treatment.

DISCUSSION

ERRα controls vast transcriptional networks pertaining to cellular energy metabolism, mitochondrial function and autophagy, among others. As dysfunctional mitochondria have a higher propensity to release danger-associated molecular patterns (DAMPs) including reactive oxygen/nitrogen species (ROS/RNS), oxidized mtDNA and cardiolipin, it is plausible that the accumulation of these DAMPs in ERRα-deficiency promote IEC death and activate innate immunity leading to inflammatory tissue damage, as observed in *Esrra*$^{-/-}$ mice. Consistently, ROS/RNS production is increased in experimental colitis and correlates with disease severity (338). ERRα has been reported to induce anti-oxidant protective genes (357), and decreased antioxidant capacity have been described in IBD patients (358). The severe colitis phenotype associated with ERRα loss may in part be due to a failure to provide adequate energy supply to maintain intestinal homeostasis. For instance, the maintenance of tight junctions (TJs) between IECs is energy dependent (359). ERRα may also be exerting its protective role through autophagy. Indeed, autophagy is central for intestinal homeostasis as evidenced in genome-wide association

studies (GWAS) of IBD and in functional studies. Autophagy plays a key role in regulating the gut microbiota, anti-microbial secretion by Paneth cells, endoplasmic reticulum stress response, and immunity, and disturbance of these processes are linked to IBD pathogenesis (360).

Among the innate immunity pathways triggered by mitochondrial DAMPs is the NLRP3 inflammasome pathway, which leads to caspase-1-dependent production of the pro-inflammatory cytokines IL-1β and IL-18, and to gasdermin D-mediated pyroptotic cell death (292). Our group has previously demonstrated the protective role of IL-18 in intestinal tissue repair in the acute model of DSS colitis (210). IL-18 engages with additional "repair cytokines", such as IL-22, to promote intestinal epithelial cell regeneration (361). In contrast, when IL-18 production is excessive, it becomes colitogenic by inhibiting goblet cell differentiation and promoting the pathological breakdown of the mucous layer (362). It is plausible that loss of ERRα results in heightened inflammasome activation and elevated production of IL-18 leading to the observed reduction in Muc-2 producing goblet cells in *Esrra*$^{-/-}$ mice. A compromised mucus layer is associated with microbiota dysbiosis and IBD. Intestinal inflammation can further this vicious cycle and create an aerobic environment further contributing to the observed microbiota alterations. An increase in *Sutterella* read counts was found in *Esrra*$^{-/-}$ mice at early time points. *Sutterella* have immunostimulatory properties when in direct contact with enterocytes, eliciting increased IL-8 production for instance (363). IEC secretion of IL-8 promotes the recruitment of neutrophils (364), which was observed in *Esrra*$^{-/-}$ mice. *Akkermansia* and *Verrucomicrobia* are associated with a healthy gut. *Akkermansia* utilises mucin as an energy source and releases metabolic by-products including short chain fatty acids (SCFA) among other factors for neighbouring gut symbionts (365). Cocultivation of *A. muciniphila* with butyrate-producing bacteria showed syntrophic interactions (366). Butyrate is catabolized by colonic epithelial cells and provides the majority of intestinal energy demands, thus, is important in the maintenance of mucosal health (367). Indeed, animals exhibited severe signs of experimental colitis when butyrate oxidation was inhibited (368). Accordingly, a decrease in *Akkermansia* read counts in *Esrra*$^{-/-}$ mice could contribute to the disruption of intestinal homeostasis by limiting butyrate production and availability.

Following DSS colitis, the genes found altered in *Esrra*$^{-/-}$ mice are implicated in diverse biological processes including immunity, metabolism, and wound healing. Several genes of the

humoral immune response were downregulated in colitic *Esrra*$^{-/-}$ mice, including immunoglobulin genes (*Ighv1-4*, *Ighv1-26*, *Igkv1-110*, *Ighv1-12*, *Ighv1-75*, *Ighv1-85*, *Ighv2-9-1*, *Igkv3-1*, *Igkv4-74*, *Igkv6-13*, *Igkv8−21*, *Igkv8-28*, *Ighv13-2*, *Igkv15-103*, *Ighv1-53*) and *C4bp*, a regulator of the complement cascade. *Zc3h12a*, encoding regnase-1, a negative regulator of TLR or IL-1R mediated cytokine production (369) was upregulated in *Esrra*$^{-/-}$ mice. CCL20, the sole ligand of CCR6, is elevated in the colon of *Esrra*$^{-/-}$ mice compared to that of WT animals. This chemokine is upregulated by inflammatory stimuli and is involved in lymphocyte trafficking and chronic intestinal inflammation (370). Consistently, *Ccr6*$^{-/-}$ mice are less susceptible to DSS colitis (371).

Several metabolic genes were also differentially expressed between the two genotypes. These included *ndufa4* (oxidative metabolism), *agpat2* (lipid metabolism), *got1* (amino acid and urea metabolism), *bhmt* and *bhmt2 (*homocysteine metabolism), *hkdc1* (glucose metabolism), *lyd* (hormone metabolism), *dmgdh* (choline metabolism) and *rdh9* and *ald1a1* (retinoic acid metabolism). *Ndufa4* encodes a subunit of complex I of the respiratory chain, and its dysregulation leads to the generation of ROS (372). Among the genes linked to amino acid metabolism, *bhmt* acts as a regulator of homocysteine metabolism (373) and hyperhomocysteinemia is associated with IBD, and suggested as a contributor of the increased thrombotic risk in IBD patients (373). The *Rhdh9* encoded enzyme converts retinol to retinal which is the rate-limiting step of retinoic acid (RA) biosynthesis, whereas the *Aldh1a1* encoded enzyme, retinal dehydrogenase 1, oxidizes retinal to retinoic acid (374, 375). RA was demonstrated to have an important role in mucosal intestinal homeostasis (374), and to mitigate intestinal inflammation (350).

Another differentially expressed gene is *plet1*, which was previously reported to play a critical role in tissue repair following DSS-induced damage (376). The remaining genes include *pon1,* which was upregulated in *Esrra*$^{-/-}$ mice. Risk variants of *pon1* have been identified to be associated with IBD (377).

The whole colon transcriptome of colitic mice at endpoint might not fully reflect direct effects of ERRα in intestinal homeostasis, due to the possibility of transcriptional activities mediated by other ERR isoforms or as a consequence of DSS-induced inflammation. Nonetheless, such analysis reaffirmed the heightened tissue damage and inflammatory pathology observed in *Esrra*$^{-/-}$ mice.

By inhibiting ERRα acutely with the inverse agonist C29, we aimed to abolish ERRα activity at an early time point in the DSS treatment regimen to maximize the discovery of genes

involved in disease induction. Within the IEC transcriptome, we observed significant alterations following ERRα acute inhibition and DSS-treatment. Among these genes are ERRα-transcriptional targets involved in lipid energy metabolism. Interestingly, *Apoc3* was identified to undergo the largest decrease in gene expression by C29. Interestingly, IEC-specific deletion of *Apoc3* resulted in increased susceptibility to acute colitis (378), and expression of this gene is significantly reduced in IBD patients (355). More recently, it was demonstrated that ApoC-III-containing lipoproteins regulate intestinal immune tolerance by through Tregs and IL-10 (379). Thus, downregulation of *Apoc3* as observed in mice following acute inhibition of ERRα can lead to exacerbated inflammation that may be influenced by impaired immune tolerance mechanisms. Next, the *apoa1* gene product is a main component of high-density lipoprotein (HDL) and is reported to have anti-inflammatory effects. In enterocytes, HDL and apoA-I supressed NF-κB-dependent activation of proinflammatory cytokines via autophagy, and apoA-I null mice were susceptible to the DSS and TNBS models of colitis (380). Therefore, decreased apoA-I as a result of *Esrra* inhibition could impair the anti-inflammatory properties of HDL leading to exacerbated inflammation. Finally, the *Scd1* gene, which encodes an enzyme involved in the synthesis of oleic acid, was also found to be downregulated following acute inhibition of ERRα and DSS treatment. When *scd1* was deleted from the intestinal epithelium, mice displayed increased gut inflammation, a phenotype that could be rescued by dietary supplementation of oleic acid (381). Oleic acid can directly or indirectly regulate the activity of other nuclear receptors, and coactivators with anti-inflammatory properties (382-384). Additional lipid metabolism genes (not known as ERRα transcriptional targets) include protein components of lipoproteins (*apoc1*, and *apoa5)*, *slc27a5* involved in fatty acid elongation and synthesis, and *Phyh* responsible for alpha-oxidation of peroxisomes.

Additional C29-modulated genes comprise genes involved in immunity, blood coagulation, amino acid metabolism, protein transport, cell adhesion and spreading, and RNA synthesis. Immune-related genes include *pigR*, *c4b*, and *itih4*. *PigR* encodes for a transmembrane receptor with a dual role in mediating transcytosis and acting as a precursor for immunoglobulin A (IgA). Downregulation of *pIgR* mRNA was observed in Crohn's disease and was associated with disease severity (385). When pIgR expression is diminished, luminal IgA accumulates in the lamina propria resulting in intestinal epithelial damage. Moreover, suboptimal levels of luminal IgA promote the direct access of bacteria to the epithelial surface. The *C4B* gene, involved in the

classical complement activation pathway, has a critical role in maintaining tissue homeostasis through microbial elimination. *C4b* genes are linked to pediatric IBD (PIBD), and their deficiency increase susceptibility to infections (386). Another immune-related gene is *itih4*, which encodes an acute phase protein involved in inflammatory responses to trauma (387). Patients with IBD have a higher risk of thrombosis (388), therefore, it is not surprising that coagulation factors are found altered following ERRα inhibition and DSS. *Serpinc1*, *serpine1,* and *plg* are regulators of the blood coagulation cascade and have been implicated in IBD. These genes products have been found elevated during intestinal inflammation and were correlated with disease severity (389-391). While our data shows decreased expression of these genes; coagulation factors are necessary for mucosal healing (392). *Vtn* has also been implicated in mucosal healing in the recovery phase of DSS-colitis (393). Additional genes repressed by C29 include *serpina1a*, which encodes alpha-1 antitrypsin, that exerts an anti-inflammatory role by neutralizing neutrophil elastase. Previous studies have demonstrated elevated elastase activity in patients with IBD and experimental models of colitis (394, 395). DSS-colitis severity was attenuated when neutrophil elastase was inhibited (395). This is consistent with increased neutrophilic infiltrates shown in our data. Overall, the biological processes associated with the ERRα-regulated genes may act in synergy with other disease-promoting mechanisms to aggravate intestinal inflammation in *Esrra*$^{-/-}$ mice.

In summary, this study reports a critical role of ERRα in the intestinal epithelium, its contribution to gut and immune homeostasis, and the maintenance of the mucosal barrier integrity. Providing insight on the role of ERRα in the development of colitis validates this nuclear transcription factor as a potential therapeutic target for clinical intervention in IBD.

MATERIALS AND METHODS

Animal Strains

WT and ERRα-deficient (*Esrra*$^{-/-}$) mice on a C57B1/6J background were bred and maintained at the McGill Comparative Medicine and Resources Centre. *Esrra*$^{-/-}$ mice were generated as previously described and were generated by crossbreeding (396). All animals used were 8–12 weeks old male mice housed under pathogen-free conditions and were fed standard laboratory

chow. All experiments were performed under guidelines of the animal ethics committee of McGill University (Canada).

Experimental colitis

Acute experimental colitis was induced by administering 3% (w/v) DSS (36,000-50,000 kDa, MP Biomedicals Cat#160110) in the drinking water for 5 days and subsequently replaced with regular water for an additional 3 days to allow for recovery. Clinical signs of colitis were monitored and recorded daily, which included weight loss, stool consistency, and rectal bleeding.

Bone Marrow Chimera

To generate bone marrow chimeras, C57BL/6 and *Esrra*$^{-/-}$ mice were lethally irradiated twice with 450 rads in a 3-h interval on X-ray RS-2000 Biological irradiator. After 3-h, lethally irradiated C57BL/6 and *Esrra*$^{-/-}$ mice were i.v injected with 10^7 red blood cell-depleted bone marrow from indicated sex-matched donors. Recipient mice were reconstituted for a total of 8 weeks, and engraftment was verified by FACs staining with anti-CD45.1 and anti-CD45.2. The efficiency of engraftment was confirmed by >90% reconstitution rate in recipient mice.

Acute Inhibition of ERRα

For acute inhibition of ERRα, C57BL/6 WT mice received i.p. injections of the ERRα inverse agonist compound 29 (C29) (Omegachem Inc.) in Ringer's solution (containing 5.2% polyethylene glycol and 5.2% tween 80) or vehicle alone (n=2/ group; 5 groups/ genotype). Mice were injected daily (10mg/kg^{-1}) for a total of 12 days prior to DSS treatment on days 10-12.

Isolation of Intestinal Epithelial Cells

Colons were flushed with cold PBS (Wisent, Cat#311-425-CL), cut into small pieces and incubated in RPMI (Wisent, Cat.350-000-CL) containing 5mM EDTA (Fisher, Cat.BP2482-500), 3% FBS (Wisent, Cat# 350-000-CL), and DTT (Fisher, Cat.BP172-5) for 45 min at 37 °C with 170 RPM of shaking. To isolate the IECs, the supernatant was filtered in 100 μM strainers (Fisher, Cat.#10282631) and centrifuged at 4°C at 1500 rpm, and cells were resuspended in 30% (vol/vol) Percoll (Sigma, Cat#P4937-100ML). The cells are spun at 4°C at 1700 rpm and the top layer is isolated, resuspended in fresh RPMI (Wisent, Cat.350-000-CL), and centrifugation is

repeated. RNA is extracted from pelleted cells using miRNeasy kit (Cat. # 217004) with RNase-free DNase Set (Qiagen, Cat. #79254).

Hematoxylin and Eosin (H&E) staining and immunofluorescence

Colons were fixed in 10% buffered formalin overnight and paraffin embedded subsequently, 4 μm thick sections were cut onto glass slides and processed for H&E staining. H&E sections were scanned using ScanScope XT digital scanner (Aperio Technologies). ImageScope software was used to quantify intestinal tissue damage and erosion by measuring the length of healthy, damaged or fully eroded sections and each category expressed as a percentage of the full colon length. Healthy crypts were defined as organized crypts being unaffected from lumen to lamina propria, damaged crypts were defined as disorganized or incomplete crypts and fully eroded sections were defined by the absence of crypts. For the immunofluorescence, the slides were de-waxed with xylene twice for 5 minutes. The sections were rehydrated in ethanol starting at 100%, followed by 90%, 70% and 50% ethanol for 5 minutes each. The slides were then incubated in water for 5 minutes and heated at 95°C for 15 minutes in a 0.1 M citrate buffer (pH 6.0) for antigen retrieval. PBS 0.05 % Tween 20 was used to wash the slides. Slides were permeabilized with 0.25% Triton X-100 in PBS for 20 min at room temperature. If required, Click-iT® TUNEL Alexa Fluor® 647 (Invitrogen, Cat# C10247) staining was performed according to manufacturer's instructions. Slides were then blocked (10% FBS, 3% BSA) for 30 minutes at 37°C and tissues were incubated with primary antibodies in PBS containing 3% BSA, overnight at room temperature. The following antibodies were used: PCNA (Abcam, Cat# AB2426), active caspse-3 (R&D Cat# AF835), E-cadherin (BD Bioscience Cat# 610182), F4/80 (Abcam, Cat #ab6640), Gr-1 (BD, Cat #557661), CD19 (eBioscience, Cat # 12-0191-81), CD3 (eBioscience, Cat# 45-0031-82), and Ly6G (Abcam, Cat #ab210204). This step was followed by a one-hour incubation with conjugated secondary antibodies coupled with Alexa Fluor® 488, 594 or 647 (Molecular Probe). The slides were rinsed with PBS and stained with Hoechst 33342 (Invitrogen, Cat# H3570). Tissues were mounted with cover slips and analyzed on a Zeiss Axioskop upright wide-field microscope (20x0.5 or 40x/0.75 Plan-Neofluar objectives) equipped with a high-resolution monochromatic AxioCam HRm camera and driven by AxioVision version 4.9.1 (Carl Zeiss Microscopy). ImageJ 1.46 (National Institute of Health) was used for processing of entire images before cropping to emphasize the main point of the image when appropriate; processing was limited to background subtraction,

brightness/contrast adjustments and pseudo colors addition to facilitate the visualization/interpretation of the results. Quantification of proliferation and cell death was performed with the ImageJ Software on epifluorescence images cleared of background using the 'BG subtraction from ROI' plugin. For proliferation, the number of PCNA-positive cells per well-oriented crypt were manually scored by visual inspection of images on screen. Quantification of epithelial cell death was performed by manually counting the number of active caspase-3, TUNEL or double-positive cells and divided by the crypt area (mm^2). Images were collected and image processing and analysis for this manuscript was performed in the McGill University Life Sciences Complex Advanced BioImaging Facility (ABIF).

Western Blot

Colon tissues were lysed in buffer B150 (20 mM Tris-HCl pH 8.0, 150 mM KCl, 10% glycerol, 5 mM MgCl2, and 0.1% NP40) supplemented with Complete-mini protease inhibitors (Roche Applied Science, Cat# 11836153001) and phosphatase inhibitors (Sigma Cat# S7920, 71768, G6376). Protein lysates were separated on SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with antibodies against cleaved caspase-3 (Cell Signaling Cat# 9661), phosphorylated RIPK1 (BD, Cat# 31122), and β-actin (Sigma, Cat# A1978).

RNA Sequencing and Analysis

mRNA was extracted from purified IEC or from a $1cm^2$ colon piece using the Qiagen miRNeasy kit (Cat. # 217004). Library construction and sequencing was conducted at Novogene Corporation Inc, Beijing, China. RNA quality and quantity were determined with Agilent Bioanalyzer 2100, with RNA integrity numbers (RIN) of 10 and quantities of ≥1μg/ sample. 1 μg RNA was used for cDNA library construction at Novogene using an NEBNext® Ultra RNA Library Prep Kit from Illumina® (Cat# NEB #E7775, New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. The resulting 250-350 bp insert libraries were quantified using a Qubit 2.0 fluorometer and quantitative PCR. Size distribution was analyzed using an Agilent 2100 Bioanalyzer. Qualified libraries were sequenced on an Illumina NovaSeq Platform using a paired-end 150 run (2×150 bases). Briefly, mRNA was enriched using oligo(dT) beads followed by two rounds of purification and fragmented randomly by adding fragmentation buffer. The first strand cDNA was synthesized using random hexamers primer, after which a custom second-strand

synthesis buffer (Illumina), dNTPs, RNase H and DNA polymerase I were added to generate the second strand (double-stranded cDNA). After a series of terminal repair, poly-adenylation, and sequencing adaptor ligation, the double-stranded cDNA library was completed following size selection and PCR enrichment. The resulting 250-350 bp insert libraries were quantified using a Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, MA, USA) and quantitative PCR. Size distribution was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Qualified libraries were sequenced on an Illumina NovaSeq 6000 Platform (Illumina, San Diego, CA, USA) using a paired-end 150 run (2×150 bases). Between 50-66 million raw reads were generated from each library. Differential gene expression analysis was done using Bioconductor DEseq2 package (v.1.24) with default parameters, namely no log fold change shrinkage, and FDR-adjusted p-value of 0.1 (heatmap, and GO enrichment analysis), or 0.05 (volcano plot) using Benjamini and Hochberg correction for multiple comparisons with at least one sample's FPKM ≥ 1. Fragments per kilobase million (FPKM) were log transformed and normalized for preparation of heatmap, and volcano plot using R programming packages ggplot2, and superheat, respectively.

Microbiota DNA Sequencing and Analysis

Fecal DNA was collected in 1.5ml eppendorf tubes (FisherBrand®) and stored at -80°C until preparation. Total DNA from fecal was extracted using QIAmp PowerFecal DNA Kit (Cat#:12830-50) in accordance with manufacturer's instructions (QIAmp DNA Stool Handbook 08/2017). DNA concentrations were measured with a NanoDrop Spectrometer and stored at -80°C. For sequencing, samples (48 total) of gDNA (10µg/ul) was delivered to Genome Quebec CES, Canada. DNA was amplified using primer pair 341F (CCTACGGGNGGCWGCAG) -805R (GACTACHVGGGTATCTAATCC) from Illumina's 16S library with sample-specific barcodes. This will amplify the 16S V4 variable region of the rRNA gene for interrogating bacterial communities. Statistics of the 48 samples show a total of 5,153,394 reads, 2,576,697,000 bases, with an average quality of 36. The 464bp PCR products are sequenced on MiSeq PE250 Illumina platform (Illumina Inc., San Diego, CA, USA) generating two FASTQ files per sample. Paired reads from MiSeq are trimmed of primers, filtered for high quality (phred score >30) and merged. Reads with at least 1"N" are discarded, and sequence chimeras are removed using Uchime2 against the "Gold" database (ref: Edgar RC. 2016a. UCHIME2: improved chimera prediction for amplicon

sequencing. [software]). Taxonomic affiliation of 16S sequencing data is performed using QIIME2 pipeline. Quality filtered sequences from pre-processing are denoised and underwent further QC filtering by DADA2 to create representative sequences for each amplicon sequence variant (AVS), and a feature table which indicates quantity of reads of each AVS observed in each sample. Feature classifier is used to assign likely taxonomies to reads through a model pretrained by a Naive Bayes classifier on GreenGenes database with 99% OTUs. Data were rarefied using the samples with the smallest number of reads and then subjected to alpha-diversity analysis employing Faith's phylogenetic diversity. Kruskal-Wallis was calculated between all groups together and for pairwise comparisons.

Quantitative Real-Time PCR

Total RNA was isolated from 1cm colon tissue by homogenization in TRIzol reagent, followed by chloroform extraction and isopropanol precipitation. Two micrograms of total RNA was reverse transcribed with M-MuLV reverse transcriptase (Protoscript, Cat# E6300S) and random hexamers in a volume of 20 µl according to the manufacturer's protocol. The primers used for qRT-PCR are available upon request. All reactions were normalized to the housekeeping gene L32 to quantify the relative gene expression and were then analyzed using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

Data is represented as average ± standard error. Two-tailed Student's t test and ANOVA were used for evaluating statistical significance between groups. *P<0.05; **P<0.01; ***P<0.001; n.s., not significant.

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