Paneth cells as the cornerstones of intestinal and organismal health: a primer

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Abstract

Paneth cells are versatile secretory cells located in the crypts of Lieberkühn of the small intestine. In normal conditions, they function as the cornerstones of intestinal health by preserving homeostasis. They perform this function by providing niche factors to the intestinal stem cell compartment, regulating the composition of the microbiome through the production and secretion of antimicrobial peptides, performing phagocytosis and efferocytosis, taking up heavy metals, and preserving barrier integrity. Disturbances in one or more of these functions can lead to intestinal as well as systemic inflammatory and infectious diseases. This review discusses the multiple functions of Paneth cells, and the mechanisms and consequences of Paneth cell dysfunction. It also provides an overview of the tools available for studying Paneth cells.

Keywords paneth cells; gut homeostasis; infection; antimicrobial peptides
Subject Category Digestive System
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See the Glossary for abbreviations used in this article.

Introduction and definition of Paneth cells

Paneth cells (PCs) are found in the crypts of the small intestine of many mammals (Lueschow & McElroy, 2020). They are highly secretory cells with a lifespan of about 2 months (Ireland *et al*, 2005). Their secretory function is hallmarked by a pyramidal-shaped morphology, abundant endoplasmic reticulum (ER), well-developed Golgi network and apically oriented secretory granules. These granules accumulate antimicrobial peptides and proteins (AMPs), enzymes and growth factors, many of which are crucial in host defense (Ayabe *et al*, 2000). Hence, PCs might control the composition of the enteric microbiota and maintain gut homeostasis. Alterations in PCs or PC dysfunction contribute to several diseases, for example, graft-versus-host disease (GVHD) and inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (Levine *et al*, 2013; Deuring *et al*, 2014). Understanding PCs and expanding the tools to study them is therefore of utmost importance. This review focusses on their biological functions, how they can be modulated by environmental conditions and disease, and the tools available to study them.

PC ontogeny

The enteric epithelium consists of a monolayer of columnar cells with diverse functions and plays a crucial role in metabolism, preserving intestinal homeostasis, and defense of the body (Ali *et al*, 2020). Its architecture is composed of upright villi interspersed by the crypts of Lieberkühn. The epithelium of the small intestine consists of five main cell types differentiated from Lgr5+ intestinal stem cells (ISCs): enterocytes, goblet cells, enteroendocrine cells, tuft cells, and PCs (Van Der Flier & Clevers, 2009). Due to the daily challenge of food components and bacteria, the epithelium is constantly renewed to maintain homeostasis and preserve the integrity of the intestinal lining (Barker *et al*, 2012). ISCs are responsible for the rapid renewal and replenishment of the epithelium by forming progenitor cells that can differentiate further. The major part of differentiated cells moves upwards in the villus, with a lifespan of 3–5 days, and are then shed into the lumen at the top of the villus, where they die by a process called anoikis (Cheng & Leblond, 1974). However, PCs move downwards into the crypts of Lieberkühn, where they exert versatile functions, including ISC support. In some mammals, such as humans and mice, PCs are abundant and easy to observe, but in other animals (e.g., pigs), their existence is controversial (Myer, 1982; van der Hee *et al*, 2018).

PC formation and differentiation

ISCs in the lower part of the crypts give rise to a large pool of transit-amplifying cells. The most important regulators of ISC activity are Wnt, bone morphogenetic protein (BMP), Notch, and epidermal growth factor (EGF) signaling pathways. These factors are gradually expressed along the villi and crypts (Malijauskaite *et al*, 2021). Transit-amplifying cells remain 2 days in the transit-amplifying zone (higher part of the crypts), where they multiply and mature into differentiated intestinal epithelial cells (IECs, Fig 1) (Van Der Flier & Clevers, 2009).

The PC differentiation is initially controlled by Notch signaling. Progenitor cells that are deficient in Notch receptor express atonal

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Glossary

PCs
Paneth cells are secretory cells located in the crypts of Lieberkühn, adjacent to the intestinal stem cells. They produce antimicrobial peptides and proteins and other components that are important in host defense and immunity.
α-defensins
Enteric α-defensins are antimicrobial peptides stored in the secretory granules of PCs. They are responsible for most of the antimicrobial activity of PCs.
LYZ
Lysozyme is the first antimicrobial peptide discovered in PCs and is widely used as a PC marker in the small intestine.
Wnt/β-catenin signaling pathway
Wingless-related integration site (Wnt)/β-catenin signaling pathway is a signal transduction pathway regulating intestinal stem cell self-renewal and differentiation. The Wnt/β-catenin pathway is most active at the intestinal crypt base.
Notch signaling
Notch signaling in the gut directs the differentiation of progenitor cells into absorptive cells (enterocytes) by inhibiting secretory cell differentiation (Goblet cells, enteroendocrine cells, tuft cells, and PCs) *via* the expression of the transcription factor Hairy and enhancer of split 1 (HES1).
miRNA
microRNA is a small single-stranded non-coding RNA molecule that regulates gene expression on a post-transcriptional level.
mTORC1
Mammalian target of rapamycin complex 1 is a serine/threonine protein kinase and serves as a sensor for a wide range of environmental factors, for example, nutrient availability. The protein responds to environmental triggers by adapting transcription, translation and autophagy, and can thereby regulate several cellular processes.
Autophagy
Autophagy is a process by which a cell removes damaged or unnecessary components via lysosome-dependent degradation. It is a fundamental cell survival mechanism contributing to the mobilization of cellular energy stores and is thus critical for maintaining the homeostasis of cells.
Endoplasmic reticulum
Endoplasmic reticulum is an intracellular organelle that plays a key role in, for example, folding, modifying, and sorting newly synthesized proteins and the synthesis of cellular lipids.
ER stress
Endoplasmic reticulum stress is induced by the accumulation of unfolded or misfolded proteins. Cells activate signaling pathways (unfolded protein response; UPR) to deal with ER stress.
Mucus
The mucus layer in the intestinal epithelium forms a physical and immunological barrier to protect the epithelium from infiltration of microorganisms and other components.

BHLH transcription factor 1 (ATOH1), the essential driver of secretory cell differentiation (Yang *et al*, 2001). Progenitor cells with high Notch activity induce HES1, a negative regulator of ATOH1, making them prone to differentiate into absorbing enterocytes. Growth factor independent 1 transcriptional repressor (GFI1) acts downstream of ATOH1 to select for differentiation of goblet cells and PCs, as it transcriptionally represses differentiation towards enteroendocrine cells by repressing the transcription factor neurogenin 3 (Bjerknes & Cheng, 2010).

Thus, factors that affect ATOH1 influence the differentiation towards PCs (and other secretory cells). $PKC_{\lambda/I}$ is such a factor, as it destabilizes EZH2, an ATOH1 suppressor co-localized with PC markers in the crypts. ${PKC_{\lambda/I}}^{\Delta IEC}$ mice (lacking *Prkci*, which encodes $PKC_{\lambda/I}$, specifically in the intestinal epithelium) had reduced ATOH1 and GFI1 crypt levels, and less mature PCs, along with an increase in an intermediate cell-type positive for both PC and goblet cell markers (Nakanishi *et al*, 2016). Erb-b2 receptor tyrosine kinase 3 (ErbB3) and serine/threonine kinase 11 (STK11) block ATOH1 *via* the PI3K–Akt pathway and PDK4, respectively, suppressing PC differentiation. Interestingly, ErbB3 knockout (KO) mice (*ErbB3*^{KO}) had increased lysozyme (LYZ, encoded in human by *LYZ*, and in mice by *Lyz1*) expressing PCs but no change in other secretory cells (Almohazey *et al*, 2017).

ATOH1/GFI1-positive precursor cells can further differentiate towards PCs, a process that is not fully understood but strongly depends on (1) transcription factors and downstream effectors of Wnt/β-catenin signaling and (2) factors that can influence Wnt/β-catenin signaling (van Es *et al*, 2005; Van Der Flier & Clevers, 2009; Grinat *et al*, 2022).

1 Active Wnt signaling promotes PC differentiation. Wnt activation causes stabilization and translocation of β-catenin to the nucleus, where it interacts with several T cell factor (TCF) molecules to induce a unique differentiation profile. TCF4 is a regulator of PC maturation and induces a PC gene program in the embryonic intestine of mice (van Es *et al*, 2005). SRY-Box transcription factor 9 (SOX9) and SAM-pointed domain containing ETS transcription factor (SPDEF) are involved in PC and goblet cells differentiation (Bastide *et al*, 2007; Gregorieff *et al*, 2009). The unique positioning of PCs towards the crypts is tightly regulated by the Wnt target ephrin receptor B3 (EphB3). Pathways downstream of ATOH1 often affect both goblet cells and PCs, and further research is needed to distinguish their differentiation. However, it is known that persistently active Wnt signaling in the secretory progenitors favors PC differentiation (van Es *et al*, 2005).
2 Spherocytosis 2 protein (SPH2), ras-related protein (RAB8A) and Krüppel-like factor 5 (KLF5) can influence Wnt/β-catenin signaling. Ablation of SPH2 reduces ERK1/2-MAPK signaling, corresponding with higher Wnt β-catenin/TCF4 signaling and differentiation towards PCs (Heuberger *et al*, 2014). Ablation of RAB8A, a protein involved in Wnt secretion, reduces Wnt signaling and PC number (Das *et al*, 2015). Deletion of the transcription factor *Klf5* reduces PC and goblet cell numbers (Nandan *et al*, 2014). Also, miRNAs can influence PC function and differentiation; for example, miRNA-802 represses TMED9, a stimulator of Wnt and LYZ/α-defensins secretion in PCs (Goga *et al*, 2021). Besides, the epigenetic factor Mll1 can influence the differentiation towards secretory cells in a dual way. Mll1 is involved in keeping stemness and preventing differentiation but also has a role in determining PC and goblet cell fate by coordinating Wnt and MAPK signaling in the progenitor cells. *Mll1*^{KO} mice had increased secretory cells, but impaired PCs and goblet cell specification, as these cells were positive for both PC and goblet cell markers (Grinat *et al*, 2022).

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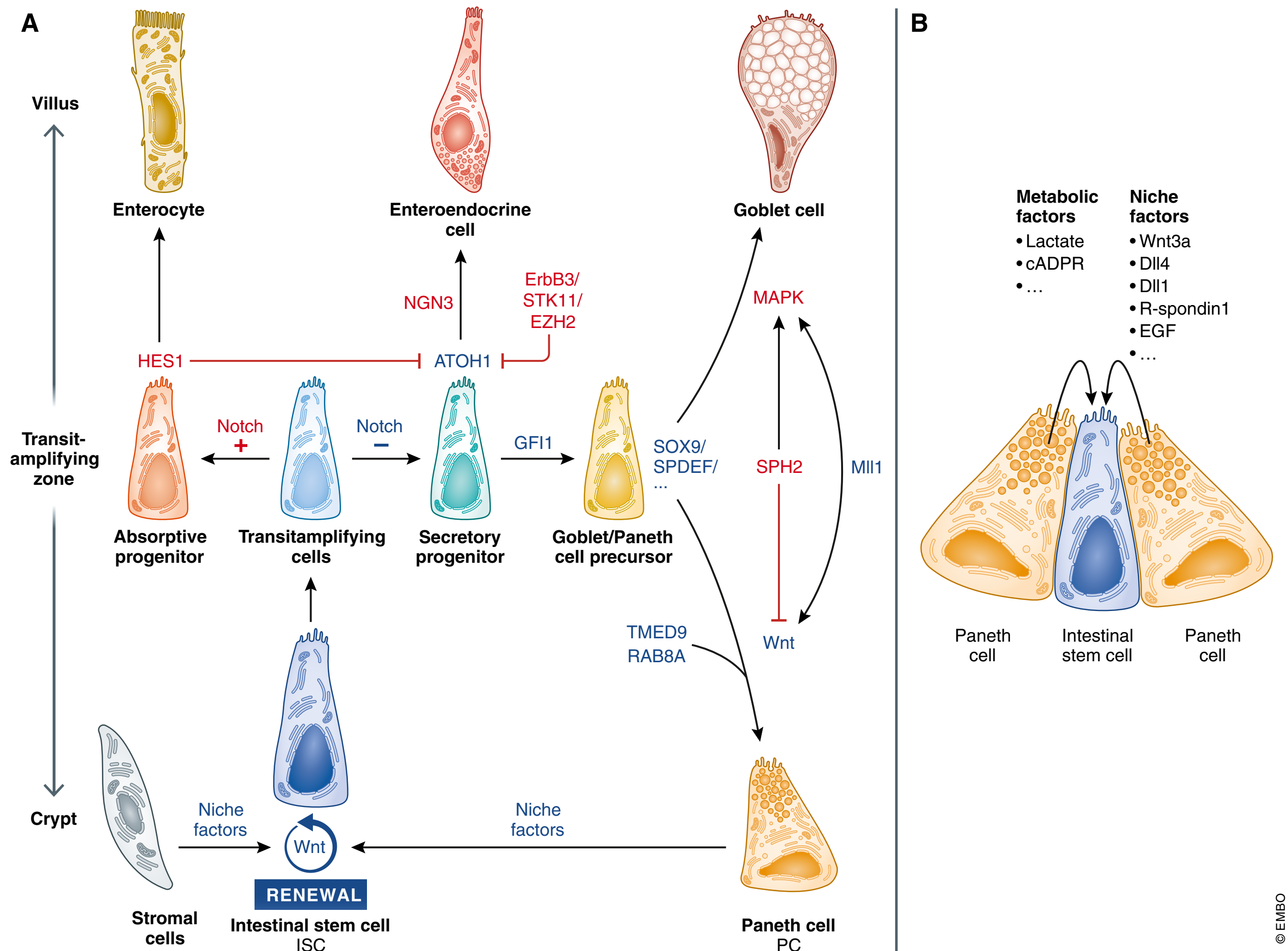

Figure 1. Intestinal cell differentiation pathways and signals.

(A) Wnt and Notch signals control ISC renewal and differentiation. PCs and for example, pericryptal stromal cells support the stemness of ISCs by providing niche factors. ISCs in the lower part of the crypt give rise to a larger pool of transit-amplifying cells in the transit-amplifying zone. Differentiation from transit-amplifying cells towards secretory or absorptive progenitors is initially controlled by Notch signaling. Cells that receive Notch signals express HES1, a negative regulator of ATOH1, leading to differentiation into absorbing enterocytes. Cells devoid of Notch express ATOH1, a hallmark of secretory cells. GFI1 acts downstream of ATOH1 to select for goblet cell and PC differentiation, as it represses neurogenin 3 (NGN3), a transcription factor involved in enteroendocrine cell differentiation. ErbB3, STK11, and EZH2 influence PC differentiation by reducing ATOH1 levels and affect the numbers and/or location of mature PCs. SOX9 and SPDEF are involved in PC and goblet cell differentiation. Reduced MAPK signaling and increased Wnt signaling (influenced by Wnt regulating factors, e.g., RAB8A, TMED9, SPH2, and Mll1) favor differentiation into PCs instead of goblet cells. (B) PCs support ISCs by providing niche and metabolic factors. Blue – factors that promote PC differentiation, red – factors that inhibit PC differentiation.

Paneth cell differentiation is a complex interplay between different factors. Reduced Notch, strong Wnt, and weak MAPK signaling in progenitor cells promote PC differentiation. Notably, most research on PC differentiation and ISC niche has been performed in mice, but there can be differences between species, for example, there are important differences in niche factors needed in human and mouse organoids (Sato *et al*, 2011b).

PC numbers

Paneth cells increase in the proximal to distal direction of the small intestine, which corresponds with increased bactericidal activity (Nakamura *et al*, 2020). The number of PCs per crypt in mice vary between strains and the techniques used but are estimated at 5–16 PCs per crypt (Nakamura *et al*, 2020). Inbred strains under identical housing conditions differ in the number of PCs and the expression of AMPs. For example, 129/SvEv mice have fewer PCs than C57BL/6J mice (Gulati *et al*, 2012). This identifies a critical role of the host genotype in PC activity and the intestinal microbiota. Such differences might be of interest to search for modifier genes that influence PC numbers and activities, but such inbred strains have been established as homozygous lines and kept in captivity over many decades. This environmental pressure may have led to genetic drifts that have little relevance to natural environments. PC studies on wild mice

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and wildling mice might be more relevant (but also more complex) than inbred lines kept in captivity.

PC functions in physiology and homeostasis

The best-known function of PCs is controlling the microbiome composition, but they have much broader functions. PCs can support and communicate with ISCs, they are involved in metal uptake, and can perform phagocytosis, efferocytosis and preserve barrier function.

PCs and the ISC niche: support and communication

The unique crypt morphology of terminally differentiated PCs interspersed between pluripotent ISCs indicates the interactivity of these two crypt cells. The study of developmental issues in villus–crypt proliferation and differentiation led to insights into ISC maintenance and plasticity. One insight is that PCs produce essential niche signals for ISCs: Wnt family member 3A (Wnt3a), EGF, R-spondin 1, Notch ligands (Dll4 and Dll1), and transforming growth factor α (TGFα) (Sato *et al*, 2011a). PCs' support of ISC stemness, and ISCs' differentiation into PCs illustrate their strong interdependence (Mei *et al*, 2020). *In vivo* PC ablation in three genetic mouse models (CR2-tox176 mice, *Gfi1* mutation, and conditional deletion of *Sox9*) leads to similar losses in ISCs (Sato *et al*, 2011a). The dependency of ISC stemness on PCs has also been shown *in vitro*, as the absence of PCs largely prevents organoid formation from ISCs. However, the effect on ISCs seems to depend on the PC-ablation model: loss of *Atoh1* in the gut induced PC ablation but did not affect the number of ISCs (Durand *et al*, 2012). Some studies indicated a role for pericryptal stromal cells in providing niche factors (Durand *et al*, 2012; McCarthy *et al*, 2020). Moreover, PC ablation by using a diphtheria toxin receptor gene inserted into the *Lyz1* locus led to the appearance of alternative niche cells (enteroendocrine and tuft cells) that provided niche factors to ISCs (Van Es *et al*, 2019).

Certain bacterial communities (such as *Bifidobacterium* spp. and *Lactobacillus* spp.) can communicate with ISCs *via* PCs (Lee *et al*, 2018). They produce lactic acid in the lumen, which binds to the recently discovered lactate G-protein-coupled receptor (Gpr81, encoded by *Hcar1*) on PCs. This interaction triggers PCs to increase *Wnt3a* expression and then stimulates Wnt signaling in ISCs (Lee *et al*, 2018). PCs can also rely on their glycolytic metabolism to produce lactate, which is then excreted and passed to ISCs. This lactate is oxidized by ISCs to pyruvate and serves as a fuel for the TCA cycle to generate ATP and reactive oxygen species (ROS). The latter is required to keep up high levels of phosphorylated P38, a MAP kinase important for regulation of ISCs self-renewal, differentiation, and crypt formation (Rodríguez-Colman *et al*, 2017). PCs can also serve as sensors of the nutritional status of the organism and communicate it to adjacent ISCs *via* mTORC1. Caloric restriction attenuates mTORC1 signaling in PCs, followed by increased bone stromal antigen (Bst1) levels. Bst1 is an ectoenzyme that converts NAD+ to cyclic ADP ribose, a paracrine product that promotes ISC self-renewal, while reducing the pool of more differentiated progenitor cells (Yilmaz *et al*, 2012). Moreover, in pathological conditions, PCs can de-differentiate into ISCs (Schmitt *et al*, 2018). PC–ISC interactions have been reviewed (Mei *et al*, 2020) and will not be discussed much in this review.

Controlling microbiome composition

Paneth cells are the main producers of AMPs in the gastrointestinal tract, making them key players in sensing and controlling the microbiome composition. Thereby, they can preserve homeostasis and prevent bacteria from crossing the intestinal barrier. PCs produce a unique repertoire of AMPs in the gut, for example, LYZ, α-defensins (called cryptdins in mice) and cryptdin-related sequence peptides. AMPs are stored in secretory granules and released at the apex of PCs into the crypt lumen. In addition, PCs also produce AMPs that are not restricted to PCs in the gastrointestinal tract, for example, Regenerating islet-derived protein 3 gamma (Reg3γ), secretory phospholipase A2 IIA ($sPLA_2$ IIA), and Angiogenin-4. A review elegantly describes the general intestinal AMPs in detail (Bevins & Salzman, 2011), so this review will focus on the AMPs that are exclusively produced by PCs in the gut.

Action mechanism of PC-specific AMPs in the gut

LYZ was the first AMP discovered in PCs and is widely used as a PC marker in the ileum (Haber *et al*, 2018). The most abundant AMPs in PCs are α-defensins, which are also produced by some myeloid-derived cells (Selsted & Ouellette, 1995). The structures, action mechanisms, and functions of both AMPs are listed in Table 1. The cryptdin-related sequence peptides share similarities with α-defensin in their prosegment but the amount and positioning of the cysteines in the mature part are different.

In mice, the MGI Genome Browser reports 43 annotated α-defensin genes, nine cryptdin-related sequence genes, and 18 α-defensin pseudogenes (Table 2). Genetic differences in α-defensins exist among mouse strains, which can affect PC studies in specific mouse backgrounds. Despite the many annotated genes in mice, not all are found at the protein level (Shanahan *et al*, 2011). The human genome encodes 10 α-defensins but produces only two enteric α-defensins (HD5 and HD6) (Patil *et al*, 2005). However, humans express diverse neutrophilic α-defensins, which is not the case in mice (Shanahan *et al*, 2011). Human HD6 differs from other α-defensins (e.g., cryptdins and HD5) by having an extra antimicrobial function, that is, the formation of self-assembled peptide nano-nets to capture bacteria (Chu *et al*, 2012; Schroeder *et al*, 2015).

The α-defensins are produced as pre-pro-peptides. First, they lose their signal peptide while moving from the ER into the secretory vesicles. Then, proteolytic cleavage turns the pro-defensin into an active α-defensin. In humans, this proteolytic maturation is executed by trypsin, which is stored as a proenzyme (trypsinogen) in the PCs and activated after or during secretion (Ghosh *et al*, 2002). In mice, proteolytic maturation is performed by matrix metalloproteinase 7 (MMP7, also known as matrilysin) (Wielockx *et al*, 2004).

Wilson *et al* (1999) reported that full-body $MMP7^{KO}$ mice do not perform terminal maturation (proteolysis) of pro-defensins in PCs. Hence, $MMP7^{KO}$ mice could be used as a mouse model without biologically active α-defensins in PCs. Although the microbiota of these mice were shifted, these mice were healthy. However, they are less able to control infection with *Salmonella typhimurium*. Also, PC-specific overexpression in mice of human α-defensin 5 (*HD5*, considered human ortholog of the mouse α-defensin genes) led to

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Table 1. The structure, mechanisms, and functions of lysozyme and α-defensins.

Lysozyme	Structure and antimicrobial activity	β-1,4-N-acetylmuramoylhydrolase: Glycosidase responsible for enzymatic hydrolysis of peptidoglycans. This causes instability in the cell wall particularly of Gram-positive bacteria	Ragland & Criss (2017)
		Cationic protein: The cationic structure leads to electrostatic interaction with phospholipids in the bacterial membrane. This results in the formation of pores in the membranes of both Gram-positive and Gram-negative bacteria, and subsequent bacterial death	Derde *et al* (2013), Ragland & Criss (2017)
	Other activities	It also has antiviral, antineoplastic and as antioxidant properties	Sava *et al* (1989), Croguennec *et al* (2000), Małaczewska *et al* (2019)
α-Defensins	Structure and antimicrobial activity	Mature peptide consisting of six conserved cysteines forming three intramolecular disulfide bonds stabilizing a β-sheet structure	Selsted & Ouellette (1995)
		The mature peptide is cationic and amphiphilic, leading to electrostatic interaction with phospholipids in the bacterial membrane. This results in the formation of membrane pores in both Gram-positive and Gram-negative bacteria, and subsequent bacterial death	Hadjicharalambous *et al* (2005)
	Other activities	It also has antiviral, antifungal and antiprotozoal activities	Daher *et al* (1986), Aley *et al* (1994), Kai-Larsen *et al* (2007)

microbial shifts and protection against *S. typhimurium* challenge (Salzman *et al*, 2003). These data reflect the importance of α-defensins in dealing with foreign bacterial invasions in the gastrointestinal tract (Wilson *et al*, 1999).

Bacterial signaling in PCs

The mechanism of bacterial sensing and signaling in PCs is incompletely understood. Yet, several pathways link bacterial signaling with increased expression and maturation of different AMPs (Fig 2). PCs might sense bacteria directly *via* myeloid differentiation primary response 88 (MYD88) and nucleotide-binding oligomerization domain containing 2 (NOD2), which trigger the expression of a different subset of AMPs (Vaishnava *et al*, 2008; Dessein *et al*, 2009). But they might also be sensed indirectly by PCs, for example, *via* the MYD88 pathway in dendritic cells (Bel *et al*, 2017).

TLR/MYD88-dependent bacterial signaling In mice with genetic MYD88 deficiency, expression of several AMPs in PCs is reduced (*Reg3*γ, *Reg3*β, *CRP-ductin*, and *RELM*) and bacterial translocation is increased (Vaishnava *et al*, 2008). Rescue of MYD88 expression only in PCs (by Defa2-MyD88 transgenic expression in MYD88^{KO} mice) rescued this phenotype. These elegant experiments illustrated that (1) intestinal bacteria can communicate directly with PCs *via* cell-autonomous MYD88 signaling and (2) the MYD88-dependent antimicrobial response in PCs can prevent bacterial translocation. MyD88-dependent pathways are essential for host defense against infections by *S. aureus*, *Toxoplasma gondii*, and *Listeria monocytogenes* (Scanga *et al*, 2002; Seki *et al*, 2002). There is also evidence for indirect extrinsic MyD88 bacterial signaling to activate the secretion of LYZ *via* secretory autophagy in PCs. Bel *et al* (2017) showed that *S. typhimurium* can invade PCs, damaging the Golgi apparatus and causing ER stress (Bel *et al*, 2017). This stress can activate secretory autophagy, only with the help of MyD88-dependent DC signaling, followed by interleukin (IL) 22 production by innate lymphoid cells (type3). The LYZ-containing secretory autophagosomes have the typical features of autophagosomes, a double membrane labeled with LC3. But, instead of fusing with lysosomes, these autophagosomes are released in the small intestinal lumen.

NOD2-dependent bacterial signaling α-Defensin gene expression seems rather NOD2 dependent, as NOD2^{KO} mice have decreased expression of multiple α-defensins (Kobayashi *et al*, 2005; Vaishnava *et al*, 2008; Tan *et al*, 2015). However, in contradiction with these previous reports, Menendez *et al* (2013) observed reduced α-defensin expression in TLR2, TLR4, and MYD88^{KO} mice, but not in NOD2^{KO} mice (Menendez *et al*, 2013). LYZ secretion in PCs is also NOD2 dependent. It is synthesized in the ER of PCs and packed in dense core secretory granules (DCSG) at the trans-Golgi network. Intestinal bacteria activate NOD2, leading to recruitment of leucine-rich repeat kinase 2 (LRRK2), receptor interacting serine/threonine kinase 2 (RIP2), and RAB2A on the surface of these DCSGs, which coordinate LYZ sorting towards the lumen (Zhang *et al*, 2015; Wang *et al*, 2017a). Yet, other proteins may end up in lysosomes and become degraded. This LYZ-sorting pathway is LYZ-specific and does not apply to other AMPs.

Degranulation of AMP-containing granules in PCs

The release of α-defensins from the PC secretory granules can be activated by Gram-negative or -positive bacteria, bacterial antigens (LPS, lipoteichoic acid, lipid A, and muramyl dipeptide), and carbamylcholine (Ayabe *et al*, 2000). This was shown by monitoring the antibacterial activity of *ex vivo* stimulated crypt cultures. Bacterial killing in the supernatant was not observed when crypts of MMP7^{KO} mice or mice devoid of PCs (CR2-tox176) were stimulated with the mentioned ligands, emphasizing the antimicrobial role of α-defensins in PCs. *In vivo* results showed that PCs are degranulated after stimulation with cholinergic agents, such as carbamylcholine, or by IL4, IL13, IL22, interferon (IFN)α, and tumor necrosis factor (TNF)α (Satoh *et al*, 1989; Ozcan *et al*, 1996; Rumio *et al*, 2012; Stockinger *et al*, 2014; Zwarycz *et al*, 2019). CpG-oligodeoxynucleotides (TLR9 antagonists) and poly(I):poly(C)

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Table 2. Annotated α-defensin genes, cryptdin-related sequence genes, and α-defensin pseudogenes in the MGI Genome Browser.

Name	MGI ID	Symbol
defensin, alpha 1	MGI:94880	Defa1
defensin, alpha, 2	MGI:94882	Defa2
defensin, alpha, 3	MGI:94883	Defa3
defensin, alpha, 4	MGI:99584	Defa4
defensin, alpha, 5	MGI:99583	Defa5
defensin, alpha, 6	MGI:99582	Defa6
defensin, alpha, 7	MGI:99581	Defa7
defensin, alpha, 8	MGI:99580	Defa8
defensin, alpha, 9	MGI:99579	Defa9
defensin, alpha, 10	MGI:99591	Defa10
defensin, alpha, 11	MGI:99590	Defa11
defensin, alpha, 12	MGI:99589	Defa12
defensin, alpha, 13	MGI:99588	Defa13
defensin, alpha, 14	MGI:99587	Defa14
defensin, alpha, 15	MGI:99586	Defa15
defensin, alpha, 16	MGI:99585	Defa16
defensin, alpha, 17	MGI:1345152	Defa17
defensin, alpha, 20	MGI:1915259	Defa20
defensin, alpha, 21	MGI:1913548	Defa21
defensin, alpha, 22	MGI:3639039	Defa22
defensin, alpha, 23	MGI:3630381	Defa23
defensin, alpha, 24	MGI:3630383	Defa24
defensin, alpha, 25	MGI:3630385	Defa25
defensin, alpha, 26	MGI:3630390	Defa26
defensin, alpha, 27	MGI:3642780	Defa27
defensin, alpha, 28	MGI:3646688	Defa28
defensin, alpha, 29	MGI:94881	Defa29, Defa-rs1
defensin, alpha, 30	MGI:3808881	Defa30
defensin, alpha, 31	MGI:102509	Defa31Defa-rs7
defensin, alpha, 32	MGI:3709042	Defa32
defensin, alpha, 33	MGI:5434357	Defa33
defensin, alpha, 34	MGI:3709048	Defa34
defensin, alpha, 35	MGI:3711900	Defa35
defensin, alpha, 36	MGI:5434853	Defa36
defensin, alpha, 37	MGI:3705236	Defa37
defensin, alpha, 38	MGI:3709605	Defa38
defensin, alpha, 39	MGI:3611585	Defa39
defensin, alpha, 40	MGI:3708769	Defa40
defensin, alpha, 41	MGI:3705230	Defa41
defensin, alpha, 42	MGI:3645033	Defa42
defensin, alpha, 43	MGI:3648003	Defa43
defensin, alpha, pseudogene 1	MGI:3630392	Defa-ps1
defensin, alpha, pseudogene 2	MGI:3832603	Defa-ps2
defensin, alpha, pseudogene 3	MGI:3705791	Defa-ps3
defensin, alpha, pseudogene 4	MGI:3705782	Defa-ps4
defensin, alpha, pseudogene 5	MGI:3705778	Defa-ps5
defensin, alpha, pseudogene 6	MGI:3705855	Defa-ps6
defensin, alpha, pseudogene 7	MGI:3705773	Defa-ps7
defensin, alpha, pseudogene 8	MGI:3832672	Defa-ps8
defensin, alpha, pseudogene 9	MGI:3705785	Defa-ps9
defensin, alpha, pseudogene 10	MGI:3705783	Defa-ps10
defensin, alpha, pseudogene 11	MGI:3705879	Defa-ps11
defensin, alpha, pseudogene 12	MGI:3647175	Defa-ps12
defensin, alpha, pseudogene 13	MGI:3705864	Defa-ps13
defensin, alpha, pseudogene 14	MGI:3705817	Defa-ps14
defensin, alpha, pseudogene 15	MGI:3705808	Defa-ps15
defensin, alpha, pseudogene 16	MGI:3705774	Defa-ps16
defensin, alpha, pseudogene 17	MGI:3705788	Defa-ps17
defensin, alpha, pseudogene 18	MGI:3642785	Defa-ps18
defensin, alpha, related sequence 2	MGI:99592	Defa-rs2
defensin, alpha, related sequence 4	MGI:102512	Defa-rs4
defensin, alpha, related sequence 5	MGI:102511	Defa-rs5
defensin, alpha, related sequence 6	MGI:102510	Defa-rs6
defensin, alpha, related sequence 8	MGI:102508	Defa-rs8
defensin, alpha, related sequence 9	MGI:102507	Defa-rs9
defensin, alpha, related sequence 10	MGI:102516	Defa-rs10
defensin, alpha, related sequence 11	MGI:102515	Defa-rs11
defensin, alpha, related sequence 12	MGI:102514	Defa-rs12

(TLR3 agonist) can also stimulate PCs, as they induce degranulation from 3 h postinjection. After oral treatment with LPS (a TLR4 agonist) and flagellin (a TLR5 agonist), late degranulation, mediated by TNFα, was observed in PCs (Rumio *et al*, 2012).

Phagocytosis of bacteria and efferocytosis in PCs

Another remarkable function of PCs is that they can digest intestinal microorganisms *via* phagocytosis. Spiral-formed bacteria and trophozoites of the flagellate *Hexamita muris* were identified in the digestive vacuoles of PCs from rats (Erlandsen & Chase, 1972). Both intact and partially digested spiral-formed bacteria and trophozoites were observed, but only in PCs in the gut. The presence of *S. typhimurium* was also demonstrated in the PCs of infected mice (Bel *et al*, 2017).

Efferocytosis was recently identified as a new PC function (Shankman *et al*, 2021) that removes apoptotic cells by phagocytes. To illustrate that PCs can effectively engulf their neighboring apoptotic IECs, they made use of organoids derived from a transgenic mouse strain, where PC membranes were labelled green, and all membranes of all other cells red, in combination with apoptotic dyes. In enteroids irradiated to induce cell death, apoptotic IECs were engulfed by PCs. Also, PC-specific ablation reduced efferocytosis in intestinal crypts. So, PCs can remove their neighboring apoptotic IECs and in this way reduce local inflammation and contribute to gut homeostasis (Shankman *et al*, 2021).

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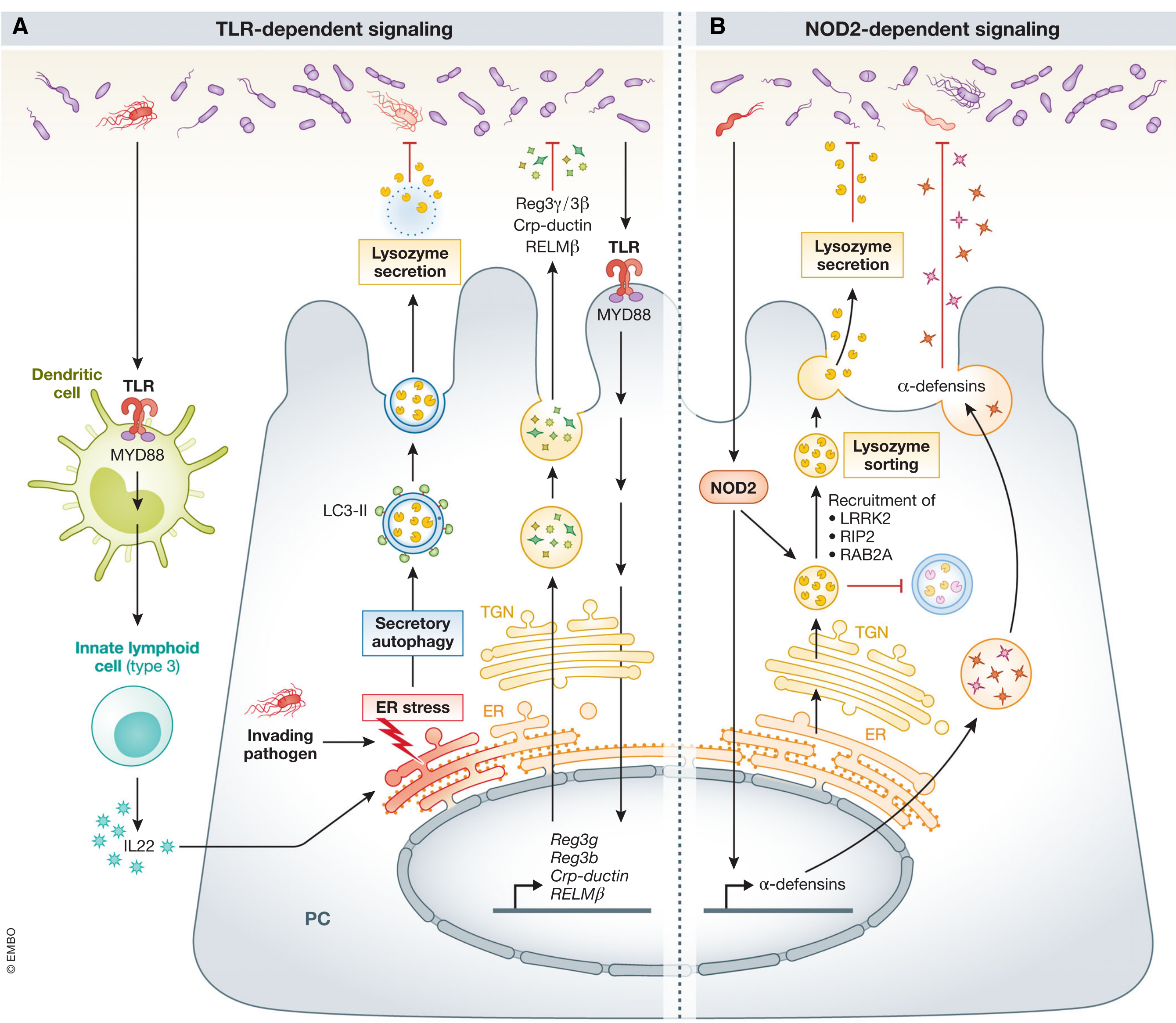

Figure 2. Bacterial stimulation of AMP expression and release in PCs.

(A) Intestinal bacteria can communicate directly with PCs *via* a PC-autonomous TLR-MYD88 pathway. This triggers the production and secretion of AMPs (measured in PCs by laser capture microdissection). There is also evidence for indirect extrinsic MyD88 bacterial signaling to activate the secretion of LYZ in PCs: *S. typhimurium* can stimulate dendritic cells *via* TLR-MYD88 signaling, leading to IL22 production in innate lymphoid cells (type 3). IL22, together with invading *S. typhimurium* in the PC, induce ER stress in these cells, leading to secretory autophagy. The LYZ-containing secretory autophagosomes have the typical features of an autophagosome, namely, a double membrane labeled with LC3. However, instead of fusing with lysosomes, these LYZ-containing autophagosomes are released in the lumen of the small intestine. (B) Intestinal bacteria can stimulate NOD2 and increase α-defensins production and secretion. Bacterial NOD2 activation can also lead to the recruitment of LRRK2, RIP2, and RAB2A onto the surface of DCSGs which coordinate lysozyme sorting.

Uptake of heavy metals

It has been established that PCs contain heavy metals (e.g., Se and Zn) (Danscher *et al*, 1985), along with heavy metal ion-binding proteins, for example, metallothioneins and the Zn-binding cysteine-rich intestinal protein (Fernandes *et al*, 1997). PCs are believed to pick up heavy metals from the lumen and utilize them.

Zn is essential for PC function, as deletion of some zinc importers/exporters causes PC defects. Fourteen Zn-importing transporters (ZIPs encoded by *Slc39a* gene family members) and 10 Zn-exporters (ZnTs, encoded by *Slc30a* family members) have been described, and several of them are expressed in PCs. An inducible loss-of-function of ZIP4 in the murine gut compromises PCs, causing abnormal expression of PC-related genes (Geiser *et al*, 2012). Another Zn transporter, ZnT2, is responsible for exporting Zn towards the secretory granules. ZnT2 full KO mice ($Slc30a2^{KO}$) have impaired PCs devoid of Zn, disturbed PC granule structure and

reduced antimicrobial activity in the ileum, leading to dysbiosis (Podany *et al*, 2016). These studies confirmed that Zn is not only directly antibacterial but also contributes to PC antimicrobial activities (Fig 3).

In PCs, Zn is stored in the secretory granules, but it is not clear why. One explanation is that Zn can stabilize HD5 and chicken egg LYZ, as shown *in vitro* (Chakraborti *et al*, 2010; Zhang *et al*, 2013). Both actions were confirmed *in vivo* in PCs (Podany *et al*, 2016; Zhong *et al*, 2020). It is also speculated that the storage of heavy metals contributes to direct antimicrobial toxicity, as Zn is released upon cholinergic PC stimulation (Giblin *et al*, 2006). A third potential function, only in mice, is that α-defensin maturation is Zn-dependent, as the final proteolytic α-defensin maturation step is performed by MMP7, a Zn-dependent metalloprotease (Wilson *et al*, 1999).

Moreover, Zn is important even in PC survival. In rats and mice, a single injection of the Zn chelator, dithizone, leads to disappearance of PCs and their reappearance after 12–24 h (Sawada *et al*, 1991). The PC-specific ablation of PCs by dithizone indicates the strong Zn dependency of PCs.

Preserving the intestinal barrier

Paneth cell-derived AMPs were found to gather in the mucus to prevent invasion and microbial attachment, augmenting the antimicrobial function of the mucus (Meyer-Hoffert *et al*, 2008). Moreover, PC-deficient mice (Cryptdin2-tox176) display increased bacterial translocation of commensal bacteria towards the mesenteric lymph nodes (Vaishnava *et al*, 2008), without adapting the luminal bacterial load in the small intestine (Vaishnava *et al*, 2008). This illustrates that PCs are essential in preventing bacterial translocation but

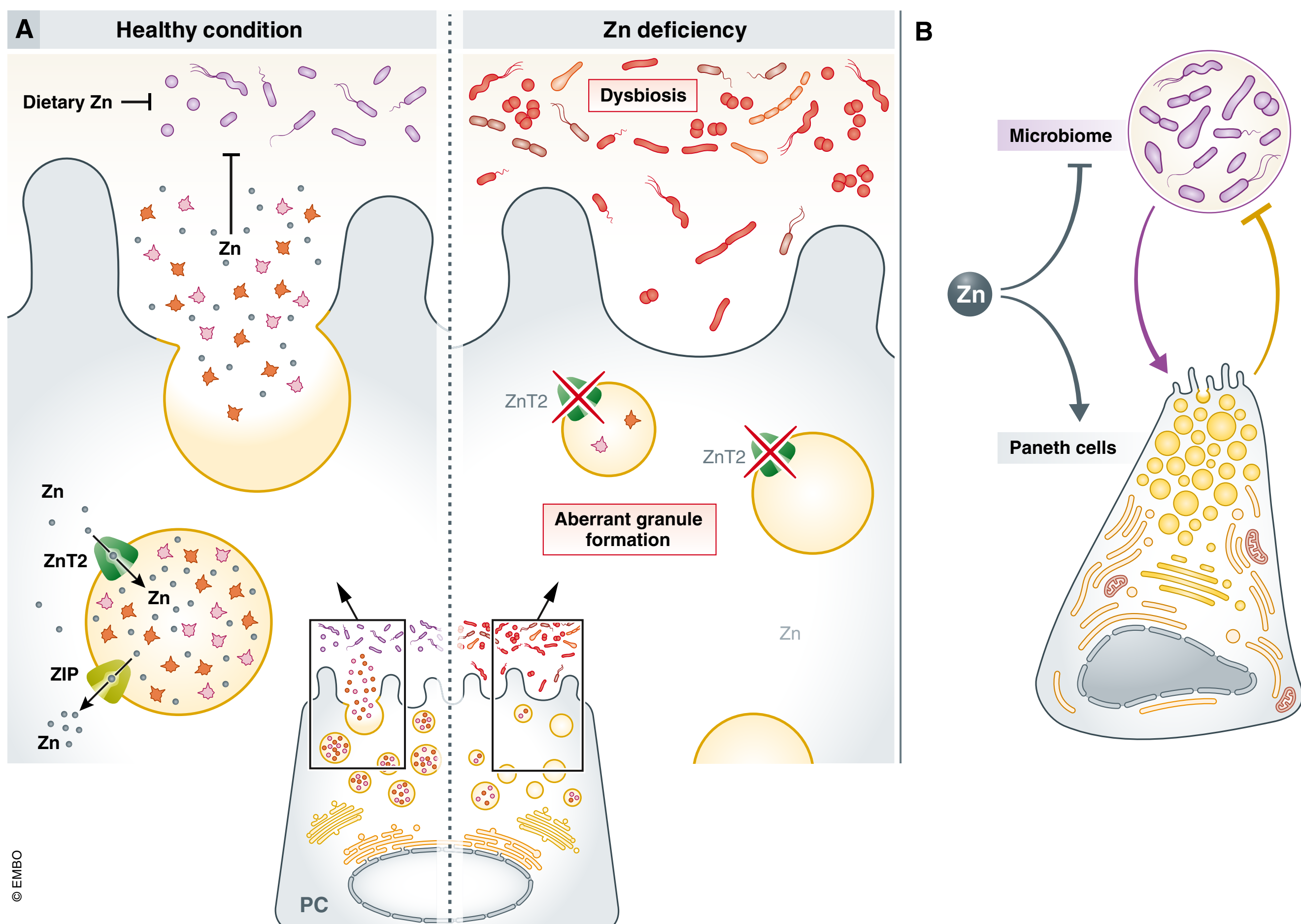

Figure 3. The direct and indirect effects (*via* PCs) of Zn on the microbiome.

(A) In healthy conditions, dietary Zn has a direct antimicrobial effect on the microbiome and inhibits growth of, for example, several *Staphylococcus* species. Moreover, ZnT2 is responsible for the accumulation of zinc in the secretory granules and for the regulation of AMP secretion in PCs. Upon degranulation, Zn is released in the lumen, leading to an indirect effect (*via* the PCs) of Zn on the microbiome. Moreover, *in vitro* assays suggest a role for Zn in stabilization of the antimicrobial HD5 and LYZ. In conditions of Zn deficiency, the direct and indirect antimicrobial effects of Zn on the microbiome are lost or reduced. Deletion of ZnT2 in mice leads to secretory granules devoid of Zn, impaired PCs, disturbed PC granule structure and reduced antimicrobial activity in the ileum, resulting in dysbiosis. (B) Zn can have an antimicrobial effect on the microbiome but is also indispensable for PC functions. PCs can further control the microbiome by the release of AMPs and Zn. The microbiome can in turn stimulate the PCs to produce AMPs.

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have no effect on the overall bacterial load in the small intestine. A possible explanation is that PCs regulate the number of mucosa-associated bacteria, which prevents close contact with the epithelium and subsequent bacterial translocation.

PCs in pathology

Correct PC functioning is important for controlling the microbiota and preserving the crypt niche. This ensures the proper metabolic environment and ISC communication needed for tissue renewal (Lueschow & McElroy, 2020). Failure or disturbance in PC morphology and activity can reduce the secretion of AMPs and stemness factors and increase bacterial translocation. This determines the severity and progression of several gastrointestinal and other disorders in distant organs such as kidney or liver (Teltschik *et al*, 2012; Cray *et al*, 2021). Likewise, there is agreement that such diseases might be prevented or even cured by correcting PC abnormalities. Examples include inflammatory bowel disease, ileal CD, and necrotizing enterocolitis (NEC) (Barreto *et al*, 2022).

The PC dysfunction can be studied by quantifying the distribution and expression pattern of cytoplasmic AMPs, the morphology of the granules, and/or the number of PCs per crypt (Stappenbeck & McGovern, 2017). In this sense, genetic disorders, environmental factors, and diet are studied and linked as inducers of PC dysfunctions.

PC numbers

It has been reported that PC numbers may decline by cell death and/or extrusion in the lumen, which aggravates intestinal and inflammatory diseases (Gassler, 2017). The reduction in PC number per crypt has been described in several pathological conditions, such as intestinal ischemia (Grootjans *et al*, 2011), pathogenic bacterial infections (White *et al*, 2017), NEC (White *et al*, 2017), and GVHD (Levine *et al*, 2013). However, in other intestinal diseases, such as ileal CD, the defect is in PC function and not in PC number (Deuring *et al*, 2014; Strigli *et al*, 2021). Moreover, changes in PC numbers are not always correlated with alterations in the expression of PC-specific AMPs (Zwarycz *et al*, 2019; Kip *et al*, 2020). Newly formed PCs after an insult could be immature and/or dysfunctional and contain fewer or aberrant granules.

Paradoxically, in some cases, like alcohol-fed animals or *Salmonella* infections, induction of cell death in the small intestine is coupled with increased PC numbers (Rodriguez *et al*, 2012). This could be explained by the fact that during intestinal inflammation, post-mitotic PCs respond by acquiring stem cell-like properties, thus contributing to the tissue regenerative response during inflammation (Schmitt *et al*, 2018). An increase in PC number per crypt has been observed after bacterial infection with *S. typhimurium* (Rodriguez *et al*, 2012). PC metaplasia has also been described in mouse models after infection (Singh *et al*, 2020). In humans, PC metaplasia has been described in NEC in premature infants (Puiman *et al*, 2011) and in CDs and ulcerative colitis patients (Tanaka *et al*, 2001).

Due to the location of PCs, the study of the number and function of these cells in humans is complex and requires tissue biopsies. The use of murine models is therefore fundamental to understanding the processes of the reduction in PC number and its association with different events.

PC cell death

The ultimate fate of IECs, death, can occur by several mechanisms, either as part of normal physiology and homeostasis (cell renewal) or due to damage-inducing events. PC death can be the result of physiological pathways defects, ER stress or autophagy, or by extrinsic cell death stimuli induced by factors such as cytokines.

Autophagy and unfolded protein response (UPR)

In PCs, autophagy is important to manage the generation and exocytosis of the secretory granules, clearance of misfolded proteins, maintenance of mitochondrial homeostasis, alleviation of ER stress, bacterial autophagy, and survival cytokine-mediated immunopathology after infections (Burger *et al*, 2018; Wang *et al*, 2018). Defects in autophagy or autophagy related-genes have been linked to abnormalities in PC morphology and function (Table 3; Fig 4) (Wang *et al*, 2018).

An important gene involved in autophagy is the autophagy-related 16 like 1 gene (*Atg16l1* in mice, *ATG16L1* in humans). The importance of *Atg16l1* is mainly in its participation in the formation of autophagosomes, but its function has been linked with other major cell activities. A study on PC-rich organoids isolated from WT and *Atg16l1*-deficient mice showed significant differences in their proteomic signature. In KO cells, 16 important functional processes were altered, the most remarkable being inhibition of the exocytosis pathway (Jones *et al*, 2019). According to one study, mice with *Atg16l1* and *Atg5*-deficient-PCs presented irregularities in the granule exocytosis pathway, which is observed in CD patients with the homozygous ATG16L1 risk allele ($Atg16l1^{T300A}$) (Cadwell *et al*, 2008). KI mice with $Atg16l1^{\mathrm{T300A}}$ have a similar phenotype, with abnormalities in PC granules, function, and PC numbers (Lassen *et al*, 2014). In mice, villin or PC-specific deletion of *Atg16l1*, *Atg5* or *Atg7*, or constitutive deletion of *Atg4b* or immunity-related GTPase family M member (*Irgm1*) result in impaired exocytosis, accumulation of dysfunctional mitochondria, accumulation of inositol-requiring enzyme 1 (IRE1) and decrease in AMP production (Fig 4A–D). Likewise, mutations of *Lrrk2* led to degradation of LYZ *via* autophagy (Fig 4E). PC-specific deletion of *Lrrk2* has also been linked with the abnormal PC-phenotype seen in Japanese CD patients (Liu *et al*, 2017b).

Paneth cells, due to their intense secretions, are very sensitive to ER stress, making autophagy and the UPR important in preventing PC death. The heavy load on the ER network in PCs makes them more prone to ER stress than other cells. Disruption of PC secretions or defects in UPR-related genes might lead to dysbiosis and inflammatory diseases (Bel *et al*, 2017; Wang *et al*, 2018). Deletion of the transcription factor X-box-binding protein 1 (*Xbp1*) specifically in PCs leads to unresolved ER stress, autophagy activation, and PC apoptosis (Table 3; Fig 4F) (Kaser *et al*, 2008; Adolph *et al*, 2013). Induction of PC-specific ER stress has also been observed in SAMP1/YitFc mice, which are a model of CD (Shimizu *et al*, 2020). The disruption of ER homeostasis in those mice leads to α-defensin misfolding associated with absence of disulfide bridges, and dysbiosis linked to progression of ileitis. The absence of disulfide bridges in HD5 (leading to reduced rather than oxidized HD5) was observed in PCs of CD patients (Tanabe *et al*, 2007). Also in CD patients, an increased abundance of enteroinvasive *Escherichia coli* and other signs of dysbiosis were associated with ER stress in PCs (Deuring *et al*, 2014).

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Table 3. Mouse studies linking defects in autophagy and/or unfolded protein response (UPR)-related genes with abnormalities in PCs' morphology and function.

	Knocked-out gene	Cell type	Mechanism	Outcome	References
UPR-related genes	X-box-binding protein 1 (*Xbp1*)	Intestinal epithelial cells	Condensed ER ER stress Abnormal secretory granules Decreased expression of alpha-defensins Paneth cell apoptosis	Susceptibility to DSS-induced colitis and bacterial infections	Kaser *et al* (2008)
		Paneth cells	Autophagy activation Abnormal secretory granules Increased cell death in crypts Unresolved ER stress UPR activation	Transmural ileitis Development of spontaneous intestinal inflammation	Adolph *et al* (2013)
		Intestinal epithelial cells	Increased total and phosphorylated IRE1α Increased NFκB activity Autophagosome formation Aberrant Paneth cell granules	Ileitis dependent on microbiota	Adolph *et al* (2013)
Autophagy-related genes	*Atg16l1*	Intestinal epithelial cells	Crypts with increased XBP1 splicing Reduction in Paneth cell size and number of granules Loss of homeostatic autophagy	Susceptibility to DSS-induced inflammation	Adolph *et al* (2013)
		Intestinal epithelial cells	Accumulation of IRE1α in Paneth cells	IRE1α-dependent spontaneous ileitis	Tschurtschenthaler *et al* (2017)
		Intestinal epithelial cells	Paneth cell depletion	Exacerbated intestinal injury in models of graft-versus-host disease	Ishimoto *et al* (2017)
	Atg16l1^{HM} (hypomorphic (HM) for expression of the ATG16L1 protein)	Constitutive	Aberrant Paneth cell granules (size, morphology, and number) Irregularities in the granule exocytosis pathway Morphological abnormalities in Paneth cells Diffuse lysozyme staining		Cadwell *et al* (2008)
	Atg16l1^{T300A} (Thr^{300} ➔ Ala^{300})	Constitutive	Abnormal Paneth cell lysozyme distribution and decreased antibacterial autophagy after infection		Bel *et al* (2017)
	Atg16l1/Xbp1	Intestinal epithelial cells	Lack of UPR-induced autophagy Increased total and phosphorylated IRE1α Increased NFκB activity Aberrant Paneth cell granules	Development of severe spontaneous ileitis Transmural inflammation	Adolph *et al* (2013)
		Intestinal epithelial cells	Impaired clearance of IrE1α aggregates ER stress Impaired Paneth cell antimicrobial function	Aggravated DSS-induced colitis	Tschurtschenthaler *et al* (2017)
	Atg5	Paneth cells	Abnormalities in number and size of Paneth cell granules Reduced expression of AMPs	Paneth cell loss upon *T. gondii* infection Increased permeability due to impaired intestinal barrier function	Burger *et al* (2018)
		Intestinal epithelial cells	Reduced expression of AMPs Impaired autophagy in Paneth cells	Susceptibility to *T. gondii*-mediated intestinal damage Paneth cell loss upon *T. gondii* infection	Burger *et al* (2018)
		Intestinal epithelial cells	Morphological abnormalities in Paneth cells Irregularities in the granule exocytosis pathway Aberrant Paneth cell granules (size, morphology, and number)		Cadwell *et al* (2008), Cadwell *et al* (2009)

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Table 3 (continued)

Knocked-out gene	Cell type	Mechanism	Outcome	References
Atg7	Intestinal epithelial cells	Deficient Paneth cell-granule formation Alterations in lysozyme storage and secretion		Wittkopf *et al* (2012)
	Intestinal epithelial cells	Morphological abnormalities in Paneth cells Aberrant Paneth cell granules (size, morphology, and number) Diffuse lysozyme staining		Cadwell *et al* (2009)
Atg7/Xbp1	Intestinal epithelial cells	Absent UPR-induced autophagy Aberrant Paneth cell granules	Spontaneous ileitis Transmural inflammation Age-dependent enteritis	Adolph *et al* (2013)
Atg4b	Constitutive KO	Abnormalities in size, morphology, and number of Paneth cell granules Autophagy impairment	Increased susceptibility to DSS-induced colitis	Cabrera *et al* (2013)
Leucine-rich kinase 2 (*Lrrk2*)	Constitutive KO	Defects in lysozyme sorting	Increased susceptibility to intestinal *Listeria monocytogenes* infections	Zhang *et al* (2015)
Immunity-related GTPase M (*Irgm1*)	Constitutive KO	Alterations in Paneth cell numbers and location	Susceptibility to dextran sodium sulfate (DSS)-induced intestinal injury	Rogala *et al* (2018)
	Constitutive KO	Decreased transcript levels of specific AMPs (*Lyz* and *Defa20*) Alterations in Paneth cell numbers and location Aberrant Paneth cell-granules (size and morphology) Abnormal Paneth cell morphology Impaired mitophagy and autophagy	Spontaneous intestinal inflammation Ileal injury	Liu *et al* (2013)
Transcription factor EB (*Tfeb*)	Intestinal epithelial cells	Abnormal morphology of Paneth cell granules	Magnified colitis response upon DSS	Murano *et al* (2017)

Cell death by ligands: IFNs

Interferons have been postulated as critical modulators of PC function and survival (Araujo *et al*, 2021). A recurrent observation is that IFNs induce PC cell death. IFNs I, II, and III have been linked to PC death and/or degranulation (Günther *et al*, 2019; Araujo *et al*, 2021). Why PCs are so IFN-sensitive and the mechanisms of their loss are unknown. However, we will explain that the effects of IFN on PCs are not necessarily direct and that indirect effects are contemplated.

Type I-IFNs (mainly IFNα and IFNβ) are involved in antimicrobial host defense due to their antiviral and antiproliferative activities. They are considered as essential in the first line of defense against viruses (Liu *et al*, 2012) and bacteria and are triggered by stimulation of TLRs (Decker *et al*, 2005) and other pathogen sensors. In PCs, TLR9 signaling might be responsible for the bacterially triggered type-I IFN production (Fig 5A). Through the IFN I receptor (a heterodimer consisting of IFNAR1 and IFNAR2), type I IFNs can activate several Janus kinases (JAKs), several signal transducers and activators of transcription (STAT), and MAPK signaling pathways (González-Navajas *et al*, 2012). Although PCs constitutively express *Ifna1* (encoding IFNα1) under homeostatic conditions, little is known about its biological effect. Feeding mice with the IFN-inducing compound, R11567DA, induced IFNα in ileum, colon, and blood, but in situ hybridization (ISH) experiments showed that IFN-dependent genes were only induced in crypts, likely in PCs (*Ifit1*, *Irf7*, and *Oas1g* genes). Perhaps PCs are unique in expressing *Ifnar1*, or the signal transduction to IFNs in PCs is much more pronounced because of the lack of inhibitors (Munakata *et al*, 2008). In another study, expansion in PC numbers in mice lacking the IFNAR1 in the gut ($Ifnar1^{\Delta IEC}$) was observed, meaning that IFNα can regulate PC numbers (Tschurtschenthaler *et al*, 2014). Finally, IFNα injection in Wistar rats was shown to cause PC degranulation and α-defensin release (Ozcan *et al*, 1996).

The type III IFN group, consisting of four IFNs generally termed IFNλ, is mainly sensed by epithelial cells. Günther *et al* (2019) reported that in the gut epithelium, IFNλ causes the expression of mixed lineage kinase domain like pseudokinase (*Mlkl*) and *Stat1*, which leads to increased necroptosis in PCs, controlled by caspase8 (CASP8) (Fig 5B) (Günther *et al*, 2019). This increased PC death has been seen in CD patients, in whom also higher levels of IFNλ were observed in serum and inflamed ileal tissue (Stolzer *et al*, 2021).

Recent findings show that of all IFNs, type II IFN (IFNγ) is the most harmful: by promoting PC loss, it is involved in intestinal inflammation (Fig 5C) (Raetz *et al*, 2013; Farin *et al*, 2014; Eriguchi *et al*, 2018). *In vitro* and *in vivo* studies have shown that IFNγ hampers the expression of α-defensins, increases degranulation, and leads to PC death (Bevins & Salzman, 2011; Raetz *et al*, 2013; Farin *et al*, 2014; Burger *et al*, 2018; Eriguchi *et al*, 2018;

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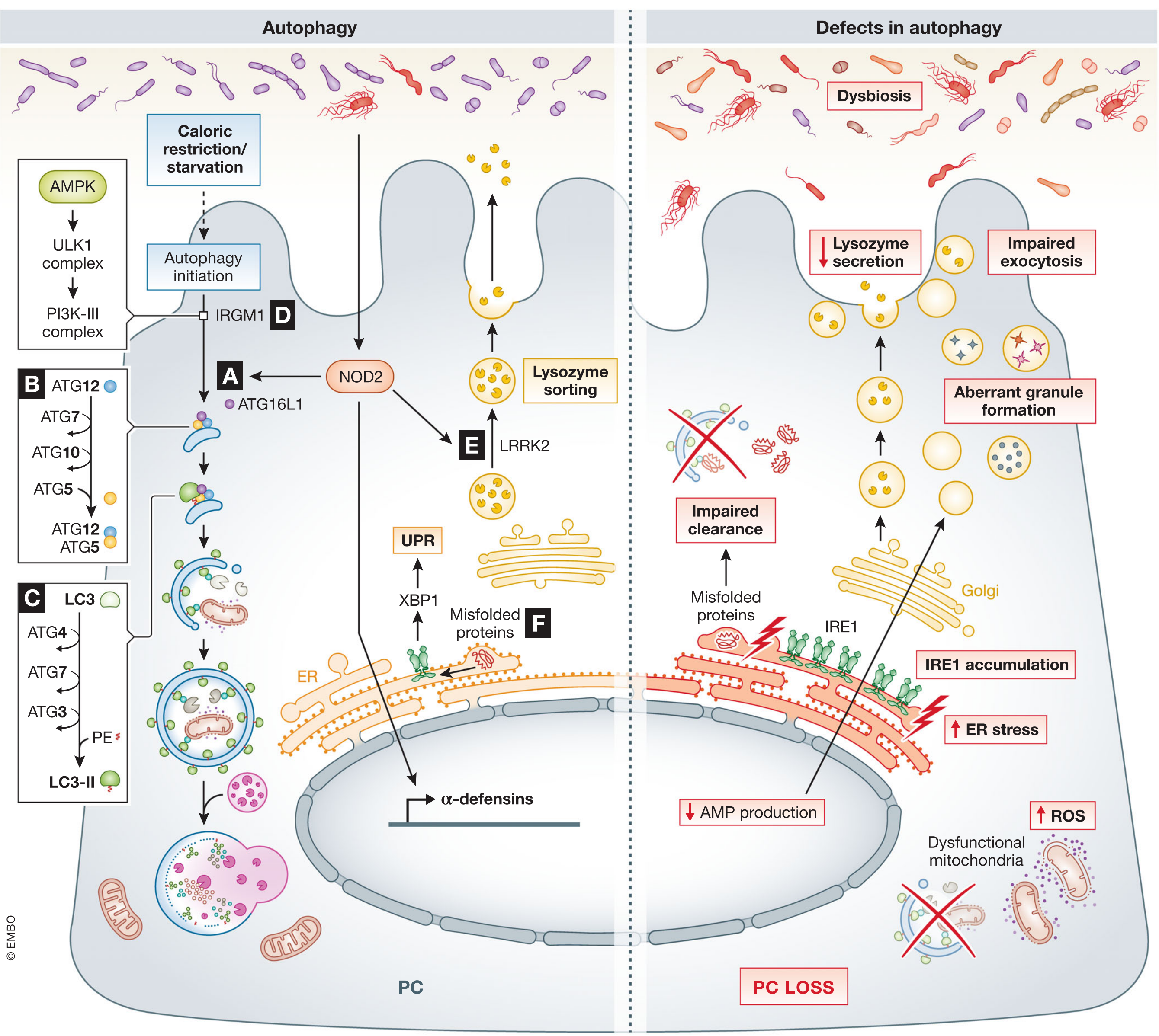

Figure 4. Role of autophagy and ER in PCs.

(A) Bacterial activation of NOD2 initiates direct autophagy by recruiting ATG16l1. Defects in *Nod2* and/or *Atg16l1* have been related to a decrease in AMP production, impaired exocytosis, accumulation of IRE1, and bacterial translocation. (B) In PCs, ATG5 binds ATG16L1 and ATG12, which is important in the early stages of autophagy, catalyzing the microtubule-associated protein light chain 3 (LC3) lipidation. Specific deletion of *Atg5* in PCs has been associated with accumulation of ROS and increased ER stress due to impaired clearance of dysfunctional mitochondria. C) ATG4 and ATG7 form a complex with ATG3, responsible for the biogenesis of the autophagosome by determining the site of LC3 lipidation. Dysfunction in autophagy, aberrant granule formation, and defects in AMP production and secretion have been observed in $Atg4b^{KO}$ and $Atg7^{\Delta IEC}$ mice. D) IRGM1 plays a direct role in organizing the autophagy process. IRGM1 can initiate the phosphorylation cascade that activates Unc-51 like autophagy activating kinase 1 (ULK1) and Beclin1 (an autophagy regulator part of the PI3K-III complex), which promotes autophagy. Defects in *Irgm1* have been linked to alterations in PC numbers and location, abnormal PC morphology and aberrant granules. E) LRRK2, together with RIP2 and RAB2A, coordinates lysozyme sorting after recruitment by bacterial-activated NOD2. Dysfunctions in *Nod2* and/or *Lrrk2* result in compromised lysozyme secretion. F) Misfolded or unfolded proteins are recognized by IRE1, which by unconventional splicing generates XBP1, activating UPR response. Specific deletion of *Xbp1* in PCs leads to PC dysfunction, a condensed ER and abnormal secretory granules. Black arrows – pathways that are active in PCs, red arrows – "up- or downregulation" of factors that negatively affect PCs function.

Araujo *et al*, 2021). In serum samples and ileal biopsies of IBD mouse models, increased IFNγ concentrations are concomitant with a higher rate of PC loss (Wang *et al*, 2018). Moreover, ileal samples from *T. gondii*-infected mice had significantly fewer PCs. This occurred entirely though events in the submucosa, namely, dendritic cell activation (Th1 activation) *via* IL12. IFNγ production by these Th

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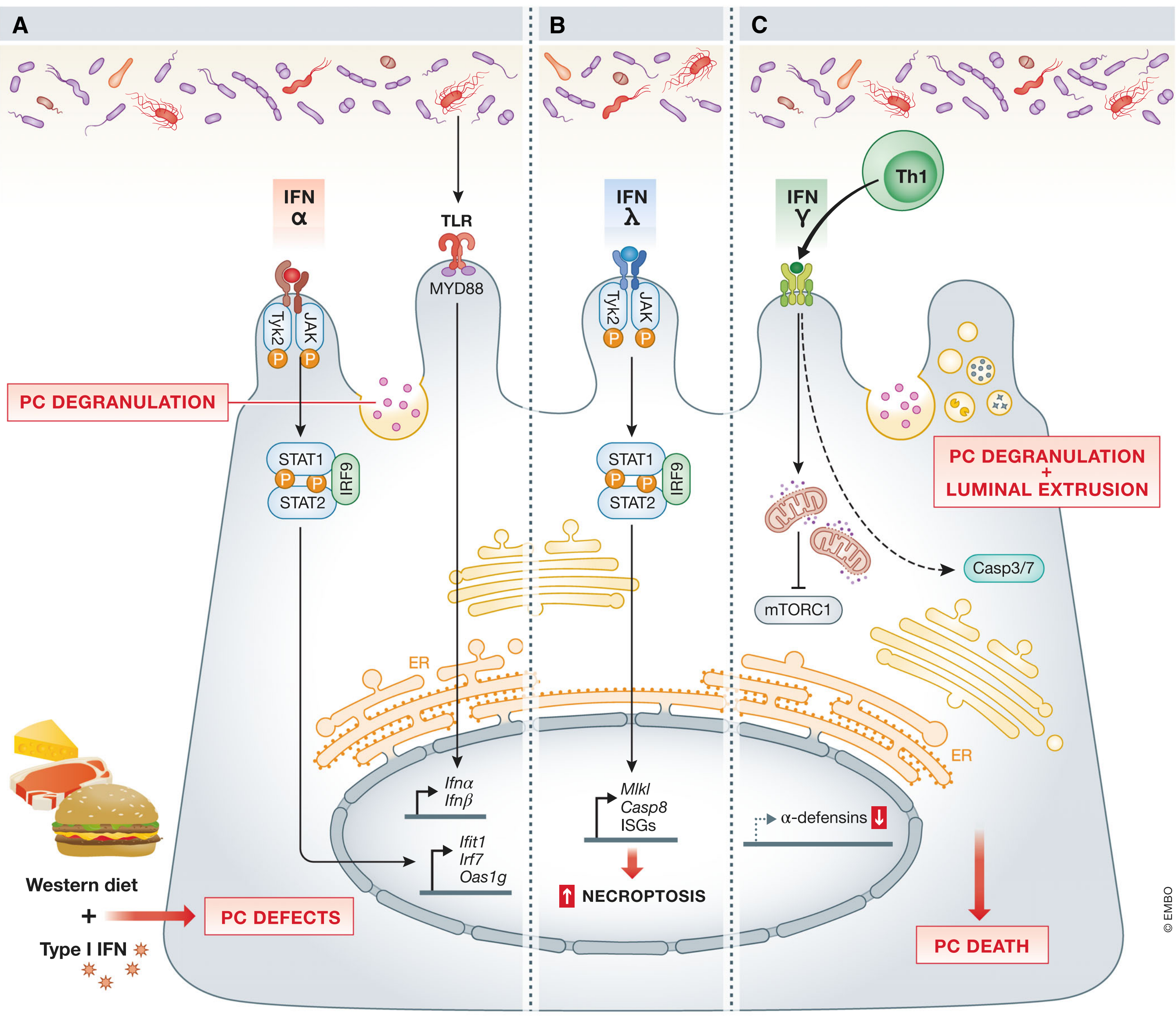

Figure 5. Role of the three types of IFNs in PCs.

(A) IFNα regulates PC-numbers *via* JAK/STAT signaling. The release of type I IFN can also be triggered by bacterial-TLR activation. The combination of Western diet and type I IFN, produced by myeloid cells, negatively affects PCs (see Figure 6 for more details). (B) Detrimental effects have been ascribed to type III IFNs, as IFNλ promotes PC necroptosis in an MLKL/CASP8-dependent manner. (C) Schematic representation of the mechanisms related to PC-loss associated with type II IFN (IFNγ). IFNγ mediates PC loss by an mTORC1-dependent mechanism after disrupting mitochondrial integrity and function. Moreover, after injury, activated Th-produced IFNγ induces PC depletion *via* a caspase3&7-dependent cell death. Black arrows – pathways that are active in PCs, red arrows – up- or downregulation" of factors that negatively affect PCs function.

cells ultimately led to PC death (Pêgo *et al*, 2019), a phenotype that was reverted in mice with an epithelial or PC-restricted IFNγRII KO (Araujo *et al*, 2021). Though it seems clear that IFNγ leads to PC death, the mechanisms remain uncertain, particularly whether this loss in PCs is a direct or indirect effect of IFNγ. Experiments on a GVHD mouse model have shown that alterations in the crypt cell niche, and consequently in PCs, were due to effects of IFNγ on ISCs (Takashima *et al*, 2019). Yokoi *et al* (2021) showed that both PCs and ISCs express IFNγRI, and that IFNγ caused cell death directly in both cells types. The same was proposed by Eriguchi *et al* (2018), who observed that IFNγ selectively induced PC death, which might impact the whole intestinal crypt (Eriguchi *et al*, 2018). While some authors proposed that IFNγ is sufficient to mediate PC loss (Farin *et al*, 2014; Eriguchi *et al*, 2018), others reported that both the microbiota and the basal microbiota-induced IFNγ are needed to cause intestinal pathologies and sustained PC death in *Atg5*-deficient mice (Burger *et al*, 2018). Recent findings have supported the idea that PCs can undergo different mechanisms of death when exposed to

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IFNγ. The study carried out by Farin *et al* (2014) confirmed that IFNγ-induced PC death was caspase3 and 7-dependent (Farin *et al*, 2014; Eriguchi *et al*, 2018). However, others stated that PC death was induced by the inhibition of mTORC1 by IFNγ (Araujo *et al*, 2021). Further investigations have confirmed that the decreased levels in mTORC1 resulted from impaired mitochondrial function as an indirect response to IFNγ (Araujo *et al*, 2021). Although autophagy can alleviate the effects of mitochondrial accumulation, it seems insufficient to rescue the loss of PCs, probably due to excessive autophagy or the prolonged inhibition of mTORC1. Thus, IFNγ-stimulated effects might be exacerbated when autophagy defects exist (Ishimoto *et al*, 2017).

We speculate that IFNs might be so cytotoxic to PCs because PCs are direct neighbors of ISCs. As PCs sustain the continuous renewal of the intestinal layer, one of their main functions could be the protection of ISCs. They may be programmed to (1) be extremely attractive to viral infection and titrate viruses away from the ISCs, and (2) die and thereby release antimicrobial peptides/proteins to control potential bacterial superinfections of the crypts. In this view, we argue that the loss of PCs associated with potential local dysbiosis may be less damaging than the loss of ISCs.

Cell death by ligands: TNF

TNF also plays an important role in autoimmune and inflammatory disorders (Puimège *et al*, 2014; Van Looveren & Libert, 2018). *In vivo* studies have shown that TNF injection can lead to systemic inflammation characterized by the secretion of proinflammatory cytokines and can induce cell death. Interestingly, in the small intestine, TNF induces apoptosis by binding exclusively to TNF receptor 1 (TNFR1) (Van Hauwermeiren *et al*, 2015; Ballegeer *et al*, 2018; Van Looveren *et al*, 2020). TNF can bind two distinct receptors, namely TNFR1, which mediates most TNF effects, and TNFR2. After TNF is recognized by TNFR1, it can activate proinflammatory and pro-survival NF-κB pathways or induce cell death by Fas-associated death domain (FADD)-CASP8-dependent apoptosis or RIPK3-MLKL-mediated necroptosis (Pasparakis & Vandenabeele, 2015). The study conducted by Van Hauwermeiren *et al* (2015) showed that TNF injection in mice induced PC dysfunction characterized by an increase of ER stress, with disturbed and dilated morphology. Moreover, PCs had fewer granules, lost cellular integrity, and became dysfunctional, which may lead to an increase in bacterial translocation induced by TNF (Van Hauwermeiren *et al*, 2015).

Over recent years, different KO mouse models of genes involved in the TNF-induced pathways have been generated to study their role and the PC status. Specific ablation of NF-κB essential modulator (NEMO) in intestinal cells caused PC apoptosis and impairments in AMP expression (Vlantis *et al*, 2016). Interestingly, the effects of NEMO$^{\Delta IEC}$-mediated PC loss were independent of microbiota, as it was also seen in GF mice, but the lack of bacterial translocation ameliorates the phenotype. In IEC-specific KO of NF-κB subunit RelA, the absence of *Ripk1* protected mice from PC death (Vlantis *et al*, 2016). Also, intestinal and myeloid deletion of A20 (TNFAIP3, encoded by *Tnfaip3* in mice, *Tnfaip3*$^{\Delta IEC/\Delta myel}$), an inhibitor of NF-κB and apoptosis, induces ileitis and severe colitis characterized by IEC apoptosis, goblet cell, and PC loss. The specific deletion of *Tnfaip3* only in epithelial cells (*Tnfaip3*$^{\Delta IEC}$) does not have a pathological phenotype but sensitize these mice to DSS-induced colitis and TNF-induced apoptosis: 24 h after injection of a sublethal TNF dose, LYZ-containing PCs and expression of PC-specific AMPs were reduced (Vereecke *et al*, 2014). Those experiments were confirmed in *Tnfaip3*-deficient organoids which were more affected than WT organoids by TNF combined with IFNγ (Vereecke *et al*, 2014). In contrast, complete ablation of *Nf-κb* in intestinal cells causes less severe PC loss, as observed in NEMO-$^{\Delta}$IEC mice, suggesting that pathways besides NF-κB are involved in prevention of PC death (Vlantis *et al*, 2016).

In CASP8$^{\Delta IEC}$ mice and FADD$^{\Delta IEC}$ mice, cell death in the intestinal crypts increased spontaneously, especially in PCs (Welz *et al*, 2011; Günther *et al*, 2012). FADD is one of the main regulators of CASP8-mediated inflammatory responses. RIPK1 and RIPK3 have found to be crucial in PC death, as deletion of these regulators lead to protection in FADD$^{\Delta IEC}$ mice (Welz *et al*, 2011; Schwarzer *et al*, 2020). Particularly in CASP8$^{\Delta IEC}$ mice but also in FADD$^{\Delta IEC}$ mice, inhibition of RIPK1 kinase was as protective as the combined deficiency of TNFR1 and Z-DNA-binding protein 1 (ZBP1), indicating that the role of RIPK1 is downstream of TNFR1 and ZBP1 activation (Schwarzer *et al*, 2020). The study by Schwarzer *et al* (2020) identified a key role of ZBP1, which appears to have similar functions as TNFR1 in the ileum. Therefore, only the double mutant can partially rescue PC loss and ileitis in FADD$^{\Delta IEC}$ mice. Günther *et al* (2012) identified a role for CASP8 in protecting IECs from TNF-induced RIPK3-mediated necroptosis. Increased PC death was observed in the ileum of CD patients, and *ex vivo* treatment with TNF on human control biopsies reduced *Lyz* in PCs, which can be rescued by an inhibitor of necroptosis. Moreover, in PCs, RIPK3 is present specifically in terminal ileum samples of human patients, but not in other intestinal cell types. These results indicate that high levels of exogenous TNF in the lamina propria, as in CD patients, can induce PC necroptosis and deficient expression of antimicrobial peptides, which may add to disease progression (Günther *et al*, 2012).

So, deficiencies in genes related to apoptosis sensitize PCs to TNF. Loss of X-linked inhibitor of apoptosis protein (XIAP) was recently reported to sensitize to TNF- and microbiota-dependent intestinal inflammation (Strigli *et al*, 2021; Wahida *et al*, 2021). Deficiency in XIAP has been linked to NF-κB impairment, alteration in the direct binding and inhibition of caspases, and altered recognition of bacteria, the last due to the critical role of XIAP in the NOD1 and NOD2 complex (Strigli *et al*, 2021). Exacerbation and development of intestinal inflammation in this mouse model were specifically due to the negative effects in PCs. Impaired expression of α-defensins-PC, aberrant granules, and reduced PCs numbers were observed in the ileum of *Xiap*^{KO} mice and *Xiap*$^{\Delta RING}$ mice (lacking the C-terminal RING domain of XIAP). Further crosses with TNFR1^{KO} and RIPK3^{KO} mice prevented PC loss in *Xiap*^{KO} mice, demonstrating that ablation of XIAP promotes TNF- and RIPK1/3-dependent PC death (Strigli *et al*, 2021). Variants in *Ripk1* and *Casp8* are observed in patients with severe immune deficiencies and IBD (Wahida *et al*, 2021).

Mice with a deletion in the TNF AU-rich elements (TNF$^{\Delta ARE}$) suffer from CD-like ileal inflammation due to loss of translational control of TNF linked to PC dysfunction. This dysfunction was characterized by decreased expression of *Lyz1* and *Defa5*, abnormal PC granularity and reduction in PC numbers. Confirming the phenotype, TEM analysis showed that remaining PCs have aberrant

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secretory granules, enlarged rough ER, and degenerative mitochondria, the latter being key in the CD-associated loss of stemness (Khaloian *et al*, 2020).

These findings indicate that deletion of specific genes involved in autophagy, MAPK and NF-κB signaling, and apoptosis, might lead to defective PCs, increase susceptibility to infection and/or IFN-/TNF insults, or even to cell death, in some cases specifically in PCs.

PCs in disease

Paneth cell (PC) dysfunction or reduced numbers per crypt have been observed in several inflammatory, infectious, or rejection diseases. CD-associated *E. coli* is able to penetrate deep in the intestinal crypts, but the link between reduced numbers of PCs, PC function, or increased resistance of the bacteria to α-defensins is not yet clear. Enterotoxigenic *E. coli* (ETEC) infection led to ileal inflammation and fewer PC markers (Ren *et al*, 2014). Mortality of ETEC-infected mice was about 30% after 24 h, and PC markers (measured by qPCR) were reduced in the ileum (Liu *et al*, 2017a). In a mouse model of *S. typhimurium* infection, PC death was associated with bacterial translocation to the spleen. The PC death was inhibited by treatment with *Lactiplantibacillus plantarum*. This effect of *Lpb. plantarum* might have been due to inhibition of pathogen colonization by competitive exclusion or mediated by TLR4/NF-κB activation (Ren *et al*, 2022).

Recent studies have shown that abnormal PC morphology and/or decreases in α-defensins are present in 50% of pediatric CD patients (Perminow *et al*, 2010; Liu *et al*, 2018). As dysfunction in autophagy-related genes trigger PC dysfunctions, it is not surprising that autophagy defects have been correlated with PC aberrations in CD patients (Table 3). Investigators recently reported that abnormalities in PCs increase the risk of ileitis and CD, and consider PCs as central players in the ileal chronic inflammation in CD patients (Wehkamp & Stange, 2020; Cray *et al*, 2021). Some genes (e.g., *Nod2*, *Atg16l1*, *Atg5*, *Xbp1*, and *Lrrk2*) described in Table 3, are identified as risk factors for CD. Moreover, ileal CD patients carrying mutations in some of these genes (e.g., *Atg16l1*, *Nod2*, and *Lrrk2*) have reduced expression of α-defensins or PC abnormalities. These results support the idea that PC functional problems are the initiators of ileal CD in patients. Moreover, in some cases those effects might be aggravated by non-specific consequences of an environmental trigger (e.g., diet, antibiotics, and tobacco) (Wehkamp *et al*, 2007; Stappenbeck & McGovern, 2017). Yet, it is still debated if, and if so, how PC dysfunction can affect inflammation in the colon. A possible explanation is that in healthy conditions, functional α-defensins from the ileum are found in the colon in an active form, where they can exert an effect (Mastroianni & Ouellette, 2009). Moreover, metaplastic PCs in the colon in IBD are considered as a repair mechanism and a useful marker of the disease (Tanaka *et al*, 2001).

Paneth cells have also been identified as sensors of viral infection. A study in male rhesus macaques infected with simian immunodeficiency virus (SIV) showed a correlation between epithelial damage and induction of IL-1b expression by PCs preceding the antiviral interferon host response (Hirao *et al*, 2014). That study reported changes in the microbial composition of the macaques (after the infection) that may promote opportunistic enteric bacterial infections (Zaragoza *et al*, 2011). Moreover, a recent study with transmissible viral gastroenteritis highlighted the negative impact of PC loss, caused by the infection, and the inhibition of Notch factors secretion on ISC self-renewal and differentiation (Wu *et al*, 2020).

In human obese patients, a shift in the microbiome, activation of the UPR in the gut, and reduced HD5 and LYZ in PCs were observed (Hodin *et al*, 2011b). Moreover, PC numbers were reduced in a human model for ischemia/reperfusion (Grootjans *et al*, 2011). In mice, clear relations between pathology, reduced PC number, and dysbiosis have been found in a model of GVHD. The rejection of tissue in the mouse model led to changes in microbiota composition. In GVHD, a convincing role of PCs in the pathology has been demonstrated, because PC numbers were greatly reduced in GVHD mice, declining from five to six PCs per crypt to just one PC per crypt. Then, *E. coli* colonization in the gut increased, followed by tissue invasion and death. The data demonstrated, indirectly, the critical role of PCs in controlling the pathological impact of graft-initiated inflammation and immune reactions (Ara & Hashimoto, 2021). Cytokines such as IL22 or proteins like R-spondin-1 showed success in preventing dysbiosis and improving PC status in *in vivo* models of GVHD (Takashima *et al*, 2011; Hanash *et al*, 2013; Hayase *et al*, 2017).

Other cytokines

Takahashi *et al* (2008) were among the first to report that PCs can produce IL17 under certain inflammatory conditions, like TNF challenge. IL17 production was shown to be PC-specific in the gut (Takahashi *et al*, 2008). This increase in TNFα-induced IL17 drives further the intestinal inflammation and is responsible for the damage observed in the small intestine (Takahashi *et al*, 2008). Others showed in mice that alcohol led to ER stress-mediated IL17 production in PCs, which increased apoptosis and permeability, leading to bacterial translocation. Normality was restored by antibody blockage of IL17A (Gyongyosi *et al*, 2019). Moreover, the effects of PC-induced IL17A have been linked to multiorgan dysfunction (Takahashi *et al*, 2008; Park *et al*, 2011, 2012). Remarkably, PCs, and not Th17 cells, have been described as the main source of IL17A, and this was also shown by the decrease of IL17A after PC ablation (Park *et al*, 2011; Gyongyosi *et al*, 2019). On the contrary, the use of IL17A inhibitors in CD and ulcerative colitis patients increased the adverse effects (Hueber *et al*, 2012). Since IL17 receptors are ubiquitously expressed, IL17 produced by PCs could be considered as a locally produced product that has systemic amplifying activities and might be considered as a therapeutic target in systemic diseases.

The roles of other cytokines, such as IL22, in PC maturation and function were recently studied (Mühl & Bachmann, 2019; Gaudino *et al*, 2021; Chiang *et al*, 2022). Early observations showed that loss of IL22 in the PCs of mice significantly compromises AMP production. A recent study published in Nature highlighted the crucial STAT3-dependent role of IL22 in crypt regeneration and PC maturation, and pointed to the activation in PCs of IL22–STAT3 signaling response after adherent-invasive *E. coli* infection (Chiang *et al*, 2022). These findings have been confirmed in organoids: production and secretion of LYZ, as well as other PCs markers, increased after IL22 supplementation (Zwarycz *et al*, 2019; Gaudino *et al*, 2021). In addition, IL22 alleviates high-fat diet effects by improving the status of PCs and increasing the production of AMP (Gaudino *et al*, 2021). Moreover, IL22 reverted the decrease of Reg3γ in PCs in a mouse model of GVHD (Zhao *et al*, 2018). However, studies on organoids

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have shown that persistent IL22 signaling can negatively impact PCs by increasing ERS, leading to a decrease in the numbers of ISCs and PCs (Zhao *et al*, 2018). Downstream of IL22–STAT3 activation is an IL18–IFNγ cascade, which might contribute to host defense against infections such as by adherent-invasive *E. coli*. Cooperation between IL22 and IL18 in a coordinated inflammatory response has been previously described in other cell types (Mühl & Bachmann, 2019). While IL22 seems to activate the cascade in response to an infection, IL18 is indispensable for the PC response and homeostasis. $IL18^{KO}$ mice displayed fewer LYZ-containing PCs and appeared to be more sensitive to bacterial infections. Moreover, organoids increased their AMP production when stimulated with IL18 (Chiang *et al*, 2022). However, there is no agreement about the role and the expression of *Il22r* and *Il18r* in PCs. Gaudino *et al* (2021) showed that PC maturation and functions depend on cell-intrinsic IL22Ra1 signaling, and this PC-specific IL22Ra1 signaling provide immunity against *S. typhimurium* (Gaudino *et al*, 2021). However, Chiang *et al* (2022) showed that the expression of *Il22r* is specific to Lgr5+ ISCs, and that the expression of *Il18r* is clearly higher in PCs (Chiang *et al*, 2022). Due to the participation of IL18 and IL22 receptors in PC antimicrobial response and homeostasis, it is important to unravel their expression profiles and mechanisms of activation.

PCs and aging

Epithelial homeostasis also depends on the balance of the ISC response between self-renewal and differentiation. The correct management of this continuous cell renewal is crucial for the intestinal epithelium's functions in absorption of nutrients and hormone secretion, while discriminating between commensal and pathogenic microbes.

In the small intestine, a decline in mucosal renewal during aging might lead to a defective immune system and an increased susceptibility to infections (Pentinmikko & Katajisto, 2020). Several authors have reported that the regenerative potential of human and mouse intestinal epithelium decreases with age due to defects in ISCs and in their niche (Nalapareddy *et al*, 2017). As stem-cell-niche supporters, PCs play a key role in tissue regeneration, regulating the number and function of ISCs by secreting niche-signaling factors, and their metabolism by producing lactate (Sato *et al*, 2011a; Rodríguez-Colman *et al*, 2017). Several studies have reported that during aging, the size and cellular composition of the crypts changes (Pentinmikko & Katajisto, 2020; Nalapareddy *et al*, 2022). Notably, the number of PCs increases in aged mice and humans, but conflicting results were reported on Lgr5+ cell numbers (Moorefield *et al*, 2017; Nalapareddy *et al*, 2017; Mihaylova *et al*, 2018; Pentinmikko & Katajisto, 2020). *In vitro* studies on aged ISCs cells and crypts from aged humans and mice have shown impaired regeneration. The impairment and reduction of the regeneration activity of ISCs in aging might be due to an increased expression in *Atoh1*, and a decrease of both *Olfm4* and *Notch1* compared with young ISCs (Nalapareddy *et al*, 2022).

Experiments on organoid cultures were used to establish the role of young and old PCs in the maintenance of the niche homeostasis (Nalapareddy *et al*, 2017; Pentinmikko *et al*, 2019). Generation of organoids from isolated ISCs and PCs have shown that the signals specifically from aged PCs compromised the niche function, probably due to reduced canonical Wnt signaling, that might be related with PC dysfunction (Pentinmikko *et al*, 2019). However, little is known about the overall function and morphology of old PCs. Differences in gene expression has been reported between old and young PCs, such as the reduced expression of *Wnt3a* (Nalapareddy *et al*, 2017). Indeed, *in vitro* supplementation with Wnt3a to aged human and murine organoids improved regeneration of old epithelia (Nalapareddy *et al*, 2017). This functional decline in stemness-maintaining Wnt signaling may be due to the production of Notum, a negative extracellular Wnt regulator produced only in aged PCs (Yilmaz *et al*, 2012). One of the main reasons for this increase in Notum activity might be the activation of mTORC1. Mechanistically, high activity of mTORC1 in aged PCs inhibits the activity of peroxisome proliferator-activated receptor α (PPARα), which ultimately leads to an increase in the expression of *Notum* (Pentinmikko *et al*, 2019). The importance of Notum was confirmed *in vitro* and *in vivo* by using ABC99, an inhibitor of Notum, which restored the Wnt-mediated PC and ISC functions, leading to increased regeneration activity in old crypts comparable to those from young mice (Pentinmikko *et al*, 2019). However, little is known about the functional effect of the bias towards the production of more PCs on the intestinal crypts and the influence on tissue regeneration (Nalapareddy *et al*, 2022). Why do aged intestines need more PCs? And what is their function in aged subjects?

Another explanation for alteration in PC numbers during aging might also be the lowered Zn availability. Several studies have shown that aging in people is associated with a decrease in blood Zn levels (Meunier *et al*, 2005). This may be partly due to changes in dietary choices and feeding behavior but could also be a reflection of reduced uptake of Zn from the food and/or the availability of the intracellular ionic Zn (Mocchegiani *et al*, 2011). As PCs are believed to be the main cells responsible for heavy metal uptake, an imbalance in PC status during aging may be a possibility. Moreover, changes in gut microbiota have been observed upon aging (Claesson *et al*, 2011). The changes in microbiota composition might affect PCs, or the increase in PCs upon aging might be related to the changes in the microbiota. Alternatively, the intestine might be trying to keep the niche homeostasis by producing more PCs, which could lead to increased secretion of AMPs.

Fasting and diet

Environmental factors such as diet can also trigger PC dysfunction or increase PC activity. In recent years, multiple studies have covered the crosstalk between diet, commensals, ingested bacteria and their byproducts, and their effect on the immune system. Diet can affect the function and number of PCs either directly or indirectly through the microbiota. The impact of diet (high-fat diet, Western diet, Zn deficiency, alcohol consumption, or others) or starvation on the status of PCs is summarized in Table 4 and Fig 6A–C.

High-fat diet induced PC defects aggravates DSS-induced colitis. Moreover, the PC defects induced by high-fat diet/Western diet can be reverted by the inclusion of vitamin D, inulin, sodium butyrate, or the apoA-I mimetic peptide 6F (Tg6F), by increasing the expression of α-defensins, or by restoring intestinal tight junctions (Su *et al*, 2016; Beisner *et al*, 2021; Mukherjee *et al*, 2022). Consumption of antibiotics or unhealthy lifestyles, also lead to changes in microbiota and PCs. Treating mice with certain antibiotics leads to decreased expression of typical PC markers (such as *Lyz1*, *Reg3γ*, and *Defa5*), suggesting that gut microbes are important modulators of PC function and

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Table 4. The impact of diet on PCs and gastrointestinal function.

Diet	Effect on Paneth cells	Outcome of the diet	Factors reinforcing the effect of the diet on Paneth cells	References
Alcohol	Reduced expression of antimicrobial peptides (α-defensins) Decreased density and size of Paneth cell granules	Increased bacterial translocation towards the liver Reduced antimicrobial activity of crypts Dysbiosis Outcome reversed by synthetic HD5 Treatment	MMP7 KO mice (α-defensin-deficient mice) Zinc deficiency	Purohit *et al* (2008), Zhong *et al* (2020)
Western diet	Paneth cell dysfunction	Reduced intestinal barrier function Dysbiosis		Liu *et al* (2021)
High-fat diet	Reduced expression of antimicrobial peptides (α-defensins and RegIIIγ) Increased Paneth cell death	Impaired intestinal barrier function Dysbiosis	Vitamin D receptor KO mice	Su *et al* (2016), Guo *et al* (2017), Lee *et al* (2017)
Oxidized n-3 polyunsaturated fatty acids (n3-PUFA)	Decreased Paneth cell numbers in the duodenum compared to unoxidized n3-PUFA	Oxidative stress and inflammation in the upper intestine		Awada *et al* (2012)
Arginine supplementation	Increased expression and secretion of AMPs	Boost of innate immune response in the small intestine		Ren *et al* (2014)
Ketogenic diet	Increased Paneth cell numbers and activity	Stimulates differentiation in the small intestine via 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2)/ and the ketone body β-hydroxybutyrate (βHB)		Wang *et al* (2017b)
Caloric restriction	Decreased mTORC1 activity in Paneth cells	Paneth cell produced cyclic ADP ribose promotes self-renewal of intestinal stem cells		Yilmaz *et al* (2012)
Starvation	Decreased expression and secretion of antimicrobial peptides (α-defensins, Lysozyme and RegIIIγ) Aberrant granule formation Increased autophagy	Increased intestinal permeability Increased bacterial translocation towards mesenteric lymph nodes		Hodin *et al* (2011a)

should be considered when placing patients on antibiotics for a long time. Moreover, in CD patients, the combination of tobacco smoking and mutations in *Atg16l1* gene triggers PC defects and apoptosis (Liu *et al*, 2018). As mentioned above, PCs are considered Zn-dependent and several studies have linked Zn deficiency and genetic deficiencies of Zn transporters with PC abnormalities (Podany *et al*, 2016). Also, Adding $ZnSO_4$ to the drinking water for a week protects PCs against the damage caused by TNF (Souffriau *et al*, 2020).

Diet can influence the health of PCs, and an imbalanced or inappropriate diet can lead to dysbiosis or aggravation of intestinal diseases such as CD and experimental DSS. Further research on how diet can influence PCs is needed.

PC tools

The small number of PCs and their location in the crypts make it difficult to study them. The development of new tools, including PC-specific mouse lines, single-cell RNA sequencing, and PC purification and development of organoid systems have expanded our knowledge in PC biology.

PC-specific transgenic tools

Several gene promoters are active exclusively in PCs. Such promoters are ideal for expressing or knocking out genes of interest in PCs *via* transgenic techniques. The most important features of such promoters are that they should be specific for PCs (no ectopic expression) and allow sufficient expression of the gene of interest.

The gene of interest is cloned behind a PC-specific promoter (several such promoters have been used), and the construct is injected in zygotes of mice. The mice are then selected for integration and PC-specific expression. Since pronucleus injection in zygotes leads to random integration of the construct, the PC-specificity of the promoter might be less apparent. This method was used by Garabedian *et al* (1997) to deplete PCs for the first time in 1997, by using a mouse *Defa2* promoter and a Diphtheria toxin A (DTA) gene (CR2-tox176) construct. Overall, PCs were reduced by 82% (Garabedian *et al*, 1997; Sato *et al*, 2011a). Cell ablation using such genetic tools is elegant and more specific than the older dithizone injection technique (Sawada *et al*, 1991) and it is also more persistent (Lueschow *et al*, 2018). In 2013, a Nature paper described a PC-specific cre line generated with a *Defa6*-promoter-driven cre construct (Adolph *et al*, 2013). Similarly, a *Defa4*-promotor-driven cre construct has been generated (Burger *et al*, 2018). Another PC cre line was created via

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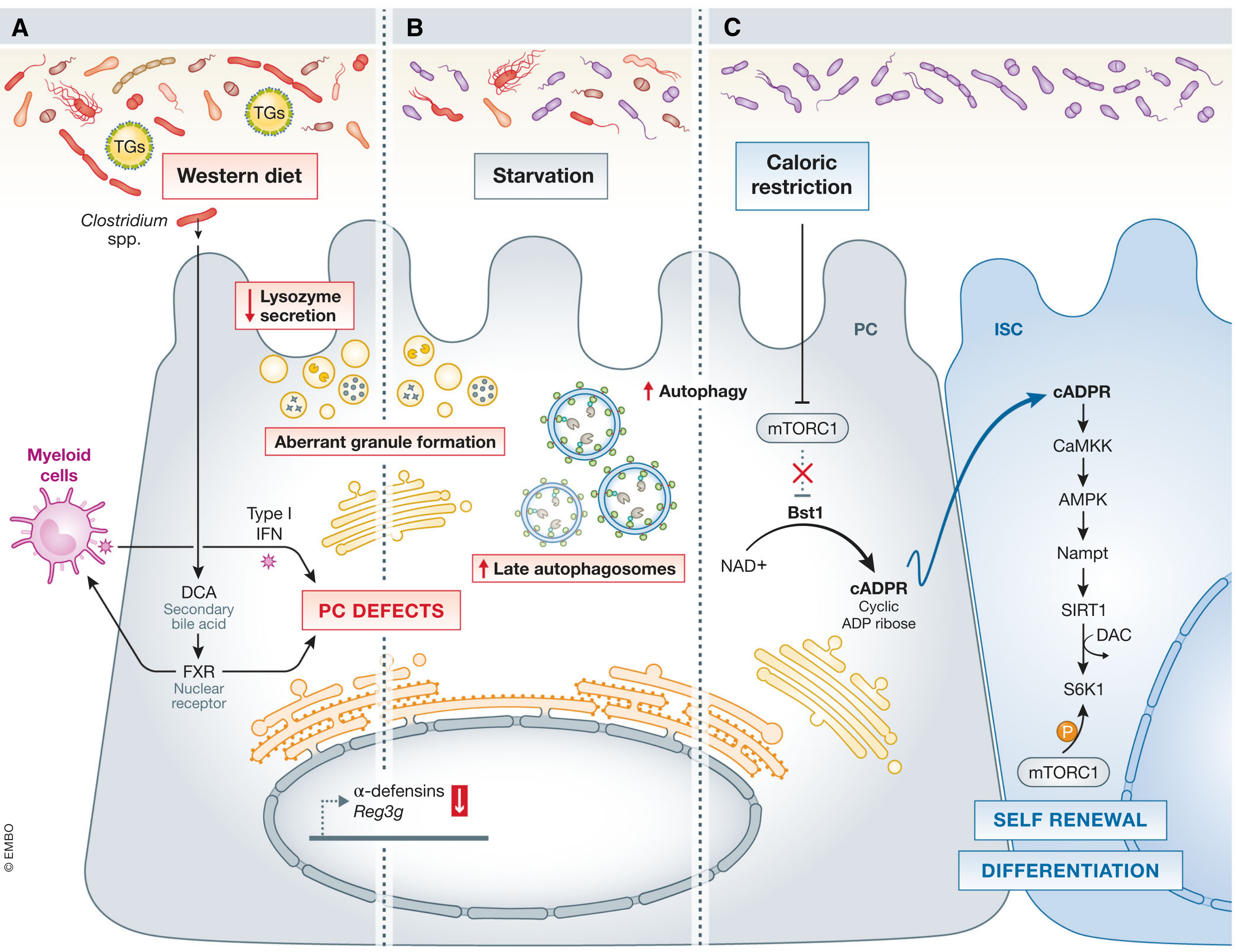

Figure 6. Effects of Western diet, starvation, and caloric restriction on PCs.

(A) The consumption of a Western diet leads to defects in PCs *via* the secondary bile acid deoxycholic acid (DCA), which is converted from cholic acid (CA) by commensal *Clostridium* spp. This increase in DCA enhances farnesoid X receptor (FXR) signaling, which also modulates the release of type I IFN by myeloid cells. Both FXR and type I IFN pathways are essential in triggering PC defects (B) Starvation has been linked to an increase in autophagy, with abundant late autophagosomes and PC defects, decreased AMP expression, and formation of aberrant granules. (C) Caloric restriction promotes ISC renewal and proliferation by inhibiting mTORC1 in PCs. mTORC1 repression enhances Bst1 and the conversion of NAD+ in cyclic ADP ribose (cADPR), which activates the AMPK/Nampt/SIRT1 pathway in ISCs. The activation of SIRT1 triggers the deacetylation of S6K1, which is further phosphorylated by mTORC1. These events are responsible for self-renewal and differentiation in the intestinal crypts. Black arrows – pathways that are active in PCs, red arrows – up- or downregulation" of factors that negatively affect PCs function.

homologous recombination of CreER in the *Lyz1* locus of embryonic stem cells (Van Es *et al*, 2019). Crossing a PC-specific cre mouse with a reporter mouse, a mouse with floxed alleles, or a floxed-STOP DTA receptor mouse, produces mice with colored PCs, PC-specific KO of the floxed alleles, or mice with PC depletion after the injection of DTA, respectively (Shankman *et al*, 2021).

Tools to increase the number of PCs have also been developed. Hayase *et al* (2017) demonstrated that injection of mice with R-spondin1, a growth and differentiation factor of PCs, led to crypt hyperproliferation and more than double the number of PCs per crypt (Hayase *et al*, 2017). The concentrations of DEFA1 and DEFA4 protein in the mouse feces was increased, and hence these two peptides might be considered as PC biomarkers. Moreover, oral supplementation of mice with certain probiotic strains, such as *Lactobacillus casei* and *Lactococcus paracasei*, led to increases in PC numbers in mouse crypts associated with increased antimicrobial activity against pathogens (Cazorla *et al*, 2018).

Single-cell sequencing data of PCs

Single-cell RNA sequencing (scRNA-seq) on the small intestine of mice and humans gave considerable information about cellular diversity and interesting insights in PCs and other intestinal cell types (Grün *et al*, 2015; Haber *et al*, 2018; Wang *et al*, 2020). Grün *et al* (2015) performed scRNA-seq on organoids of mice and developed an algorithm (RaceID) to identify rare cell types in complex populations (Grün *et al*, 2015). Haber *et al*, 2018 were the first to

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perform scRNA-seq on cells extracted from the small intestine of mice and listed the signature genes for each identified IEC type. For PCs, they identified 82 signature genes, 12 of them being α-defensins, three cryptdin-related sequence peptide-coding genes, and some known PC markers (e.g. *Lyz1*). Haber *et al*, 2018 identified in this way a new PC-marker, *Mptx2*, which colocalized *via* ISH with LYZ in the PCs, and nowhere else in the small intestine. Furthermore, they identified 2 PC subtypes in the small intestine. These two populations represent regional variation as all the marker genes from the first subtype were enriched in PCs of the ileum, while 70% of the PC marker genes of the second subtype were enriched in duodenum and jejunum. The first subtype expressing more α-defensins, suggesting that PCs located in the ileum are more antimicrobial active. Single cell data on sorted PCs could learn us more about the intrinsic variability of these cells, for example, identifying heterogeneous PC populations or PCs in a different differentiation status, as was performed on sorted mucin2 (MUC2)-positive goblet cells with the use of a MUC2 reporter mouse line (Nyström *et al*, 2021). In this study several distinct goblet cell populations (after the removal of MUC2-expressing PCs), correlating with different functions, were identified. The authors described a subpopulation of goblet cells, with a clear microbial defense profile (e.g., antimicrobial genes), suggesting at least a partial overlapping gene signature between goblet cells and PCs (Nyström *et al*, 2021).

In 2020, a scRNA-seq experiment was performed on three compartments of the human gastrointestinal tract, ileum, colon, and rectum (Wang *et al*, 2020), which shed more light on the differential nutrient absorption functions in the different compartments. Moreover, the authors identified Paneth-like cells in the large intestine that shared a group of highly expressed genes with PCs. These genes encode some AMPs (e.g., *LYZ*), genes that serve as niche factors for the ISCs (e.g., *EGF*, *WNT3A*, and *Notch*) and transcription factors involved in differentiation. However, Paneth-like cells in the large intestine, unlike PCs, do not express *HD5*, *HD6*, *REG1A*, and *REG3A*, suggesting a more prominent role of PCs in microbial defense. However, another recent scRNAseq performed on different compartments of the human gut shows that *LYZ* expression is not unique to PCs in the gut, and that PCs are devoid of some niche factors for ISCs (e.g., *EGF* and *WNT3A*) (Burclaff *et al*, 2022).

Purification of PCs and organoids

Before 2009, PC studies were practically limited to *in vivo* studies (e.g., antimicrobial activity assays, microscopy, and qPCR on PC markers) as no culture system was available. Clevers *et al* published in 2009 a new 3D *in vitro* culture system containing a mix of all the differentiated IECs found in the small intestine, named mini-gut organoids or enteroids (Sato *et al*, 2009). A major advantage of such models is that specific growth factors can be added to skew the differentiation to the desired lineage. Differentiation of organoids can be forced towards PCs by adding a notch inhibitor (DAPT) and an inhibitor of GSK3β-mediated β-catenin degradation (CHIR99021) (Treveil *et al*, 2020). This is an elegant model to study PCs in the context of other epithelial cells, but it is not possible to study direct effects exclusively on PCs. A 2D monolayer culture method starting from porcine organoids has also been described. It consists of granular PCs and other cell types. This method has the advantage that the apical surface is easily accessible, and that it has high TEER values (1000 Ω/cm^2), making it of interest for testing the effect of drugs and nutrients on epithelial membrane integrity (van der Hee *et al*, 2018).

A protocol to purify PCs was initially described by Clevers' group (Sato *et al*, 2011a) and further optimized (Schmitt *et al*, 2018). Briefly, crypts are purified from the small intestine and dissociated to obtain a single-cell suspension. Single cells are then stained for two PC makers, CD24 and C-kit, and hematopoietic lineage antibodies (CD31, CD45, and Ter119). PCs are gated on $CD24^+$C-$kit^+SCC^{Medium\text{-}High}$ $CD31^-CD45^-TER119^-$ cells and sorted. The sorted PCs can be kept in culture for a short time or used to perform RNAseq (Yu *et al*, 2018). Another way to study or sort PCs is by using PC-specific fluorescent reporter lines.

Laser capture dissection is another method that can be used to isolate a region tissue of interest (e.g., PCs from small intestinal cryosections) with a laser beam. This method enables the study of RNA expression in PCs (Vaishnava *et al*, 2008). There is still no (immortalized) PC line commercially available, hampering *in vitro* studies on pure PCs.

Pending issues

i Genetic disorders, environmental factors, and diet have been linked to Paneth cell dysfunctions in mice. However, these factors have not been adequately studied in human samples or biopsies.

ii Studying Paneth cells in human patients is problematic because it requires invasive biopsies. A good biomarker that represents the health state of Paneth cells and can be measured in body fluids (urine or blood) or feces would therefore be of value.

iii The challenges in Paneth cell studies relate to their limited number and deep location. Establishing a pure Paneth cell-line would make it possible to directly study the effects on Paneth cells and to use them as an assay for screening compounds.

iv Different inbred mouse strains under identical housing conditions differ in the number of Paneth cells, the expression of antimicrobial peptides and in α-defensin genes and proteins. Moreover, these strains are kept in captivity over many decades, making them vulnerable to genetic drift. To better mimic the human situation, Paneth cell studies on wild mice or wildling mice might be more relevant, but they are also more complex.

Conclusions

Paneth cells play a key role in preserving gut homeostasis. Their decisive functions include (i) providing niche factors to the ISC compartment, (ii) regulating the composition of the microbiome by producing AMPs, (iii) dampening inflammation by phagocytosis and efferocytosis, (iv) uptake of heavy metals, and (v) preserving barrier integrity. Dysfunction of PCs can disturb one or more of these functions, leading to an imbalance in the gastrointestinal tract. Decreased or dysfunctional PCs, followed by escape of bacteria towards different organs, is often observed in several diseases, such as infectious diseases, IBD (CD and ulcerative colitis) and GVHD. Genetic disorders or environmental triggers can cause ERS or autophagy deficits in PCs, which are clearly linked with intestinal

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diseases. The challenges in PC studies relate to the location of PCs and their limited number. In particular, the lack of a pure PC line to study direct effects is a major impediment. Until recently, PC expression studies have relied on ISH or the use of PC-specific markers on ileum biopsies. Fortunately, PCs can now be sorted to study the expression signature of pure PCs in different (disease) models. Moreover, the PC-specific cre tool has recently been used to confirm earlier PC results obtained in villin^{KO} models. The recent development of new tools and techniques has led to a more rapid expansion of knowledge on PCs.

Author contributions

Charlotte Wallaeys: Writing—original draft; writing—review and editing; figures and tables—design and original draft, figures and tables—review and editing. **Natalia Garcia Gonzalez:** Writing—original draft; writing—review and editing; figures and tables—design and original draft, figures and tables—review and editing. **Claude Libert:** Writing—original draft; writing—review and editing.

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