UNIVERSIDADE DE LISBOA

Faculdade de Medicina de Lisboa

GLIAL CELL-DERIVED NEUROREGULATORS CONTROL TYPE 3 INNATE LYMPHOID CELLS AND GUT DEFENCE

Bethania García Cassani

Orientador: Doutor José Henrique Veiga Fernandes

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas e especialidade em Imunologia

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TABLE OF CONTENTS

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RESUMO

No intestino, três tipos celulares são fundamentais na defensa do organismo perante as agressões externas: as células linfóides inatas, as células epiteliais e as células da glia. Esta relação funcional entre tecidos sugere a existência de um mecanismo de regulação entre o sistema imunitário, o epitélio e o sistema nervoso.

As células linfóides inatas (ou *innate lymphoid cells*-ILCs) são os novos membros na família dos linfócitos. Como tais, apresentam morfologia linfóide mas por outro lado as ILCs não possuem os genes activadores da recombinação (ou *recombination activating genes*-RAG) nem marcadores específicos de outras linhagens celulares. As ILCs integram sinais do ambiente e respondem a citoquinas, alarminas e moléculas produzidas pelas células epiteliais, as células do estroma e outras células do sistema imunológico. Podem ser divididas em três grupos dependendo dos factores de transcrição que regulam o seu desenvolvimento e a sua função.

O grupo 3 das ILCs têm como função regular os tecidos revestidos por mucosas sendo de grande importância na preservação da homeostasia. Este grupo compreende células indutoras de tecidos linfóides (ou *lymphoid tissue inducer cells*- LTis) e as células inatas linfóides do grupo 3 (ou *innate lymphoid cells group 3* – ILC3s e possuem RORγt como factor de transcrição fundamental no seu desenvolvimento e função. No intestino, as ILC3s encontram-se principalmente agrupadas, junto com outras células do sistema imunológico e células do estroma, em estruturas chamadas cryptopatches. As ILC3s são de grande importância na formação de tecidos linfóides (LTis), na protecção frente a bactérias e são cruciais durante doenças inflamatórias devido ao seu grande potencial para produzir citoquinas importantes na protecção da barreira epitelial. Citoquinas como a IL-22 vão activar as células epiteliais que são as responsáveis de produzir proteínas antimicrobianas e mucus.

No nosso laboratório, foi descoberto que as ILC3s expressam elevados níveis da proteína tirosina cinase RET, o receptor dos factores neurotróficos da família do GDNF (*glial cell line-dereived neurotrophic factor*) (GDNF *family ligands* – GFLs). Isto foi confirmado tanto ao nível de RNA como de proteína utilizando um modelo animal GFP (green fluorescent protein) para RET.

Ret gene - em inglês "***r***earranged during ***t***ransfection"- foi descrito em 1985 como um proto-oncogene que codifica para a proteína RET essencial para o correto

desenvolvimento, maturação e manutenção de diferentes tipos celulares e tecidos. RET é expresso em tecidos embrionários, está envolvido na formação dos rins, manutenção das células estaminais e no desenvolvimento do sistema nervoso. Para além disso, foi também demonstrado que possui um papel crucial na formação de tecidos linfóides, em particular nas células hematopoiéticas envolvidas na organogénese das placas de Peyer no intestino durante a vida embrionária.

Dado que a deficiência em RET é embrionariamente letal, para poder perceber o papel do RET nas ILC3s foram desenvolvidas quimeras com o fígado fetal de ratinhos deficientes nesta proteína. Curiosamente, apesar de não terem sido encontradas diferenças no número de células nem de cryptopatches foi observada uma deficiência na função das ILC3s, em particular uma diminuição na produção da citoquina IL-22. Em concordância, no modelo animal em que há uma activação constitutiva de RET (*Ret*MEN2B) encontramos um aumento na citoquina IL-22 procedente das ILC3s, o que se traduz numa maior protecção da barreira epitelial.

Para conseguir estudar o papel de RET nas ILC3s foi desenvolvido no laboratório um modelo de ratinho com uma ablação específica de RET nas células com expressão de Rorγt. No modelo RorγtCre$Ret^{fl/fl}$ (ou Ret^{Δ}) encontramos uma diminuição da IL-22 e uma redução dos genes envolvidos na reactividade epitelial: mucinas e defensinas.

Com o fim de perceber o papel de RET não só em condições basais mas também em estados de inflamação, os animais Ret^{Δ} foram tratados com DSS (dextran sodium sulfate) para induzir colite. Nestes animais observamos uma redução na produção de IL-22 e consequentemente uma maior ruptura da barreira epitelial o que ao mesmo tempo produz maior translocação bacteriana desde o intestino para outros órgãos, tais como, nódulos linfáticos e o fígado. Do mesmo modo, os animais *Ret*MEN2B foram tratados com DSS e após tratamento, mostraram uma maior produção de IL-22 junto com uma maior protecção da barreira epitelial e menor translocação bacteriana para órgãos periféricos. Estes resultados mostram o papel protector das RET^{+}ILC3s em modelos de inflamação.

De forma a estudar o processo inflamatório num contexto mais fisiológico, os animais Ret^{Δ} foram infectados com a bactéria *Citrobacter rodentium* que adere às células epiteliais e causa colite. Nestes animais, tanto a produção de IL-22 como a expressão dos genes de defesa aparecem diminuídos. Além disso observamos um aumento da inflamação intestinal, infecção e mortalidade.

Com o objectivo de perceber a relação de RET e de GFLs na produção de IL-22 por parte das ILC3s foi usado um sistema *in vitro* de organóides epiteliais ou “mini intestinos”.

Observamos que quando são adicionados GFLs aumenta a expressão de genes de reactividade epitelial de forma dependente de IL-22. Para além disso a presença de GFLs nas ILC3s deficientes em RET (Ret^{Δ}) não afecta a expressão destes genes em relação aos controlos. Estes resultados mostram o papel fundamental de RET na activação das ILC3s através de factores neurotróficos.

Por outro lado, descobrimos que a regulação molecular da produção de IL-22 tem lugar após a activação das RET^{+}ILC3s por GFLs e da fosforilação da cascata de sinalização p38 MAPK/ERK-AKT. Isto leva a activação de STAT3 que se liga ao promotor da *Il22* iniciando a sua transcrição.

O sistema nervoso entérico ou também chamado "segundo cérebro" contem uma enorme rede de células nervosas. Em particular, nas células da glia do intestino, foi descrita a presença de receptores de reconhecimento de patógenos tais como "*toll like receptors* ou TLRs". Isto juntamente com o facto das células da glia produzirem GDNF – necessários para a sinalização de RET- levou-nos a pensar numa regulação nervosa das células do sistema imunitário, neste caso, das RET^{+}ILC3s.

As células da glia exprimem a proteína GFAP (glial fibrillary acidic protein) e para identificar a sua localização em relação às ILC3s foi preciso desenvolver um modelo de ratinho duplo fluorescente GFP/RFP para as proteínas RET e GFAP. Com este modelo, conseguimos perceber que as projecções das células da glia estão localizadas muito próximas as *cryptopatches*, na lâmina própria do intestino.

Por outro lado, foram desenvolvidas co-culturas de ILC3s e células da glia que mostraram a capacidade para produzir GFLs em resposta à activação por TLR2 e TLR4 e também por IL-1β e IL-33 promovendo a produção de IL-22. Estudos *in vivo* com ratinhos deficientes em *Myd88* (molécula adaptadora para a sinalização por TLRs) nas células da glia e tratados com DSS, manifestaram um aumento da inflamação e uma redução na expressão de GFLs e IL-22 para além de sofrer uma maior perda de peso em comparação com os controlos. Quando infectados com *Citrobacter Rodentium* os animais deficientes em *Myd88* foram mais susceptíveis a inflamação intestinal e a infecção.

Resumindo, as células de glia processam os sinais do ambiente via MYD88 para produzir GFLs que ao mesmo tempo activam as RET^{+}ILC3s e pela cascata das MAP cinases e STAT3 induzem a transcrição de *Il22.* A produção de IL-22 por sua vez promove a produção de péptidos antimicrobianos e genes de defesa e reparação do epitélio. A unidade glia-ILC3-epitélio é crítica na manutenção da homeostasia intestinal sendo fundamental na protecção barreira epitelial. Esta descoberta abre mais uma porta na

compreensão e nos possíveis tratamentos de doenças inflamatórias intestinais tais como doença de Crohn ou cancro.

SUMMARY

In this thesis we demonstrate that three distinct players form a novel multi-tissue defence unit in the intestinal wall: group 3 of innate lymphoid cells (ILC3s), intestinal epithelial cells and enteric glial cells (EGCs). This interplay reveals a neuro-immune interaction unit that regulates epithelial homeostasis and mucosal defense.

ILC3s are major regulators at mucosal surfaces being critical in tissue repair and in the maintenance of gut homeostasis. Intestinal ILC3 – that mainly aggregate into cryptopatches - integrate environmental signals leading to the production of the pro-inflammatory cytokines IL-22 and IL-17. IL-22 in turn induces intestinal epithelial cells to produce antimicrobial peptides and mucus.

We found that ILC3s express high levels of RET, a neuroregulatory receptor for GDNF family ligands (GFLs). In order to address the effect of RET in ILC3s development and function RET-deficient mice foetal liver chimeras were analyzed. Interestingly a decrease of IL-22 expressing ILC3s was observed when compared to WT controls. In addition a RET gain of function model (*Ret*MEN2B) resulted in increased IL-22 expressing ILC3s.

In line with these experiments, cell-autonomous ablation of RET in Rorγt expressing cells was performed. RorγtCre$Ret^{fl/fl}$, (Ret^{Δ}) mice had decrease IL-22 expressing ILC3s and a reduction of epithelial reactivity genes such as mucins and defensins comparing with their littermate controls. Upon infection with the attaching and effacing bacteria *Citrobacter rodentium*, Ret^{Δ} mice had marked gut inflammation, reduced IL-22 producing ILC3, increased *C. rodentium* infection and translocation, reduced epithelial reactivity genes, increased weight loss and reduced survival. All these data together, suggest that RET cell autonomous ILC3 signals regulate IL-22 production.

Signals downstream of *Ret* were regulated via GFLs which directly controled rapid phosphorylation of the p38 MAPK/ERK-AKT cascade and STAT3 activation in ILC3s. In turn, STAT3 bound to the *Il22* promoter to induce transcription.

Finally, we found that enteric glial cells integrated commensal and environmental signals to produce GFLs that control IL-22 production. Physical localization of glial cells in the vicinity of ILC3 was observed taking advantage of double reporter mice for GFAP (glial fibrillary acidic protein) and RET. Enteric glial cells had a stellate shape morphology, projecting into cryptopatches.

In vitro co-culture studies showed EGCs capacity to produce GFLs in response to TLR2 and TLR4 activation and IL-1β and IL-33 stimulation, promoting IL-22 production by ILC3s. *In vivo* studies with DSS induced colitis in glial specific *Myd88* deficient mice (*Gfap*-Cre.*Myd88*$^{\Delta}$) showed an increase of gut inflammation and weight loss along with a reduced expression of intestinal GFLs and ILC3-derived IL-22 levels compared with their littermate controls. When infected with *Citrobacter Rodentium Myd88* deficient mice exhibited a pronounced susceptibility to bowel inflammation and infection.

In summary, we were able to show that the enteric glial cells sense environmental cues through MYD88 to produce GFLs that in turn activate RET expressing ILC3s and via MAP kinase and STAT3 induce the transcription of *Il22*. The production of IL-22 promotes the expression of defence and repair genes. Thus, this novel glial-ILC3-epithelial unit is critical in the maintenance of intestinal homeostasis providing protection and repairing the epithelial barrier after injury.

INTRODUCTION

The intestinal mucosal barrier isolates the internal body from the external environment and is necessary for nutrient absorption and waste secretion[1,2]. Epithelial cells in the mucosa are in contact with the commensal gut microbiota and through recognition of multiple receptor molecules are able to identify danger signals from the environment, thus avoiding pathogen penetration into the gut wall. Furthermore, the gut mucosa contains a vast number of immune cells responsible for the maintenance of tissue homeostasis. Importantly, disruptions of the intestinal mucosal barrier and dysfunction of any of its components can produce disease[3].

Malfunction of immune cells and their secreted molecules can increase gut permeability, altering nutrient absorption and allowing microbial product translocation that further increases inflammation[4]. However, many other factors are involved in the progression of intestinal pathologies. As such, we need to take into consideration not only immune alterations, but also environmental changes, genetic backgrounds, alterations in the gut microbiota and the extensive cell network that constitutes the enteric nervous system, which was shown to be altered in inflammatory bowel pathologies[3,5]. Interactions between the central and the enteric nervous system with the intestinal microbiota, diet products and immune responses are currently being investigated[6]. Nevertheless, the mechanisms by which the nervous system contribute to inflammatory intestinal pathologies remains unclear[7–9].

In Europe approximately 3 million people suffer from inflammatory bowel diseases (IBD) costing to the health-care system billions of euros annually[10]. Bowel inflammation pathologies are a worldwide group of diseases that cause patients significant suffering due to gastrointestinal tract inflammation and tissue damage. There are two major clinical manifestations: Ulcerative colitis (UC) and Crohn´s disease (CD). Despite all efforts made so far trying to eradicate these pathologies, there is still no cure for IBD[3].

Since IBD is a chronic inflammation pathology actual treatments are focused on immune suppressive drugs that target cytokines and their receptors[3]. To improve therapeutic design and implement new effective treatments to IBD, a better understanding of the mechanisms that regulate intestinal barrier homeostasis is needed.

The intestinal barrier

After birth, new-borns are exposed to millions of different microorganisms through contact, inhalation and ingestion[11]. Body surfaces such as the skin, the respiratory tract and the intestine start to be colonised. From then on, the intestine and its epithelia will act as barriers and as a reservoir for trillions of microorganisms from hundreds of different species: the gut microbiota[12–16]. Different organs, cells and molecules are involved in the maintenance of a healthy tissue microenvironment and to ensure that, not only the immune system but also the nervous system is intimately connected to provide protection against infection[17,18,6].

The intestinal epithelial barrier (IEB) is the largest exchange surface between the body and the external environment. The IEB consists of a monolayer of epithelial cells organized into invaginations, or Lieberkühn crypts (proliferation compartment), and finger-like projections or villi (differentiation compartment)[19]. The brush border of epithelial cells constitutes an essential interface during infection with adhering bacterial pathogens[20,21]. Importantly, epithelial cells have the ability to repair wounds after an insult[22,23], proliferating and migrating into the injured tissue while integrating microbial signals through pattern recognition receptors. These receptors can sense a wide range of microbial products such as peptidoglycans, flagellins, amino acids, microbiota derived short-chain fatty acids and butyrate, among others[57].

The IEB allows for absorption of nutrients, but it also controls the passage of pathogens and toxins; therefore, changes in paracellular permeability can lead to multiple inflammatory and digestive diseases. The IEB plays a critical role in the protection against aggressions by deploying different mechanisms: *i.* the production of antimicrobial peptides by enterocytes and Paneth cells; *ii.* electrolyte production by enterocytes; *iii.* hormone secretion by enteroendocrine cells; and *iv.* mucus production by goblet cells (Figure 1). Epithelial cell renewal in the small intestine relies on pluripotent intestinal stem cells located in the base of the crypts with a high rate of cell turnover. Differentiation occurs while they migrate upwards into the villi, acquiring their specific absorption and secretion functions[24]. In contrast, Paneth cells migrate downwards to the base of the crypt where they become majors elements of the epithelial stem cell niche[21]. In conclusion, the intestinal epithelial barrier is the first line of defence against enteric pathogens and the maintenance of an intact IEB function is crucial to ensure health[17].

Goblet cells and mucus production

The intestinal mucus is produced by specialized mucin secreting cells, or Goblet cells, derived from the secretory epithelial cell lineage[25]. Mucins are synthesized in the endoplasmic reticulum and final glycosylations occurs in the Golgi[26]. Mature mucins are then packed into secretory granules to be secreted into the lumen[27–30].

Mucins are proteins with serines, prolines, threonines and carbohydrate chains attached by glycoside bonds[31]. There is a wide range of different mucins classified into three groups: *i.* gel forming; *ii.* soluble; and *iii.* membrane-bound mucins[21]. Changes in the composition of mucus and goblet cell function was reported in response to changes in the microbiota or in the presence of pathogens, but the mechanism behind these changes remains poorly understood[26].

The intestinal mucus allows for protection and lubrication of the epithelium but it is also known to be involved in the regulation of foetal development, epithelial renewal and carcinogenesis[32]. Moreover, some enteric bacteria can use mucus for nutrition purposes, which facilitates their growth and colonization of the gut[33,34]. As the mucus layer acts as a physical barrier, enteric pathogens have developed mechanisms to interfere with mucin production and to cross the mucus barrier[34]. However, resident commensal bacteria have also developed mechanisms to inhibit the adherence of pathogens by means of increasing the production of mucins[35].

Paneth cells and antimicrobial peptides

Antimicrobial peptides (AMPs) are mostly produced by Paneth cells at the base of the crypts of Lieberkühn and are part of the innate immune response being the front line of enteric defence[36]. This antimicrobial system is also present in the airways, gingival epithelium, cornea, reproductive tract, urinary and gastrointestinal tract where they can develop a rapid response before the adaptive immune system become active[37,38].

Paneth cells are pyramid shaped exocrine cells containing secretory granules with AMPs and other antimicrobial molecules such as lysozyme and phospholipase A[21,39]. Antimicrobial peptides are able to control the bacterial composition and, in conjunction with the innate immune system, contribute to maintain the crypt microenvironment free from pathogens[39–42]. AMPs are small hydrophobic peptides of 20 to 40 amino acids and

are subdivided in two groups: the defensins and the cathelicidins[43–45]. The antimicrobial peptides have also been subdivided according to their secondary molecular structure as α-defensins (α helical structure) and β-defensins (β -sheet structure)[45]. AMPs have a wide spectrum of microbicide activity against gram negative and gram positive bacteria, fungi, protozoa and viruses[21]. AMPs can induce damage to microorganisms, binding to their surface and forming transmembrane pores[46,47]. Nevertheless, certain enteric pathogens have developed AMPs resistance[48].

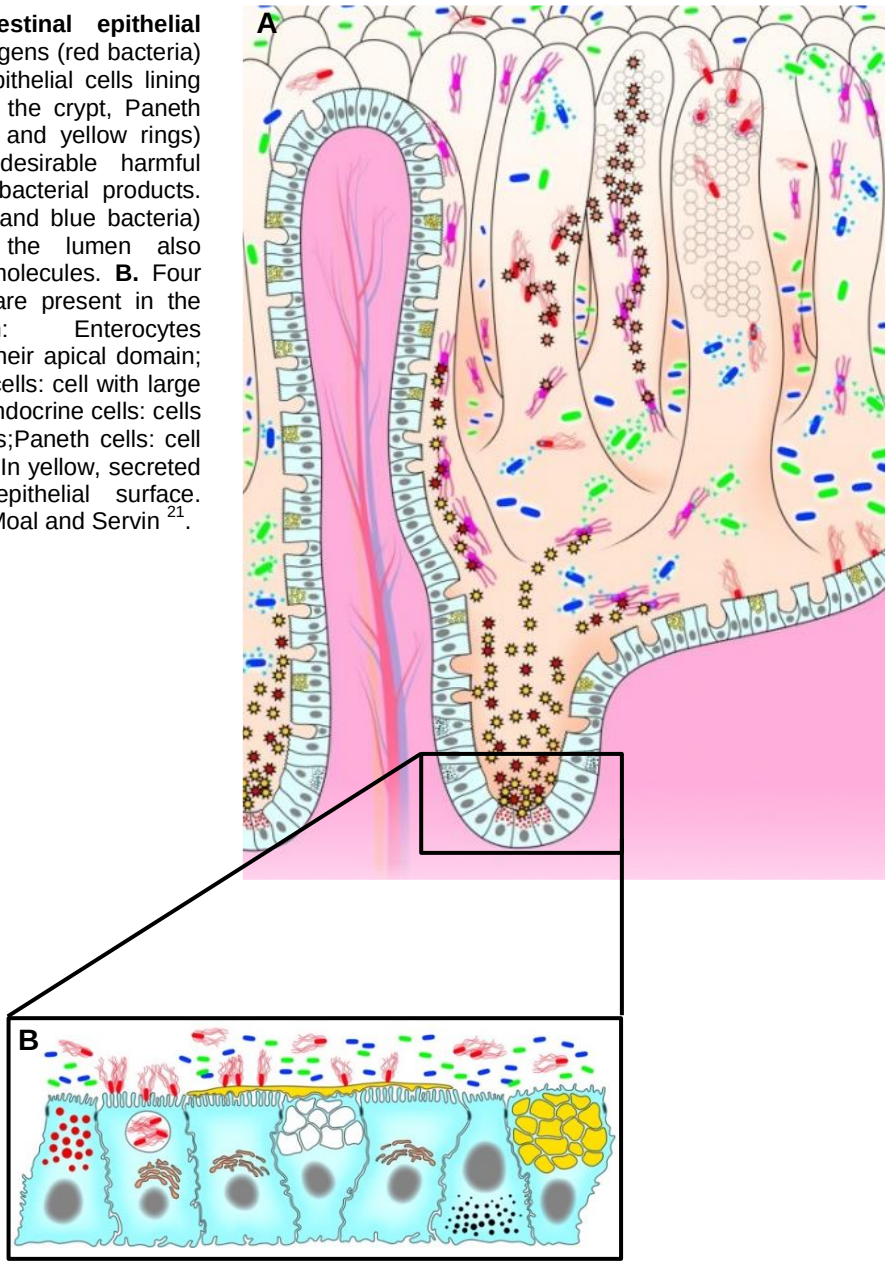

Figure 1 - The intestinal epithelial barrier. A. Enteric pathogens (red bacteria) interact with the host epithelial cells lining the villi. At the base of the crypt, Paneth cells releas AMPs (red and yellow rings) upon exposure to undesirable harmful pathogens and/or their bacterial products. The commensal (green and blue bacteria) intestinal bacteria in the lumen also produced antibacterial molecules. **B.** Four epithelial cell lineages are present in the intestinal epithelium: Enterocytes harbouring microvilli at their apical domain; mucus-secreting goblet cells: cell with large yellow granules; enteroendocrine cells: cells with small, dark granules;Paneth cells: cell with small, red granules. In yellow, secreted mucins coating the epithelial surface. Adapted from Liévin-Le Moal and Servin [21].

Gut associated lymphoid tissue

The gastrointestinal tract is considered the largest immune organ due to its well-organized immune system: the gut associated lymphoid tissue (GALT). The GALT (Figure 2) is composed by different lymphoid organs, such as mesenteric Lymph Nodes (mLN), Peyer's Patches (PP), Cryptopatches (CP) and Isolate Lymphoid Follicles (ILF) where T and B cells can interact and become activated[49]. Development of secondary lymphoid organs is a well characterized[50] process that starts between day 12.5 and 15.5 during embryogenesis where foetal liver hematopoietic progenitors start to colonize the gut[51,52].

Enteric secondary lymphoid organ development depends on lymphoid tissue inducer (LTi) cells[53,54] and it was shown that LTi deficient animals fail to develop LNs, PPs, CPs and ILFs[55–59]. Peyer´s patches formation occurs due to interactions between RET^+ lymphoid tissue initiator (LTin) and LTi cells with $VCAM^+ICAM^+$ lymphoid tissue organiser (LTo) stromal cells[60]. RET and LTo-derived ARTN signalling is crucial in the formation or PPs[60]. Nevertheless, it was shown that RET is not required for the development of LTin or LTi cells, but *Ret* deficient LTin and LTi cells fail to aggregate into PPs[51,52]. Different cytokines such as TRANCE or IL-7 induce LTi differentiation into $LT\alpha\beta$ expressing cells which are attracted by chemokines such as CXCL13, CCL19 and CCL21 into the emergent anlagen structures[60].

Similar to Peyer´s patches, during the formation of lymphoid nodes LTi cells aggregate close to endothelial cells regulating the differentiation of LTo cells[61]. In this process, $CD11c^+$ LTin cells are observed in the primitive lymph node but these cells and RET-ARTN interactions are dispensable for their development[61].

In the lamina propria of the murine intestine we can also found clusters of group 3 (ILC3s) surrounded by dendritic cells (DC) named as cryptopatches. Although CPs start to aggregate around day 14 after birth[49,62–65], they do not rely on microbiota, thus, it was observed that in germ free mice the number of CPs is unperturbed[66,67]. After contact with bacteria or during an inflammation, B cells are recruited to CP where critical IgA mediated-responses develop[49,68,69]. As occurs in PP, follicular B cells become plasma cells controlling microbiota in the lumen of the gut[68,70,71]. These mature follicles are named isolate lymphoid follicles (ILFs) and are large lymphoid structures composed by a single B cell follicle surrounded by ILC3[69]. In contrast to LNs and PPs, ILFs maturation depends on microbiota[62,72], notably in germ free mice that CPs fail to recruit B cells[66,67]. Thus, ILFs only form around weaning time and continuously throughout life when solid food intake increase bacterial load[67]. Pathogen associated molecular patterns induce ILFs

aggregation via NOD-1-dependent recognition by epithelial cells[67]. In addition, expression of $LT\alpha_1\beta_2$ and CXCL13 by ILCs regulates ILFs development. $LT\alpha_1\beta_2$ binds to LTβR on stromal cells[73] up-regulating the expression of the chemokine CXCL13 and adhesion molecules in order to recruit and retain lymphocytes[72,74]. In agreement, LTα and LTβ-deficient mice do not have CPs and ILFs [14,75–77].

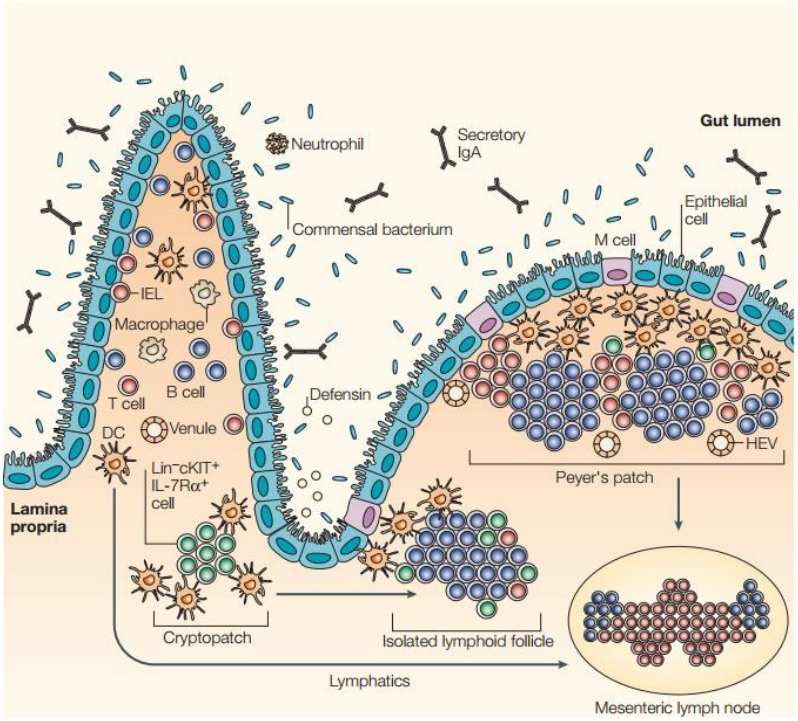

Figure 2 - Associated lymphoid tissue or GALT. The lymphoid structures of the gastrointestinal tract include mesenteric lymphoid nodes, Peyer´s patches, cryptopatches and ILFs. Development of PPs and MLNs occurs during embryogenesis. After birth, ILC3s induce the formation of cryptopatches that, upon challenging with microbiota and recruitment of B cells may develop into ILFs[66].

Intestinal microbiota

Presumably, when thinking of a human body the first thing that comes into mind is the idea of a separate and unique entity. Far from this, we are the result of millions of years of co-evolution with prokaryotic entities which resulted into a balanced host-microbial symbiotic relationship. In the last decade the intestinal microbiota got our attention due to its capacity to affect the host´s metabolism, immune system and its response to infection. Recent studies also showed that the microbiota can affect and be affected by factors such as the diet, genetics and immune signals, thus, microbiota sensing by the host may have a critical impact in organismic homeostasis, in health and disease[78–80]. As an example,

chronic infectious, inflammatory and metabolic diseases in humans have been associated with alterations in the composition or localization of commensal bacteria[81].

The intestinal bacterial load is 10 times more abundant than the total number of cells in the body. The microbiome - the collective genome of the microbiota - contains at least 100 times more genes than the human genome[12], therefore, the microbial composition and its metabolites can significantly shape gut microenvironment and dysbiosis can relate to disease[82]. Importantly, the gut microbiota, the epithelial cells and the immune system in the gut act together in order to provide protection against deviating pathobionts and pathogenic microbes, ensuring the maintenance of a balanced commensal bacterial population[49,83,84].

The enteric immune system

It is well-established that a healthy immune system is necessary to prevent disease and immune cells are fundamental to limit pathogen invasion. For that purpose, the immune system deploys two major arms of response: the innate and the adaptive immune system.

The innate immune system first appeared 750 million years ago at almost every level of the evolutionary tree of life, that is, vertebrates, invertebrates and plants. It has been remarkably conserved throughout the evolution. From a Darwinian point of view innate immunity has been crucial for surviving in the so-called "struggle for existence" [85–87].

The innate immune system is composed by different cell types such as phagocytic cells, antigen presenting cells, killer cells and we should also take in consideration epithelial cells that are physical barriers, cytokine and chemokines producers, and express a broad repertoire of innate immune receptors[88]. This arm of the immune system also includes soluble recognition molecules and the complement system[89]. Innate immune sensing mechanisms include pattern recognition molecules that are able to integrate and perceive danger signals from the microenvironment.

The adaptive immune system arose 500 million years ago and is restricted to vertebrates. Its response is mediated by T and B lymphocytes that evolved to express a high number of recombinant receptors: the T-cell receptor and the immunoglobulins, respectively, and which are capable to recognize defined antigens. B and T lymphocytes have the ability to recall previous antigens encounters and mount memory responses that are stronger and faster that previous primary immune responses[11,86,87,90].

Although adaptive immune responses are highly specific, they are slow as a consequence of the requirement for specific B and T cell clones to be activated and expanded after the first contact with a pathogen. The orchestration of immune responses and the production of cytokines is crucial for adaptive immunity to infection[91]. Since single bacteria can produce a 20 million progeny in an hour, the adaptive immune system can take a week before the response is effective. Accordingly, during the first critical hours and days after exposure to a new pathogen we mostly rely on our innate immune system to protect us from infection. As such, the innate immune system acts instantaneously as a first line of defence and is important until the adaptive immune system become efective[11,89].

Adaptive T cells are activated after binding of their T cell receptor (TCR) with an antigen presenting cell. However, in order to control their response many different signals are integrated by the T cell; notably, diet derived products, cytokines, microbial products and

oxygen levels[92]. As an example, recent work showed T cell oxygen sensing by prolyl-4-hydroxylase domain (PHD) proteins as being critical for tumour metastasis into the lung[93]. Interestingly, B cells can also sense environmental cues, such as calcium. Notably, B cells can sense calcium levels fluctuations shaping B cell function[94].

Pattern recognition receptors

The expression of microbe-associated molecular patterns or MAMPs in endogenous bacterial populations and pathogen-associated molecular patterns or PAMPs in enteric pathogens can be distinguish through pattern recognition receptors (PRRs)[95]. Sensing is one of the capacities that the organism implements to protect itself from aggressions. PAMPS can be recognized by different types of PRRs depending on their localization. They can be sub-divided in 3 groups: secreted, cytosolic and transmembrane receptors[96].

- The secreted PRRs are the collectins, ficolins and pentraxins and are involved in virus, bacteria and fungi infections, clearance of apoptotic and necrotic cells, resolution of allergic processes and inflammation[96]. These proteins can bind to the pathogen cell surface, activate the complement or opsonize the pathogen for phagocytosis by neutrophils and macrophages[96].

- The cytosolic PRRs include the retinoic acid inducible gene I (RIG-I) like receptors (RLRs) and the nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs). These receptors can detect viral nucleic acids, microbial products and a variety of different stress signals[95,97].

- The transmembrane PRRs are the family of toll-like receptors (TLR) (Figure 3) and C-type lectin receptors that are express either in endosomal organelles or in the plasma membrane. Mammals possess 10–13 types of TLRs and each of them depends on the PAMP that can recognize. Thus, TLR4 recognizes lipopolysaccharide (LPS) of gram$^-$ bacteria; TLR1/TLR2 and TLR2/TLR6 lipoteichoic acids of gram$^+$ bacteria and bacterial lipoproteins respectively and TLR5 recognizes flagellin. In addition, endosomal TLRs can sense microbial nucleic acids. For instance, dsRNA can be recognized by TLR3, ssRNA by TLR7 and dsDNA by TLR9. The C-type lectin family of receptors include Dectin-1 that recognizes β-glucans and Dectin-2 which senses fungi[98].

TLRs are membrane-bound glycoproteins with leucine-rich repeats and cysteine-rich repeats ligand-binding motifs and with a Toll/IL-1 receptor [TIR] as cytoplasmic signalling domain[99]. TIR domains are involved in inflammation[100]. After activation, TIR domains use the adaptor molecule myeloid differentiation primary response gene 88 (MyD88) or the TIR domain–containing adapter-inducing INF- β (TRIF). Essentially, TLR responses are subdivided in two types depending on the molecular pathway that is used: Myd88 or TRIF[100]. For instance, TLR3 exclusively signal through TRIF but other TLRs signal primarily through MyD88. As an exception, TLR4 can activate both signalling pathways[95,97,99]. Interestingly was shown that TLRs are express not only in immune cells but also in non-immune cells as the enteric nervous cells in the intestine[101–103]

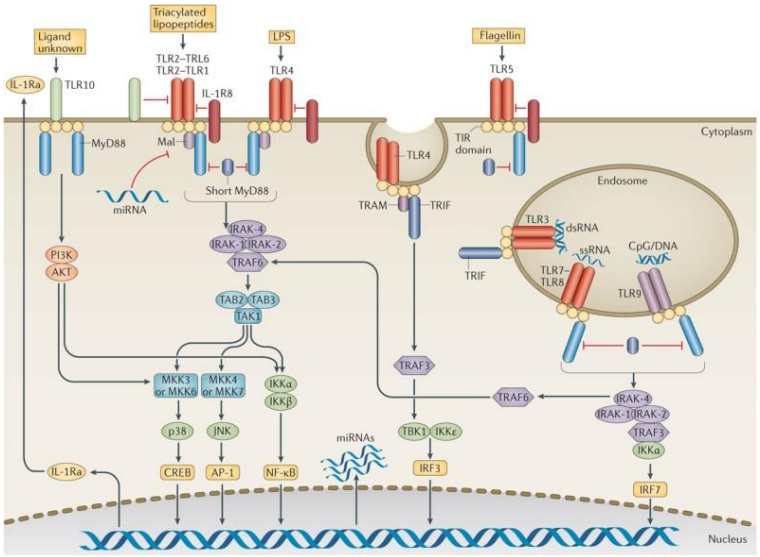

Figure 3 - Localization, signaling pathways and regulation of Toll-like receptors (TLRs). TLR1, TLR2, TLR4, TLR5 and TLR6 localize in the cell surface, TLR3, TLR7, TLR8 and TLR9 localize in the endosomes and sense microbial and host-derived nucleic acids. TLRs are membrane-bound glycoproteins with a Toll/IL-1 receptor [TIR] as cytoplasmic signalling domain. TIR domains use the adaptor molecule myeloid differentiation primary response gene 88 (MyD88) or the TIR domain–containing adapter-inducing INF- β (TRIF). TLR responses are subdivided depending on the molecular pathway that is used: Myd88 or TRIF. dsRNA, double-stranded RNA; LPS, lipopolysaccharide; miRNA, microRNA; ssRNA, single-stranded RNA. Adapted from O'Neill, L. A. *et al.*[403].

Innate lymphoid cells

In addition to adaptive T lymphocytes, the immune system also harbours the recently identified family of innate lymphoid cells (ILCs). ILCs are members of the lymphoid lineage and are characterized by their lymphoid morphology, the absence of recombination activating genes (RAG) and the lack of cell lineage phenotypical markers[83,104]. ILC responses are important to resolve inflammation and infection[53,91]. Contrary to T cells, ILCs mainly reside in non-lymphoid tissues[105]. Importantly, ILCs can also express MHC class II and present antigens to adaptive lymphocytes[106,107]. Thus, ILCs can interact with other immune cells, such as T cells being important in the coordination of adaptive immune responses[108–111]. Deregulated ILC functions were also shown to contribute to chronic inflammatory diseases, metabolic disorders and cancer[83]. Several studies have investigated the role of ILCs in humans[112], but little is known on their role in inflammatory bowel pathologies.

Different nomenclatures have been proposed for innate lymphoid cells[53], nevertheless, there is a consensus in which ILCs can be sub-divided in three main groups based on their cytokine production and the expression of transcription factors that regulate their development and function (Figure 4). Innate lymphoid cell subsets mirror the T helper cell compartments[104]. As such, ILCs are the innate counterparts of the Th1, Th2 and Th17 subsets.

- Group 1 ILCs (ILC1) comprises the cytotoxic NK cells[113] - INFγ, TNF, perforin and granzyme producers- and helper ILC1s – INFγ and TNF producers - that express the transcription factor T-bet. Group 1 ILCs participate in immunity to viruses, intracellular pathogens and in tumour surveillance being involved in inflammatory diseases[114,115].

- Group 2 ILCs (ILC2) comprises IL-5, IL-9, IL-13 and amphiregulin producing cells. ILC2 dependent on GATA3, RORα and Notch for their development and function. ILC2s are involved in helminthic infections, wound healing and are associated with allergy and asthma[116–118].

- Group 3 ILCs (ILC3) comprises the foetal Lymphoid Tissue inducer cells (LTis) and helper ILC3 - IL-17 and/or IL-22, GM-CSF, TNF and lymphotoxin producers - that require RORγt, AHR and Notch for their development and function. Group 3 ILCs are involved in lymphoid tissue development, intestinal homeostasis, immunity to

extracellular bacteria and are associated with inflammation bowel disease[91,119–122]. Crohn´s disease patients were shown to have altered innate IL-17 [119] and reduced ILC3 numbers together with increased ILC1s, possibly suggesting that ILC1s may derived from the ILC3s counterparts[115].

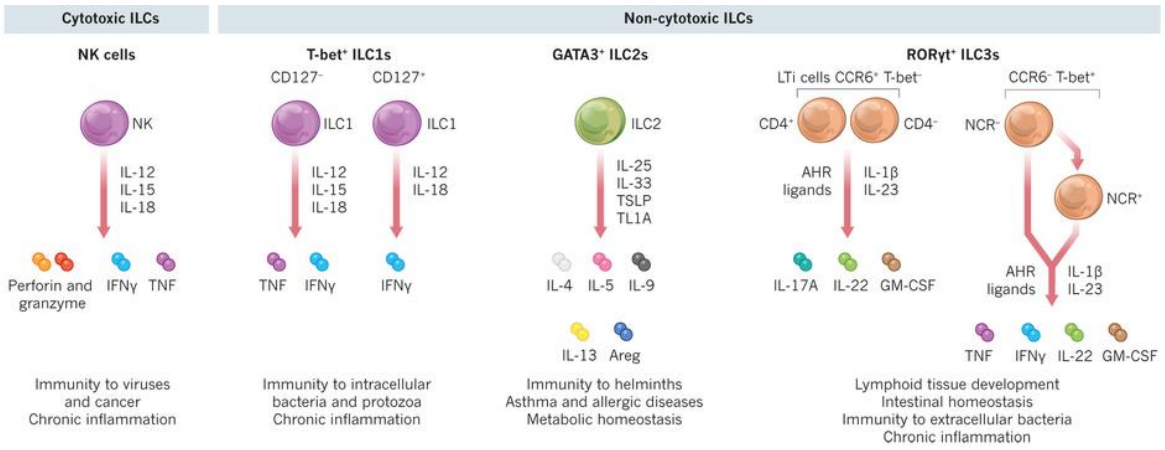

Figure 4 – Innate lymphoid cells. ILCs can be subdivided in cytotoxic ILCs or NK cells and in non-cytotoxic or helper ILCs which include ILC1s, ILC2s and ILC3s. These different subsets were grouped depending on their transcription factors and cytokines that regulate their development and function. ILCs respond to a diverse range of signals such as neuropeptides, hormones or cytokines contributing to immunity, inflammation and homeostasis. However, dysregulated ILC responses can also contribute to chronic inflammatory diseases, metabolic disorders and cancer. AHR, aryl hydrocarbon receptor; Areg, amphiregulin; GM-CSF, granulocyte macrophage colony-stimulating factor; IFNγ, interferon-γ; IL, interleukin; LTi, lymphoid tissue inducer; NCR, natural cytotoxicity receptor; NK, natural killer; TNF, tumour necrosis factor; TSLP, thymic stromal lymphopoietin[83]

Development of Innate Lymphoid cells

Innate lymphoid cells derive from a $Flt3^{+}IL7R\alpha^{+}$ Common Lymphoid Progenitor (CLP)[123] (Figure 5). Therefore, CLPs in either adult bone marrow or foetal liver can give rise of all ILC subsets[124]. Downstream of CLPs, a common lymphoid precursor (αLP) and the early innate lymphoid progenitor (EILPs) are further committed to the helper ILC and NK cell lineages[125,126]. αLP was described as $IL7R\alpha^{+}CXCR6^{+}NFIL3^{+}TOX^{+}\alpha4\beta7^{+}$ [127,128] and EILPs as $NFIL3^{+}IL7R\alpha^{-}TCF\text{-}1^{+}$[129]. EILPs express Id2 which is not required for their development[129]. NFIL3 regulates the expression of TOX, that is required for the development of NK cells and ILC subsets[130] and also promotes the expression of the transcriptional repressor inhibitor of DNA binding 2 (Id2)[131]. Differentiation of ILC subsets requires ID2 mediated suppression of lymphoid cell fates (as B or T cells) [132]. Restriction of αLPs into the specific ILC lineages occurs after losing NK-cell and LTi-cell potential[131].

ILC1s, ILC2s and ILC3s subsets differentiate via a common helper innate cell precursor (ChILP) present in adult bone marrow and foetal which expresses IL-7Rα, α4β7 and ID2. A subset of ChILP that express promyelocytic leukemia zinc finger (PLZF) and GATA3 was designated as helper ILC progenitors (ILCP). ILCP give rise to all helper ILC subsets. In contrast, NK cells differentiate from a NK progenitor (NKP) whereas LTi cells from a $PLZF^{-}$ChiLP[83,124,127,133]. Recently, it was demonstrated the role of PD-1 in ILC progenitors. A committed ILC progenitor identical to ILCP was described as $PD\text{-}1^{hi}IL\text{-}25R^{hi}$ and is critical in ILC2 development[134,135].

ILC progenitors can be found in both in the embryo and adult sharing similar phenotypes and developmental programs[136]. The vast majority of peripheral ILCs are tissue resident cells and it was established that these cells have a slow cell turnover in steady state, thus suggesting that the majority of ILCs may arise during foetal life[137]. Nevertheless, it is believed that ILC replacement occurs in inflammation or stress situations[136]. In contrast, NK cells continuously move from the blood to tissues regenerating their peripheral pool[137].

The exact mechanism of how resident ILCs and NK cells are regenerated and the pathways leading to cytotoxic or helper potential are not fully understood. It was proposed that ILCs may share the same factors that regulate T cell differentiation programs[138]. However, there are at least some differences between both systems such as the requirement for NFIL3 and ID2[136]. It remains unknown how different cell progenitors differentiate into adaptive lymphocytes, NK cells or LTis and how they acquired or lose their transcription factors. As an example, ILC3 were shown to loose RORγt expression and give rise to IFNγ producing ILC1 (ex-ILC3s) in certain conditions[136].

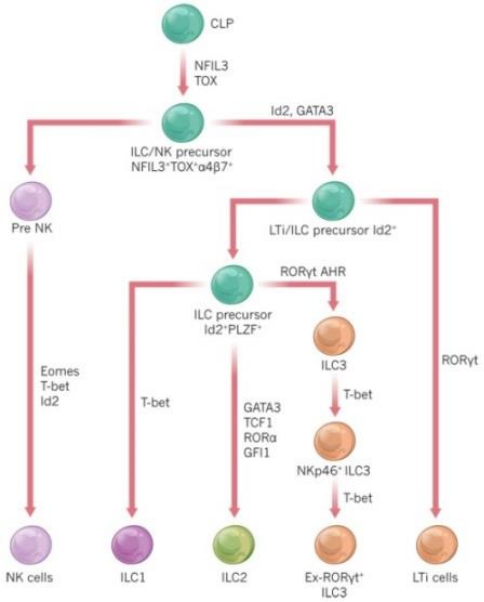

Figure 5 - Development of Innate lymphoid cells. Innate lymphoid cells derived from a common lymphoid progenitor (CLP). The common ILC/NK precursor also express the α4β7 integrin. Downstream is an Id2-expressing precursor and a $Id2^{+}PLZF^{+}$ precursor for non-cytotoxic ILCs. However, LTi cells are not derived from that $Id2^{+}PLZF^{+}$. AHR, aryl hydrocarbon receptor[83].

Group 3 Innate lymphoid cells

ILC3s are key regulators of mucosal barrier defence, notably for the maintenance of a healthy gut microbial environment contributing for tissue repair and homeostasis[83,53,132]. ILC3s are required for the proliferation of intestinal stem cells and replenishing the epithelial barrier after tissue damage during inflammation[139]. Interestingly, during chemotherapy ILC3s were shown to be radio-resistant mediating interleukin 22 (IL-22) production required for tissue protection[139]. ILC3s have been most studied in the intestinal mucosa where they aggregate into cryptopatches and isolate lymphoid follicles[134].

ILC3s rely on the transcription factor RORγt[140] which is necessary to regulate critical genes such as *Il17* or *Il22*. ILC3 are also regulated by the transcription factor AHR, that is essential for Th17 and ILC3 survival and function in particular the production of IL-22 by NCR^+ILC3 cells[141] . Noteworthy, AHR is activated by environmental and endogenous signals such as diet-derived ligands and microbial products[141,142]. Another master regulator required for ILC3 development is ID2, which acts upstream of RORγt and it was shown to affect the susceptibility to *C. rodentium* colonization[143]. RUNX3 is also required for the development of ILC1s and ILC3s[144]. RUNX3 binds to *Rorc* (which encodes RORγt) and it is necessary for optimal RORγt expression and also for *Ahr* transcription[144]. Likewise GATA3 regulates ILC3 development through regulation of CD127 (which encodes IL-7Rα) and controls RORγt and T-bet in ILC3s[145–147].

ILC3s can be further subdivided according to the expression of the chemokine receptor CCR6: *i.* $CCR6^+$ ILC3 include LTi cells ($CD4^+$ and $CD4^-$); *ii.* $CCR6^-$ ILC3s. $CCR6^+$ LTi cells were discover in 1997[148] and are crucial in the formation of secondary lymphoid organs during embryogenesis[60,149]. In the embryo, they are known for their Lin^- $CD45^+$ $ROR\gamma t^+$ $CD4^+LT\alpha^+LT\beta^+CD127^+CD117^+$ phenotype[150]. In adulthood they mature into LTi-like cells and are capable of producing IL-22 and IL-17[104]. LTi-like cells may be fundamental in the reconstruction of lymph nodes after acute viral infection[151]. However, their exact immune function in adulthood is still unknown[53,152,153].

$CCR6^-$ ILC3s can express the expression of the natural cytotoxicity receptor (NCR) NKp46. NCR^+ ILC3 are specific IL-22 producers while NCR^- can express IL-22 and IL-17[154]. Nevertheless, NCR^- IL-17 producers can also produce GM-CSF having an important role in colitis[154]. NCR^+ cells reside predominantly in mucosal tissues like the skin, lungs and intestinal tract mediating the balance between the symbiotic microbiota and the immune system[91]. These ILC populations also rely on signalling through IL-7Rα [53]. Innate

IL-22- producers have also been shown to play a critical role against bacterial infections such as *Citrobacter rodentium*[83,156]. *C.rodentium* is an attaching and effacing bacteria that induce colitis in mice and mirrors the effect of *E.coli* in humans[157]. ILC3s were shown to have an essential function in *C. Rodentium* infection model being critical for IL-22 production[121,158]. In this model, ILC3s are the predominant population of IL-22 producers in the first weeks of infection[121,159]. Nevertheless, whether innate and adaptive lymphocyte functions are redundant is not fully understood. A specific mouse model for ILC3 deletion will be extremely useful to understand the exact contributions of T cells and ILC3s. Nevertheless, recent work of ILC3 deficiency in humans demonstrated that the functions of this subset may be redundant with T cells[112], while in long-term-treated HIV-1-infected individuals with reduced T cell-derived IL-22 but preserved innate IL-22 displayed intact sigmoid mucosa integrity [160].

Environmental sensing by ILC3s

ILCs lack pattern recognition receptors but instead, they can sense cytokines, alarmins, lipids, hormones, dietary and microbial products and other molecules released by epithelial cells, stromal cells and other immune cells[161]. In contrast to mouse, human ILC3s can also be activated by recognition of pathogen-associated molecular patterns via the expression of Toll-like receptors[162]. As such, ILC3 have the capacity to sense their environment and integrate these signals in their functions. For instance, ILC1s can sense IL-12[161]; ILC2s, IL-33 or IL-25[163–165] and ILC3s can sense IL-23 and IL-1β among others[162,165]. Innate lymphoid cells were also shown to work as hubs in the crosstalk between different systems. As an example, ILC2s can be activated by enteric neurons through the neuropeptide VIP possibly regulated by the circadian clock[166] and ILC3s were shown to express molecules that might allow them to interact with the nervous system[167.]

ILC3s can respond to a sort of environmental signals, such as diet-derived molecules, microbial products and cytokines[165]. Several sensing mechanisms were shown to affect the development and function of different immune cells and organs.

Vitamin A-derived retinoic acid (RA), can bind to RAR or RXR nuclear receptors in the ILCs. Vitamin A is crucial for LTi cell differentiation and for the development of lymphoid organs in the embryo[168] acting upstream of RORγt[169]. As was previously mention, in the formation of lymphoid nodes, LTis are attracted by the chemokine CXCL13 from mesenchymal lymphoid organizer cells through LTβR[61,170]. For this to happen, stimulation

by TRANCE, IL-7 and RA is required[72,165]. This interaction promote the production of chemokines which will attract T cells and B cells into the follicle increasing the expression adhesion molecules[72]. In adult mice, RA signals promote ILC3 responses contrary to ILC2s that are supressed[171]. Hence, vitamin A deficient mice fail to resolve *Citrobacter rodentium* infection[168,171] although they can effectively resolve helminth infections.

Another dietary product sense by ILCs is the indole-3-carbinol (I3C) derived from cruciferous vegetables such as broccoli[172]. I3C ligands bind to the nuclear receptor aryl hydro-carbon receptor or AHR expressed in the ILC3s[172]. $CCR6^{-}$ILC3s are dependent on *Ahr* while $CCR6^{+}$ILC3s can develop in its absence although their function is affected producing less IL-22[173]. Accordingly, *Ahr* deficient mice failed to develop cryptopatches and to control *C. Rodentium* infection[142,174] .

ILC3s can sense IL-23 produced by dendritic cells (DCs) or mononuclear phagocytes inducing the production of IL-22 and IL-17, the master pro-inflammatory cytokines of the ILC3 lineage[175]. Stimulation of DCs is initiated by the LTβR-LTαβ binding on the ILC3s within the cryptopatches and is crucial for IL-23 production[176]. DCs-derived IL-23 is dependent of *Notch2* and is required for ILC3 activation. Accordingly IL-23 deficient mice were shown to fail in resolving C. *Rodentium* infection[165,175].

Another important environmental factor is represented by the microbiota. Commensals can induce IL-25 production in intestinal epithelial cells and IL-25 acts on DCs to limit ILC3s derived IL-22[159]. In addition, microbial products can be presented by $CCR6^{+}$ILC3s to $CD4^{+}$T cells through MHC class II inducing apoptotic cell death of activated commensal bacteria-specific T cells in the mesenteric lymph node[111]. Furthermore, in Crohn´s disease patients it was observed a reduction of ILC3s $MHCII^{+}$ cells suggesting a role of this pathway in inflammatory bowel pathologies[111].

In conclusion, a better understanding of ILC biology will certainly encompass the discovery of metabolites, microbial products and neuron-derived factors that are sensed by ILCs and can affect their function.

Dual effects of Interleukin 22

Interleukin (IL) -22 belongs to the IL-10 cytokine family (IL-10, IL-19, IL-20, IL-24 and IL-26)[177] and its expressed by innate (DCs, natural killer (NK) cells and ILCs) and adaptive (T cells) immune cells[178]. IL-22 can be regulated by many cytokines such as IL-23, IL-6 or IL-1β and transcription factors such as STAT3, RORγt or AHR. In this way, ILCs can respond to cytokines and produce IL-22 due to their expression of IL-1R and IL-23R[104]. IL-22 receptor is an heterodimer composed by IL-22R1 and IL10R2[179]. In contrast to IL10R2, which is expressed by most cells types, IL-22R1 is restricted to non-hematopoietic cells such as epithelial cells, stromal cells, keratinocytes or hepatocytes[179,180]. In enteric epithelial cells, IL22-IL-22R1-IL10R2 complex induces the activation of the STAT3 cascade that regulates many repairing genes, notably MYC, cyclin D1 and other proteins involved in proliferation and cell cycle as well as the expression of anti-apoptotic genes such as *Bcl2*, *Bcl2l1*, and *Mcl1*[181–183]. IL-22-derived ILC3 is necessary and sufficient for induction of epithelial cell fucosylation, a type of protein glycosylation which protects against enteric pathogens, possibly inducing epithelial cells to produce antimicrobial molecules[158,184–188]. The expression of IL22R1 in non-hematopoietic cells makes IL-22 an ideal therapeutic candidate specifically affecting tissue responses without targeting immune cells. Since Interleukin 22 is associated with IBD, the usage of IL-22 recombinant protein was proposed as an anti-inflammatory treatment[189].

A dual “beneficial vs pathogenic” role was assigned to IL-22 due its ability to induce pro-inflammatory (IL-8 or IL-6)[190,191] and anti-inflammatory or regulatory (antibacterial peptides or IL-10) molecules. IL-22 promotes epithelial cell proliferation to restore the epithelial barrier after an insult. IL-22 function is tissue specific contributing to the regulation of diseases like hepatitis or IBD[187,192] and IL-22 is critical for mucosal homeostasis and tissue repair. Nevertheless, exacerbated production of IL-22 can also lead to inflammation[188,190,193–195]. Thus, IL-22 can be beneficial for the host in many diseases but in some others it can also be pathogenic showing a strong pro-inflammatory effect that can be enhanced when acting together with other pro-inflammatory cytokines such as IL-17.

In the *C. rodentium* colitis model, IL-22 deficient mice fail to resolve infection and to induce the expression of antimicrobial proteins, such as epithelial cell-derived defensins[156], and mucus[192]. During colitis, IL-22 also induces wound healing through the STAT3 pathway[196]. IL-22 often acts together with IL-17; nevertheless the main role of IL-

17 is to recruit neutrophils and to induce the expression of pro-inflammatory cytokines[197,198]. In the DSS model, overexpression of IL-22 in T-cell receptor (TCRα)-deficient mice reduces disease[192]. Accordingly, blocking of the IL-22 pathway increases DSS induced colitis score[183,192]. Interestingly, IL-23R was shown to be a susceptible gene during Crohn's disease and elevated levels of IL-22 were found in IBD patients, correlating levels of cytokine with disease severity[199].

Constitutive expression of IL-22 in the small intestine contributes to the anatomical containment of commensal bacteria. While IL-22 is produced constitutively by ILC3s in the small intestine, depletion of ILC3s resulted in peripheral dissemination of commensal bacteria and systemic inflammation, which could be prevented by administration of IL-22 recombinant protein[200].

Additionally, IL-22 can also be beneficial in allergy airway inflammation, graft-versus-host disease (GFHD) and autoimmune diseases[201–203]. In liver diseases, IL-22 stimulates the proliferation of hepatocytes and regenerates the tissue[204]. However, in many situations, IL-22 acts as a pro-inflammatory cytokine and the dysregulation of its expression can lead to pathology. During *Toxoplasma gondii* infection, IL22-deficient mice show decreased signs of inflammation in the small intestine[205] while wild type IL-22 sufficient mice die of necrosis in the ileum.

A clear example of the dual effect of IL-22 is found in the skin. IL-22 induces tissue defence and wound healing; nevertheless, overexpression of IL-22 can cause skin inflammation. In fact, this overexpression produces psoriasis; an hyper proliferation and abnormal differentiation of keratinocytes (thickness of the skin) and infiltration of leukocytes[206]. This beneficial or pathogenic role of IL-22 depends on different factors such as the concentration and duration of local cytokine, the target tissues and the cytokine microenvironment. For instance IFN-α and TNF-α increase IL-22R and IL-10R2 expression on keratinocytes, thus increasing the capacity to respond to IL-22[194,207]. In addition IFN-α induce differentiation of monocytes into DCs that via IL-23 leads to IL-22 production[208].

IL-17 has a pro-inflammatory role, but both IL22 and IL-17 can act together to determine the protective or pathogenic effect of IL-22[198]. Acute airway inflammation is lethal in wild type mice but is ameliorated in *Il22* deficient mice, indicating a pro-inflammatory role of IL-22. In contrast, *Il17* deficient mice are more protected from airway inflammation despite higher IL-22 production, thus indicating that IL-17 regulates the pro-inflammatory function of IL-22[181].

Finally, the IL-22 effects can be neutralize by the soluble-secreted receptor IL-22 binding protein (IL-22BP)[209–211] mainly produced by colon DCs[212,213]. During DSS-induced colitis IL-22 beneficial role was supressed in the presence of IL-22BP, nevertheless during acute colitis IL-22BP was downregulated[192]. It is known that inflammation may predispose to the development of colon cancer. In this way, IL-22BP regulates epithelial cell proliferation limiting colon tumorigenesis[212,214]. In a model of IL-22BP deficient mouse tumour induction was linked to the insufficiency to control IL-22 effects[212]. After tissue damage, the inflammasomes NLRP3 and NLRP6 downregulate the expression of IL-22BP, which increases the proliferative effect of IL-22 promoting tumour development[212].

Inflammatory bowel pathologies

Malfunction of the intestinal epithelial barrier is related with ulcerative colitis (UC) and Crohn´s disease (CD). Ulcerative colitis was first described in 1859 by Samuel Wilks while Crohn´s disease was first reported in 1932[215]. Although UC and CD are different entities from a pathophysiological point of view, both pathologies share clinical manifestations including bowel inflammation. Inflammatory bowel diseases (IBD) can course with bloody diarrhoea, weight loss, fever, abdominal pain, anxiety, depression and other psychological issues[216,217]. Unfortunately, these diseases are incurable and the available treatments are focused on reducing bowel inflammation and infection control[218]. Nevertheless, different factors are involved on the onset and progression of IBD, including environmental conditions, lifestyle, genetics and gut microbiota content.

Inflammatory bowel pathologies were shown to correlate with smoking, high fat and high sugar diets, stress and drug consumption[219]. As such, IBD is a “modern pathology” as its incidence has increased in the last century[220]. Interestingly, smokers have milder disease, fewer hospitalizations and less need of medications[221].

A large number of genes are involved in the modulation of UC and CD, as an example, defects in the recognition molecule NOD2 were found in Crohn´s disease patients[222,223]. It is currently known that genetic factors greatly influence Crohn´s disease while their role in ulcerative colitis might be less pronouced[224]. Nevertheless, genetic screenings are not indicated to assess the risk of these pathologies[218]. Another abnormality in this pathologies is the high production of pro-inflammatory cytokines such as TNF-α, IL1-β and IL-6, but there is no specific pattern allowing to discriminate UC from CD[222,223,225,226]. Differences in humoral and cellular immunity such as IgM, IgA and IgG were found in IBD,

while specific increase of IgG1 was found in UC patients[227]. An important immunological feature that distinguishes UC from CD are $CD4^+$ T cell responses. Crohn´s disease patients exhibit Th1 responses producing INFγ while ulcerative colitis patients are characterised by atypical Th2 response (with NK cells) producing IL-13 and IL-5[227–230].

While intestinal microbial communities are essential for efficient digestion and protection against invasive microbes, there is limited evidence on what defines a beneficial gut microbiota for intestinal health. Different studies showed that patients with UC or CD have a higher density of particular microbes when comparing with healthy patients[231], nevertheless no particular group of commensal or pathogenic microbes was identified as the cause of IBD[232].

Faecal microbiota transplantation studies

Microbiota is a key component in inflammation bowel pathologies shaping the response of the immune system. Some bacteria populations can affect the development of T regulatory cells and pro-inflammatory cells, therefore, dysbiosis can be critical for immune responses and any alteration in the microbiota composition can led to disease. Dysbiosis implies changes in microbial metabolic products affecting environmental sensing and the recognition of pathogenic microbes[233]. Altered microbial composition was reported in Crohn´s disease patients showing invasion of pathogenic enterobacterias, such as *E.coli*[231,234–236].

The fact that dysbiosis is related with disease makes microbial manipulation an interesting tool to treat inflammatory bowel pathologies. Gut microbiota is now considered as a virtual organ and faecal microbiota transplantation (FMT) has emerged recently as a new strategy to ameliorate IBD symptoms[237]. Nevertheless, clinical trials with IBD patients showed diverse results that is in some cases a reduction of inflammation after FMT[238] and in some others no symptoms remission[239].

Other studies with Irritable bowel syndrome (IBS) patients focused in bacterial genomic DNA from faecal samples[240]. As observed in IBD, microbiota of healthy donors was distinct from the one in IBS patients, which had increased *Firmicutes* and *Bacteroidetes*[241].

Interestingly, many studies have associated microbiota with mental disorders such as Parkinson, Multiple sclerosis or autism[242]. After alteration of gut microbiota with antibiotic treatments Parkinson symptoms resolved[9,243–245]. Coffee and smoking were considered

neuroprotective against Parkinson disease, yet through an unknown mechanism[243]. Additionally, autism was shown to be associated with intestinal microbes. In many patients suffering of chronic diarrhoea, antimicrobial treatments led to the appearance of autism and distinct bacteria populations where found when compared to healthy controls[246].

The brain-gut axis association was also considered to be involved in many gastrointestinal and neurodegenerative disorders. For instance, germ free mice have an over activation of the hypothalamic-pituitary-adrenal axis when responding to stress and this was reversed only with microbial treatment[247]. Several studies showed how FMT can alter behaviour, however, there is still no evidence on what type of beneficial microbiota may ameliorate these kinds of disorders[248–250].

Despite the large amount of correlative data on microbiota and disease, the physiology of this process and the exact healthy bacterial populations for each disease remain elusive. Nevertheless, the use of FMT could be in the near future an exciting treatment for both bowel and mental disorders.

Enteric nervous system

The gastrointestinal tract harvests the largest number of nerves and glial cells outside the central nervous system (CNS). Our “second brain” is the enteric nervous system[251].

The ENS (Figure 6) is composed by more than 100 million neurons and 400 million glial cells organized in two major plexus: the myenteric plexus (Auerbach´s plexus) that controls gut motor activity and the submucosal plexus (Meissner´s plexus) which role is to regulate mucosal processes[251,252]. The axons that reach the intestinal mucosa are critical contributors of neuromediators such as acetylcholine, vasoactive intestinal peptide (VIP), substance P and neuropeptide Y. While the ENS has the ability of regulate the gastrointestinal functions independently from the central nervous system (CNS), the CNS can modulate the activity of the ENS affecting digestive functions[253].

The ENS controls a large number of processes including intestinal motility and vascular blood flow. However, recent evidence indicates that enteric neurons and enteric glial cells can also control intestinal epithelial barrier functions[251]. Signals released by the microbiota were shown to influence the interactions between macrophages and enteric neurons in the intestinal muscular layer contributing to gastrointestinal motility[254]. Thus, the ENS together with the immune system and the microbiota should be consider as major actors in the maintenance of the intestinal epithelial barrier [17].

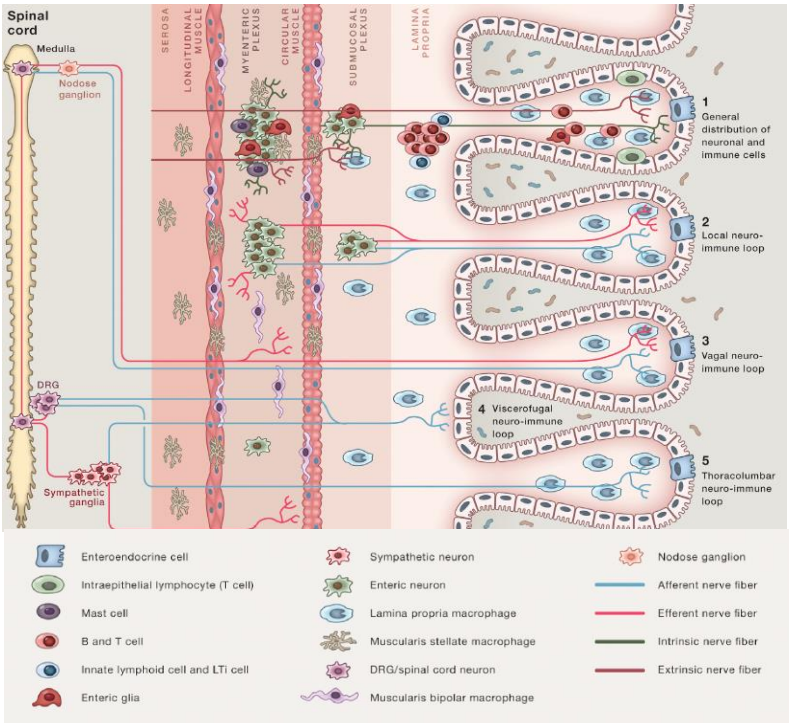

Figure 6 - The enteric nervous system. The ENS has ganglionated plexuses, the myenteric plexus between the longitudinal and circular layers of the external musculature and the submucosal plexus that has outer and inner components. Nerve fibres connect the ganglia and form plexuses that innervate the longitudinal muscle, circular muscle, muscularis mucosae, intrinsic arteries and the mucosa. Most immune cells accumulate in the lamina propria of the mucosa regions, while neuronal cell bodies are restricted to the submucosal and myenteric plexuses. Red processes, afferent; light-blue processes, efferent[6,404].

Enteric glial cells

Enteric glial cells (EGCs) were first identified in 1899 by Dogiel[255] as nucleated satellite cells localized in the proximity of enteric neurons[17]. As astrocytes from the CNS, enteric glial cells express the glial fibrillary acidic protein (GFAP), S100β [256,257] and can also express SOX10 [258,259] in mature differentiation stages. Different levels of expression of those proteins were found in distinct glial cells from the same layer or from different compartments, thus suggesting the existence of enteric glial cell subpopulations[260,261].

The colon and the small intestinal mucosa are highly innervated by neurons and glial cells that derived from neural crest progenitors and colonize the gut during embryogenesis[262,263]. In the intestinal lamina propria a dense network of glial cells is known to locate predominantly at the base of the crypts[185] and their close proximity to intestinal epithelial cells and axons suggests the possibility of paracrine communication between these cell types[265,266].

In the past decade the knowledge on enteric glial cells increased dramatically thanks to new tool implementations[6]. EGCs have an important role in the maintenance of the IEB integrity and paracellular permeability, demonstrated by *in vivo* ablation of those cells in mouse models[17,267,268].

Previous studies have also shown regulation of GFAP during intestinal inflammation. For instance, upregulation of GFAP in inflamed areas was observed in patients suffering from Crohn´s disease and ulcerative colitis[268,269]. This upregulation of GFAP was also associated with an increase in GDNF expression[269] and was correlated with a protective role on both epithelial cells and enteric neurons[270,271]. In addition, the RET ligand GDNF was described to be synthetized and secreted by enteric glial cells contributing to the maintenance of intestinal homeostasis and being critical in colitis and inflammation processes. In fact, it was shown that GDNF helps to resolve inflammation after dextran sodium sulphate (DSS) induced colitis via regulation of enteric neurons[269,270,272,273].

In the intestinal mucosa TLRs are critical for environmental sensing mediating inflammation and protection against pathogens[274–276]. Notably, TLR2 was found to play a crucial role in gut homeostasis and its absence relates with inflammatory bowel pathologies[277,278]. Interestingly, TLRs are also expressed in tissues that are not usually exposed to microbes such as the nervous system and the smooth muscle. Interestingly, enteric glial cells also express TLR2[99,101,279–281].

The lack of murine TLR2 was demonstrated to produce alterations in the enteric nervous system together with a dysfunction in mucus secretion and a low production of GDNF[101]. Interestingly, in TLR2 deficient mice those abnormalities were corrected by the administration of GDNF[101]. In addition after DSS induced colitis, TLR2 deficient mice were more susceptible to inflammation when compared their wild-type counterparts[101]. Nevertheless, the mechanisms that regulate GDNF expression and its specific target cells still needs to be elucidated.

RET tyrosine kinase receptor

The *Ret* gene - ***r***earranged during ***t***ransfection- was first described as a proto-oncogene in 1985[282–284]. The *Ret* proto-oncogene encodes the protein RET, a tyrosine kinase receptor essential for neural crest cell migration and normal proliferation, maturation and survival of the enteric nervous system[285–287]. RET is highly express during development[288,289] and additionally it has been implied in kidney development, spermatogonial stem cell maintenance and it also was recently established a role for RET in the survival and function of hematopoietic stem cells[290–294]. Furthermore, RET is expressed in neuroendocrine cells of the thyroid and chromaffin cells of the adrenal gland[288,289].

In humans, *Ret* is located in the chromosome 10q11.2[295] and due to alternative splicing it can generate three different protein isoforms RET9, RET43 and RET51 differing on their C-terminal amino acid composition[296]. RET is composed by a large extracellular domain with four cadherin-like repeats, which are important to stabilize RET dimers; a cysteine-rich domain crucial for protein conformation and ligand binding; a transmembrane domain and an intracellular kinase domain[284,297,298]. In the endoplasmic reticulum RET proteins are glycosylated to produce immature proteins which have to be processed to obtain the final mature protein.

Increasing evidence indicates an important role of RET in the immune system and its relevance in the formation of Peyer´s Patches in the intestine[60,299]. Interestingly, RET is express in a subset of lymphoid tissues initiator cells (LTin) ($CD45^+CD3^-CD4^-IL\text{-}7R\alpha^-c\text{-}kit^+CD11c^+$) cells. LTin cells are able to interact with stromal cells via ARTN/GFRa3 axis attracting LTi ($CD45^+CD3^-CD4^+IL\text{-}7R\alpha^+c\text{-}kit^+$) cells which cluster into Peyer's patches primordia in a chemokine-dependent manner[60]. RET is also express in hematopoietic stem cells (HSC) and it was shown to regulate HSC survival and function[293].

The expression of RET in human B cells, T cells and monocytes was reported several years ago[300]. Similarly to nervous cells in the central nervous system (CNS), immune cells were also shown to produce the RET ligands NRTN and GDNF. CNS-derived RET ligands were thought to affect immune cells and vice versa, but at that time, the exact mechanisms that regulated those processes was unclear[300].

Years later another set of data indicated that patients suffering from Hirschsprung disease (HSCR), and with hypomorphic mutations of *Ret*, had increased inflammatory cytokines in peripheral blood mononuclear cells (PBMCs). Notably, PBMC stimulated with the RET

ligand GDNF had increased chemokines and inflammatory cytokines such as IL-1β, IL-6 and IL-8[301].

Activation of the RET receptor requires a group of soluble proteins of the glial cell line neurotrophic factor (GDNF) family ligands (GFL). GFL protein components includes neurturin (NRTN), artemin (ARTN), persephin (PSPN) as well as GDNF first described in 1993 by Li and colaborators[302,303] (Figure 7). However, RET does not bind directly to GFLs, it requires additional co-receptors, the GDNF family receptor-α (GFRα) members located in the cell surface through a glycosylphosphatidylinositol (GPI) anchor[298,302]. GFRα-GFLs form binary complexes that bind to RET recruiting it into lipid rafts where RET signaling takes place[304].

Despite some possible cross-talk, GDNF, NRTN, ARTN and PSPN bind preferably to GFRα1, GFRα2, GFRα3 and GFRα4, respectively[285]. This provides specificity and selectivity for RET-activating complexes in different cells. Furthermore RET can also crosstalk with other tyrosine kinase receptors, adhesion molecules and other cell surface proteins helping to stabilize signalling complexes and increase RET activity[305–308].

After binding of the GFRα-GFLs complex to RET, the latter dimerizes and auto-phosphorylation of intracellular tyrosine residues occurs[285,309]. This leads to the recruitment of adaptor and signalling proteins and the stimulation of multiple downstream pathways[302,309] along with the activation of RAS–MAPK and PI3K–AKT signalling pathways or to the recruitment of the CBL family of ubiquitin ligases that functions in RET downregulation[290,310,311].

RET controls important biological processes such as cell proliferation, migration, invasion and survival and accordingly act as a potent oncogene being critical in the initiation and progression of multiple human malignancies associated to both, loss and gain of RET function [285].

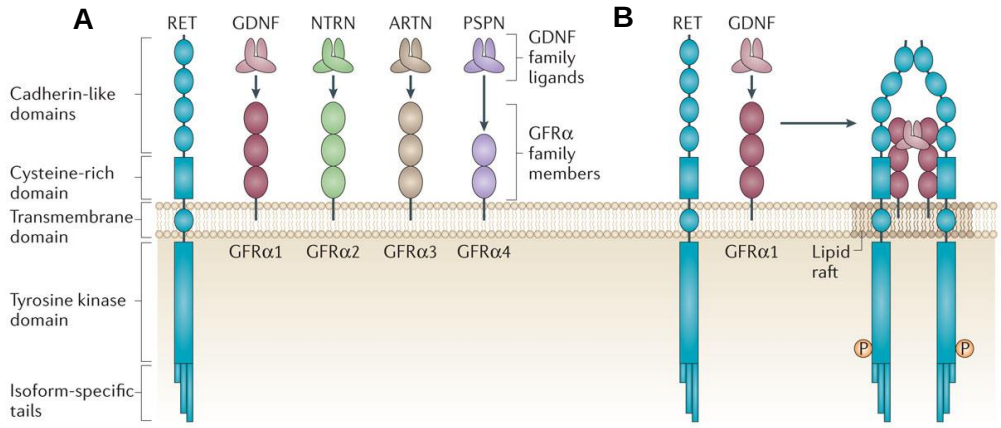

Figure 7 - The RET tyrosine kinase receptor. A. RET is the receptor for a family of soluble neurotrophic factor ligands, the glial cell line-derived neurotrophic factors (GFLs). Each of these ligands interacts with RET via a cell surface co-receptor, which consists of two to three globular protein domains linked to the cell membrane by a glycosylphosphatidylinositol anchor. **B.** Once RET bounds to the ligand–co-receptor complex, conformational changes facilitate RET monomer association via the cadherin homology domains and lead to receptor dimerization and autophosphorylation (P)[285].

RET associated pathologies

The dominantly inherited multiple endocrine neoplasia type 2 (MEN2), a cancer syndrome characterized by medullary thyroid carcinoma, is caused by amino acid substitutions in the RET protein leading to a constitutive ligand-independent RET aberrant activation and stimulation of downstream signals[312]. There are three clinical types of MEN2 syndromes: MEN2A, MEN2B and FMTC (familiar medullary thyroid carcinoma). In particular, MEN2B is caused usually by the substitution of a threonine for a Met918 converting the *Ret* gene in an oncogene[313]. MEN2B is characterized by developmental abnormalities including neuromas of the lips, tongue and conjunctivas and intestinal ganglioneuromas[289,314]. Those RET activations are mostly found in thyroid and lung carcinomas[285,315–317]. Although RET mutations are rare in other neoplasms, these were also detected in patients with chronic myelomonocytic leukemia and colorectal cancer[285].

Other tumours such as pancreatic carcinoma are associated with RET wild-type protein expression[318]. RET ligands are secreted by pancreatic nerves and stimulate in a paracrine manner migration of RET-expressing tumour cells[319]. It was also found in breast cancer that co-expression of RET and GFLs leads to an autocrine activation of tumour cells relating with metastasis[320]. Somatic mutations in RET were also identify in sporadic tumours and are the consequence of *Ret* locus rearrangements as occur in papillary thyroid carcinoma (PTC), lung adenocarcinoma or chronic myelomonocytic leukaemia[321,322]. Inversions or translocations that involve the *Ret* locus can produce chimeric proteins resulting in constitutive cytosolic activated proteins[323].

Since RET is express in a wide range of cells, loss of function mutations can also give rise to different pathologies including Hirschsprung disease (HSCR) and congenital abnormalities of the kidney (CAKUT). HSCR is characterized by abnormalities in the embryo neuroblasts migration into the gut leading to the incapacity of neurons and ganglia to mature[324–326] and producing intestinal motility alterations, aganglionosis and myenteric disorders[287]. Strikingly, fulminant gastroenteritis is frequently associated with paediatric cases of HSCR[285].

Neuro-immune interactions at barrier sites

The Immune and the nervous system recognize, integrate and respond to different signals and are the main sensory interfaces between the internal and the external environment[18]. Although well established that these systems can share similar regulatory mechanisms[327].

Every day organisms are faced with external aggressions and the immune and nervous systems are continuously evolving to guarantee protection. The immune and the nervous system form sophisticated barriers such as the blood/brain and the intestine/body barriers that share similar principles: they are physical barriers supported by cells with functional and morphological similarities. Despite their distinct embryological origin, nervous and immune cells have identical features suggesting shared mechanisms that may have evolved in different compartments of the body [328].

Both systems have a range of similarities such as the usage of transmitters and signalling molecules[329] being capable of communicate via cytokines, chemokines, neuropeptides, neurotransmitters and their receptors[330–332]. Cytokines and their receptors are produced not only by immune cells but also by nervous cells such as microglia, astrocytes and neurons[333].

The immune and the nervous system can recognize environmental cues via surface receptors. As an example, the TCR in T cells or GABBA receptors in neurons and they have the ability to connect and carry information from and to distant parts of the body using dendrites and axons in the case of neurons and active or passive mobility in the immune cells[327]. As already referred, TLRs are found in immune and nervous cells and are a clear example of a common feature between these two systems[103]. Another example is the neuropeptide Y that is produced by the central nervous system but also by lymphocytes affecting cell migration, cytokine release and antibody production [334].

In contrast, the interplay between the two systems can also be based on synaptic signals. Thus, the information is passed through specialized membrane interactions between cells of the nervous and immune systems[335,336]. Strong evidence is emerging suggesting that the nervous and the immune system are communicating through synapsis[337–339]. The immune and nervous system functions have somehow converged, raising questions about a possible common evolution from an ancestral progenitor cell [327,340].

AIMS OF THIS THESIS

The maintenance of a healthy intestinal epithelial barrier is critical to prevent pathologies. Previous studies have demonstrated that the commensal microbiota and enteric tissues play a critical role in the protection against aggressions. In particular, group 3 innate lymphoid cells are main regulators of mucosal barriers, sensing the environment and producing cytokines that in turn activate the epithelium to produce antimicrobial peptides and mucus[156,180].

Innate lymphoid cells (ILC) are an emergent family of effectors abundantly present at mucosal sites. Group 3 ILC (ILC3) produce pro-inflammatory cytokines and regulate mucosal homeostasis, anti-microbial defence and adaptive immune responses. ILC development and function have been widely perceived to be programmed. However, recent evidence indicates that ILC are also controlled by dietary signals. Nevertheless, how ILC3 perceive, integrate and respond to environmental cues remains utterly unexplored.

In this thesis, we hypothesise that ILC3 sense their environment and exert their function as part of a novel epithelial-glial-ILC unit orchestrated by neurotrophic factors. Thus, we employed genetic, cellular and molecular approaches to decipher how this unconventional multi-cellular unit is controlled and how glial-derived factors set ILC3 function and intestinal homeostasis.

In order to achieve this, we assessed ILC3-autonomous functions of neurotrophic factor receptors. ILC3-specific loss and gain of function mutant mice for neuroregulatory receptors were used to define the role of these molecules in ILC3 function, mucosal homeostasis, gut defence and microbial ecology. Sequentially we deciphered the anatomical and functional basis for the enteric epithelial-glial-ILC units. To this end we employed high-resolution imaging and glial-specific mutants for define target genes.

In conclusion, we aimed at establishing a novel sensing program by which ILC3 integrate environmental cues, focusing our efforts in defining a key multi-cellular unit at the core of intestinal homeostasis and defence. Finally, we believe that these combined approaches may have revealed new target pathways in inflammatory diseases that are major Public Health concerns.

GLIAL CELL-DERIVED NEUROREGULATORS CONTROL TYPE 3 INNATE LYMPHOID CELLS AND GUT DEFENCE

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Abstract

Group 3 innate lymphoid cells (ILC3) are major regulators of inflammation and infection at mucosal barriers[83]. ILC3 development has been considered to be programmed[83]. Nevertheless, how ILC3 perceive, integrate and respond to local environmental signals remains unclear. Here we show that ILC3 sense their environment and control gut defence as part of a novel glial-ILC3-epithelial cell unit orchestrated by neurotrophic factors. We found that enteric ILC3 express the neuroregulatory receptor RET. ILC3-autonomous *Ret* ablation led to decreased innate interleukin-22 (IL-22), impaired epithelial reactivity, dysbiosis and increased susceptibility to bowel inflammation and infection. Neurotrophic factors directly controlled innate *Il22,* downstream of p38 MAPK/ERK-AKT cascade and STAT3 activation. Strikingly, ILC3 were adjacent to neurotrophic factor expressing glial cells that exhibited stellate-shaped projections into ILC3 aggregates. Glial cells sensed microenvironmental cues in a MYD88 dependent manner to control neurotrophic factors and innate IL-22. Accordingly, glial-intrinsic *Myd88* deletion led to impaired ILC3-derived IL-22 and pronounced propensity to gut inflammation and infection. Our work sheds light into a novel multi-tissue defence unit, revealing glial cells as central hubs of neuron and innate immune regulation via neurotrophic factor signals.

Methods

Mice: C57BL/6J mice were purchased from Charles River. *Ret*^{GFP}[341], *Rag1*$^{-/-}$*γc*$^{-/-}$[342,343], *Ret*^{MEN2B}[317], *Rosa26*^{YFP} [344], *Rosa26*^{RFP}[345], *Ret*$^{fl/fl}$[346], *Rorgt*-Cre[347], *Il1b*$^{-/-}$ [348] and *Myd88*$^{-/-}$ [349] were in a full C57BL/6J background. *Gfap*-Cre[350] bred to *Myd88*$^{fl/fl}$ [351] were in F8-F9 to a C57Bl/6J background. All lines were bred and maintained at IMM Lisboa animal facility. Mice were systematically compared with co-housed littermate controls. Both males and females were used in this study. Randomization and blinding were not used unless stated otherwise. All animal experiments were approved by national and institutional ethical committees, respectively Direção Geral de Veterinária and iMM Lisboa ethical committee. Germ-free mice were housed at Instituto Gulbenkian de Ciência, Portugal, and Institut Pasteur, France, in accordance to institutional guidelines for animal care. Power analysis was performed to estimate the number of experimental mice.

Generation of foetal liver chimeras: For reconstitution experiments, 5x10^6 foetal liver cells were isolated from E14.5 *Ret*$^{WT/GFP}$ or *Ret*$^{GFP/GFP}$ mice and injected intravenously into non-lethally irradiated (200rad) alymphoid *Rag1*$^{-/-}$*γc*$^{-/-}$ hosts. Mice were analysed 8 weeks post-transplantation.

Dextran Sodium Sulphate-induced colitis: Dextran Sodium Sulphate (DSS) (molecular mass 36,000-50,000 Da; MP Biomedicals) was added into drinking water 3% (w/v) for 5 days followed by 2 days of regular water. Mice were analysed at day 7. Body weight, presence of blood and stool consistency was assessed daily.

***Citrobacter rodentium* infection:** Infection with *Citrobacter rodentium* ICC180 (derived from DBS100 strain)[352] was performed by gavage inoculation of 10^9 colony forming units[352,353]. Acquisition and quantification of luciferase signal was performed in an IVIS system (Caliper Life Sciences). Throughout infection, weight loss, diarrhoea and bloody stools were monitored daily.

Antibiotic treatment: Pregnant females or new born mice were treated with streptomycin 5g/L, ampicillin 1g/L and colistin 1g/L (Sigma-Aldrich) into drinking water with 3% sucrose. Control mice were given 3% sucrose in drinking water as previously described[22].

Microscopy: Intestines from *Ret*^{GFP} and *Ret*^{GFP} chimeras were imaged in a Zeiss Lumar V12 fluorescence stereo microscope with a NeoLumar S 0.8x objective using the GFP filter. Whole-mount analysis was performed as previously described[60,168]. Briefly, adult intestines were flushed with cold PBS (Gibco) and opened longitudinally. Mucus and

epithelium was removed and intestines were fixed in 4% PFA (Sigma-Aldrich) at room temperature for 10 minutes and incubated in blocking/permeabilising buffer solution (PBS containing 2% BSA, 2% goat serum, 0.6% Triton X-100). To visualise three-dimensional structures of the small intestine, samples were cleared with benzyl alcohol-benzyl benzoate (Sigma-Aldrich) prior dehydration in methanol[60,168]. For analysis of thick gut sections intestines were fixed with 4% PFA at 4°C overnight and were then included in 4% low-melting temperature agarose (Invitrogen). Sections of 100µm were obtained with a Leica VT1200/VT1200 S vibratome and embedded in Mowiol (Calbiochem)[168]. Slides or whole-mount samples were incubated overnight or for 1–2 days respectively at 4°C using the following antibodies: rat monoclonal anti-B220 (RA3-6B2) (eBioscience), mouse monoclonal anti-RORγt (Q31-378) (BD Pharmigen), mouse monoclonal anti-GFAP (GA-5) (Sigma-Aldrich), mouse monoclonal anti-GFAP Cy3 (GA-5) (Abcam), anti-GDNF antibody (Abcam), DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Invitrogen). A647 goat anti-rat, A568 goat anti-rat, A647 goat anti-mouse, A488 rabbit anti-GFP, and A488 goat anti-rabbit secondary antibodies were purchased from Invitrogen. Neurospheres and cultured glial cells were fixed in PFA 4% 10 minutes at room temperature and permeabilised in PBS-Triton 0.1% during 30 seconds. After several washing steps with PBS cells were incubated with antibodies during 3h at room temperature and then mounted in Mowiol[354]. Samples were acquired on a Zeiss LSM710 confocal microscope using EC Plan-Neofluar 10x/0.30 M27, Plan Apochromat 20x/0.8 M27 and EC Plan-Neofluar 40x/1.30 objectives. Three-dimensional reconstruction of images was achieved using Imaris software and snapshot pictures were obtained from the three-dimensional images. For analysis of confocal images, cells were counted using in-house software, written in MATLAB (Mathworks, Natick, MA). Briefly, single-cell ILC3 nuclei were identified via RORγt by thresholding and particle analysis. Regions of interest (ROIs) (Extended Data Fig.1i; Bottom panels) were defined from each nucleus for analysis in the GFP channel, where staining was considered positive if a minimum number of pixels (usually 20) were above a given threshold. The software allows for batch processing of multiple images and generates individual report images for user verification of cell-counting results and co-expression analysis: (https://imm.medicina.ulisboa.pt/en/servicos-e-recursos/technical-facilities/bioimaging).

Histopathology analysis: Colon samples were fixed in 10% neutral buffered formalin. The colon was prepared in multiple cross-sections or ‘‘Swiss roll’’ technique[355], routine-processed for paraffin embedding and 3-4µm sections were stained with haematoxylin and eosin. Enteric lesions were scored by a pathologist blinded to experimental groups, according to previously published criteria[356–358]. Briefly, lesions were individually scored (0-

4 increasing severity) for the following criteria: 1-mucosal loss; 2-mucosal epithelial hyperplasia, 3-degree of inflammation, 4-extent of the section affected in any manner and 5-extent of the section affected in the most severe manner as previously described[358]. Final scores were derived by summing the individual lesion and the extent scores. The internal diameter of the crypts was measured in at least five fields (10x magnification), corresponding to the hotspots in which the most severe changes in crypt architecture were seen. Measurements were performed in an average of 35 crypts per sample/mouse, from proximal to distal colon. Intestinal villus height was measured in the jejunum. Measurements were performed in slides scanned using a Hamamatsu Nanozoomer SQ digital slide scanner running NDP Scan software.

Enteric glial cell isolation: Enteric glial cells isolation was adapted from previously described protocols[254,359]. Briefly, the muscularis layer was separated from the submucosa with surgical forceps under a dissection microscope (SteREO Lumar.V12, Zeiss). The lamina propria was scraped mechanically from the underlying submucosa using 1.5mm cover-slips (Thermo Scientific). Isolated tissues were collected and digested with Liberase TM (7,5 µg/mL; Roche) and DNase I (0.1mg/ mL; Roche) in RPMI supplemented with 1% hepes, sodium pyruvate, glutamine, streptomycin and penicillin and 0.1% β-mercaptoethanol (Gibco) for approximately 40min at 37°C. Single-cell suspensions were passed through a 100µm cell strainer (BD Biosciences) to eliminate clumps and debris.

Flow cytometry and cell sorting: Lamina propria cells were isolated as previously described[360]. Briefly, intestines were digested with collagenase D (0.5mg/mL; Roche) and DNase I (0.1mg/ mL; Roche) in RPMI supplemented with 10% FBS, 1% hepes, sodium pyruvate, glutamine, streptomycin and penicillin and 0.1% β-mercaptoethanol (Gibco) for approximately 30min at 37°C under gentle agitation. For cytokine analysis, cell suspensions were incubated 4h in PMA/Ionomycin (Sigma-Aldrich) and Brefeldin A (eBioscience) at 37ºC. Intracellular staining was performed using IC fixation/permeabilisation kit (eBioscience). Cells were stained using PBS, 1% FBS, 1% hepes and 0.6% EDTA (Gibco). Flow cytometry analysis and cell sorting were performed using FORTESSA and FACSAria flow cytometers (BD Biosciences). Data analysis was done using FlowJo software (Tristar). Sorted populations were >95% pure. Cell suspensions were stained with anti-CD45 (30-F11), anti-TER119 (TER-119), TCRβ (H57-597), anti-CD3ε (eBio500A2), anti-CD19 (eBio1D3), anti-NK1.1 (PK136), anti-CD11c (N418), anti-Gr1 (RB6-8C5), anti-CD11b (Mi/70), anti-CCR6 (29-2L17), anti-CD127 (IL-7Rα; A7R34), anti-Thy1.2 (53-2.1), anti-CD49b (DX5), anti-TCRδ (GL3), anti-NKp46 (29A1.4), anti-IL-17 (eBio17B7), anti-IL-22 (1H8PWSR), Rat IgG1 isotype control

(eBRG1) antibodies, 7AAD viability dye, anti-Mouse CD16/CD32 (Fc block), anti-RORγt (AFKJS-9); Rat IgG2a$_\kappa$ Isotype Control (eBR2a) and streptavidin fluorochrome conjugates all from eBioscience; anti-CD4 (GK1.5), anti-CD31 (390), anti-CD8α (53-6.7), anti-CD24 (M1/69), anti-Epcam (G8.8) antibodies were purchased from Biolegend. Anti-RET (IC718A) antibody was purchased from R&D Systems. LIVE/DEAD Fixable Aqua Dead Cell Stain Kit was purchased from Invitrogen. Cell populations were defined as: ILC3 - CD45$^+$Lin$^-$Thy1.2^{hi}IL7Rα$^+$RORγt$^+$; For ILC3 subsets additional markers were employed: LTi - CCR6$^+$Nkp46$^-$; ILC3 NCR$^-$ - CCR6$^-$Nkp46$^-$; ILC3 NCR$^+$ - CCR6$^-$Nkp46$^+$; Lineage was composed by CD3ε, CD8α, TCRβ, TCRγδ, CD19, Gr1, CD11c and TER119; Glial cells - CD45$^-$CD31$^-$TER119$^-$CD49b$^+$ [47]; T cells - CD45$^+$CD3ε$^+$; γδ T cells - CD45$^+$CD3ε$^+$γδTCR$^+$; B cells - CD45$^+$CD19$^+$B220$^+$; Macrophages - CD45$^+$CD11b$^+$F4/80$^+$; Dendritic cells - CD45$^+$CD19$^-$CD3ε$^-$MHCII$^+$CD11c$^+$; enteric neurons - CD45$^-$RET/GFP$^+$[341], Epithelial cells - CD45$^-$CD24$^+$Epcam$^+$.

Quantitative RT-PCR: Total RNA was extracted using RNeasy micro kit (Qiagen) or Trizol (Invitrogen) according to the manufacturer's protocol. RNA concentration was determined using Nanodrop Spectrophotometer (Nanodrop Technologies). Quantitative real-time RT–PCR was performed as previously described[60,168,293]. *Hprt* and *Gapdh* were used as housekeeping genes. For TaqMan assays (Applied Biosystems) RNA was retro-transcribed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems), followed by a pre-amplification PCR using TaqMan PreAmp Master Mix (Applied Biosystems). TaqMan Gene Expression Master Mix (Applied Biosystems) was used in real-time PCR. TaqMan Gene Expression Assays (Applied Biosystems) were the following: *Gapdh* Mm99999915_g1; *Hprt* Mm00446968_m1; *Artn* Mm00507845_m1; *Nrtn* Mm03024002_m1; *Gdnf* Mm00599849_m1; *Gfra1 Mm00439086_m1; Gfra2 Mm00433584_m1; Gfra3 Mm00494589_m1; Ret* Mm00436304_m1; *Il22* Mm01226722_g1; *Il17a* Mm00439618_m1; *Il23r* Mm00519943_m1; *Rorgt* Mm01261022_m1; *Il7ra* Mm00434295_m1; *Ahr* Mm00478932_m1; *Stat3* Mm01219775_m1; *Cxcr6* Mm02620517_s1; *Nfkbiz* Mm_00600522_m1; *RegIIIa* Mm01181787_m1; *RegIIIb* Mm00440616_g1; *RegIIIg* Mm00441127_m1; *Defa1* Mm02524428_g1; *Defa-rs1* Mm00655850_m1; *Defa5* Mm00651548_g1; *Defa21* Mm04206099_gH; *Muc1* Mm00449599_m1; *Muc3* Mm01207064_m1; *Muc13* Mm00495397_m1; *Gfap* Mm01253033_m1*; Ascl2* Mm01268891_g*; Tff3* Mm00495590_m1*; Relm-b* Mm00445845_m1*; Pla2g2a* Mm00448160_m1*; Pla2g5* Mm00448162_m1*; Wnt3* Mm00437336_m1*; Ctnnb1* Mm00483039_ m1*; Axin2* Mm00443610_m1*; Dll1b* Mm01279269_m1*; Il18* Mm00434225_m1*; Tnfa* Mm00443260_g1*; Lyz1* Mm00657323_m1*; Lrg5* Mm00438890_m1*; Tbx21*

Mm00450960_m1*;* *Id2* Mm00711781_m1*;* *Runx1* Mm01213404_m1*;* *Notch1* Mm00435249_m1*;* *Notch2* Mm00803077_m1*;* *Gata3* Mm00484683_m1*;* *Bcl2* Mm00477631_m1*;* *Bcl2l1* Mm00437783_m1*;* *Arntl* Mm00500226_m1; *Glpr2* Mm01329475_m1; *Gja1* Mm01179639_s1; *Ednrb* Mm00432989; *S100b* Mm00485897_m1; *Sox10* Mm00569909_m1. Real-time PCR analysis was performed using ABI Prism 7900HT Sequence Detection System or StepOne Real-Time PCR system (Applied Biosystems).

ILC3 activation and cell signalling: Sorted intestinal ILC3 cells were starved for 3 hours in RPMI at 37°C in order to ensure ILC3 viability. *Ret*^{fl} or *Ret*$^{\Delta}$ were analysed directly *ex vivo*. To test ERK, AKT, p38-MAPK (Cell Signaling Technology) and STAT3 (BD Pharmigen) upon GFL stimulation WT ILC3 were activated with 500ng/mL (each GFL) and co-receptors (rrGFR-α1, rmGFR-α2, rrGFR-α3 and rrGNDF from R&D Systems; rhNRTN and rhARTN from PeproTech) for 10 and 30min. When referring to the use of 'GFL', we have employed GDNF, NRTN, ARTN and their specific co-receptors in combination. For inhibition experiments cells were incubated 1h at 37°C before GFL stimulation, to test ERK, AKT, p38/MAPK and STAT3 phosphorylation, or during overnight stimulation with GFLs, to determine *Il22* expression levels. Inhibitors were purchased from Sigma-Aldrich: p38 MAPK/ERK-AKT - LY294002 (LY); ERK - PD98059 (PD); AKT - AKT Inhibitor VIII (VIII); p38 MAPK - SB 202190 (SB); and pSTAT3 – S3I-201 (S3I).

Chromatin immunoprecipitation (ChIP) assay: Enteric ILC3 from adult C57BL/6J mice were isolated by flow cytometry. Cells were starved for 3h with RPMI supplemented with 1% hepes, sodium pyruvate, glutamine, streptomycin and penicillin and 0.1% β-mercaptoethanol (Gibco) at 37°C. Cells were stimulated with GFLs (500ng/mL each)[293], lysed, cross-linked and chromosomal DNA-protein complex sonicated to generate DNA fragments ranging from 100-300 base pairs. DNA/protein complexes were immunoprecipitated, using LowCell# ChIP kit (Diagenode)[130], with 3µg of rabbit polyclonal antibody against anti-pSTAT3 (Cell Signalling Technology), rabbit control IgG (Abcam) or H3K36me3 (07-030; Millipore). Immunoprecipitates were uncross-linked and analysed by quantitative PCR using primer pairs (5'-3') flanking putative sites on *Il22*. Vehicle (BSA) stimulated ILC3s were used as controls. *Il22* primer sequences were previously described[361–363], briefly: a, F-TGCAATCAATCCCAGTATTTTG and R-CTTGTGCAAGCATAAGTCTCAA; b, F-GAAGTTGGTGGGAAAATGAGTCCGTGA and R-GCCATGGCTTTGCCGTAGTAGATTCTG; c, F-ACGGGAGATCAAAGGCTGCTCT and R-GCCAACAAGGTGCTTTTGC; d, F-CTCACCGTGACGTTTTAGGG and R-GTGAATGATATGACATCAGAC; e, F-CGACGAACATGCTCCCCTGATGTTTTT and R-

AAACTCATAGATTTCTGCAGGACAGCC; f, F-AGCTGCATCTCTTTCTCTCCA and R-TATCCTGAAGGCCAAAATAGGA; g, F-ACGACCAGAACATCCAGAAGA and R-GCAGAGAAAGAAATCCCCGC; h, F-AGGGGGACTTGCTTTGCCATTT and R-AACACCCCTTCTTTCCTCCTCCAT; i, F-CTGCTCCTTCCTGCCTTCTA and R-CTGAGCCAGGTTTCATGTGA. Primer positions are shown in Fig.3i relative to the transcription start codon of *Il22*.

Colony forming units and paracellular permeability: Organs were harvested, weighed, and brought into suspension. Bacterial colony forming units (CFU) were determined per gram of tissue and total organ. CFU were determined via serial dilutions on Luria Broth (LB) agar and MacConkey agar (Sigma-Aldrich). Colonies were counted after 2 days of culture at 37ºC. To address intestinal paracellular permeability 16 mg per mouse of Dextran-Fitc (Sigma Aldrich) were administrated by gavage after overnight starvation. Plasma was analysed after 4 hours of Dextran-Fitc administration using a Microplate Reader TECAN Infinity F500.

BrdU administration and Ki-67 labeling: BrdU was administrated by i.p. injection (1.25 mg/mouse). For flow cytometric analysis of epithelial cell proliferation anti-BrdU (Staining Kit for flow Cytometry- eBioscience) and anti-mouse Ki-67 antibody (BioLegend) were employed.

Quantitative PCR analysis of bacteria in stool at the Phylum level: DNA from faecal pellet samples was isolated with ZR Fecal DNA MicroPrep™ (Zymo Research). Quantification of bacteria were determined from standard curves established by qPCR. qPCR were performed with Power SYBR® Green PCR Master Mix (Applied Biosystems) and different primer sets using a StepOne Plus (Applied Biosystems) thermocycler. Samples were normalized to 16S rDNA and reported according to the $2^{-\Delta\Delta CT}$ method. Primer sequences were: 16S rDNA, F- ACTCCTACGGGAGGCAGCAGT and R-ATTACCGCGGCTGCTGGC; *Firmicutes*, F- ACTCCTACGGGAGGCAGC and R-GCTTCTTAGTCAGGTACCGTCAT; *Bacteroidetes*, F- GGTTCTGAGAGGAGGTCCC and R-GCTGGCTCCCGTAGGAGT; *Proteobacteria*, F- GGTTCTGAGAGGAGGTCCC and R-GCTGGCTCCCGTAGGAGT.

16S rRNA quantification and gene sequencing: Faeces were isolated from co-

housed *Ret*^{fl} or *Ret*$^{\Delta}$ littermates. Sequencing of the 16S *rRNA* gene was performed as previously described[364]. Briefly, barcoded primers were used to amplify the V4 region of the 16S *rRNA* gene, and the amplicons were sequenced on a MiSeq instrument (Illumina, San Diego, USA) using 150 bp, paired-end chemistry at the University of Pennsylvania

Next Generation Sequencing Core. The paired ends were assembled and quality filtered, selecting for reads with a quality score ≥30. Reads with >10 bp homopolymers and reads shorter than 248 bp or longer than 255 bp were removed from the analysis. 16S rRNA sequence data were processed using mothur v 1.25.0[365] and QIIME v 1.8[366]. Chimeric sequences were removed with ChimeraSlayer[367]. Operational taxonomic units (OTUs) were defined with CD-HIT[368] using 97% sequence similarity as a cut-off. Only OTUs containing ≥2 sequences were retained; OTUs assigned to Cyanobacteria or which were not assigned to any phylum were removed from the analysis. Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier v 2.2[369], multiple sequence assignment was performed with PyNAST (v 1.2.2)[370], and FastTree[371]was used to build the phylogeny. Samples were rarified to 22,000 sequences per sample for alpha- and beta-diversity analyses. Taxonomic relative abundances are reported as the median with standard deviation. P values were calculated using the Wilcoxon rank-sum test. Statistical tests were conducted in R v. 3.2.0. To determine which factors were associated with microbial community composition, statistical tests were performed using the non-parametric analysis of similarities (ANOSIM) with weighted UniFrac distance metrics[372].

Data accession: The sequencing data generated in this study have been submitted to the NCBI Sequence Read Archive under BioProject PRJNA314493 (SRA: http://www.ncbi.nlm.nih.gov/sra/?term=PRJNA314493).

Intestinal organoids: IntestiCult™ Organoid Growth Medium and Gentle Cell Dissociation Reagent were purchased from StemCell. Intestinal crypts were isolated from C57BL/6J mice according to the manufacturer's instructions and were added to previously thawed, ice-cold Matrigel at a 1:1 ratio and at a final concentration of 5,000-7,000 crypts/mL. 15µL of this mix was plated per well of a 96 well round-bottom plate. After Matrigel solidification 100µL of growth medium (100U/mL penicillin/streptomycin) was added and replaced every 3 days. Organoids were grown at 37ºC with 5% CO2 and passaged according to the manufacturer's instructions. Freshly sorted intestinal ILC3 were added to 5-8 days old epithelial organoids after plating for 24 hours with or without anti-mouse IL-22 antibody (R&D Systems).

IL-22 agonist administration *in vivo*: 150 µg of anti-IL-22 antibody (8E11; gift from Genentech, South San Francisco, CA) or mouse IgG1 isotype control (MOPC-21; Bio X Cell) was administered i.p. to *Ret*^{MEN2B} mice every 2 days. Animals were analysed 2 weeks after the first administration.

Neurosphere-derived glial cells: Neurosphere-derived glial cells were obtained as previously described[373]. Briefly, total intestines from E14.5 C57BL/6J and *Myd88*$^{-/-}$ mice were digested with collagenase D (0.5mg/mL; Roche) and DNase I (0.1 mg/ mL; Roche) in DMEM/F-12, GlutaMAX, supplemented with 1% hepes, streptomycin/penicillin and 0.1% β-mercaptoethanol (Gibco) for approximately 30 minutes at 37°C under gentle agitation. Cells were cultured during 1 week in a CO2 incubator at 37 °C in DMEM/F-12, GlutaMAX™, streptomycin and penicillin and 0.1% β-mercaptoethanol (Gibco) supplemented with B27 (Gibco), EGF (Gibco) and FGF2 (Gibco) 20ng/mL. After 1 week of culture cells were treated with 0.05% trypsin (Gibco), transferred into PDL (Sigma-Aldrich) coated plates and culture in DMEM supplemented with 10% FBS, 1% hepes, glutamine, streptomycin and penicillin and 0.1% β-mercaptoethanol (Gibco) until confluence. Glial cells were activated with TLR2 (5µg/ml) (Pam3CSK4), TLR3 (100µg/ml) (PolyI:C), TLR4 (50ng/ml) (LPS), TLR9 (50µg/ml) (DsDNA-EC) ligands from Invivogen and IL-1β (10ng/mL) (401ML005), IL-18 (50ng/mL) (B002-5), IL-33 (0.1 ng/mL) (3626ML) recombinant proteins from R&D Systems. Cells were also co-cultured with purified ILC3 from WT and *Il1b* deficient mice. IL-22 expression in glial-ILC3 co-cultures upon TLR4 activation was also performed using GDNF (2µg/mL) (AB-212–NA), NRTN (2µg/mL) (AF-387sp) and ARTN (0.3µg/mL) (AF-1085-sp) blocking antibodies. Cells were analysed after 24 hours of co-culture.

Statistics: Results are shown as mean ± SEM. Statistical analysis used Microsoft Excel. Variance was analysed using F-test. Student's t-test was performed on homocedastic populations, and Student's t-test with Welch correction was applied on samples with different variances. Analysis of survival curves was performed using a MAntel-Cox test. Results were considered significant at *$p \leq 0.05$; **$p \leq 0.01$. Statistical treatment of metagenomics analysis is described in the methods section: 16S rRNA gene sequencing and analysis.

Results

Group 3 innate lymphoid cells (ILC3) produce pro-inflammatory cytokines, regulate mucosal homeostasis and anti-microbial defence[83]. In addition to their well-established developmentally regulated program, ILC3 are also controlled by microbial and dietary signals[83,142,168,171,173,174] raising the hypothesis that ILC3 possess other unexpected environmental sensing strategies. Neurotrophic factors are extra-cellular environmental cues to neurons and include the glial-derived neurotrophic factor (GDNF) family ligands (GFL) that activate the tyrosine kinase receptor RET in the nervous system, kidney and haematopoietic progenitors[60,285,293,299,374].

Analysis of gut lamina propria revealed that ILC3 express high levels of *Ret* (Fig.1a)[161,285], a finding confirmed at the protein level and by *Ret*^{GFP} knock-in mice (Fig.1b-d and Extended Data Fig.1a-d)[341]. ILC3 subsets expressed *Ret*^{GFP} and aggregated in Cryptopatches (CP) and Isolated Lymphoid Follicles (ILF), suggesting a role of neuroregulators in ILC3 (Fig.1b-d and Extended Data Fig.1b-j). To explore this hypothesis, we transplanted foetal liver cells from *Ret* competent (*Ret*$^{WT/GFP}$) or deficient (*Ret*$^{GFP/GFP}$)[341] animals into alymphoid *Rag1*$^{-/-}$*γc*$^{-/-}$ hosts. *Ret* deficient chimeras revealed unperturbed ILC3 and CP development (Fig.1e). Strikingly, IL-22 expressing ILC3 were largely reduced despite normal IL-22 producing T cells (Fig.1f,g). In contrast, innate IL-17 was unaffected by *Ret* ablation (Fig.1f and Extended Data Fig.2a). In agreement, analysis of gain-of-function *Ret*^{MEN2B} mice[317] revealed a selective increase of IL-22 producing ILC3 while their IL-17 counterparts were unaffected (Fig.1h and Extended Data Fig.2b). To more specifically evaluate the effects of RET in ILC3, we deleted *Ret* in RORγt expressing cells by breeding *Rorgt*-Cre to *Ret*$^{fl/fl}$ mice[346,347] (Extended Data Fig.3a,b). Analysis of *Rorgt*-Cre.*Ret*$^{fl/fl}$ (*Ret*^{Δ}) mice revealed selective and large reduction of ILC3-derived IL-22, but normal IL-22 producing T cells (Fig.2a and Extended Data Fig.3c,d). IL-22 acts on epithelial cells to induce reactivity and repair genese[83]. When compared to their wild-type (WT) littermate controls, the *Ret*^{Δ} epithelium revealed normal morphology, proliferation and paracellular permeability, but a profound reduction of epithelial reactivity and repair genes (Fig.2b and Extended Data Fig.3e-h). Accordantly, the *Ret*^{MEN2B} epithelium displayed increased levels of these molecules in an IL-22 dependent manner (Fig.2b and Extended data Fig.3i). These results indicate that RET signals selectively control innate IL-22 and shape intestinal epithelial reactivity.

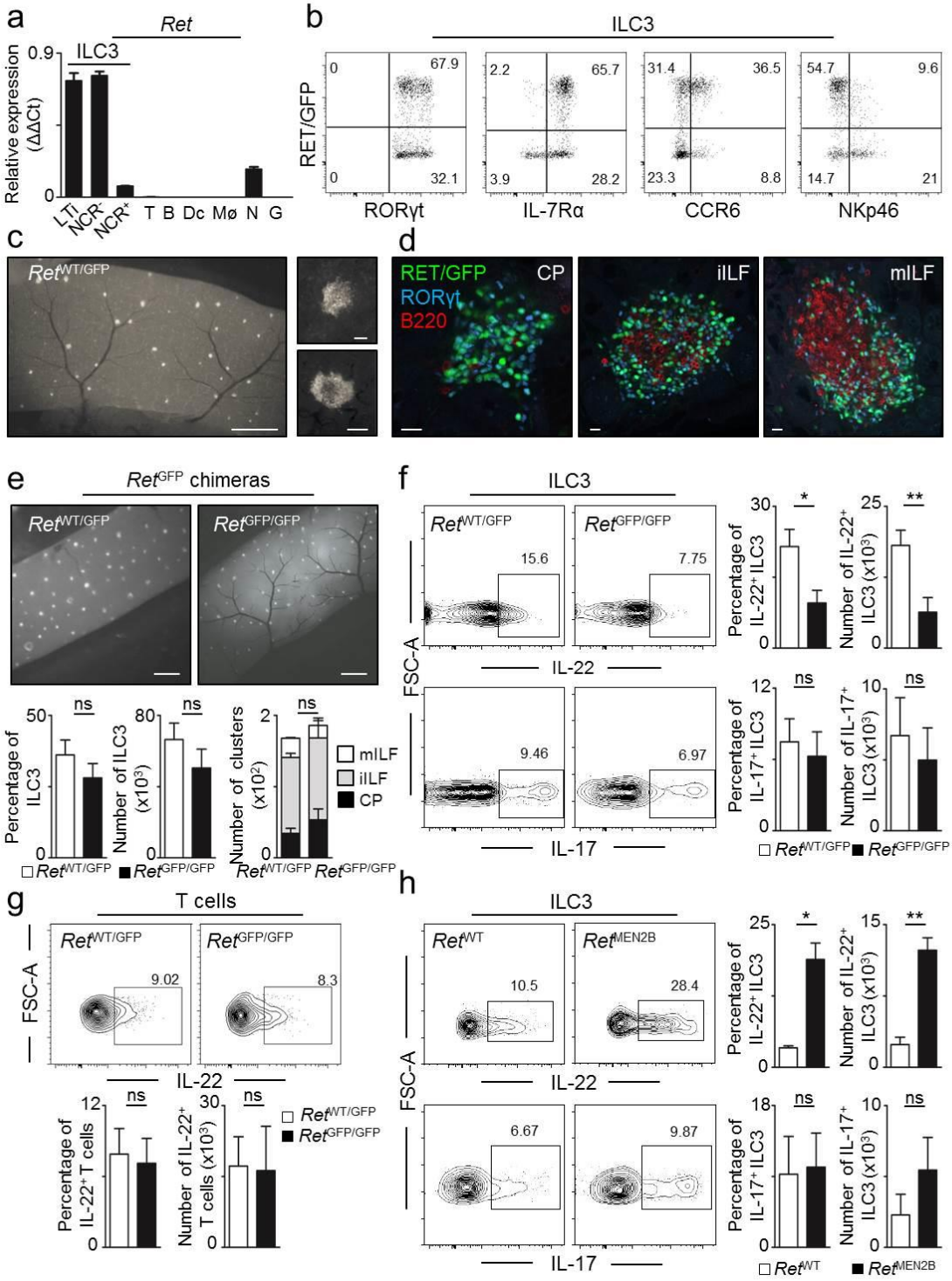


Figure 1. The neurotrophic factor receptor RET drives enteric ILC3-derived IL-22.a, LTi, NCR$^-$ and NCR$^+$ ILC3 subsets, T cells (T), B cells (B), Dendritic cells (Dc), Macrophages (Mø), enteric Neurons (N) and mucosal Glial cells (G). **b**, *Ret*^{GFP} ILC3. **c**, Left: *Ret*^{GFP} gut. White: GFP. Right: ILC3 aggregates. **d**, Cryptopatches (CP), immature (iILF) and mature (mILF) isolated lymphoid follicles. Green: RET/GFP; Blue: RORγt; Red: B220. **e**, *Ret*^{GFP} chimeras. n=15. **f,g**, *Ret*^{GFP} chimeras. *Ret*$^{WT/GFP}$ n=25; *Ret*$^{GFP/GFP}$ n=22. **h**, *Ret*^{MEN2B} mice. n=7. Scale bars: 1mm (c left, e); 50µm (c right); 30µm (d). Data are representative of 4 independent experiments. Error bars show s.e.m. *P<0.05; **P<0.01; ns not significant.

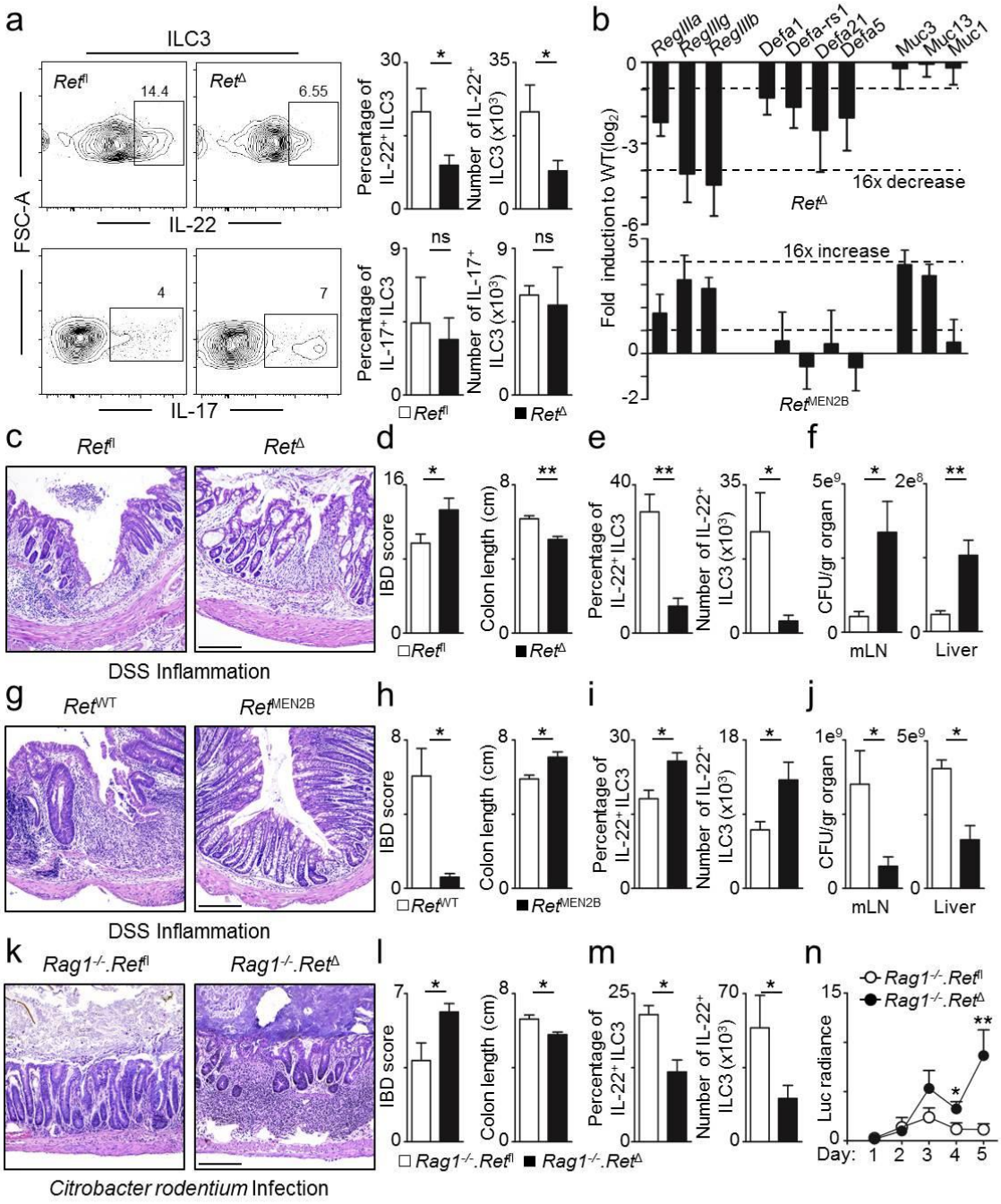


Figure 2. **ILC3-intrinsic RET signals regulate gut defence. a**, ILC3-derived cytokines. n=11. **b**, *Ret*$^{\Delta}$ and *Ret*^{MEN2B} mice compared to their WT littermate controls. n=7. **c-f**, DSS treatment. *Ret*^{fl} n=8; *Ret*$^{\Delta}$ n=8. **c**, Histopathology. **d**, Inflammation score and colon length. **e**, Innate IL-22. **f**, Bacterial translocation. **g-j**, DSS treatment. *Ret*^{WT} n=8; *Ret*^{MEN2B} n=8. **g**, Histopathology. **h**, Inflammation score and colon length. **i**, Innate IL-22. **j**, Bacterial translocation. **k-n**, *C. rodentium* infection. *Rag1*$^{-/-}$.*Ret*^{fl} n=15; *Rag1*$^{-/-}$.*Ret*$^{\Delta}$ n=17. **k**, Histopathology. **l**, Inflammation score and colon length. **m**, Innate IL-22. **n**, Infection burden. Scale bars: 200µm. Data are representative of 4 independent experiments. Error bars show s.e.m. *P<0.05; **P<0.01; ns not significant.

To interrogate whether neurotrophic factors regulate intestinal defence we tested how varying degrees of RET signals control enteric aggressions. While *Ret*$^{\Delta}$ mice treated with Dextran Sodium Sulfate (DSS) had increased weight loss and inflammation, reduced IL-22 producing ILC3, decreased epithelial reactivity/repair genes and pronounced bacterial translocation from the gut, *Ret*^{MEN2B} mutants were highly protected over their WT littermate controls (Fig.2c-j and Extended Data Fig.4). Since DSS mostly causes epithelial injury, we tested whether ILC3-autonomous RET signals are required to control infection. To this end, *Ret*$^{\Delta}$ mice were bred to *Rag1*$^{-/-}$ mice to formally exclude adaptive T cell effects. *Rag1*$^{-/-}$*.Ret*$^{\Delta}$ mice were infected with the attaching and effacing bacteria *Citrobacter rodentium*. When compared to their littermate controls, *Rag1*$^{-/-}$*.Ret*$^{\Delta}$ mice had marked gut inflammation, reduced IL-22 producing ILC3, increased *C. rodentium* infection and translocation, reduced epithelial reactivity genes, increased weight loss and reduced survival (Fig.2k-n and Extended Data Fig.5). Altogether, these data indicate that ILC3-intrinsic neurotrophic factor cues regulate gut defence and homeostasis.

Formal definition that IL-22 is the molecular link between RET-dependent ILC3 activation and epithelial reactivity was provided by a multi-tissue organoid system. Addition of GFL to ILC3/epithelial organoids strongly induced epithelial reactivity genes in an IL-22 and RET dependent manner (Fig.3a,b and Extended Data Fig.6a). To further examine how RET signals control innate IL-22 we investigated a gene signature associated with ILC identity[83]. While most of those genes were unperturbed, notably the master ILC transcription factors *Runx1*, *Id2*, *Gata3*, *Rora, Rorgt*, *Ahr and Stat3*, *Il22* was significantly reduced in *Ret*$^{\Delta}$ ILC3 (Fig.3c and Extended Data Fig.6b). In agreement, activation of ILC3 with all or distinct GFL/GFRα pairs *in trans* efficiently increased *Il22* despite normal expression of other ILC3-related genes (Fig.3d and Extended Data Fig.6c). Activation of RET by GFL leads to p38 MAPK/ERK-AKT cascade activation in neurons, while phosphorylation of STAT3 shapes *Il22* expression[285,375]. Analysis of *Ret*$^{\Delta}$ ILC3 revealed hypo-phosphorylated ERK1/2, AKT, p38/MAP kinase and STAT3 (Fig.3e and Extended Data Fig.6d). Accordantly, GFL-induced RET activation in ILC3 led to rapid ERK1/2, AKT, p38/MAP kinase and STAT3 phosphorylation and increased *Il22* transcription (Fig.3d,f and Extended Data Fig.6e,f). In agreement, inhibition of ERK, AKT or p38/MAP kinase upon GFL activation led to impaired STAT3 activation and *Il22* expression (Fig.3g,h). Finally, inhibition of STAT3 upon GFL-induced RET activation led to decreased *Il22* (Fig.3h). To examine whether GFL directly regulate *Il22* we performed chromatin immunoprecipitation (ChIP) (Fig.3i,j)[130]. Stimulation of ILC3 with GFL resulted in increased binding of pSTAT3 in the *Il22* promoter and increased trimethyl-H3K36 at the 3' end of *Il22,* indicating active *Il22* transcribed regions (Fig.3d,j)[376]. Thus, cell-autonomous RET

signals control ILC3 function and gut defence via direct regulation of *Il22* downstream of TAT3 activation.

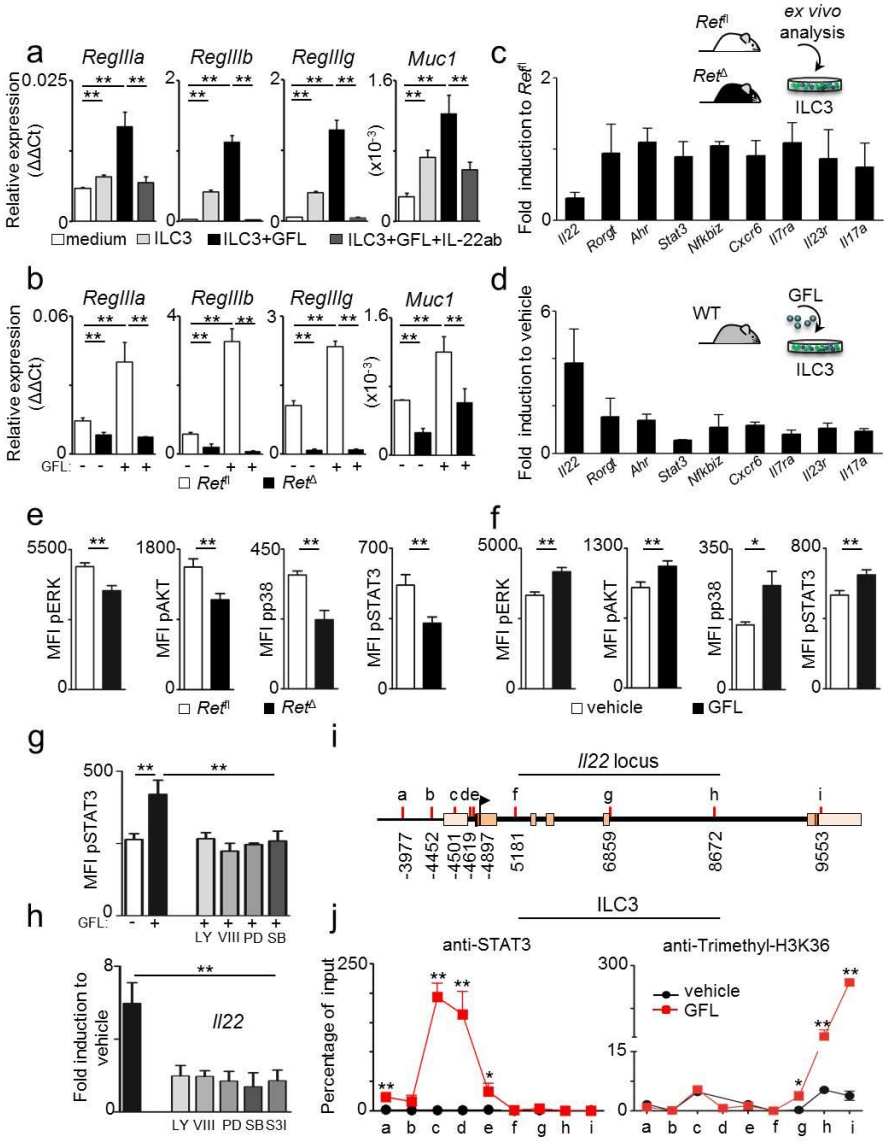


Figure 3. ILC3-autonomous RET signals directly control *Il22* downstream of pSTAT3. a,b, Epithelial/ILC3 organoids. n=9. **c**, *Ret*$^{\Delta}$ ILC3 compared to their WT controls. n=4. **d**, ILC3 activation by GFL. n=4. **e**, *Ret*$^{\Delta}$ ILC3. pERK n=8; pAKT n=12; phosphorylated p38/MAP kinase n=6; pSTAT3 n=14. **f**, ILC3 activation by GFL. pERK n=10; pAKT n=16; phosphorylated p38/MAP kinase n=3; pSTAT3 n=15. **g**, pSTAT3 in ILC3 cultured with medium (n=7), GFL (n=11) or GFL and inhibitors for: p38 MAPK/ERK-AKT (LY) (n=7); ERK (PD) (n=7); AKT (VIII) (n=8); and p38 MAPK (SB) (n=6). **h**, *Il22* in ILC3 cultured with GFL (n=17) or GFL and the inhibitors LY (n=18); PD (n=16); VIII (n=15); SB (n=15); and the STAT3 inhibitor (S3I) (n=8). **i**, *Il22* locus. **j.** ChIP analysis of ILC3 stimulated with GFL. n=10. Data are representative of 3 independent experiments. Error bars show s.e.m. *P<0.05; **P<0.01; ns not significant.

Propensity to inflammation and dysregulation of intestinal homeostasis have been associated to dysbiosis[377,378]. When compared to their WT littermates, *Ret*$^{\Delta}$ mice have altered microbial communities as evidenced by quantitative analysis, weighted UniFrac analysis and significantly altered levels of *Sutterella*, unclassified *Clostridiales* and *Bacteroides* (Fig.4a and Extended Data Fig.7). Discrete microbial communities may have transmissible colitogenic potential[377,378]. Nevertheless, germ-free mice colonised with the microbiota of *Ret*$^{\Delta}$ or their control littermates revealed similar susceptibility to DSS-induced colitis and identical innate IL-22 (Fig.4b-d). In agreement, co-housed *Ret*$^{\Delta}$ and WT littermates had differential propensity to intestinal inflammation (Fig.2c,d). Together, these data indicate that dysbiosis *per se* is insufficient to cause altered innate IL-22 and susceptibility to gut inflammation as observed in *Ret*$^{\Delta}$ mice (Fig.2c-f). Thus, we hypothesised that GFL producing cells integrate commensal and environmental signals to control innate IL-22. Accordingly, antibiotic treatment of *Ret*$^{\Delta}$ and their WT littermate controls resulted in similar ILC3-derived IL-22 (Fig.4e)[379].

Neurotrophic factors of the GDNF family were shown to be produced by enteric glial cells, which are neuron-satellites expressing the glial fibrillary acidic protein (GFAP)[17,285]. Strikingly, double reporter mice for ILC3 (*Ret*^{GFP}) and glial cells (*Gfap*-Cre.*Rosa26*^{RFP}) revealed that stellate-shaped projections of glial cells are adjacent (4.35µm±1.42) to RORγt^{+} ILC3 within CP (Fig.4f and Extended Data Fig.8a). These data suggest a paracrine glial-ILC3 crosstalk orchestrated by neurotrophic factors. In agreement, lamina propria glial cells were main producers of GFL (Extended Data Fig.8b). Recent studies have shown that glial cells express pattern recognition receptors, notably Toll-like receptors (TLRs)[101,263]. Activation of neurosphere-derived glial cells revealed they specifically respond to TLR2, TLR4, and the alarmins IL-1β and IL-33, which efficiently controlled GFL expression and induced robust innate *Il22* in a MYD88 dependent manner (Fig.4g-i and Extended Data Fig.8c-g). To formally demonstrate the physiological importance of MYD88-dependent glial cell sensing on innate IL-22, we deleted *Myd88* in GFAP expressing glial cells by breeding *Gfap*-Cre to *Myd88*$^{fl/fl}$ mice[350,351]. Remarkably, glial-intrinsic deletion of *Myd88* resulted in decreased intestinal GFL, increased gut inflammation, impaired ILC3-derived IL-22, and increased weight loss (Fig.4j-m; Extended Data Fig.9a-d). In agreement, *Gfap*-Cre.*Myd88*$^{\Delta}$ mice had increased susceptible to *C.rodentium* infection (Extended Data Fig.9e-h). Thus, mucosal glial cells orchestrate innate IL-22 via neurotrophic factors, downstream of MYD88-dependent sensing of commensal products and alarmins.

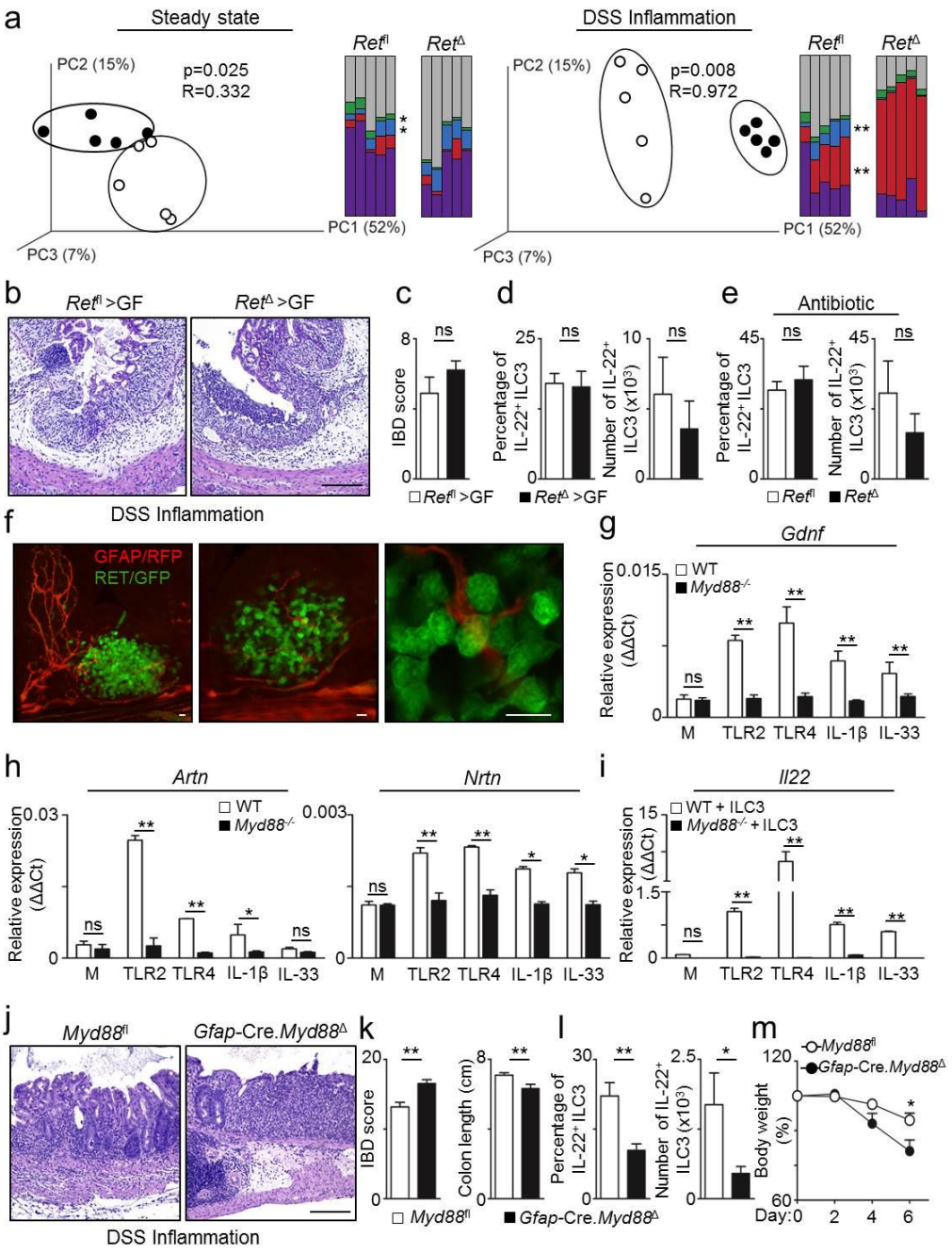


Figure 4. Glial cells set GFL expression and innate IL-22, via MYD88-dependent sensing of the microenvironment. a, Weighted Unifrac PCoA analysis and genus-level comparisons from co-housed *Ret*^{fl} (white circles) and *Ret*$^{\Delta}$ (black circles) littermates. n=5. Purple: Unclassified *S24-7*; Red: *Bacteroides*; Green: *Sutterella*; Blue: Unclassified *Clostridiales*; Grey: Other. **b-d**, DSS treatment of colonised germ-free (GF) mice. n=5. **b**, Histopathology. **c**, Inflammation score. **d**, Innate IL-22. **e**, Innate IL-22 after antibiotic treatment. n=8. **f**, *Ret*^{GFP}.*Gfap*-Cre.*Rosa26*^{RFP} mice. Green: RET/GFP; Red: GFAP/RFP. **g,h**, Glial cell activation with TLR2, TLR4, IL-1β receptor and IL-33 receptor ligands. n=6. **i**, TLR ligands, IL-1β and IL-33 activation of co-cultured ILC3 with WT (white bars) or *Myd88*$^{-/-}$ glial cells (black bars). n=6. **j-m**, DSS treatment of *Gfap*-Cre.*Myd88*$^{\Delta}$ mice. n=12. **j**, Histopathology. **k**, Inflammation score and colon length. **l**, Innate IL-22. **m**, Body weight. Scale bars: 200μm (b, j); 10μm (f). Data are representative of 3-4 independent experiments. Error bars show s.e.m. *P<0.05; **P<0.01; ns not significant.

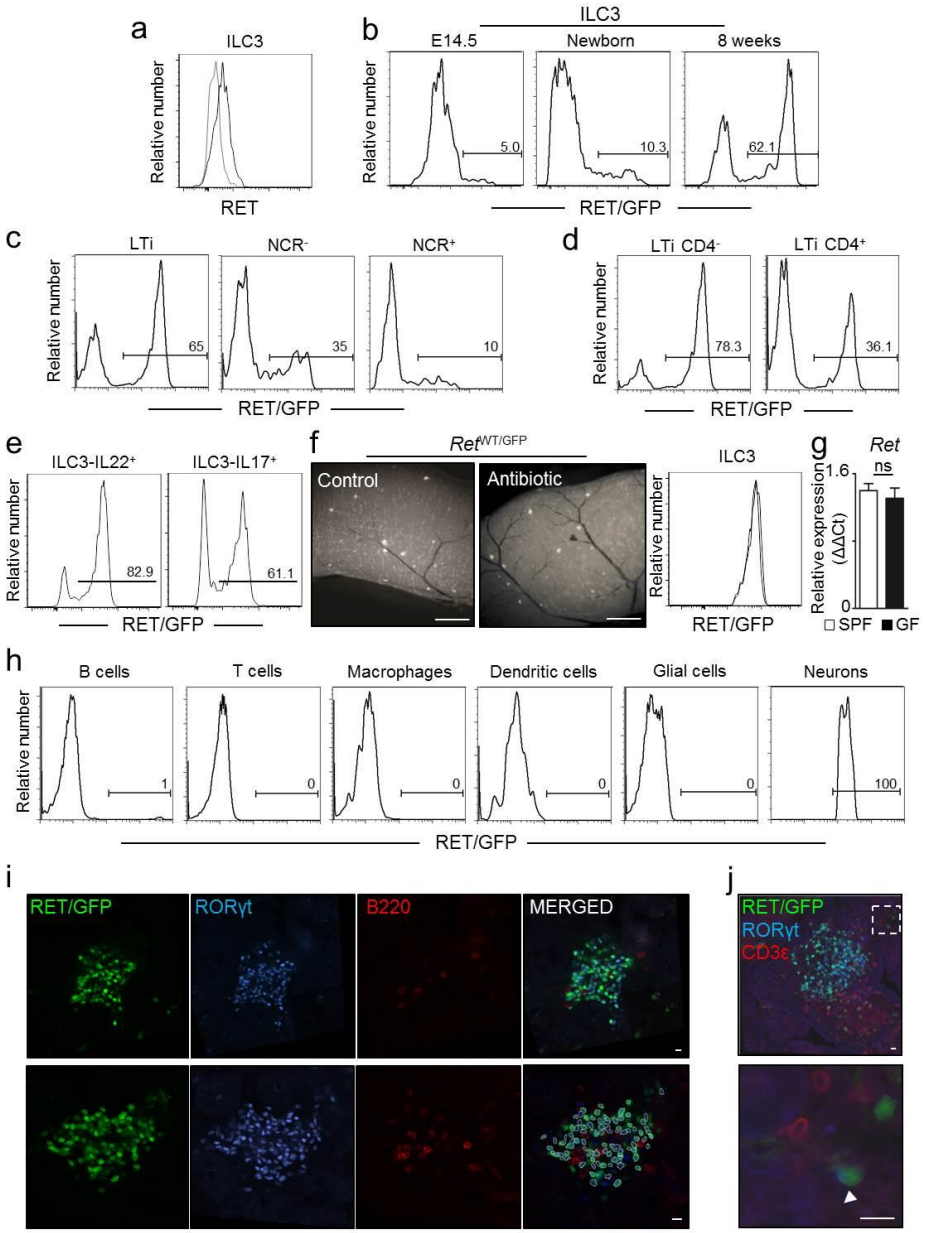
Discussion

Defining the mechanisms by which ILC3 integrate environmental cues is critical to understand mucosal homeostasis. Our work sheds light on the relationships between ILC3 and their microenvironment, notably through decoding a novel glial-ILC3-epithelial cell unit orchestrated by neurotrophic factors (Extended Data Fig.10). Glial-derived neurotrophic factors operate in an ILC3-intrinsic manner by activating the tyrosine kinase RET, which directly regulates innate IL-22 downstream of p38 MAPK/ERK-AKT and STAT3 phosphorylation (Extended Data Fig.10). Future studies will elucidate further the mechanisms inducing RET expression in ILC3.

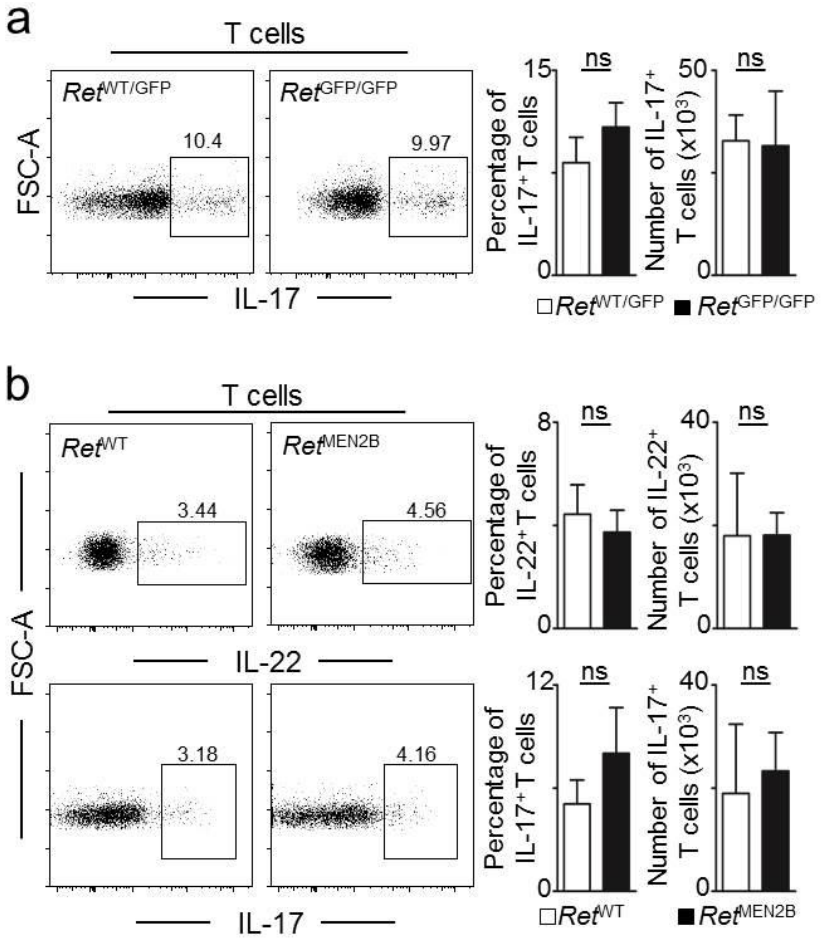
Our data demonstrate that in addition to their well-established capacity to integrate dendritic cell-derived cytokines[380], ILC3 perceive distinct multi-tissue regulatory signals leading to STAT3 activity and IL-22 expression, notably via integration of glial cell-derived neuroregulators. Thus, rather than providing hard-wired signals for ILC3-immunity, we propose that RET signals critically fine-tune innate IL-22 leading to efficient gut homeostasis and defence.

Previous studies demonstrated that neurons can indirectly shape foetal lymphoid tissue inducer cells and that ablation of glial cells leads to gut inflammation[267,381]; here we reveal glial cells as central hubs of neuronal and innate immune regulation. Notably, neurotrophic factors are the molecular link between glial cell sensing, innate IL-22 and intestinal epithelial defence. Thus, it is tempting to speculate that glial/immune cell units might be also critical to the homeostasis of other barriers, notably in the skin, lung and brain[6]. From an evolutionary perspective, coordination of innate immunity and neuronal function may ensure efficient mucosal homeostasis and a co-regulated neuro-immune response to various environmental challenges, including xenobiotics, intestinal infection, dietary aggressions and cancer.

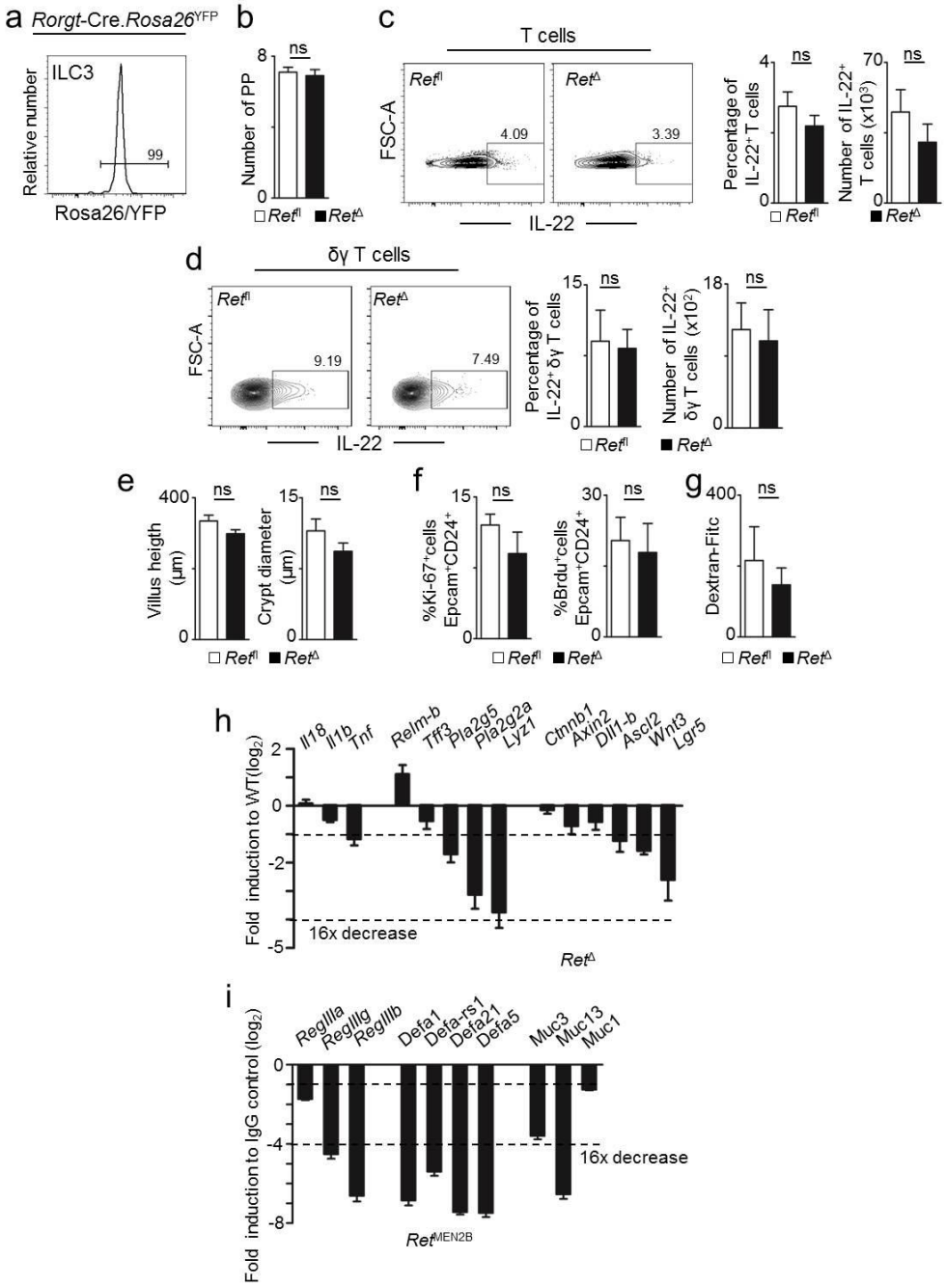
SUPPLEMENTARY FIGURES



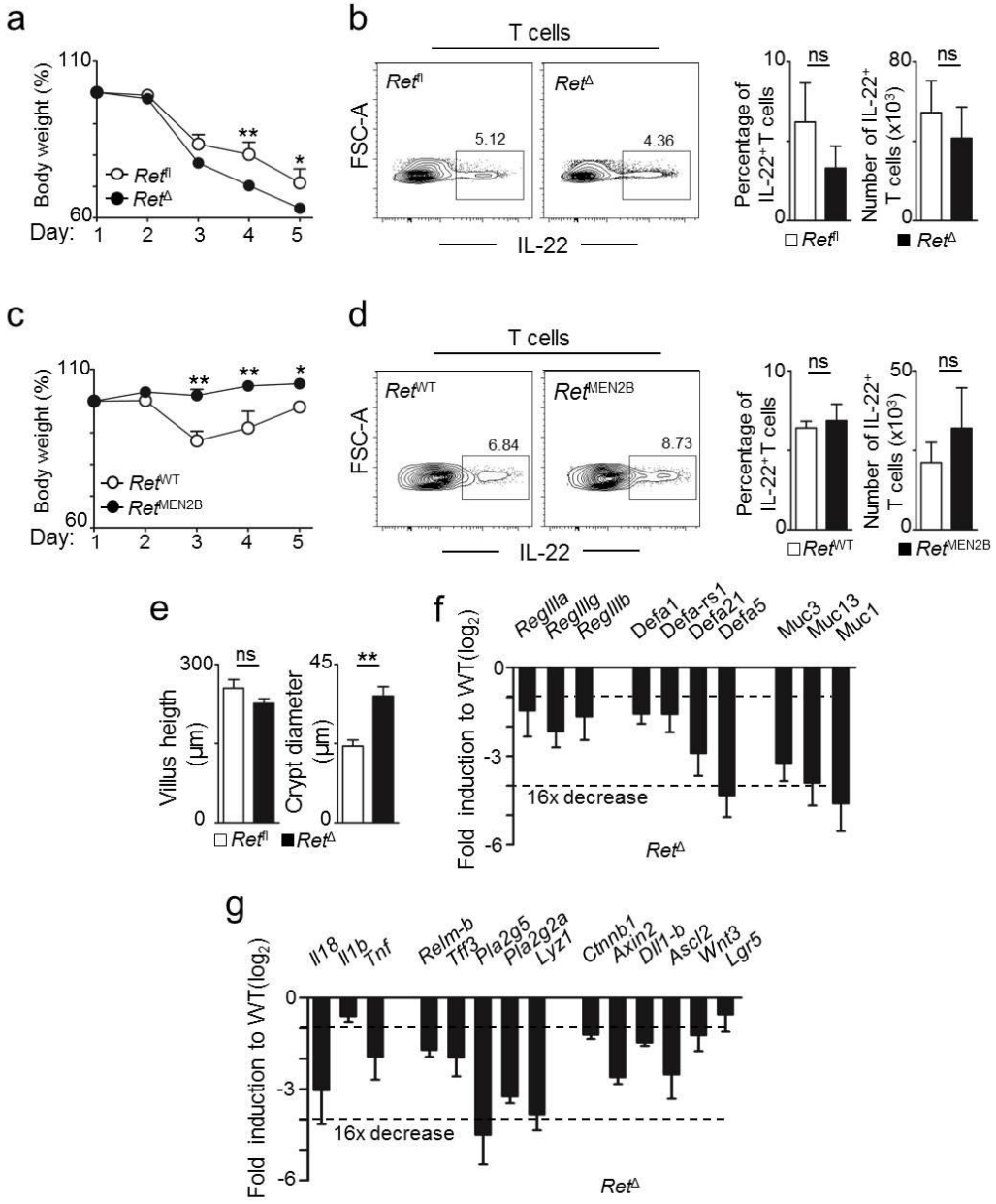
Extended Data Figure 1. ILC3 selectively express the neurotrophic factor receptor RET. a, Expression of RET protein in gut CD45^{+}Lin^{-}Thy1.2^{hi}IL7Rα^{+}RORγt^{+} ILC3. **b**, Analysis of gut ILC3 from *Ret*^{GFP} mice. Embryonic day 14.5 (E14.5). **c,d** Analysis of enteric ILC3 subsets from *Ret*^{GFP} mice. **e**, Analysis of cytokine producing ILC3 from *Ret*^{GFP} mice. **f**, Pregnant *Ret*^{GFP} mice were provided with antibiotic cocktails that were maintained after birth until analysis at 6 weeks of age. Left: RET/GFP (white). Right: flow cytometry analysis of RET/GFP expression in ILC3. Thin line: Ab treated; Bold line: SPF. **g**, *Ret* expression in enteric ILC3 from Germ-Free (GF) mice and Specific Pathogen Free (SPF) controls. n=4. **h**, Analysis of lamina propria populations from *Ret*^{GFP} mice. **i**, Enteric ILC3 clusters. Green: RET/GFP; Blue: RORγt; Red: B220. Bottom: quantification analysis for RET/GFP and RORγt co-expression (79,97 ±4,72%). **j**, Rare RET expressing ILC3 in intestinal villi. Green: RET/GFP; Blue: RORγt; Red: CD3ε. Scale bars: 10μm. Data are representative of 4 independent experiments. Error bars show s.e.m. ns not significant.



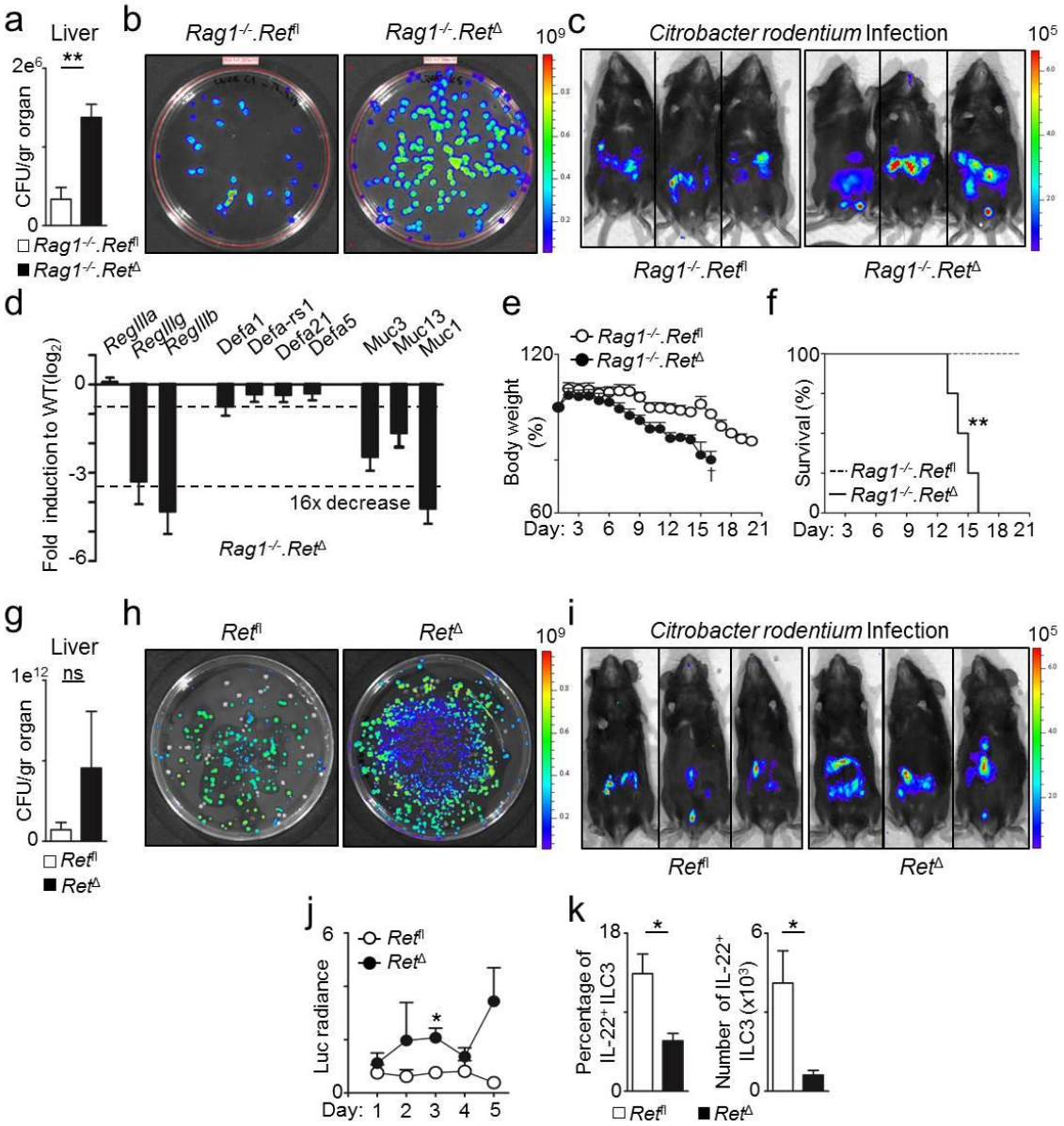
Extended Data Figure 2. T cell-derived IL-22 and IL-17 in *Ret*^{GFP} chimeras and *Ret*^{MEN2B} mice. a, T cell derived IL-17 in *Ret*^{GFP} chimeras. *Ret*$^{WT/GFP}$ n=25; *Ret*$^{GFP/GFP}$ n=22. **b**, T cell derived IL-22 and IL17 in the intestine of *Ret*^{MEN2B} mice and their WT littermate controls. *Ret*^{WT} n=7; *Ret*^{MEN2B} n=7. Data are representative of 4 independent experiments. Error bars show s.e.m. ns not significant.



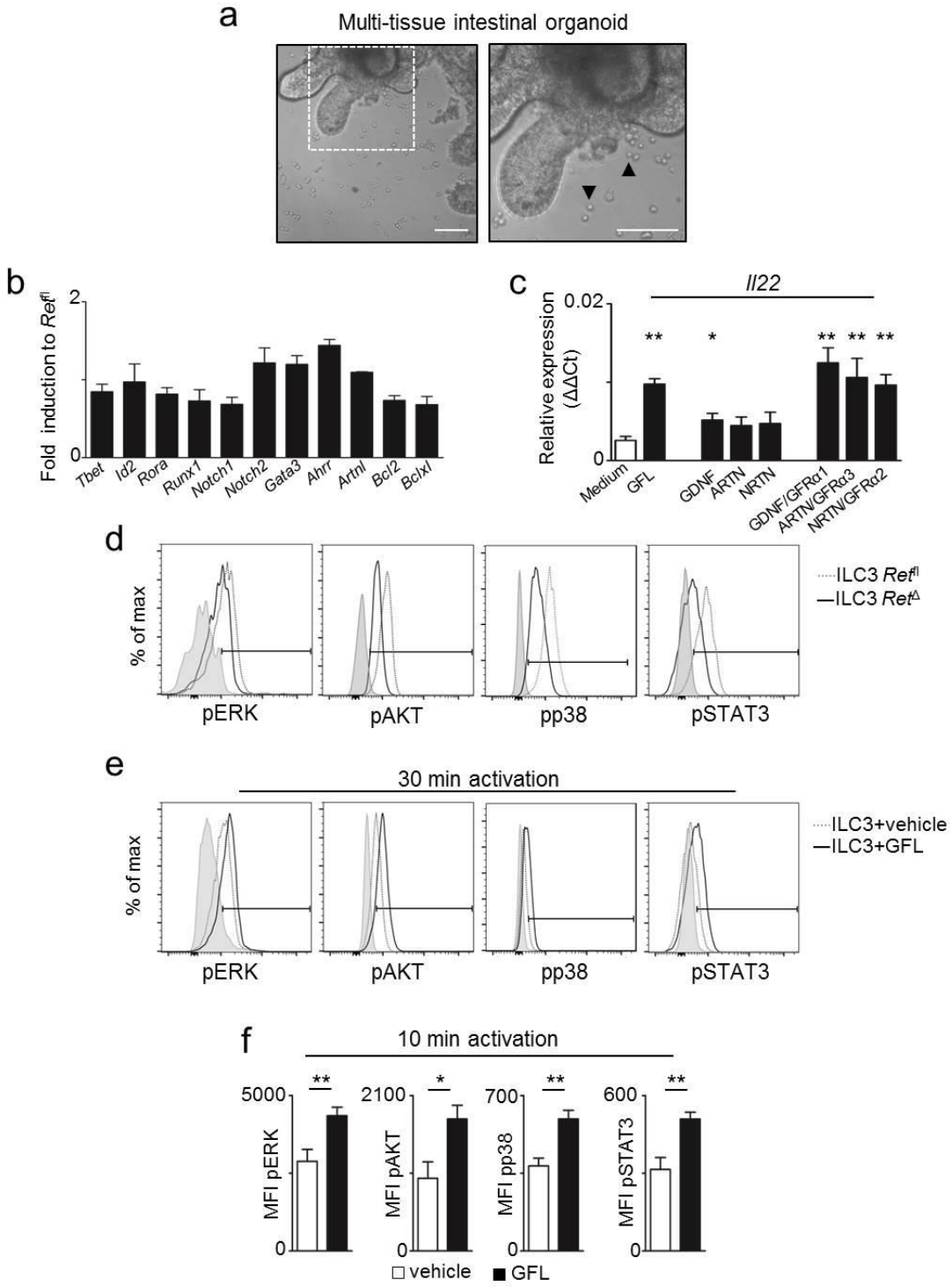
Extended Data Figure 3. Enteric homeostasis in steady-state *Ret*$^{\Delta}$ mice. a, *Rorgt*-Cre mice were bread to *Rosa26*^{YFP}. Analysis of Rosa26/YFP expression in gut ILC3 from *Rorgt*-Cre.*Rosa26*^{YFP} mice. **b**, Number of Peyer's patches (PP). *Ret*^{fl} n=10; *Ret*$^{\Delta}$ n=10. **c**, T cell derived IL-22 in *Ret*$^{\Delta}$ mice and their WT littermate controls. *Ret*^{fl} n=11; *Ret*$^{\Delta}$ n=11. **d**, γδ T cell derived IL-22 in *Ret*$^{\Delta}$ mice and their WT littermate controls. *Ret*^{fl} n=4; *Ret*$^{\Delta}$ n=4. **e**, Intestinal villus and crypt morphology. *Ret*^{fl} n=6; *Ret*$^{\Delta}$ n=6. **f**, Epithelial cell proliferation. *Ret*^{fl} n=5; *Ret*$^{\Delta}$ n=5. **g**, Intestinal paracellular permeability measured by Dextran-Fitc in the plasma. *Ret*^{fl} n=5; *Ret*$^{\Delta}$ n=5. **h**, Tissue repair genes in *Ret*$^{\Delta}$ intestinal epithelium in comparison to their WT littermate controls. n=8. **i**, Reactivity genes in *Ret*^{MEN2B} mice treated with anti-IL-22 blocking antibodies in comparison to *Ret*^{MEN2B} intestinal epithelium. *Ret*^{MEN2B} n=4; *Ret*^{MEN2B} + anti-IL-22 n=4. Data are representative of 3 independent experiments. Error bars show s.e.m. ns not significant.



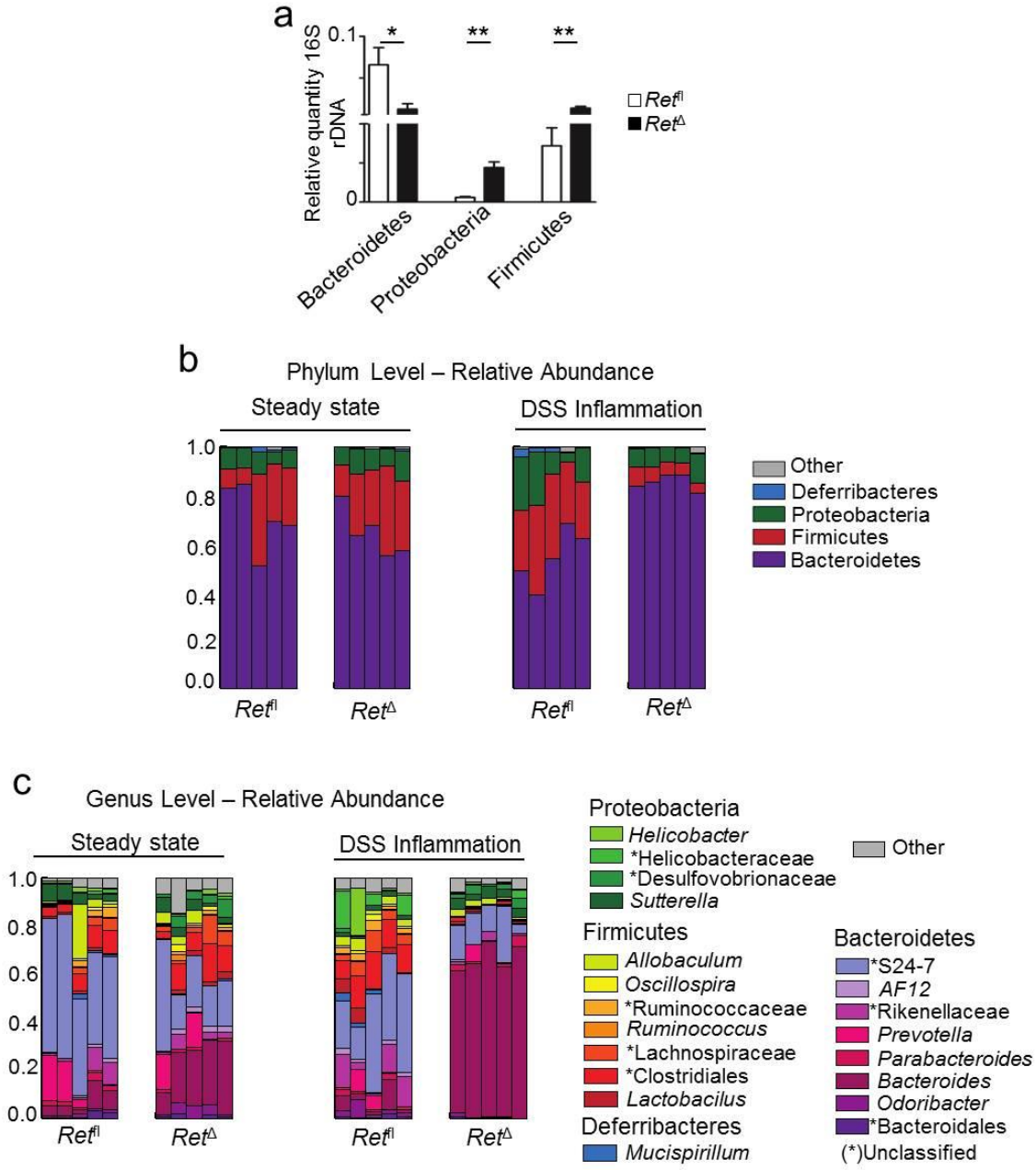
Extended Data Figure 4. Enteric inflammation in mice with altered RET signals. Mice were treated with DSS in the drinking water. **a**, Weight loss of DSS treated *Ret*$^{\Delta}$ mice and their littermate controls. *Ret*^{fl} n=8; *Ret*$^{\Delta}$ n=8. **b**, T cell derived IL-22 in *Ret*$^{\Delta}$ mice and their WT littermate controls after DSS treatment. *Ret*^{fl} n=8; *Ret*$^{\Delta}$ n=8. **c**, Weight loss of DSS treated *Ret*^{MEN2B} mice and their WT littermate controls. *Ret*^{WT} n=8; *Ret*^{MEN2B} n=8. **d**, T cell derived IL-22 in *Ret*^{MEN2B} mice and their WT littermate controls. *Ret*^{WT} n=8; *Ret*^{MEN2B} n=8. **e**, Intestinal villi and crypt morphology. *Ret*^{fl} n=6; *Ret*$^{\Delta}$ n=6. **f**, Epithelial reactivity gene expression in DSS treated *Ret*$^{\Delta}$ mice in comparison to their WT littermate controls. n=8. **g**, Tissue repair gene expression in DSS treated *Ret*$^{\Delta}$ mice in comparison to their WT littermate controls. n=4. Data are representative of 3-4 independent experiments. Error bars show s.e.m. ns not significant. Error bars show s.e.m. *P<0.05; **P<0.01; ns not significant.



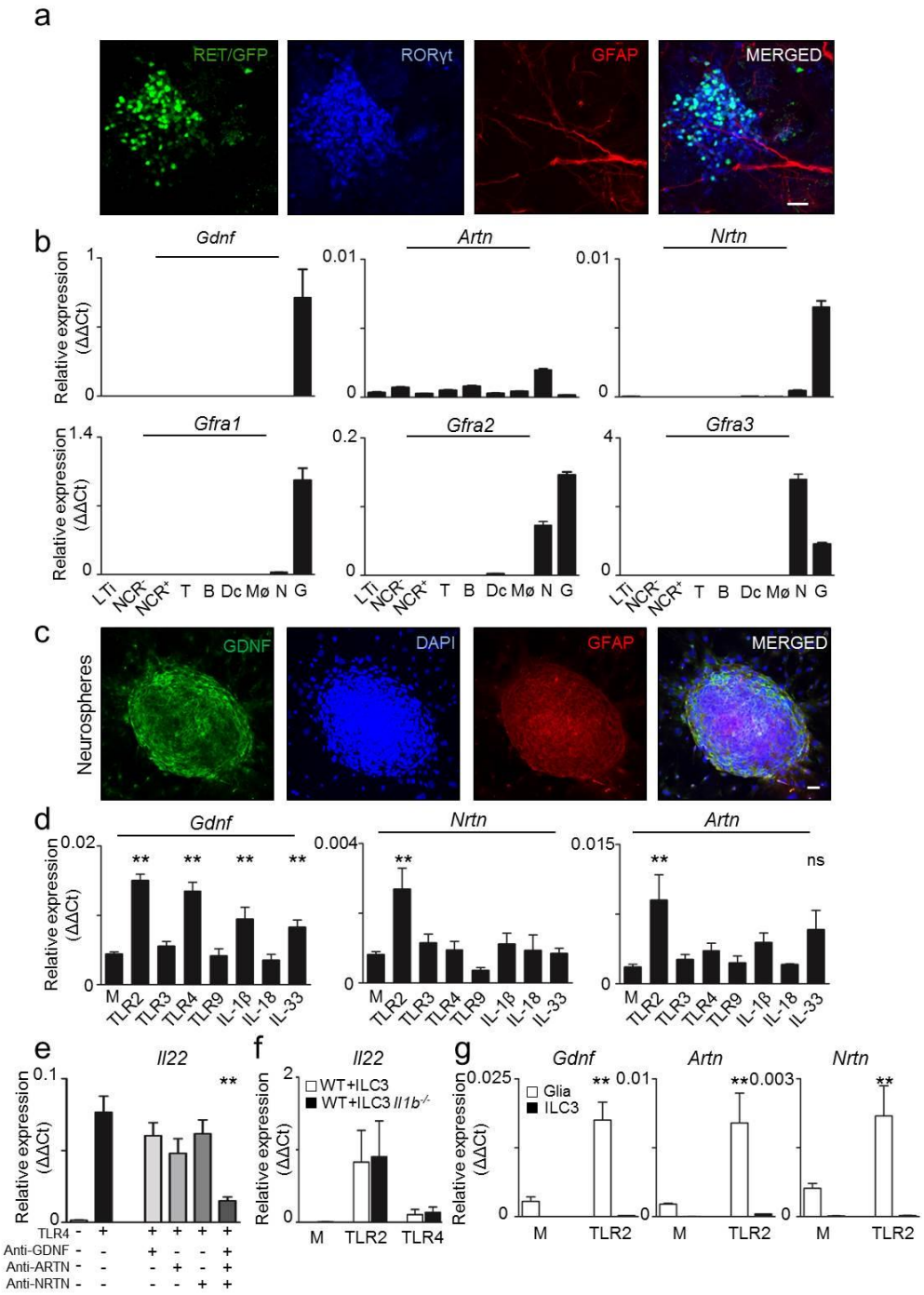
Extended Data Figure 5. *Citrobacter rodentium* infection in *Ret*$^{\Delta}$ mice. a, *C. rodentium* translocation to the liver of *Rag1*$^{-/-}$.*Ret*$^{\Delta}$ and their *Rag1*$^{-/-}$.*Ret*^{fl} littermate controls at day 6 post-infection. n=15. **b**, MacConkey plates of liver cell suspensions from *Rag1*$^{-/-}$.*Ret*$^{\Delta}$ and their *Rag1*$^{-/-}$.*Ret*^{fl} littermate controls at day 6 after *C. rodentium* infection. **c**, Whole-body imaging of *Rag1*$^{-/-}$.*Ret*$^{\Delta}$ and their *Rag1*$^{-/-}$.*Ret*^{fl} littermate controls at day 6 after luciferase-expressing *C. rodentium* infection. **d**, Epithelial reactivity gene expression in *C. rodentium* infected *Rag1*$^{-/-}$.*Ret*$^{\Delta}$ mice and their *Rag1*$^{-/-}$.*Ret*^{fl} littermate controls. *Rag1*$^{-/-}$.*Ret*^{fl} n=15; *Rag1*$^{-/-}$.*Ret*$^{\Delta}$ n=17. **e**, Weight loss in *C. rodentium* infected *Rag1*$^{-/-}$.*Ret*$^{\Delta}$ mice and their *Rag1*$^{-/-}$.*Ret*^{fl} littermate controls. *Rag1*$^{-/-}$.*Ret*^{fl} n=8; *Rag1*$^{-/-}$.*Ret*$^{\Delta}$ n=8. **f**, Survival curves in *C. rodentium* infected *Rag1*$^{-/-}$.*Ret*$^{\Delta}$ mice and their *Rag1*$^{-/-}$.*Ret*^{fl} littermate controls. *Rag1*$^{-/-}$.*Ret*^{fl} n=8; *Rag1*$^{-/-}$.*Ret*$^{\Delta}$ n=8. **g**, *C. rodentium* translocation to the liver of *Ret*$^{\Delta}$ and their *Ret*^{fl} littermate controls at day 6 post-infection. n=6. **h**, MacConkey plates of liver cell suspensions from *Ret*$^{\Delta}$ and their *Ret*^{fl} littermate controls at day 6 after *C. rodentium* infection. **i**, Whole-body imaging of *Ret*$^{\Delta}$ and their *Ret*^{fl} littermate controls at day 6 after luciferase-expressing *C. rodentium* infection. **j**, *C. rodentium* infection burden. *Ret*^{fl} n=8; *Ret*$^{\Delta}$ n=8. **k**, Innate IL-22 in in *C. rodentium* infected *Ret*$^{\Delta}$ mice and their *Ret*^{fl} littermate controls. *Ret*^{fl} n=8; *Ret*$^{\Delta}$ n=8. Data are representative of 3-4 independent experiments. Error bars show s.e.m. ns not significant. Error bars show s.e.m. *P<0.05; **P<0.01; ns not significant.



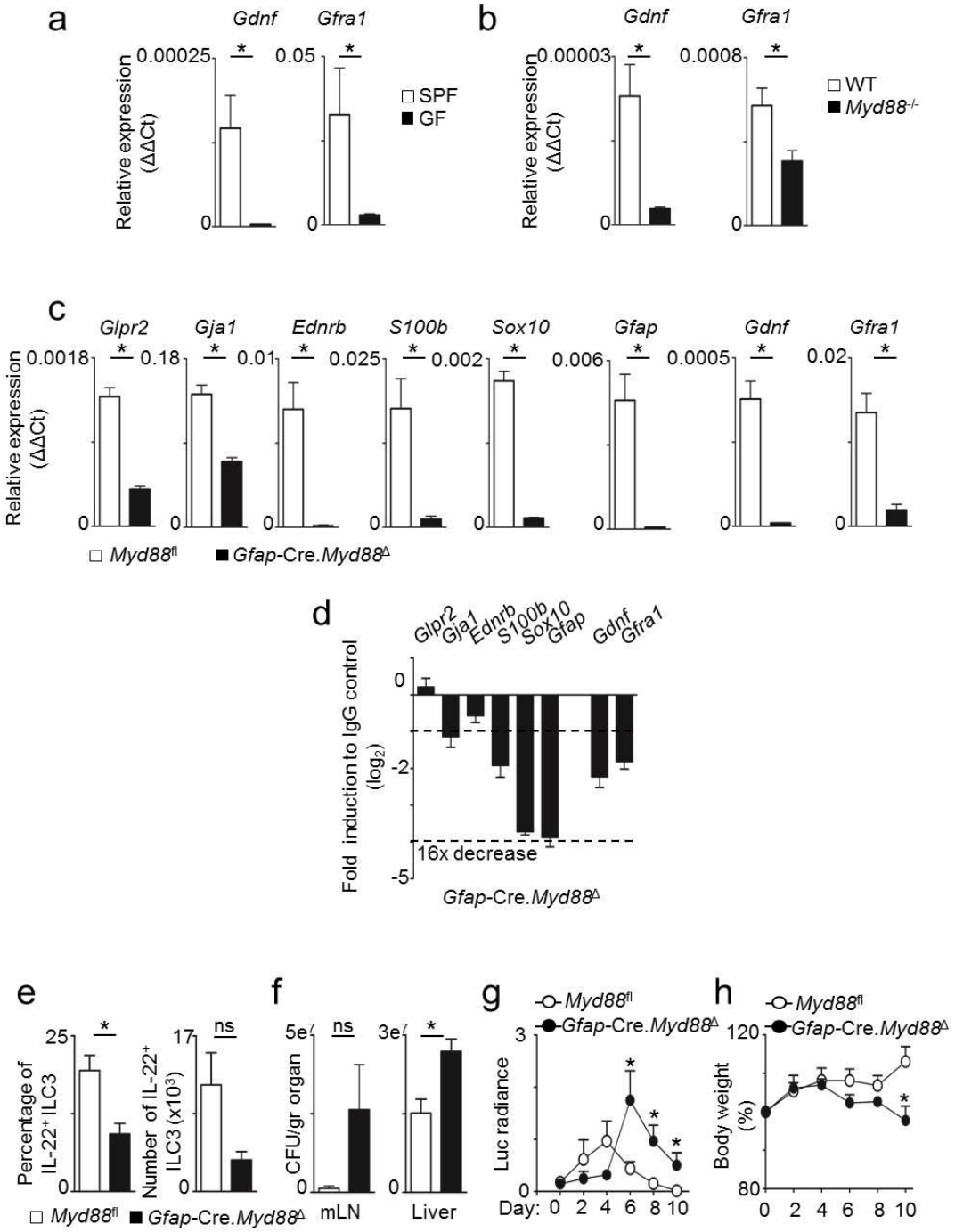
Extended Data Figure 6. Glial-derived neurotrophic factor family ligand (GFL) signals in ILC3. a, Multi-tissue intestinal organoid system. Scale bar: 20µm. Black arrows: ILC3. **b**, Expression of ILC-related genes in ILC3 from *Ret*$^{\Delta}$ mice in comparison to their littermate controls. n=4. **c**, ILC3 activation with all GFL/GFRα pairs (GFL); single GDNF family ligand (GDNF, ARTN or NRTN); or single GFL/GFRα pairs (GDNF/GFRα1, ARTN/GFRα3 or NRTN/GFRα2) compared to vehicle BSA. n=5. **d**, ILC3 from *Ret*$^{\Delta}$ mice (open black) and their littermate controls (open dash). Isotype (closed grey). **e**, 30 minutes activation of ILC3 by GFL (open black) compared to vehicle BSA (open dash). Isotype (closed grey). **f**, 10 minutes activation of ILC3 by GFL. pERK n=8; pAKT n=8; phosphorylated p38/MAP kinase n=8; pSTAT3 n=8. Similar results were obtained in at least 3-4 independent experiments. Error bars show s.e.m. *P<0.05; **P<0.01; ns not significant.



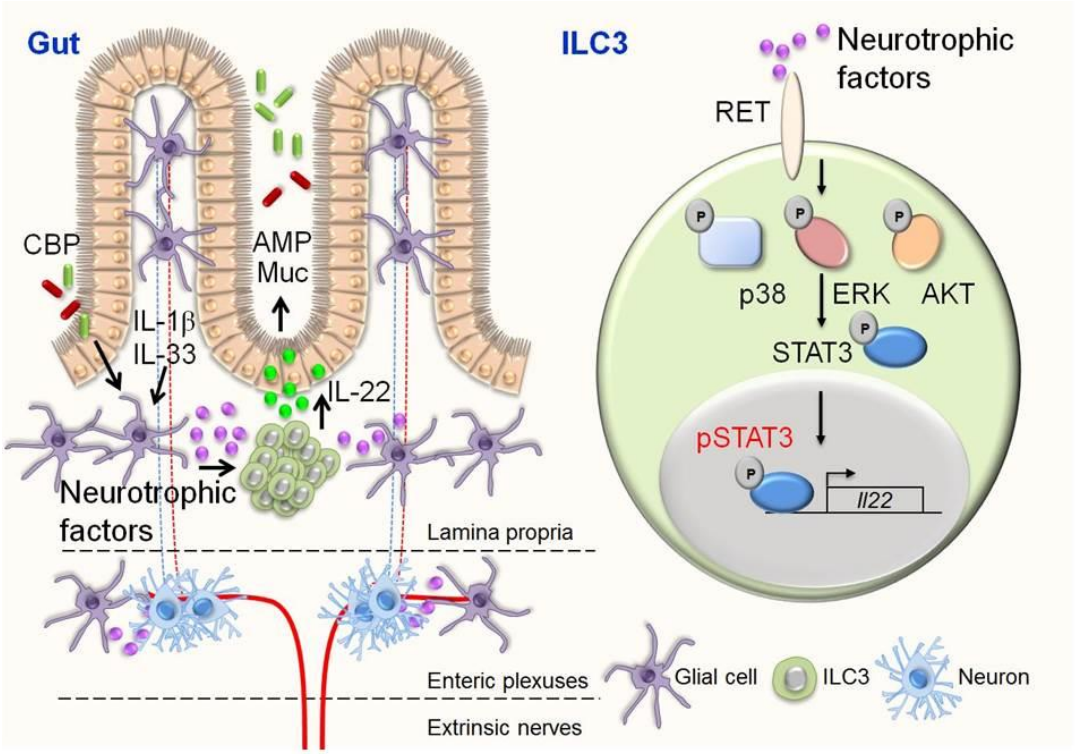
Extended Data Figure 7. Alterations in the diversity of intestinal commensal bacteria of *Ret*$^{\Delta}$ mice. a, Quantitative PCR analysis at the Phylum level in stool bacterial from co-housed *Ret*^{fl} and *Ret*$^{\Delta}$ littermates in steady state. n=5. **b**, Metagenomic Phylum level comparisons in stool bacterial from co-housed *Ret*^{fl} and *Ret*$^{\Delta}$ littermates in steady state (left) and after DSS treatment (right). n=5. **c**, Genus level comparisons in stool bacterial from co-housed *Ret*^{fl} and *Ret*$^{\Delta}$ littermates in steady state (left) and after DSS treatment (right). n=5. Error bars show s.e.m. *P<0.05; **P<0.01; ns not significant.



Extended Data Figure 8. GFL expressing glial cells anatomically co-localise with ILC3. a, Intestine of *Ret*^{GFP} mice. Green: RET/GFP; Red: GFAP; Blue: RORγt. Similar results were obtained in three independent experiments. **b**, Purified lamina propria LTi, NCR^{-} and NCR^{+} ILC3 subsets, T cells (T), B cells (B), Dendritic cells (Dc), Macrophages (Mø), enteric Neurons (N) and mucosal Glial cells (G). **c**, Neurosphere-derived glial cells. **d**, M: medium. Activation of neurosphere-derived glial cells with TLR2 (Pam3CSK4), TLR3 (Poli I:C), TLR4 (LPS) and TLR9 (DsDNA-EC) ligands, as well as IL-1β, IL-18 and IL-33. n=6. **e**, *Il22* in co-cultures of glial and ILC3 using single or combined GFL antagonists. n=6. **f**, *Il22* in co-cultures of ILC3 and glial cells from *Il1b*$^{-/-}$ or their WT controls. n=3. **g**, *Gdnf, Artn* and *Nrtn* expression in glial cells and ILC3 upon TLR2 stimulation. n=3. Scale bar: 30µm.Similar results were obtained in at least 4 independent experiments.



Extended Data Figure 9. Glial cell sensing via MYD88 signals. a-c, Intestinal glial cells were purified by flow cytometry. **a**, Germ-free (GF) and their respective Specific Pathogen Free (SPF) controls. n=3. **b**, Myd88$^{-/-}$ and their respective WT littermate controls. n=3. **c**, *Gfap*-Cre.*Myd88*$^{\Delta}$ and their littermate controls (*Myd88*^{fl}). n=3. **d**, Total lamina propria cells of *Gfap*-Cre.*Myd88*$^{\Delta}$ and their littermate controls (*Myd88*^{fl}). n=6. **e-h**, *Citrobacter rodentium* infection of *Gfap*-Cre.*Myd88*$^{\Delta}$ mice and their littermate controls (*Myd88*^{fl}). n=6. **e**, Innate IL-22. **f**, *Citrobacter rodentium* translocation. **g**, Infection burden. **h**, Weight loss. Data are representative of 3 independent experiments. Error bars show s.e.m. *P<0.05; **P<0.01; ns not significant.



Extended Data Figure 10. A novel glial-ILC3-epithelial cell unit orchestrated by neurotrophic factors. Lamina propria glial cells sense microenvironmental products, that control neurotrophic factor expression. Glial-derived neurotrophic factors operate in an ILC3-intrinsic manner by activating the tyrosine kinase RET, which directly regulates innate IL-22 downstream of a p38 MAPK/ERK-AKT cascade and STAT3 phosphorylation. GFL induced innate IL-22 acts on epithelial cells to induce reactivity gene expression (CBP: Commensal bacterial products; AMP: antimicrobial peptides; Muc: mucins). Thus, neurotrophic factors are the molecular link between glial cell sensing, innate IL-22 production and intestinal epithelial barrier defence.

GENERAL DISCUSSION AND PERSPECTIVES

Non-scientific articles are published every day in popular media such as health magazines, pseudo-medicine journals or internet "bio-guides", among others. This information is usually based on people's experiences and passed down through generations. Acupuncture, herbal or traditional Chinese medicine have been widely used for thousands of years as a way to cure inflammation bowel disorders and mental disorders such as depression or anxiety. Body-brain interactions are the basis of that pseudo-scientific knowledge in which scientists are often sceptic. This lack of credibility is due to the absence of scientific mechanistically studies and reproducibility. Nevertheless, an increase amount of data has recently emerged to support an important role of neuro-immune interactions in physiology and organismic homeostasis. From an evolutionary perspective, we can consider that the immune and the nervous systems evolved as interfaces between the inner body and the external environment. This immune/nervous co-regulation is becoming nowadays one of the most popular fields of study in the biological sciences.

Many studies have shown the relevance of immune cytokines and chemokines in the central nervous system and their relation with neuronal functions and behaviour[382–385]. On the other hand, immune cells can also respond to neurotransmitters from neurons[386–388]. Moreover, both systems are located in close proximity within different organs as it was recently shown with the muscularis macrophages in the gut[254]. Taking this together with the role of glial cells in the modulation of ILC3 function, we can hypothesise that ILC3s play a role in neural function. ILC3s release cytokines to their microenvironment that may modulate the central and the enteric nervous system. An interesting approach would be to identify the expression of different pro-inflammatory cytokine receptors such as the IL-22 or IL-17 receptors in some of the cells that compose the enteric nervous system. In this scenario, regulation of the nervous system through innate cytokines would have a direct impact in neurological responses, possibly working as a relay station for dietary and microbial products.

How neurons and immune cells cooperate to regulate physiological processes and to maintain tissue homeostasis is a fascinating issue. Neurological states can negatively impact in immunological activities[389]. As an example, prolonged periods of stress were related to viral infections in the respiratory tract[390,391]. Moreover, chronic stress or depression can activate the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) axis[392]. In this context, both axis can persistent

secrete glucocorticoids and noradrenaline respectively which would impact on the immune functions[393]. In addition, neurotransmitters such as acetylcholine (Ach), dopamine, serotonin and noradrenaline were shown to be implicated in neuro-immune interactions[394395]. Although is not fully characterized, adrenaline and noradrenaline can control different immune processes such as cell proliferation, cytokine and antibody production[396] and acetylcholine (Ach) plays also an important role during inflammation binding to macrophage Ach receptors[395].

The blood brain barrier (BBB) separates the brain from the invasion of pathogens. The entrance of lymphocytes in steady state conditions is very low; however, in inflammatory diseases it was observed that lymphocytes and macrophages can cross the BBB. Lymphocytes can lead to an inflammatory response, releasing cytokines and chemokines that can modulate the central nervous system in ways still very poorly understood. Thus, it is fundamental to understand the presence of neuro-immune units in steady state and inflammatory conditions. As an example, the identification of CNS lymphatics that drain the brain parenchyma into the deep cervical lymph nodes[397] provides an anatomical link between behavioural traits and the immune system. Furthermore, single-cell transcriptomic analysis and injury models have suggested that interferon-γ (IFN-γ) signalling is likely to control the homeostasis of neural stem cells in the adult brain[398] and appears to serve as a mediator of the effects of meningeal immunity on brain function[399].

The immune system can respond to a wide range of signals. In particular, innate lymphoid cells can be activated by diet products, cytokines, hormones and other factors modulating their IL-22 production[56,400]. Microbiota can induce not only IL-23 by dendritic cells, thus indirectly inducing IL-22, but microbial products can also induce IL-25 expression by enterocytes. Interestingly, it was shown that IL-25 represses ILC3 derived-IL-22[159]. Therefore, this pathway is critical to establish a microbiota-immune-epithelial balance in the gastrointestinal tract. Thus it would be interesting to study this balance in the susceptibility to inflammation and colon cancer.

Our data confirm that *Ret* is expressed in ILC3s while RET ligands and its co-receptors are expressed in enteric glial cells located in their close proximity. This allows ILC3s to crosstalk in a paracrine manner with enteric glial cells. Furthermore, our findings reveal that in addition to their capacity to sense dendritic cell-derived cytokines, ILC3 can be also activated by neurotrophic factors. Specific ablation of *Ret* in ILC3s resulted in cytokine malfunction, translated by decreased IL-22 cytokine. In agreement, constitutive activation of *Ret* produce increase ILC3-derived IL-22, indicating that RET has a critical role in ILC3 function. Interestingly, enteric glial cells were main producers of ligands and co-receptors

of RET, and their close proximity to ILC3s made them perfect candidates for ILC3 regulators. In agreement, we demonstrated that glial cells sense environmental products through TLR2 and TLR4, thus increasing RET ligand production. Specific ablation of *Myd88* -adaptor molecule for TLR sensing- in glial cells produced a decrease in IL-22 producing and infection with *C. Rodentium of* mice lacking *Myd88* in glial cells resulted in pronounced pathology.

Mucosal surfaces are protected with epithelial cells that constitute the first line of defence against invading pathogens[380]. Noteworthy, ILCs are also found abundantly at mucosal sites[83]. Thus, it is likely that epithelial-glia-ILC units may also exist beyond the gut. Preliminary data in our laboratory revealed that the expression of the neurotrophic factor receptor *Ret* is found not only in ILC3s, but also in ILC2s. Since RET^{+}ILC2s were found to be present in the gut and in the lungs these may be important for the resolution of respiratory and intestinal disorders such as asthma or allergy. The mucosal epithelia of the lungs act as barrier which has to block the passage of invasive pathogens. Thus, we can hypothesise that a glial-ILC2 unit may regulate important aspects of innate type 2 cytokine programmes.

The fact that the enteric nervous system senses environmental signals through pattern recognition receptors, while neurotrophic factors regulate the function of haematopoietic cells, strongly indicates that microbe-neuro-immune interaction may control intestine physiology. An increasing number of studies are currently emerging showing that the intestinal microbiota can influence metabolic, immune and nervous pathways in the host. In our study, we have analysed bacterial populations present in faeces. We showed that in steady state Ret^{Δ} mice had increase the Proteobacteria (increased in IBD patients[401]) and upon DSS-induced colitis the phylum Firmicutes (present in healthy individuals[402]) was preferentially reduced in Ret^{Δ} mice. As such, we speculated that a reduction of this beneficial microbiota, although not colitogenic, may enhance enteric inflammation in Ret^{Δ} mice. In line with this idea, treatments with pre- and pro-biotics have been developed in order to ameliorate the symptoms of inflammation bowel disease patients and to treat mental disorders. However, is still unknown what defines a beneficial microbiota that could act as a possible treatment. A better knowledge of the population pool of microbes existing in each pathology will be extremely important to understand their particular role in health and disease.

In this thesis we described an alternative pathway in which ILC3s can perform their function crosstalking with the enteric nervous system and enteric epithelial cells. We found a new mechanism by which ILC3 can be activated by glial derived neurotrophic factors in

order to control inflammation. This new discovery further supports a neuroregulatiory axis of immunity, suggesting that inflammation bowel pathologies might be affected by the nervous system acting as one of the main players involved in their onset and progression. It is still unclear in which physiological conditions the organism predominantly use the IL-23 or the glial-ILC3 pathway. Are all of those pathways acting simultaneously? Further studies will be required to better understand the exact mechanisms that regulate innate IL-22 production.

The fact that ILC3s are necessary in human health and disease is being discussed in recently studies showing some redundancy of T cells and ILC3s. Here, we showed the effect of ILC3s in T cell sufficient and deficient mice, showing the critical importance of ILC3s in the progression of inflammatory conditions, notably as critical regulators in DSS and *C. Rodentium* colitis.

Finally, from my point of view and as future directions, I consider that we need to better characterize the different enteric glial cell populations and how they may regulate mucosal immunity and physiology. Notably, which glial-derived factors may modulate and resolve mucosal pathologies and how these factors relate with specific microbiota entities. In a developmental point of view, another issue would be to understand the role of glial cells in the formation of innate lymphoid structures throughout embryonic life. These neuro-immune interactions could be also regulating the formation of lymphoid follicles such as cryptopatches and Peyer´s patches already known to contain RET^+ cells.

Currently there is no cure for IBD patients and nowadays medical doctors are focused on the improvement of patient's quality of life reducing their symptoms. Innate lymphoid cells changes were observed in human inflamed tissues within different diseases, thus, a precisely study of the role of the different subsets of ILCs will allow us to target them and eventually prevent disease. In this way, ILCs could be the missing pieces that are still lacking in the inflammation bowel pathology puzzle.

Our finding that neurotrophic factors control innate IL-22 may pave the way for novel therapeutic approaches in IBD. It is crucial to identify and understand the role of RET in human ILC3s and its influence in inflammatory pathologies. Can we modulate the production of neurotrophic factors by faecal transplantation treatments? Additionally, the interaction of glial cells with other types of RET^+ cells in the gut, such as T cells, ILC2s or enteroendocrine cells could modulate the onset, progression and resolution of different pathologies. If so, we might be able to ameliorate bowel inflammation conditions that are major Public Health concerns.

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ANNEX

S. Ibiza*- **B. García-Cassani*** Hélder Ribeiro, Tânia Carvalho, Luís Almeida, Rute Marques,Ana M. Misic, Casey Bartow-McKenney, Denise M. Larson, William J. Pavan, Gérard Eberl,Elizabeth A. Grice & Henrique Veiga-Fernandes. Glial cell-derived neuroregulators control type 3 innate lymphoid cells and gut defence. *Nature* 535, 440–443 (21 July 2016) doi:10.1038/nature18644
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LETTER

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Glial–cell–derived neuroregulators control type 3 innate lymphoid cells and gut defence

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Group 3 innate lymphoid cells (ILC3) are major regulators of inflammation and infection at mucosal barriers[1]. ILC3 development is thought to be programmed[1], but how ILC3 perceive, integrate and respond to local environmental signals remains unclear. Here we show that ILC3 in mice sense their environment and control gut defence as part of a glial–ILC3–epithelial cell unit orchestrated by neurotrophic factors. We found that enteric ILC3 express the neuroregulatory receptor RET. ILC3-autonomous *Ret* ablation led to decreased innate interleukin-22 (IL-22), impaired epithelial reactivity, dysbiosis and increased susceptibility to bowel inflammation and infection. Neurotrophic factors directly controlled innate *Il22* downstream of the p38 MAPK/ERK-AKT cascade and STAT3 activation. Notably, ILC3 were adjacent to neurotrophic-factor-expressing glial cells that exhibited stellate-shaped projections into ILC3 aggregates. Glial cells sensed microenvironmental cues in a MYD88-dependent manner to control neurotrophic factors and innate IL-22. Accordingly, glial-intrinsic *Myd88* deletion led to impaired production of ILC3-derived IL-22 and a pronounced propensity towards gut inflammation and infection. Our work sheds light on a novel multi-tissue defence unit, revealing that glial cells are central hubs of neuron and innate immune regulation by neurotrophic factor signals.

ILC3 produce proinflammatory cytokines, and regulate mucosal homeostasis and anti-microbial defence[1]. In addition to their well-established developmentally regulated program, ILC3 are also controlled by microbial and dietary signals[1–6], suggesting that ILC3 possess other unexpected environment-sensing strategies. Neurotrophic factors are extracellular environmental cues to neurons and include the glial-derived neurotrophic factor (GDNF) family ligands (GFL) that activate the tyrosine kinase receptor RET in the nervous system, kidney and haematopoietic progenitors[7–11].

Analysis of gut lamina propria revealed that ILC3 express high levels of *Ret*[7,12] (Fig.1a), a finding confirmed at the protein level and by reporter Ret^{GFP} knock-in mice[13] (Fig. 1b–d and Extended Data Fig. 1a–d). ILC3 subsets expressed Ret^{GFP} and aggregated in cryptopatches and isolated lymphoid follicles (ILF), suggesting a role of neuroregulators in ILC3 (Fig.1b–d and Extended Data Fig. 1b–j). To explore this hypothesis, we transplanted fetal liver cells from *Ret* competent ($Ret^{WT/GFP}$) or deficient ($Ret^{GFP/GFP}$)[13] animals into alymphoid $Rag1^{-/-}\gamma c^{-/-}$ hosts. *Ret*-deficient chimaeras revealed unperturbed ILC3 and cryptopatch development (Fig. 1e). Notably, IL-22-expressing ILC3 were largely reduced despite normal IL-22 production by T cells (Fig.1f, g). In contrast, innate IL-17 was unaffected by *Ret* ablation (Fig.1f and Extended Data Fig. 2a). In agreement with this, analysis of gain-of-function Ret^{MEN2B} mice[14] revealed a selective

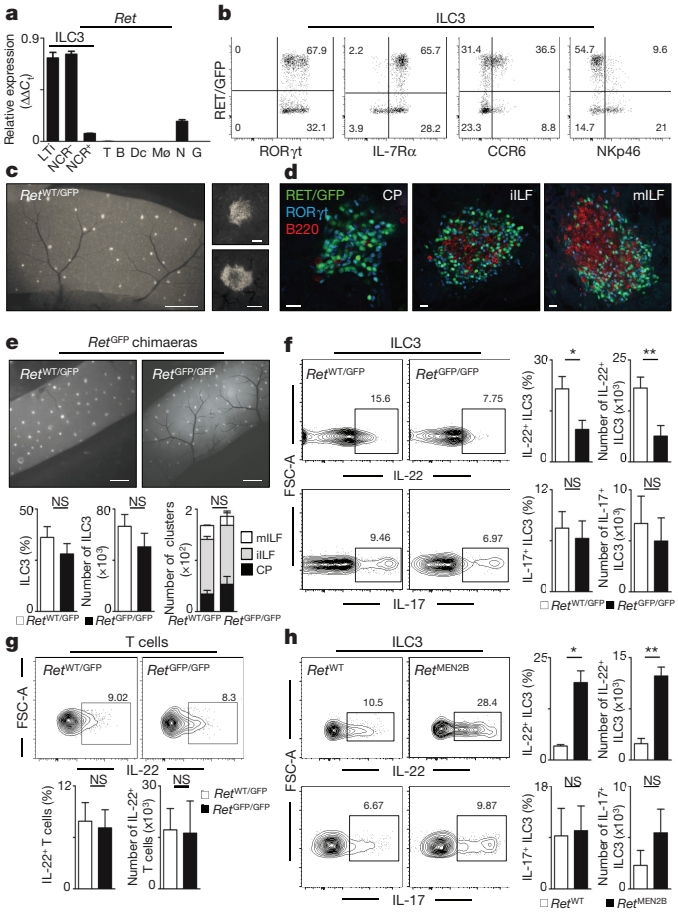

Figure 1 | The neurotrophic factor receptor RET drives enteric ILC3-derived IL-22. a, Relative expression of *Ret* in lymphoid tissue initiator cells (LTi), natural cytotoxicity receptor $(NCR)^-$ and NCR^+ ILC3 subsets, T cells (T), B cells (B), dendritic cells (Dc), macrophages (Mø), enteric neurons (N) and mucosal glial cells (G). **b**, Ret^{GFP} ILC3 subsets in the gut. **c**, Left, Ret^{GFP} gut. Right, ILC3 aggregates. White, GFP. **d**, Enteric cryptopatches (CP), immature (iILF) and mature (mILF) isolated lymphoid follicles. Green, RET/GFP; blue, RORγt; red, B220. **e**, Small intestine of Ret^{GFP} chimaeras ($n = 15$). **f**, **g**, Enteric ILC3 and T cells from Ret^{GFP} chimaeras. $Ret^{WT/GFP}$, $n = 25$; $Ret^{GFP/GFP}$, $n = 22$. **h**, Enteric ILC3 from Ret^{MEN2B} mice ($n = 7$). Scale bars, 1 mm (**c** left, **e**); 50 μm (**c** right); 30 μm (**d**). Data are representative of 4 independent experiments. Error bars show s.e.m. $*P < 0.05$, $**P < 0.01$; NS, not significant.

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increase of IL-22-producing ILC3, whereas their IL-17-producing counterparts were unaffected (Fig.1h and Extended Data Fig. 2b). To more specifically evaluate the effects of RET in ILC3, we deleted *Ret* in RORγt-expressing cells by breeding *Rorgt*-Cre with $Ret^{fl/fl}$ mice[15,16] (Extended Data Fig. 3a, b). Analysis of *Rorgt*-Cre$Ret^{fl/fl}$ (Ret^{Δ}) mice revealed selective and extensive reduction of ILC3-derived IL-22, but normal IL-22-producing T cells (Fig. 2a and Extended Data Fig. 3c, d). IL-22 acts on epithelial cells to induce reactivity and repair genes[1]. When compared to their wild-type littermate controls, the Ret^{Δ} epithelium revealed normal morphology, proliferation and paracellular permeability, but a marked reduction of epithelial reactivity and repair genes (Fig. 2b and Extended Data Fig. 3e–h). Accordingly, the Ret^{MEN2B} epithelium displayed increased levels of these molecules in an IL-22-dependent manner (Fig. 2b and Extended Data Fig. 3i). These results indicate that RET signals selectively control innate IL-22 and shape intestinal epithelial reactivity.

To determine whether neurotrophic factors regulate intestinal defence, we tested how varying degrees of RET signals control enteric aggressions. Whereas Ret^{Δ} mice treated with dextran sodium sulfate (DSS) had increased weight loss and inflammation, reduced IL-22-producing ILC3, decreased epithelial reactivity/repair genes and pronounced bacterial translocation from the gut, Ret^{MEN2B} mutants were highly protected compared to their wild-type littermate controls (Fig. 2c–j and Extended Data Fig. 4). As DSS largely causes epithelial injury, we tested whether ILC3-autonomous RET signals are required to control infection. To this end, Ret^{Δ} mice were bred with $Rag1^{-/-}$ mice to formally exclude adaptive T-cell effects. The resulting $Rag1^{-/-}Ret^{\Delta}$ mice were infected with the attaching and effacing bacteria *Citrobacter rodentium*. When compared to their littermate controls, $Rag1^{-/-}Ret^{\Delta}$ mice had marked gut inflammation, reduced IL-22-producing ILC3, increased *C. rodentium* infection and translocation, reduced epithelial reactivity genes, increased weight loss and reduced survival (Fig. 2k–n and Extended Data Fig. 5). Altogether, these data indicate that ILC3-intrinsic neurotrophic factor cues regulate gut defence and homeostasis.

We used a multi-tissue organoid system to show that IL-22 is the molecular link between RET-dependent ILC3 activation and epithelial reactivity. Addition of GFL to ILC3–epithelial organoids strongly induced epithelial reactivity genes in an IL-22- and RET-dependent manner (Fig. 3a, b and Extended Data Fig. 6a). To further examine how RET signals control innate IL-22, we investigated a gene signature

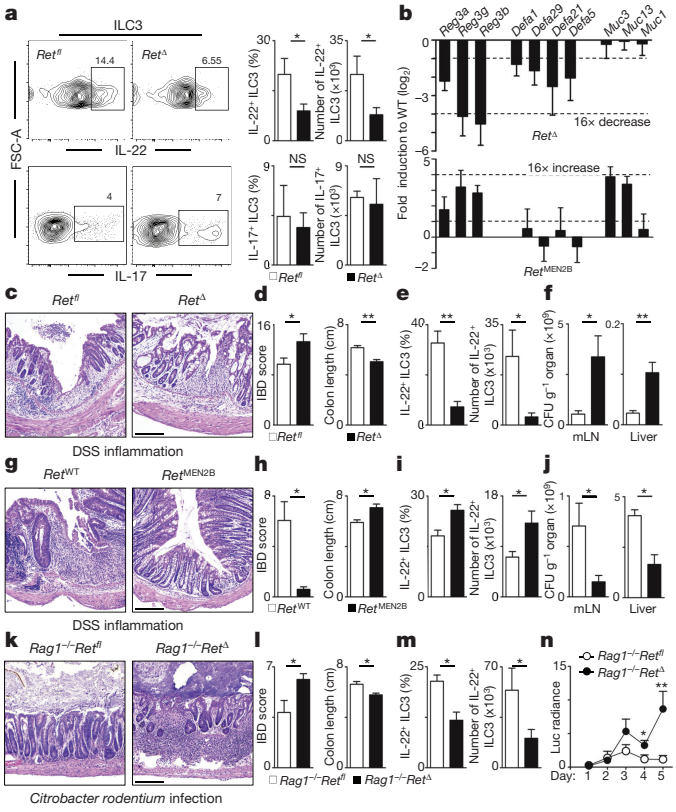

Figure 2 | ILC3-intrinsic RET signals regulate gut defence. a, ILC3-derived cytokines ($n = 11$). **b**, Gene expression in the epithelium of Ret^{Δ} and Ret^{MEN2B} mice compared to their wild-type littermate controls ($n = 7$). **c**–**f**, DSS treatment in Ret^{fl} and Ret^{Δ} mice ($n = 8$). **c**, Histopathology. **d**, Inflammation score and colon length. **e**, Innate IL-22. **f**, Bacterial translocation. CFU, colony-forming unit. **g**–**j**, DSS treatment in Ret^{WT} and Ret^{MEN2B} mice ($n = 8$). **g**, Histopathology. **h**, Inflammation score and colon length. **i**, Innate IL-22. **j**, Bacterial translocation. mLN, mesenteric lymph node. **k**–**n**, *C. rodentium* infection in $Rag1^{-/-}Ret^{fl}$ ($n = 15$) and $Rag1^{-/-}Ret^{\Delta}$ ($n = 17$) mice. **k**, Histopathology. **l**, Inflammation score and colon length. **m**, Innate IL-22. **n**, Infection burden. Scale bars, 200 μm. Data are representative of 4 independent experiments. Error bars show s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

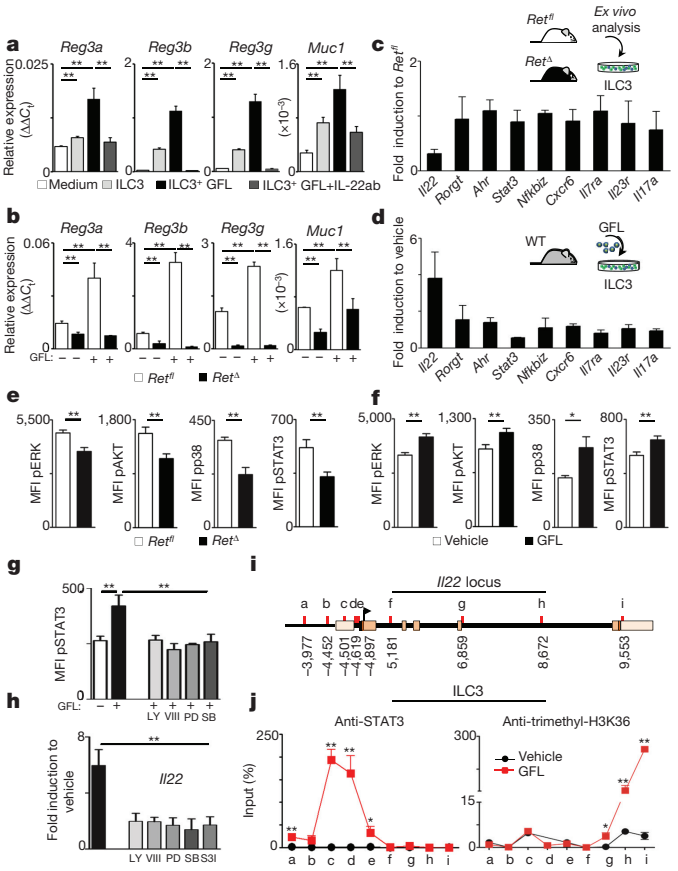

Figure 3 | ILC3-autonomous RET signals directly control *Il22* downstream of pSTAT3. a, **b**, Epithelial-ILC3 organoids ($n = 9$). **c**, Gene expression in Ret^{Δ} ILC3 compared to their wild-type controls ($n = 4$). **d**, Gene expression in ILC3 after activation by GFL ($n = 4$). **e**, Enteric Ret^{Δ} ILC3. pERK, $n = 8$; pAKT, $n = 12$; phosphorylated p38/MAP kinase, $n = 6$; pSTAT3, $n = 14$. MFI, mean fluorescence intensity. **f**, Enteric ILC3 activation by GFL. pERK, $n = 10$; pAKT, $n = 16$; phosphorylated p38/MAP kinase, $n = 3$; pSTAT3, $n = 15$. **g**, pSTAT3 expression in enteric ILC3 cultured with medium ($n = 7$), GFL ($n = 11$) or GFL and inhibitors for: p38 MAPK/ERK-AKT (LY) ($n = 7$); ERK (PD) ($n = 7$); AKT (VIII) ($n = 8$); and p38 MAPK (SB) ($n = 6$). **h**, *Il22* in enteric ILC3 cultured with GFL ($n = 17$) or GFL and the inhibitors LY ($n = 18$); PD ($n = 16$); VIII ($n = 15$); SB ($n = 15$); and the STAT3 inhibitor (S3I) ($n = 8$). **i**, *Il22* locus. The letters a–i in this panel indicate putative STAT3 binding sites and primer location (see Methods). **j**, ChIP analysis of ILC3 stimulated with GFL ($n = 10$). Data are representative of 3 independent experiments. Error bars show s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

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associated with ILC identity[1]. Whereas the master ILC transcription factors *Runx1*, *Id2*, *Gata3*, *Rora, Rorgt*, *Ahr* and *Stat3* were unperturbed, *Il22* was significantly reduced in *Ret*$^{\Delta}$ ILC3 (Fig. 3c and Extended Data Fig. 6b). Accordingly, activation of ILC3 with all or distinct GFL–GFRα pairs *in trans* efficiently increased *Il22* despite normal expression of other ILC3-related genes (Fig. 3d and Extended Data Fig. 6c). Activation of RET by GFL leads to p38 MAPK/ERK-AKT cascade activation in neurons, whereas phosphorylation of STAT3 shapes *Il22* expression[7,17]. Analysis of *Ret*$^{\Delta}$ ILC3 revealed hypo-phosphorylated ERK1/2, AKT, p38/MAP kinase and STAT3 (Fig. 3e and Extended Data Fig. 6d). Accordingly, GFL-induced RET activation in ILC3 led to rapid ERK1/2, AKT, p38/MAP kinase and STAT3 phosphorylation and increased *Il22* transcription (Fig. 3d, f and Extended Data Fig. 6e, f). Accordingly, inhibition of ERK, AKT or p38/MAP kinase upon GFL activation led to impaired STAT3 activation and *Il22* expression (Fig. 3g, h). Finally, inhibition of STAT3 upon GFL-induced RET activation led to decreased *Il22* (Fig. 3h). To examine whether GFL directly regulate *Il22*, we performed chromatin immunoprecipitation (ChIP)[18] (Fig. 3i, j). Stimulation of ILC3 with GFL resulted in increased binding of pSTAT3 in the *Il22* promoter and increased trimethyl-H3K36 at the 3' end of *Il22,* indicating active *Il22*-transcribed regions[19] (Fig. 3d, j). Thus, cell-autonomous RET signals control ILC3 function and gut defence by direct regulation of *Il22* downstream of STAT3 activation.

Propensity towards inflammation and dysregulation of intestinal homeostasis have been associated with dysbiosis[20,21]. When compared to their wild-type littermates, *Ret*$^{\Delta}$ mice have altered microbial communities as evidenced by quantitative analysis, weighted UniFrac analysis and significantly altered levels of *Sutterella*, unclassified *Clostridiales* and *Bacteroides* (Fig. 4a and Extended Data Fig. 7). Discrete microbial communities may have transmissible colitogenic potential[20,21]. Nevertheless, germ-free mice colonized with the microbiota of *Ret*$^{\Delta}$ or their control littermates revealed similar susceptibility to DSS-induced colitis and identical innate IL-22 production (Fig. 4b–d). In agreement, co-housed *Ret*$^{\Delta}$ and wild-type littermates had different propensity towards intestinal inflammation (Fig. 2c, d). Together, these data indicate that dysbiosis *per se* is insufficient to cause altered innate IL-22 and susceptibility to gut inflammation as observed in *Ret*$^{\Delta}$ mice (Fig. 2c–f). Thus, we hypothesised that GFL-producing cells integrate commensal and environmental signals to control innate IL-22. Accordingly, antibiotic treatment of *Ret*$^{\Delta}$ and their wild-type littermate controls resulted in similar ILC3-derived IL-22 (ref. 22) (Fig. 4e).

Neurotrophic factors of the GDNF family were shown to be produced by enteric glial cells, which are neuron-satellites expressing the glial fibrillary acidic protein (GFAP)[7,23]. Notably, double reporter mice for ILC3 (*Ret*^{GFP}) and glial cells (*Gfap*-Cre*Rosa26*^{RFP}) revealed that stellate-shaped projections of glial cells are adjacent ($4.35 \pm 1.42\,\mu m$) to RORγt^{+} ILC3 within cryptopatches (Fig. 4f and Extended Data Fig. 8a). These data suggest the existence of paracrine glial–ILC3 cross-talk orchestrated by neurotrophic factors. Accordingly, lamina propria glial cells were the main producers of GFL (Extended Data Fig. 8b). Recent studies have shown that glial cells express pattern recognition receptors, notably Toll-like receptors (TLRs)[24,25]. Activation of neurosphere-derived glial cells revealed they specifically respond to TLR2, TLR4, and the alarmins IL-1β and IL-33, which efficiently controlled GFL expression and induced robust innate *Il22* in a MYD88-dependent manner (Fig. 4g–i and Extended Data Fig. 8c–g). To formally demonstrate the physiological importance of MYD88-dependent glial cell sensing on innate IL-22 production, we deleted *Myd88* in GFAP-expressing glial cells by breeding *Gfap*-Cre with *Myd88*$^{fl/fl}$ mice[26,27]. Remarkably, glial-intrinsic deletion of *Myd88* resulted in decreased intestinal GFL, increased gut inflammation, impaired ILC3-derived IL-22, and increased weight loss (Fig. 4j–m; Extended Data Fig. 9a–d). In agreement, *Gfap*-Cre*Myd88*$^{\Delta}$ mice had increased susceptible to *C. rodentium* infection (Extended Data Fig. 9e–h). Thus, mucosal glial cells orchestrate innate IL-22 via neurotrophic factors, downstream of MYD88-dependent sensing of commensal products and alarmins.

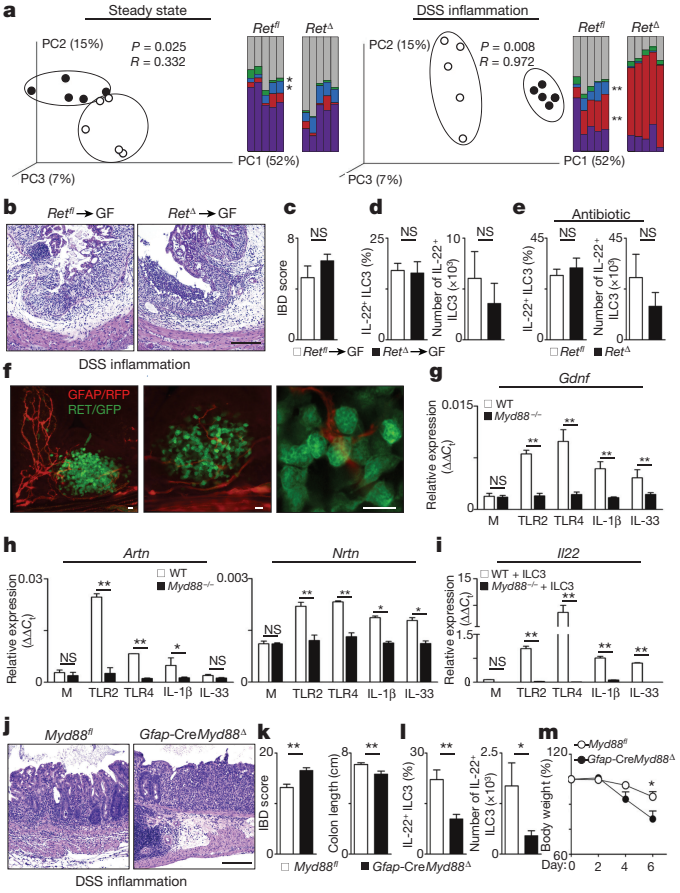

Figure 4 | Glial cells set GFL expression and innate IL-22 via MYD88-dependent sensing of the microenvironment. a, Weighted Unifrac PCoA analysis and genus-level comparisons from co-housed *Ret*^{fl} (white circles) and *Ret*$^{\Delta}$ (black circles) littermates ($n = 5$). Purple, unclassified *S24-7*; red, *Bacteroides*; green, *Sutterella*; blue, unclassified *Clostridiales*; grey, other. **b**–**d**, DSS treatment of germ-free (GF) mice ($n = 5$) colonized with microbiota from *Ret*^{fl} or *Ret*$^{\Delta}$ mice. **b**, Histopathology. **c**, Inflammation score. **d**, Innate IL-22. **e**, Innate IL-22 after antibiotic treatment ($n = 8$). **f**, *Ret*^{GFP}*Gfap*-Cre*Rosa26*^{RFP} mice. Green, RET/GFP; red, GFAP/RFP. **g**, **h**, Glial cell activation with TLR2, TLR4, IL-1β receptor and IL-33 receptor ligands ($n = 6$). **i**, TLR ligands, IL-1β and IL-33 activation of co-cultured ILC3 with wild-type (white bars) or *Myd88*$^{-/-}$ glial cells (black bars) ($n = 6$). **j**–**m**, DSS treatment of *Gfap*-Cre*Myd88*$^{\Delta}$ mice ($n = 12$). **j**, Histopathology. **k**, Inflammation score and colon length. **l**, Innate IL-22. **m**, Body weight. Scale bars, 200 μm (**b**, **j**); 10 μm (**f**). Data are representative of 3–4 independent experiments. Error bars show s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

Defining the mechanisms by which ILC3 integrate environmental cues is critical to understanding mucosal homeostasis. Our work sheds light on the relationships between ILC3 and their microenvironment, notably through decoding a novel glial–ILC3–epithelial cell unit orchestrated by neurotrophic factors (Extended Data Fig. 10). Glial-derived neurotrophic factors operate in an ILC3-intrinsic manner by activating the tyrosine kinase RET, which directly regulates innate IL-22 downstream of p38 MAPK/ERK-AKT and STAT3 phosphorylation (Extended Data Fig. 10). Future studies will further elucidate the mechanisms inducing RET expression in ILC3.

Our data demonstrate that, in addition to their well-established capacity to integrate dendritic-cell-derived cytokines[1], ILC3 perceive distinct multi-tissue regulatory signals leading to STAT3 activity and IL-22 expression, notably by integration of glial-cell-derived neuroregulators. Thus, rather than providing hard-wired signals for

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ILC3-immunity, we propose that RET signalling induces fine-tuned innate IL-22 production that leads to efficient gut homeostasis and defence.

Previous studies demonstrated that neurons may indirectly shape fetal lymphoid tissue inducer cell aggregation via regulation of mesenchymal cells and that ablation of glial cells leads to gut inflammation[28,29]; here we reveal that glial cells are central hubs of neuronal and innate immune regulation. Notably, neurotrophic factors are the molecular link between glial cell sensing, innate IL-22 and intestinal epithelial defence. Thus, it is tempting to speculate that glial–immune cell units might be also critical to the homeostasis of other barriers, notably in the skin, lung and brain[30]. From an evolutionary perspective, coordination of innate immunity and neuronal function may ensure efficient mucosal homeostasis and a co-regulated neuro-immune response to various environmental challenges, including xenobiotics, intestinal infection, dietary aggressions and cancer.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions S.I. and B.G.-C. designed, performed and analysed the experiments in Figs 1–4 and Extended Data Figs 1–9. T.C. analysed the experiments in Figs 2c, d, g, h, k, l, 4j, k, and Extended Data Figs 3e and 4e. H.R. performed and analysed the experiments in Figs 2f, j, n, 4a, m, Extended Data Figs 5a–c, e–j, 7a and 9f–h. L.A. contributed to experiments in Fig. 3a, b and Extended Data Fig. 6a. D.M.L., W.J.P., A.M.M., C.B.M. and E.A.G. performed and analysed the experiments in Fig. 4a and Extended Data Fig. 7b, c. R.M. and G.E. designed, performed and analysed the experiments in Fig. 4b–d. H.V.-F. supervised the work, planned the experiments and wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to H.V.-F. (jhfernandes@medicina.ulisboa.pt or henrique.veigafernandes@research.fchampalimaud.org).

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METHODS

Data reporting. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment, unless stated otherwise. Power analysis was used to estimate sample size.

Mice. C57BL/6J mice were purchased from Charles River. *Ret*^{GFP} (ref. 13), *Rag1*$^{-/-}\gamma c^{-/-}$ (refs 31, 32), *Ret*^{MEN2B} (ref. 14), *Rosa26*^{YFP} (ref. 33), *Rosa26*^{RFP} (ref. 34), *Ret*$^{fl/fl}$ (ref. 16), *Rorgt*-Cre[15], *Il1b*$^{-/-}$ (ref. 35) and *Myd88*$^{-/-}$ (ref. 36) were in a full C57BL/6J background. *Gfap*-Cre[26] bred to *Myd88*$^{fl/fl}$ (ref. 27) were in F8–F9 to a C57Bl/6J background. All lines were bred and maintained at IMM Lisboa animal facility. Mice were systematically compared with co-housed littermate controls. Both males and females were used in this study. Randomization and blinding were not used unless stated otherwise. All animal experiments were approved by national and institutional ethical committees, respectively, Direção Geral de Veterinária and iMM Lisboa ethical committee. Germ-free mice were housed at Instituto Gulbenkian de Ciência, Portugal, and Institut Pasteur, France, in accordance to institutional guidelines for animal care. Power analysis was performed to estimate the number of experimental mice.

Generation of fetal liver chimeras. For reconstitution experiments, 5×10^6 fetal liver cells were isolated from E14.5 *Ret*$^{WT/GFP}$ or *Ret*$^{GFP/GFP}$ mice and injected intravenously into non-lethally irradiated (200 rad) alymphoid *Rag1*$^{-/-}\gamma c^{-/-}$ hosts. Mice were analysed 8 weeks after transplantation.

DSS-induced colitis. Dextran sodium sulfate (DSS) (molecular mass 36,000–50,000 Da; MP Biomedicals) was added into drinking water 3% (w/v) for 5 days followed by 2 days of regular water. Mice were analysed at day 7. Body weight, presence of blood and stool consistency was assessed daily.

***Citrobacter rodentium* infection.** Infection with *Citrobacter rodentium* ICC180 (derived from DBS100 strain)[37] was performed by gavage inoculation of 10^9 colony-forming units[37,38]. Acquisition and quantification of luciferase signal was performed in an IVIS system (Caliper Life Sciences). Throughout infection, weight loss, diarrhoea and bloody stools were monitored daily.

Antibiotic treatment. Pregnant females or newborn mice were treated with streptomycin 5 g l^{-1}, ampicillin 1 g l^{-1} and colistin 1 g l^{-1} (Sigma-Aldrich) into drinking water with 3% sucrose. Control mice were given 3% sucrose in drinking water as previously described[22].

Microscopy. Intestines from *Ret*^{GFP} and *Ret*^{GFP} chimaeras were imaged in a Zeiss Lumar V12 fluorescence stereo microscope with a NeoLumar S 0.8× objective using the GFP filter. Whole-mount analysis was performed as previously described[2,9]. Briefly, adult intestines were flushed with cold PBS (Gibco) and opened longitudinally. Mucus and epithelium was removed and intestines were fixed in 4% PFA (Sigma-Aldrich) at room temperature for 10 mins and incubated in blocking/permeabilizing buffer solution (PBS containing 2% BSA, 2% goat serum, 0.6% Triton X-100). To visualise three-dimensional structures of the small intestine, samples were cleared with benzyl alcohol-benzyl benzoate (Sigma-Aldrich) before dehydration in methanol[2,9]. For analysis of thick gut sections, intestines were fixed with 4% PFA at 4 °C overnight and were then included in 4% low-melting temperature agarose (Invitrogen). Sections of 100 μm were obtained with a Leica VT1200/VT1200 S vibratome and embedded in Mowiol (Calbiochem)[2]. Slides or whole-mount samples were incubated overnight or for 1–2 days respectively at 4 °C using the following antibodies: rat monoclonal anti-B220 (RA3-6B2) (eBioscience), mouse monoclonal anti-RORγt (Q31-378) (BD Pharmigen), mouse monoclonal anti-GFAP (GA-5) (Sigma-Aldrich), mouse monoclonal anti-GFAP Cy3 (GA-5) (Abcam), anti-GDNF antibody (Abcam), DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Invitrogen). A647 goat anti-rat, A568 goat anti-rat, A647 goat anti-mouse, A488 rabbit anti-GFP, and A488 goat anti-rabbit secondary antibodies were purchased from Invitrogen. Neurospheres and cultured glial cells were fixed in PFA 4% for 10 minutes at room temperature and permeabilized in PBS-Triton 0.1% within 30 seconds. After several washing steps with PBS, cells were incubated with antibodies for 3 h at room temperature and then mounted in Mowiol[39]. Samples were acquired on a Zeiss LSM710 confocal microscope using EC Plan-Neofluar 10×/0.30 M27, Plan Apochromat 20×/0.8 M27 and EC Plan-Neofluar 40×/1.30 objectives. Three-dimensional reconstruction of images was achieved using Imaris software and snapshot pictures were obtained from the three-dimensional images. For analysis of confocal images, cells were counted using in-house software, written in MATLAB (Mathworks, Natick, MA). Briefly, single-cell ILC3 nuclei were identified by RORγt by thresholding and particle analysis. Regions of interest (ROIs) (Extended Data Fig. 1i; bottom panels) were defined from each nucleus for analysis in the GFP channel, where staining was considered positive if a minimum number of pixels (usually 20) were above a given threshold. The software allows for batch processing of multiple images and generates individual report images for user verification of cell-counting results and co-expression analysis (https://imm.medicina.ulisboa.pt/en/servicos-e-recursos/technical-facilities/bioimaging).

Histopathology analysis. Colon samples were fixed in 10% neutral buffered formalin. The colon was prepared in multiple cross-sections or 'swiss roll' technique[40], routine-processed for paraffin embedding and 3–4 μm sections were stained with haematoxylin and eosin. Enteric lesions were scored by a pathologist blinded to experimental groups, according to previously published criteria[41–43]. Briefly, lesions were individually scored (0–4 increasing severity) for the following criteria: 1, mucosal loss; 2, mucosal epithelial hyperplasia; 3, degree of inflammation; 4, extent of the section affected in any manner; and 5, extent of the section affected in the most severe manner as previously described[43]. Final scores were derived by summing the individual lesion and the extent scores. The internal diameter of the crypts was measured in at least five fields (10× magnification), corresponding to the hotspots in which the most severe changes in crypt architecture were seen. Measurements were performed in an average of 35 crypts per mouse, from proximal to distal colon. Intestinal villus height was measured in the jejunum. Measurements were performed in slides scanned using a Hamamatsu Nanozoomer SQ digital slide scanner running NDP Scan software.

Enteric glial cell isolation. Enteric glial cells isolation was adapted from previously described protocols[44,45]. Briefly, the muscularis layer was separated from the submucosa with surgical forceps under a dissection microscope (SteREO Lumar. V12, Zeiss). The lamina propria was scraped mechanically from the underlying submucosa using 1.5-mm cover-slips (Thermo Scientific). Isolated tissues were collected and digested with Liberase TM (7,5 μg ml^{-1}; Roche) and DNase I (0.1 mg ml^{-1}; Roche) in RPMI supplemented with 1% HEPES, sodium pyruvate, glutamine, streptomycin and penicillin and 0.1% β-mercaptoethanol (Gibco) for approximately 40 min at 37 °C. Single-cell suspensions were passed through a 100-μm cell strainer (BD Biosciences) to eliminate clumps and debris.

Flow cytometry and cell sorting. Lamina propria cells were isolated as previously described[46]. Briefly, intestines were digested with collagenase D (0.5 mg ml^{-1}; Roche) and DNase I (0.1 mg ml^{-1}; Roche) in RPMI supplemented with 10% FBS, 1% HEPES, sodium pyruvate, glutamine, streptomycin and penicillin and 0.1% β-mercaptoethanol (Gibco) for approximately 30 min at 37 °C under gentle agitation. For cytokine analysis, cell suspensions were incubated 4 h in PMA/ionomycin (Sigma-Aldrich) and brefeldin A (eBioscience) at 37 °C. Intracellular staining was performed using IC fixation/permeabilization kit (eBioscience). Cells were stained using PBS, 1% FBS, 1% HEPES and 0.6% EDTA (Gibco). Flow cytometry analysis and cell sorting was performed using FORTESSA and FACSAria flow cytometers (BD Biosciences). Data analysis was performed using FlowJo software (Tristar). Sorted populations were >95% pure. Cell suspensions were stained with anti-CD45 (30-F11), anti-TER119 (TER-119), TCRβ (H57-597), anti-CD3ε (eBio500A2), anti-CD19 (eBio1D3), anti-NK1.1 (PK136), anti-CD11c (N418), anti-Gr1 (RB6-8C5), anti-CD11b (Mi/70), anti-CCR6 (29-2L17), anti-CD127 (IL-7Rα; A7R34), anti-Thy1.2 (53-2.1), anti-CD49b (DX5), anti-TCRδ (GL3), anti-NKp46 (29A1.4), anti-IL-17 (eBio17B7), anti-IL-22 (1H8PWSR), rat IgG1 isotype control (eBRG1) antibodies, 7AAD viability dye, anti-mouse CD16/CD32 (Fc block), anti-RORγt (AFKJS-9); rat IgG2a$_\kappa$ isotype control (eBR2a) and streptavidin fluorochrome conjugates all from eBioscience; anti-CD4 (GK1.5), anti-CD31 (390), anti-CD8α (53-6.7), anti-CD24 (M1/69), anti-Epcam (G8.8) antibodies were purchased from Biolegend. Anti-RET (IC718A) antibody was purchased from R&D Systems. LIVE/DEAD Fixable Aqua Dead Cell Stain Kit was purchased from Invitrogen. Cell populations were defined as: ILC3 - CD45$^+$Lin$^-$Thy1.2^{hi}IL7Rα$^+$RORγt$^+$. For ILC3 subsets, additional markers were employed: LTi - CCR6$^+$Nkp46$^-$; ILC3 NCR$^-$ - CCR6$^-$Nkp46$^-$; ILC3 NCR$^+$ - CCR6$^-$Nkp46$^+$. Lineage was composed from CD3ε, CD8α, TCRβ, TCRγδ, CD19, Gr1, CD11c and TER119. Glial cells - CD45$^-$CD31$^-$TER119$^-$CD49b$^+$ (ref. 47); T cells - CD45$^+$CD3ε$^+$; γδ T cells - CD45$^+$CD3ε$^+$γδTCR$^+$; B cells - CD45$^+$CD19$^+$B220$^+$; macrophages - CD45$^+$CD11b$^+$F4/80$^+$; dendritic cells - CD45$^+$CD19$^-$CD3ε$^-$MHCII$^+$CD11c$^+$; enteric neurons - CD45$^-$RET/GFP$^+$ (ref. 13); epithelial cells - CD45$^-$CD24$^+$Epcam$^+$.

Quantitative RT–PCR. Total RNA was extracted using RNeasy micro kit (Qiagen) or Trizol (Invitrogen) according to the manufacturer's protocol. RNA concentration was determined using Nanodrop Spectrophotometer (Nanodrop Technologies). Quantitative real-time reverse transcription (RT)–PCR was performed as previously described[2,8,9]. *Hprt* and *Gapdh* were used as housekeeping genes. For TaqMan assays (Applied Biosystems), RNA was retro-transcribed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems), followed by a pre-amplification PCR using TaqMan PreAmp Master Mix (Applied Biosystems). TaqMan Gene Expression Master Mix (Applied Biosystems) was used in real-time PCR. TaqMan Gene Expression Assays (Applied Biosystems) were the following: *Gapdh* Mm99999915_g1; *Hprt* Mm00446968_m1; *Artn* Mm00507845_m1; *Nrtn* Mm03024002_m1; *Gdnf* Mm00599849_m1; *Gfra1 Mm00439086_m1*; *Gfra2 Mm00433584_m1*; *Gfra3 Mm00494589_m1*; *Ret* Mm00436304_m1; *Il22* Mm01226722_g1; *Il17a* Mm00439618_m1; *Il23r* Mm00519943_m1; *Rorgt* Mm01261022_m1; *Il7ra* Mm00434295_m1; *Ahr* Mm00478932_m1; *Stat3* Mm01219775_m1; *Cxcr6* Mm02620517_s1; *Nfkbiz* Mm_00600522_m1; *Reg3a* Mm01181787_m1; *Reg3b* Mm00440616_g1; *Reg3g* Mm00441127_m1;

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Defa1 Mm02524428_g1; *Defa29* Mm00655850_m1; *Defa5* Mm00651548_g1; *Defa21* Mm04206099_gH; *Muc1* Mm00449599_m1; *Muc3* Mm01207064_m1; *Muc13* Mm00495397_m1; *Gfap* Mm01253033_m1; *Ascl2* Mm01268891_g; *Tff3* Mm00495590_m1; *Relm-b* Mm00445845_m1; *Pla2g2a* Mm00448160_m1; *Pla2g5* Mm00448162_m1; *Wnt3* Mm00437336_m1; *Ctnnb1* Mm00483039_ m1; *Axin2* Mm00443610_m1; *Dll1b* Mm01279269_m1; *Il18* Mm00434225_m1; *Tnfa* Mm00443260_g1; *Lyz1* Mm00657323_m1; *Lrg5* Mm00438890_m1; *Tbx21* Mm00450960_m1; *Id2* Mm00711781_m1; *Runx1* Mm01213404_m1; *Notch1* Mm00435249_m1; *Notch2* Mm00803077_m1; *Gata3* Mm00484683_m1; *Bcl2* Mm00477631_m1; *Bcl2l1* Mm00437783_m1; *Arntl* Mm00500226_m1; *Glpr2* Mm01329475_m1; *Gja1* Mm01179639_s1; *Ednrb* Mm00432989; *S100b* Mm00485897_m1; *Sox10* Mm00569909_m1. Real-time PCR analysis was performed using ABI Prism 7900HT Sequence Detection System or StepOne Real-Time PCR system (Applied Biosystems).

ILC3 activation and cell signalling. Sorted intestinal ILC3 cells were starved for 3 hours in RPMI at 37 °C in order to ensure ILC3 viability. Ret^{fl} or Ret^{Δ} were analysed directly *ex vivo*. To test ERK, AKT, p38-MAPK (Cell Signaling Technology) and STAT3 (BD Pharmigen) upon GFL stimulation wild-type ILC3 were activated with 500 ng ml^{-1} (each GFL) and co-receptors (recombinant rat (rr) GFR-α1, recombinant mouse (rm) GFR-α2, rrGFR-α3 and rrGNDF from R&D Systems; recombinant human (rh) NRTN and rhARTN from PeproTech) for 10 and 30 min. When referring to the use of 'GFL' we have employed GDNF, NRTN, ARTN and their specific co-receptors in combination. For inhibition experiments cells were incubated 1 h at 37 °C before GFL stimulation, to test ERK, AKT, p38/MAPK and STAT3 phosphorylation, or during overnight stimulation with GFLs, to determine *Il22* expression levels. Inhibitors were purchased from Sigma-Aldrich; p38 MAPK/ERK-AKT, LY294002 (LY); ERK, PD98059 (PD); AKT, AKT Inhibitor VIII (VIII); p38 MAPK, SB 202190 (SB); and pSTAT3, S3I-201 (S3I).

Chromatin immunoprecipitation (ChIP) assay. Enteric ILC3 from adult C57BL/6J mice were isolated by flow cytometry. Cells were starved for 3 h with RPMI supplemented with 1% HEPES, sodium pyruvate, glutamine, streptomycin and penicillin and 0.1% β-mercaptoethanol (Gibco) at 37 °C. Cells were stimulated with GFLs (500 ng ml^{-1} each)[8], lysed, cross-linked and chromosomal DNA–protein complex sonicated to generate DNA fragments ranging from 100–300 base pairs. DNA–protein complexes were immunoprecipitated, using LowCell ChIP kit (Diagenode)[18], with 3 μg of rabbit polyclonal antibody against anti-pSTAT3 (Cell Signalling Technology), rabbit control IgG (Abcam) or H3K36me3 (07-030; Millipore). Immunoprecipitates were uncross-linked and analysed by quantitative PCR using primer pairs (5'–3') flanking putative sites on *Il22*. Vehicle (BSA)-stimulated ILC3s were used as controls. *Il22* primer sequences were previously described[48–50], briefly: (a) forward: 5′-TGCAATCAATCCCAGTATTTTG-3′ and reverse: 5′-CTTGTGCAAGCATAAGTCTCAA-3′; (b) forward: 5′-GAAGTTGGTGG GAAAATGAGTCCGTGA-3′ and reverse: 5′-GCCATGGCTTTGCCGTAG TAGATTCTG-3′; (c) forward: 5′-ACGGGAGATCAAAGGCTGCTCT-3′ and reverse: 5′-GCCAACAAGGTGCTTTTGC-3′; (d) forward: 5′-CTCACC GTGACGTTTTAGGG-3′ and reverse: 5′-GTGAATGATATGACATCAGAC-3′; (e) forward 5′-CGACGAACATGCTCCCCTGATGTTTTT-3′ and reverse: 5′-AAACTCATAGATTTCTGCAGGACAGCC-3′; (f) forward: 5′-AGCTG CATCTCTTTCTCTCCA-3′ and reverse: 5′-TATCCTGAAGGCCAA AATAGGA-3′; (g) forward: 5′-ACGACCAGAACATCCAGAAGA-3′ and reverse: 5′-GCAGAGAAAGAAATCCCCGC-3′; (h) forward: 5′-AGGGGGAC TTGCTTTGCCATTT-3′ and reverse: 5′-AACACCCCTTCTTTCC TCCTCCAT-3′; (i) forward: 5′-CTGCTCCTTCCTGCCTTCTA-3′ and reverse: 5′-CTGAGCCAGGTTTCATGTGA-3′. Primer positions are shown in Fig. 3i relative to the transcription start codon of *Il22*.

Colony-forming units and paracellular permeability. Organs were collected, weighed, and brought into suspension. Bacterial colony-forming units were determined per gram of tissue and total organ. Colony-forming units were determined by serial dilutions on Luria Broth (LB) agar and MacConkey agar (Sigma-Aldrich). Colonies were counted after 2 days of culture at 37 °C. To address intestinal paracellular permeability 16 mg per mouse of Dextran-Fitc (Sigma Aldrich) were administrated by gavage after overnight starvation. Plasma was analysed after 4 hours of Dextran-Fitc administration using a Microplate Reader TECAN Infinity F500.

BrdU administration and Ki-67 labeling. BrdU was administrated by intraperitoneal injection (1.25 mg per mouse). For flow cytometric analysis of epithelial cell proliferation anti-BrdU (Staining Kit for flow Cytometry, eBioscience) and anti-mouse Ki-67 antibody (BioLegend) were used.

Quantitative PCR analysis of bacteria in stool at the phylum level. DNA from faecal pellet samples was isolated with ZR Fecal DNA MicroPrep (Zymo Research). Quantification of bacteria was determined from standard curves established by qPCR. qPCR were performed with Power SYBR Green PCR Master Mix (Applied Biosystems) and different primer sets using a StepOne Plus (Applied Biosystems) thermocycler. Samples were normalized to 16S rDNA and reported according to the $2^{\Delta\Delta Ct}$ method. Primer sequences were: 16S rDNA, forward: 5′-ACTCCTACGGGAGGCAGCAGT-3′ and reverse: 5′-ATTACCGCGGCTGCTGGC-3′; *Firmicutes*, forward: 5′-ACTCCT ACGGGAGGCAGC-3′ and reverse: 5′-GCTTCTTAGTCAGGTACCGTCAT-3′; *Bacteroidetes*, forward: 5′-GGTTCTGAGAGGAGGTCCC-3′ and reverse: 5′-GCTGGCTCCCGTAGGAGT-3′; *Proteobacteria*, forward: 5′-GGTTC TGAGAGGAGGTCCC-3′ and reverse 5′-GCTGGCTCCCGTAGGAGT-3′.

16S rRNA quantification and gene sequencing. Faeces were isolated from co-housed Ret^{fl} or Ret^{Δ} littermates. Sequencing of the 16S *rRNA* gene was performed as previously described[51]. Briefly, barcoded primers were used to amplify the V4 region of the 16S rRNA gene, and the amplicons were sequenced on a MiSeq instrument (Illumina, San Diego, USA) using 150 bp, paired-end chemistry at the University of Pennsylvania Next Generation Sequencing Core. The paired ends were assembled and quality filtered, selecting for reads with a quality score ≥30. Reads with >10 bp homopolymers and reads shorter than 248 bp or longer than 255 bp were removed from the analysis. 16S rRNA sequence data were processed using mothur version 1.25.0 (ref. 52) and QIIME version 1.8 (ref. 53). Chimaeric sequences were removed with ChimeraSlayer[54]. Operational taxonomic units (OTUs) were defined with CD-HIT[55] using 97% sequence similarity as a cut-off. Only OTUs containing ≥2 sequences were retained; OTUs assigned to cyanobacteria or those which were not assigned to any phylum were removed from the analysis. Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier v 2.2 (ref. 56), multiple sequence assignment was performed with PyNAST (v 1.2.2)[57], and FastTree[58] was used to build the phylogeny. Samples were rarified to 22,000 sequences per sample for alpha- and beta-diversity analyses. Taxonomic relative abundances are reported as the median with standard deviation. *P* values were calculated using the Wilcoxon rank-sum test. Statistical tests were conducted in R version 3.2.0. To determine which factors were associated with microbial community composition, statistical tests were performed using the non-parametric analysis of similarities (ANOSIM) with weighted UniFrac distance metrics[59].

Data accession. The sequencing data generated in this study have been submitted to the NCBI Sequence Read Archive under BioProject PRJNA314493 (SRA: http://www.ncbi.nlm.nih.gov/sra/?term=PRJNA314493).

Intestinal organoids. IntestiCult Organoid Growth Medium and Gentle Cell Dissociation Reagent were purchased from StemCell. Intestinal crypts were isolated from C57BL/6J mice according to the manufacturer's instructions and were added to previously thawed, ice-cold Matrigel at a 1:1 ratio and at a final concentration of 5,000–7,000 crypts per ml. 15 μl of this mix was plated per well of a 96-well round-bottom plate. After Matrigel solidification 100 μl of growth medium (100 U ml^{-1} penicillin/streptomycin) was added and replaced every 3 days. Organoids were grown at 37 °C with 5% CO_2 and passaged according to the manufacturer's instructions. Freshly sorted intestinal ILC3 were added to 5–8 days old epithelial organoids after plating for 24 hours with or without anti-mouse IL-22 antibody (R&D Systems).

IL-22 agonist administration *in vivo*. 150 μg of anti-IL-22 antibody (8E11; gift from Genentech) or mouse IgG1 isotype control (MOPC-21; Bio X Cell) was administered by intraperitoneal injection to Ret^{MEN2B} mice every 2 days. Animals were analysed 2 weeks after the first administration.

Neurosphere-derived glial cells. Neurosphere-derived glial cells were obtained as previously described[60]. Briefly, total intestines from E14.5 C57BL/6J and $Myd88^{-/-}$ mice were digested with collagenase D (0.5 mg ml^{-1}; Roche) and DNase I (0.1 mg ml^{-1}; Roche) in DMEM/F-12, GlutaMAX, supplemented with 1% HEPES, streptomycin/penicillin and 0.1% β-mercaptoethanol (Gibco) for approximately 30 min at 37 °C under gentle agitation. Cells were cultured for 1 week in a CO_2 incubator at 37 °C in DMEM/F-12, GlutaMAX, streptomycin and penicillin and 0.1% β-mercaptoethanol (Gibco) supplemented with B27 (Gibco), EGF (Gibco) and FGF2 (Gibco) 20 ng ml^{-1}. After 1 week of culture cells were treated with 0.05% trypsin (Gibco), transferred into PDL (Sigma-Aldrich) coated plates and culture in DMEM supplemented with 10% FBS, 1% HEPES, glutamine, streptomycin and penicillin and 0.1% β-mercaptoethanol (Gibco) until confluence. Glial cells were activated with TLR2 (5 μg ml^{-1}) (Pam3CSK4), TLR3 (100 μg ml^{-1}) (PolyI:C), TLR4 (50 μg ml^{-1}) (LPS), TLR9 (50 μg ml^{-1}) (DsDNA-EC) ligands from Invivogen and IL-1β (10 μg ml^{-1}) (401ML005), IL-18 (50 μg ml^{-1}) (B002-5), IL-33 (0.1 μg ml^{-1}) (3626ML) recombinant proteins from R&D Systems. Cells were also co-cultured with purified ILC3 from wild-type and *Il1b*-deficient mice. IL-22 expression in glial-ILC3 co-cultures upon TLR4 activation was also performed using GDNF (2 μg ml^{-1}) (AB-212–NA), NRTN (2 μg ml^{-1}) (AF-387sp) and ARTN (0.3 μg ml^{-1}) (AF-1085-sp) blocking antibodies. Cells were analysed after 24 hours of co-culture.

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Statistics. Results are shown as mean ± s.e.m. Statistical analysis used Microsoft Excel. Variance was analysed using *F*-test. Student's *t*-test was performed on homoscedastic populations, and Student's *t*-test with Welch correction was applied on samples with different variances. Analysis of survival curves was performed using a MAntel–Cox test. Results were considered significant at $*P \leq 0.05$ and $**P \leq 0.01$. Statistical treatment of metagenomics analysis is described in the Methods section '16S rRNA gene sequencing and analysis'.

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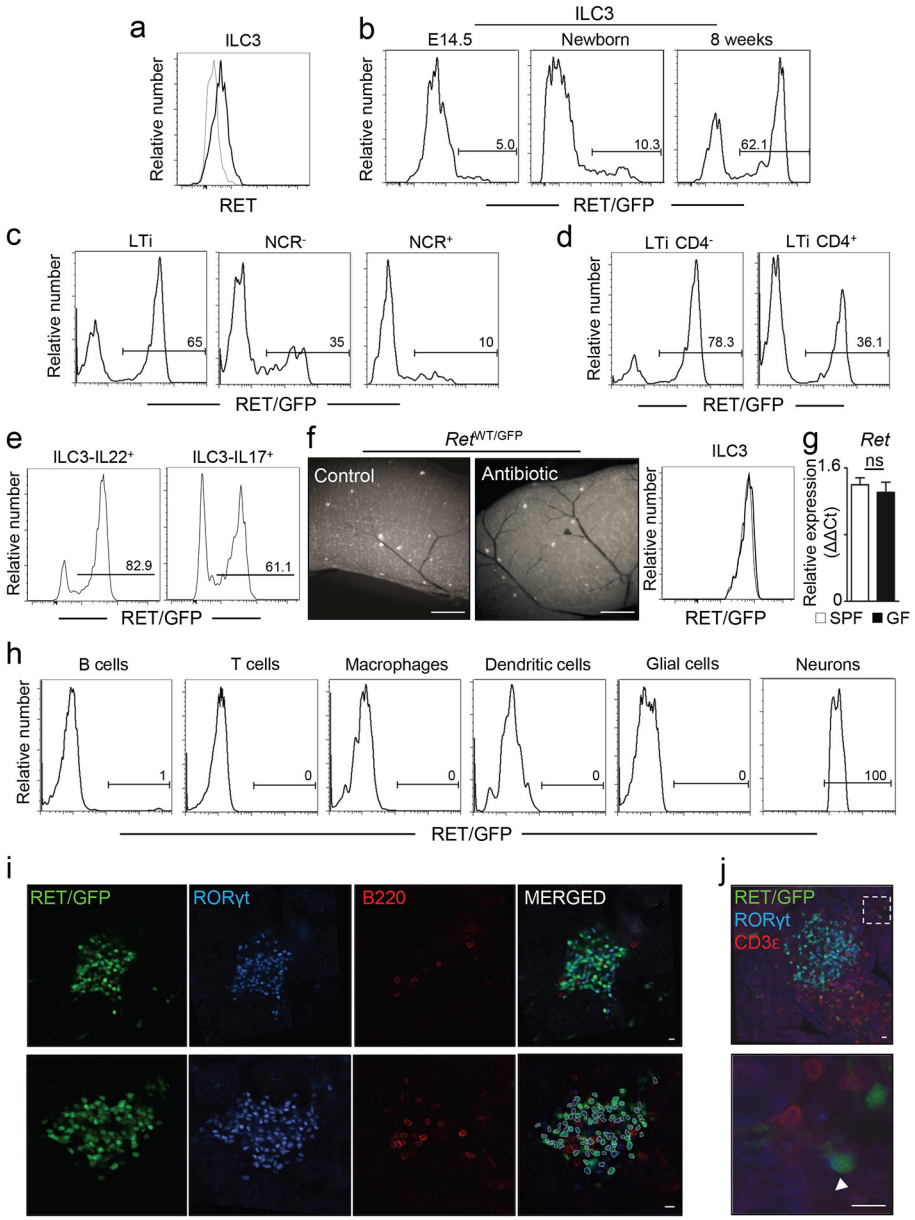

Extended Data Figure 1 | ILC3 selectively express the neurotrophic factor receptor RET. a, Expression of RET protein in gut CD45$^+$Lin$^-$Thy1.2^{hi}IL7Rα$^+$RORγt$^+$ ILC3. **b**, Analysis of gut ILC3 from *Ret*^{GFP} mice. Embryonic day 14.5 (E14.5). **c**, **d**, Analysis of enteric ILC3 subsets from *Ret*^{GFP} mice. **e**, Analysis of cytokine-producing ILC3 from *Ret*^{GFP} mice. **f**, Pregnant *Ret*^{GFP} mice were provided with antibiotic cocktails that were maintained after birth until analysis at 6 weeks of age. Left, RET/GFP (white); right, flow cytometry analysis of RET/GFP expression in ILC3. Thin line, Ab-treated; bold line, specific pathogen free (SPF). **g**, *Ret* expression in enteric ILC3 from germ-free (GF) mice and SPF controls ($n = 4$). **h**, Analysis of lamina propria populations from *Ret*^{GFP} mice. **i**, Enteric ILC3 clusters. Green, RET/GFP; blue, RORγt; red, B220. Bottom, quantification analysis for RET/GFP and RORγt co-expression (79.97 ± 4.72%). **j**, Rare RET-expressing ILC3 in intestinal villi. Green, RET/GFP; blue, RORγt; red, CD3ε. Scale bars, 10μm. Data are representative of 4 independent experiments. Error bars show s.e.m.

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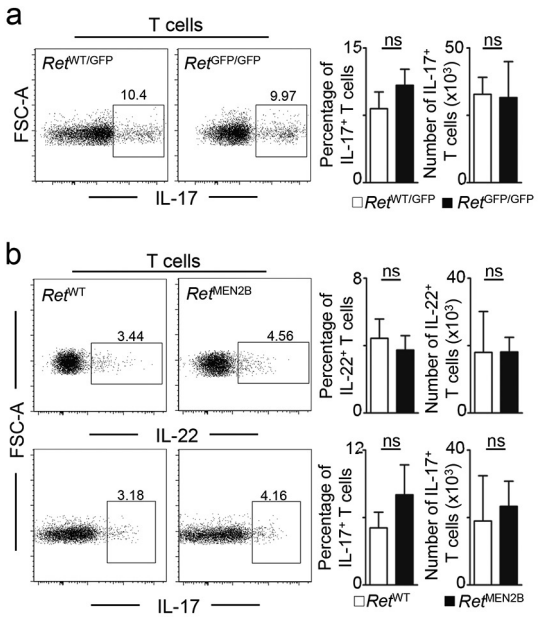

Extended Data Figure 2 | T cell-derived IL-22 and IL-17 in *Ret*^{GFP} chimaeras and *Ret*^{MEN2B} mice. a, T-cell-derived IL-17 in *Ret*^{GFP} chimaeras. *Ret*$^{WT/GFP}$, $n = 25$; *Ret*$^{GFP/GFP}$, $n = 22$. **b**, T-cell-derived IL-22 and IL-17 in the intestine of *Ret*^{MEN2B} mice and their wild-type littermate controls ($n = 7$). Data are representative of 4 independent experiments. Error bars show s.e.m.

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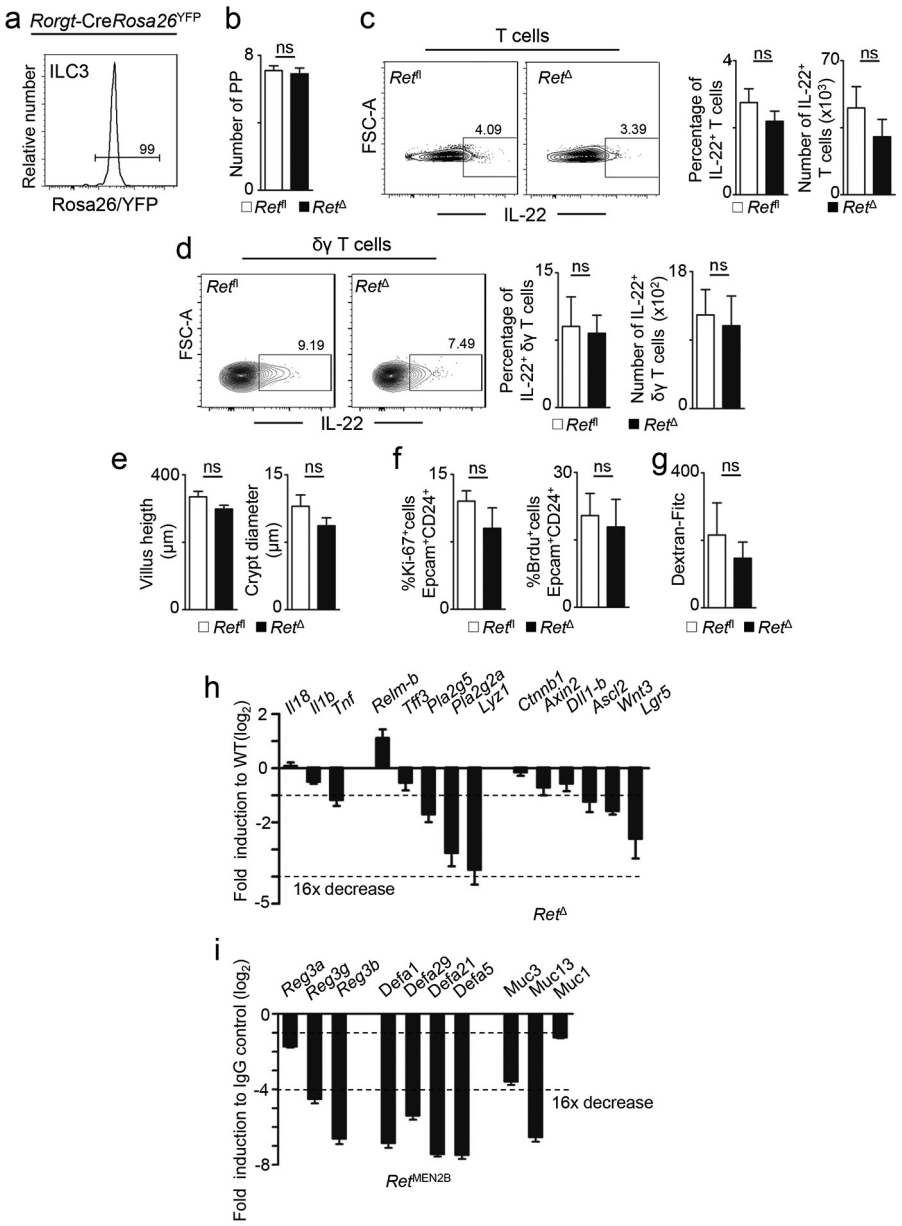

Extended Data Figure 3 | Enteric homeostasis in steady-state *Ret*$^{\Delta}$ mice. **a**, *Rorgt*-Cre mice were bread to *Rosa26*^{YFP} mice. Analysis of Rosa26/YFP expression in gut ILC3 from *Rorgt*-Cre*Rosa26*^{YFP} mice. **b**, Number of Peyer's patches (PP) ($n = 10$). **c**, T-cell-derived IL-22 in *Ret*$^{\Delta}$ mice and their wild-type littermate controls. ($n = 11$). **d**, γδ T-cell-derived IL-22 in *Ret*$^{\Delta}$ mice and their wild-type littermate controls ($n = 4$). **e**, Intestinal villus and crypt morphology ($n = 6$). **f**, Epithelial cell proliferation ($n = 5$). **g**, Intestinal paracellular permeability measured by Dextran-Fitc in the plasma ($n = 5$). **h**, Tissue repair genes in *Ret*$^{\Delta}$ intestinal epithelium in comparison to their wild-type littermate controls ($n = 8$). **i**, Reactivity genes in *Ret*^{MEN2B} mice treated with anti-IL-22 blocking antibodies compared to *Ret*^{MEN2B} intestinal epithelium. *Ret*^{MEN2B}, $n = 4$; *Ret*^{MEN2B} + anti-IL-22, $n = 4$. Data are representative of 3 independent experiments. Error bars show s.e.m.

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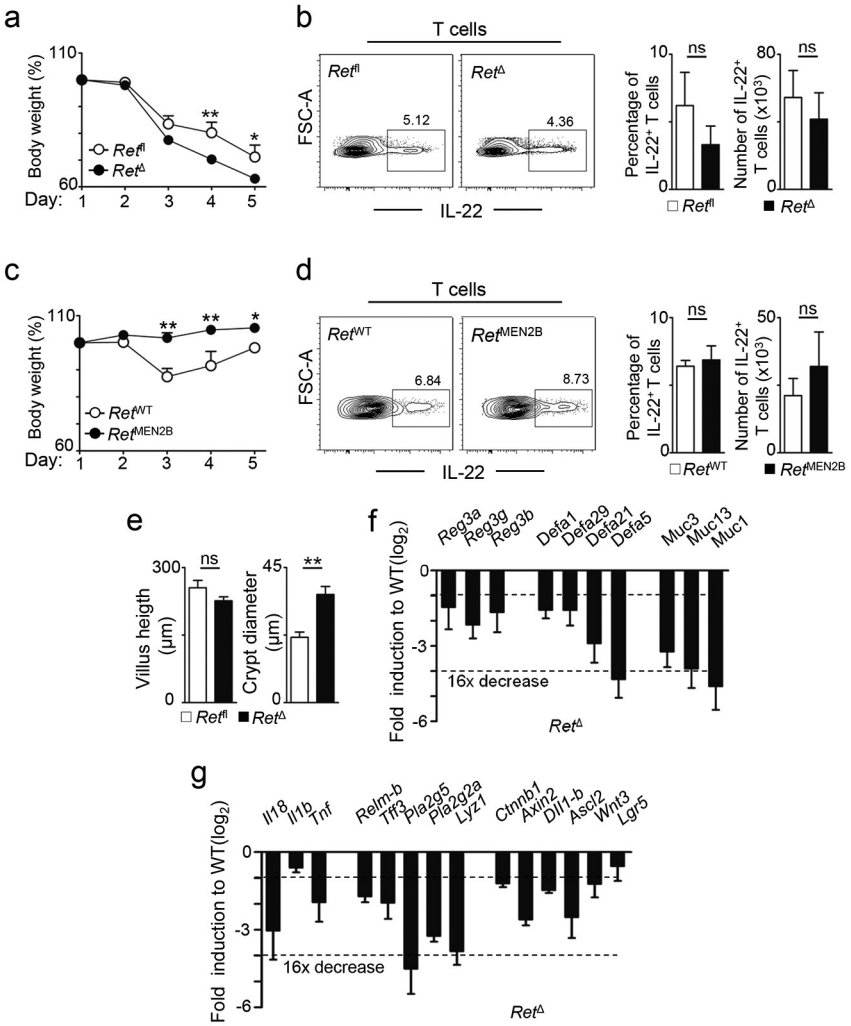

Extended Data Figure 4 | Enteric inflammation in mice with altered RET signals. Mice were treated with DSS in the drinking water. **a**, Weight loss of DSS-treated Ret^{Δ} mice and their littermate controls ($n = 8$). **b**, T-cell-derived IL-22 in Ret^{Δ} mice and their wild-type littermate controls after DSS treatment ($n = 8$). **c**, Weight loss of DSS treated Ret^{MEN2B} mice and their wild-type littermate controls ($n = 8$). **d**, T-cell-derived IL-22 in Ret^{MEN2B} mice and their wild-type littermate controls ($n = 8$). **e**, Intestinal villi and crypt morphology ($n = 6$). **f**, Epithelial reactivity gene expression in DSS treated Ret^{Δ} mice in comparison to their wild-type littermate controls ($n = 8$). **g**, Tissue repair gene expression in DSS treated Ret^{Δ} mice in comparison to their wild-type littermate controls ($n = 4$). Data are representative of 3–4 independent experiments. Error bars show s.e.m. $^{*}P < 0.05$; $^{**}P < 0.01$.

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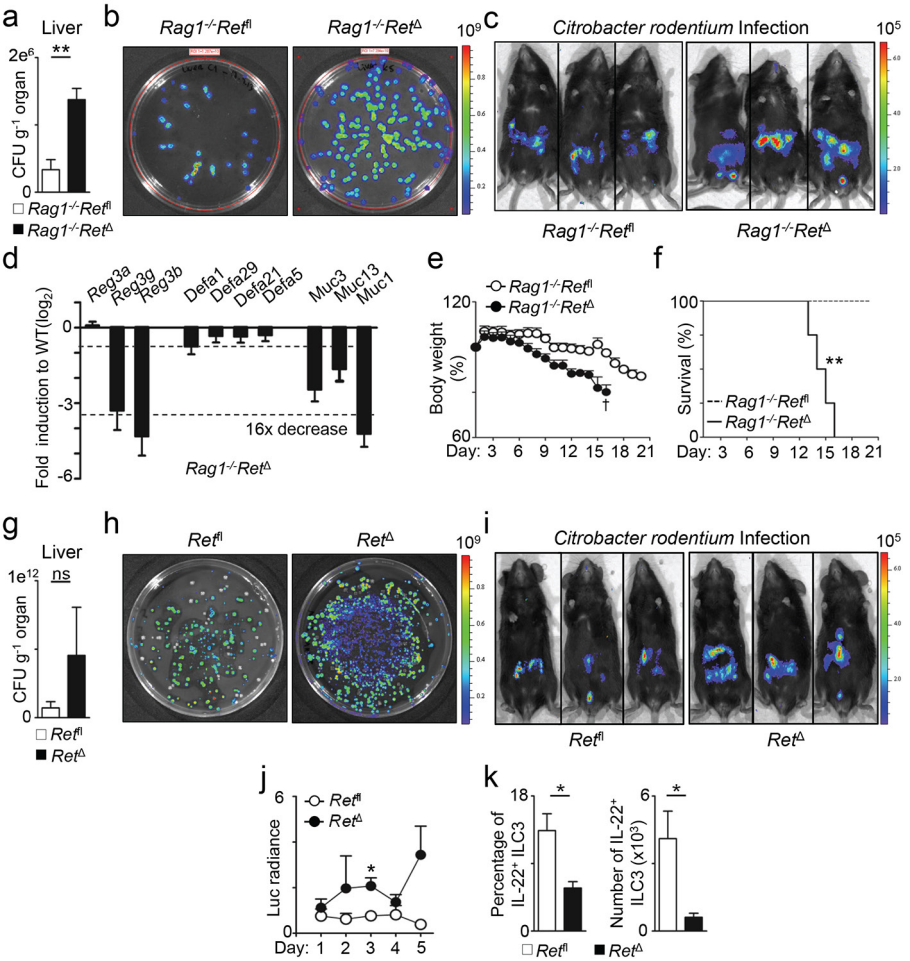

Extended Data Figure 5 | *Citrobacter rodentium* infection in *Ret*$^{\Delta}$ mice. a, *C. rodentium* translocation to the liver of *Rag1*$^{-/-}$*Ret*$^{\Delta}$ and their *Rag1*$^{-/-}$*Ret*^{fl} littermate controls at day 6 after infection ($n = 15$). **b**, MacConkey plates of liver cell suspensions from *Rag1*$^{-/-}$*Ret*$^{\Delta}$ and their *Rag1*$^{-/-}$*Ret*^{fl} littermate controls at day 6 after *C. rodentium* infection. **c**, Whole-body imaging of *Rag1*$^{-/-}$*Ret*$^{\Delta}$ and their *Rag1*$^{-/-}$*Ret*^{fl} littermate controls at day 6 after luciferase-expressing *C. rodentium* infection. **d**, Epithelial reactivity gene expression in *C. rodentium* infected *Rag1*$^{-/-}$*Ret*$^{\Delta}$ mice (n = 17) and their *Rag1*$^{-/-}$*Ret*^{fl} littermate controls ($n = 15$). **e**, Weight loss in *C. rodentium*-infected *Rag1*$^{-/-}$*Ret*$^{\Delta}$ mice and their *Rag1*$^{-/-}$*Ret*^{fl} littermate controls ($n = 8$). **f**, Survival curves in *C. rodentium* infected *Rag1*$^{-/-}$*Ret*$^{\Delta}$ mice and their *Rag1*$^{-/-}$*Ret*^{fl} littermate controls ($n = 8$). **g**, *C. rodentium* translocation to the liver of *Ret*$^{\Delta}$ and their *Ret*^{fl} littermate controls at day 6 after infection ($n = 6$). **h**, MacConkey plates of liver cell suspensions from *Ret*$^{\Delta}$ and their *Ret*^{fl} littermate controls at day 6 after *C. rodentium* infection. **i**, Whole-body imaging of *Ret*$^{\Delta}$ and their *Ret*^{fl} littermate controls at day 6 after luciferase-expressing *C. rodentium* infection. **j**, *C. rodentium* infection burden ($n = 8$). **k**, Innate IL-22 in in *C. rodentium* infected *Ret*$^{\Delta}$ mice and their *Ret*^{fl} littermate controls ($n = 8$). Data are representative of 3–4 independent experiments. Error bars show s.e.m. ns, not significant. $^{*}P < 0.05$; $^{**}P < 0.01$.

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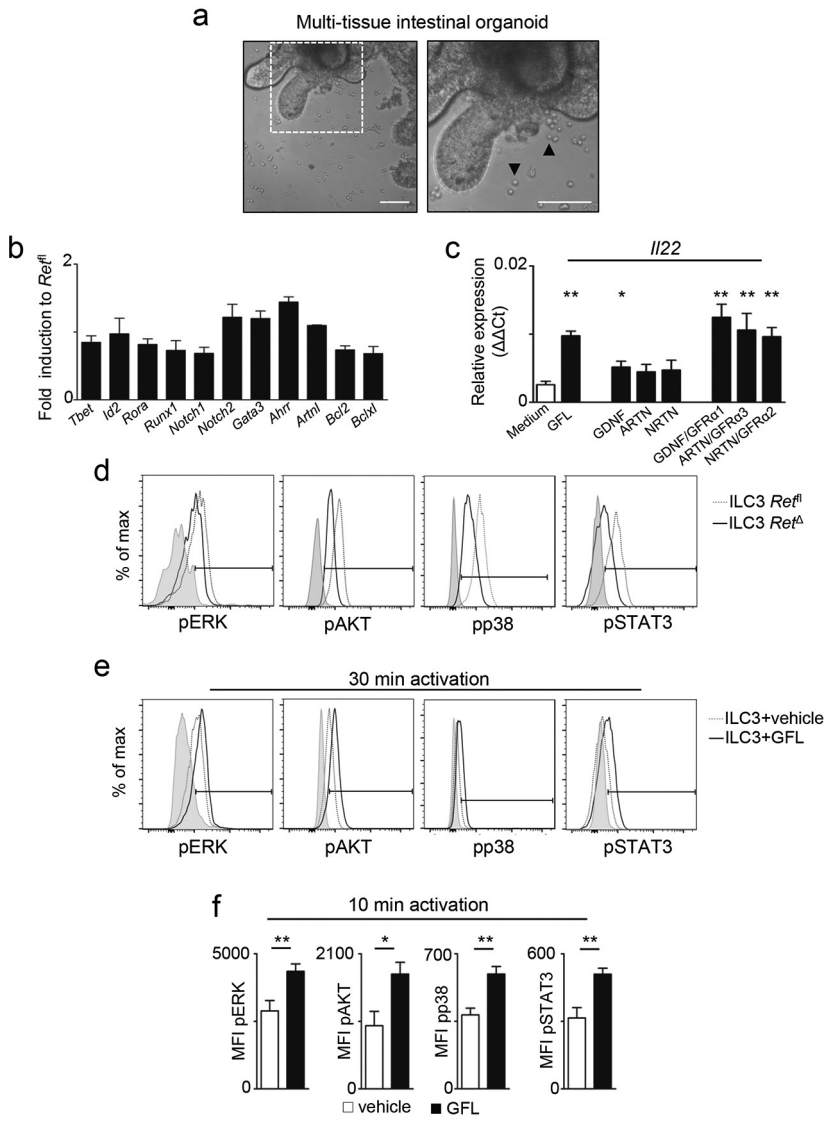

Extended Data Figure 6 | Glial-derived neurotrophic factor family ligand (GFL) signals in ILC3. a, Multi-tissue intestinal organoid system. Scale bar, 20μm. Black arrows, ILC3. **b**, Expression of ILC-related genes in ILC3 from Ret^{Δ} mice in comparison to their littermate controls ($n = 4$). **c**, ILC3 activation with all GFL/GFRα pairs (GFL); single GDNF family ligand (GDNF, ARTN or NRTN); or single GFL/GFRα pairs (GDNF/GFRα1, ARTN/GFRα3 or NRTN/GFRα2) compared to vehicle BSA ($n = 5$). **d**, ILC3 from Ret^{Δ} mice (open black) and their littermate controls (open dash). Isotype (closed grey). **e**, 30-min activation of ILC3 by GFL (open black) compared to vehicle BSA (open dash). Isotype (closed grey). **f**, 10-min activation of ILC3 by GFL. pERK, $n = 8$; pAKT, $n = 8$; phosphorylated p38/MAP kinase, $n = 8$; pSTAT3, $n = 8$. Similar results were obtained in at least 3–4 independent experiments. Error bars show s.e.m. $^{*}P < 0.05$; $^{**}P < 0.01$.

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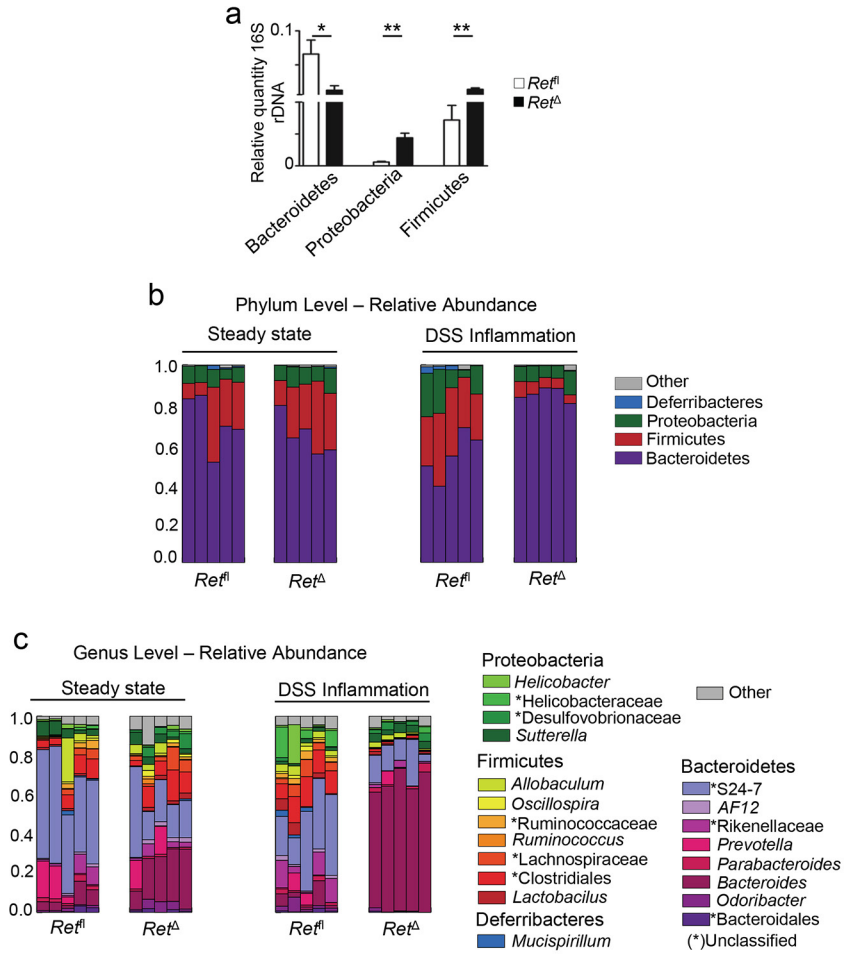

Extended Data Figure 7 | Alterations in the diversity of intestinal commensal bacteria of *Ret*$^{\Delta}$ mice. a, Quantitative PCR analysis at the phylum level in stool bacteria from co-housed *Ret*^{fl} and *Ret*$^{\Delta}$ littermates in steady state ($n = 5$). **b**, Metagenomic phylum level comparisons in stool bacterial from co-housed *Ret*^{fl} and *Ret*$^{\Delta}$ littermates in steady state (left) and after DSS treatment (right) ($n = 5$). **c**, Genus-level comparisons in stool bacteria from co-housed *Ret*^{fl} and *Ret*$^{\Delta}$ littermates in steady state (left) and after DSS treatment (right) ($n = 5$). Error bars show s.e.m. $^{*}P < 0.05$; $^{**}P < 0.01$.

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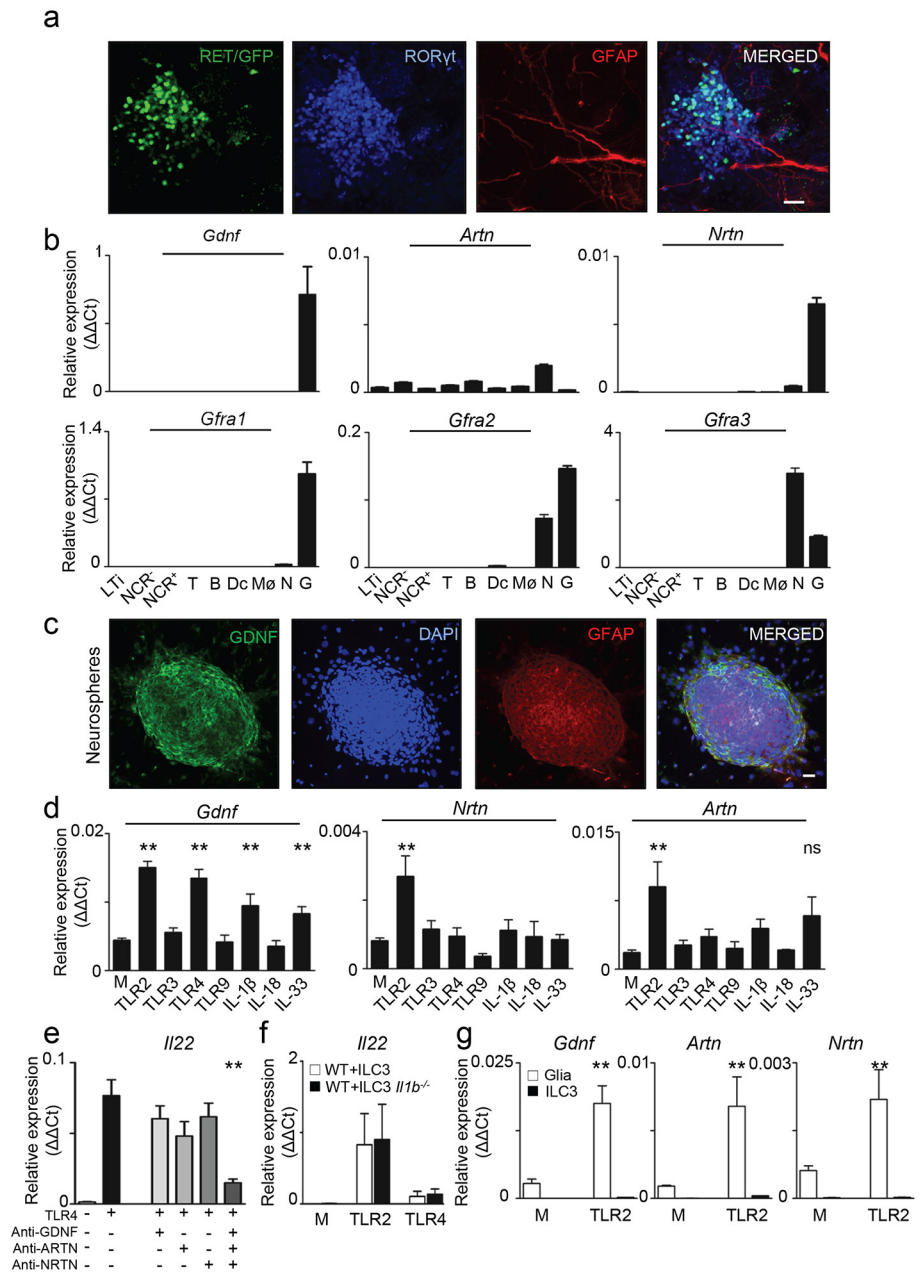

Extended Data Figure 8 | GFL-expressing glial cells anatomically co-localize with ILC3. a, Intestine of *Ret*^{GFP} mice. Green, RET/GFP; red, GFAP; blue, RORγt. Similar results were obtained in 3 independent experiments. **b**, Purified lamina propria LTi, NCR^{-} and NCR^{+} ILC3 subsets, T cells (T), B cells (B), dendritic cells (Dc), macrophages (Mø), enteric neurons (N) and mucosal glial cells (G). **c**, Neurosphere-derived glial cells. **d**, Activation of neurosphere-derived glial cells with TLR2 (Pam3CSK4), TLR3 (Poli I:C), TLR4 (LPS) and TLR9 (DsDNA-EC) ligands, as well as IL-1β, IL-18 and IL-33 ($n = 6$). M, medium. **e**, *Il22* in co-cultures of glial and ILC3 using single or combined GFL antagonists ($n = 6$). **f**, *Il22* in co-cultures of ILC3 and glial cells from *Il1b*$^{-/-}$ or their wild-type controls ($n = 3$). **g**, *Gdnf, Artn* and *Nrtn* expression in glial cells and ILC3 upon TLR2 stimulation ($n = 3$). Scale bar, 30 μm. Similar results were obtained in at least 4 independent experiments.

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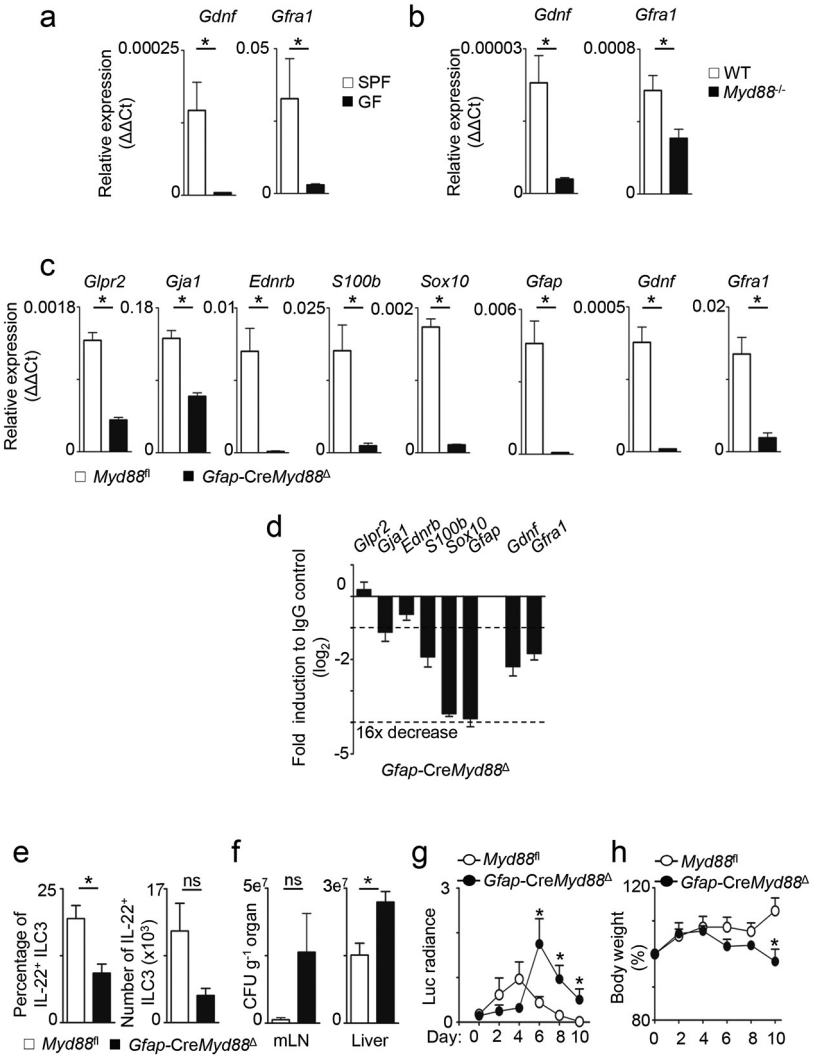

Extended Data Figure 9 | Glial cell sensing via MYD88 signals. **a**–**c**, Intestinal glial cells were purified by flow cytometry. **a**, Germ-free and their respective SPF controls ($n = 3$). **b**, Myd88$^{-/-}$ and their respective wild-type littermate controls ($n = 3$). **c**, *Gfap*-Cre*Myd88*$^{\Delta}$ and their littermate controls (*Myd88*^{fl}) ($n = 3$). **d**, Total lamina propria cells of *Gfap*-Cre*Myd88*$^{\Delta}$ and their littermate controls (*Myd88*^{fl}) ($n = 6$). **e**–**h**, *Citrobacter rodentium* infection of *Gfap*-Cre*Myd88*$^{\Delta}$ mice and their littermate controls (*Myd88*^{fl}) ($n = 6$). **e**, Innate IL-22. **f**, *Citrobacter rodentium* translocation. **g**, Infection burden. **h**, Weight loss. Data are representative of 3 independent experiments. Error bars show s.e.m. $^{*}P < 0.05$; $^{**}P < 0.01$.

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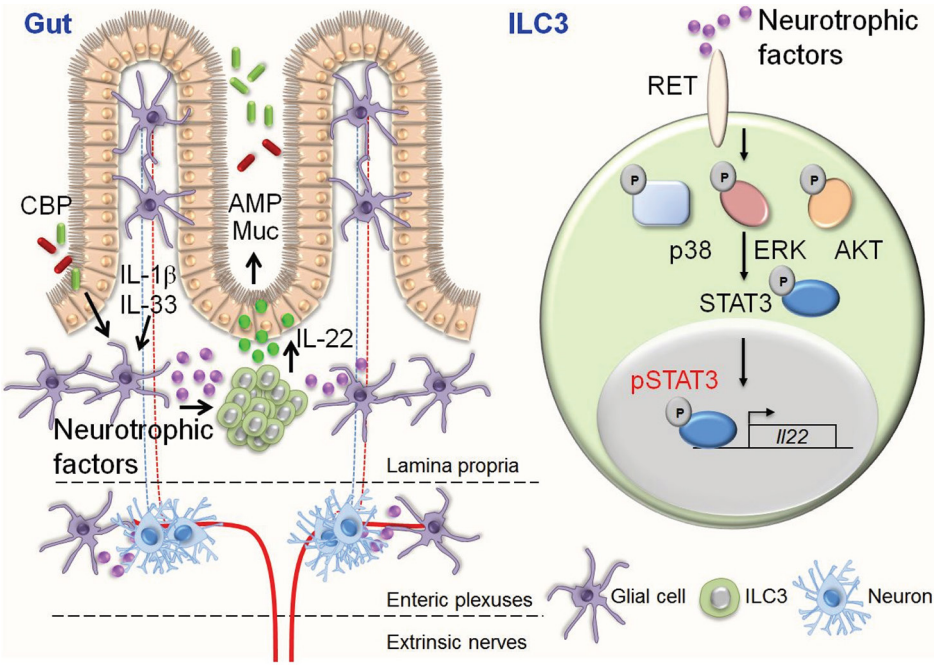

Extended Data Figure 10 | A novel glial-ILC3-epithelial cell unit orchestrated by neurotrophic factors. Lamina propria glial cells sense microenvironmental products that control neurotrophic factor expression. Glial-derived neurotrophic factors operate in an ILC3-intrinsic manner by activating the tyrosine kinase RET, which directly regulates innate IL-22 downstream of a p38 MAPK/ERK-AKT cascade and STAT3 phosphorylation. GFL induced innate IL-22 acts on epithelial cells to induce reactivity gene expression (CBP, commensal bacterial products; AMP, antimicrobial peptides; Muc, mucins). Thus, neurotrophic factors are the molecular link between glial cell sensing, innate IL-22 production and intestinal epithelial barrier defence.

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