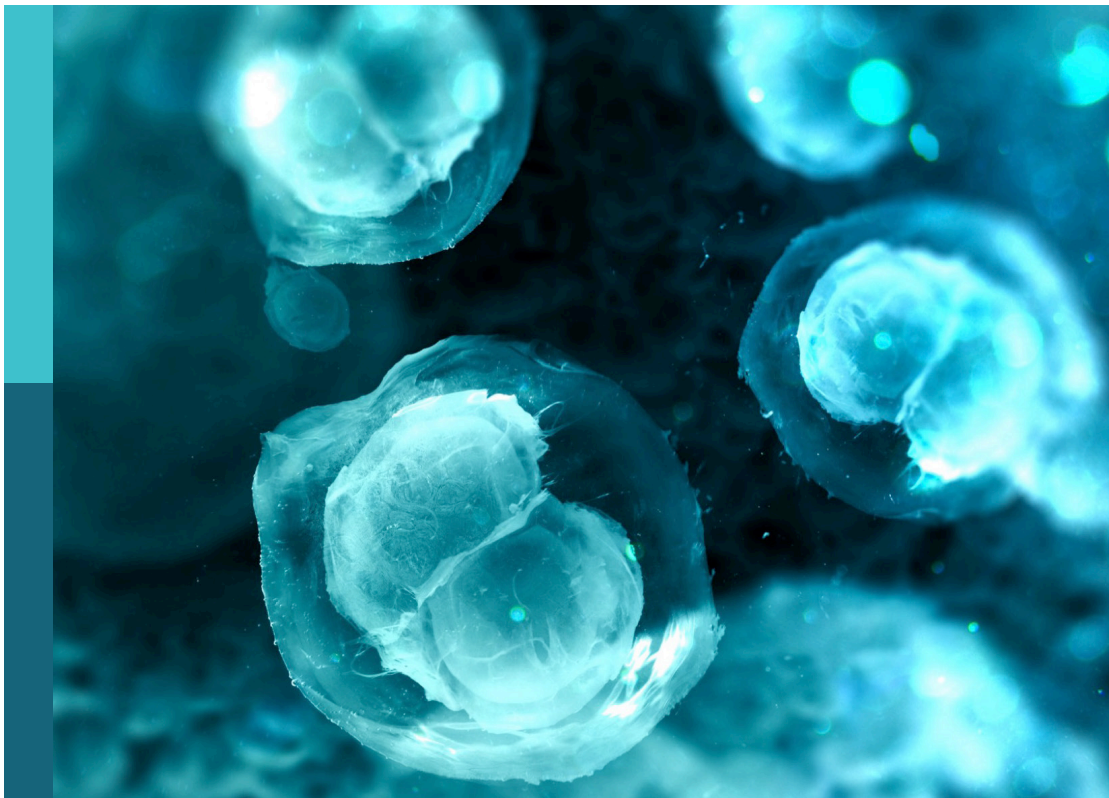
frontiers | Research Topics

Epithelial barrier dysfunction in disease

Edited by
Carlos Pardo-Pastor, Rocío López Posadas
and Elena Ortiz Zapater

Coordinated by
Dustin Bagley

Published in
Frontiers in Cell and Developmental Biology
Frontiers in Medicine



ISSN 1664-8714
ISBN 978-2-8325-4669-7
DOI 10.3389/978-2-8325-4669-7

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Epithelial barrier dysfunction in disease

Topic editors

Carlos Pardo-Pastor — Randall Centre for Cell & Molecular Biophysics, School of Basic & Medical Biosciences, Faculty of Life Sciences & Medicine, King's College London, United Kingdom

Rocío López Posadas — University Hospital Erlangen, Germany

Elena Ortiz Zapater — University of Valencia, Spain

Topic coordinator

Dustin Bagley — King's College London, United Kingdom

Citation

Pardo-Pastor, C., Posadas, R. L., Zapater, E. O., Bagley, D., eds. (2024). *Epithelial barrier dysfunction in disease*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-4669-7

Table of contents

frontiers | Frontiers in Cell and Developmental Biology

TYPE Mini Review
PUBLISHED 10 July 2023
DOI 10.3389/fcell.2023.1225677

OPEN ACCESS

EDITED BY
Carlos Pardo-Pastor,
King's College London, United Kingdom

REVIEWED BY
Sharon Celeste Morley,
Washington University in St. Louis,
United States

*CORRESPONDENCE
Andrew G. Clark,
andrew.clark@srcsb.uni-stuttgart.de

RECEIVED 19 May 2023
ACCEPTED 27 June 2023
PUBLISHED 10 July 2023

CITATION
Saha S, Müller D and Clark AG (2023), Mechanosensory feedback loops during chronic inflammation.
Front. Cell Dev. Biol. 11:1225677.
doi: 10.3389/fcell.2023.1225677

Mechanosensory feedback loops during chronic inflammation

Sarbari Saha[1,2,3], Dafne Müller[1] and Andrew G. Clark[1,2,3]*

[1]University of Stuttgart, Institute of Cell Biology and Immunology, Stuttgart, Germany, [2]University of Stuttgart, Stuttgart Research Center Systems Biology, Stuttgart, Germany, [3]University of Tübingen, Center for Personalized Medicine, Tübingen, Germany

Epithelial tissues are crucial to maintaining healthy organization and compartmentalization in various organs and act as a first line of defense against infection in barrier organs such as the skin, lungs and intestine. Disruption or injury to these barriers can lead to infiltration of resident or foreign microbes, initiating local inflammation. One often overlooked aspect of this response is local changes in tissue mechanics during inflammation. In this mini-review, we summarize known molecular mechanisms linking disruption of epithelial barrier function to mechanical changes in epithelial tissues. We consider direct mechanisms, such as changes in the secretion of extracellular matrix (ECM)-modulating enzymes by immune cells as well as indirect mechanisms including local activation of fibroblasts. We discuss how these mechanical changes can modulate local immune cell activity and inflammation and perturb epithelial homeostasis, further dysregulating epithelial barrier function. We propose that this two-way relationship between loss of barrier function and altered tissue mechanics can lead to a positive feedback loop that further perpetuates inflammation. We discuss this cycle in the context of several chronic inflammatory diseases, including inflammatory bowel disease (IBD), liver disease and cancer, and we present the modulation of tissue mechanics as a new framework for combating chronic inflammation.

KEYWORDS

epithelial barrier, inflammation, immune cells, extracellular matrix, tissue mechanics, chronic inflammatory diseases, immuno-biophysics, immuno-mechanobiology

Introduction

Epithelial barrier tissues maintain a tight seal between the outside environment and the inside of the body. Loss of barrier integrity leads to local activation of immune cells and fibroblasts, which can remodel local ECM networks, the major determinants of tissue mechanics. Over time, these structural and molecular changes result in tissue stiffening (Barron and Wynn, 2011; Chrysanthopoulou et al, 2014; Curaj et al, 2020). During acute inflammation, increased tissue stiffness can be beneficial for regeneration and wound healing, for example, by enhancing immune cell activity and stimulating immune cell migration and infiltration (Sridharan et al, 2019; Gaertner et al, 2022; Millán-Salanova and Vicente-Manzanares, 2022; Nalkurthi et al, 2022). However, during chronic inflammation, modifications in local ECM networks can become permanent, leading to irreversible stiffening of the tissue and culminating in fibrosis (Jeljeli et al, 2019; Velotti et al, 2020).

Pathologically stiff tissue can promote immune cell recruitment and activation via mechanosensing pathways, leading to increased immune cell migration and differentiation and activation of fibroblasts (Chen et al, 2020; Atcha et al, 2021;

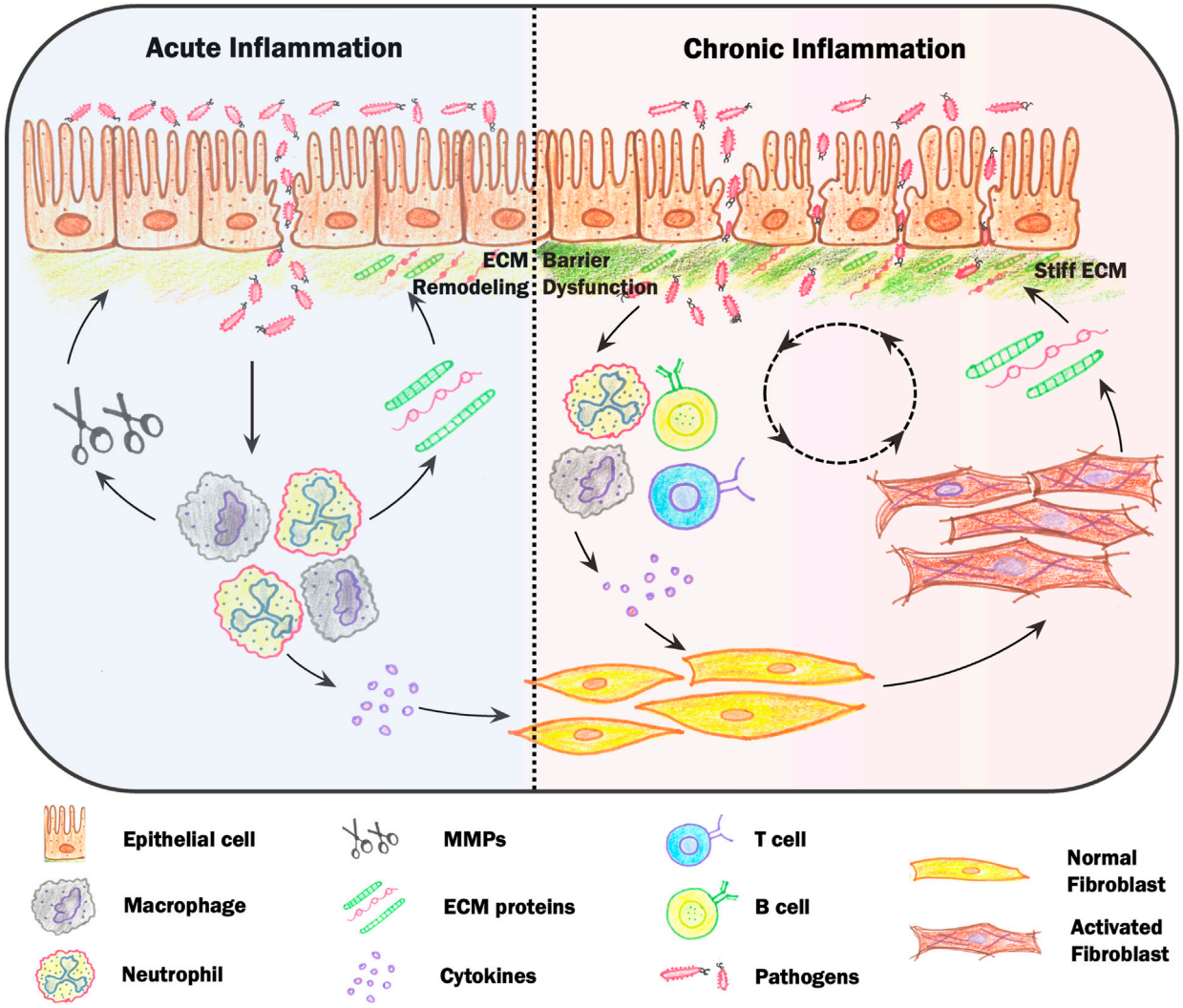


FIGURE 1
Mechanosensory feedback loops during inflammation. Loss of barrier integrity results in infiltration of microbes that initiates acute inflammation. Inflammation is led by neutrophils and macrophages, which release cytokines and chemokines and modify local extracellular matrix (ECM) structures by secretion of ECM proteins and matrix metalloproteinases (MMPs). Activated immune cells also stimulate fibroblasts, which secrete, assemble and physically remodel ECM networks, resulting in a stiffening of ECM networks. During chronic inflammation, pathologically stiff tissue can lead to over-activation of immune cells via mechanosensing pathways, resulting in increased immune cell migration and differentiation. Increases in tissue stiffness can also lead to epithelial cell depolarization, reduced cell-cell junctions and increased migration. While in the short-term this may aid in wound resealing, epithelial cells on stiff environments are less able to maintain a tight barrier, creating a positive feedback loop between increased barrier permeability and inflammation mediated by changes in tissue mechanics.

Chirivì et al, 2021; Jiang et al, 2022). Increased tissue stiffness also results in epithelial cell depolarization, reduced cell-cell junctions and increased migration (Discher et al, 2005; Aparicio-Yuste et al, 2022). While in the short-term this may aid in wound resealing, epithelial cells on stiff environments are less able to maintain a tight barrier, creating a feedback loop between increased barrier permeability and inflammation mediated by changes in tissue mechanics (Figure 1). Such mechanical feedback can ultimately disrupt organ function and presents a major risk factor for cancer development. Here, we discuss the molecular mechanisms that contribute to these feedback loops as well as pathologies where such mechanical feedback can play a role in disease progression.

Epithelial barrier disruption leads to inflammation and local ECM remodelling

Loss of barrier integrity leads to infiltration of microbes, initiating a cascade of immune reactions whereby neutrophils and monocytes are first recruited to the site of infection (Jenne et al, 2018; Herrero-Cervera et al, 2022). These first responders not only trigger inflammation by releasing cytokines and chemokines, but also modify local extracellular matrix (ECM) structures by secretion of neutrophil elastase (NE), cathepsins, gelatinases and matrix metalloproteinases (MMPs; Delclaux et al, 1996; Ong et al, 2015; 2017; Medeiros et al, 2017). These enzymes promote the

degradation of ECM components such as collagen, laminin, elastin, fibronectin and matrix bound glycoproteins (Ong et al, 2015; Xu et al, 2020). Activated neutrophils release exosomes and Neutrophil Extracellular Traps (NETs) rich in NE. NE-rich exosomes can bind to the ECM via the integrin Mac1 and degrade Collagen-I (Genschmer et al, 2019). NEs found in NETs have been shown to degrade cartilage matrix synovium, resulting in synovial joint injury (Carmona-Rivera et al, 2020). Collagenase and elastase treatment in lung tissues causes a loss and shortening of ECM fibers and decreases mechanical tissue stiffness by up to 50% (Mariano et al, 2023). In addition to degrading local ECM networks, neutrophils are also involved in tissue repair and scar formation. In response to liver injury, neutrophils physically transport existing ECM fibers to the wound site, leading to ECM accumulation at the site of damage (Fischer et al, 2022).

Similar to neutrophils, macrophages also produce and secrete various ECM-degrading enzymes (Sutherland et al, 2023). In addition, macrophages ingest and degrade ECM structures by integrin-mediated phagocytosis and receptor-mediated endocytosis (McKleroy et al, 2013; Zhao et al, 2022). On the other hand, macrophages also secrete ECM proteins including fibronectin, laminin and versican, which can help to provide a mechanical scaffold following injury and aid in the renewal of tissue architecture (Tomlin and Piccinini, 2018). Exposure to inflammatory cytokines including Transforming growth factor beta (TGF-β), Interleukin (IL)-10 and IL-13 can stimulate secretion of collagen-IV in macrophages (Schnoor et al, 2008). Differentiation of macrophages to myofibroblasts results in the production of fibrillar collagen during scar formation and ECM remodelling (Simões et al, 2020). Both macrophages and neutrophils are thus involved in degradation, production and remodeling of ECM networks and are crucial to maintaining a proper balance during homeostasis and regeneration.

When this balance is disturbed, for example, during chronic inflammation, macrophages and neutrophils can activate fibroblasts, which secrete, assemble and physically remodel ECM networks (Jeljeli et al, 2019). Culturing fibroblasts in conditioned medium from M2-like macrophages causes an increase in *Col5a1* and *Col6a1* production, leading to the production of thinner and more aligned collagen matrices. On the other hand, treating fibroblasts with hybrid M1/M2-conditioned medium results in the production of thicker, randomly oriented collagen networks. This suggests that shifting the phenotype of macrophages can promote architectural changes in the ECM via modulation of fibroblast activity (Witherel et al, 2021). In addition to molecular signals, physical cues from the microenvironment can also influence fibroblast-mediated ECM remodeling. When fibroblasts treated with M1/M2 conditioned medium are cultured on stiff substrates, they produce more aligned collagen networks compared to when they are cultured on softer hydrogels (Li and Bratlie, 2021). Fibroblasts also regulate their own activity via autocrine signaling. For example, during the inflammatory phase of myocardial infarction, activated fibroblasts produce and assemble fibrin and fibronectin and begin secreting TGF-β1, leading to a positive feedback loop of enhanced fibroblast differentiation, collagen synthesis and macrophage polarization. After reaching a stable state, a negative feedback loop is initiated, reducing TGF-β1 expression and resulting in completion of the mature scar (Curaj et al, 2020). Repeated injury and scarring can lead to a build-up of stiff fibrotic tissue that triggers fibroblasts to secrete more collagen, further driving the cycle of ECM deposition (Liu et al, 2010). Interestingly, a number of inflammatory conditions can also lead to tissue hypoxia, which, at least in tumors, can stimulate fibroblast-mediated collagen deposition and secretion of collagen-modifying enzymes including prolyl and lysyl hydroxylases (Gilkes et al, 2013). Together, these studies suggest that in various inflammatory conditions, activation of immune cells and fibroblasts leads to the reorganization of local ECM structures. During chronic inflammation, this results in a build-up of ECM and stiffening of the tissue, which can in turn stimulate immune cell activity via various mechanosensitive pathways.

Immune cell activation by mechanosensing pathways

The innate immune system forms the first line of defense against pathogens entering the body. Leucocytes involved in the innate immune response, or myeloid cells, including macrophages, dendritic cells and mast cells, are adherent and contact-dependent, making them sensitive to changes in tissue mechanics. In particular, increased substrate stiffness, which is a result of long-term chronic inflammation, leads to increased immune cell activation and secretion of inflammatory cytokines. Lipopolysaccharide (LPS)-Activated macrophages and bone-marrow derived dendritic cells (DCs) both display enhanced production of inflammatory cytokines when cultured on mechanically stiff substrates as compared to soft hydrogels (Meli et al, 2023). DCs cultured on stiff substrates also show increased expression of glucose metabolism genes and an overall increase in their glycolytic rate, suggesting that DCs are more metabolically active on stiff substrates (Chakraborty et al, 2021). Mast cells, which are implicated in pulmonary fibrosis, are also mechanosensitive. Reseeding of healthy mast cells onto decellularized fibrotic lung tissue leads to increased degranulation and secretion of histamine and TGF-β1 compared to mast cells reseeded on healthy decellularized lung. Mechanical stretching of mast cells can produce a similar phenotype, further implicating mechanosensing in this response (Shimbori et al, 2019). The regulation of immune cell activity by increased substrate stiffness and mechanical stress is mediated by various mechanosensitive pathways including Yes-associated protein 1 (YAP) and Transcriptional coactivator with PDZ-binding motif (TAZ). High substrate stiffness leads to increased stress on the nuclear envelope, resulting in the accumulation of nuclear YAP and activation of downstream targets (Elosegui-Artola et al, 2017). In addition to YAP/TAZ signaling, stretch-activated ion channels such as piezo type mechanosensitive ion channel component 1 (PIEZO1) and Transient Receptor Potential Cation Channel Subfamily V Member 4 (TRPV4) are also involved in mechanosensing responses (reviewed in Du et al, 2022). Together, these studies suggest that immune cells involved in the innate immune response are mechanosensitive and display pro-inflammatory phenotypes in response to increased mechanical stiffness.

Cells involved in the adaptive immune response are also mechanosensitive. In order to carry out their effector functions,

naïve B cells and T cells must first be activated, or "primed," by antigen presenting cells (APCs) such as DCs. Increased stiffness of substrates designed to mimic the APC cell surface has been shown to facilitate the activation of B cells, T cells and Natural Killer (NK) cells (Judokusumo et al, 2012; Comrie et al, 2015; Meng et al, 2020). Similarly, increased stiffness of the actomyosin cortex of antigen presenting DCs enhances T cell activation (Blumenthal et al, 2020). Experiments using optical tweezers or fluid flow have demonstrated that direct application of mechanical force on T cell receptors (TCRs) can induce T cell activation (Kim et al, 2009; Li et al, 2010). Although adaptive immune cell activation is clearly mechanosensitive, it is not clear how tissue stiffness influences adaptive immune cell activity. Furthermore, B cell and T cell priming typically occurs in lymph nodes, not in the inflamed tissue. The relationship between tissue stiffness and adaptive immune priming therefore remains an open question. However, recent studies have suggested that T cell migration, along with the migration of DCs and mast cells is increased on stiff environments (Meng et al, 2020; Yu et al, 2021). This suggests that increased tissue stiffness may enhance local immune activity by stimulating both innate and adaptive immune cell migration. Increased mechanical stiffness during inflammation not only affects immune cell activity but can also have an impact on epithelial barrier integrity by directly regulating epithelial cells.

Modulation of epithelial cell behavior by mechanical cues

The maintenance of epithelial barrier integrity is most commonly associated with tight junctions (TJs), which provide a tight seal at cell-cell boundaries and prevent the passage of materials across the epithelial layer. Recent work also suggests that adherens junctions (AJs) play a major role in epithelial integrity, either directly through mechanosensing pathways or by mediating TJ stability (Yap et al, 2018). A number of studies have demonstrated that both AJs and TJs are mechanosensitive in response to in-plane stresses arising from actomyosin contraction or external stretch, whereby moderate amounts of tensile stress led to junction reinforcement, while very high stresses cause epithelial tearing and rupture (Spadaro et al, 2017; Acharya et al, 2018; Schwayer et al, 2019). In addition to in-plane stresses, mechanosensing at cell-substrate adhesions can also affect cell-cell junction integrity. The balance between cell-cell and cell-substrate adhesions has been described as an "active wetting" phenomenon (Gonzalez-Rodriguez et al, 2012; Beaune et al, 2014; Pérez-González et al, 2019). For surfaces where cell-substrate adhesion is low, for example, very soft substrates, cell-cell adhesions dominate, leading to rounding and aggregation. This is analogous to water droplet formation on a hydrophobic surface, where liquid-substrate interactions are unfavorable and the surface tension of the droplet dominates. On substrates where cell-substrate adhesions are high, for example, on very stiff substrates, cell-substrate adhesions dominate, causing the multicellular structure to spread, or "wet" (Gonzalez-Rodriguez et al, 2012). Softer substrates therefore favor stable junctions and a tight barrier, whereas a stiff substrate favors more loosely attached cells and can also lead to dispersal into individual cells (Gonzalez-Rodriguez et al, 2012; Pérez-González et al, 2019; Ilina et al, 2020). In addition to mechanical wetting/dewetting resulting from the balance between cell-cell and cell-substrate adhesions, molecular cross-talk between different adhesion structures has also been shown to regulate cell-cell junction integrity in a substrate stiffness-dependent manner (Haas et al, 2020).

In addition to stabilization of junction proteins, efficient wound healing is a crucial aspect of tissue barrier maintenance. Wound healing requires cell migration to rapidly infiltrate the wound and actomyosin contraction to reseal the damaged area (Martin and Leibovich, 2005; Rodrigues et al, 2019). Higher substrate stiffness leads to faster wound closure mediated by increased collective migration speed and more coordinated cell movements. On stiffer substrates, actomyosin contraction slows down due to increased drag from the substrate, while crawling migration is independent of the substrate mechanics (Staddon et al, 2018; Ajeti et al, 2019). Other reports have suggested that higher stiffness can increase collective migration speeds and correlation in wound healing assays (Ng et al, 2012). It is likely that the dependence on stiffness is biphasic and highly cell-type dependent. In addition to elastic stiffness of tissues and cellular substrates, viscoelastic properties of ECM networks also influence coordinated cell movements. Crosslinking of collagen networks leads to increased network stiffness and reduces viscoelasticity, resulting in reduced collective migration (Murrell et al, 2011; Clark et al, 2022). Interestingly, changes in tissue viscoelasticity have also recently been shown to regulate collective cell behavior during development and cell invasion (Barriga and Mayor, 2019; Elosegui-Artola, 2021; Elosegui-Artola et al, 2023). Along with cell rearrangements to seal the wound, increased cell division is required to repopulate the wounded area. Substrate mechanics also regulates this process by modulating in-plane stresses generated during the resealing response, which can stimulate cell division (Zhang et al, 2003; Gudipaty et al, 2017; Donker et al, 2022). The mechanisms underlying the regulation of epithelial cell division and turnover in response to in-plane forces has been studied in several contexts (reviewed in Ragkousi and Gibson, 2014). Taken together, these studies indicate that increased substrate stiffness can perturb cell-cell junctions and cell polarity and impair the wound healing response. This suggests that the mechanical changes induced during inflammation can feed back onto epithelial cell function, resulting in further loss of barrier integrity.

Mechanosensing feedback loops in chronic inflammatory diseases

Mechanical feedback loops are likely to play a role in a number of chronic inflammatory diseases including IBD, liver disease and cancer. IBD is characterized by a cycle of increased intestinal barrier permeability and inflammation. Both immune cells and fibroblasts participate in ECM deposition and reorganization in IBD, leading to the onset of pathological tissue stiffening (Wang et al, 2022). Once tissue stiffening has begun, additional feedback mechanisms drive further tissue stiffening, leading to fibrosis and stricture formation (Figure 2A). During intestinal fibrosis, mast cell infiltration and degranulation leads to the release of large amounts of tryptase through the PAR-2/Akt/mTOR pathway, which converts fibroblasts into activated myofibroblasts. This results in deposition of collagen and fibronectin to promote intestinal fibrosis (Liu et al, 2021). Other recent work has suggested that ubiquitin-specific protease 2 (USP2), which is upregulated in

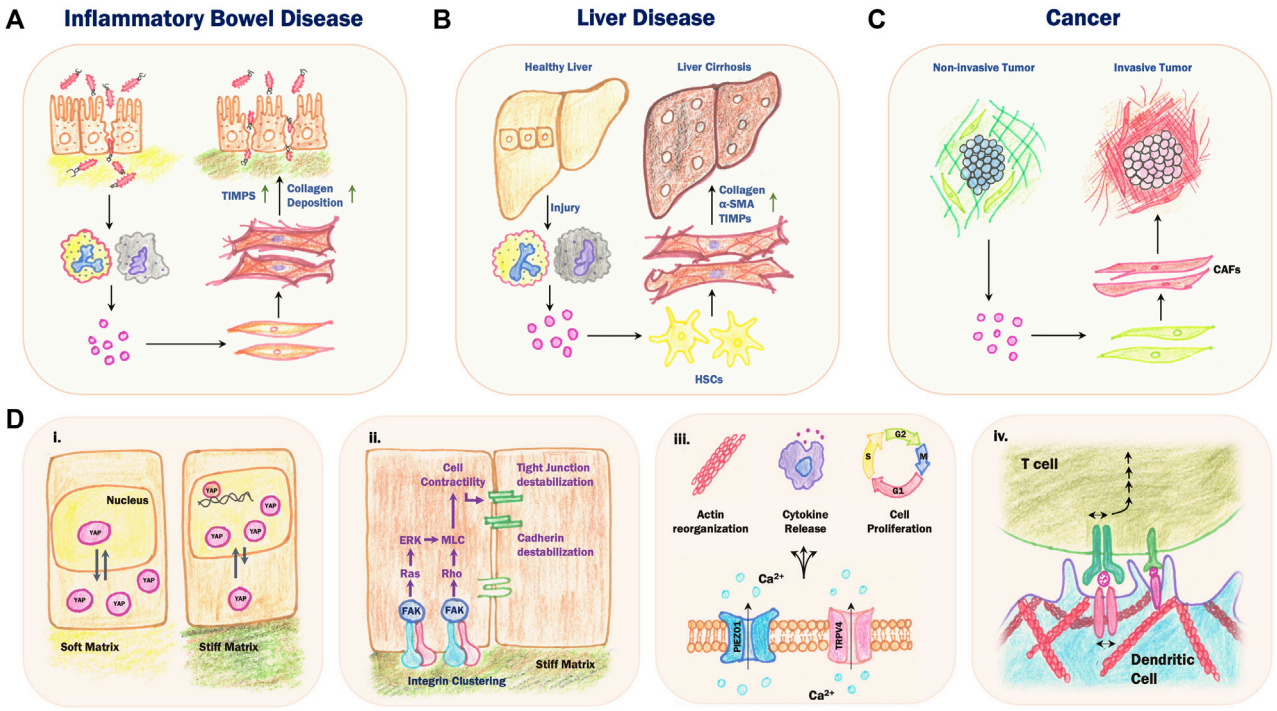


FIGURE 2
Mechanosensory feedback loops in chronic inflammatory diseases. **(A)** During Inflammatory Bowel Disease (IBD), a cycle of reduced intestinal barrier function and chronic inflammation results in increased collagen deposition and secretion of tissue inhibitors of metalloproteinases (TIMPs) by local activated fibroblasts, leading to stiffening of the underlying ECM and tissue fibrosis. **(B)** Persistent injury and inflammation in the liver results in the differentiation of hepatic stellate cells (HSCs) into activated fibroblast-like cells which secrete collagen and TIMPs and express α-SMA. This results in a replacement of the normal liver parenchyma with fibrotic scar tissue, eventually leading to fibrosis and cirrhosis. **(C)** The cross-talk between cancer cells and stromal cells leads to the activation of cancer-associated fibroblasts (CAFs), which secrete ECM proteins and matrix remodelling enzymes that contribute to increased stromal stiffness and invasion and metastasis. **(D)** Mechanochemical feedback during chronic inflammation involves various mechanosensing pathways. (i) Increased substrate stiffness results in mechanical stress on the nucleus, which inhibits nuclear export of yes-associated protein (YAP). In the nucleus, YAP acts as transcriptional coactivator to increase expression of downstream genes involved in cell proliferation and migration. (ii) High substrate stiffness leads to increased integrin clustering, which activates downstream signal transduction pathways leading to destabilization of cell-cell junctions. (iii) Mechanical stretching of the plasma membrane opens stretch-activated channels including PIEZO1 and TRPV4, leading to an influx of Ca^{2+} ions and several downstream effects including changes in actin dynamics, cytokine release and cell proliferation. (iv) Activation, or "priming", of B cells and T cells involves heterotypic binding of membrane receptors between the B or T cell and an antigen presenting cell such as a dendritic cell. Increased stiffness of the actomyosin cortex in the dendritic cell limits mobility of the membrane receptors, resulting in increased mechanical stress on the B or T cell receptor, which enhances the activation process.

intestinal myeloid cells during IBD and mouse models of colitis, increases the expression of collagen and alpha smooth muscle actin (αSMA), leading to further ECM remodeling and tissue stiffening (An et al, 2022). Collagen-I deposition in the intestine also activates the YAP/TAZ pathway in epithelial cells through Fak/Src signaling to initiate a regenerative cascade to induce a fetal-like state in the colonic epithelium, where cells become more motile and prone to reorganization compared to homeostatic conditions (Yui et al, 2018). Downstream effects of YAP/TAZ also induce the secretion of IL-33 and IL-18 and lead to cytoskeletal re-organization (Kobayashi et al, 2022). Together, these studies suggest that mechanical reorganization of ECM networks during IBD can drive further tissue stiffening, prolonged inflammation and reduced barrier function.

The liver is also exposed to various external stresses arising from dietary factors, exposure to gut microbe metabolites and alcohol and drug use, leading to tissue damage and inflammation (Lang and Schnabl, 2020; Yahoo et al, 2023). In the case of persistent inflammation, increased accumulation of ECM results in the replacement of healthy liver parenchyma with fibrotic scar tissue, further driving mechanosensitive feedback pathways (Figure 2B; Dhar et al, 2020). In mouse models of liver fibrosis, excess deposition of collagen and fibronectin along with accumulation of αSMA-expressing myofibroblasts leads to cirrhosis and increased expression of ECM genes, which correlates with poor patient prognosis (Wu et al, 2021). During this process, hepatic stellate cells (HSCs) transdifferentiate into fibroblast-like cells that express αSMA and secrete ECM components such as collagen-I and-III, fibronectin and laminin, contributing to the development of fibrosis (Friedman, 2008). HSCs also produce MMPs and Tissue Inhibitors of Metalloproteinases (TIMPs) which are the major drivers of ECM remodelling during hepatic fibrosis (Duarte et al, 2015). Chronic overexpression of TIMPs prevents normal collagen remodeling, leading to an increased collagen build-up that drives liver fibrosis (Benyon and Arthur, 2021). The resulting altered biomechanical environment can also drive liver tumorigenesis by activation of integrin-β1 and focal adhesion kinase, leading to increased cell proliferation (Schrader et al, 2011).

Chronic inflammation is a risk factor for tumorigenesis and cancer not only in the liver, but also in other tissues. The evolution of the tumor microenvironment shares many similarities with chronic inflammation, and tumors have been notably characterized as "wounds that never heal" (Dvorak, 1986; Hua and Bergers, 2019). One prominent feature of tumor progression is the cross-talk between tumor cell behavior and the increased stiffening of connective tissue surrounding the tumor (the "stroma"; Figure 2C). High stromal stiffness can lead to increased cytoskeletal activity and migration, reduced polarity and epithelial-mesenchymal transition (EMT; Clark and Vignjevic, 2015). Changes in stromal network architecture and mechanics are mediated primarily by cancer-associated fibroblasts (CAFs), which share many common features with activated fibroblasts during chronic inflammation. CAFs display increased secretion of cytokines, growth factors and matrix remodeling enzymes as well as increased mechanical force production (Sahai et al, 2020). Together, these factors drive changes in ECM organization that contribute to increased stromal stiffness, tumor invasion and metastasis. In addition, CAFs secrete proteases that cleave and activate ECM-bound cytokines and cell adhesion molecules, promoting increased migration of cancer cells and EMT (Fiori et al, 2019). The mechanical properties of the tumor stroma are also thought to contribute to immune escape mechanisms during cancer and could interfere with cancer immunotherapy (Denton et al, 2018; Ollauri-Ibáñez et al, 2021). Together, these studies suggest that similar to chronic inflammatory diseases, mechanosensory feedback loops can drive local tissue stiffening and cancer progression.

Conclusion and outlook

Disruption of epithelial barrier tissues leads to local inflammation and activation of immune cells and fibroblasts that modify local ECM structures. Repeated injury or chronic inflammation can lead to permanent ECM remodeling and tissue stiffening, which can further exacerbate inflammation, excess fibroblast activity and barrier disruption via various mechanosensing pathways (Figure 2D). Altered tissue mechanics represents a common and general feature of chronic inflammatory diseases, despite differences in the molecular profiles of these pathologies. Future translational studies aimed at modulating tissue mechanics therefore have the potential to identify exciting new therapeutic approaches with broad applications from chronic inflammation to cancer.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

AGC and SS are funded by the Federal Ministry of Education and Research (BMBF) and the Baden-Württemberg Ministry of Science (MWK BW) as part of the Excellence Strategy of the German Federal and State Governments (NWG-GastroTumors to AGC). DM is supported by the German Cancer Aid (Grant 70114233). This work was also supported by the Biomedical Systems Seed Funding from the University of Stuttgart.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Acharya, B. R., Nestor-Bergmann, A., Liang, X., Gupta, S., Duszyc, K., Gauquelin, E., et al. (2018). A Mechanosensitive RhoA Pathway that Protects Epithelia against Acute Tensile Stress. *Dev. Cell* 47, 439–452.e6. doi:10.1016/j.devcel.2018.09.016

Ajeti, V., Tabatabai, A. P., Fleszar, A. J., Staddon, M. F., Seara, D. S., Suarez, C., et al. (2019). Wound Healing Coordinates Actin Architectures to Regulate Mechanical Work. *Nat. Phys.* 15, 696–705. doi:10.1038/S41567-019-0485-9

An, R., Wang, P., Guo, H., Liuyu, T., Zhong, B., and Zhang, Z.-D. (2022). USP2 promotes experimental colitis and bacterial infections by inhibiting the proliferation of myeloid cells and remodeling the extracellular matrix network. *Cell Insight* 1, 100047. doi:10.1016/j.cellin.2022.100047

Aparicio-Yuste, R., Muenkel, M., Clark, A. G., Gómez-Benito, M. J., and Bastounis, E. E. (2022). A Stiff Extracellular Matrix Favors the Mechanical Cell Competition that Leads to Extrusion of Bacterially-Infected Epithelial Cells. *Front. Cell Dev. Biol.* 10, 912318. doi:10.3389/fcell.2022.912318

Atcha, H., Jairaman, A., Holt, J. R., Meli, V. S., Nagalla, R. R., Veerasubramanian, P. K., et al. (2021). Mechanically activated ion channel Piezo1 modulates macrophage polarization and stiffness sensing. *Nat. Commun.* 12, 3256. doi:10.1038/s41467-021-23482-5

Barriga, E. H., and Mayor, R. (2019). Adjustable viscoelasticity allows for efficient collective cell migration. *Semin. Cell Dev. Biol.* 93, 55–68. doi:10.1016/j.semcdb.2018.05.027

Barron, L., and Wynn, T. A. (2011). Fibrosis is regulated by Th2 and Th17 responses and by dynamic interactions between fibroblasts and macrophages. *Am. J. Physiol. Gastrointest. Liver Physiol.* 300, 723–728. doi:10.1152/ajpgi.00414.2010

Beaune, G., Stirbat, T. V., Khalifat, N., Cochet-Escartin, O., Garcia, S., Gurchenkov, V. V., et al. (2014). How cells flow in the spreading of cellular aggregates. *Proc. Natl. Acad. Sci.* 111, 8055–8060. doi:10.1073/pnas.1323788111

Benyon, R. C., and Arthur, M. J. P. (2001). Extracellular Matrix Degradation and the Role of Hepatic Stellate Cells. *Semin. Liver Dis.* 21 (3), 373–384. doi:10.1055/s-2001-17552

Blumenthal, D., Chandra, V., Avery, L., and Burkhardt, J. K. (2020). Mouse T cell priming is enhanced by maturation-dependent stiffening of the dendritic cell cortex. *Elife* 9, e55995. doi:10.7554/eLife.55995

Carmona-Rivera, C., Carlucci, P. M., Goel, R. R., James, E., Brooks, S. R., Rims, C., et al. (2020). Neutrophil extracellular traps mediate articular cartilage damage and

enhance cartilage component immunogenicity in rheumatoid arthritis. *JCI Insight* 5, e139388. doi:10.1172/jci.insight.139388

Chakraborty, M., Chu, K., Shrestha, A., Revelo, X. S., Zhang, X., Gold, M. J., et al. (2021). Mechanical Stiffness Controls Dendritic Cell Metabolism and Function. *Cell Rep.* 34, 108609. doi:10.1016/j.celrep.2020.108609

Chen, M., Zhang, Y., Zhou, P., Liu, X., Zhao, H., Zhou, X., et al. (2020). Substrate stiffness modulates bone marrow-derived macrophage polarization through NF-κB signaling pathway. *Bioact. Mater* 5, 880–890. doi:10.1016/j.bioactmat.2020.05.004

Chirivì, M., Maiullari, F., Milan, M., Presutti, D., Cordiglieri, C., Crosti, M., et al. (2021). Tumor extracellular matrix stiffness promptly modulates the phenotype and gene expression of infiltrating T lymphocytes. *Int. J. Mol. Sci.* 22, 5862. doi:10.3390/ijms22115862

Chrysanthopoulou, A., Mitroulis, I., Apostolidou, E., Arelaki, S., Mikroulis, D., Konstantinidis, T., et al. (2014). Neutrophil extracellular traps promote differentiation and function of fibroblasts. *J. Pathol.* 233, 294–307. doi:10.1002/path.4359

Clark, A. G., Maitra, A., Jacques, C., Bergert, M., Pérez-González, C., Simon, A., et al. (2022). Self-generated gradients steer collective migration on viscoelastic collagen networks. *Nat. Mater* 21 (10), 1200–1210. doi:10.1038/s41563-022-01259-5

Clark, A. G., and Vignjevic, D. M. (2015). Modes of cancer cell invasion and the role of the microenvironment. *Curr. Opin. Cell Biol.* 36, 13–22. doi:10.1016/j.ceb.2015.06.004

Comrie, W. A., Li, S., Boyle, S., and Burkhardt, J. K. (2015). The dendritic cell cytoskeleton promotes T cell adhesion and activation by constraining ICAM-1 mobility. *J. Cell Biol.* 208, 457–473. doi:10.1083/jcb.201406120

Curaj, A., Schumacher, D., Rusu, M., Staudt, M., Li, X., Simsekyilmaz, S., et al. (2020). Neutrophils modulate fibroblast function and promote healing and scar formation after murine myocardial infarction. *Int. J. Mol. Sci.* 21 (10), 3685. doi:10.3390/ijms21103685

Delclaux, C., Delacourt, C., D'Ortho, M. P., Boyer, V., Lafuma, C., and Harf, A. (1996). Role of Gelatinase B and Elastase in Human Polymorphonuclear Neutrophil Migration across Basement Membrane. *Am. J. Respir. Cell Mol. Biol.* 14, 288–295. doi:10.1165/ajrcmb/.14.3.8845180

Denton, A. E., Roberts, E. W., and Fearon, D. T. (2018). "Stromal Cells in the Tumor Microenvironment," in *Stromal Immunology*. Editors B. M. J. Owens and M. A. Lakins (Cham: Springer International Publishing), 99–114. doi:10.1007/978-3-319-78127-3_6

Dhar, D., Baglieri, J., Kisseleva, T., and Brenner, D. A. (2020). Mechanisms of liver fibrosis and its role in liver cancer. *Exp. Biol. Med.* 245, 96–108. doi:10.1177/1535370219898141

Discher, D. E., Janmey, P., and Wang, Y.-L. (2005). Tissue cells feel and respond to the stiffness of their substrate. *Science* 310, 1139–1143. doi:10.1126/science.1116995

Donker, L., Houtekamer, R., Vliem, M., Trepat, X., Borghi, N., Gloerich, M., et al. (2022). A mechanical G2 checkpoint controls epithelial cell division through E-cadherin-mediated regulation of Wee1-Cdk1. *Cell Rep.* 41, 111475. doi:10.1016/j.celrep.2022.111475

Du, H., Bartleson, J. M., Butenko, S., Alonso, V., Liu, W. F., Winer, D. A., et al. (2022). Tuning immunity through tissue mechanotransduction. *Nat. Rev. Immunol.* 23, 174–188. doi:10.1038/s41577-022-00761-w

Duarte, S., Baber, J., Fujii, T., and Coito, A. J. (2015). Matrix metalloproteinases in liver injury, repair and fibrosis. *Matrix Biol.* 44-46, 147–156. doi:10.1016/j.matbio.2015.01.004

Dvorak, H. F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* 315, 1650–1659. doi:10.1056/NEJM198612253152606

Elosegui-Artola, A., Andreu, I., Beedle, A. E. M., Lezamiz, A., Uroz, M., Kosmalska, A. J., et al. (2017). Force triggers YAP nuclear entry by regulating transport across nuclear pores. *Cell* 171, 1397–1410.e14. doi:10.1016/j.cell.2017.10.008

Elosegui-Artola, A., Gupta, A., Najibi, A. J., Seo, B. R., Garry, R., Tringides, C. M., et al. (2023). Matrix viscoelasticity controls spatiotemporal tissue organization. *Nat. Mater* 22, 117–127. doi:10.1038/s41563-022-01400-4

Elosegui-Artola, A. (2021). The extracellular matrix viscoelasticity as a regulator of cell and tissue dynamics. *Curr. Opin. Cell Biol.* 72, 10–18. doi:10.1016/j.ceb.2021.04.002

Fiori, M. E., Di Franco, S., Villanova, L., Bianca, P., Stassi, G., and De Maria, R. (2019). Cancer-associated fibroblasts as abettors of tumor progression at the crossroads of EMT and therapy resistance. *Mol. Cancer* 18, 70. doi:10.1186/s12943-019-0994-2

Fischer, A., Wannemacher, J., Christ, S., Koopmans, T., Kadri, S., Zhao, J., et al. (2022). Neutrophils direct preexisting matrix to initiate repair in damaged tissues. *Nat. Immunol.* 23, 518–531. doi:10.1038/s41590-022-01166-6

Friedman, S. L. (2008). Hepatic stellate cells: Protean, multifunctional, and enigmatic cells of the liver. *Physiol. Rev.* 88, 125–172. doi:10.1152/physrev.00013.2007

Gaertner, F., Reis-Rodrigues, P., de Vries, I., Hons, M., Aguilera, J., Riedl, M., et al. (2022). WASp triggers mechanosensitive actin patches to facilitate immune cell migration in dense tissues. *Dev. Cell* 57, 47–62.e9. doi:10.1016/j.devcel.2021.11.024

Genschmer, K. R., Russell, D. W., Lal, C., Szul, T., Bratcher, P. E., Noerager, B. D., et al. (2019). Activated PMN Exosomes: Pathogenic Entities Causing Matrix Destruction and Disease in the Lung. *Cell* 176, 113–126.e15. doi:10.1016/j.cell.2018.12.002

Gilkes, D. M., Bajpai, S., Chaturvedi, P., Wirtz, D., and Semenza, G. L. (2013). Hypoxia-inducible Factor 1 (HIF-1) Promotes Extracellular Matrix Remodeling under Hypoxic Conditions by Inducing P4HA1, P4HA2, and PLOD2 Expression in Fibroblasts. *J. Biol. Chem.* 288, 10819–10829. doi:10.1074/jbc.M112.442939

Gonzalez-Rodriguez, D., Guevorkian, K., Douezan, S., and Brochard-Wyart, F. (2012). Soft Matter Models of Developing Tissues and Tumors. *Science* 338, 910–917. doi:10.1126/science.1226418

Gudipaty, S. A., Lindblom, J., Loftus, P. D., Redd, M. J., Edes, K., Davey, C. F., et al. (2017). Mechanical stretch triggers rapid epithelial cell division through Piezo1. *Nature* 543, 118–121. doi:10.1038/nature21407

Haas, A. J., Zihni, C., Ruppel, A., Hartmann, C., Ebnet, K., Tada, M., et al. (2020). Interplay between Extracellular Matrix Stiffness and JAM-A Regulates Mechanical Load on ZO-1 and Tight Junction Assembly. *Cell Rep.* 32, 107924. doi:10.1016/j.celrep.2020.107924

Herrero-Cervera, A., Soehnlein, O., and Kenne, E. (2022). Neutrophils in chronic inflammatory diseases. *Cell Mol. Immunol.* 19, 177–191. doi:10.1038/s41423-021-00832-3

Hua, Y., and Bergers, G. (2019). Tumors vs. Chronic Wounds: An Immune Cell's Perspective. *Front. Immunol.* 10, 2178. doi:10.3389/fimmu.2019.02178

Ilina, O., Gritsenko, P. G., Syga, S., Lippoldt, J., La Porta, C. A. M., Chepizhko, O., et al. (2020). Cell–cell adhesion and 3D matrix confinement determine jamming transitions in breast cancer invasion. *Nat. Cell Biol.* 22 (9), 1103–1115. doi:10.1038/s41556-020-0552-6

Jeljeli, M., Riccio, L. G. C., Doridot, L., Chêne, C., Nicco, C., Chouzenoux, S., et al. (2019). Trained immunity modulates inflammation-induced fibrosis. *Nat. Commun.* 10, 5670. doi:10.1038/s41467-019-13636-x

Jenne, C. N., Liao, S., and Singh, B. (2018). Neutrophils: multitasking first responders of immunity and tissue homeostasis. *Cell Tissue Res.* 371, 395–397. doi:10.1007/s00441-018-2802-5

Jiang, Y., Zhang, H., Wang, J., Liu, Y., Luo, T., and Hua, H. (2022). Targeting extracellular matrix stiffness and mechanotransducers to improve cancer therapy. *J. Hematol. Oncol.* 15, 34. doi:10.1186/s13045-022-01252-0

Judokusumo, E., Tabdanov, E., Kumari, S., Dustin, M. L., and Kam, L. C. (2012). Mechanosensing in T Lymphocyte Activation. *Biophys. J.* 102, L5–L7. doi:10.1016/J.BPJ.2011.12.011

Kim, S. T., Takeuchi, K., Sun, Z. Y. J., Touma, M., Castro, C. E., Fahmy, A., et al. (2009). The alphabeta T cell receptor is an anisotropic mechanosensor. *J. Biol. Chem.* 284, 31028–31037. doi:10.1074/jbc.M109.052712

Kobayashi, S., Ogasawara, N., Watanabe, S., Yoneyama, Y., Kirino, S., Hiraguri, Y., et al. (2022). Collagen type I-mediated mechanotransduction controls epithelial cell fate conversion during intestinal inflammation. *Inflamm. Regen.* 42, 49. doi:10.1186/S41232-022-00237-3

Lang, S., and Schnabl, B. (2020). Microbiota and Fatty Liver Disease—the Known, the Unknown, and the Future. *Cell Host Microbe* 28, 233–244. doi:10.1016/j.chom.2020.07.007

Li, Y.-C., Chen, B.-M., Wu, P.-C., Cheng, T.-L., Kao, L.-S., Tao, M.-H., et al. (2010). Cutting Edge: Mechanical Forces Acting on T Cells Immobilized via the TCR Complex Can Trigger TCR Signaling. *J. Immunol.* 184, 5959–5963. doi:10.4049/jimmunol.0900775

Li, Z., and Bratlie, K. M. (2021). Fibroblasts treated with macrophage conditioned medium results in phenotypic shifts and changes in collagen organization. *Mater Sci. Eng. C* 122, 111915. doi:10.1016/j.msec.2021.111915

Liu, B., Yang, M. Q., Yu, T. Y., Yin, Y. Y., Liu, Y., Wang, X. D., et al. (2021). Mast Cell Tryptase Promotes Inflammatory Bowel Disease-Induced Intestinal Fibrosis. *Inflamm. Bowel Dis.* 27, 242–255. doi:10.1093/ibd/izaa125

Liu, F., Mih, J. D., Shea, B. S., Kho, A. T., Sharif, A. S., Tager, A. M., et al. (2010). Feedback amplification of fibrosis through matrix stiffening and COX-2 suppression. *J. Cell Biol.* 190, 693–706. doi:10.1083/jcb.201004082

Mariano, C. A., Sattari, S., Ramirez, G. O., and Eskandari, M. (2023). Effects of tissue degradation by collagenase and elastase on the biaxial mechanics of porcine airways. *Respir. Res.* 24, 105. doi:10.1186/s12931-023-02376-8

Martin, P., and Leibovich, S. J. (2005). Inflammatory cells during wound repair: The good, the bad and the ugly. *Trends Cell Biol.* 15, 599–607. doi:10.1016/j.tcb.2005.09.002

McKleroy, W., Lee, T. H., and Atabai, K. (2013). Always cleave up your mess: targeting collagen degradation to treat tissue fibrosis. *Am. J. Physiol. Lung Cell Mol. Physiol.* 304, L709–L721. doi:10.1152/ajplung.00418.2012

Medeiros, N. I., Fares, R. C. G., Franco, E. P., Sousa, G. R., Mattos, R. T., Chaves, A. T., et al. (2017). Differential Expression of Matrix Metalloproteinases 2, 9 and Cytokines by Neutrophils and Monocytes in the Clinical Forms of Chagas Disease. *PLoS Negl. Trop. Dis.* 11, e0005284. doi:10.1371/journal.pntd.0005284

Meli, V. S., Atcha, H., Veerasubramanian, P. K., Nagalla, R. R., Luu, T. U., Chen, E. Y., et al. (2023). YAP-mediated mechanotransduction tunes the macrophage inflammatory response. *Sci. Adv.* 6, eabb8471. doi:10.1126/sciadv.abb8471

Meng, K. P., Majedi, F. S., Thauland, T. J., and Butte, M. J. (2020). Mechanosensing through YAP controls T cell activation and metabolism. *J. Exp. Med.* 217, e20200053. doi:10.1084/jem.20200053

Millán-Salanova, M., and Vicente-Manzanares, M. (2022). The interface between biochemical signaling and cell mechanics shapes T lymphocyte migration and activation. *Eur. J. Cell Biol.* 101, 151236. doi:10.1016/j.ejcb.2022.151236

Murrell, M., Kamm, R., and Matsudaira, P. (2011). Substrate Viscosity Enhances Correlation in Epithelial Sheet Movement. *Biophys. J.* 101, 297–306. doi:10.1016/j.bpj.2011.05.048

Nalkurthi, C., Schroder, W. A., Melino, M., Irvine, K. M., Nyuydzefe, M., Chen, W., et al. (2022). ROCK2 inhibition attenuates profibrogenic immune cell function to reverse thioacetamide-induced liver fibrosis. *JHEP Rep.* 4, 100386. doi:10.1016/j.jhepr.2021.100386

Ng, M. R., Besser, A., Danuser, G., and Brugge, J. S. (2012). Substrate stiffness regulates cadherin-dependent collective migration through myosin-II contractility. *J. Cell Biol.* 199, 545–563. doi:10.1083/jcb.201207148

Ollauri-Ibáñez, C., Ayuso-íñigo, B., and Pericacho, M. (2021). Hot and Cold Tumors: Is Endoglin (CD105) a Potential Target for Vessel Normalization? *Cancers (Basel)* 13, 1552. doi:10.3390/cancers13071552

Ong, C. W. M., Elkington, P. T., Brilha, S., Ugarte-Gil, C., Tome-Esteban, M. T., Tezera, L. B., et al. (2015). Neutrophil-Derived MMP-8 Drives AMPK-Dependent Matrix Destruction in Human Pulmonary Tuberculosis. *PLoS Pathog.* 11, e1004917. doi:10.1371/journal.ppat.1004917

Ong, C. W. M., Pabisiak, P. J., Brilha, S., Singh, P., Roncaroli, F., Elkington, P. T., et al. (2017). Complex regulation of neutrophil-derived MMP-9 secretion in central nervous system tuberculosis. *J. Neuroinflammation* 14, 31. doi:10.1186/s12974-017-0801-1

Pérez-González, C., Alert, R., Blanch-Mercader, C., Gómez-González, M., Kolodziej, T., Bazellieres, E., et al. (2019). Active wetting of epithelial tissues. *Nat. Phys.* 15, 79–88. doi:10.1038/s41567-018-0279-5

Ragkousi, K., and Gibson, M. C. (2014). Cell division and the maintenance of epithelial order. *J. Cell Biol.* 207, 181–188. doi:10.1083/jcb.201408044

Rodrigues, M., Kosaric, N., Bonham, C. A., and Gurtner, G. C. (2019). Wound Healing: A Cellular Perspective. *Physiol. Rev.* 99, 665–706. doi:10.1152/physrev.00067.2017

Sahai, E., Astsaturov, I., Cukierman, E., DeNardo, D. G., Egeblad, M., Evans, R. M., et al. (2020). A framework for advancing our understanding of cancer-associated fibroblasts. *Nat. Rev. Cancer* 20 (3 20), 174–186. doi:10.1038/s41568-019-0238-1

Schnoor, M., Cullen, P., Lorkowski, J., Stolle, K., Robenek, H., Troyer, D., et al. (2008). Production of type VI collagen by human macrophages: a new dimension in macrophage functional heterogeneity. *J Immunol* 180, 5707–5719. doi:10.4049/jimmunol.180.8.5707

Schrader, J., Gordon-Walker, T. T., Aucott, R. L., van Deemter, M., Quaas, A., Walsh, S., et al. (2011). Matrix Stiffness Modulates Proliferation, Chemotherapeutic Response and Dormancy in Hepatocellular Carcinoma Cells. *Hepatology* 53, 1192–1205. doi:10.1002/hep.24108

Schwayer, C., Shamipour, S., Pranjic-Ferscha, K., Schauer, A., Balda, M., Tada, M., et al. (2019). Mechanosensation of Tight Junctions Depends on ZO-1 Phase Separation and Flow. *Cell* 179, 937–952.e18. doi:10.1016/j.cell.2019.10.006

Shimbori, C., Upagupta, C., Bellaye, P.-S., Ayaub, E. A., Sato, S., Yanagihara, T., et al. (2019). Mechanical stress-induced mast cell degranulation activates TGF-β1 signalling pathway in pulmonary fibrosis. *Thorax* 74, 455–465. doi:10.1136/thoraxjnl-2018-211516

Simões, F. C., Cahill, T. J., Kenyon, A., Gavriouchkina, D., Vieira, J. M., Sun, X., et al. (2020). Macrophages directly contribute collagen to scar formation during zebrafish heart regeneration and mouse heart repair. *Nat. Commun.* 11 (1), 600–617. doi:10.1038/s41467-019-14263-2

Spadaro, D., Le, S., Laroche, T., Mean, I., Jond, L., Yan, J., et al. (2017). Tension-Dependent Stretching Activates ZO-1 to Control the Junctional Localization of Its Interactors. *Curr. Biol.* 27, 3783–3795.e8. doi:10.1016/j.cub.2017.11.014

Sridharan, R., Cavanagh, B., Cameron, A. R., Kelly, D. J., and O'Brien, F. J. (2019). Material stiffness influences the polarization state, function and migration mode of macrophages. *Acta Biomater.* 89, 47–59. doi:10.1016/j.actbio.2019.02.048

Staddon, M. F., Bi, D., Tabatabai, A. P., Ajeti, V., Murrell, M. P., and Banerjee, S. (2018). Cooperation of dual modes of cell motility promotes epithelial stress relaxation to accelerate wound healing. *PLoS Comput. Biol.* 14, e1006502. doi:10.1371/journal.pcbi.1006502

Sutherland, T. E., Dyer, D. P., and Allen, J. E. (2023). The extracellular matrix and the immune system: A mutually dependent relationship. *Science* 379, eabp8964. doi:10.1126/science.abp8964

Tomlin, H., and Piccinini, A. M. (2018). A complex interplay between the extracellular matrix and the innate immune response to microbial pathogens. *Immunology* 155, 186–201. doi:10.1111/imm.12972

Velotti, F., Barchetta, I., Cimini, F. A., and Cavallo, M. G. (2020). Granzyme B in Inflammatory Diseases: Apoptosis, Inflammation, Extracellular Matrix Remodeling, Epithelial-to-Mesenchymal Transition and Fibrosis. *Front. Immunol.* 11, 587581. doi:10.3389/fimmu.2020.587581

Wang, Y., Huang, B., Jin, T., Ocansey, D. K. W., Jiang, J., and Mao, F. (2022). Intestinal Fibrosis in Inflammatory Bowel Disease and the Prospects of Mesenchymal Stem Cell Therapy. *Front. Immunol.* 13, 835005. doi:10.3389/fimmu.2022.835005

Witherel, C. E., Sao, K., Brisson, B. K., Han, B., Volk, S. W., Petrie, R. J., et al. (2021). Regulation of extracellular matrix assembly and structure by hybrid M1/M2 macrophages. *Biomaterials* 269, 120667. doi:10.1016/j.biomaterials.2021.120667

Wu, Y., Cao, Y., Xu, K., Zhu, Y., Qiao, Y., Wu, Y., et al. (2021). Dynamically remodeled hepatic extracellular matrix predicts prognosis of early-stage cirrhosis. *Cell Death Dis.* 12, 163. doi:10.1038/s41419-021-03443-y

Xu, J., Mao, X., Jin, R., Yin, J., Lu, K., Guo, Y., et al. (2020). Neutrophil extracellular traps degrade fibronectin in a rat model of bronchopulmonary dysplasia induced by perinatal exposure to lipopolysaccharide. *J. Cell Mol. Med.* 24, 14645–14649. doi:10.1111/jcmm.15842

Yahoo, N., Dudek, M., Knolle, P., and Heikenwälder, M. (2023). Role of immune responses in the development of NAFLD-associated liver cancer and prospects for therapeutic modulation. *J. Hepatol.* doi:10.1016/j.jhep.2023.02.033

Yap, A. S., Duszyc, K., and Viasnoff, V. (2018). Mechanosensing and mechanotransduction at cell –cell junctions. *Cold Spring Harb. Perspect. Biol.* 10, a028761. doi:10.1101/cshperspect.a028761

Yu, Y., Ren, L.-J., Liu, X.-Y., Gong, X.-B., and Yao, W. (2021). Effects of substrate stiffness on mast cell migration. *Eur. J. Cell Biol.* 100, 151178. doi:10.1016/j.ejcb.2021.151178

Yui, S., Azzolin, L., Maimets, M., Pedersen, M. T., Fordham, R. P., Hansen, S. L., et al. (2018). YAP/TAZ-Dependent Reprogramming of Colonic Epithelium Links ECM Remodeling to Tissue Regeneration. *Cell Stem Cell* 22, 35–49.e7. doi:10.1016/j.stem.2017.11.001

Zhang, J., Li, W., Sanders, M. A., Sumpio, B. E., Panja, A., and Basson, M. D. (2003). Regulation of the intestinal epithelial response to cyclic strain by extracellular matrix proteins. *FASEB J.* 17, 926–928. doi:10.1096/fj.02-0663fje

Zhao, X., Chen, J., Sun, H., Zhang, Y., and Zou, D. (2022). New insights into fibrosis from the ECM degradation perspective: the macrophage-MMP-ECM interaction. *Cell Biosci.* 12, 117. doi:10.1186/S13578-022-00856-w

frontiers | Frontiers in Cell and Developmental Biology

TYPE Review
PUBLISHED 14 July 2023
DOI 10.3389/fcell.2023.1228283

OPEN ACCESS

EDITED BY
Carlos Pardo-Pastor,
King's College London, United Kingdom

REVIEWED BY
Elizabeth A. Novak,
University of Pittsburgh, United States
Chhinder Sodhi,
Johns Hopkins University, United States

*CORRESPONDENCE
Christoph Becker,
christoph.becker@uk-erlangen.de

RECEIVED 24 May 2023
ACCEPTED 07 July 2023
PUBLISHED 14 July 2023

CITATION
Iyer K, Erkert L and Becker C (2023), Know your neighbors: microbial recognition at the intestinal barrier and its implications for gut homeostasis and inflammatory bowel disease.
Front. Cell Dev. Biol. 11:1228283.
doi: 10.3389/fcell.2023.1228283

Know your neighbors: microbial recognition at the intestinal barrier and its implications for gut homeostasis and inflammatory bowel disease

Krishna Iyer[1,2], Lena Erkert[1] and Christoph Becker[1]*

[1]Department of Medicine 1, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany, [2]Evergrande Center for Immunologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, MA, United States

Intestinal epithelial cells (IECs) perform several physiological and metabolic functions at the epithelial barrier. IECs also play an important role in defining the overall immune functions at the mucosal region. Pattern recognition receptors (PRRs) on the cell surface and in other cellular compartments enable them to sense the presence of microbes and microbial products in the intestinal lumen. IECs are thus at the crossroads of mediating a bidirectional interaction between the microbial population and the immune cells present at the intestinal mucosa. This communication between the microbial population, the IECs and the underlying immune cells has a profound impact on the overall health of the host. In this review, we focus on the various PRRs present in different cellular compartments of IECs and discuss the recent developments in the understanding of their role in microbial recognition. Microbial recognition and signaling at the epithelial barrier have implications in the maintenance of intestinal homeostasis, epithelial barrier function, maintenance of commensals, and the overall tolerogenic function of PRRs in the gut mucosa. We also highlight the role of an aberrant microbial sensing at the epithelial barrier in the pathogenesis of inflammatory bowel disease (IBD) and the development of colorectal cancer.

KEYWORDS

intestinal epithelial cells (IECs), pattern recognition receptors (PRRs), mucosal immunity, intestinal homeostasis, microbiome, inflammatory bowel disease (IBD)

1 Introduction

Intestinal epithelial cells (IECs) form a dynamic monolayer called the epithelial barrier, and together with the muscular layer, and connective tissue, make up the intestinal mucosa. A variety of absorptive and secretory cells, including the absorptive enterocytes, goblet cells, Paneth cells, tuft cells, and enteroendocrine cells, make up the intestinal epithelial layer. M or microfold cells contribute to an active microbial recognition and sampling in the intestinal lumen. These cells perform several metabolic and immune functions that are involved in maintaining intestinal homeostasis and overall epithelial barrier function. The intestinal epithelial layer is constantly exposed to a large population of microbes, both as part of the microbiota and potential pathogens, and pathobionts. IECs are thus at the crossroads of mediating a bidirectional interaction between the immune cells present at the mucosal layer, and the environment. However, to ensure a symbiotic relationship between the host and the

indigenous commensal microorganisms, while allowing for efficient recognition and clearance of invading pathogens, microbial sensing at the mucosal surface of the gut must be tightly controlled (Shibolet and Podolsky, 2007). This is primarily achieved by a complex recognition system via pattern recognition receptors (PRRs), including the toll-like receptors (TLRs), NOD and NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), and Alpha-protein kinase 1 (ALPK1).

Microbial interaction with IECs also define several developmental and physiological functions in the host, and dysbiosis has been implicated in the pathogenesis of inflammatory bowel disease (IBD) and colorectal cancer (Tamboli et al., 2004; Carvalho et al., 2012). Of the trillions of microbes that occupy the habitat in close proximity to the epithelial barrier, both the microbial population and their interactions with different epithelial cells vary largely along the lengths of the small and the large intestine. PRR signaling, mediated by the MyD88 pathway in IECs, play a key role in controlling both the spatial segregation and composition of commensals. Secretion of mucus from the goblet cells in the colon and antimicrobial peptides from Paneth cells in the small intestine facilitate spatial segregation of commensals in the respective regions (Johansson et al., 2008; Vaishnava et al., 2011), suggesting that diverse mechanisms are involved in the establishment of an immunologically tolerated interaction between the IECs and the commensal microbes. Studies in germ-free mice demonstrate the beneficial effects of microbial colonization of the gut lumen on intestinal epithelial metabolism, proliferation, survival, barrier function, and on IEC communication with immune cells (Smith et al., 2007). This interaction at the epithelial barrier also promotes the development and the maturation of diverse immune cell populations residing in the underlying lymphoid tissues. Thus, the overall crosstalk between microbes, IECs, and the underlying immune cells define the immune responses in the region and shapes the overall metabolic and physiological processes in the host tissue.

2 Microbial recognition at the intestinal epithelial barrier

A number of somatically encoded PRRs are expressed in different cellular compartments of the IECs. The juxtaposition and varying expression patterns of these receptors long the small and the large intestine determine the microbial recognition and effector function of IECs at the epithelial barrier. Based on their location in the cell, PRRs can be broadly divided into two classes—those located on the cell membrane and those localized within cellular compartments such as the cytosol and endosomes.

2.1 Toll-like receptors, NOD and NOD-like receptors

Toll-like receptors (TLRs) are found on the cell surface as well as in cytosolic compartments like the endosomes. It has been reported that TLR expression varies significantly along the length of the small and large intestine (Price et al., 2018). *In-situ* and organoid-based studies of the differential expression patterns of TLRs reveal very low or no expression of TLR(s)- 2, −4, −5, −7, and −9 in IECs of the small intestine, while very high expression of TLR(s)- 2, −4, and −5 along colonic epithelial cells (Price et al., 2018). This differential pattern of expression of PRRs along the small and the large intestine defines an immunologically important effector function of epithelial cells in the context of their interaction with the microbial population. Classically, the majority of cell surface TLRs are involved in the recognition of bacterial surface structures such as lipopolysaccharide (LPS), lipoproteins, or flagellins. TLRs located in cytosolic compartments such as endosomes are involved in the recognition of nucleic acids such as microbial dsRNA, ssRNA, and dsDNA.

Nucleotide oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid inducible gene-I (RIG-1)-like receptors (RLRs) are found in the cytosolic compartments of epithelial cells (Martinon and Tschopp, 2005; Thompson et al., 2011). NOD1 and NOD2 have been intensively studied in the gut and are responsible for recognition of bacterial cell wall peptidoglycan (PGN). NOD1 senses the meso-diaminopimelic type of PGN, which is most commonly found in Gram-negative bacteria (Girardin et al., 2003a; Girardin et al., 2003b). NOD2 has a broader sensing spectrum, recognizing the muramyl dipeptide N-acetylmuramyl-L-alanyl-D-glutamate, which is common to both Gram-negative and Gram-positive bacteria (Girardin et al., 2003b; Girardin et al., 2003c). NOD2 is highly expressed in Paneth cells of the small intestine and leads to cellular responses such as antimicrobial peptide (AMP) production, cytokine secretion, induction of autophagy, intracellular trafficking, and activation of epithelial regeneration (Couturier-Maillard et al., 2013; Nigro et al., 2014a; Ramanan et al., 2014). NOD1 and NOD2 have also been implicated in mediating beneficial interactions with the commensal flora (Eberl and Boneca, 2010).

Activation of PRRs leads to a signaling cascade that triggers a transcriptional program, and many of these receptors share a common downstream signaling pathway. Nuclear factor kappa light chain enhancer of B cells (NF-κB) is known as a master transcription factor involved in immune signaling. In a resting state, it is sequestered in the cytosol. Activation of the NF-κB cascade leads to release of NF-κB from its inhibitors, resulting in nuclear translocation and transcription of genes. TLR signaling also activates MAPKs, which synergise with NF-κB to express cytokines, chemokines and antimicrobial effectors (Kagan et al., 2008) (Figure 1). TLRs, with the exception of TLR3, transmit signaling information through the recruitment of the adaptor molecule myeloid differentiation primary response gene 88 (MyD88). In contrast, TLR3 has been shown to induce IRF3 activation through the TRIF pathway. TLR4 and TLR5 are also involved in TRIF pathway signaling in IECs, activating IRF3 and type I interferon production. Notably, while TLR4 signaling via MyD88 not always requires plasma membrane trafficking to endocytic vesicles, it has been shown that TRIF-mediated signaling of TLR4 requires internalization of the receptor (Kagan et al., 2008). NOD1 and NOD2 also activate NF-κB through the receptor-interacting serine/threonine kinase (RIPK1) signaling pathway. NOD1 and NOD2 in the cytosol signal through MAPK and NF-κB and their activation requires both molecules to get recruited to the plasma membrane (Barnich et al., 2005; Lecine et al., 2007; Kufer et al., 2008). NF-κB activation by NOD1/2 requires the adaptor molecule RIPK2 (Park et al., 2007), whereas the MAPK pathway is mediated by CARD9 (Hsu et al., 2007).

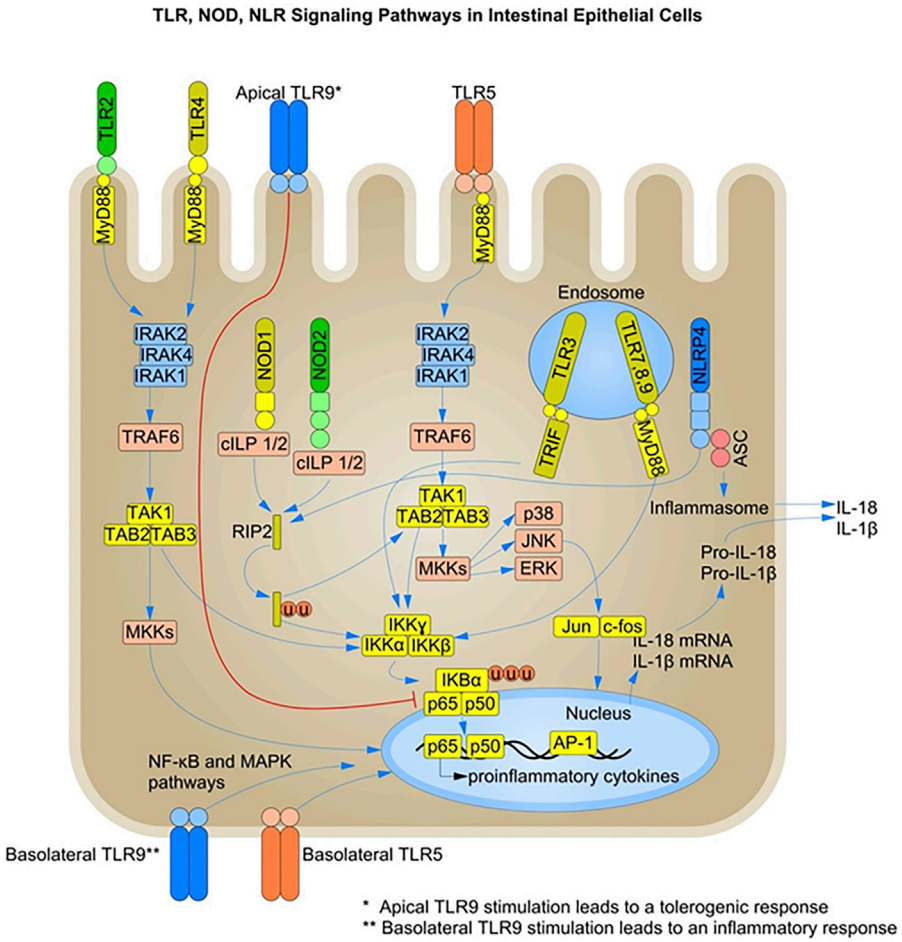


FIGURE 1
TLR-, NOD-, and NLR-mediated Signaling Pathways in Intestinal Epithelial Cells: Pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are present on intestinal epithelial cells (IECs). TLR recognition of MAMPs such as unmethylated CpG-containing DNA, flagellin, lipopolysaccharide (LPS) and lipoproteins, induces the recruitment of adaptor proteins like MyD88 and TRIF, leading to activation of the NF-κB and MAPK signaling pathways. NLRs such as NOD1 and NOD2 recognize bacterial peptidoglycans and activate the NF-κB and MAPK pathways through the recruitment of RIPK2. Members of the NLR family also initiate a pro-inflammatory response by activating the inflammasome complex and secreting active forms of IL-1β and IL-18. Under steady-state conditions, PRR stimulation in IECs leads to the production of AMPs and other mediators of gut homeostasis. Under inflammatory conditions, both surface and endosomal PRRs are stimulated, leading to a pro-inflammatory response and pathogen clearance. The basolateral and intracellular localization of PRRs is one of the mechanisms of immune response dampening and microbial tolerance at the epithelial barrier. TLR, Toll-like receptors; NOD, nucleotide-binding oligomerization domain; RIPK2, Receptor-interacting protein 2; MyD88, myeloid differentiation primary-response gene 88; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kB; NLR, nucleotide-binding oligomerization domain (NOD)-like receptors; TRIF, TIR-domain-containing adaptor protein inducing interferon-β; NLRP, NLR family pyrin domain-containing.

NLRs are involved in the intracellular recognition and sensing of microbes or microbial products via the formation of molecular scaffold complexes called inflammasomes. Inflammasomes are macromolecular complexes that initiate an inflammatory response by activating caspase-1 in response to microbial recognition, cellular stress, or cellular damage (Von-Moltke et al., 2013). Inflammasome complexes contain a sensor protein from the nucleotide-binding domain and leucine-rich repeat protein (NLR) family, an adaptor protein such as apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1 (Figure 1). The activation of the inflammasome complex leads to the induction of inflammatory signals through the cleavage of pro-IL-1β and IL-18 into their active forms (Kamada et al., 2013).

Microbial recognition leads to the activation and expression of inflammasome components, and substrate cytokines in the infected cells. Several other cues, such as cellular insults like loss of membrane integrity due to pathogen invasion, can provide the second signal for activation, as in the case of the (NLR) family pyrin domain-containing 3 (NLRP3) inflammasome. This leads to caspase-1 cleavage and cytokine release. NLRP3 senses danger signals such as ATP release or potassium imbalance during infection (Petrilli et al., 2007). NLRP1 is expressed in glandular epithelial structures in the intestine (Kummer et al., 2007) and is involved in the detection of cellular toxins. The NLRP6-inflammasome is also highly expressed in IECs and drives mucus secretion from goblet cells by promoting autophagy (Wlodarska et al., 2014). However, the specific NLRs involved, the mechanism of

10.3389/fcell.2023.1228283

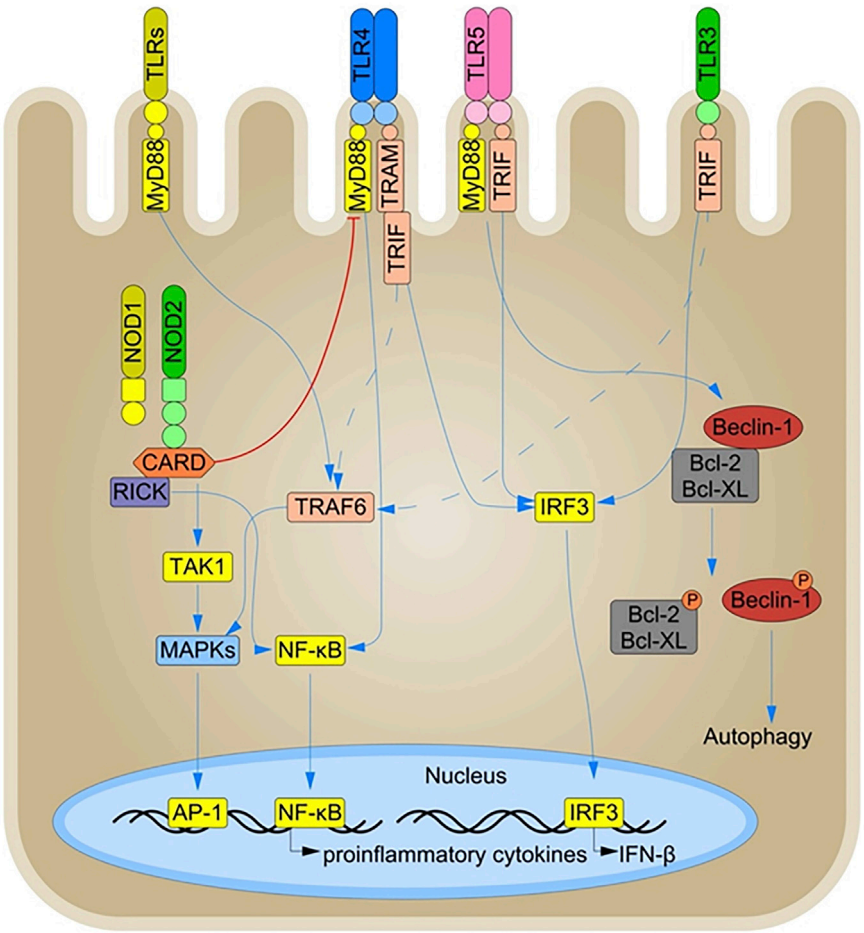


FIGURE 2
Immune Signaling Pathways shared by the PRRs in Intestinal Epithelial Cells: The PRRs present in the different cellular compartments of IECs, such as the cell surface, and endosomes, share a common downstream signaling pathway and thus have an overall complementary or inhibitory effect on each other. The TLRs signal downstream through the common MyD88, TRIF, and IRF3 signaling molecules. Signaling via the MyD88 and TRIF signaling components leads to nuclear translocation of NF-κB via the common TRAF6 molecule and production of pro-inflammatory cytokines. In addition, both TLR and NOD signaling converge to activate the MAPK pathway, amplifying the inflammatory response. Conversely, NOD2 activation is known to dampen TLR signaling such as TLR4 in certain inflammatory conditions. TLR signaling via the MyD88 and TRIF pathways also influences the induction of autophagy by interacting with Beclin-1, a key inducer of autophagosome formation. Beclin-1 directly interacts with the anti-apoptotic proteins Bcl-X_L and Bcl-2, and TLR activation leads to the phosphorylation and subsequent disassociation of Beclin-1 from Bcl-2 and Bcl-X_L, resulting in the initiation of autophagy.

activation of different inflammasomes and their exact role in intestinal epithelial function remain poorly understood (Cario, 2010).

Autophagy is a crucial cellular defense mechanism involving microbial recognition and clearance by targeting them to the lysosomal compartment for degradation. Activation of NOD1 and NOD2 induces autophagy in response to pure microbe-associated molecular patterns (MAMPs) as well as bacterial infections such as *Listeria monocytogenes* and *Shigella* spp. in a RIPK2-independent manner (Travassos et al., 2010). TLR signaling also induces autophagy via the engagement of the adaptor molecule MyD88 and the TRIF pathway in macrophages and epithelial cells (Shi and Kehrl, 2008; Benjamin et al., 2013). Both MyD88 and the TRIF molecule interact with Beclin-1, the primary inducer of autophagosome formation, leading to its dissociation from the anti-apoptotic proteins Bcl-2 and Bcl-X_L, thereby promoting autophagy (Figure 2) (Shi and Kehrl, 2008).

TLR and NLR activation lead to the recruitment of different adaptor molecules but result in similar downstream signaling with activation of NF-κB, MAPK, the inflammasome, and autophagosome formation. There is a dynamic crosstalk between the individual signaling pathways through the TLRs and NLRs that can be either synergistic or antagonistic in effect (Figure 2). For example, NOD2 activation in IECs has been shown to dampen TLR2 and TLR4 signaling, thereby preventing enhanced inflammation in the gut (Barreau et al., 2010). This suggests an

interplay between PRRs present in different cellular compartments at the intestinal barrier, and an unequal division of the labor between them could explain the tolerogenic effect towards the commensal MAMPS at the epithelial barrier. However, the interplay between different PRRs has primarily been documented in immune cells (O'Neill, 2008) and their role in IECs needs further investigation (Rosenstiel et al., 2003).

2.2 C-type lectin receptors and RIG-1-like receptors

Members of the C-type lectin receptor (CLR) family recognize fungal pathogens and are also responsible for maintaining the fungal microbiota in the gut. Dectin1/2/3 and Mincle are the most notable members of the CLR family and are involved in the recognition of specific carbohydrate motifs on the fungal cell surface (Geijtenbeek and Gringhuis, 2016; Goyal et al., 2018). CLRs have been mainly characterized to be expressed on myeloid cells in the lamina propria, where the fungal molecules rarely come into direct contact during homeostasis (Volman et al., 2010). However, the role of CLRs in maintaining epithelial integrity and homeostasis cannot be undermined. Dysbiosis, in terms of loss of both bacterial and fungal populations from the gut, leads to the manifestation of IBD, and thus CLRs may play a critical role in microbial recognition and signaling even at steady-state (Belkaid and Hand, 2014; Francescone et al., 2014; Meng et al., 2018).

CLRs have been shown to exert a host-protective function in the context of dysbiosis. Dectin-2 knockout mice were found to be more susceptible to *Candida albicans* infections, an opportunistic pathobiont. This suggests a role for dectin-2 in recognizing the yeast and in suppressing the overgrowth of *C. albicans* (Ifrim et al., 2016). There are several factors that can lead to a breach in epithelial barrier integrity during different physiological states in the gut. A lack of, or over-activation of, certain immune responses due to a disturbance in the gut flora can damage the intestinal epithelial barrier, compromising the spatial separation between microbes and host tissue (Candela et al., 2014). This is one of the many plausible scenarios for the interaction of microbes with CLRs residing in the epithelial cells. Alternatively, microfold (M) cells serve as a portal for microbes to cross the barrier and induce subsequent immune responses in the lamina propria (Mabbott et al., 2013). CLRs are thought to play an important role in mediating pathogen recognition and immune response, and dectin-1 has been shown to play a role in defining the first line of defense in the gut. Dectin-1 pairs with Siglec-5 receptors and mediates the delivery of soluble immunoglobulin A (sIgA) via M cells (Rochereau et al., 2013). Members of the CLR family also perform immune functions by cooperating with other PRRs in the cell. Dectin-1 activation has been shown to act synergistically with TLR2, and TLR4 (Taylor et al., 2007; Ferwerda et al., 2008) in immune cells, and thus the possibility of similar associations in IECs may provide a basis by which CLRs mediate fungal recognition and signaling in epithelial cells.

Retinoic acid-inducible gene (RIG-I)-like receptors (RLRs) are a family of RNA helicases that recognize viral RNAs and induce innate antiviral responses via activation of pro-inflammatory cytokines and type-I interferon (IFN) (Rehwinkel and Reis-e Sousa, 2010). RLRs, RIG-I, and MDA-5 are RNA helicases containing a DExD/H box RNA helicase domain and two CARD-like domains and are located in the cytoplasm (Kawai and Akira, 2008). These protein domains can recognize viral RNA molecules and signal via NF-κB, MAPK, and IRFs. These translocate to the nucleus to promote transcription of genes encoding type I IFN and other proinflammatory cytokines. RIG-I, through the induction of an RNA polymerase III-transcribed RNA intermediate, has also been shown to sense AT-rich double-stranded DNA (Ablasser et al., 2009). However, the role of RLRs in viral DNA and RNA sensing and the effects of RLR-specific knockouts in viral microbiome maintenance and inflammation have only been studied in immune cells in the lamina propria, and their role in IECs requires further investigation. Furthermore, the ligands involved as well as the exact mechanism of activation of different RLRs like the RIG-I are not fully understood.

2.3 Alpha-protein kinase 1 (ALPK1)

The ALPK-1-TIFA-dependent cytosolic surveillance pathway is efficient at sensing the bacterial metabolites heptose-1,7-bisphosphate (HBP) and ADP-β-d-manno-heptose (ADP-heptose), both of which are an intermediate in bacterial LPS secretion (Gaudet et al., 2017; Pfannkuch et al., 2019) (Figure 3). HBP and ADP-heptose are produced by both pathogenic and commensal bacteria. A recent study showed that ADP-heptose has significantly higher activity than HBP and that cells are specifically able to detect the presence of the β-form, even when the compound is added extracellularly (Pfannkuch et al., 2019). The same study also found lower levels of HBP in *Helicobacter pylori* lysates, suggesting their inability to successfully activate the NF-κB pathway during infection and cytosolic invasion.

Pathogenic bacteria such as *Yersinia pseudotuberculosis* and *H. pylori* inject ADP-heptose into host cells using type III and IV secretion systems (Zimmermann et al., 2017; Zhou et al., 2018). In contrast, HBP recognition in the cytoplasm requires translocation by bacterial injection systems and must be enzymatically converted to ADP-heptose-7P to be detected by the cytoplasmic ALPK1-TIFA-associated surveillance system. However, ADP-heptose can freely cross the host cell membranes and enter the host cytoplasm (Xue and Man, 2018). HBP and ADP-heptose are metabolic intermediates in bacterial LPS biosynthesis and represent novel PAMPs, specific to Gram-negative bacteria (Gaudet et al., 2015). Host recognition of HBP requires its release from the bacterial cytosol by extracellular or intra-phagosomal bacteriolysis in case of enteric bacteria (Gaudet et al., 2015; Gaudet and Gray-Owen, 2016).

Thus, the alpha-protein kinase-1 (ALPK1) is primarily involved in the detection of freely replicating cytosolic bacteria and elicits a robust NF-κB response following activation of the peptidoglycan sensor NOD1 (Gaudet et al., 2017). NOD1 is known to mediate an initial transient burst of NF-κB activation during bacterial invasion (Girardin et al., 2003a). The ALPK1-TIFA-mediated pathogen recognition system is thought to play a role in supporting a sustained inflammatory response during the later stages of bacterial infection after the initial transient NOD1-mediated NF-κB activation.

Contamination of the cytosol with HBP/ADP-heptose has been shown to induce oligomerization of TIFA dependent on Thr-9 phosphorylation, which recruits and activates the E3 ubiquitin

10.3389/fcell.2023.1228283

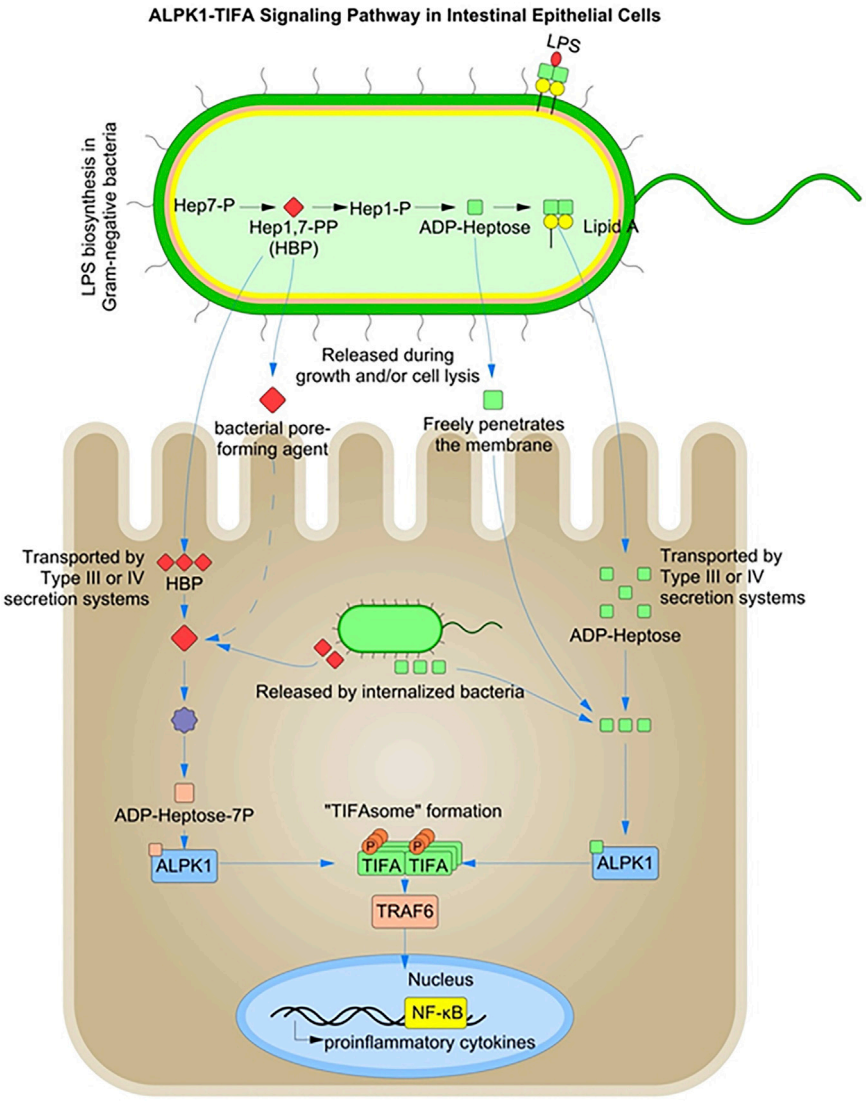


FIGURE 3
ALPK1-TIFA Signaling Pathway in Intestinal Epithelial Cells: Heptose 1,7-Bisphosphate (HBP) and ADP-β-d-manno-heptose (ADP-heptose) are intermediates in the biosynthesis of lipopolysaccharide (LPS). Both HBP, and ADP-heptose can activate a pro-inflammatory signaling pathway via ALPK1-dependent TIFA oligomerization in the cytosol.

ligase TRAF6 to initiate a NF-κB-dependent pro-inflammatory transcriptional response. ALPK1 is responsible for TIFA oligomerization and IL-8 expression in response to the infection. The ALPK1-TIFA cytosolic surveillance pathway thus represents a NOD-independent mechanism for detecting invasive Gram-negative bacteria (Milivojevic et al., 2017). Intracellular infections, such as *Shigella flexneri,* can also be detected indirectly via damage-associated molecular patterns (DAMPs). Membrane vacuolar remnants produced after vacuolar lysis have been shown to be detected by host cells and the signals produced contribute to enhanced inflammation (Dupont et al., 2009). Accumulation of diacylglycerol around the bacterial entry site and within membrane remnants has been shown to activate NF-κB via a mechanism dependent on the CARD–BCL10–MALT1 complex and TRAF6 (Sanada et al., 2012).

The exact mechanism by which HBP and/or ADP-heptose induces TIFA-dependent activation in both infected and bystander cells is not fully understood. Gaudet *et al.* demonstrated IL-8 production in response to *S. flexneri* and *Salmonella typhimurium* infection due to HBP endocytosis (Gaudet et al., 2015). However, studies by Kasper *et al.* have shown that non-invasive *S. flexneri* bacteria do not induce an IL-8 expression (Kasper et al., 2010). Studies by Lippmann *et al.* also show that IL-8 expression in bystander cells requires bacterial internalization and that mere diffusion of HBP does not lead to TIFA activation (Lippmann et al., 2015).

Therefore, a more detailed investigation of intracellular HBP/ADP-heptose detection and its ability to induce TIFA activation is warranted. ADP-heptose can be classified as a small-diffusing molecule, but its role as a potent PAMP for PRR activation

depends on its ability to serve as a marker of microbial invasion and cytosolic proliferation.

2.4 Other receptors involved in microbial recognition

There are other receptors on IECs that are involved in the detection of various microbial components and metabolites, and their role is of paramount importance in both defense and maintenance of intestinal homeostasis.

IECs are actively involved in the sensing of microbial metabolites produced by bacterial fermentation of dietary components in the gut, which may be particularly important for establishing a symbiotic relationship with the commensals and thereby defining a number of physiological roles in the gut. The pregnane X receptor (PXR) (Venkatesh et al., 2014) and the aryl hydrocarbon receptor (AhR) (Zelante et al., 2013; Metidji et al., 2018) are involved in the sensing of tryptophan catabolites produced by microbes in the lumen. PXR is sensitive to indole-3-propionic acid, a metabolite of tryptophan that is produced by the commensal *Clostridium* sporogenes. PXR knockout mice have increased inflammatory damage to the epithelium and decreased expression of the tight junction protein (TJP) (Venkatesh et al., 2014), supporting its role in a variety of anti-inflammatory and protective barrier functions in the gut.

IECs infected with *Yersinia enterocolitica* have been shown to use β1 integrins as Pathogen Recognition Receptor that recognize the bacterial adhesin called invasin. The invasin-integrin interaction provides an initial signal for activation of the NLRP3 inflammasome (Thinwa et al., 2014). Hydroxycarboxylic acid receptor-2 (GPCR-109A) is involved in the sensing of butyrate and niacin. Together with AIM-2 (Singh et al., 2014; Macia et al., 2015) and NLRP3, hydroxycarboxylic acid receptor-2 is involved in fine-tuning of IL-18 levels in the intestine. Epithelial IL-18 plays a central role in orchestrating the intestinal host–microbial homeostasis, and genetic deletion of these receptors results in intestinal inflammation, tumorigenesis, and increased susceptibility to enteric infections (Song-Zhao et al., 2014; Man et al., 2015).

More recently, the role of tuft cells in eliciting type 2-mediated immunity to allergens, helminth and protist infestations has been linked to their chemosensory capabilities in the small intestine. Tuft cells are a part of an elaborate tuft type 2 innate lymphoid cell (ILC-2) network and are critical for the activation of ILC-2 cells through the secretion of IL-25 and its downstream adaptor Act-1 (Kang et al., 2012). Tuft cells are thought to have distinct sensing mechanisms for both helminth and protist infestations with specific enrichment in G protein-coupled sensory receptor(s) and transmit downstream signals to activate type 2 immune cells (Nadjsombati et al., 2018). The extracellular succinate receptor (SUCNR1) has been identified to be expressed in both IL-25+ and TRPM5+ tuft cells in the small intestine (Bezencon et al., 2008; Lei et al., 2018; Nadjsombati et al., 2018), and succinate has been shown to act as an innate immune ligand sufficient to activate type 2 inflammation in mice. Furthermore, tuft cells also express other metabolic sensing receptors such as free fatty acid receptor 3 (FFAR3) (Schneider et al., 2018), but their role in orchestrating the type 2-mediated immune response is not fully understood. Tuft cells also express enzymes involved in the biosynthesis of eicosanoids, such as 5-lipoxygenase (Alox5), Cox-1, Cox-2, and hematopoietic PG-D synthase (HPGDS), and proliferate in an inflammatory environment (Gerbe et al., 2011; Von-Moltke et al., 2016).

3 Impact of microbial recognition at the intestinal epithelial barrier

3.1 Barrier function and maintenance of intestinal homeostasis

Upon detection of microbial patterns in the intestinal lumen, IECs enhance intestinal barrier functions, including mucus and AMP production, improved tight junction integrity, and mediating cell proliferation and differentiation to protect the bowel wall from microbial infiltration. We summarize different PRR mediated downstream signaling in cells on the epithelial barrier and their role in mediating either a pro- or anti-inflammatory response in Table 1.

IECs secrete a variety of AMPs through PRR/MyD88-dependent mechanisms (Vaishnava et al., 2008), which accumulate in the mucus layer and exert antimicrobial activities (Fahlgren et al., 2003). Paneth cells are involved in the secretion of α-defensins such as HD-5/6 in humans and cryptidins and CRS in mice. They also secrete other AMPs like RegIIIα/β/γ, sPLA2, and lysozyme-C in humans and mice (Brandl et al., 2007; Rakoff-Nahoum and Medzhitov, 2007; Gong et al., 2010). Enterocytes are primarily involved in the secretion of β-defensins in both humans and mice (O'Neil et al., 1999; Simmons et al., 2002). During an infection with *Citrobacter rodentium*, MyD88 signaling in IECs alone was found to be sufficient to improve epithelial barrier integrity and to increase production of RegIII-γ and the acute phase protein serum amyloid A1 (SAA1) (Friedrich et al., 2017).

Goblet cells form a viscous layer of mucus on the epithelial surface by secreting mucin glycoproteins. A discontinuous mucus layer in the mouse cecum and corresponding areas of the epithelium have been shown to form hotspots for microbial infection (Furter et al., 2019). Recognition of microbial LPS by LPS binding protein (LBP) and TLR4 elicits a pro-inflammatory response that induces expression of mucins in goblet cells (Smirnova et al., 2003). Inflammasome-mediated activation of NLRP6 has also been implicated in goblet cell mucus secretion through the promotion of autophagy (Cario, 2010).

The interaction of PRRs with commensal MAMPs also has a positive effect on mucus production by goblet cells at the epithelial barrier under homeostatic conditions. Amuc-1100 is a membrane protein of the commensal *Akkermansia muciniphila* that actively interacts with TLR2, resulting in increased mucus thickness and TJP expression at the epithelial barrier (Plovier et al., 2017). Microbiota-derived short-chain fatty acids (SCFAs) have been shown to regulate a number of IEC functions, including cell turnover (Park et al., 2016), TJP expression (Zheng et al., 2017), and upregulation of inflammasome- or hypoxia-inducible factor (HIF)-mediated epithelial integrity (Kelly et al., 2015; Macia et al., 2015).

Bacterial LPS can directly stimulate Paneth cells and apical stimulation of TLRs-2/3/4, NOD1/2 and NLRP3 leads to secretion of AMPs via immediate degranulation (Yokoi et al.,

TABLE 1 Pathogen Recognition Receptors (PRRs) expressed on different cells at the intestinal epithelial barrier and their downstream signaling effect in mediating a pro- or anti-inflammatory response in IECs.

Pathogen Recognition Receptor (PRR)	Expressed in IEC cell-type	PRR activation results in	Pro/Anti-inflammatory	References
Toll-like Receptors (TLRs)				
TLR-1	Enteroendocrine cells	NF-icB and MAPK activation; TNF-a expression	Pro-inflammatory	Bogunovic et al. (2007)
TLR-2	Enteroendocrine cells	NF-icB and MAPK activation; TNF-a expression	Pro-inflammatory	Bogunovic et al. (2007)
TLR-3	Paneth cells	Paneth cell degranulation	Anti-inflammatory	Rumio et al. (2011)
TLR-4	Enteroendocrine cells	NF-icB and MAPK activation; TNF-a expression Chemokine induction	Pro-inflammatory	Bogunovic et al. (2007); Selleri et al. (2008)
	Paneth cells	Paneth Cell Degranulation	Anti-inflammatory	Rumio et al. (2011)
TLR-5	Enteroendocrine cells	Chemokine induction	Pro-inflammatory	Selleri et al. (2008)
	Paneth cells	Paneth cell degranulation	Anti-inflammatory	Rumio et al. (2011)
		Chemokine and cytokine production	Pro-inflammatory	Price et al. (2018)
TLR-9	Enteroendocrine cells	Secretion of cholecystokinin	Anti-inflammatory	Daly et al. (2020)
NOD and NOD-like receptors (NLRs)				
NOD-1	Paneth Cells	NF-icB and MAPK activation; TNF-a expression Paneth Cell degranulation; induction of autophagy	Pro-inflammatory	Barnich et al. (2005), Lecine et al. (2007), Kufer et al. (2008)
NOD-2	Paneth Cells	NF-icB and MAPK activation; TNF-a expression Paneth Cell degranulation; induction of autophagy	Pro-inflammatory	Couturier-Maillard et al. (2013); Nigro et al. (2014a); Ramanan et al. (2014); Barnich et al. (2005); Lecine et al. (2007); Kufer et al. (2008)
NLRP3	Paneth Cells	Paneth Cell degranulation	Pro-inflammatory	Yokoi et al. (2019)
NLRP6	Goblet Cells	Inflammasome activation; Induction of autophagy leading to mucus secretions	Anti/Pro inflammatory	Cario (2010)
Other Receptors on IECs				
SUCNR1	Tuft Cells	Induction of type-2 inflammatory reaction	Pro-inflammatory	Bezencon et al. (2008); Lei et al. (2018); Nadjsombati et al. (2018)

2019). MyD88-deficient mouse models have shown decreased production of RegIIIγ, RELMβ, and RegIIIβ in the intestinal epithelium (Gong et al., 2010), suggesting a correlation between PRR stimulation and AMP secretion. Paneth cells also interact with both pathogenic and commensal microbes in an alternative, indirect manner through the release of pro-inflammatory IFN-γ (Farin et al., 2014; Burger et al., 2018) to further secrete antimicrobial peptides.

The production of mucus and AMPs such as RegIIIγ in response to the microbial recognition by epithelial cells is critical for maintaining overall immune homeostasis and defining the spatial segregation between the host tissue and the commensals (Vaishnava et al., 2011). MyD88-dependent TLR signaling has been shown to be critical for protection against mucosal damage (Fukata et al., 2005; Choi et al., 2010). TLR2 signaling in IECs induce the expression of the TJP ZO-1, strengthening the epithelial barrier integrity and providing resistance to apoptosis (Cario et al., 2004; Cario et al., 2007). TLR2 signaling also induces the production of cytoprotective trefoil factor, involved in mucosal tissue repair (Podolsky et al., 2009). TLR4-mediated signaling in IECs via MyD88 further induces cyclooxygenase 2 (COX-2), which enhances prostaglandin E2 (PGE2) synthesis, thereby promoting epithelial cell survival (Pull et al., 2005; Fukata et al., 2006; Brown et al., 2007; Hernandez et al., 2010). TLR4 signaling in IECs has further been shown to induce the secretion of amphiregulin and epiregulin, which activate epidermal growth factor (EGF) receptors (Fukata et al., 2007).

Intestinal stem cells (ISCs) located at the bottom of crypts of Lieberkühn play a critical role in the maintenance of epithelial barrier integrity due to their ability to propagate progeny that differentiate into diverse cell-types depending on the physiological demands. The Lgr5+ ISCs are known to express PRRs, in particular TLR4 and NOD2. These play important roles in the maintenance of intestinal homeostasis by effecting stem cell survival, proliferation, and apoptosis (Nigro et al., 2014b). TLR4 signaling has been demonstrated to affect ISC proliferation and differentiation by influencing Wnt and Notch signaling in the intestinal crypts (Sodhi et al., 2011; Sodhi et al., 2012). Stem cells are key to warrant repopulation of the intestinal epithelium during the resolution phase of inflammation when the inflammation-induced damage has to be repaired. Although stem cells reside deep within the crypts in an area considered largely inaccessible for microbes in healthy individuals, in the inflamed gut with epithelial erosions and barrier dysfunction, TLR-mediated signaling in stem cells driving proliferation and inhibiting cell death becomes highly relevant for tissue recovery.

TLR signaling in Paneth cells has been shown to regulate the release of antimicrobial peptides and to play an important functional role in host defense and in the maintenance of gut homeostasis. For

example, Rumio and others showed that engagement of TLR9, using the agonist CpG oligodeoxynucleotide (ODN) *in vivo*, leads to Paneth cell degranulation. Similarly, the TLR3 agonist polyinosinic-polycytidylic acid induced a strong and rapid degranulation, whereas the TLR4 agonist LPS and the TLR5 agonist flagellin induced only a late degranulation of Paneth cells (Rumio et al., 2011). Interestingly, a recent study by Price et al. examined TLR expression along the intestine and villus-crypt axis and showed that TLR5 expression in the small intestine is restricted to the Paneth cells, suggesting that TLR5 is particularly important for microbial sensing via Paneth cells. Remarkably, in this study, TLR5 signaling in Paneth cells did not induce antimicrobial peptides itself, but rather elicited chemokine and cytokine responses via Ccl20 and TNF-α, as well as NF-κB pathway-related molecules, including A20, Iκbα and Nfκb2. Antimicrobial peptide production was then shown to be indirectly induced by these inflammatory cytokines (Price et al., 2018). Paneth cells not only express TLR, but also express NLRs such as NOD2 (Lala et al., 2003) and NOD2$^{-/-}$ mice have reduced Paneth cell-related α-defensin transcripts, including cryptidin-4 and cryptidin-10, and show increased susceptibility to infection when challenged with *Listeria monocytogenes* (Kobayashi et al., 2005).

Enteroendocrine cells also express several functional TLRs, including TLR1, TLR2, and TLR4, ultimately leading to NF-κB and MAPK activation and TNF-α expression (Bogunovic et al., 2007). Interestingly, a study by Selleri et al. showed that the TLR agonists LPS and flagellin are able to induce pro-inflammatory chemokines, such as CXCL1 and IL-32 specifically within enteroendocrine cells, suggesting this rare cell type as an important contributor in inflammatory processes in the gut (Selleri et al., 2008). More recently, TLR9 has been shown to be specifically expressed by enteroendocrine cells of the proximal intestine, where it leads to the secretion of cholecystokinin upon stimulation, which could ultimately lead to the elimination of pathogens through cholecystokinin-stimulated emesis, demonstrating a critical role for enteroendocrine cells in enteric infections (Daly et al., 2020). Altogether, these data demonstrate multiple layers of PRR-mediated interaction between the microbiota and the gut epithelium with its diverse cell types. Signaling via different PRR located in different compartments on the cell surface but also within the cell and its organelles allows epithelial cells to detect the nature of the microbial signal and the potential challenge it might pose for host defense. Receptor activation induces signaling cascades that regulate diverse epithelial cell functions, including proliferation and cell death, barrier integrity, metabolism and innate immunity.

3.2 Impact on the diversity of the gut microbiota

The presence of PRRs in different cellular compartments of the IECs has a profound effect on the overall maintenance of the commensal microbial population at the intestinal barrier. In exchange for this symbiotic relationship, several factors produced by the microbial population shape the overall immune system at the mucosal region and influence the various developmental and metabolic processes in the host tissue.

Metabolites of certain commensal spore-forming bacteria such as *Clostridium spp*. are known to promote serotonin (5-hydroxytryptamine (5-HT)) secretion from a subtype of enteroendocrine cells called enterochromaffin cells in colonized mice (Yano et al., 2015). The neurotransmitter serotonin is an important regulator of enteric nervous system development, gastrointestinal tract motility, and inflammation (Terry and Margolis, 2017). Microbial sensing by TLRs present on enteroendocrine cells also promotes the secretion of several other hormones such, as glucagon-like peptide 1 (GLP-1) (Lebrun et al., 2017) and peptide tyrosine-tyrosine (PYY) (Larraufie et al., 2017). Collectively, these hormones increase insulin secretion (Grøndahl et al., 2017), regulate mood, and induce satiety (Loh et al., 2015), thereby influencing overall host physiology.

Stimulation of the innate immune response by the microbiota also provides indirect resistance to infection in the gut. The depletion of commensal microbes in the gut has a direct effect on the viral immunity (Ichinohe et al., 2011; Abt et al., 2012). Induction of IFN-λ and IL-18 or IL-22 is essential for an effective antiviral innate immunity in the gut (Zhang et al., 2014; Nice et al., 2015). Commensals stimulate the production of IL-18 and IL-22, but actively suppress IFN-λ production, promoting viral persistence as a part of the gut microbial population (Baldridge et al., 2015). However, dysbiosis can lead to an imbalance in this complex loop, resulting in pronounced antiviral immunity in the gut.

Alterations in the normal resident microbial flora alter the normal gut immune response, as has been shown in the case of altered antimicrobial response following antibiotic treatment (Cash et al., 2006). As the production of AMPs requires TLR-dependent stimulation of Paneth cells, an imbalance in the microbiota population has been associated with impaired resistance to bacterial infections (Brandl et al., 2007; Brandl et al., 2008). MyD88-dependent TLR signaling at the intestinal barrier is also essential for maintaining the spatial segregation of commensal microbes and host tissues (Vaishnava et al., 2011). MyD88-mediated secretion of RegIIIγ anti-bacterial lectin has been shown to define both the composition and the spatial localization of the intestinal microbiome. This could fundamentally determine the balance between tolerogenic and pro-inflammatory immune responses in the gut. In the absence of MyD88, commensal bacteria have been shown to gain proximity to the intestinal surface, resulting in a manifold increase in mucosa-associated bacteria compared to that in wild type mice (Vaishnava et al., 2011).

PRR signaling also plays a pivotal role in defining the diversity of the commensal population at the gut. This has been suggested by the occurrence of dysbiosis (Tamboli et al., 2004), its association with polymorphisms in the gene encoding for NOD2, and its overall impact on host susceptibility to IBD such as Crohn's disease (Hugot et al., 2001; Ogura et al., 2001). Thus, the antimicrobial peptides and secretory IgA produced in response to microbial sensing, balance the microbial composition, thereby limiting the penetration of commensal bacteria into the gut (Macpherson and Uhr, 2004). This regulation of bacterial load and composition may be one of the primary functions of PRRs in maintaining intestinal homeostasis.

3.3 IEC-microbiota-immune cells cross-talk

The signaling at the epithelial layer is not limited to the barrier region, but the resulting effector molecules are actively disseminated to the underlying mucosal layer. This, in turn influences the development and maturation of the underlying immune cells.

The presence and function of M-cells at the epithelial barrier suggests a much more dynamic role for epithelial cells in microbial sensing and sampling than that of a rigid barrier system. M cells are specialized cells that mediate a direct uptake of antigens and intact microbes from the intestinal lumen and transport them for presentation to resident immune cells. This effectively activates the adaptive immune system. M-cells initiate phagocytosis of the pathogen at the intestinal barrier upon recognition via PRRs. GP2 functions as a receptor for type I pili on a subset of Gram-negative enterobacilli (Hase et al., 2009) and is essential for immune surveillance at mucosal surfaces. Cellular prion protein (PrPc) is a glycosylphosphatidylinositol (GPI)-anchored protein that is expressed on the apical surface of M cells (Nakato et al., 2009). PrPc interacts with pathogens that contain heat shock protein 60 (HSP-60), a conserved surface protein with immunogenic properties (Kaufmann, 1990).

Specific microbial signaling at the epithelial barrier also plays a role in immune cell development. In particular, epithelial NOD1 signaling has been shown to be important for C-C motif chemokine 20 (CCL20)-mediated generation of isolated lymphoid follicles from cryptopatches (CPs) in the gut (Bouskra et al., 2008). Likewise, signaling via the innate receptors present on myeloid cells of the lamina propria also affects epithelial cells and the microbial population further in the intestinal lumen. Myeloid cells modulate key pathways such as IL-22 cytokine expression by innate lymphoid cells (ILCs) and induce the production of the antimicrobial peptides RegIIIβ and RegIIIγ, which are important for maintaining a spatial separation between the commensals and the intestinal epithelial layer. This modulation plays an integral role in supporting the spatial separation between commensals and the intestinal mucosa (Zheng et al., 2008; Vaishnava et al., 2011; Sonnenberg et al., 2012).

Another direct consequence of microbial sensing at the epithelial layer is related to the secretion of immunoglobulin A (IgA) in the gut. IgA has a specific role in the mucosal immune system and also plays an important role in maintaining the spatial segregation and composition of luminal microorganisms. TLR signaling in IECs induces expression of B cell activating factors that induce immunoglobulin class switch recombination in lamina propria B cells in a T cell-independent manner (He et al., 2007; Shang et al., 2008). TLR signaling in IECs also results in the secretion of April and BAFF. April directly induces IgA class switching recombination, while BAFF promotes B cell proliferation and survival. Furthermore, IECs can also indirectly induce class switching recombination by secretion of TSLP, which stimulates dendritic cells in the lamina propria to secrete April (He et al., 2007).

Activation of TLR3, and TLR4 has also been shown to induce the expression of polymeric immunoglobulin receptors involved in the epithelial transport of immunoglobulin, thereby enhancing the luminal IgA secretion (Schneeman et al., 2005). Thus, TLR signaling in IECs is actively involved in multiple steps of intestinal IgA secretion.

3.4 Tolerogenic effect of microbial recognition at the epithelial barrier

A very important aspect of the overall immune function at the intestinal mucosa is the ability of both the epithelial and immune cells to discriminate microbial cues from commensals as opposed to invasive pathogens. This is critical for maintaining a symbiotic relationship between the host and the microbial population present at the epithelial barrier. The successful establishment of the microbial niches along the elaborate spaces of the epithelial barrier is the result of the selective tolerogenic effect of the immune system towards commensal microbes. A number of topological, metabolic and genetic factors play an important role in establishing a fine line between the tolerogenic and defensive functions of the immune system.

The juxtaposition and polarity of PRR expression on epithelial cells play a very primitive but consequential role in determining the tolerogenic effect at the epithelial barrier. The unequal division of labor between the PRRs present on the epithelial cell surface and those in the cytosolic cellular compartments also provides a basis for tolerance. Only invasive microbes that manage to penetrate the barrier are detected by cytosolic and endosomal PRRs and trigger an inflammatory response. TLR9 is expressed on both the apical and basolateral sides of IECs (Figure 1). Ligand recognition on the apical side activates a tolerogenic effect, whereas stimulation of TLR9 on the basolateral side induces a robust inflammatory response (Lee et al., 2006). However, apical stimulation of TLRs primed by LPS leads to immediate degranulation and secretion of AMPs in Paneth cells (Yokoi et al., 2019).

IECs also have an overall muted response to LPS due to a low expression of TLR2, TLR4, the co-receptor MD-2, and CD14 (Abreu et al., 2001; Funda et al., 2001). Many of the TLRs are expressed and localized exclusively in the crypt epithelial cells of both the stomach and intestine (Cario et al., 2000; Kawai and Akira, 2008; Abreu, 2010), where they are inaccessible to commensal bacteria. Small intestinal IECs have been found to express very low levels of several TLRs that are normally highly expressed in the colonic IECs (Price et al., 2018). Furthermore, TLR4 has been reported to be sequestered in the Golgi apparatus (Hornef et al., 2002) and requires prior internalization of LPS to induce an immune response (Hornef et al., 2003).

There are other mechanisms that have been investigated to better understand commensal tolerance at the epithelial barrier. It has long been speculated that bacterial pathogens may modulate host epigenomics as a part of their virulence to establish contact during invasion. In the case of commensal microbes, the analysis of epigenetic modifications in the IECs of germ-free mice revealed a low level of methylation on the genes encoding for the LPS sensor TLR4, suggesting that commensal bacteria may be able to induce tolerance through epigenetic repression of genes encoding for PRRs (Takahashi et al., 2011). Commensal microbes have been shown to actively dampen the overall immune response to their MAMPs by suppressing the activation of pro-inflammatory pathways upon PRR sensing. In resting cells, NF-κB is sequestered in the cytoplasm by IκB, which masks NF-κB's nuclear localization sequences. When the receptor is stimulated, classical NF-κB activation occurs by phosphorylating IκB, targeting it for ubiquitination and subsequent proteasomal degradation (Nigro et al., 2014a).

Commensal bacteria have been shown to prevent the degradation of phosphorylated IκB by interfering with the host cellular machinery that controls the processes of ubiquitination and degradation (Neish et al., 2000; Collier Hyams et al., 2005; Tien et al., 2006).

A variety of inhibitors such as IRAK-M, Tollip, SIGIRR, and A20 regulate TLR and NLR responses in IECs (Shibolet and Podolsky, 2007). These inhibitors regulate the potential for chronic inflammation in the gut by dampening the response of the PRRs. SIGIRR has been characterized to play an active role specifically in the IECs (Shibolet and Podolsky, 2007). Inhibitors such as A20 have been studied to regulate NLRs in immune cells, but the same has not yet been demonstrated in IECs (Hitotsumatsu et al., 2008). miRNA-mediated regulation has also emerged as a central regulatory mechanism supporting tolerance to commensal MAMPs in the gut. In particular, MiR-155 plays an important role in attenuating *Helicobacter pylori*–induced inflammation in gastric epithelial cells (Xiao et al., 2009).

4 Epithelial recognition of microbial signals and its implications in diseases

4.1 Infection and inflammatory state

Despite an overall muted response to the presence of microbial cues and a tolerogenic response to MAMPs at the epithelial barrier, the primary function of IECs remain to be the identification and expulsion of any invading pathogen. The inflammasome-forming NLRC4 is a sensor of flagellin and bacterial secretion systems, and its epithelial expression promotes the extrusion of infected IECs from the epithelial layer (Nordlander et al., 2014; Sellin et al., 2014). NLRC4 has also been implicated for its role in protecting the host from intestinal carcinogenesis (Hu et al., 2010; Allam et al., 2015).

Interestingly, PRR signaling is tightly connected to mitochondria and the cellular response to microbial stimulation can be orchestrated at mitochondria. For example, it is well established that activation of various TLRs, including TLR1, TLR2, and TLR4, induce translocation of TRAF6 to mitochondria, resulting in increased ROS production, thereby promoting an inflammatory response (West et al., 2011). Interestingly, in addition to mitochondrial ROS production under pro-inflammatory conditions, mitochondria are also capable of releasing mitochondrial DNA, which acts as a DAMP, thereby promoting inflammatory pathways mediated by TLR9-dependent mechanisms. These pathways might play a role in the pathophysiology of chronic inflammation in humans, as mitochondrial DNA levels are significantly elevated in plasma samples of both UC and CD patients and correlate with disease severity (Boyapati et al., 2018). Pro-inflammatory mechanisms are also mediated by the binding of oxidized mitochondrial DNA to the NLRP3 inflammasome, which induces inflammasome activation during apoptosis (Shimada et al., 2012; Iyer et al., 2013).

Mouse models of ulcerative colitis (UC) are effective tools for studying inflammation in the context of diseases, and several models have successfully established the relationship between excessive NF-κB activation and the pathogenesis of IBD. However, several mouse models have also demonstrated a beneficial role for NF-κB (Wullaert et al., 2011) in maintaining intestinal homeostasis. NEMO is involved in the activation of the canonical NF-κB signaling pathway, and a mouse model with IEC-specific deletion of NEMO shows spontaneous development of severe chronic colitis, characterized by epithelial ulceration, infiltration of immune cells, increased expression of pro-inflammatory mediators, impaired expression of antimicrobial peptides, and translocation of bacteria into the bowel wall (Nenci et al., 2007). This is thought to be due to the fact that NF-κB deficiency leads to apoptosis of colonic epithelial cells, triggering a chronic inflammatory response in the colon. NEMO deficiency also sensitizes epithelial cells to tumor necrosis factor (TNF)-induced apoptosis, triggering inflammation even in the absence of NF-κB activation (Nenci et al., 2007). Mice lacking TAK1, a molecule that acts upstream of the IKK complex in IECs, have also been shown to develop spontaneous intestinal inflammation, supporting the role of NF-κB activation in maintaining intestinal mucosal homeostasis (Nenci et al., 2007). These phenomena suggest a double-edged function of inflammatory pathways in epithelial cells, as they both contribute to the maintenance of intestinal homeostasis and facilitate a rapid detection and clearance of pathogens upon invasion.

4.2 Inflammatory bowel disease (IBD)

Chronic inflammation in the gut is a causative factor in the pathogenesis of IBD, including Crohn's disease and ulcerative colitis. Several factors related to PRR signaling at the epithelial barrier are involved in the induction and development of an inflammatory state in the gut.

Dysbiosis is strongly implicated in causing IBD. Exogenous introduction of an *Escherichia coli* strain associated with Crohn's disease into TLR5 KO mice has been shown to promote disease pathogenesis (Carvalho et al., 2012), suggesting that immune dysfunction is an adjunct to specific microbial alterations in the development of IBD. A number of genetic aberrations, including NOD2 (Ogura et al., 2001; Macpherson and Uhr, 2004), which is associated with immune activation by peptidoglycans, and ATG16L1 (Hampe et al., 2007; Rioux et al., 2007), which plays a role in autophagy, have been implicated in the development of chronic inflammation. Variations in the TLR(s)- 2, −4, −5, and −9 genes have also been implicated in the pathogenesis of Crohn's disease (Pierik et al., 2006; Toeroek et al., 2009; Eberl and Boneca, 2010; Chassaing et al., 2014). Epithelial-specific deletion of TLR5 has also been shown to result in microbial dysbiosis and low-grade chronic inflammation (Chassaing et al., 2014).

The individual role of TLR signaling in the development of gut inflammation may depend on the cell type and the interactions between individual TLRs. In the case of colitis development associated with TLR4 signaling, constitutively active TLR4 in epithelial cells did not induce mucosal inflammation in villin-TLR4 transgenic mice (Shang et al., 2008; Fukata et al., 2011). Although selective deletion of MyD88 in IECs results in spontaneous inflammation of the small intestine (Gong et al., 2010), suggesting a protective function of MyD88, MyD88 signaling in myeloid cells was found to be a driver of intestinal inflammation (Asquith et al., 2010).

One of the major factors contributing to the development of chronic inflammation in IBD is the loss of tolerance to commensal

MAMPs at the epithelial barrier. Suppression of TLR9 signaling by adenoviral oligodeoxynucleotides has been shown to suppress intestinal inflammation in several mouse models of chronic colitis (Obermeier et al., 2005). Adenoviral oligodeoxynucleotides block the effect of bacterial cytosine-phosphate-guanosine oligodeoxynucleotides, and therefore innate immune signaling by commensal-derived DNA has been demonstrated as one of the factors inducing intestinal inflammation through activation of TLR9 during chronic colitis. Thus, while TLR signaling contributes to cytoprotection and mucosal restitution in the DSS colitis model, it may also be involved in promoting persistent mucosal inflammation in response to commensal bacteria (Obermeier et al., 2005).

NOD2 regulates the intestinal commensal flora through the secretion of bactericidal factors (Petnicki-Ocwieja et al., 2009). Impaired NOD2 signaling has been shown to alter commensal composition and increase susceptibility to intestinal inflammation due to defective secretion of antimicrobial peptides in the gut, followed by an abnormal immune response to the altered commensal flora (Petnicki-Ocwieja et al., 2009). Impairment of epithelial repair due to certain polymorphisms associated with TLRs is one of the factors contributing to the development and progression of IBD. TLR2 has been shown to play an important role in the induction of connexin-43 (Cx43)-mediated intracellular communication through intracellular gap junctions and controls IEC barrier function and restitution during acute and chronic inflammatory injury (Ey et al., 2009).

The nuclear receptor peroxisome proliferator-activated receptor-γ (PPAR-γ) regulates the expression of NLRP6 in IECs. PPAR-γ, in turn, is known to be induced by TLR4 signaling. This provides a potential anti-inflammatory role for TLR4 as it indirectly regulates NLRP6-mediated protection against DSS-induced mucosal damage (Dubuquoy et al., 2003; Kempster et al., 2011). Furthermore, NLRP3 has a prominent role in intestinal stromal cells in providing resistance to DSS-induced colitis. Both *Caspase-1*$^{-/-}$ and *NLRP3*$^{-/-}$ mice have impaired epithelial proliferation and increased mucosal permeability accompanied by defective healing responses to mucosal damage during DSS-induced colitis (Zaki et al., 2010a). However, the mechanism of NLRP3-mediated regulation of epithelial proliferation is still unknown, although it may involve IFN-γ and IL-18 in the regulation of cell proliferation (Fantuzzi et al., 1998; Nava et al., 2010; Zaki et al., 2011). Dectin-3 deficiency was also found to promote colitis development through severe colonic epithelial cell damage and impaired mucosal healing in the DSS colitis model. This suggests that CLRs may have additional roles in disease pathogenesis (Wang et al., 2016).

4.3 Colorectal carcinoma

Chronic intestinal inflammation triggers tissue transformation to become neoplastic and promotes a higher incidence of colorectal cancer in patients with IBD. Abnormal PRR signaling is thought to result in the dysregulated expression of genes and enzymes that regulate cell apoptosis, proliferation, and DNA repair. Frequent cycles of epithelial injury and repair, as in the case of chronic intestinal inflammation, in the presence of tumor-promoting cytokines, chemokines, and prostaglandins, may also act as a predisposition to genetic mutations, thereby increasing the risk of neoplastic transformation (Kundu and Surh, 2008; Ono, 2008). TLR4 stimulation has been shown to promote the proliferation of human IECs via epidermal growth factor receptor ligand expression (Hsu et al., 2010). Abnormal signaling via TLR2 and TLR4 in both IECs and sub-epithelial macrophages has been shown to induce dysregulated epithelial proliferation and therefore may promote the development of malignancies in the setting of chronic intestinal inflammation. Dysbiosis that arises in the absence of NLRP6 has also been demonstrated to promote cancer development through IL6-induced epithelial proliferation (Hu et al., 2013).

In the AOM-DSS-induced colitis-associated cancer model in mice, a single injection of azoxymethane (AOM) followed by repeated cycles of DSS treatment and periods of recovery is used to model colitis-associated cancer. The model represents recurrent mucosal injury and repair leading to dysplastic transformation in the colon (Suzuki et al., 2005). Some of the work in this mouse model provides a better understanding of the interplay between the various IEC-expressed TLRs in inducing epithelial hyperplasia under chronic inflammatory conditions. The *TLR4*$^{-/-}$ mouse has been shown to be protected against tumor development with reduced expression of mucosal COX-2, PGE2 and amphiregulin (Fukata et al., 2007). Bone marrow transplant-based analysis of the role of selective TLR4 signaling in colonic epithelial cells *versus* the myeloid cells showed a seminal role TLR4 signaling in epithelial cells in the development of dysplastic lesions (Fukata et al., 2009). This highlights the central role of innate immune signaling in epithelial cells in the formation of dysplastic lesions, as well as the recruitment of Cox-2-expressing macrophages and neutrophils during the development and progression of colorectal cancer. Conversely, *TLR2*$^{-/-}$ mice treated with AOM-DSS have been shown to have an increased tumor incidence with rampant proliferation and dampened apoptosis, although TLR2 deletion under normal conditions shows a reduced proliferation and increased apoptosis in IECs (Lowe et al., 2010). This increased tumor burden in *TLR2*$^{-/-}$ mice was further explained by the overactivation of signal transducer and activator of transcription 3 (STAT3) in epithelial cells and the elevated expression of tumor-promoting cytokines, such as IL-6, IL-17A and TNF-α in the gut mucosa.

MyD88$^{-/-}$ mouse models show variable responses to carcinoma challenge, depending on the differential inflammation in the models (Uronis et al., 2009; Salcedo et al., 2010; Schiechl et al., 2011). *MyD88*$^{-/-}$ mice show no proliferation in IECs after AOM-DSS treatment (Schiechl et al., 2011), however, *MyD88*$^{-/-}$ mice show an overall increased susceptibility to AOM-DSS induced intestinal tumors due to an upregulation of Wnt signaling associated genes, angiogenesis and DNA repair. *MyD88*$^{-/-}$ mice also show a higher mutation rate in the β-catenin gene in IECs as a result of AOM-DSS treatment, explaining the susceptibility to tumor pathogenesis (Salcedo et al., 2010). *MyD88*$^{-/-}$ mouse models further explain the development of tumorigenesis in the context of chronic inflammation. In the absence of a chronic inflammation, MyD88 deficiency has been shown to result in resistance to intestinal tumor development in the *Apc*$^{Min/+}$ and AOM mouse models (Rakoff-Nahoum and Medzhitov, 2007; Salcedo et al., 2010), demonstrating that MyD88 signaling can have both tumorigenic

and anti-tumorigenic effects depending on the inflammatory context (Brandl et al., 2010).

The *NLRP3*$^{-/-}$ as well as the *NLRP6*$^{-/-}$ mice have been shown to have a higher incidence of intestinal tumors in the AOM-DSS model due to their inability to produce mature forms of IL-18 and IL-1β (Allen et al., 2010; Zaki et al., 2010b; Hu et al., 2010; Chen et al., 2011). Deletion of functional NLRC4, NLRP12, and caspase-1 also results in increased incidence of tumorigenesis in mouse models (Chen et al., 2008; Hu et al., 2010). The exact mechanism underlying increased IEC proliferation in NLR-deficient mice remains largely unknown, but *NLRP6*$^{-/-}$ mice have shown increased expression of proto-oncogenic genes such as *Mycl1* involved in the Wnt pathway in the AOM-DSS model (Normand et al., 2011), highlighting the possibility of similar mechanisms involved in the tumorigenesis in other knock-out models.

MUC2 deficiency in mice results in an increased predisposition to inflammation-induced colorectal cancer due to the inability to produce mucin via goblet cell stimulation (Velcich et al., 2002; Vander Sluis et al., 2006). The specific role of epithelial cell signaling in the pathogenesis of colorectal cancer, independent of myeloid cells, is a very interesting facet of studying the immunogenic role of IECs in the gut. Deficiency of the epithelial cell-specific MyD88-dependent MMP7 molecule in the *Apc*$^{Min/+}$ mouse model of human familial adenomatous polyposis has been shown to reduce the incidence of tumorigenesis by more than 60% (Wilson et al., 1997; Rakoff-Nahoum and Medzhitov, 2007). Furthermore, MyD88-mediated tumorigenesis driven by epithelial cell signaling has been shown to result in the post-transcriptional stabilization of the c-myc protein, which is involved in the upregulation of anti-apoptotic mechanisms, proliferation and angiogenesis (Lee et al., 2010). This study using the *Apc*$^{Min/+}$ mouse model provides direct evidence of the IEC-dependent signaling pathway leading to rampant IEC proliferation and tumor growth.

The relative role of epithelial cells *versus* the myeloid cells in colitis-associated tumor development is further validated by the observed reduction in tumor incidence in IEC-specific IKK-β deletion, without affecting the overall intestinal inflammation of both AOM and *Apc*$^{Min/+}$ mouse models (Greten et al., 2004). NOD1 signaling has been shown to be protective against colon tumor development in both AOM-DSS and *Apc*$^{Min/+}$ models, where NOD1 plays a pivotal role in maintaining the intestinal epithelial barrier against chemically induced chronic injury, as in the case of these mouse models (Chen et al., 2008). In humans, NOD2 mutations have been associated with a significant risk of developing colorectal cancer (Mockelmann et al., 2009; Tian et al., 2010).

5 Summary

PRR-mediated microbial recognition and signaling at the intestinal epithelial barrier plays a multifaceted role in maintaining epithelial barrier function and homeostasis, microbial composition and localization, development of overall mucosal immune functions, and defines a number of host physiological and metabolic functions. Several mouse model-based studies using IEC-specific PRR knockouts have demonstrated the importance of PRR signaling at the epithelial barrier and its impact on the various immune and metabolic functions in this region. However, further investigation is required to characterize the expression and specific roles of a number of these PRRs in IECs, independent of their roles in myeloid cells. Recently, the β-glucan receptor Dectin-1 has been shown to be a positive inducer of intestinal prostaglandin E2 (PGE2) secretion by myeloid-derived suppressor cells (MDSCs), leading to enhanced colorectal tumorigenesis in human colorectal cancer patient cohorts as well as in AOM-DSS and *Apc*$^{Min/+}$ mouse models. Dectin-1 signaling was correlated with increased PGE2-synthase expression and suppressed *IL22RA2* in human CRC-infiltrating cells (Tang et al., 2023). The intestinal epithelial barrier is the primary site of CRC disease pathogenesis and progression, and thus it is intriguing to question the exact role of Dectin-1 signaling, among other innate immune signaling in IECs in CRC development and pathogenesis. Several innate immune receptors and the resulting crosstalk between the drivers of these signaling cascades have so far only been characterized in myeloid cells, and their independent roles on IECs warrants further investigation. However, in the above-mentioned study, *Tang et al.* could not specifically determine the effect of Dectin-1 receptor ablation on PGE2, as well as *Il22ra2* expression in the intestinal epithelial cells (Tang et al., 2023).

Innate immune signaling at the epithelial barrier has a complex role due to its proximity to the gut microbiota. Although dysbiosis, or a shift in the overall microbial population at the mucosal region, has been implicated in the pathogenesis of several inflammatory diseases as discussed in this review, there is no consensus on the exact composition of the microbiota in health *versus* disease (McBurney et al., 2019). In addition, it would be critical to understand the multitude of factors that lead to the loss of an overall tolerogenic immune response in the mucosal region, leading up to a chronic inflammatory state. Dysbiosis, as a pathologically relevant factor in intestinal inflammatory disorders, is further hampered by the limitations in understanding weather a change in the commensal microbial population is a prerequisite for the development of inflammation or whether the onset of an inflammatory setting leads to a shift in the microbial population. Furthermore, the labeling of certain commensals involved in the development of inflammatory diseases as pathobionts has been questioned and the inclusion of additional factors such as the 'microbial context' has been emphasized to play a key role in defining the opportunistic properties of the gut microbiota in inflammation (Jochum and Stecher, 2020). A deeper understanding of the role of different commensal strains in different contexts of infection and inflammation would allow a broader definition of their contribution to disease pathogenesis.

Crosstalk between the various drivers of innate immune signaling pathways is another very interesting component of PRR signaling at the epithelial barrier. The mechanistic activation of IEC-bound PRRs by their cognate ligands and how activation of these PRRs influence the various canonical and non-canonical signaling pathways requires further investigation. In this context, an orphan nuclear receptor, Nur77, has recently been shown to sense intracellular LPS, leading to non-canonical NLRP3 inflammasome activation via gasdermin-D (GSDMD) processing in macrophages (Zhu et al., 2023). Nur77 expression in macrophages was shown to increase upon treatment with various cognate TLR ligands,

suggesting a possible crosstalk between the canonical and non-canonical signaling pathways of LPS sensing. Further studies investigating such convergent signaling pathways would highlight such crosstalk and their net physiological impact on innate immune signaling in IECs.

Author contributions

Manuscript drafting—KI; Intellectual contributions and manuscript editing—CB, LE. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by DFG projects 375876048 (A03), SFB1181 (C05) and individual grants with project numbers 418055832 and 510624836. The work was also supported by the Interdisciplinary Centre for Clinical Research (IZKF: A76, A93).

Acknowledgments

The authors thank Eva Liebing for critical reading of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Ablasser, A., Bauernfeind, F., Hartmann, G., Latz, E., Fitzgerald, K. A., and Hornung, V. (2009). RIG-I-dependent sensing of poly (dA: dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nat. Immunol.* 10, 1065–1072. doi:10.1038/ni.1779

Abreu, M. T. (2010). Toll-like receptor signalling in the intestinal epithelium: How bacterial recognition shapes intestinal function. *Nat. Rev. Immunol.* 10, 131–144. doi:10.1038/nri2707

Abreu, M. T., Vora, P., Faure, E., Thomas, L. S., Arnold, E. T., and Arditi, M. (2001). Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *J. Immunol.* 167, 1609–1616. doi:10.4049/jimmunol.167.3.1609

Abt, M. C., Osborne, L. C., Monticelli, L. A., Doering, T. A., Alenghat, T., Sonnenberg, G. F., et al. (2012). Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity* 37, 158–170. doi:10.1016/j.immuni.2012.04.011

Allam, R., Maillard, M. H., Tardivel, A., Chennupati, V., Bega, H., Yu, C. W., et al. (2015). Epithelial NAIPs protect against colonic tumorigenesis. *J. Exp. Med.* 212, 369–383. doi:10.1084/jem.20140474

Allen, I. C., TeKippe, E. M., Woodford, R. M. T., Uronis, J. M., Holl, E. K., Rogers, A. B., et al. (2010). The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J. Exp. Med.* 207, 1045–1056. doi:10.1084/jem.20100050

Asquith, M. J., Boulard, O., Powrie, F., and Maloy, K. J. (2010). Pathogenic and protective roles of MyD88 in leukocytes and epithelial cells in mouse models of inflammatory bowel disease. *Gastroenterology* 139, 519–529. e511-512. doi:10.1053/j.gastro.2010.04.045

Baldridge, M. T., Nice, T. J., McCune, B. T., Yokoyama, C. C., Kambal, A., Wheadon, M., et al. (2015). Commensal microbes and interferon-λ determine persistence of enteric murine norovirus infection. *Science* 347, 266–269. doi:10.1126/science.1258025

Barnich, N., Aguirre, J. E., Reinecker, H. C., Xavier, R., and Podolsky, D. K. (2005). Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-{kappa}B activation in muramyl dipeptide recognition. *J. Cell Biol.* 170, 21–26. doi:10.1083/jcb.200502153

Barreau, F., Madre, C., Meinzer, U., Berrebi, D., Dussaillant, M., Merlin, F., et al. (2010). Nod2 regulates the host response towards microflora by modulating T cell function and epithelial permeability in mouse Peyer's patches. *Gut* 59, 207–217. doi:10.1136/gut.2008.171546

Belkaid, Y., and Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell* 157, 121–141. doi:10.1016/j.cell.2014.03.011

Benjamin, J. L., Sumpter, R., Jr., Levine, B., and Hooper, L. V. (2013). Intestinal epithelial autophagy is essential for host defense against invasive bacteria. *Cell Host Microbe* 13, 723–734. doi:10.1016/j.chom.2013.05.004

Bezencon, C., Fuerholz, A., Raymond, F., Mansourian, R., Metairon, S., Le-Coutre, J., et al. (2008). Murine intestinal cells expressing Trpm5 are mostly brush cells and express markers of neuronal and inflammatory cells. *J. Comp. Neurol.* 509, 514–525. doi:10.1002/cne.21768

Bogunovic, M., Davé, S. H., Tilstra, J. S., Chang, D. T. W., Harpaz, N., Xiong, H., et al. (2007). Enteroendocrine cells express functional Toll-like receptors. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292 (6), G1770–G1783. doi:10.1152/ajpgi.00249.2006

Bouskra, D., Brézillon, C., Bérard, M., Werts, C., Varona, R., Boneca, I. G., et al. (2008). Lymphoid tissue Genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature* 456, 507–510. doi:10.1038/nature07450

Boyapati, R. K., Dorward, D. A., Tamborska, A., Kalla, R., Ventham, N. T., Doherty, M. K., et al. (2018). Mitochondrial DNA is a pro-inflammatory damage-associated molecular pattern released during active IBD. *Inflamm. Bowel Dis.* 24 (10), 2113–2122. doi:10.1093/ibd/izy095

Brandl, K., Plitas, G., Mihu, C. N., Ubeda, C., Jia, T., Fleisher, M., et al. (2008). Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature* 455, 804–807. doi:10.1038/nature07250

Brandl, K., Plitas, G., Schnabl, B., DeMatteo, R. P., and Pamer, E. G. (2007). MyD88-mediated signals induce the bactericidal lectin RegIII gamma and protect mice against intestinal Listeria monocytogenes infection. *J. Exp. Med.* 204, 1891–1900. doi:10.1084/jem.20070563

Brandl, K., Sun, L., Neppl, C., Siggs, O. M., Le Gall, S. M., Tomisato, W., et al. (2010). MyD88 signaling in non-hematopoietic cells protects mice against induced colitis by regulating specific EGF receptor ligands. *Proc. Natl. Acad. Sci. U. S. A.* 107, 19967–19972. doi:10.1073/pnas.1014669107

Brown, S. L., Riehl, T. E., Walker, M. R., Geske, M. J., Doherty, J. M., Stenson, W. F., et al. (2007). Myd88-dependent positioning of Ptgs2-expressing stromal cells maintains colonic epithelial proliferation during injury. *J. Clin. Invest.* 117, 258–269. doi:10.1172/JCI29159

Burger, E., Araujo, A., López-Yglesias, A., Rajala, M. W., Geng, L., Levine, B., et al. (2018). Loss of paneth cell autophagy causes acute susceptibility to toxoplasma gondii-mediated inflammation. *Cell Host Microbe* 23, 177–190.e4. doi:10.1016/j.chom.2018.01.001

Candela, M., Turroni, S., Biagi, E., Carbonero, F., Rampelli, S., Fiorentini, C., et al. (2014). Inflammation and colorectal cancer, when microbiota–host mutualism breaks. *World J. Gastroenterol.* 20, 908–922. doi:10.3748/wjg.v20.i4.908

Cario, E., Gerken, G., and Podolsky, D. K. (2007). Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. *Gastroenterology* 132, 1359–1374. doi:10.1053/j.gastro.2007.02.056

Cario, E., Gerken, G., and Podolsky, D. K. (2004). Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. *Gastroenterology* 127, 224–238. doi:10.1053/j.gastro.2004.04.015

Cario, E. (2010). Heads up! How the intestinal epithelium safe-guards mucosal barrier immunity through the inflammasome and beyond. *Curr. Opin. Gastroenterol.* 26, 583–590. doi:10.1097/MOG.0b013e32833d4b88

Cario, E., Rosenberg, I. M., Brandwein, S. L., Beck, P. L., Reinecker, H. C., and Podolsky, D. K. (2000). Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J. Immunol.* 164, 966–972. doi:10.4049/jimmunol.164.2.966

Carvalho, F. A., Koren, O., Goodrich, J. K., Johansson, M. E. V., Nalbantoglu, I., Aitken, J. D., et al. (2012). Transient inability to manage proteobacteria promotes chronic gut inflammation in TLR5-deficient mice. *Cell Host Microbe* 12, 139–152. doi:10.1016/j.chom.2012.07.004

Cash, H. L., Whitham, C. V., Behrendt, C. L., and Hooper, L. V. (2006). Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* 313, 1126–1130. doi:10.1126/science.1127119

Chassaing, B., Ley, R. E., and Gewirtz, A. T. (2014). Intestinal epithelial cell toll-like receptor 5 regulates the intestinal microbiota to prevent low-grade inflammation and metabolic syndrome in mice. *Gastroenterology* 147, 1363–1377. e17. doi:10.1053/j.gastro.2014.08.033

Chen, G. Y., Liu, M., Wang, F., Bertin, J., and Nunez, G. A. (2011). A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. *J. Immunol.* 186, 7187–7194. doi:10.4049/jimmunol.1100412

Chen, G. Y., Shaw, M. H., Redondo, G., and Núñez, G. (2008). The innate immune receptor Nod1 protects the intestine from inflammation-induced tumorigenesis. *Cancer Res.* 68, 10060–10067. doi:10.1158/0008-5472.CAN-08-2061

Choi, Y. J., Im, E., Chung, H. K., Pothoulakis, C., and Rhee, S. H. (2010). TRIF mediates Toll-like receptor 5-induced signaling in intestinal epithelial cells. *J. Biol. Chem.* 285, 37570–37578. doi:10.1074/jbc.M110.158394

Collier Hyams, L. S., Sloane, V., Batten, B. C., and Neish, A. S. (2005). Cutting edge: Bacterial modulation of epithelial signaling via changes in neddylation of cullin-1. *J. Immunol.* 175, 4194–4198. doi:10.4049/jimmunol.175.7.4194

Couturier-Maillard, A., Secher, T., Rehman, A., Normand, S., De Arcangelis, A., Haesler, R., et al. (2013). NOD2-mediated dysbiosis predisposes mice to transmissible colitis and colorectal cancer. *J. Clin. Invest.* 123, 700–711. doi:10.1172/JCI62236

Daly, K., Burdyga, G., Al-Rammahi, M., Moran, A. W., Eastwood, C., and Shirazi-Beechey, S. P. (2020). Toll-like receptor 9 expressed in proximal intestinal enteroendocrine cells detects bacteria resulting in secretion of cholecystokinin. *Biochem. Biophys. Res. Commun.* 525 (4), 936–940. doi:10.1016/j.bbrc.2020.02.163

Dubuquoy, L., Jansson, E. A., Deeb, S., Rakotobe, S., Karoui, M., Colombel, J. F., et al. (2003). Impaired expression of peroxisome proliferator-activated receptor gamma in ulcerative colitis. *Gastroenterology* 124, 1265–1276. doi:10.1016/S0016-5085(03)00271-3

Dupont, N., Lacas-Gervais, S., Bertout, J., Paz, I., Freche, B., Van-Nhieu, G. T., et al. (2009). Shigella phagocytic vacuolar membrane remnants participate in the cellular response to pathogen invasion and are regulated by autophagy. *Cell Host Microbe* 6 (2), 137–149. doi:10.1016/j.chom.2009.07.005

Eberl, G., and Boneca, I. G. (2010). Bacteria and MAMP-induced morphogenesis of the immune system. *Curr. Opin. Immunol.* 22, 448–454. doi:10.1016/j.coi.2010.06.002

Ey, B., Eyking, A., Gerken, G., Podolsky, D. K., and Cario, E. (2009). TLR2 mediates gap junctional intercellular communication through connexin-43 in intestinal epithelial barrier injury. *J. Biol. Chem.* 284, 22332–22343. doi:10.1074/jbc.M901619200

Fahlgren, A., Hammarstrom, S., Danielsson, A., and Hammarstrom, M. L. (2003). Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clin. Exp. Immunol.* 131, 90–101. doi:10.1046/j.1365-2249.2003.02035.x

Fantuzzi, G., Puren, A. J., Harding, M. W., Livingston, D. J., and Dinarello, C. A. (1998). Interleukin-18 regulation of interferon γ production and cell proliferation as shown in interleukin-1β–converting enzyme (Caspase-1)-Deficient mice. *Blood* 91, 2118–2125. doi:10.1182/blood.V91.6.2118

Farin, H. F., Karthaus, W. R., Kujala, P., Rakhshandehroo, M., Schwank, G., Vries, R. G., et al. (2014). Paneth cell extrusion and release of antimicrobial products is directly controlled by immune cell-derived IFN-γ. *J. Exp. Med.* 211, 1393–1405. doi:10.1084/jem.20130753

Ferwerda, G., Meyer-Wentrup, F., Kullberg, B. J., Netea, M. G., and Adema, G. J. (2008). Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. *Cell Microbiol.* 10, 2058–2066. doi:10.1111/j.1462-5822.2008.01188.x

Francescone, R., Hou, V., and Grivennikov, S. I. (2014). Microbiome, inflammation, and cancer. *Cancer J.* 20, 181–189. doi:10.1097/PPO.0000000000000048

Friedrich, C., Mamareli, P., Thiemann, S., Kruse, F., Wang, Z., Holzmann, B., et al. (2017). MyD88 signaling in dendritic cells and the intestinal epithelium controls immunity against intestinal infection with C. rodentium. *Rodentium. PLoS Pathog.* 13, e1006357. doi:10.1371/journal.ppat.1006357

Fukata, M., Chen, A., Klepper, A., Krishnareddy, S., Vamadevan, A. S., Thomas, L. S., et al. (2006). Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: Role in proliferation and apoptosis in the intestine. *Gastroenterology* 131, 862–877. doi:10.1053/j.gastro.2006.06.017

Fukata, M., Chen, A., Vamadevan, A. S., Cohen, J., Breglio, K., Krishnareddy, S., et al. (2007). Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. *Gastroenterology* 133, 1869–1881. doi:10.1053/j.gastro.2007.09.008

Fukata, M., Hernandez, Y., Conduah, D., Cohen, J., Chen, A., Breglio, K., et al. (2009). Innate immune signaling by Toll-like receptor-4 (TLR4) shapes the inflammatory microenvironment in colitis-associated tumors. *Inflamm. Bowel Dis.* 15, 997–1006. doi:10.1002/ibd.20880

Fukata, M., Michelsen, K. S., Eri, R., Thomas, L. S., Hu, B., Lukasek, K., et al. (2005). Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 288, G1055–G1065. doi:10.1152/ajpgi.00328.2004

Fukata, M., Shang, L., Santaolalla, R., Sotolongo, J., Pastorini, C., España, C., et al. (2011). Constitutive activation of epithelial TLR4 augments inflammatory responses to mucosal injury and drives colitis-associated tumorigenesis. *Inflamm. Bowel Dis.* 17, 1464–1473. doi:10.1002/ibd.21527

Funda, D. P., Tuckova, L., Farre, M. A., Iwase, T., Moro, I., and Tlaskalova-Hogenova, H. (2001). CD14 is expressed and released as soluble CD14 by human intestinal epithelial cells *in vitro*: Lipopolysaccharide activation of epithelial cells revisited. *Infect. Immun.* 69, 3772–3781. doi:10.1128/IAI.69.6.3772-3781.2001

Furter, M., Sellin, M. E., Hansson, G. C., and Hardt, W. D. (2019). Mucus architecture and near-surface swimming affect distinct *Salmonella typhimurium* infection patterns along the murine intestinal tract. *Cell Rep.* 27, 2665–2678.e3. doi:10.1016/j.celrep.2019.04.106

Gaudet, R. G., and Gray-Owen, S. D. (2016). Heptose sounds the alarm: Innate sensing of a bacterial sugar stimulates immunity. *PLoS Pathog.* 12 (9), e1005807. doi:10.1371/journal.ppat.1005807

Gaudet, R. G., Guo, C. X., Molinaro, R., Kottwitz, H., Rohde, J. R., Dangeard, A. S., et al. (2017). Innate recognition of intracellular bacterial growth is driven by the TIFA-dependent cytosolic surveillance pathway. *Cell Rep.* 19, 1418–1430. doi:10.1016/j.celrep.2017.04.063

Gaudet, R. G., Sintsova, A., Buckwalter, C. M., Leung, N., Cochrane, A., Li, J., et al. (2015). Innate Immunity. Cytosolic detection of the bacterial metabolite HBP activates TIFA-dependent innate immunity. *Science* 348 (6240), 1251–1255. doi:10.1126/science.aaa4921

Geijtenbeek, T. B., and Gringhuis, S. I. (2016). C-type lectin receptors in the control of T helper cell differentiation. *Nat. Rev. Immunol.* 16, 433–448. doi:10.1038/nri.2016.55

Gerbe, F. J., Van Es, H., Makrini, L., Brulin, B., Mellitzer, G., Robine, S., et al. (2011). Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium. *J. Cell Biol.* 192, 767–780. doi:10.1083/jcb.201010127

Girardin, S. E., Boneca, I. G., Carneiro, L. A. M., Antignac, A., Jéhanno, M., Viala, J., et al. (2003a). Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science* 300, 1584–1587. doi:10.1126/science.1084677

Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., et al. (2003c). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278, 8869–8872. doi:10.1074/jbc.C200651200

Girardin, S. E., Travassos, L. H., Hervé, M., Blanot, D., Boneca, I. G., Philpott, D. J., et al. (2003b). Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *J. Biol. Chem.* 278, 41702–41708. doi:10.1074/jbc.M307198200

Gong, J., Xu, J., Zhu, W., Gao, X., Li, N., and Li, J. (2010). Epithelial-specific blockade of MyD88-dependent pathway causes spontaneous small intestinal inflammation. *Clin. Immunol.* 136, 245–256. doi:10.1016/j.clim.2010.04.001

Goyal, S., Castrillon-Betancur, J. C., Klaile, E., and Slevogt, H. (2018). The interaction of human pathogenic fungi with C-type lectin receptors. *Front. Immunol.* 9, 1261. doi:10.3389/fimmu.2018.01261

Greten, F. R., Eckmann, L., Greten, T. F., Park, J. M., Li, Z. W., Egan, L. J., et al. (2004). IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* 118, 285–296. doi:10.1016/j.cell.2004.07.013

Grøndahl, M. F., Keating, D. J., Vilsbøll, T., and Knop, F. K. (2017). Current therapies that modify glucagon secretion: What is the therapeutic effect of such modifications. *Curr. Diab. Rep.* 17, 128. doi:10.1007/s11892-017-0967-z

Hampe, J., Franke, A., Rosenstiel, P., Till, A., Teuber, M., Huse, K., et al. (2007). A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat. Genet.* 39, 207–211. doi:10.1038/ng1954

Hase, K., Kawano, K., Nochi, T., Pontes, G. S., Fukuda, S., Ebisawa, M., et al. (2009). Uptake through glycoprotein 2 of FimH+ bacteria by M cells initiates mucosal immune response. *Nature* 462, 226–230. doi:10.1038/nature08529

He, B., Xu, W., Santini, P. A., Polydorides, A. D., Chiu, A., Estrella, J., et al. (2007). Intestinal bacteria trigger T cell-independent immunoglobulin A (2) class switching by inducing epithelial cell secretion of the cytokine APRIL. *Immunity* 26, 812–826. doi:10.1016/j.immuni.2007.04.014

Hernandez, Y., Sotolongo, J., Breglio, K., Conduah, D., Chen, A., Xu, R., et al. (2010). The role of prostaglandin E2 (PGE 2) in toll-like receptor 4 (TLR4)-mediated colitis-associated neoplasia. *BMC Gastroenterol.* 10, 82. doi:10.1186/1471-230X-10-82

Hitotsumatsu, O., Ahmad, R. C., Tavares, R., Wang, M., Philpott, D., Turer, E. E., et al. (2008). The ubiquitin-editing enzyme A20 restricts nucleotide-binding oligomerization domain containing 2-triggered signals. *Immunity* 28, 381–390. doi:10.1016/j.immuni.2008.02.002

Hornef, M. W., Frisan, T., Vandewalle, A., Normark, S., and Richter-Dahlfors, A. (2002). Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J. Exp. Med.* 195, 559–570. doi:10.1084/jem.20011788

Hornef, M. W., Normark, B. H., Vandewalle, A., and Normark, S. (2003). Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells. *J. Exp. Med.* 198, 1225–1235. doi:10.1084/jem.20022194

Hsu, D., Fukata, M., Hernandez, Y. G., Sotolongo, J. P., Goo, T., Maki, J., et al. (2010). Toll-like receptor 4 differentially regulates epidermal growth factor-related growth factors in response to intestinal mucosal injury. *Lab. Invest.* 90, 1295–1305. doi:10.1038/labinvest.2010.100

Hsu, Y. M., Zhang, Y., You, Y., Wang, D., Li, H., Duramad, O., et al. (2007). The adaptor protein CARD9 is required for innate immune responses to intracellular pathogens. *Nat. Immunol.* 8, 198–205. doi:10.1038/ni1426

Hu, B., Elinav, E., Huber, S., Booth, C. J., Strowig, T., Jin, C., et al. (2010). Inflammation-induced tumorigenesis in the colon is regulated by caspase-1 and NLRC4. *Proc. Natl. Acad. Sci. U. S. A.* 107, 21635–21640. doi:10.1073/pnas.1016814108

Hu, B., Elinav, E., Huber, S., Strowig, T., Hao, L., Hafemann, A., et al. (2013). Microbiota-induced activation of epithelial IL-6 signaling links inflammasome-driven inflammation with transmissible cancer. *Proc. Natl. Acad. Sci. U. S. A.* 110 (24), 9862–9867. doi:10.1073/pnas.1307575110

Hugot, J. P., Chamaillard, M., Zouali, H., Lesage, S., Cézard, J. P., Belaiche, J., et al. (2001). Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411, 599–603. doi:10.1038/35079107

Ichinohe, T., Pang, I. K., Kumamoto, Y., Peaper, D. R., Ho, J. H., Murray, T. S., et al. (2011). Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc. Natl. Acad. Sci. U. S. A.* 108, 5354–5359. doi:10.1073/pnas.1019378108

Ifrim, D. C., Quintin, J., Courjol, F., Verschueren, I., van Krieken, J. H., Koentgen, F., et al. (2016). The role of Dectin-2 for host defense against disseminated candidiasis. *J. Interferon Cytokine Res.* 36, 267–276. doi:10.1089/jir.2015.0040

Iyer, S. S., He, Q., Janczy, J. R., Elliott, E. I., Zhong, Z., Olivier, A. K., et al. (2013). Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* 39 (2), 311–323. doi:10.1016/j.immuni.2013.08.001

Jochum, L., and Stecher, Baerbel. (2020). Label or concept- what is a pathobiont? *Trends Microbiol.* 28 (10), 789–792. doi:10.1016/j.tim.2020.04.011

Johansson, M. E. V., Phillipson, M., Petersson, J., Velcich, A., Holm, L., and Hansson, G. C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *PNAS* 105 (39), 15064–15069. doi:10.1073/pnas.0803124105

Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S., and Medzhitov, R. (2008). TRAM couples' endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat. Immunol.* 9, 361–368. doi:10.1038/ni1569

Kamada, N., Seo, S. U., Chen, G. Y., and Nunez, G. (2013). Role of the gut microbiota in immunity and inflammatory disease. *Nat. Rev. Immunol.* 13, 321–335. doi:10.1038/nri3430

Kang, Z., Swaidani, S., Yin, W., Wang, C., Barlow, J. L., Gulen, M. F., et al. (2012). Epithelial cell-specific Act1 adaptor mediates interleukin-25-dependent helminth expulsion through expansion of Lin(-)c-Kit(+) innate cell population. *Immunity* 36, 821–833. doi:10.1016/j.immuni.2012.03.021

Kasper, C. A., Sorg, I., Schmutz, C., Tschon, T., Wischnewski, H., Kim, M. L., et al. (2010). Cell-cell propagation of NF-κB transcription factor and MAP kinase activation amplifies innate immunity against bacterial infection. *Immunity* 33 (5), 804–816. doi:10.1016/j.immuni.2010.10.015

Kaufmann, S. H. (1990). Heat shock proteins and the immune response. *Immunol. Today* 11, 129–136. doi:10.1016/0167-5699(90)90050-J

Kawai, T., and Akira, S. (2008). Toll-like receptor and RIG-I like receptor signaling. *Ann. N.Y. Acad. Sci.* 1143, 1–20. doi:10.1196/annals.1443.020

Kelly, C. J., Zheng, L., Campbell, E. L., Saeedi, B., Scholz, C. C., Bayless, A. J., et al. (2015). Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell Host Microbe* 17, 662–671. doi:10.1016/j.chom.2015.03.005

Kempster, S. L., Belteki, G., Forhead, A. J., Fowden, A. L., Catalano, R. D., Lam, B. Y., et al. (2011). Developmental control of the Nlrp6 inflammasome and a substrate, IL-18, in mammalian intestine. *Am. J. Physiol. Gastrointest. Liver Physiol.* 300, G253–G263. doi:10.1152/ajpgi.00397.2010

Kobayashi, K. S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nuñez, G., et al. (2005). Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307 (5710), 731–734. doi:10.1126/science.1104911

Kufer, T. A., Kremmer, E., Adam, A. C., Philpott, D. J., and Sansonetti, P. J. (2008). The pattern-recognition molecule Nod1 is localized at the plasma membrane at sites of bacterial interaction. *Cell Microbiol.* 10, 477–486. doi:10.1111/j.1462-5822.2007.01062.x

Kummer, J. A., Broekhuizen, R., Everett, H., Agostini, L., Kuijk, L., Martinon, F., et al. (2007). Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. *J. Histochem Cytochem* 55, 443–452. doi:10.1369/jhc.6A7101.2006

Kundu, J. K., and Surh, Y. J. (2008). Inflammation: Gearing the journey to cancer. *Mutat. Res.* 659, 15–30. doi:10.1016/j.mrrev.2008.03.002

Lala, S., Ogura, Y., Osborne, C., Hor, S. Y., Bromfield, A., Davies, S., et al. (2003). Crohn's disease and the NOD2 gene: A role for paneth cells. *Gastroenterology* 125 (1), 47–57. doi:10.1016/S0016-5085(03)00661-9

Larraufie, P., Doré, J., Lapaque, N., and Blottière, H. M. (2017). TLR ligands and butyrate increase Pyy expression through two distinct but inter-regulated pathways. *Cell Microbiol.* 19, e12648. doi:10.1111/cmi.12648

Lebrun, L. J., Lenaerts, K., Kiers, D., Pais de Barros, J. P., Le Guern, N., Plesnik, J., et al. (2017). Enteroendocrine L cells sense LPS after gut barrier injury to enhance GLP-1 secretion. *Cell Rep.* 21, 1160–1168. doi:10.1016/j.celrep.2017.10.008

Lecine, P., Esmiol, S., Métais, J. Y., Nicoletti, C., Nourry, C., McDonald, C., et al. (2007). The NOD2-RICK complex signals from the plasma membrane. *J. Biol. Chem.* 282, 15197–15207. doi:10.1074/jbc.M606242200

Lee, J., Mo, J. H., Katakura, K., Alkalay, I., Rucker, A. N., Liu, Y. T., et al. (2006). Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nat. Cell Biol.* 8, 1327–1336. doi:10.1038/ncb1500

Lee, S. H., Hu, L. L., Gonzalez-Navajas, J., Seo, G. S., Shen, C., Brick, J., et al. (2010). ERK activation drives intestinal tumorigenesis in *Apc*$^{min/+}$ mice. *Nat. Med.* 16, 665–670. doi:10.1038/nm.2143

Lei, W., Ren, W., Ohmoto, M., Urban, J. F., Jr, Matsumoto, M., Margolskee, R. F., et al. (2018). Activation of intestinal tuft cell expressed Sucnr1 triggers type 2 immunity in the mouse small intestine. *Proc. Natl. Acad. Sci. USA.* 115, 5552–5557. doi:10.1073/pnas.1720758115

Lippmann, J., Gwinner, F., Rey, C., Tamir, U., Law, H. K., Schwikowski, B., et al. (2015). Bacterial internalization, localization, and effectors shape the epithelial immune response during Shigella flexneri infection. *Infect. Immun.* 83 (9), 3624–3637. doi:10.1128/IAI.00574-15

Loh, K., Herzog, H., and Shi, Y. C. (2015). Regulation of energy homeostasis by the NPY system. *Trends Endocrinol. Metab.* 26, 125–135. doi:10.1016/j.tem.2015.01.003

Lowe, E. L., Crother, T. R., Rabizadeh, S., Hu, B., Wang, H., Chen, S., et al. (2010). Toll-like receptor 2 signaling protects mice from tumor development in a mouse model of colitis-induced cancer. *PLoS One* 5, e13027. doi:10.1371/journal.pone.0013027

Mabbott, N. A., Donaldson, D. S., Ohno, H., Williams, I. R., and Mahajan, A. (2013). Microfold (M) cells: Important immunosurveillance posts in the intestinal epithelium. *Mucosal Immunol.* 6, 666–677. doi:10.1038/mi.2013.30

Macia, L., Tan, J., Vieira, A. T., Leach, K., Stanley, D., Luong, S., et al. (2015). Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nat. Commun.* 6, 6734. doi:10.1038/ncomms7734

Macpherson, A. J., and Uhr, T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303, 1662–1665. doi:10.1126/science.1091334

Man, S. M., Zhu, Q., Zhu, L., Liu, Z., Karki, R., Malik, A., et al. (2015). Critical role for the DNA sensor AIM2 in stem cell proliferation and cancer. *Cell* 162, 45–58. doi:10.1016/j.cell.2015.06.001

Martinon, F., and Tschopp, J. (2005). NLRs join TLRs as innate sensors of pathogens. *Trends Immunol.* 26, 447–454. doi:10.1016/j.it.2005.06.004

McBurney, M. I., Davis, C., Fraser, C. M., Schneeman, B. O., Huttenhower, C., Verbeke, K., et al. (2019). Establishing what constitutes a healthy human gut microbiome: State of the science, regulatory considerations, and future directions. *J. Nutr.* 149, 1882–1895. doi:10.1093/jn/nxz154

Meng, C., Bai, C., Brown, T. D., Hood, L. E., and Tian, Q. (2018). Human gut microbiota and gastrointestinal cancer. *Genome Proteomics Bioinform* 16, 33–49. doi:10.1016/j.gpb.2017.06.002

Metidji, A., Omenetti, S., Crotta, S., Li, Y., Nye, E., Ross, E., et al. (2018). The environmental sensor AHR protects from inflammatory damage by maintaining intestinal stem cell homeostasis and barrier integrity. *Immunity* 49, 353–362.e5. doi:10.1016/j.immuni.2018.07.010

Milivojevic, M., Dangeard, A. S., Kasper, C. A., Tschon, T., Emmenlauer, M., Pique, C., et al. (2017). ALPK1 controls TIFA/TRAF6-dependent innate immunity against heptose-1,7-bisphosphate of gram-negative bacteria. *PLOS Pathog.* 13, e1006224. doi:10.1371/journal.ppat.1006224

Mockelmann, N., von Schönfels, W., Buch, S., von Kampen, O., Sipos, B., Egberts, J. H., et al. (2009). Investigation of innate immunity genes CARD4, CARD8 and CARD15 as germline susceptibility factors for colorectal cancer. *BMC Gastroenterol.* 9, 79. doi:10.1186/1471-230X-9-79

Nadjsombati, M. S., McGinty, J. W., Lyons-Cohen, M. R., Jaffe, J. B., DiPeso, L., Schneider, C., et al. (2018). Detection of succinate by intestinal tuft cells triggers a type 2 innate immune circuit. *Immunity* 49, 33–41. doi:10.1016/j.immuni.2018.06.016

Nakato, G., Fukuda, S., Hase, K., Goitsuka, R., Cooper, M. D., and Ohno, H. (2009). New approach for M-cell-specific molecules screening by comprehensive transcriptome analysis. *DNA Res.* 16, 227–235. doi:10.1093/dnares/dsp013

Nava, P., Koch, S., Laukoetter, M. G., Lee, W. Y., Kolegraff, K., Capaldo, C. T., et al. (2010). Interferon-gamma regulates intestinal epithelial homeostasis through

converging beta-catenin signaling pathways. *Immunity* 32, 392–402. doi:10.1016/j.immuni.2010.03.001

Neish, A. S., Gewirtz, A. T., Zeng, H., Young, A. N., Hobert, M. E., Karmali, V., et al. (2000). Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. *Science* 289, 1560–1563. doi:10.1126/science.289.5484.1560

Nenci, A., Becker, C., Wullaert, A., Gareus, R., van Loo, G., Danese, S., et al. (2007). Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* 446, 557–561. doi:10.1038/nature05698

Nice, T. J., Baldridge, M. T., McCune, B. T., Norman, J. M., Lazear, H. M., Artyomov, M., et al. (2015). Interferon-λ cures persistent murine norovirus infection in the absence of adaptive immunity. *Science* 347, 269–273. doi:10.1126/science.1258100

Nigro, G., Rossi, R., Commere, P. H., Jay, P., and Sansonetti, P. J. (2014a). The cytosolic bacterial peptidoglycan sensor Nod2 affords stem cell protection and links microbes to gut epithelial regeneration. *Cell Host Microbe* 15, 792–798. doi:10.1016/j.chom.2014.05.003

Nigro, G., Rossi, R., Commere, P. H., Jay, P., and Sansonetti, P. J. (2014b). The cytosolic bacterial peptidoglycan sensor Nod2 affords stem cell protection and links microbes to gut epithelial regeneration. *Cell Host Microbe* 15, 792–798. doi:10.1016/j.chom.2014.05.003

Nordlander, S., Pott, J., and Maloy, K. J. (2014). NLRC4 expression in intestinal epithelial cells mediates protection against an enteric pathogen. *Mucosal Immunol.* 7, 775–785. doi:10.1038/mi.2013.95

Normand, S., Delanoye-Crespin, A., Bressenot, A., Huot, L., Grandjean, T., Peyrin-Biroulet, L., et al. (2011). Nod-like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. *Proc. Natl. Acad. Sci. U. S. A.* 108, 9601–9606. doi:10.1073/pnas.1100981108

Obermeier, F., Dunger, N., Strauch, U. G., Hofmann, C., Bleich, A., Grunwald, N., et al. (2005). CpG motifs of bacterial DNA essentially contribute to the perpetuation of chronic intestinal inflammation. *Gastroenterology* 129, 913–927. doi:10.1053/j.gastro.2005.06.061

Ogura, Y., Bonen, D. K., Inohara, N., Nicolae, D. L., Chen, F. F., Ramos, R., et al. (2001). A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603–606. doi:10.1038/35079114

O'Neil, D. A., Porter, E. M., Elewaut, D., Anderson, G. M., Eckmann, L., Ganz, T., et al. (1999). Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. *J. Immunol.* 163, 6718–6724. doi:10.4049/jimmunol.163.12.6718

O'Neill, L. A. (2008). When signaling pathways collide positive and negative regulation of Toll-like receptor signal transduction. *Immunity* 29, 12–20. doi:10.1016/j.immuni.2008.06.004

Ono, M. (2008). Molecular links between tumor angiogenesis and inflammation: Inflammatory stimuli of macrophages and cancer cells as targets for therapeutic strategy. *Cancer Sci.* 99, 1501–1506. doi:10.1111/j.1349-7006.2008.00853.x

Park, J. H., Kim, Y. G., McDonald, C., Kanneganti, T. D., Hasegawa, M., Body-Malapel, M., et al. (2007). RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. *J. Immunol.* 178, 2380–2386. doi:10.4049/jimmunol.178.4.2380

Park, J. H., Kotani, T., Konno, T., Setiawan, J., Kitamura, Y., Imada, S., et al. (2016). Promotion of intestinal epithelial cell turnover by commensal bacteria: Role of short-chain fatty acids. *PLoS One* 11, e0156334. doi:10.1371/journal.pone.0156334

Petnicki-Ocwieja, T., Hrncir, T., Liu, Y. J., Biswas, A., Hudcovic, T., Tlaskalova-Hogenova, H., et al. (2009). Nod2 is required for the regulation of commensal microbiota in the intestine. *Proc. Natl. Acad. Sci. USA.* 106, 15813–15818. doi:10.1073/pnas.0907722106

Petrilli, V., Papin, S., Dostert, C., Mayor, A., Martinon, F., and Tschopp, J. (2007). Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ.* 14, 1583–1589. doi:10.1038/sj.cdd.4402195

Pfannkuch, L., Hurwitz, R., Traulsen, J., Sigulla, J., Poeschke, M., Matzner, L., et al. (2019). ADP heptose, a novel pathogen-associated molecular pattern identified in *Helicobacter pylori*. *FASEB J.* 33, 9087–9099. doi:10.1096/fj.201802555R

Pierik, M., Joossens, S., Van-Steen, K., Van-Schuerbeek, N., Vlietinck, R., Rutgeerts, P., et al. (2006). Toll-like receptor-1, -2, and -6 polymorphisms influence disease extension in inflammatory bowel diseases. *Inflamm. Bowel Dis.* 12, 1–8. doi:10.1097/01.MIB.0000195389.11645.ab

Plovier, H., Everard, A., Druart, C., Depommier, C., Van-Hul, M., Geurts, L., et al. (2017). A purified membrane protein from Akkermansia muciniphila or the pasteurized bacterium improves metabolism in obese and diabetic mice. *Nat. Med.* 23, 107–113. doi:10.1038/nm.4236

Podolsky, D. K., Gerken, G., Eyking, A., and Cario, E. (2009). Colitis-associated variant of TLR2 causes impaired mucosal repair because of TFF3 deficiency. *Gastroenterology* 137, 209–220. doi:10.1053/j.gastro.2009.03.007

Price, A. E., Shamardani, K., Lugo, K. A., Deguine, J., Roberts, A. W., Lee, B. L., et al. (2018). A map of toll-like receptor expression in the intestinal epithelium reveals distinct spatial, cell type-specific, and temporal patterns. *Immunity* 49, 560–575.e6. doi:10.1016/j.immuni.2018.07.016

Pull, S. L., Doherty, J. M., Mills, J. C., Gordon, J. I., and Stappenbeck, T. S. (2005). Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. *Proc. Natl. Acad. Sci. U. S. A.* 102, 99–104. doi:10.1073/pnas.0405979102

Rakoff-Nahoum, S., and Medzhitov, R. (2007). Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. *Science* 317, 124–127. doi:10.1126/science.1140488

Ramanan, D., Tang, M. S., Bowcutt, R., Loke, P., and Cadwell, K. (2014). Bacterial sensor Nod2 prevents inflammation of the small intestine by restricting the expansion of the commensal Bacteroides vulgatus. *Immunity* 41, 311–324. doi:10.1016/j.immuni.2014.06.015

Rehwinkel, J., and Reis-e Sousa, C. (2010). RIGorous detection: Exposing virus through RNA sensing. *Science* 327, 284–286. doi:10.1126/science.1185068

Rioux, J. D., Xavier, R. J., Taylor, K. D., Silverberg, M. S., Goyette, P., Huett, A., et al. (2007). Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat. Genet.* 39, 596–604. doi:10.1038/ng2032

Rochereau, N., Drocourt, D., Perouzel, E., Pavot, V., Redelinghuys, P., Brown, G. D., et al. (2013). Dectin-1 is essential for reverse transcytosis of glycosylated sIgA-antigen complexes by intestinal M cells. *PLoS Biol.* 11, e1001658. doi:10.1371/journal.pbio.1001658

Rosenstiel, P., Fantini, M., Bräutigam, K., Kühbacher, T., Waetzig, G. H., Seegert, D., et al. (2003). TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology* 124, 1001–1009. doi:10.1053/gast.2003.50157

Rumio, C., Sommariva, M., Sfondrini, L., Palazzo, M., Morelli, D., Viganò, L., et al. (2011). Induction of Paneth cell degranulation by orally administered Toll-like receptor ligands. *J. Cell. Physiology* 227 (3), 1107–1113. doi:10.1002/jcp.22830

Salcedo, R., Worschech, A., Cardone, M., Jones, Y., Gyulai, Z., Dai, R. M., et al. (2010). MyD88-mediated signaling prevents development of adenocarcinomas of the colon: Role of interleukin-18. *J. Exp. Med.* 207, 1625–1636. doi:10.1084/jem.20100199

Sanada, T., Kim, M., Mimuro, H., Suzuki, M., Ogawa, M., Oyama, A., et al. (2012). The Shigella flexneri effector OspI deamidates UBC13 to dampen the inflammatory response. *Nature* 483 (7391), 623–626. doi:10.1038/nature10894

Schiechl, G., Bauer, B., Fuss, I., Lang, S. A., Moser, C., Ruemmele, P., et al. (2011). Tumor development in murine ulcerative colitis depends on MyD88 signaling of colonic F4/80+CD11b(high)Gr1(low) macrophages. *J. Clin. Invest.* 121, 1692–1708. doi:10.1172/JCI42540

Schneeman, T. A., Bruno, M. E. C., Schjerven, H., Johansen, F. E., Chady, L., and Kaetzel, C. S. (2005). Regulation of the polymeric ig receptor by signaling through TLRs 3 and 4: Linking innate and adaptive immune responses. *J. Immunol.* 175, 376–384. doi:10.4049/jimmunol.175.1.376

Schneider, C., O'Leary, C. E., von Moltke, J., Liang, H. E., Ang, Q. Y., Turnbaugh, P. J., et al. (2018). A metabolite-triggered tuft cell-ILC2 circuit drives small intestinal remodeling. *Cell* 174, 271–284.e14. doi:10.1016/j.cell.2018.05.014

Selleri, S., Palazzo, M., Deola, S., Wang, E., Balsari, A., Marincola, F. M., et al. (2008). Induction of pro-inflammatory programs in enteroendocrine cells by the Toll-like receptor agonists flagellin and bacterial LPS. *Int. Immunol.* 20 (8), 961–970. doi:10.1093/intimm/dxn055

Sellin, M. E., Müller, A. A., Felmy, B., Dolowschiak, T., Diard, M., Tardivel, A., et al. (2014). Epithelium-intrinsic NAIP/NLRC4 inflammasome drives infected enterocyte expulsion to restrict Salmonella replication in the intestinal mucosa. *Cell Host Microbe* 16, 237–248. doi:10.1016/j.chom.2014.07.001

Shang, L., Fukata, M., Thirunarayanan, N., Martin, A. P., Arnaboldi, P., Maussang, D., et al. (2008). Toll-like receptor signaling in small intestinal epithelium promotes B-cell recruitment and IgA production in lamina propria. *Gastroenterology* 135, 529–538. doi:10.1053/j.gastro.2008.04.020

Shi, C. S., and Kehrl, J. H. (2008). MyD88 and trif target Beclin 1 to trigger autophagy in macrophages. *J. Biol. Chem.* 283 (48), 33175–33182. doi:10.1074/jbc.M804478200

Shibolet, O., and Podolsky, D. K. (2007). TLRs in the gut. IV. Negative regulation of toll-like receptors and intestinal homeostasis: Addition by subtraction. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292, G1469–G1473. doi:10.1152/ajpgi.00531.2006

Shimada, K., Crother, T. R., Karlin, J., Dagvadorj, J., Chiba, N., Chen, S., et al. (2012). Oxidized Mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 36 (3), 401–414. doi:10.1016/j.immuni.2012.01.009

Simmons, C. P., Goncalves, N. S., Ghaem-Maghami, M., Bajaj-Elliott, M., Clare, S., Neves, B., et al. (2002). Impaired resistance and enhanced pathology during infection with a non-invasive, attaching effacing enteric bacterial pathogen, Citrobacter rodentium, in mice lacking IL-12 or IFN-gamma. *J. Immunol.* 168, 1804–1812. doi:10.4049/jimmunol.168.4.1804

Singh, N., Gurav, A., Sivaprakasam, S., Brady, E., Padia, R., Shi, H., et al. (2014). Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* 40, 128–139. doi:10.1016/j.immuni.2013.12.007

Smirnova, M. G., Guo, L., Birchall, J. P., and Pearson, J. P. (2003). LPS up-regulates mucin and cytokine mRNA expression and stimulates mucin and cytokine secretion in goblet cells. *Cell Immunol.* 221, 42–49. doi:10.1016/S0008-8749(03)00059-5

Smith, K., McCoy, K. D., and Macpherson, A. J. (2007). Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin. Immunol.* 19, 59–69. doi:10.1016/j.smim.2006.10.002

Sodhi, C. P., Neal, M. D., Siggers, R., Sho, S., Ma, C., Branca, M. F., et al. (2012). Intestinal epithelial toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice. *Gastroenterology* 143(3), 708–718. doi:10.1053/j.gastro.2012.05.053

Sodhi, C. P., Shi, X. H., Richardson, W. M., Grant, Z. S., Shapiro, R. A., Prindle, T., Jr, et al. (2011). Toll-like-receptor-4 inhibits enterocyte proliferation via impaired β-catenin signaling in necrotizing enterocolitis. *Gastroenterology* 138 (1), 185–196. doi:10.1053/j.gastro.2009.09.045

Song-Zhao, G. X., Srinivasan, N., Pott, J., Baban, D., Frankel, G., and Maloy, K. J. (2014). Nlrp3 activation in the intestinal epithelium protects against a mucosal pathogen. *Mucosal Immunol.* 7, 763–774. doi:10.1038/mi.2013.94

Sonnenberg, G. F., Monticelli, L. A., Alenghat, T., Fung, T. C., Hutnick, N. A., Kunisawa, J., et al. (2012). Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science* 336, 1321–1325. doi:10.1126/science.1222551

Suzuki, R., Kohno, H., Sugie, S., and Tanaka, T. (2005). Dose-dependent promoting effect of dextran sodium sulfate on mouse colon carcinogenesis initiated with azoxymethane. *Histol. Histopathol.* 20, 483–492. doi:10.14670/HH-20.483

Takahashi, K., Sugi, Y., Nakano, K., Tsuda, M., Kurihara, K., Hosono, A., et al. (2011). Epigenetic control of the host gene by commensal bacteria in large intestinal epithelial cells. *J. Biol. Chem.* 286, 35755–35762. doi:10.1074/jbc.M111.271007

Tamboli, C. P., Neut, C., Desreumaux, P., and Colombel, J. F. (2004). Dysbiosis in inflammatory bowel disease. *Gut* 53 (1), 1–4. doi:10.1136/gut.53.1.1

Tang, C., Sun, H., Kadoki, M., Han, W., Ye, X., Makusheva, Y., et al. (2023). Blocking Dectin-1 prevents colorectal tumorigenesis by suppressing prostaglandin E2 production in myeloid-derived suppressor cells and enhancing IL-22 binding protein expression. *Nat. Commun.* 14, 1493. doi:10.1038/s41467-023-37229-x

Taylor, P. R., Tsoni, S. V., Willment, J. A., Dennehy, K. M., Rosas, M., Findon, H., et al. (2007). Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* 8, 31–38. doi:10.1038/ni1408

Terry, N., and Margolis, K. G. (2017). Serotonergic mechanisms regulating the GI tract: Experimental evidence and therapeutic relevance. *Handb. Exp. Pharmacol.* 239, 319–342. doi:10.1007/164_2016_103

Thinwa, J., Segovia, J. A., Bose, S., and Dube, P. H. (2014). Integrin-mediated first signal for inflammasome-activation in intestinal epithelial cells. *J. Immunol.* 193 (3), 1373–1382. doi:10.4049/jimmunol.1400145

Thompson, M. R., Kaminski, J. J., Kurt-Jones, E. A., and Fitzgerald, K. A. (2011). Pattern recognition receptors and the innate immune response to viral infection. *Viruses* 3, 920–940. doi:10.3390/v3060920

Tian, Y., Li, Y., Hu, Z., Wang, D., Sun, X., and Ren, C. (2010). Differential effects of NOD2 polymorphisms on colorectal cancer risk: A meta-analysis. *Int. J. Colorectal Dis.* 25, 161–168. doi:10.1007/s00384-009-0809-9

Tien, M. T., Girardin, S. E., Regnault, B., Le Bourhis, L., Dillies, M. A., Coppée, J. Y., et al. (2006). Anti-inflammatory effect of Lactobacillus casei on Shigella-infected human intestinal epithelial cells. *J. Immunol.* 176, 1228–1237. doi:10.4049/jimmunol.176.2.1228

Toeroek, H. P., Glas, J., Endres, I., Tonenchi, L., Teshome, M. Y., Wetzke, M., et al. (2009). Epistasis between Toll-like receptor-9 polymorphisms and variants in NOD2 and IL23R modulates susceptibility to Crohn's disease. *Am. J. Gastroenterol.* 104, 1723–1733. doi:10.1038/ajg.2009.184

Travassos, L. H., Carneiro, L. A. M., Ramjeet, M., Hussey, S., Kim, Y. G., Magalhães, J. G., et al. (2010). Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat. Immunol.* 11, 55–62. doi:10.1038/ni.1823

Uronis, J. M., Mühlbauer, M., Herfarth, H. H., Rubinas, T. C., Jones, G. S., and Jobin, C. (2009). Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility. *PLoS One* 4, e6026. doi:10.1371/journal.pone.0006026

Vaishnava, S., Behrendt, C. L., Ismail, A. S., Eckmann, L., and Hooper, L. V. (2008). Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host–microbial interface. *Proc. Natl. Acad. Sci. U. S. A.* 105, 20858–20863. doi:10.1073/pnas.0808723105

Vaishnava, S., Yamamoto, M., Severson, K. M., Ruhn, K. A., Yu, X., Koren, O., et al. (2011). The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. *Science* 334, 255–258. doi:10.1126/science.1209791

Van-der Sluis, M., De-Koning, B. A., De-Bruijn, A. C., Velcich, A., Meijerink, J. P., Van-Goudoever, J. B., et al. (2006). Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology* 131, 117–129. doi:10.1053/j.gastro.2006.04.020

Velcich, A., Yang, W., Heyer, J., Fragale, A., Nicholas, C., Viani, S., et al. (2002). Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science* 295, 1726–1729. doi:10.1126/science.1069094

Venkatesh, M., Mukherjee, S., Wang, H., Li, H., Sun, K., Benechet, A. P., et al. (2014). Symbiotic bacterial metabolites regulate gastrointestinal barrier function via the xenobiotic sensor PXR and Toll-like receptor 4. *Immunity* 41, 296–310. doi:10.1016/j.immuni.2014.06.014

Volman, J. J., Mensink, R. P., Buurman, W. A., Onning, G., and Plat, J. (2010). The absence of functional dectin-1 on enterocytes may serve to prevent intestinal damage. *Eur. J. Gastroenterol. Hepatol.* 22, 88–94. doi:10.1097/MEG.0b013e32832a20dc

Von-Moltke, J., Ayres, J. S., Kofoed, E. M., Chavarria-Smith, J., and Vance, R. E. (2013). Recognition of bacteria by inflammasomes. *Annu. Rev. Immunol.* 31, 73–106. doi:10.1146/annurev-immunol-032712-095944

Von-Moltke, J., Ji, M., Liang, H. E., and Locksley, R. M. (2016). Tuft-cell-derived IL-25 regulates an intestinal ILC2-epithelial response circuit. *Nature* 529, 221–225. doi:10.1038/nature16161

Wang, T. T., Pan, D., Zhou, Z. C., You, Y., Jiang, C., Zhao, X., et al. (2016). Dectin-3 deficiency promotes colitis development due to impaired antifungal innate immune responses in the gut. *PLoS Pathog.* 12, e1005662. doi:10.1371/journal.ppat.1005662

West, A. P., Brodsky, I. E., Rahner, C., Woo, D. K., Erdjument-Bromage, H., Tempst, P., et al. (2011). TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature* 472, 476–480. doi:10.1038/nature09973

Wilson, C. L., Heppner, K. J., Labosky, P. A., Hogan, B. L., and Matrisian, L. M. (1997). Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. *Proc. Natl. Acad. Sci. U. S. A.* 94, 1402–1407. doi:10.1073/pnas.94.4.1402

Wlodarska, M., Thaiss, C. A., Nowarski, R., Henao-Mejia, J., Zhang, J. P., Brown, E. M., et al. (2014). NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. *Cell* 156, 1045–1059. doi:10.1016/j.cell.2014.01.026

Wullaert, A., Bonnet, M. C., and Pasparakis, M. (2011). NF-κB in the regulation of epithelial homeostasis and inflammation. *Cell Res.* 21, 146–158. doi:10.1038/cr.2010.175

Xiao, B., Liu, Z., Li, B. S., Tang, B., Li, W., Guo, G., et al. (2009). Induction of microRNA-155 during *Helicobacter pylori* infection and its negative regulatory role in the inflammatory response. *J. Infect. Dis.* 200, 916–925. doi:10.1086/605443

Xue, Y., and Man, S. M. (2018). ALPK1: Innate attraction to the sweetness of bacteria. *Cell Res.* 28, 1125–1126. doi:10.1038/s41422-018-0100-0

Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., et al. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell* 161, 264–276. doi:10.1016/j.cell.2015.02.047

Yokoi, Y., Nakamura, K., Yoneda, T., Kikuchi, M., Sugimoto, R., Shimizu, Y., et al. (2019). Paneth cell granule dynamics on secretory responses to bacterial stimuli in enteroids. *Sci. Rep.* 9, 2710. doi:10.1038/s41598-019-39610-7

Zaki, M. H., Boyd, K. L., Vogel, P., Kastan, M. B., Lamkanfi, M., and Kanneganti, T. D. (2010a). The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity* 32, 379–391. doi:10.1016/j.immuni.2010.03.003

Zaki, M. H., Lamkanfi, M., and Kanneganti, T. D. (2011). The Nlrp3 inflammasome: Contributions to intestinal homeostasis. *Trends Immunol.* 32, 171–179. doi:10.1016/j.it.2011.02.002

Zaki, M. H., Vogel, P., Body-Malapel, M., Lamkanfi, M., and Kanneganti, T. D. (2010b). IL-18 production downstream of the Nlrp3 inflammasome confers protection against colorectal tumor formation. *J. Immunol.* 185, 4912–4920. doi:10.4049/jimmunol.1002046

Zelante, T., Iannitti, R. G., Cunha, C., De-Luca, A., Giovannini, G., Pieraccini, G., et al. (2013). Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity* 39, 372–385. doi:10.1016/j.immuni.2013.08.003

Zhang, B., Chassaing, B., Shi, Z., Uchiyama, R., Zhang, Z., Denning, T. L., et al. (2014). Viral infection. Prevention and cure of rotavirus infection via TLR5/NLRC4-mediated production of IL-22 and IL-18. *Science* 346, 861–865. doi:10.1126/science.1256999

Zheng, L., Kelly, C. J., Battista, K. D., Schaefer, R., Lanis, J. M., Alexeev, E. E., et al. (2017). Microbial derived butyrate promotes epithelial barrier function through IL-10 receptor-dependent repression of claudin-2. *J. Immunol.* 199, 2976–2984. doi:10.4049/jimmunol.1700105

Zheng, Y., Valdez, P. A., Danilenko, D. M., Hu, Y., Sa, S. M., Gong, Q., et al. (2008). Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat. Med.* 14, 282–289. doi:10.1038/nm1720

Zhou, P., She, Y., Dong, N., Li, P., He, H., Borio, A., et al. (2018). Alpha-kinase 1 is a cytosolic innate immune receptor for bacterial ADP-heptose. *Nature* 561, 122–126. doi:10.1038/s41586-018-0433-3

Zhu, F., Ma, J., Li, W., Liu, Q., Qin, X., Qian, Y., et al. (2023). The Orphan receptor Nur77 binds cytoplasmic LPS to activate the non-canonical NLRP3 inflammasome. *Immunity* 56, 753–767.e8. doi:10.1016/j.immuni.2023.03.003

Zimmermann, S., Pfannkuch, L., Al-Zeer, M. A., Bartfeld, S., Koch, M., Liu, J., et al. (2017). ALPK1- and TIFA-dependent innate immune response triggered by the *Helicobacter pylori* type IV secretion system. *Cell Rep.* 20, 2384–2395. doi:10.1016/j.celrep.2017.08.039

frontiers | Frontiers in Cell and Developmental Biology

TYPE Perspective
PUBLISHED 14 August 2023
DOI 10.3389/fcell.2023.1213383

OPEN ACCESS

EDITED BY
Carlos Pardo-Pastor,
King's College London, United Kingdom

REVIEWED BY
Triet M. Bui,
Dana–Farber Cancer Institute,
United States

*CORRESPONDENCE
Michael Stürzl,
michael.stuerzl@uk-erlangen.de

†These authors have contributed equally to this work

RECEIVED 22 May 2023
ACCEPTED 31 July 2023
PUBLISHED 14 August 2023

CITATION
Naschberger E, Flierl C, Huang J, Erkert L, Gamez-Belmonte R, Gonzalez-Acera M, Bober M, Mehnert M, Becker C, Schellerer VS, Britzen-Laurent N and Stürzl M (2023), Analysis of the interferon-γ-induced secretome of intestinal endothelial cells: putative impact on epithelial barrier dysfunction in IBD.
Front. Cell Dev. Biol. 11:1213383.
doi: 10.3389/fcell.2023.1213383

Analysis of the interferon-γ-induced secretome of intestinal endothelial cells: putative impact on epithelial barrier dysfunction in IBD

Elisabeth Naschberger[1], Christian Flierl[1], Jinghao Huang[1], Lena Erkert[2], Reyes Gamez-Belmonte[2], Miguel Gonzalez-Acera[2], Magdalena Bober[3], Martin Mehnert[3], Christoph Becker[2], Vera S. Schellerer[4], Nathalie Britzen-Laurent[5†] and Michael Stürzl[1,6*†]

[1]Division of Molecular and Experimental Surgery, Department of Surgery, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany, [2]Department of Medicine I, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany, [3]Biognosys AG, Schlieren, Switzerland, [4]Department of Pediatric Surgery, University Medicine Greifswald, Greifswald, Germany, [5]Division of Surgical Research, Department of Surgery, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany, [6]Comprehensive Cancer Center Erlangen-EMN, Universitätsklinikum Erlangen, Erlangen, Germany

The development of inflammatory bowel diseases (IBD) involves the breakdown of two barriers: the epithelial barrier and the gut-vascular barrier (GVB). The destabilization of each barrier can promote initiation and progression of the disease. Interestingly, first evidence is available that both barriers are communicating through secreted factors that may accordingly serve as targets for therapeutic modulation of barrier functions. Interferon (IFN)-γ is among the major pathogenesis factors in IBD and can severely impair both barriers. In order to identify factors transmitting signals from the GVB to the epithelial cell barrier, we analyzed the secretome of IFN-γ-treated human intestinal endothelial cells (HIEC). To this goal, HIEC were isolated in high purity from normal colon tissues. HIEC were either untreated or stimulated with IFN-γ (10 U/mL). After 48 h, conditioned media (CM) were harvested and subjected to comparative hyper reaction monitoring mass spectrometry (HRM™ MS). In total, 1,084 human proteins were detected in the HIEC-CM. Among these, 43 proteins were present in significantly different concentrations between the CM of IFN-γ- and control-stimulated HIEC. Several of these proteins were also differentially expressed in various murine colitis models as compared to healthy animals supporting the relevance of these proteins secreted by inflammatory activated HIEC in the inter-barrier communication in IBD. The angiocrine pathogenic impact of these differentially secreted HIEC proteins on the epithelial cell barrier and their perspectives as targets to treat IBD by modulation of trans-barrier communication is discussed in detail.

KEYWORDS

IBD-inflammatory bowel disease, cytokines, interferon, secretion, angiocrine, paracrine, barrier, endothelial

Introduction

Inflammatory bowel diseases (IBD) affect several million individuals worldwide, with Crohn's disease (CD) and ulcerative colitis (UC) being the clinically predominant forms. IBD similarities are based on their common presentation as intestinal chronic inflammatory disorders characterized by cyclic flares of destructive inflammation resulting in severe impact on the intestinal barrier functions (Zhang and Li, 2014). Heterogeneity is present at the levels of clinical presentation, immune reactions, molecular-genetic components and microbial players involved (Lloyd-Price et al., 2019).

The intestinal barrier serves manifold tasks, which is also evident from its complex structure composed of two sequential physical barriers. The first barrier from the intestinal lumen is established by the epithelial barrier that consists of a single cell layer of epithelial cells overlaid by a mucus layer, which physically separates the microbiota in the gut lumen from epithelial cells (Stürzl et al., 2021). Directly below this epithelial barrier lies the gut-vascular barrier (GVB) controlling the entry of molecules and cells into the portal circulation and their subsequent delivery to the liver (Spadoni et al., 2015; Spadoni et al., 2017).

The structure and functions of the epithelial barrier have been comprehensively described in previous work (López-Posadas et al., 2017). In contrast, the existence and significant contribution of the GVB to IBD has been recognized only recently. Clinical evidence for a role of the GVB in IBD was obtained by the observation that the vasculature in patients exhibits increased permeability during acute phases of the disease, which is decreasing or absent in remission phases (Langer et al., 2019). In addition, studies in preclinical mouse models revealed that a breakdown of the GVB in the colon allows the permeation of bacteria into the blood with access to distant organs, including the liver, with significant impact on IBD pathogenesis (Spadoni et al., 2015). In own studies, we detected that IFN-γ, an immune-modulatory cytokine with driver activity in IBD pathogenesis, increases vascular permeability in the dextran sodium sulfate (DSS)-induced colitis model (Langer et al., 2019). Increased intestinal blood vessel permeability was associated with structural and functional perturbations of the adherens junction protein vascular endothelial (VE)-cadherin and significant worsening of the disease. An endothelial specific knock-out of the IFN-γ-receptor 2 (IFNγR2) as well as pharmacological vessel stabilization in mouse models suppressed vascular permeability and the development of acute and chronic DSS-colitis (Langer et al., 2019). These results provided clear evidence for the importance of the vascular barrier in IBD.

Effective cooperation of two different barriers requires coordinated action and communication. Well-established communication pathways between the epithelial barrier and the GVB are indicated by the observation that nutrient composition in the gut can affect the blood flow (Stan et al., 2012; Gentile and King, 2018). In addition, epithelial cells can secrete factors in response to pathogens such as cytokines, chemokines, reactive oxygen species, and lipid mediators, which can activate endothelial cells (Boueiz and Hassoun, 2009; Franze et al., 2016; Ferrari et al., 2017; Gentile and King, 2018).

However, the endothelium is not only a passive tube system transporting blood and receiving signals from surrounding cells, but exerts perfusion-independent functions, which actively contribute to the tissue microenvironment in organ development and diseases. In IBD, the intestinal microvasculature is notably involved in immune cell recruitment through expression of cell adhesion molecules (CAMs), such as VCAM1 or MadCAM1 (Binion et al., 1998). The inhibition of T-cell recruitment by targeting the binding of α4β7 integrins to endothelial MadCAM1 represents a new therapeutic axis in IBD (Neurath, 2017).

The first hint for an active paracrine function of the endothelium within the tissue microenvironment was derived from cancer research (Butler et al., 2010). Subsequent studies identified tumor repressive molecules that are expressed and released from endothelial cells, including the slit homolog 2 protein (Slit 2), perlecan, thrombospondin and SPARCL1 (Butler et al., 2010; Franses et al., 2011; Naschberger et al., 2016; Hinshaw and Shevde, 2019). Now, it is generally accepted that endothelial cells can actively trigger the microenvironment via so called "angiocrine factors" -a term that. includes secreted and membrane-bound inhibitory or stimulatory growth factors, trophogens, chemokines, cytokines, extracellular matrix components, exosomes, and other cellular products expressed by endothelial cells (Rafii et al., 2016).

Angiocrine functions in IBD have not been extensively investigated as yet. Only recently, we performed a meta-analysis to investigate whether angiocrine signaling in the colon may impact epithelial barrier functions (Stürzl et al., 2021). This approach yielded six putative candidates that are secreted from endothelial cells and may contribute to IBD pathogenesis, including proteins of the von Willebrand factor domain superfamily (VWA1, vWF), tissue inhibitor of metalloproteinases (TIMP)-1, matrix metalloproteinase (MMP)-14, the chemokine CXCL10, and the matricellular protein SPARCL1 (Stürzl et al., 2021). The expression and known functions of these proteins supported the hypothesis that they may be active in IBD. However, the bioinformatical analysis also showed that the overlap of genes retrieved from the different studies was very low, which was well in agreement with the high variation of activation and organ-dependent plasticity of endothelial cells (Stürzl et al., 2021).

Analysis of the IFN-γ-induced secretome in HIEC

Here we aimed to determine putative angiocrine factors released from cultivated primary human intestinal endothelial cells (HIEC) under pathogenically relevant stimulation in an experimental approach. Based on own previous results we used IFN-γ as a model cytokine for stimulation (Langer et al., 2019). In order to reduce pathogenesis-related heterogeneity we refrained from using patient-derived human HIEC but focused on highly pure cultures of healthy HIEC instead. To this goal, HIEC were isolated from healthy colon areas of five patients who underwent surgical therapy for colorectal cancer (CRC) (see Supplementary Methods). Endothelial cells were isolated by FACS-based cell sorting following previously established protocols (Naschberger et al., 2016; Naschberger et al., 2018). A purity above 98% of all five cultures was determined with reverse transcription quantitative polymerase chain reaction (RT-qPCR) and cytochemistry as described previously (Naschberger et al., 2016; Naschberger et al., 2018) and is exemplarily shown

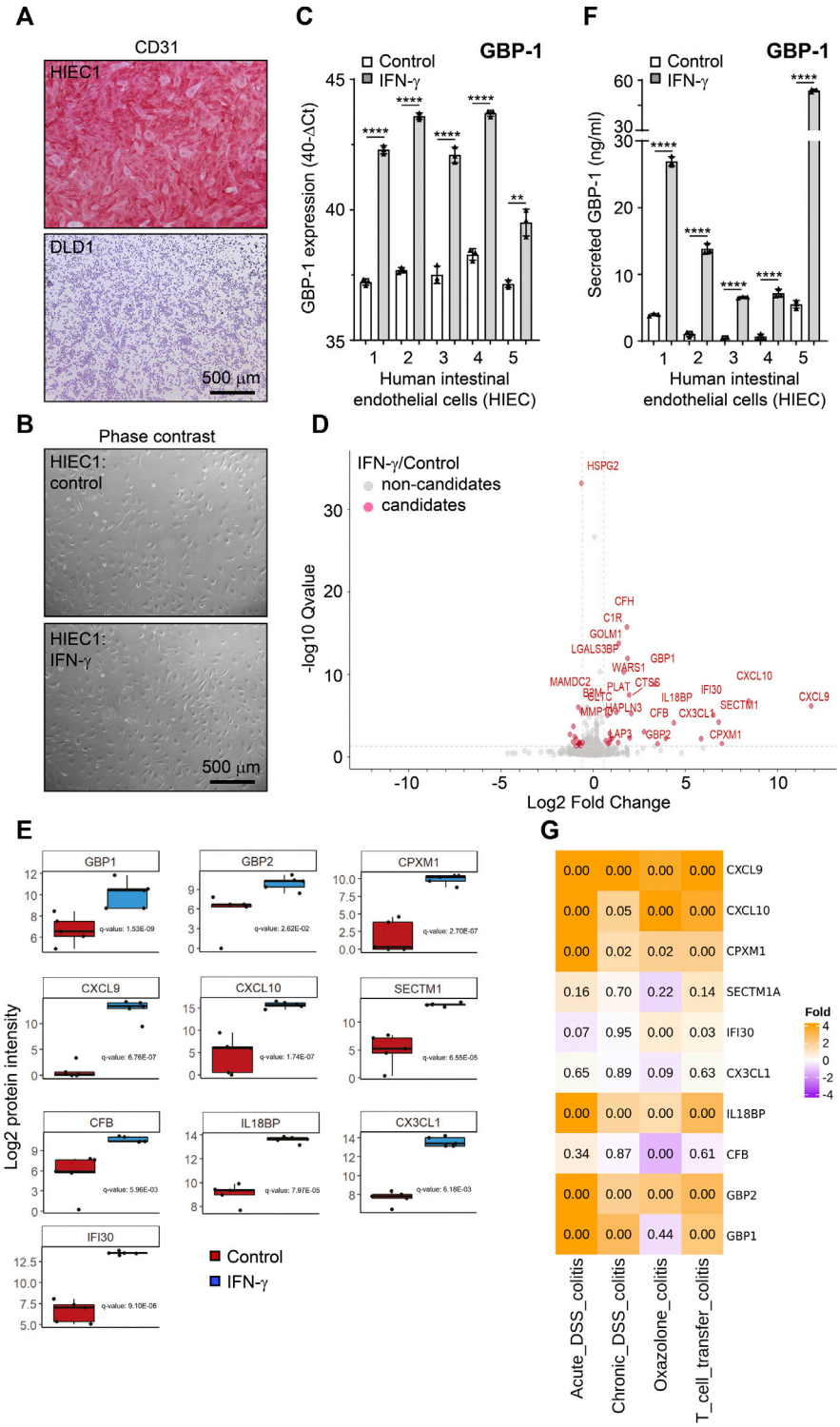


FIGURE 1
The secretome of IFN-γ-treated human intestinal endothelial cells. **(A)** Cultivated human intestinal endothelial cells (HIEC) uniformly express the endothelial cell-specific CD31 antigen whereas the epithelial colorectal cancer cell line DLD1 is negative. **(B)** No difference in the cell phenotype is detected in untreated and IFN-γ-treated HIEC. **(C)** IFN-γ treatment (10 U/mL, 48 h) induces expression of GBP-1 in all HIEC cultures as determined by RT-qPCR. **(D)** Volcano blot of the secretome of IFN-γ-treated HIEC. Proteins present in significantly different concentrations in the cell culture supernatants of IFN-γ-treated and untreated HIEC are indicated in red. **(E)** Box blots showing differential secretion of the different factors in all HIEC cultures (n = 5) in response to IFN-γ. *p*-values were calculated with the one sample *t*-test (μ = 0) and were corrected for overall FDR using the q-value approach (Storey and Tibshirani, 2003). **(F)** IFN-γ treatment (10 U/mL, 48 h) induces secretion of GBP-1 in all HIEC cultures as determined by GBP-1-specific ELISA. **(G)** Expression of genes encoding the top ten secreted proteins from IFN-γ-treated HIEC in different experimentally induced murine colitis models. Expression relative to healthy control mice is indicated by color code. Numbers are representing adjusted *p*-values of statistical differences. **(A,B)** Scale bars correspond to 500 µm. **(C,F)** *p* values: *** = $p < 0.001$, ** = $p < 0.01$, and * = $p < 0.05$, paired *t*-test.

TABLE 1 Significantly changed proteins between the supernatants of IFN-γ treated and untreated HIEC.

Protein description	Gene ID	Number of precursors	Ratio	*p*-value
C-X-C motif chemokine 9	CXCL9	3	3625.19	8.43E-09
C-X-C motif chemokine 10	CXCL10	8	348.02	2.00E-09
Probable carboxypeptidase X1	CPXM1	1	126.95	3.15E-03
Secreted and transmembrane protein 1	SECTM1	4	112.59	1.70E-06
Gamma-interferon-inducible lysosomal thiol reductase	IFI30	5	92.04	1.89E-07
Fractalkine	CX3CL1	2	58.11	3.79E-04
Interleukin-18-binding protein	IL18BP	3	20.93	2.32E-06
Complement factor B	CFB	2	15.52	3.60E-04
Guanylate-binding protein 2	GBP2	3	11.41	2.94E-03
Guanylate-binding protein 1	GBP1	14	10.09	1.27E-11
Hyaluronan and proteoglycan link protein 3	HAPLN3	7	6.86	3.88E-05
Cathepsin S	CTSS	11	4.23	1.13E-07
Signal transducer and activator of transcription 1-alpha/beta	STAT1	3	4.00	2.74E-04
Tryptophan-tRNA ligase. cytoplasmic	WARS1	12	3.91	2.85E-10
Golgi membrane protein 1	GOLM1	16	3.67	6.27E-15
Complement factor H	CFH	31	3.57	5.47E-19
Galectin-3-binding protein	LGALS3BP	15	3.20	3.72E-13
Complement C1r subcomponent	C1R	11	2.65	6.89E-17
Cytosol aminopeptidase	LAP3	3	2.58	1.86E-03
Tissue-type plasminogen activator	PLAT	20	2.41	7.70E-08
Legumain	LGMN	11	2.07	3.38E-04
HLA class I histocompatibility antigen. C alpha chain	HLA-C	13	1.91	5.51E-04
Cystatin-C	CST3	10	1.88	1.88E-03
Keratin. type I cytoskeletal 14	KRT14	10	1.88	6.29E-05
Midkine	MDK	8	1.74	3.10E-03
Beta-2-microglobulin	B2M	5	1.72	2.06E-07
Procathepsin L	CTSL	7	1.61	8.39E-04
Glypican-1	GPC1	7	0.67	1.87E-03
Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	170	0.64	7.03E-37
Clathrin heavy chain 1	CLTC	30	0.64	5.30E-08
X-ray repair cross-complementing protein 6	XRCC6	6	0.63	5.72E-03
Endothelial cell-specific molecule 1	ESM1	6	0.60	1.67E-03
Ephrin type-B receptor 4	EPHB4	2	0.60	1.71E-03
Ephrin-A1	EFNA1	4	0.59	2.42E-03
MAM domain-containing protein 2	MAMDC2	20	0.58	1.55E-08
Eukaryotic translation initiation factor 3 subunit B	EIF3B	4	0.57	4.86E-03
Vesicle-trafficking protein SEC22b	SEC22B	2	0.57	4.10E-03
Lymphatic vessel endothelial hyaluronic acid receptor 1	LYVE1	4	0.53	4.67E-04

(Continued on following page)

TABLE 1 (*Continued*) **Significantly changed proteins between the supernatants of IFN-γ treated and untreated HIEC.**

Protein description	Gene ID	Number of precursors	Ratio	*p*-value
Splicing factor. proline- and glutamine-rich	SFPQ	4	0.52	6.29E-03
Annexin A6	ANXA6	6	0.50	1.91E-04
Stromelysin-2	MMP10	9	0.48	7.77E-06
Placenta growth factor	PGF	3	0.47	1.22E-03
Protein SETSIP; Protein SET	SETSIP, SET	2	0.42	8.19E-05

here by the uniform expression of the endothelial marker CD31 in all HIEC of the different patients but not in the CRC tumor cell line DLD1 (Figure 1A, Supplementary Figures S1A).

The five HIEC cultures were treated with IFN-γ which did not induce different morphology in untreated as compared to treated HIEC (Figure 1B, quantitative evaluation Supplementary Figures S1B). However, successful stimulation of all five cultures was indicated by the expression of IFN-γ-induced guanylate binding protein-1 (GBP-1), a well-established marker for IFN-γ stimulation of eukaryotic cells (Guenzi et al., 2001; Lubeseder-Martellato et al., 2002), which was highly increased in all stimulated HIEC (Figure 1C).

Next, cell culture supernatants were harvested from the IFN-γ-treated and untreated HIEC and subjected to hyper reaction monitoring mass spectrometry (HRM™ MS). Comparison of stimulated and unstimulated cultures identified 1,713 proteins detected by a mean of 5.79 peptides per protein. From all proteins identified, 1,084 were of human origin (629 from medium FBS) with 43 proteins differentially secreted between the CM of IFN-γ-stimulated and unstimulated HIEC (Figure 1D, red; Table 1). The top ten differentially secreted proteins included the chemokines CXCL9, CXCL10 and fractalkine as well as the IFN-γ-induced secreted proteins secreted and transmembrane protein 1 (SECTM1 or K12), gamma-interferon-inducible lysosomal thiol reductase (IFI30) and GBP-1 (Figure 1E). Of note, increased secretion of GBP-1 by IFN-γ-treated HIECs as detected by mass spectrometry was confirmed by independent ELISA (Figure 1F).

Interestingly, with the exception of CXCL10 no further top candidate of our previous meta-analysis was detected in the present study confirming that endothelial cells exhibit high tissue-related heterogeneity and suggesting that HIEC should be preferentially used in order to obtain data of relevance for IBD.

Pathogenic impact of the vascular IFN-γ-secretome in IBD

In order to determine the pathogenic impact of the intestinal vascular IFN-γ-induced secretome, the expression of the top ten secreted proteins was examined in different murine models of experimentally induced colitis, including acute and chronic DSS-colitis as well as oxazolone-induced colitis and T-cell transfer colitis in a next step. The expression of the genes encoding CXCL9, CXCL10, CPMX1, IL18BP and GBP2 was highly significantly increased in each of the different models (Figure 1G). Moreover, GBP-1 and IFI30 showed an increased expression in three and two of the models, respectively (Figure 1G). Only SECTM1, fractalkine and CFB did not show a significant increase of expression in any of the different colitis models (Figure 1G). Altogether, seven of the ten genes, encoding for the most differentially secreted proteins from HIEC in the presence of IFN-γ, also showed significantly increased expression in experimentally induced colitis models, supporting their function in pathogenesis. In the following, the most relevant top candidates involved in IBD pathogenesis retrieved by our screening are discussed in detail.

Discussion

CXCL10

CXCL10 is an 8.7 kDa non-glutamic acid leucine-arginine (ELR)-CXC chemokine, which acts as a ligand for the CXCR3 receptor (Singh et al., 2007). CXCL10 is secreted by several cell types, including endothelial cells, in response to IFN-γ to induce the recruitment and activation of CXCR3+ cells (Singh et al., 2007). CXCL10 is upregulated in colonic tissues of patients with UC and CD compared to control non-IBD tissues (Uguccioni et al., 1999; Zahn et al., 2009; Schroepf et al., 2010; Hosomi et al., 2011; Ostvik et al., 2013). Accordingly, the number of CXCR3-expressing immune cells is increased in the lamina propria of IBD patients (Singh et al., 2007). Expression of CXCL10 in colon biopsies correlates with secondary loss of response to anti-TNF-α therapy after achieving an initial response (Luther et al., 2018). Elevated CXCL10 serum levels correlate with extra-intestinal manifestations indicating that CXCL10 is released into the circulation during IBD (Martinez-Fierro et al., 2019). Furthermore, CXCL10 serum levels are increased in IBD patients with unstable remission compared to patients with stable remission (Kessel et al., 2021). Based on these findings, several clinical trials were performed to test the efficacy of eldelumab, a human monoclonal antibody against CXCL10, as treatment for UC (Trivedi and Adams, 2018). Despite trends towards clinical response and remission, the primary and secondary end points were not met and further dose-response or combination studies are warranted (Danese and Panés, 2014; Mayer et al., 2014; Sandborn et al., 2016). In murine colitis models, inhibition of CXCL10 reduces intestinal inflammation (Sasaki et al., 2002; Singh et al., 2003; Hyun et al., 2005; Suzuki et al., 2007; Zhao et al., 2017) but also had unexpected effects on intestinal epithelial cells (Sasaki et al., 2002; Singh et al., 2003; Hyun et al.,

2005; Suzuki et al., 2007; Zhao et al., 2017). Neutralization of CXCL10 resulted in increased epithelial cell proliferation and decreased apoptosis, which resulted in reduced epithelial ulceration and longer colon crypts (Sasaki et al., 2002; Suzuki et al., 2007). In addition, CD patients with the highest levels of the IFN-γ-induced chemokines CXCL9, CXCL10 and CXCL11 showed hypertrophied epithelial layers at multiple sites (Singh et al., 2007). These findings suggest that CXCL10 secreted by endothelial cells during intestinal inflammation is not only involved in immune cell recruitment but also crypt cell growth regulation and extra-intestinal manifestations.

CXCL9

Similarly to CXCL10, CXCL9 is a CXC-chemokine induced by IFN-γ in numerous cell types. It also binds to the CXCR3 receptor, and is involved in the recruitment of granulocytes and mononuclear cells. CXCL9 expression is increased in mucosal samples of UC and CD patients (Hosomi et al., 2011; Elia and Guglielmi, 2018; Caruso, 2019) and positively correlates with disease activity and negatively with response to treatment using corticosteroids in UC or anti-TNF-α in CD (Egesten et al., 2007; Lacher et al., 2007; Luther et al., 2018; Zhong et al., 2022). Serum CXCL9 levels also reflect disease activity in both UC and CD (Caruso, 2019; Bergemalm et al., 2021; Boucher et al., 2022; Chen et al., 2022) and circulating CXCL9 was identified in preclinical CD and UC as an IBD-risk biomarker (Bergemalm et al., 2021; Leibovitzh et al., 2023) that predicts relapse in UC and CD (Kessel et al., 2021; Walshe et al., 2022). At the molecular level, CXCL9 has been shown to inhibit the reconstitution of the intestinal mucosa after injury (Lu et al., 2015) and to control *E. coli* overgrowth through the pyruvate dehydrogenase-encoding *aceE* gene in a DSS-induced colitis model (Wei et al., 2022). Hence, CXCL9 released by endothelial cells might not only increase immune cell recruitment but also may compromise the epithelial barrier and alter the microbiota in intestinal inflammation.

Fractalkine/CX3CL1

Fractalkine (FKN/CX3CL1) is a transmembrane protein which mediates leukocyte adhesion to endothelial cells (Sans et al., 2007). In addition, a soluble form of fractalkine with chemoattractive properties is secreted by cleavage. Its receptor, CX3CR1, is expressed primarily on the surface of monocytes, natural killer cells, and $CD8^+$ T cells and mediates both adhesive and chemoattractive functions (Sans et al., 2007). Fractalkine expression is upregulated by inflammatory cytokines (IFN-γ, IL-1β and TNF-α) or by direct leukocyte contact (Muehlhoefer et al., 2000; Sans et al., 2007), and has been detected in intestinal epithelial cells and endothelial cells both in normal small intestine and in active Crohn's disease mucosa (Muehlhoefer et al., 2000). However, significantly higher levels of fractalkine mRNA were found in the intestine during active CD and UC (Muehlhoefer et al., 2000; Brand et al., 2006; Kobayashi et al., 2007). Similarly, HIECs isolated from IBD patients exhibited significantly stronger fractalkine expression as compared to control HIECs (Sans et al., 2007). This correlated with significantly higher numbers of mucosal circulating CX3CR1+ T cells in active IBD compared to inactive IBD or healthy subjects (Kobayashi et al., 2007; Sans et al., 2007). The presence of two *CX3CR1* polymorphisms (T280M and V249I) has been associated with intestinal stenosis in CD patients (Brand et al., 2006; Sabate et al., 2008). The knockout/blockade of fractalkine attenuated mucosal inflammation in murine colitis models and showed a moderate clinical response in CD patients (Wakita et al., 2017; Kuboi et al., 2019; Tabuchi et al., 2019; Matsuoka et al., 2021). Targeting endothelial fractalkine might be particularly important to block leukocyte adhesion and migration, platelet adhesion and even angiogenesis (Scaldaferri et al., 2009; Rutella et al., 2011).

GBP-1 and GBP-2

Two members of the guanylate binding protein family, GBP-1 and GBP-2, were detected in our analysis. GBPs are large GTPases, which are expressed in response to stimulation by inflammatory cytokines (Britzen-Laurent et al., 2016). GBP-1 is among the most highly induced proteins by IFN-γ in eukaryotic cells. *In vivo*, a strong expression of GBP-1 is associated with the presence of inflammation and was detected in inflamed tissues during autoimmune diseases or IBD, where it is mostly associated with blood vessels (Lubeseder-Martellato et al., 2002; Haep et al., 2015; Ning et al., 2023). In pediatric patients with IBD, a high expression of GBP-1 was associated with an absence of early response to anti-TNF treatment (Salvador-Martín et al., 2021). Murine GBP-1/GBP-2b is also upregulated during experimental colitis (de Buhr et al., 2006). Intracellular expression of GBP-1 inhibits angiogenesis in endothelial cells (Guenzi et al., 2001), and inhibits proliferation and migration in tumor cells and intestinal epithelial cells, while preventing cell apoptosis (Schnoor et al., 2009; Britzen-Laurent et al., 2013; Ostler et al., 2014). GBP-1 is also able to regulate T-cell receptor signaling (Forster et al., 2014). Interestingly, GBP-1 is specifically and efficiently secreted from endothelial cells by a non-classical, likely ABC transporter-dependent, pathway (Naschberger et al., 2006; Naschberger et al., 2017; Carbotti et al., 2020). GBP-1 has been detected in the serum or cerebrospinal fluid during infectious and inflammatory diseases including bacterial meningitis, systemic lupus erythematosus, rheumatoid arthritis and systemic sclerosis (Naschberger et al., 2006; Hammon et al., 2011; Naschberger et al., 2017). The functions of secreted GBP-1 and GBP-2 remain unknown and further studies are warranted to investigate their potential as blood biomarkers in IBD, as well as their function on the intestinal epithelial barrier.

IL-18BP

IL-18 binding protein (IL-18BP) is a natural circulating high-affinity antagonist of interleukin-18 (IL-18), which belongs to the IL-1 superfamily. While IL-18 is produced by a range of immune and non-immune cells including macrophages, dendritic cells (DCs), fibroblasts and intestinal epithelial cells, its receptor (IL-18R) is expressed by T cells, macrophages, NK-cells or endothelial

cells (Kaplanski, 2018). IL-18BP blocks the binding of IL-18 to IL-18R, thereby dampening IFN-γ production. In children and adult CD patients, elevated expression of both IL-18 and IL-18BP has been detected in mucosal samples, with intestinal endothelial cells and macrophages being the major sources of IL-18BP (Corbaz et al., 2002). Higher IL-18 and IL-18BP levels have also been observed in the serum of IBD patients as compared to controls, which might be attributed to secretion by endothelial cells (Corbaz et al., 2002; Ludwiczek et al., 2005; Naftali et al., 2007; Leach et al., 2008). In particular in CD, circulating levels of both IL-18 and IL-18BP correlated with disease activity, which is well in agreement with the exacerbated Th1 immune response characteristic of the disease (Corbaz et al., 2002; Ludwiczek et al., 2005; Naftali et al., 2007; Leach et al., 2008). However, high levels of free unbound IL-18 are still detectable in CD patients, suggesting that IL-18BP is not produced in sufficient amounts to compensate the effects of IL-18 (Corbaz et al., 2002; Ludwiczek et al., 2005; Naftali et al., 2007; Leach et al., 2008). In DSS-induced experimental colitis the administration of IL-18BP or the neutralization of IL-18 was able to attenuate intestinal inflammation and weight loss (Siegmund et al., 2001; Sivakumar et al., 2002; Siegmund et al., 2004). IL-18BP may act anti-inflammatory not only by inhibition of immune cell recruitment but also through inhibition of IL-18-induced intestinal epithelial permeability (Allam et al., 2018). This is supported by the fact that, the knock-out of IL-18 in endothelial cells, hematopoietic cells or in intestinal epithelial cells was found to abrogate DSS-induced colitis, while the knock-out of IL-18R was only protective when present in intestinal epithelial cells (Nowarski et al., 2015). Overall, IL-18BP is produced and released during IBD, notably by endothelial cells, where it exerts protective effects by dampening the pro-inflammatory effects of IL-18.

Complement factors

Our analysis has revealed an increased secretion of three complement system members in IFN-γ-stimulated intestinal endothelial cells: the complement C1r subcomponent (C1R) from the classical pathway and the complement factor B (CFB) and H (CFH) from the alternative pathway (Lubbers et al., 2017). Complement proteins are produced and secreted mostly by hepatocytes but also by endothelial cells, epithelial cells and leukocytes (Morgan and Gasque, 1997; Lubbers et al., 2017). IBD patients exhibit increased levels of circulating CFB (Nielsen et al., 1978; Campbell et al., 1982; Adinolfi and Lehner, 1988) and a similar increase of serum CFB has been observed in DSS-induced and bisphenol A (BPA)-induced experimental colitis in mice (Huang et al., 2022). More recently, genome-wide association studies (GWAS) have identified one SNP (rs4151657) at the CFB locus, which represents a risk variant for UC susceptibility (Juyal et al., 2015; Gupta et al., 2016; Shi et al., 2020; Mortlock et al., 2023). The presence of the rs4151651 SNP was associated with increased CFB expression, and CFB expression was shown to correlate with disease activity (Shi et al., 2020; Mortlock et al., 2023). CFB expression can be induced in human glomerular endothelial cells and intestinal epithelial cells by different inflammatory cytokines and is found in increased concentrations in the jejunal fluid of IBD patients (Ahrenstedt et al., 1990; Ostvik et al., 2014; Sartain et al., 2016). In contrast to CFB, very little is known about the role of CFH and C1r in IBD. C1r concentration was significantly increased in the serum of CD patients in clinical and serological remission in response to treatment with the anti-TNF-α antibody infliximab, suggesting an inverse correlation between C1r production and disease activity (Gazouli et al., 2013).

Potential new angiocrine factors in IBD

Little is known about the role of CPXM1, IFI30 and SECTM1 in IBD, which were also among the top ten candidates of our screening. SECTM1 is an IFN-γ-regulated molecule acting as a co-stimulatory molecule in T and NK cells, where it binds CD7 (Lyman et al., 2000; Wang et al., 2012; Hubel et al., 2019). SECTM1 is expressed by antigen-presenting cells and epithelial cells that may also secrete a soluble form (Lam et al., 2005; Kamata et al., 2016). Carboxypeptidase X-1 (CPX-1), an inactive member of the metallocarboxypeptidase family encoded by the CPXM1 gene, is also a secreted protein (Reznik and Fricker, 2001; Kim et al., 2015). CPXM1 expression is upregulated in the inflamed intestinal mucosa of CD patients (Hong et al., 2017). Finally, IFN-γ-inducible lysosomal thiol reductase (IFI30/GILT) is a thiol reductase involved in the processing of antigenic proteins for antigen presentation by MHC class II molecules (Barjaktarević et al., 2006). Upregulation of IFI30 has been observed in uterine microvascular endothelial cells in response to IFN-γ (Kitaya et al., 2007).

In conclusion, the important pathogenic role of the vasculature in IBD has been appreciated only recently. Here, we identified and discussed factors secreted from HIEC in the presence of IFN-γ stimulation. Among these factors, CXCL9, CXCL10 and fractalkine have been already described to be closely associated with IBD pathogenesis either in preclinical murine models or in patients. Moreover, we identified novel factors secreted from IFN-γ-activated HIEC including GBP-1, GBP-2, CPXM1, IFI30 and SECTM1. These factors may warrant further studies on their role in IBD pathogenesis and as target for disease monitoring.

Data availability statement

Datasets are available on request: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study involving humans was approved by the Ethics committee of the Friedrich-Alexander-Universität Erlangen-Nürnberg (AZ 159_15B, TuMiC Study) and written informed consent was provided by the patients. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from primarily isolated as part of your previous study for which ethical approval was obtained. Written informed consent

for participation was not required from the participants or the participants' legal guardians/next of kin. The animal study was approved by the Institutional Animal Care and Use Committee of the University of Erlangen and the Animal Experiment Committee of the State Government of Lower Franconia, Würzburg, Germany (55.2 2532–2-1137 and -1178). The studies were conducted in accordance with the local legislation and institutional requirements.

Author contributions

Concept of manuscript: MS and NB-L. Experimental work: CB, CF, EN, LE, JH, MB, MG-A, RG-B, and VSS. Data analyses: CB, EN, LE, MG-A, MB, MM, MS, RG-B, and NB-L. Writing of manuscript: EN, MS, and NB-L. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) Project-ID 375876048—TRR 241 subproject A03 to CB and A06 to NB-L and MS, Project-ID 429280966—TRR 305 subproject B07 to NB-L and B08 to EN, Project-ID 437201724—STU 238/10-1 to MS, Project-ID 280163318—FOR 2438-2 to EN and MS, Project-ID 292410854—BR 5196/2-1 to NB-L; by the W. Lutz Stiftung, the Forschungsstiftung Medizin am Universitätsklinikum Erlangen and a start-up funding from the TRR 241 to NB-L and MS.

Conflict of interest

Authors MB and MM were employed by Biognosys AG.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2023.1213383/full#supplementary-material

References

Adinolfi, M., and Lehner, T. (1988). C9 and factor B as acute phase proteins and their diagnostic and prognostic value in disease. *Exp. Clin. Immunogenet* 5 (2-3), 123–132.

Ahrenstedt, O., Knutson, L., Nilsson, B., Nilsson-Ekdahl, K., Odlind, B., and Hällgren, R. (1990). Enhanced local production of complement components in the small intestines of patients with Crohn's disease. *N. Engl. J. Med.* 322 (19), 1345–1349. doi:10.1056/NEJM199005103221903

Allam, O., Samarani, S., Mehraj, V., Jenabian, M. A., Tremblay, C., Routy, J. P., et al. (2018). HIV induces production of IL-18 from intestinal epithelial cells that increases intestinal permeability and microbial translocation. *PLoS One* 13 (3), 0194185. doi:10.1371/journal.pone.0194185

Barjaktarević, I., Rahman, A., Radoja, S., Bogunović, B., Vollmer, A., Vukmanović, S., et al. (2006). Inhibitory role of IFN-gamma-inducible lysosomal thiol reductase in T cell activation. *J. Immunol.* 177 (7), 4369–4375. doi:10.4049/jimmunol.177.7.4369

Bergemalm, D., Andersson, E., Hultdin, J., Eriksson, C., Rush, S. T., Kalla, R., et al. (2021). Systemic inflammation in preclinical ulcerative colitis. *Gastroenterology* 161 (5), 1526–1539.e9. doi:10.1053/j.gastro.2021.07.026

Binion, D. G., West, G. A., Volk, E. E., Drazba, J. A., Ziats, N. P., Petras, R. E., et al. (1998). Acquired increase in leucocyte binding by intestinal microvascular endothelium in inflammatory bowel disease. *Lancet* 352 (9142), 1742–1746. doi:10.1016/S0140-6736(98)05050-8

Boucher, G., Paradis, A., Chabot-Roy, G., Coderre, L., Hillhouse, E. E., Bitton, A., et al. (2022). Serum analyte profiles associated with Crohn's disease and disease location. *Inflamm. Bowel Dis.* 28 (1), 9–20. doi:10.1093/ibd/izab123

Boueiz, A., and Hassoun, P. M. (2009). Regulation of endothelial barrier function by reactive oxygen and nitrogen species. *Microvasc. Res.* 77 (1), 26–34. doi:10.1016/j.mvr.2008.10.005

Brand, S., Hofbauer, K., Dambacher, J., Schnitzler, F., Staudinger, T., Pfennig, S., et al. (2006). Increased expression of the chemokine fractalkine in Crohn's disease and association of the fractalkine receptor T280M polymorphism with a fibrostenosing disease Phenotype. *Am. J. Gastroenterol.* 101 (1), 99–106. doi:10.1111/j.1572-0241.2005.00361.x

Britzen-Laurent, N., Herrmann, C., Naschberger, E., Croner, R. S., and Stürzl, M. (2016). Pathophysiological role of guanylate-binding proteins in gastrointestinal diseases. *World J. Gastroenterol.* 22 (28), 6434–6443. doi:10.3748/wjg.v22.i28.6434

Britzen-Laurent, N., Lipnik, K., Ocker, M., Naschberger, E., Schellerer, V. S., Croner, R. S., et al. (2013). GBP-1 acts as a tumor suppressor in colorectal cancer cells. *Carcinogenesis* 34 (1), 153–162. doi:10.1093/carcin/bgs310

Butler, J. M., Kobayashi, H., and Rafii, S. (2010). Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nat. Rev. Cancer* 10 (2), 138–146. doi:10.1038/nrc2791

Campbell, C. A., Walker-Smith, J. A., Hindocha, P., and Adinolfi, M. (1982). Acute phase proteins in chronic inflammatory bowel disease in childhood. *J. Pediatr. Gastroenterol. Nutr.* 1 (2), 193–200. doi:10.1097/00005176-198201020-00007

Carbotti, G., Petretto, A., Naschberger, E., Stürzl, M., Martini, S., Mingari, M. C., et al. (2020). Cytokine-induced guanylate binding protein 1 (GBP1) release from human ovarian cancer cells. *Cancers (Basel)* 12 (2), 488. doi:10.3390/cancers12020488

Caruso, C. (2019). MIG in Crohn's disease. *Clin. Ter.* 170 (3), e206–e210. doi:10.7417/CT.2019.2134

Chen, J., Zhou, Y., Sun, Y., Yuan, S., Kalla, R., Sun, J., et al. (2022). Bi-directional Mendelian randomization analysis provides evidence for the causal involvement of dysregulation of CXCL9, CCL11 and CASP8 in the pathogenesis of ulcerative colitis. *J. Crohns Colitis* 17 (5), 777–785. doi:10.1093/ecco-jcc/jjac191

Corbaz, A., ten Hove, T., Herren, S., Graber, P., Schwartsburd, B., Belzer, I., et al. (2002). IL-18-binding protein expression by endothelial cells and macrophages is up-regulated during active Crohn's disease. *J. Immunol.* 168 (7), 3608–3616. doi:10.4049/jimmunol.168.7.3608

Danese, S., and Panés, J. (2014). Development of drugs to target interactions between leukocytes and endothelial cells and treatment algorithms for inflammatory bowel diseases. *Gastroenterology* 147 (5), 981–989. doi:10.1053/j.gastro.2014.08.044

de Buhr, M. F., Mähler, M., Geffers, R., Hansen, W., Westendorf, A. M., Lauber, J., et al. (2006). Cd14, Gbp1, and Pla2g2a: three major candidate genes for experimental IBD identified by combining QTL and microarray analyses. *Physiol. Genomics* 25 (3), 426–434. doi:10.1152/physiolgenomics.00022.2005

Egesten, A., Eliasson, M., Olin, A. I., Erjefält, J. S., Bjartell, A., Sangfelt, P., et al. (2007). The proinflammatory CXC-chemokines GRO-alpha/CXCL1 and MIG/CXCL9 are concomitantly expressed in ulcerative colitis and decrease during treatment with topical corticosteroids. *Int. J. Colorectal Dis.* 22 (12), 1421–1427. doi:10.1007/s00384-007-0370-3

Elia, G., and Guglielmi, G. (2018). CXCL9 chemokine in ulcerative colitis. *Clin. Ter.* 169 (5), e235–e241. doi:10.7417/CT.2018.2085

Ferrari, D., Cimino, F., Fratantonio, D., Molonia, M. S., Bashllari, R., Busà, R., et al. (2017). Cyanidin-3-O-Glucoside modulates the *in vitro* inflammatory crosstalk between intestinal epithelial and endothelial cells. *Mediat. Inflamm.* 2017, 3454023. doi:10.1155/2017/3454023

Forster, F., Paster, W., Supper, V., Schatzlmaier, P., Sunzenauer, S., Ostler, N., et al. (2014). Guanylate binding protein 1-mediated interaction of T cell antigen receptor signaling with the cytoskeleton. *J. Immunol.* 192 (2), 771–781. doi:10.4049/jimmunol.1300377

Franses, J. W., Baker, A. B., Chitalia, V. C., and Edelman, E. R. (2011). Stromal endothelial cells directly influence cancer progression. *Sci. Transl. Med.* 3 (66), 66ra5. doi:10.1126/scitranslmed.3001542

Franze, E., Marafini, I., De Simone, V., Monteleone, I., Caprioli, F., Colantoni, A., et al. (2016). Interleukin-34 induces cc-chemokine ligand 20 in gut epithelial cells. *J. Crohns Colitis* 10 (1), 87–94. doi:10.1093/ecco-jcc/jjv181

Gazouli, M., Anagnostopoulos, A. K., Papadopoulou, A., Vaiopoulou, A., Papamichael, K., Mantzaris, G., et al. (2013). Serum protein profile of Crohn's disease treated with infliximab. *J. Crohns Colitis* 7 (10), e461–e470. doi:10.1016/j.crohns.2013.02.021

Gentile, M. E., and King, I. L. (2018). Blood and guts: the intestinal vasculature during health and helminth infection. *PLoS Pathog.* 14 (7), 1007045. doi:10.1371/journal.ppat.1007045

Guenzi, E., Töpolt, K., Cornali, E., Lubeseder-Martellato, C., Jörg, A., Matzen, K., et al. (2001). The helical domain of GBP-1 mediates the inhibition of endothelial cell proliferation by inflammatory cytokines. *EMBO J.* 20 (20), 5568–5577. doi:10.1093/emboj/20.20.5568

Gupta, A., Juyal, G., Sood, A., Midha, V., Yamazaki, K., Vich Vila, A., et al. (2016). A cross-ethnic survey of CFB and SLC44A4, Indian ulcerative colitis GWAS hits, underscores their potential role in disease susceptibility. *Eur. J. Hum. Genet.* 25 (1), 111–122. doi:10.1038/ejhg.2016.131

Haep, L., Britzen-Laurent, N., Weber, T. G., Naschberger, E., Schaefer, A., Kremmer, E., et al. (2015). Interferon gamma counteracts the angiogenic switch and induces vascular permeability in dextran sulfate sodium colitis in mice. *Inflamm. Bowel Dis.* 21 (10), 2360–2371. doi:10.1097/MIB.0000000000000490

Hammon, M., Herrmann, M., Bleiziffer, O., Pryymachuk, G., Andreoli, L., Munoz, L. E., et al. (2011). Role of guanylate binding protein-1 in vascular defects associated with chronic inflammatory diseases. *J. Cell Mol. Med.* 15 (7), 1582–1592. doi:10.1111/j.1582-4934.2010.01146.x

Hinshaw, D. C., and Shevde, L. A. (2019). The tumor microenvironment innately modulates cancer progression. *Cancer Res.* 79 (18), 4557–4566. doi:10.1158/0008-5472.CAN-18-3962

Hong, S. N., Joung, J. G., Bae, J. S., Lee, C. S., Koo, J. S., Park, S. J., et al. (2017). RNA-Seq reveals transcriptomic differences in inflamed and noninflamed intestinal mucosa of Crohn's disease patients compared with normal mucosa of healthy controls. *Inflamm. Bowel Dis.* 23 (7), 1098–1108. doi:10.1097/MIB.0000000000001066

Hosomi, S., Oshitani, N., Kamata, N., Sogawa, M., Okazaki, H., Tanigawa, T., et al. (2011). Increased numbers of immature plasma cells in peripheral blood specifically overexpress chemokine receptor CXCR3 and CXCR4 in patients with ulcerative colitis. *Clin. Exp. Immunol.* 163 (2), 215–224. doi:10.1111/j.1365-2249.2010.04290.x

Huang, C., Wang, Y., Lin, X., Chan, T. F., Lai, K. P., and Li, R. (2022). Uncovering the functions of plasma proteins in ulcerative colitis and identifying biomarkers for BPA-induced severe ulcerative colitis: a plasma proteome analysis. *Ecotoxicol. Environ. Saf.* 242, 113897. doi:10.1016/j.ecoenv.2022.113897

Hubel, P., Urban, C., Bergant, V., Schneider, W. M., Knauer, B., Stukalov, A., et al. (2019). A protein-interaction network of interferon-stimulated genes extends the innate immune system landscape. *Nat. Immunol.* 20 (4), 493–502. doi:10.1038/s41590-019-0323-3

Hyun, J. G., Lee, G., Brown, J. B., Grimm, G. R., Tang, Y., Mittal, N., et al. (2005). Anti-interferon-inducible chemokine, CXCL10, reduces colitis by impairing T helper-1 induction and recruitment in mice. *Inflamm. Bowel Dis.* 11 (9), 799–805. doi:10.1097/01.mib.0000178263.34099.89

Juyal, G., Negi, S., Sood, A., Gupta, A., Prasad, P., Senapati, S., et al. (2015). Genome-wide association scan in north Indians reveals three novel HLA-independent risk loci for ulcerative colitis. *Gut* 64 (4), 571–579. doi:10.1136/gutjnl-2013-306625

Kamata, H., Yamamoto, K., Wasserman, G. A., Zabinski, M. C., Yuen, C. K., Lung, W. Y., et al. (2016). Epithelial cell-derived secreted and transmembrane 1a signals to activated neutrophils during pneumococcal pneumonia. *Am. J. Respir. Cell Mol. Biol.* 55 (3), 407–418. doi:10.1165/rcmb.2015-0261OC

Kaplanski, G. (2018). Interleukin-18: biological properties and role in disease pathogenesis. *Immunol. Rev.* 281 (1), 138–153. doi:10.1111/imr.12616

Kessel, C., Lavric, M., Weinhage, T., Brueckner, M., de Roock, S., Däbritz, J., et al. (2021). Serum biomarkers confirming stable remission in inflammatory bowel disease. *Sci. Rep.* 11 (1), 6690. doi:10.1038/s41598-021-86251-w

Kim, Y. H., O'Neill, H. M., and Whitehead, J. P. (2015). Carboxypeptidase X-1 (CPX-1) is a secreted collagen-binding glycoprotein. *Biochem. Biophys. Res. Commun.* 468 (4), 894–899. doi:10.1016/j.bbrc.2015.11.053

Kitaya, K., Yasuo, T., Yamaguchi, T., Fushiki, S., and Honjo, H. (2007). Genes regulated by interferon-gamma in human uterine microvascular endothelial cells. *Int. J. Mol. Med.* 20 (5), 689–697.

Kobayashi, T., Okamoto, S., Iwakami, Y., Nakazawa, A., Hisamatsu, T., Chinen, H., et al. (2007). Exclusive increase of CX3CR1+CD28-CD4+ T cells in inflammatory bowel disease and their recruitment as intraepithelial lymphocytes. *Inflamm. Bowel Dis.* 13 (7), 837–846. doi:10.1002/ibd.20113

Kuboi, Y., Nishimura, M., Ikeda, W., Nakatani, T., Seki, Y., Yamaura, Y., et al. (2019). Blockade of the fractalkine-CX3CR1 axis ameliorates experimental colitis by dislodging venous crawling monocytes. *Int. Immunol.* 31 (5), 287–302. doi:10.1093/intimm/dxz006

Lacher, M., Kappler, R., Berkholz, S., Baurecht, H., von Schweinitz, D., and Koletzko, S. (2007). Association of a CXCL9 polymorphism with pediatric Crohn's disease. *Biochem. Biophys. Res. Commun.* 363 (3), 701–707. doi:10.1016/j.bbrc.2007.09.020

Lam, G. K., Liao, H. X., Xue, Y., Alam, S. M., Scearce, R. M., Kaufman, R. E., et al. (2005). Expression of the CD7 ligand K-12 in human thymic epithelial cells: regulation by IFN-gamma. *J. Clin. Immunol.* 25 (1), 41–49. doi:10.1007/s10875-005-0356-5

Langer, V., Vivi, E., Regensburger, D., Winkler, T. H., Waldner, M. J., Rath, T., et al. (2019). IFN-gamma drives inflammatory bowel disease pathogenesis through VE-cadherin-directed vascular barrier disruption. *J. Clin. Invest.* 129 (11), 4691–4707. doi:10.1172/JCI124884

Leach, S. T., Messina, I., Lemberg, D. A., Novick, D., Rubenstein, M., and Day, A. S. (2008). Local and systemic interleukin-18 and interleukin-18-binding protein in children with inflammatory bowel disease. *Inflamm. Bowel Dis.* 14 (1), 68–74. doi:10.1002/ibd.20272

Leibovitzh, H., Lee, S. H., Raygoza Garay, J. A., Espin-Garcia, O., Xue, M., Neustaeter, A., et al. (2023). Immune response and barrier dysfunction-related proteomic signatures in preclinical phase of Crohn's disease highlight earliest events of pathogenesis. *Gut* 72, 1462–1471. doi:10.1136/gutjnl-2022-328421

Lloyd-Price, J., Arze, C., Ananthakrishnan, A. N., Schirmer, M., Avila-Pacheco, J., Poon, T. W., et al. (2019). Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* 569 (7758), 655–662. doi:10.1038/s41586-019-1237-9

López-Posadas, R., Stürzl, M., Atreya, I., Neurath, M. F., and Britzen-Laurent, N. (2017). Interplay of GTPases and cytoskeleton in cellular barrier defects during gut inflammation. *Front. Immunol.* 8, 1240. doi:10.3389/fimmu.2017.01240

Lu, H., Liu, H., Wang, J., Shen, J., Weng, S., Han, L., et al. (2015). The chemokine CXCL9 exacerbates chemotherapy-induced acute intestinal damage through inhibition of mucosal restitution. *J. Cancer Res. Clin. Oncol.* 141 (6), 983–992. doi:10.1007/s00432-014-1869-y

Lubbers, R., van Essen, M. F., van Kooten, C., and Trouw, L. A. (2017). Production of complement components by cells of the immune system. *Clin. Exp. Immunol.* 188 (2), 183–194. doi:10.1111/cei.12952

Lubeseder-Martellato, C., Guenzi, E., Jörg, A., Töpolt, K., Naschberger, E., Kremmer, E., et al. (2002). Guanylate-binding protein-1 expression is selectively induced by inflammatory cytokines and is an activation marker of endothelial cells during inflammatory diseases. *Am. J. Pathol.* 161 (5), 1749–1759. doi:10.1016/S0002-9440(10)64452-5

Ludwiczek, O., Kaser, A., Novick, D., Dinarello, C. A., Rubinstein, M., and Tilg, H. (2005). Elevated systemic levels of free interleukin-18 (IL-18) in patients with Crohn's disease. *Eur. Cytokine Netw.* 16 (1), 27–33.

Luther, J., Gala, M., Patel, S. J., Dave, M., Borren, N., Xavier, R. J., et al. (2018). Loss of response to anti-tumor necrosis factor alpha therapy in Crohn's disease is not associated with emergence of novel inflammatory pathways. *Dig. Dis. Sci.* 63 (3), 738–745. doi:10.1007/s10620-018-4932-8

Lyman, S. D., Escobar, S., Rousseau, A. M., Armstrong, A., and Fanslow, W. C. (2000). Identification of CD7 as a cognate of the human K12 (SECTM1) protein. *J. Biol. Chem.* 275 (5), 3431–3437. doi:10.1074/jbc.275.5.3431

Martinez-Fierro, M. L., Garza-Veloz, I., Rocha-Pizaña, M. R., Cardenas-Vargas, E., Cid-Baez, M. A., Trejo-Vazquez, F., et al. (2019). Serum cytokine, chemokine, and growth factor profiles and their modulation in inflammatory bowel disease. *Med. Baltim.* 98 (38), e17208. doi:10.1097/MD.0000000000017208

Matsuoka, K., Naganuma, M., Hibi, T., Tsubouchi, H., Oketani, K., Katsurabara, T., et al. (2021). Phase 1 study on the safety and efficacy of E6011, antifractalkine antibody, in patients with Crohn's disease. *J. Gastroenterol. Hepatol.* 36 (8), 2180–2186. doi:10.1111/jgh.15463

Mayer, L., Sandborn, W. J., Stepanov, Y., Geboes, K., Hardi, R., Yellin, M., et al. (2014). Anti-IP-10 antibody (BMS-936557) for ulcerative colitis: a phase II randomised study. *Gut* 63 (3), 442–450. doi:10.1136/gutjnl-2012-303424

Morgan, B. P., and Gasque, P. (1997). Extrahepatic complement biosynthesis: where, when and why? *Clin. Exp. Immunol.* 107 (1), 1–7. doi:10.1046/j.1365-2249.1997.d01-890.x

Mortlock, S., Lord, A., Montgomery, G., Zakrzewski, M., Simms, L. A., Krishnaprasad, K., et al. (2023). An extremes of phenotype approach confirms significant genetic heterogeneity in patients with ulcerative colitis. *J. Crohns Colitis* 17 (2), 277–288. doi:10.1093/ecco-jcc/jjac121

Muehlhoefer, A., Saubermann, L. J., Gu, X., Luedtke-Heckenkamp, K., Xavier, R., Blumberg, R. S., et al. (2000). Fractalkine is an epithelial and endothelial cell-derived chemoattractant for intraepithelial lymphocytes in the small intestinal mucosa. *J. Immunol.* 164 (6), 3368–3376. doi:10.4049/jimmunol.164.6.3368

Naftali, T., Novick, D., Gabay, G., Rubinstein, M., and Novis, B. (2007). Interleukin-18 and its binding protein in patients with inflammatory bowel disease during remission and exacerbation. *Isr. Med. Assoc. J.* 9 (7), 504–508.

Naschberger, E., Geißdörfer, W., Bogdan, C., Tripal, P., Kremmer, E., Stürzl, M., et al. (2017). Processing and secretion of guanylate binding protein-1 depend on inflammatory caspase activity. *J. Cell Mol. Med.* 21 (9), 1954–1966. doi:10.1111/jcmm.13116

Naschberger, E., Liebl, A., Schellerer, V. S., Schütz, M., Britzen-Laurent, N., Kölbel, P., et al. (2016). Matricellular protein SPARCL1 regulates tumor microenvironment-dependent endothelial cell heterogeneity in colorectal carcinoma. *J. Clin. Invest.* 126 (11), 4187–4204. doi:10.1172/JCI78260

Naschberger, E., Lubeseder-Martellato, C., Meyer, N., Gessner, R., Kremmer, E., Gessner, A., et al. (2006). Human guanylate binding protein-1 is a secreted GTPase present in increased concentrations in the cerebrospinal fluid of patients with bacterial meningitis. *Am. J. Pathol.* 169 (3), 1088–1099. doi:10.2353/ajpath.2006.060244

Naschberger, E., Regensburger, D., Tenkerian, C., Langheinrich, M., Engel, F. B., Geppert, C., et al. (2018). Isolation of human endothelial cells from normal colon and colorectal carcinoma - an improved protocol. *J. Vis. Exp.* 4 (134), 57400. doi:10.3791/57400

Neurath, M. F. (2017). Current and emerging therapeutic targets for IBD. *Nat. Rev. Gastroenterol. Hepatol.* 14 (5), 269–278. doi:10.1038/nrgastro.2016.208

Nielsen, H., Petersen, P. H., and Svehag, S. E. (1978). Circulating immune complexes in ulcerative colitis--II Correlation with serum protein concentrations and complement conversion products. *Clin. Exp. Immunol.* 31 (1), 81–91.

Ning, Y., Lin, K., Fang, J., Chen, X., Hu, X., Liu, L., et al. (2023). Pyroptosis-related signature predicts the progression of ulcerative colitis and colitis-associated colorectal cancer as well as the anti-TNF therapeutic response. *J. Immunol. Res.* 2023, 7040113. doi:10.1155/2023/7040113

Nowarski, R., Jackson, R., Gagliani, N., de Zoete, M. R., Palm, N. W., Bailis, W., et al. (2015). Epithelial IL-18 equilibrium controls barrier function in colitis. *Cell* 163 (6), 1444–1456. doi:10.1016/j.cell.2015.10.072

Ostler, N., Britzen-Laurent, N., Liebl, A., Naschberger, E., Lochnit, G., Ostler, M., et al. (2014). Gamma interferon-induced guanylate binding protein 1 is a novel actin cytoskeleton remodeling factor. *Mol. Cell Biol.* 34 (2), 196–209. doi:10.1128/MCB.00664-13

Ostvik, A. E., Granlund, A. V. B., Bugge, M., Nilsen, N. J., Torp, S. H., Waldum, H. L., et al. (2013). Enhanced expression of CXCL10 in inflammatory bowel disease: potential role of mucosal toll-like receptor 3 stimulation. *Inflamm. Bowel Dis.* 19 (2), 265–274. doi:10.1002/ibd.23034

Ostvik, A. E., Granlund, A. v., Gustafsson, B. I., Torp, S. H., Espevik, T., Mollnes, T. E., et al. (2014). Mucosal toll-like receptor 3-dependent synthesis of complement factor B and systemic complement activation in inflammatory bowel disease. *Inflamm. Bowel Dis.* 20 (6), 995–1003. doi:10.1097/MIB.0000000000000035

Rafii, S., Butler, J. M., and Ding, B. S. (2016). Angiocrine functions of organ-specific endothelial cells. *Nature* 529 (7586), 316–325. doi:10.1038/nature17040

Reznik, S. E., and Fricker, L. D. (2001). Carboxypeptidases from A to z: implications in embryonic development and wnt binding. *Cell Mol. Life Sci.* 58 (12-13), 1790–1804. doi:10.1007/PL00000819

Rutella, S., Vetrano, S., Correale, C., Graziani, C., Sturm, A., Spinelli, A., et al. (2011). Enhanced platelet adhesion induces angiogenesis in intestinal inflammation and inflammatory bowel disease microvasculature. *J. Cell Mol. Med.* 15 (3), 625–634. doi:10.1111/j.1582-4934.2010.01033.x

Sabate, J. M., Ameziane, N., Lamoril, J., Jouet, P., Farmachidi, J. P., Soulé, J. C., et al. (2008). The V249I polymorphism of the CX3CR1 gene is associated with fibrostenotic disease behavior in patients with Crohn's disease. *Eur. J. Gastroenterol. Hepatol.* 20 (8), 748–755. doi:10.1097/MEG.0b013e3282f824c9

Salvador-Martín, S., Kaczmarczyk, B., Álvarez, R., Navas-López, V. M., Gallego-Fernández, C., Moreno-Álvarez, A., et al. (2021). Whole transcription profile of responders to anti-TNF drugs in pediatric inflammatory bowel disease. *Pharmaceutics* 13 (1), 77. doi:10.3390/pharmaceutics13010077

Sandborn, W. J., Colombel, J. F., Ghosh, S., Sands, B. E., Dryden, G., Hébuterne, X., et al. (2016). Eldelumab [Anti-IP-10] induction therapy for ulcerative colitis: a randomised, placebo-controlled, phase 2b study. *J. Crohns Colitis* 10 (4), 418–428. doi:10.1093/ecco-jcc/jjv224

Sans, M., Danese, S., de la Motte, C., de Souza, H. S. P., Rivera-Reyes, B. M., West, G. A., et al. (2007). Enhanced recruitment of CX3CR1+ T cells by mucosal endothelial cell-derived fractalkine in inflammatory bowel disease. *Gastroenterology* 132 (1), 139–153. doi:10.1053/j.gastro.2006.10.010

Sartain, S. E., Turner, N. A., and Moake, J. L. (2016). TNF regulates essential alternative complement pathway components and impairs activation of protein C in human glomerular endothelial cells. *J. Immunol.* 196 (2), 832–845. doi:10.4049/jimmunol.1500960

Sasaki, S., Yoneyama, H., Suzuki, K., Suriki, H., Aiba, T., Watanabe, S., et al. (2002). Blockade of CXCL10 protects mice from acute colitis and enhances crypt cell survival. *Eur. J. Immunol.* 32 (11), 3197–3205. doi:10.1002/1521-4141(200211)32:11<3197::AID-IMMU3197>3.0.CO;2-1

Scaldaferri, F., Sans, M., Vetrano, S., Correale, C., Arena, V., Pagano, N., et al. (2009). The role of MAPK in governing lymphocyte adhesion to and migration across the microvasculature in inflammatory bowel disease. *Eur. J. Immunol.* 39 (1), 290–300. doi:10.1002/eji.200838316

Schnoor, M., Betanzos, A., Weber, D. A., and Parkos, C. A. (2009). Guanylate-binding protein-1 is expressed at tight junctions of intestinal epithelial cells in response to interferon-gamma and regulates barrier function through effects on apoptosis. *Mucosal Immunol.* 2 (1), 33–42. doi:10.1038/mi.2008.62

Schroepf, S., Kappler, R., Brand, S., Prell, C., Lohse, P., Glas, J., et al. (2010). Strong overexpression of CXCR3 axis components in childhood inflammatory bowel disease. *Inflamm. Bowel Dis.* 16 (11), 1882–1890. doi:10.1002/ibd.21312

Shi, D., Zhong, Z., Wang, M., Cai, L., Fu, D., Peng, Y., et al. (2020). Identification of susceptibility locus shared by IgA nephropathy and inflammatory bowel disease in a Chinese Han population. *J. Hum. Genet.* 65 (3), 241–249. doi:10.1038/s10038-019-0699-9

Siegmund, B., Fantuzzi, G., Rieder, F., Gamboni-Robertson, F., Lehr, H. A., Hartmann, G., et al. (2001). Neutralization of interleukin-18 reduces severity in murine colitis and intestinal IFN-gamma and TNF-alpha production. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 281 (4), R1264–R1273. doi:10.1152/ajpregu.2001.281.4.R1264

Siegmund, B., Sennello, J. A., Lehr, H. A., Senaldi, G., Dinarello, C. A., and Fantuzzi, G. (2004). Frontline: interferon regulatory factor-1 as a protective gene in intestinal inflammation: role of TCR gamma delta T cells and interleukin-18-binding protein. *Eur. J. Immunol.* 34 (9), 2356–2364. doi:10.1002/eji.200425124

Singh, U. P., Singh, S., Taub, D. D., and Lillard, J. W. (2003). Inhibition of IFN-gamma-inducible protein-10 abrogates colitis in IL-10-/- mice. *J. Immunol.* 171 (3), 1401–1406. doi:10.4049/jimmunol.171.3.1401

Singh, U. P., Venkataraman, C., Singh, R., and Lillard, J. W. (2007). CXCR3 axis: role in inflammatory bowel disease and its therapeutic implication. *Endocr. Metab. Immune Disord. Drug Targets* 7 (2), 111–123. doi:10.2174/187153007780832109

Sivakumar, P. V., Westrich, G. M., Kanaly, S., Garka, K., Born, T. L., Derry, J. M. J., et al. (2002). Interleukin 18 is a primary mediator of the inflammation associated with dextran sulphate sodium induced colitis: blocking interleukin 18 attenuates intestinal damage. *Gut* 50 (6), 812–820. doi:10.1136/gut.50.6.812

Spadoni, I., Fornasa, G., and Rescigno, M. (2017). Organ-specific protection mediated by cooperation between vascular and epithelial barriers. *Nat. Rev. Immunol.* 17 (12), 761–773. doi:10.1038/nri.2017.100

Spadoni, I., Zagato, E., Bertocchi, A., Paolinelli, R., Hot, E., Di Sabatino, A., et al. (2015). A gut-vascular barrier controls the systemic dissemination of bacteria. *Science* 350 (6262), 830–834. doi:10.1126/science.aad0135

Stan, R. V., Tse, D., Deharvengt, S. J., Smits, N. C., Xu, Y., Luciano, M. R., et al. (2012). The diaphragms of fenestrated endothelia: gatekeepers of vascular permeability and blood composition. *Dev. Cell* 23 (6), 1203–1218. doi:10.1016/j.devcel.2012.11.003

Storey, J. D., and Tibshirani, R. (2003). Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. U. S. A.* 100 (16), 9440–9445. doi:10.1073/pnas.1530509100

Stürzl, M., Kunz, M., Krug, S. M., and Naschberger, E. (2021). Angiocrine regulation of epithelial barrier integrity in inflammatory bowel disease. *Front. Med. (Lausanne)* 8, 643607. doi:10.3389/fmed.2021.643607

Suzuki, K., Kawauchi, Y., Palaniyandi, S. S., Veeraveedu, P. T., Fujii, M., Yamagiwa, S., et al. (2007). Blockade of interferon-gamma-inducible protein-10 attenuates chronic experimental colitis by blocking cellular trafficking and protecting intestinal epithelial cells. *Pathol. Int.* 57 (7), 413–420. doi:10.1111/j.1440-1827.2007.02117.x

Tabuchi, H., Katsurabara, T., Mori, M., Aoyama, M., Obara, T., Yasuda, N., et al. (2019). Pharmacokinetics, pharmacodynamics, and safety of E6011, a novel humanized antifractalkine (CX3CL1) monoclonal antibody: a randomized, double-blind, placebo-controlled single-ascending-dose study. *J. Clin. Pharmacol.* 59 (5), 688–701. doi:10.1002/jcph.1361

Trivedi, P. J., and Adams, D. H. (2018). Chemokines and chemokine receptors as therapeutic targets in inflammatory bowel disease; pitfalls and promise. *J. Crohns Colitis* 12 (2), 1508–s652. doi:10.1093/ecco-jcc/jjy130

Uguccioni, M., Gionchetti, P., Robbiani, D. F., Rizzello, F., Peruzzo, S., Campieri, M., et al. (1999). Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis. *Am. J. Pathol.* 155 (2), 331–336. doi:10.1016/S0002-9440(10)65128-0

Wakita, H., Yanagawa, T., Kuboi, Y., and Imai, T. (2017). E6130, a novel CX3C chemokine receptor 1 (CX3CR1) modulator, attenuates mucosal inflammation and reduces CX3CR1(+) leukocyte trafficking in mice with colitis. *Mol. Pharmacol.* 92 (5), 502–509. doi:10.1124/mol.117.108381

Walshe, M., Nayeri, S., Ji, J., Hernandez-Rocha, C., Sabic, K., Hu, L., et al. (2022). A role for CXCR3 ligands as biomarkers of post-operative Crohn's disease recurrence. *J. Crohns Colitis* 16 (6), 900–910. doi:10.1093/ecco-jcc/jjab186

Wang, T., Huang, C., Lopez-Coral, A., Slentz-Kesler, K. A., Xiao, M., Wherry, E. J., et al. (2012). K12/SECTM1, an interferon-γ regulated molecule, synergizes with CD28 to costimulate human T cell proliferation. *J. Leukoc. Biol.* 91 (3), 449–459. doi:10.1189/jlb.1011498

Wei, J., Zhang, C., Gao, Y., Li, Y., Zhang, Q., Qi, H., et al. (2022). Gut epithelial-derived CXCL9 maintains gut homeostasis through preventing overgrown *E. coli*. *J. Crohns Colitis* 16 (6), 963–977. doi:10.1093/ecco-jcc/jjab234

Zahn, A., Giese, T., Karner, M., Braun, A., Hinz, U., Stremmel, W., et al. (2009). Transcript levels of different cytokines and chemokines correlate with clinical and endoscopic activity in ulcerative colitis. *BMC Gastroenterol.* 9, 13. doi:10.1186/1471-230X-9-13

Zhang, Y. Z., and Li, Y. Y. (2014). Inflammatory bowel disease: pathogenesis. *World J. Gastroenterol.* 20 (1), 91–99. doi:10.3748/wjg.v20.i1.91

Zhao, Q., Kim, T., Pang, J., Sun, W., Yang, X., Wang, J., et al. (2017). A novel function of CXCL10 in mediating monocyte production of proinflammatory cytokines. *J. Leukoc. Biol.* 102 (5), 1271–1280. doi:10.1189/jlb.5A0717-302

Zhong, W. M., Qian, X. H., and Jin, Z. W. (2022). Identification of potential predictive biomarkers and biological pathways and the correction with immune infiltration in the activation of Crohn's disease. *Immunogenetics* 74 (6), 527–537. doi:10.1007/s00251-022-01274-5

frontiers | Frontiers in Cell and Developmental Biology

TYPE Original Research
PUBLISHED 22 August 2023
DOI 10.3389/fcell.2023.1240289

OPEN ACCESS

EDITED BY
Elena Ortiz Zapater,
University of Valencia, Spain

REVIEWED BY
Raul Ramos,
University of California, Irvine,
United States
Sheng-Jie Yu,
Taichung Veterans General Hospital,
Taiwan

*CORRESPONDENCE
Karin Pfisterer,
karin.pfisterer@meduniwien.ac.at
Wolfgang Weninger,
w.weninger@meduniwien.ac.at

RECEIVED 14 June 2023
ACCEPTED 04 August 2023
PUBLISHED 22 August 2023

CITATION
Pfisterer K, Wielscher M, Samardzic D, Weinzettl P, Symmank D, Shaw LE, Campana R, Huang H-J, Farlik M, Bangert C, Vrtala S, Valenta R and Weninger W (2023), Non-IgE-reactive allergen peptides deteriorate the skin barrier in house dust mite-sensitized atopic dermatitis patients.
Front. Cell Dev. Biol. 11:1240289.
doi: 10.3389/fcell.2023.1240289

Non-IgE-reactive allergen peptides deteriorate the skin barrier in house dust mite-sensitized atopic dermatitis patients

Karin Pfisterer[1]*, Matthias Wielscher[1], David Samardzic[1], Pauline Weinzettl[1], Dorte Symmank[1], Lisa E. Shaw[1], Raffaela Campana[2], Huey-Jy Huang[2,3], Matthias Farlik[1], Christine Bangert[1], Susanne Vrtala[2], Rudolf Valenta[2] and Wolfgang Weninger[1]*

[1]Department of Dermatology, Medical University of Vienna, Vienna, Austria, [2]Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology, and Immunology, Medical University of Vienna, Vienna, Austria, [3]Karl Landsteiner University of Health Sciences, Krems, Austria

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by type 2 cytokine-driven skin inflammation and epithelial barrier dysfunction. The latter is believed to allow the increased penetration of chemicals, toxins, and allergens into the skin. House dust mite allergens, particularly Der p 2, are important triggers in sensitized individuals with AD; the precise actions of these allergens in epithelial biology remain, however, incompletely understood. In this study, we compared the effects of the protein allergen Der p 2 and a mix of non-IgE-reactive Der p 2 peptides on skin cells using patch tests in AD patients and healthy participants. We then analyzed mRNA expression profiles of keratinocytes by single-cell RNA-sequencing. We report that existing barrier deficiencies in the non-lesional skin of AD patients allow deep penetration of Der p 2 and its peptides, leading to local microinflammation. Der p 2 protein specifically upregulated genes involved in the innate immune system, stress, and danger signals in suprabasal KC. Der p 2 peptides further downregulated skin barrier genes, in particular the expression of genes involved in cell–matrix and cell–cell adhesion. Peptides also induced genes involved in hyperproliferation and caused disturbances in keratinocyte differentiation. Furthermore, inflammasome-relevant genes and IL18 were overexpressed, while KRT1 was downregulated. Our data suggest that Der p 2 peptides contribute to AD initiation and exacerbation by augmenting hallmark features of AD, such as skin inflammation, barrier disruption, and hyperplasia of keratinocytes.

KEYWORDS

skin barrier, epithelial barrier disruption, atopic dermatitis, Der p 2, house dust mite, allergy, allergen response in the skin, keratinocytes

1 Introduction

The human skin shields the body against physical, chemical, and immunological threats, preventing the loss of body fluids and the entry of substances from the environment. The epidermis is the outermost layer of the skin and builds the first line of defense. It consists of keratinocytes (KCs) that are continuously renewed and maintained by proliferating cells in the basal layer (Blanpain and Fuchs, 2009). Basal KCs are located on the basement membrane (BM), a sheet-like extracellular matrix (ECM) that separates the dermis and epidermis. The BM is composed of collagen type IV (Col IV), laminin, and fibronectin. Basal KCs strongly adhere to ECM molecules of the BM through supramolecular cell adhesion complexes called hemidesmosomes that contain α6β4 integrin receptors (Garrod, 1993). These complexes establish connections with keratin intermediate filaments (KRT5 and KRT14) and transmembrane Col XVII (Nahidiazar et al., 2015). Basal KCs also exhibit focal adhesion through α/β1 integrins, linking F-actin fibers to the ECM and allowing cell attachment to the BM (Muroyama and Lechler, 2012).

When differentiating into suprabasal KCs, encompassing cells of the stratum spinosum and granulosum, basal KCs weaken their connection with the BM and migrate to the upper layers of the skin. During this process, differentiating KCs reduce their mitotic activity and increase cell–cell interactions in a calcium-dependent mechanism (Carter et al., 1990). Suprabasal KCs downregulate the expression of KRT5 and KRT14 and instead express KRT1 and KRT10, which are connected to robust intercellular junction complexes called desmosomes that stabilize cells and the epithelium (Garrod, 1993; Candi et al., 2005; Blanpain and Fuchs, 2009). Another stabilizing cell–cell connection in the epidermis is adherens junctions, which connect the actin cytoskeleton to the plasma membrane through cadherins (Candi et al., 2005). Additionally, KCs in the granular layer form tight junctions, which contribute to the formation of a dense skin barrier, limiting the entry of molecules that are larger than 500 Da (Da) (Kabashima et al., 2019; Beck et al., 2022). Tight junctions are intercellular belt-like adhesion complexes that connect to F-actin and consist of occludins, claudins, and junctional adhesion molecules (Mertens et al., 2005). They regulate the paracellular molecule passage and prevent transepidermal water loss (Tsakok et al., 2019; Yazici et al., 2022). The barrier function of the skin is mostly dependent on tight junctions and the stratum corneum, the outermost epidermal layer. The stratum corneum is formed through crosslinking of structural proteins and lipids during the terminal differentiation of KCs (Zeeuwen, 2004). Desmosomes, hemidesmosomes, and focal adhesions further regulate the skin barrier function and must be dynamically controlled during cell proliferation and differentiation (Huber et al., 2023).

Atopic dermatitis (AD) is a chronic or relapsing inflammatory skin disease affecting 10%–20% of people in the Global North (Weidinger and Novak, 2016). AD typically develops in early childhood and can persist into adulthood, manifesting in dry, itchy, and inflamed skin lesions (Weidinger and Novak, 2016). This skin disease is characterized by elevated type 2 cytokines, high allergen-specific IgE (in atopic forms), an imbalance of skin microbiota, and a pronounced disruption of the epidermal barrier. In 20%–40% of patients, the barrier dysfunction is caused by a loss-of-function mutation of filaggrin, which is mainly expressed in granular KCs (Langan et al., 2020). Filaggrin binds keratin filaments and is secreted into the stratum corneum, contributing to the barrier formation along with lipids (Langan et al., 2020). Even in the absence of this mutation, filaggrin expression is often decreased in AD patients through downregulation by type 2 cytokines such as IL-4 and IL-13 (Moosbrugger-Martinz et al., 2022). Type 2 immune responses also weaken the skin barrier by reducing the expression of stratum corneum lipids and genes involved in the formation of tight junctions (Langan et al., 2020; Beck et al., 2022). A dysfunctional epidermal barrier induces stress on KCs, resulting in the secretion of proinflammatory cytokines and chemokines, DAMPs, and alarmins, such as IL-1 family cytokines, KRT6, and KRT16 (Lessard et al., 2013; Leung et al., 2020). Pruritus-induced scratching and an imbalance in the bacterial skin microbiome can also induce alarmins, which, in turn, can provoke type 2-mediated immune responses (Leung et al., 2020). This often results in chronic inflammation through a positive feedback loop.

The factors responsible for initiating the development of AD and triggering relapse after the clearance of inflammation are yet not well understood. However, it has been suggested that a dysfunctional epidermal barrier plays a crucial role by allowing the penetration of chemicals or allergens. House dust mite (HDM) allergens, in particular, can induce skin inflammation in allergic patients with AD, but the exact mechanisms are not fully understood (Kaplan et al., 2012; Serhan et al., 2019). Der p 2 from *Dermatophagoides pteronyssinus* is one of the major air-borne HDM allergens (Huang et al., 2019). It can trigger type I hypersensitivity reactions, resulting in IgE responses, high type 2 cytokine production, and histamine release. HDM allergens can also act as a contact allergen and cause local skin inflammation in sensitized individuals, which has been shown in isolated human cells and mouse models (Liedén et al., 2009; Kaplan et al., 2012; Stremnitzer et al., 2014). In healthy skin, allergens with molecular weights of >500 Da cannot pass through the epidermal barrier due to size constraints (Smith et al., 2017). The HDM allergen Der p 1 is approximately 25 kDa in size and can directly bypass the skin barrier using its intrinsic papain-like proteases to disrupt cell–cell adhesions between KCs (Reithofer and Jahn-Schmid, 2017). This is in contrast to Der p 2 (15 kDa), which has no protease activity but may be fragmented into peptides through highly abundant proteases in HDM feces. Furthermore, the skin of people with AD is believed to be more permissive due to a barrier deficiency and may allow deeper penetration of allergens compared to healthy skin.

To date, there is limited understanding of the mechanisms underlying the transition from a non-lesional skin with a barrier deficiency to acute lesional skin with a pronounced barrier disruption in AD. In this study, we compared the ability of the recombinant HDM allergen Der p 2 and a mix of hypoallergenic Der p 2 peptides, which lack IgE reactivity and do not induce basophil activation (Huang et al., 2019), to induce skin inflammation in both AD patients and healthy participants using single-cell RNA sequencing (scRNA-seq). We report here that the existing barrier deficiency in the non-lesional skin of AD patients allowed deep penetration of both protein and peptide allergens. Der p 2 protein caused local microinflammations, as evidenced by the activation of immune system-relevant genes. Non-IgE-reactive Der p 2 peptides downregulated the expression of KRT1 and upregulated the

expression of the inflammasome gene *PYCARD* and the alarmin IL18. Furthermore, Der p 2 peptides significantly disrupted the skin barrier by downregulating the expression of cell–cell and cell–matrix adhesion genes and induced genes associated with hyperproliferation in KCs. Based on our observations, we propose that Der p 2 peptides are involved in disrupting the skin barrier in AD patients sensitized to HDM and thereby contribute to acute disease exacerbations.

2 Materials and methods

2.1 Study participants and ethics statement

The study was approved by the Ethics Committee of the Medical University of Vienna (#2472/2020) and is in accordance with the Declaration of Helsinki principles. Patients were informed about the study procedures, benefits, and risks and gave their written informed consent. Four patients, who were diagnosed with atopic dermatitis according to the Hanifin–Rajka criteria and with reported allergy to house dust mite (HDM), and four healthy participants without any records of chronic inflammatory skin diseases and HDM sensitization were recruited to participate in this study. All participants were aged between 18 and 80 years (Supplementary Figure S1A). Adult human skin samples were obtained as discarded materials from routine plastic surgery in accordance with the Declaration of Helsinki principles and after approval by the Ethics Committee of the Medical University of Vienna.

2.2 Expression and purification of recombinant Der p 2 and synthesis of Der p 2-derived peptides

For the expression and purification of recombinant Der p 2 (Der p 2 rec), the cDNA coding for Der p 2 (GenBank accession number AF276239) was amplified by RT-PCR using mite RNA, as previously described (Chen et al., 2008). PCR products of Der p 2 cDNA contained NdeI and EcoRI sites in the upstream region and an EcoRI site in the downstream region, as well as six His codons, and were subcloned into the plasmid pET-17b expression vector (GenScript, United States). The vector was introduced into ClearColi™ BL21 (DE3) electrocompetent cells (Lucigen, Wisconsin, United States) by electroporation using a MicroPulser Electroporator (program Ec2: 0.2-cm cuvette and 2.5 kV) (Bio-Rad, United States). Expression of Der p 2 rec was induced by adding 1 mM isopropyl-β-thiogalactopyranoside (IPTG) at an OD_{600} of 0.6 for 4 h at 37°C in the LB medium with 100 μg/mL ampicillin. Cells from 500 mL cultured medium were harvested by centrifugation (2,000 g for 20 min at 4°C), and cell pellets were then dissolved in 15 mL lysis buffer (25 mM imidazole, 0.1% Triton X-100, and pH 7.4) by mixing for 20 min at room temperature. Cell lysates were obtained by three consecutive freeze–thaw cycles (−70°C/+50°C). Incubation with 2 μg DNase I for 10 min at room temperature was performed to remove DNA, and thereafter, 100 mM NaCl was added to the lysates. Der p 2 rec was detected in the pellets (inclusion body) after centrifugation (38,900 g for 20 min at 4°C) and solubilized by mixing with a buffer containing 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris-Cl (pH 8) for 3 h at room temperature. After centrifugation (38,900 g for 20 min at 4°C), Der p 2 rec protein in the supernatant was incubated with Ni-NTA resin overnight at 4°C and bound Der p 2 rec was eluted from Ni-NTA resin affinity columns (QIAGEN, Hilden, GER) using 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris-Cl (pH 4.5). Purified Der p 2 rec was then dialyzed with 10 mM NaH_2PO_4 (pH 4.7) to increase solubility and prevent precipitation. Endotoxin of Der p 2 rec was <10 EU/mL determined by LAL assays (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific, United States).

Five overlapping peptides derived from Der p 2 with a length between 31 and 42 amino acids covering the full sequence of Der p 2 were synthesized using a peptide synthesizer (Liberty, CEM Corporation, Kamp-Lintfort, GER) and reconstituted in sterile endotoxin-free water, as previously described (Huang et al., 2019). Der p 2 peptides were defined as hypoallergenic peptides due to the lack of IgE reactivity and the disability to induce basophil activation. Five Der p 2-derived peptides (Der p 2 pep) and Der p 2 rec were filtered using 0.2-μm sterile syringe filters (Thermo Scientific), and the concentration of protein and peptides was determined using the BCA Protein Assay Kit (Pierce, Rockford, Illinois, United States).

2.3 Patch test, tissue sampling, and cell isolation

Non-inflamed skin on the upper back of AD patients and healthy participants was tape-stripped 10 times, and 40 μg of Der p 2 rec, and an equimolar mix of Der p 2 pep, saline (0.9% NaCl), and water (all sterile) were applied onto four separate nonwoven fabric spots of adhesive strips for patch tests (Curatest®, Lohmann & Rauscher, GER). The adhesive strips were further secured using a water-repellent plaster. Participants were invited back to the clinics 72 h later, and patch test areas were evaluated for inflammation. One 6-mm biopsy was taken from the skin treated with allergen and one from the skin treated with allergen-derived peptides (two biopsies in total per participant), and biopsies were separately stored in sterile, precooled phosphate-buffered saline for a maximum of 20 min until further processing. Skin biopsies were cut into small pieces using a scalpel, and cells were isolated via enzymatic digestion for 1.5 h using the Gentle MACS Whole Skin Dissociation Kit, according to the manufacturer's protocol (Miltenyi Biotec, GER). Single cells were resuspended in scRNA-seq resuspension buffer (1x PBS +0.04% BSA (w/vol), sterile), and counted using 0.4% trypan blue solution in saline (Corning, United States). Overall, all samples were processed within 3 h from taking the biopsy until processing for next-generation sequencing.

2.4 Single-cell RNA sequencing (scRNA-seq)

In total, 30,000 living cells were loaded into a 10x Genomics Chromium Controller for the generation of single-cell droplets. DNA and transcriptome libraries were generated following the 10x Genomics Next GEM Single Cell 5′ V2 protocol. Quality control was performed using Qubit (Invitrogen #Q33231) and the TapeStation system (Agilent). Libraries of six samples from

three donors were sequenced on two lanes of one NovaSeq SP flow cell using a NovaSeq 6000 system with a read length of 2 × 50 bp, resulting in an average of 100 million reads per sample. Reads were demultiplexed and analyzed using Cell Ranger.

2.5 EmptyDrops, doublet removal, and quality control (QC) for scRNA-seq

To differentiate between background noise and cell containing droplets, we used emptyDrops (Lun et al., 2019), which models the ambient RNA background within the dataset and identifies deviations from this background RNA. We applied a false discovery rate cut off of 0.05 to identify cells to be included into further analysis. To eliminate droplets containing more than one cell, we utilized the scran package from Bioconductor (Lun et al., 2016). The doublet score was calculated based on the simulation of thousands of doublet cells by adding together two randomly chosen single-cell profiles. For the doublet score calculation, the cells and the set of randomly generated doublet cells were clustered. Then, for each cell, the number of simulated doublets in its neighborhood was recorded and used as the input for score calculations. We used 200 nearest neighbors for each cell and applied a threshold of doublet score >4 to identify doublets in each dataset separately. The doublet score was defined as log10 of the ratio between simulated doublet cells and the total number of neighbors taken into consideration for each cell. Following the quality control process, we obtained a count of more than 3,000 cells for each sample (total of 50,000 cells), with approximately 1,690 genes per cell and 169 UMI per gene for the final analysis (Supplementary Table S1).

2.6 Analysis of scRNA-seq data

After performing individual quality control of the samples, the raw read counts from all datasets were merged into one count matrix. To conduct principal component analysis and differential gene expression analysis, we used Pearson residuals that were derived from a generalized negative binomial model of UMI counts, which is implemented in the R package sctransform (Hafemeister and Satija, 2019) using Seurat (Satija et al., 2015). In addition, we adjusted the regression model for sequencing depth, mitochondrial RNA content, and experimental batch effects. We removed cells with the mitochondrial RNA content above 15%. Furthermore, batch correction across individual datasets was performed using the Harmony algorithm (Korsunsky et al., 2019). Harmony uses batch information provided by the user and then utilizes fuzzy clustering to assign cells to multiple clusters in a manner that maximized batch diversity within each cluster. Correction factors for each cell were obtained by calculating global and batch-specific centroids for each cluster, and the procedure was repeated until convergence of global and batch-specific centroids. tSNE analysis of whole skin samples was performed using PCs 1–15. Clusters were assigned based on the nearest-neighbor-based clustering analysis. We observed a saturation of possible generated cluster at a resolution of 0.7, which was then chosen for further analysis. To assign clusters to known cell types in the skin (Tan et al., 2013; Shih et al., 2017; Cheng et al., 2018; Haensel et al., 2020; Wang et al., 2020; Reynolds et al., 2021; Polkoff et al., 2022), we visualized known cell type-specific markers within the tSNE plot. We also performed a cluster-specific regression analysis, providing us with a list of specific markers for each cluster. Out of approximately 50,000 analyzed cells, we identified 18 skin cell clusters, among which we found 4 KC clusters that were extracted for further analysis (data not shown). Extracted KC cells were re-analyzed including filtering out low expressed genes, calculating Pearson residuals from count data (i.e., data normalization), PC calculation, and clustering. Due to less variability in the keratinocyte dataset, it was sufficient to include PC 1–5 into cluster analysis and tSNE projections. We performed cluster assignment at a resolution of 0.2 to identify eight separate KC clusters. Based on the visual inspection of gene expression of cluster-specific markers (Supplementary Figure S1C) and analysis of gene lists from the Wilcoxon rank-sum test specific for each KC cluster, we defined the following KC cell clusters: basal 1, basal 2, proliferating/mitotic, granular 1, granular 2, spinous, hair follicle, and sebaceous gland KC clusters. To see whether or not the numbers of cells per cluster differ between AD patients and healthy controls, we counted the cells per cluster separately for AD and healthy control samples and performed a chi-squared test to evaluate whether cell numbers differed significantly.

2.7 Trajectory analysis

Trajectory analysis was performed using scVelo (La Manno et al., 2018). The analysis framework is based on the abundance of unspliced and mature (spliced) mRNA. This method assumes that differentiation takes place on a timescale similar to the typical half-life of mRNA. The ratio of spliced and unspliced mRNAs in each cell is used to model the progression of cell states. Thus, the arrows in the trajectory analysis plot (Figure 2) indicate a decrease in unspliced mRNA and/or an increase in spliced mRNA, as shown in Supplementary Tables S11–S13.

2.8 Pathway analysis

Downregulated genes in AD versus healthy skin (Supplementary Table S6) and differentially regulated genes in AD_pep versus AD_rec (Supplementary Table S10) were used to identify gene sets enriched in GO biological process pathways in the STRING database (Snel et al., 2000). Furthermore, pathway analyses of all genes differentially regulated in AD_pep versus AD_rec were analyzed using the fold change values for gene expression data in the Reactome database (Fabregat et al., 2015). Genes driving RNA velocity trajectories for the merged dataset comprising AD_rec, AD_pep, H_rec, and H_pep were analyzed using Enrichr (Chen et al., 2013). Identified pathway genes were used for bubble plots and to visualize the percentage of cells expressing involved genes in AD, H, AD_rec, or AD_pep using Prism (GraphPad Software, United States).

2.9 Intercellular communication network analysis

The probability of cell–cell communications via soluble cytokines and ligands was analyzed using CellChat (Jin et al., 2021). Briefly, gene expression data from our scRNA-seq dataset were integrated with the published data on signaling ligands and receptors to model the probability of cell–cell communications. We modeled the intercellular communication probability for keratinocyte and fibroblast subsets of the merged AD or H datasets for CCL and CXCL signaling pathways to investigate signals from the epidermis to the dermis and *vice versa* using hierarchy plots.

2.10 *In vitro* barrier disruption assay

KCs were isolated from human skin after 16–18 h incubation with 2.4 U/mL dispase II (Roche Diagnostics) in PBS at 4°C. The epidermis was separated from the dermis, and single cells were prepared using 0.5% trypsin-EDTA (Invitrogen). KCs were expanded using a serum-free Ca^{2+} low growth medium (PromoCell). For imaging, KCs were seeded into eight-well imaging dishes (IBIDI) and differentiated using 2 mM Ca^{2+}. KC sheets were incubated with 5 µg/mL sterile Der p 2 rec or Der p 2 pep (or equal volumes of the solvents PBS or H_2O) for 24 h and thereafter washed with 1x PBS, fixed with 4% PFA, permeabilized with 0.2% Triton X-100, blocked for 1 h with 5% BSA–PBS, incubated for 2 h with primary Ab, (monoclonal mouse anti-human claudin 1, clone 2H10D10, Invitrogen), and detected with an anti-mouse secondary Ab conjugated to AF488 (Invitrogen). Cell nuclei were stained with DAPI, and cells were imaged using a confocal microscope (FV3000 from Olympus). Images were analyzed using Fiji (Schindelin et al., 2012), and intensities were expressed as arbitrary units (mean fluorescence intensity) normalized to the respective control.

2.11 ELISA

IL18 was detected and measured in the serum from HDM-sensitized AD patients or non-sensitized healthy participants using an ELISA Kit (Cloud-Clone Corp.), according to the manufacturer's instructions.

2.12 Statistical analysis

Statistical tests were performed using Prism 9 (GraphPad). Unless stated otherwise, statistical differences were evaluated using the Student's *t*-test. Data are expressed as mean ± SD, and a p-value < 0.05 was considered significant.

3 Results

3.1 KC subsets in the human skin

Allergic diseases and chronic inflammatory skin pathologies are increasing, but whether skin flares and epithelial barrier impairment can be directly triggered by aeroallergens is still elusive. Here, we investigated the effect of the house dust mite allergen Der p 2 on the skin of AD patients sensitized to house dust mite (HDM) and healthy non-sensitized individuals (Supplementary Figure S1A). Recombinant Der p 2 allergens (Der p 2 rec) or a mix of hypoallergenic Der p 2 peptides (Der p 2 pep) were applied onto the non-inflamed back skin of HDM-allergic AD patients or non-allergic healthy individuals (Figure 1A; Supplementary Figure S1B). Biopsies for rec and pep-exposed skin were taken after 72 h, and KCs were analyzed by single-cell RNA sequencing (scRNA-seq).

We identified eight KC clusters (Figure 1B; Supplementary Figure S1C) in our merged dataset with a unique expression pattern of specific genes (Supplementary Tables S2–S5 and Figure 1D). Cells of all KC clusters were found in the skin of AD patients (AD) and healthy participants (H) 72 h after Der p 2 protein and peptide exposure at slightly varying amounts (Figure 1B). KCs from the clusters basal 1, granular 2, and sebaceous gland were reduced, whereas KCs from the clusters basal 2, proliferating/mitotic, and spinous were increased in AD compared to H skin. The basal 1 cluster was defined by high expression of hemidesmosome genes, such as alpha 6 and beta 4 integrins (ITGA6 and ITGB4), which bind to laminin, and collagen type 17 (COL17A1, Figure 1D). Both basal 1 and basal 2 expressed high amounts of the intermediate filaments keratin KRT5 and KRT15 and the integrins ITGA2 and ITGB1, which localize to focal adhesions and bind to collagen but have also been shown to be important for cell–cell interactions (Carter et al., 1990). KCs of the basal 2 cluster expressed high amounts of ASS1, which is enriched between rete ridges and may play a role in regulating dermal papilla integrity (Wang et al., 2020). The proliferating/mitotic KC cluster was defined by elevated expression of cell cycle regulator genes, such as *RRM2*, *PTTG1*, *CDC20*, *TYMS*, and *HIST1H4C* (Wang et al., 2020). *KRT1* gene expression was already detectable in mitotic KCs and was most abundant in granular KCs (clusters granular 1 and 2), as described previously (Cohen et al., 2022). The granular KC clusters granular 1 and granular 2 and spinous KC specifically expressed KRT10, CDH1, EPHA2, and the desmosome-specific genes desmoglein (DSG1) and desmocollin (DSC1) at various levels (Figure 1D) (Wang et al., 2020). Spinous KC expressed high levels of the differentiation marker cornifin-B (SPRR1B), CD24, involucrin (IVL), and mesotrypsin (PRSS3) (Tan et al., 2013; Miyai et al., 2014; Cheng et al., 2018; Wang et al., 2020). The spinous cluster also expressed KRT6A, KRT6C, and KRT16, which have been associated with inflammatory skin diseases and barrier dysfunction (Lessard et al., 2013; Cheng et al., 2018; Leung et al., 2020). Both spinous and hair follicle KCs expressed the gap junction genes *GJB2* and *KRT17*. KCs in the hair follicle cluster expressed the stem cell marker SOX9, galectin 1 (LGALS1), basonuclin 2 (BNC2), and latrophilin 3 (ADGRL3), and KCs from the sebaceous gland cluster expressed KRT79, fatty acid-binding protein FABP7, fatty acid hydroxylase FA2H, fatty acid synthase FASN, and the sebocyte marker MGST1 (Shih et al., 2017; Cheng et al., 2018). Taken together, we found all major KC subsets that have been previously identified in the adult human skin (Cheng et al., 2018; Reynolds et al., 2021).

3.2 Trajectory and pathway analyses identify AD-specific perturbations in KCs

To understand the relationship between different KC clusters, we analyzed KC data using the trajectory analysis pipeline scVelo.

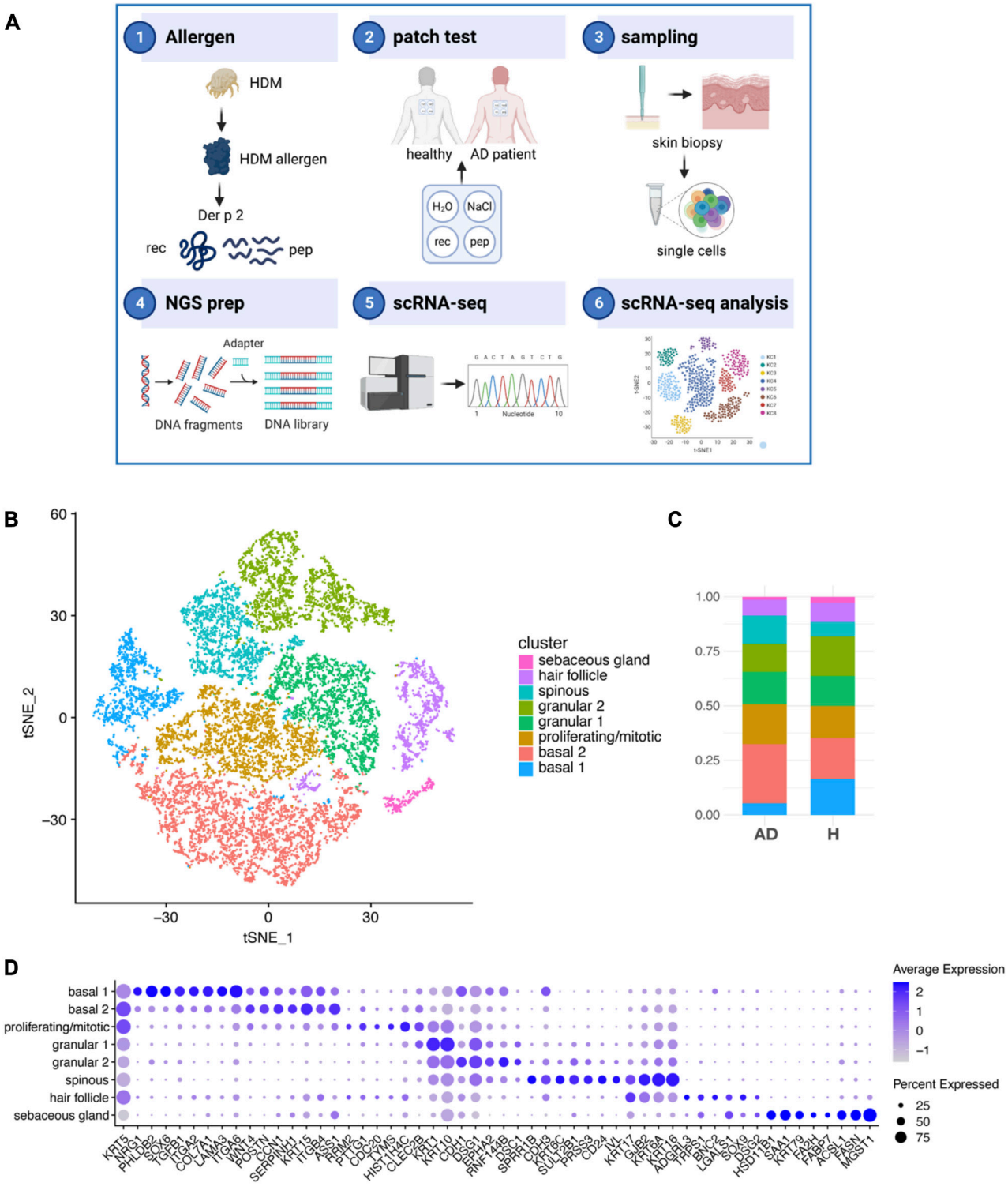


FIGURE 1
Keratinocyte subsets from AD and H after exposure to HDM allergens. **(A)** Schematic outline of the study strategy: 1) the recombinant HDM allergen Der p 2 (Der p 2 rec), a mix of five hypoallergenic Der p 2 peptides (Der p 2 pep) and negative controls were 2) applied onto the non-lesional back skin of four non-sensitized participants without AD (healthy) and four HDM sensitized participants with AD using patch tests. 3) Biopsies of Der p 2 rec- and pep-treated skin were taken after 72 h, and single cells were produced by the digestion of whole skin tissue. 4) NGS libraries were prepared and 5) sequenced using scRNA-seq technology. 6) Sequencing data from 16 skin samples were processed and KC analyzed. **(B)** tSNE plot showing eight different KC clusters from the merged dataset comprising 16 skin biopsies (4x AD and 4x H, each 1x rec and 1x pep; summing up to 52,975 KC in total). **(C)** Bar charts showing the relative abundance of KC subsets within AD and H samples (n = 8 biopsies for AD and H). **(D)** Bubble plot shows the expression of cluster specific markers (x-axis) for KC subsets (y-axis). The average expression is represented by blue values (low expression, light blue; high expression, dark blue). The percentage of cells expressing the respective marker is represented by the size of the circles. AD, atopic dermatitis; H, healthy; HDM, house dust mite; KC, keratinocyte; scRNA-seq, single-cell RNA sequencing; rec, recombinant; pep, peptide; tSNE, t-distributed stochastic neighbor embedding.

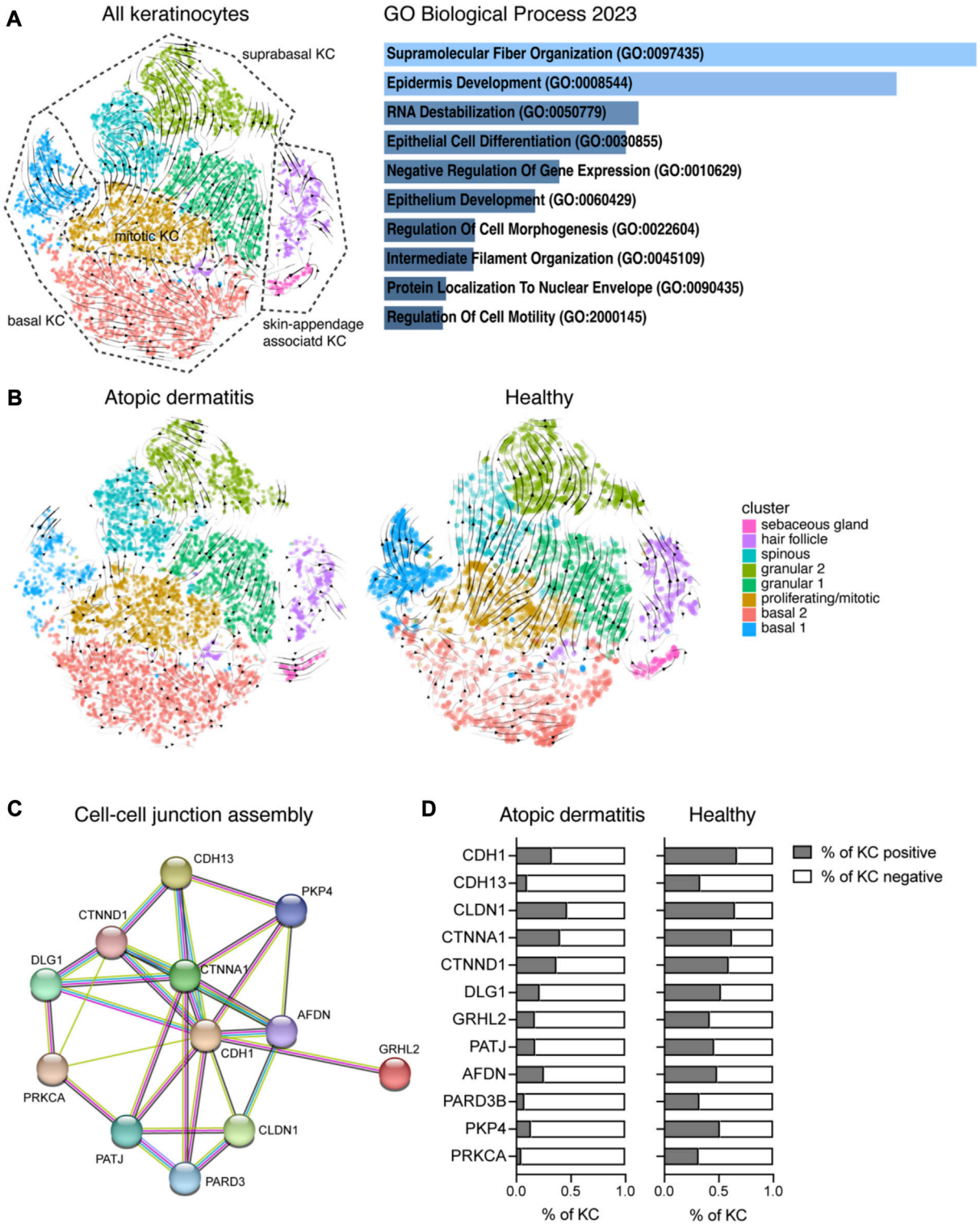


FIGURE 2
Analysis of the ratio between unspliced and spliced mRNAs reveals AD-specific disturbances in KC differentiation. **(A)** Left: RNA velocity vectors were projected on the tSNE plot from Figure 1B showing KC clusters of the whole dataset (cells from 16 biopsies, AD and H). Right: 140 genes that were driving RNA dynamics were analyzed using Enrichr (Chen et al., 2013). The bar graph shows gene set enrichments in the GO biological process 2023 database (sorted by *p*-value ranking). **(B)** RNA velocity vector projections are shown for KC from treated AD (left) and H (right) skin. **(C)** Functional enrichment analysis identified cell–cell junction perturbations in AD. Downregulated genes in AD were analyzed using the STRING network database The network view of gene names (circles) and their predicted associations (lines) is shown. **(D)** Percentage of KC from AD and H skin expressing genes listed in **(C)** (eight biopsies per group). AD, atopic dermatitis; H, healthy; KC, keratinocyte; tSNE, t-distributed stochastic neighbor embedding.

This tool identifies cell differentiation paths and cell states by analyzing the ratio of unspliced to spliced mRNAs in each cell for a gene transcript (Supplementary Tables S11–S13). When all KCs were used for analysis (AD and H; rec and pep), we found that basal and suprabasal KCs originate from the mitotic cell cluster, and hair follicle and sebaceous gland KCs (skin appendage-associated KCs) have their own progenitor cells (Figure 2A). The four major pathways that were identified as drivers of the trajectory analysis were "supramolecular fiber organization," "epidermis development," "RNA destabilization," and "epithelial cell differentiation," suggesting that ECM organization, keratinocyte differentiation, and epidermis development are the main pathways associated with RNA dynamics in the whole dataset comprising AD and H samples. When we analyzed KC data from AD and healthy participants separately, we found that trajectories from healthy KC (Figure 2B, right panel) showed again a clear separation between basal and suprabasal KCs. Data from healthy skin highlighted a clear dependency between cell division and KC differentiation and a fluid transition between spinous and granular KCs. A closer look on AD-derived KCs (Figure 2B, left panel) revealed highly disordered trajectories. Mitotic cells were again the origin of basal and suprabasal KCs, but there was higher entropy within different clusters, in particular for the KC clusters basal 1 and basal 2 and the spinous KC cluster. Interestingly, KRT5 and KRT14 expression levels, which is highest in basal KCs in healthy skin, were not efficiently downregulated in differentiating cells in AD patients, further suggesting a disturbance in KC differentiation pathways in AD skin (Supplementary Figure S2A). Suprabasal KCs comprising clusters granular 1 and granular 2 and spinous KC maintained high expression levels of KRT5/14. Our data confirm disturbances in KC cell differentiation pathways in AD skin, which contributes to the disease-specific weakened skin barrier function.

To further understand preexisting perturbations in AD skin, we compared the gene expression profile of AD with healthy skin (pooled data from rec and pep for each group, Supplementary Table S6; see also Supplementary Tables S7, S8 for individual analysis of separate KC clusters). We identified over 2,000 significant genes that were differentially regulated between AD and H (Supplementary Table S6). As we aimed to identify pathways involved in AD barrier dysfunction, we further analyzed genes that were downregulated in AD using the STRING database. We identified functional enrichments in GO biological process pathways, such as the "cell–cell junction assembly," (Figure 2C) "tight junctions," and "cell–cell adhesion mediated by cadherin" (Supplementary Figure S1D), which are essential components of a functional epidermal barrier. When we had a closer look at genes that are specific for the cell–cell junction assembly (Figure 2C), we identified downregulated gene expression for adherens junction markers, such as E-cadherin (CDH1), alpha- and delta-catenin (CTNNA1 and CTNND1), afadin (AFDN), DLG1, GRHL2, and plakoglobin 4 (PKP4). T-cadherin (CDH13) was previously described to be expressed in basal KCs, and it has been suggested that it plays a role in cell–matrix adhesion (Zhou et al., 2002; Mukoyama et al., 2007). Furthermore, we identified downregulated genes that are involved in the tight junction assembly, such as claudin 1 (CLDN1), PATJ, and PARD3 (Figure 2C; Supplementary Figure S1D). Reduced protein kinase C alpha (PRKCA) expression may downregulate both adherens and the tight junction assembly. Gene expression for cell–cell junction genes was not only downregulated, but also the percentage of KCs expressing those genes was reduced in AD compared to H skin (Figure 2D). Furthermore, we analyzed cell–cluster-specific expression of the tight junction genes *CLDN1*, *PARD3*, and *TIAM1* (Mertens et al., 2005) in KCs from AD patients treated with recombinant Der p 2 (AD_rec) and Der p 2 peptides (AD_pep) and compared it with KCs from healthy individuals that were treated with the same allergens and allergen peptides (H_rec and H_pep) (Supplementary Figure S2B). Tight junction gene expression was highest in granular, spinous, and hair follicle KCs from healthy skin and clearly downregulated in AD patients, independent of allergen exposure. Our data reveal preexisting epithelial barrier damage in AD patients in suprabasal KCs, which is mainly driven by downregulation of cell–cell adhesion genes and confirms previously published data for chronic type 2-driven inflammation (Akdis, 2021).

3.3 Der p 2 induces a pro-inflammatory gene expression signature in granular and spinous KCs

We then wanted to investigate the potential of HDM allergens and allergen peptides to induce the expression of inflammation markers in AD and H skin (Figure 3A). Analysis of relevant immune system-related genes in KC from AD_rec, AD_pep, H_rec, and H_pep revealed an upregulation of pro-inflammatory markers that can be assigned to the innate immune system (*POLR2L*, *PYCARD*, *PI3*, *CST3*, *FABP5*, *FLT*, *SERPINB1*, *SERPINB3*, and *CXCL1*) in AD_rec and AD_pep, compared to H_rec and H_pep. Furthermore, markers reported to be relevant for adaptive immune responses (CALR, CLEC2B, AP2S1, and CCL27) and genes encoding for interleukin signaling molecules (*NMU*, *IL18*, and *HMGV1*) showed increased expression, and a high number of KCs from Der p 2 rec- and Der p 2 pep-treated AD skin were expressing these genes. Remarkably, there was also a tendency for increased IL18 levels in the serum of AD patients (Supplementary Figure S3E). Markers that are relevant for stress response and danger signaling, such as the alarmins S100A7, S100A8, and S100A9, the antimicrobial peptide lactotransferrin (LTF), and the interferon inducible protein (IFI27) were upregulated in KCs from AD patient skin treated with either Der p 2 rec or Der p 2 pep. In contrast, expression of the actomyosin stabilizing non-muscle myosin IIA (MYH9) and moesin (MSN), and the anti-inflammatory gene *ANXA1* were downregulated by Der p 2 rec and pep treatment (Figure 3A). Some of the genes with increased expression levels have been reported to be specifically upregulated in lesional skin in AD. For instance, the serine protease inhibitor SERPINB3 is upregulated in AD patients, where its expression correlates with skin inflammation and returns to baseline levels in non-inflamed skin upon treatment (Kawashima et al., 2000; Mitsuishi et al., 2005). Interestingly, despite only mild inflammation after patch testing (Supplementary Figure S1B), SERPIN levels were strongly induced in KC upon Der p 2 rec and pep exposure in AD patients (Figure 3A). Further analysis of SERPINB1 and SERPINB3 in KC clusters revealed that SERPIN was highly upregulated in both AD_rec and AD_pep (Figure 3B), but the

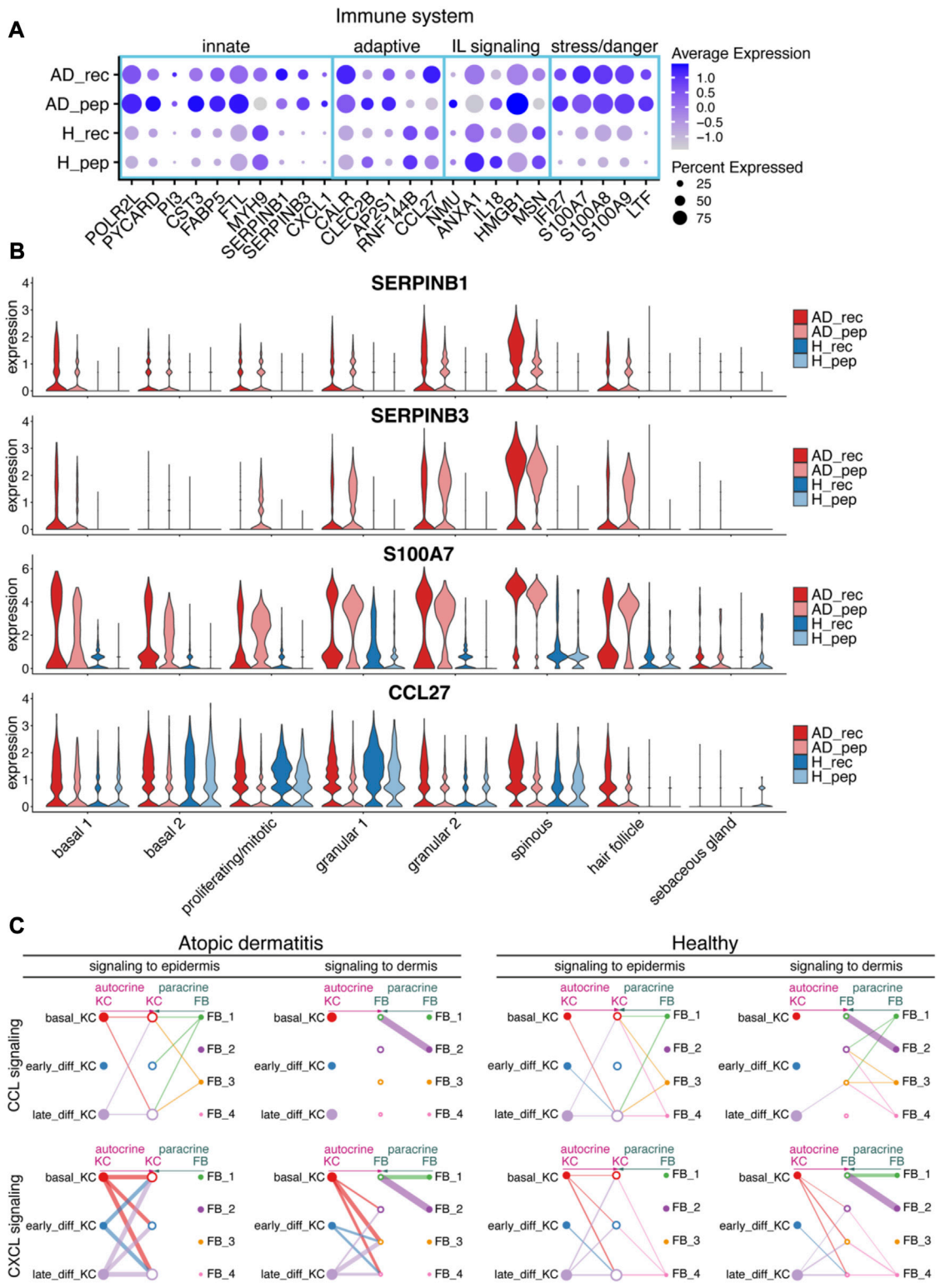


FIGURE 3
House dust mite allergen Der p 2 initiates inflammatory pathways in KC. **(A)** Bubble plot depicting the expression of immune system-relevant genes in KC from skin exposed to Der p 2 protein (AD_rec, H_rec) and Der p 2 peptides (AD_pep, H_pep). Blue rectangles highlight genes relevant for the innate IS, the adaptive IS, interleukin (IL), and stress/danger signaling. **(B)** Violin plots show the average gene expression in KC clusters from AD_rec (dark pink), AD_pep (light pink), H_rec (dark blue), and H_pep (light blue) skin samples (n = 4). **(C)** Analysis of signaling crosstalk via soluble and membrane-bound factors in KC and FB. Hierarchy plots with the signal source plotted left for autocrine signaling (pink) and right for paracrine signaling (green) are shown. The receiving cell subsets (signal target) were plotted in the middle (left plot: signaling to epidermis; right plot: signaling to dermis). The plots illustrate the probability of cell–cell communications in AD and H for CCL (top panel) and CXCL signaling pathways (lower panel). Thick lines represent high probability of cell–cell interactions. AD, atopic dermatitis; H, healthy; FB, fibroblast; IL, interleukin; IS, immune system; KC, keratinocyte; rec, recombinant; pep, peptide.

fully functional protein Der p 2 rec induced the strongest response in KCs from AD patients, particularly in suprabasal KC (Figure 3B). This suggests that Der p 2 rec induced sub-clinical inflammation, which is called microinflammation in AD skin (Akdis, 2021). It has been shown in an AD mouse model that SERPINB3 regulates the epidermal barrier function, and its overexpression is accompanied by increased expression of S100 proteins (Sivaprasad et al., 2015). Especially, the onset of acute skin lesions in AD is associated with elevated S100A7, S100A8, and S100A9 levels in humans (Gittler et al., 2012). We found that S100A7 expression was highly upregulated in all KC clusters, except sebaceous glands (Figure 3B). S100A7 expression was highest in granular and spinous KC clusters of AD patients upon exposure to Der p 2 rec. Moreover, genes that are relevant for adaptive immune responses appeared to be specifically induced by recombinant Der p 2. CCL27, for instance, acts as a chemotactic signal for T cells, enabling KCs to recruit T cells into the skin upon an allergen encounter. CCl27 expression was upregulated in KCs of the spinous and granular 2 clusters in AD patients through the recombinant Der p 2 protein and remained lower in AD_pep, H_rec, and H_pep samples, reinforcing the idea that the Der p 2 protein can efficiently induce a pro-inflammatory environment in suprabasal KCs.

KCs are one of the first cells that encounter environmental allergens and respond quickly by secreting cytokines that may distribute into deeper layers of the skin. To investigate transcriptional changes in the dermis in response to epidermis-derived signals and *vice versa*, we analyzed CCL and CXCL signaling interaction pathways between KC and fibroblasts (FB) using CellChat (Jin et al., 2021). Interestingly, we found that in AD, FB did not respond to KC-derived CCL signals with altered gene expression, whereas FB from healthy skin responded to signals originating from late differentiating KCs (Figure 3C, upper panel). In contrast, KC-derived signals assigned to CXCL signaling pathways could be associated with gene expression responses in FB clusters 2, 3, and 4 with a high probability for AD (Figure 3C, lower panel). Interestingly, there was no association of KC responses to CXCL signaling derived from FB in AD, whereas KC received signals from FB cluster 4 in healthy skin (Figure 3C, lower panel). This suggests that CCL and CXCL signaling crosstalk pathways between KC and FB may be disrupted in AD.

3.4 Der p 2-derived peptides downregulate cell–cell and cell–matrix adhesion genes in AD patients

Our next aim was to understand the differences between the effects of Der p 2 rec and Der p 2 pep on the skin of AD patients and healthy participants. We did not observe any differential gene expression for the comparison of H_pep versus H_rec, suggesting that recombinant Der p 2 protein and Der p 2 peptides did not induce any significant changes in healthy skin. The global comparison of gene expression data from AD_rec versus AD_pep revealed 19 differentially expressed genes and an enrichment for genes belonging to cell cycle and mitosis pathways (Supplementary Tables S9, S14). In contrast, when we calculated average log2 fold changes between AD_pep and AD_rec for each KC cluster separately, we identified in total 98 significantly down- and 197 significantly upregulated genes (Supplementary Table S10). Downregulated genes were imported into the STRING database and lead to the identification of genes enriched in the GO pathway "cell–matrix adhesion," such as TIAM1, LYPD3, β4 integrin (ITGB4), β1 integrin (ITGB1), beta-catenin (CTNNB1), COL17A1, BCAM, and alpha-actinin-1 (ACTN1). (Figure 4A). When we had a closer look at the percentage of KC expressing cell–matrix adhesion genes, we saw a reduction in the percentage of KC expressing these marker genes in AD_pep compared to AD_rec (Figure 4B). TIAM1 (T-lymphoma invasion and metastasis) is a Rac-specific guanine nucleotide exchange factor and has been shown to control tight junction biogenesis in KC, thereby controlling barrier formation (Mertens et al., 2005). Alpha actinin-1 (ACTN1) is an F-actin crosslinking protein, which connects F-actin fibers to focal adhesions and hemidesmosomes through interaction with integrins and Col XVII and thereby promotes matrix adhesion (Carter et al., 1990; Hamill et al., 2015). Both BCAM (laminin α5 receptor) and LYPD3 (Ly6/PLAUR domain-containing protein 3) bind laminin in the BM, whereas beta-catenin (CTNNB1) indirectly regulates cell–matrix adhesions by controlling the hemidesmosome assembly through WNT signaling (Kosumi et al., 2022). In addition, the expression of known cell–cell adhesion and ECM organization genes was even further downregulated upon AD skin exposure to Der p 2 pep compared to Der p 2 rec (Supplementary Figures S3A,B). To investigate whether Der p 2 rec or Der p 2 pep could reduce cell–cell adhesion molecules *in vitro*, isolated primary KC were differentiated to form tight junctions incubated with Der p 2 rec or Der p 2 pep, and claudin 1 (CLDN1) expression was analyzed by confocal microcopy (Supplementary Figure S3C). Interestingly, we found that Der p 2 pep significantly reduced CLDN1 protein expression after 24 h of incubation (Supplementary Figure S3D), suggesting a potential direct effect of Der p 2 peptides on keratinocytes.

For a more comprehensive observation at cell–cell and cell–matrix adhesion molecules, we included additional pathway-specific markers into our analysis and compared the average expression and percentage of cells expressing the respective genes for AD_rec, AD_pep, H_rec, and H_pep (Figure 4C). The majority of cell–matrix and cell–cell adhesion genes and genes encoding for ECM organizational proteins (see also Supplementary Figure S3B) was downregulated in KC from the skin of AD patients exposed to Der p 2 pep (AD_pep) compared to AD_rec with the exception of the upregulation of cathepsin L (CTSL) and cathepsin D (CTSD) expression (Figure 4C, blue rectangle). Our data suggest an IgE-independent downregulation of cell–cell and cell–matrix gene expression in the KCs of AD skin, identifying a yet unknown effect of Der p 2 peptides on the skin barrier function.

3.5 Der p 2-derived peptides upregulate IL18 and downregulate KRT1 gene expression in AD patients

Cathepsins are proteases, which are considered to be involved in various biological processes such as proenzyme

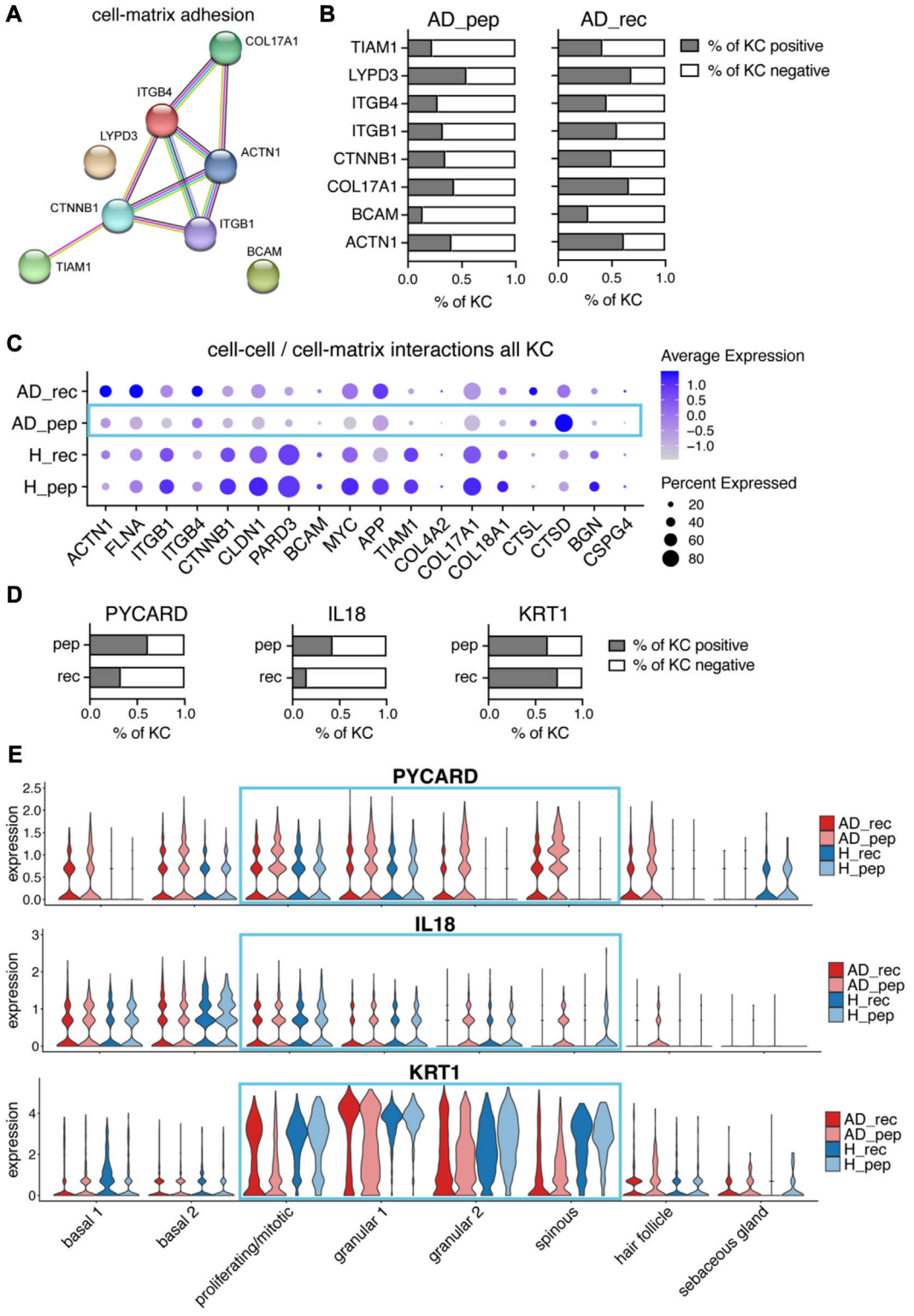


FIGURE 4
Der p 2 peptides downregulate cell–matrix adhesion genes and upregulate alarmin IL18 in AD. **(A)** Differential gene expression analysis of AD_pep and AD_rec revealed the downregulation of cell–matrix adhesion genes by Der p 2 peptides in KC. The STRING network and predicted functional associations of cell–matrix adhesion genes (n = 4) are shown. **(B)** Percentage of KC-expressing genes **(A)** from AD_pep and AD_rec skin (four biopsies per group). **(C)** Bubble plot showing the expression of cell–cell and cell–matrix interaction genes in KC from skin exposed to Der p 2 protein (AD_rec and H_rec) and Der p 2 peptides (AD_pep and H_pep). **(D)** Percentage of KC expressing *PYCARD*, *IL18*, and *KRT1* from AD skin exposed to Der p 2 peptides (pep) and recombinant protein (rec). **(E)** Violin plots depicting the average gene expression of PYCARD (upper plot), IL18 (middle plot), and KRT1 (bottom plot) in KC clusters from AD_rec (dark pink), AD_pep (light pink), H_rec (dark blue), and H_pep (light blue) skin samples (n = 4). AD, atopic dermatitis; H, healthy; KC, keratinocyte; KRT, keratin; rec, recombinant; pep, peptide.

and enzyme activation, tissue remodeling, and matrix remodeling (Zeeuwen, 2004). In particular, lysosomal cathepsins have been shown to activate NLRP3 inflammasomes, which are involved in the recognition of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (Li et al., 2021). This prompted us to further investigate inflammasome-related genes that may be involved in danger sensing in the human skin. When we plotted the percentages of KC expressing PYCARD, we found higher amounts of KC expressing this NLRP3-inflammasome adapter protein, which is involved in recruiting the protease caspase-1, in AD_pep compared to AD_rec (Figure 4D, left plot). Interestingly, all KC subsets expressed high levels of PYCARD in AD_pep, except sebaceous gland KCs (Figure 4E, upper plot). In addition, the percentage of KCs expressing the alarmin IL18, a substrate of caspase-1, was increased in KC from the skin of AD patients exposed to Der p 2 pep (Figures 4D and E, middle plots). The upregulation of NLRP3 inflammasome and IL18 gene expression suggests the activation of PAMP or DAMP receptors by Der p 2 pep, and enhanced cathepsin expression may further boost NLRP3 inflammasome activity. Pro-IL18 is processed by activated inflammasomes into its biologically active form. A mouse study has shown that downregulation of KRT1 can further upregulate IL18 expression and diminish the skin barrier function (Roth et al., 2012). In accordance with this study, KRT1 expression was downregulated in KCs from AD_pep (Supplementary Figures S4C, E), and we found elevated IL18 protein levels in the serum from AD patients, although not significant (Supplementary Figure S3E). However, we only identified a minor reduction in the percentage of KC expressing KRT1 (Figure 4D, right plot), suggesting that KRT1 expression was decreased specifically in KRT1-expressing KC subsets. When observing different KC clusters, we identified the Der p 2 pep-specific downregulation of KRT1 expression in proliferating/mitotic, granular, and spinous KCs (Figure 4E, bottom plot). In addition to PYCARD, IL18, and CTSD, we found several other leukocyte-related activation genes to be upregulated in KCs from AD_pep compared to AD_rec such as S100A7-9, which is reported to be upregulated in KRT1 knock-out mice alongside IL18 (Roth et al., 2012) (Supplementary Figures S4A, B). Furthermore, several genes involved in KC differentiation and epidermal keratinization were differentially regulated in AD_pep versus AD_rec (Supplementary Figure S4C). For instance, KRT5, KRT6A/B/C, KRT14, and KRT16 gene expression was upregulated, whereas KRT1, KRT10, and KRT15 expression was downregulated. KRT6 and KRT16 are involved in danger sensing via DAMPs and their expression levels have been shown to be elevated in stressed KC during wound healing and chronic skin inflammation (DePianto and Coulombe, 2004; Hobbs et al., 2012; Rotty and Coulombe, 2012; Lessard et al., 2013). KRT15, in contrast, is expressed in the hair bulge and in undifferentiated KCs of the basal layer (Cheng et al., 2018; Cohen et al., 2022). Hence, our data suggest that Der p 2 peptides induce perturbations in KC differentiation and keratinization in basal, suprabasal, and hair follicle KCs.

3.6 Der p 2-derived peptides upregulate the expression of mitotic and cell cycle progression genes in AD patients

Overexpression of alarmin IL18 can promote hallmark features of AD, such as type 2 skin inflammation and IL4- and IL13-induced epidermal hyperplasia (Leung et al., 2020; Beck et al., 2022). As hyperplasia is the result of increased cell proliferation, we investigated the expression of cell cycle and mitotic genes in KCs from AD_rec, AD_pep, H_rec, and H_pep samples (Figure 5A). Interestingly, a plethora of cell cycle and mitotic genes were upregulated in KCs from AD patients exposed to Der p 2 pep (Figure 5A, blue rectangle), such as DNA topoisomerase 2 (TOP2A) and centromere protein F (CENPF), which are important for chromosome segregation in mitosis; cytoskeletal genes such as tubulin beta (TUBB2); microtubule-stabilizing NUSAP1 and microtubule-destabilizing stathmin (STMN1); the cyclin-dependent kinase CKS2; and CDC20, UBE2C, and UBE2S, which encode for anaphase-promoting complex/cyclosome-regulating genes. When we analyzed genes that were specifically upregulated in AD_pep using STRING, we identified an enrichment of genes in the GO biological process pathway “mitosis” (Figure 5B) and found that a higher percentage of KCs from AD_pep expressed mitosis relevant genes compared to AD_rec (Figure 5C). This suggests that Der p 2-derived peptides can activate KC hyper-proliferation and thereby increase epidermal hyperplasia. Further investigation of genes reportedly upregulated in hyperplasia (Beck et al., 2022) revealed that Ki-67 (MKI67) expression is increased upon Der p 2 pep exposure in KC clusters basal 1 and basal 2, mitotic and hair follicle KC, and also in the suprabasal KC clusters granular 1 and granular 2 but not in spinous and sebaceous gland KCs (Figure 5D, upper panel). We found a similar expression pattern for CDC20 and the G2/M-specific cell cycle gene *CCNB1*, which both showed increased expression for AD_pep in basal, mitotic, and hair follicle KCs. Similar to MKI67, CDC20 was upregulated in granular KCs as well (Figure 5D, middle and bottom panel). Our data reveal the specific induction of hyperplasia-related genes in KC through Der p 2-derived peptides that were applied to non-inflamed skin of AD patients.

4 Discussion

The epidermal barrier plays a crucial role in preventing the entry of microbes, chemical irritants, and allergens into the skin. To date, there is limited information available on how allergens affect human tissue-resident skin cells at the single-cell level in terms of gene expression. To address this, we conducted patch tests on the non-lesional skin of patients with AD and healthy participants using the HDM allergen Der p 2. Although Der p 2 itself does not possess proteolytic activity like Der p 1, proteases present on the skin or in HDM feces can break down Der p 2 into smaller fragments. Therefore, we chose to compare gene expression responses to both Der p 2 protein and a mix of Der p 2 peptides in KCs from AD patients and healthy individuals using single-cell RNA sequencing technology.

Our KC cluster analysis of allergen-exposed skin confirmed the presence of all major KC subsets reported for adult skin

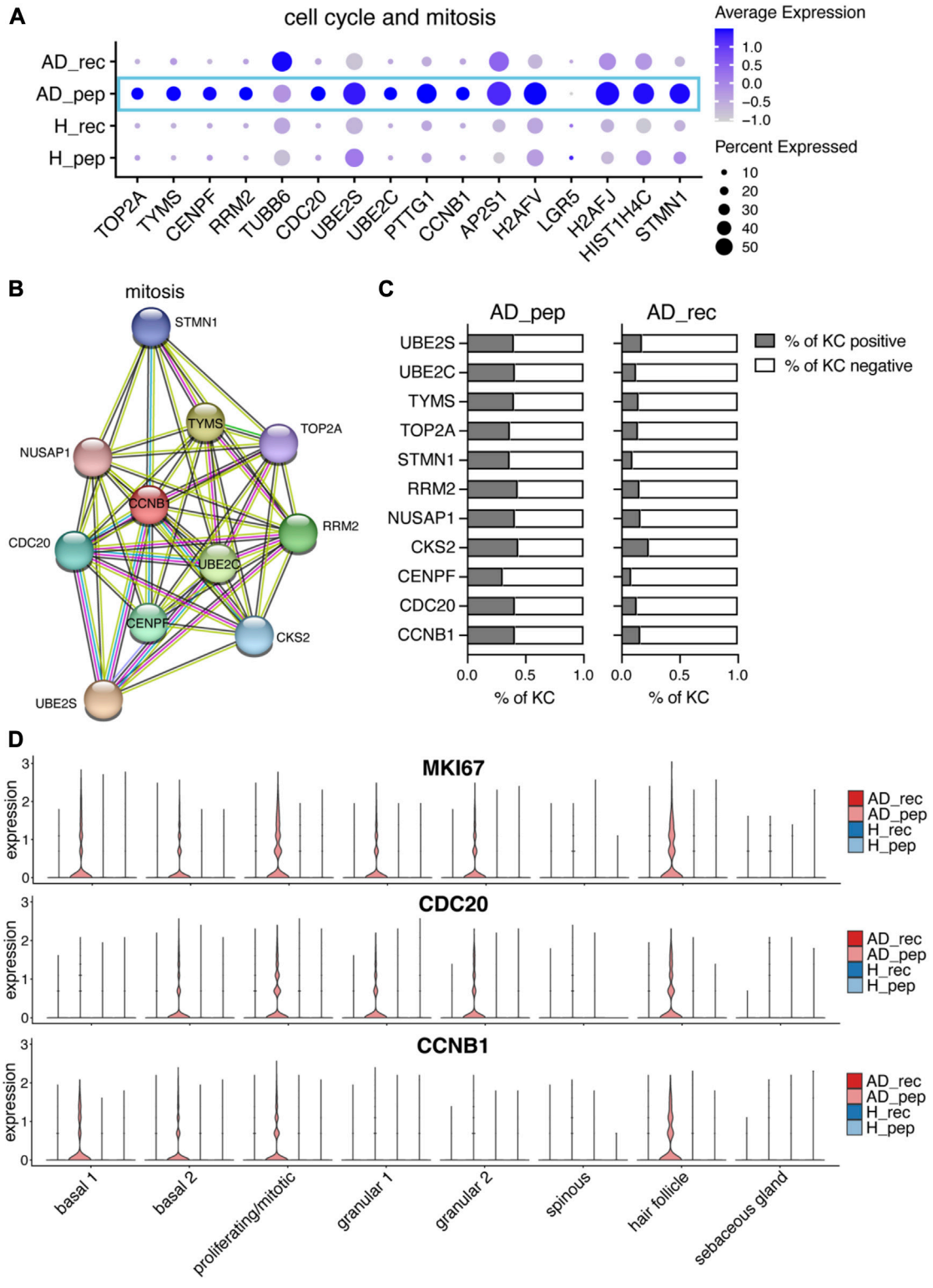


FIGURE 5
Der p 2 peptides upregulate KC hyperproliferation in AD. **(A)** Bubble plot showing the expression of cell cycle and mitotic genes in KC from skin exposed to Der p 2 protein (AD_rec and H_rec) and Der p 2 peptides (AD_pep and H_pep). **(B)** Functional enrichment analysis identified mitotic genes enriched in AD skin exposed to Der p 2 peptides. Genes identified by differential gene expression between AD_pep and AD_rec were further analyzed using the STRING network database. Identified genes are visualized by circles and their predicted associations with lines. **(C)** Percentage of KC-expressing genes identified with STRING is shown in **(B)** for AD skin exposed to Der p 2 peptides (pep) and recombinant protein (rec). **(D)** Violin plots show the average gene expression in KC clusters from AD_rec (dark pink), AD_pep (light pink), H_rec (dark blue), and H_pep (light blue) skin samples (n = 4 per group). AD, atopic dermatitis; H, healthy; KC, keratinocyte; rec, recombinant; pep, peptide.

(Cheng et al., 2018; Reynolds et al., 2021) (Figure 1). However, we could not identify a decrease in the percentage of spinous KCs in AD compared to healthy individuals, as previously suggested (Leung et al., 2020). Overall, our data revealed a clear AD-specific signature in KC from patients compared to healthy participants, confirming that skin cell differentiation is disrupted even in the absence of severe skin inflammation, as described previously (Suárez-Fariñas et al., 2011). This finding was confirmed by the global comparison of gene expression data from AD and H, where we found a significant impairment of the skin barrier in AD patients (Akdis, 2021; Mitamura et al., 2021) characterized by the downregulation of genes involved in the cell–cell junction assembly (Figures 2B, C). Consistent with this, we also observed a profound downregulation of genes regulating tight junctions, namely, CLDN1 (De Benedetto et al., 2011), PARD3 (Ali et al., 2016), and TIAM1 (Mertens et al., 2005), another hallmark of AD (Supplementary Figure S2B).

Recombinant Der p 2, but not hypoallergenic Der p 2 peptides, can be presented by IgE-facilitated mechanisms. This may explain the specific upregulation of certain immune system relevant genes by the full protein, such as SERPINB1, CALR, CCL27, and S100A7 (Figure 3). Furthermore, Der p 2 protein has been shown to activate the innate immune system by mimicking MD2-related lipid recognition domains (Trompette et al., 2009; Kaplan et al., 2012; Eyerich and Novak, 2013; Reithofer and Jahn-Schmid, 2017; Smith et al., 2017). The protease inhibitor SERPINB3 has been found to be upregulated in the lesional skin of AD patients, and its expression is induced by type 2 cytokines (Mitsuishi et al., 2005). Despite low inflammation scores in our patch tests, we observed an upregulation of SERPINB3 in basal, spinous, granular, and hair follicle KCs of AD skin (Figure 3). Previous studies in mice have shown that SERPINB3 likely contributes to early skin inflammation in AD (Sivaprasad et al., 2015). Interestingly, exposure to Der p 2 recombinant proteins further increased SERPINB3 expression, particularly in spinous KC. This suggests that even without proteolytic cleavage of tight junctions, allergens can overcome the skin barrier in AD, induce inflammatory pathways, and activate an early inflammatory gene expression signature in KC. This fact can be attributed to the preexisting barrier dysfunction in AD, allowing proteins such as allergens to penetrate deep into the skin.

Increased expression of S100A7 in suprabasal KCs has been reported after barrier disruption by tape stripping of the healthy skin (Gläser et al., 2009). Analysis of S100A7 expression in our samples using feature plots (data not shown) and violin plots (Figure 3B) revealed that S100A7 was not uniformly expressed in KCs. In particular, granular and hair follicle KCs illustrated a bimodal expression pattern of S100A7. We speculate that certain KC cells that reside on the outer surface of the skin may be more prone to experiencing environmental impacts and consequently respond with similar gene expression patterns compared to KC in deeper epidermal layers. Further studies will show whether cells expressing high levels of S100A7 localize to the same niche within the skin.

Comparing gene expression changes induced by Der p 2 protein and Der p 2 peptides, we found a marked reduction in the expression of genes associated with hemidesmosomes and focal adhesions in KCs from AD skin treated with Der p 2 peptides (Figures 4A, B). In a mouse model, the disruption of the anchorage of intestinal epithelial cells to the BM through loss of hemidesmosomes led to caspase-1 activation and increased IL18 secretion (De Arcangelis et al., 2017). Similarly, exposure of skin in AD patients to Der p 2 peptides reduced the expression of hemidesmosome genes and increased PYCARD and IL18 expression in proliferating/mitotic, spinous, and granular KCs (Figures 4D, E). This suggests a similar activation pathway in skin KC, which may be further enhanced by the disruption of ECM organization (Supplementary Figure S3B) (Bhattacharjee et al., 2019; Pfisterer et al., 2021). Another study in mice demonstrated that the downregulation of KRT1, along with increased IL18, S100A8, and S100A9, caused barrier defects in the skin (Roth et al., 2012). In AD patients, HDM extracts have been shown to increase the Th2 signature and upregulate S100A7 and S100A8 in the skin (Malik et al., 2017). Our *in vitro* barrier disruption experiment suggests that Der p 2 peptides can directly affect tight junctions by downregulating claudin 1 expression in the absence of other cells. Although the mechanism remains elusive, our findings suggest that Der p 2 peptides in HDM feces may play a role in driving this switch.

KRT1 expression was particularly downregulated in KC clusters, showing the upregulation of PYCARD and IL18 upon skin exposure to Der p 2 peptides (Figure 4E). *In vitro* studies with isolated KC have shown that Der p 1, but not Der p 2, induced the assembly of the NLRP3 inflammasome, leading to caspase-1 activation, and IL-1β and IL18 secretion (Dai et al., 2011). In contrast, we observed a clear upregulation of PYCARD and IL18 by Der p 2 peptides and a less pronounced effect by the full protein. This discrepancy may be due to the higher sensitivity of scRNA-seq, which can detect subtle changes in gene expression on the single-cell level. Moreover, our data suggest that the effect seen for recombinant Der p 2 protein may depend on IgE-dependent immune system activation, whereas Der p 2 peptides may directly affect KCs in the absence of IgE reactivity. High levels of IL4 and IL13 have been reported to reduce KRT1 expression in the lesional skin of AD patients (Beck et al., 2022). Interestingly, we found in our study that KRT1 expression was particularly downregulated upon AD skin exposure to Der p 2 peptides (Supplementary Figure S4C). In contrast, KRT6A/B/C and KRT16 were upregulated, which, along with KRT1 downregulation, have been implicated in causing barrier disruptions (Hobbs et al., 2012).

IL18, a proinflammatory pleiotropic cytokine, can modulate both the innate and the adaptive immune systems. When IL18 was identified and named interferon-gamma-inducing factor, it was found to induce type 1 cytokine production in the presence of IL12. However, in the absence of IL12, IL18 enhances type 2 cytokine production by helper T cells, mast cells, and basophils (Yoshimoto et al., 1999). Our data suggest that Der p 2 peptides induced IL18 and may skew the immune system toward an elevated type 2 response as we detected IL13 but not IL12 in our dataset comprising all skin cells (data not shown). As IL18 can potentially regulate both type 1 and type 2 cytokine productions, it makes it a prominent candidate to control the switch between acute and chronic AD, which are characterized by type 2 and type 1 cytokines, respectively (Langan et al., 2020).

IL4 and IL13 promote epithelial cell proliferation and hyperplasia, which are specifically triggered during AD initiation and acute lesions (Gittler et al., 2012; Beck et al., 2022). We examined the expression of cell cycle and mitosis genes and found a specific upregulation of mitosis genes in KC from AD skin exposed to Der p 2 peptides (Figure 4). Further analysis of

upregulated genes in Der p 2 peptide-treated skin revealed an enrichment of genes involved in the mitosis pathway and a higher percentage of KC expressing mitosis genes in AD_pep compared to AD_rec. Known markers for hyperplasia, such as MKI67, S100A8, S100A9 (Beck et al., 2022), and K16, were all upregulated. Interestingly, MKI67 was upregulated in most KC clusters, indicating a proliferative response to Der p 2 peptides in both basal and suprabasal KC, with the exception of spinous and sebaceous KCs.

In conclusion, our findings suggest that Der p 2 peptides may contribute to initiating major hallmark features of AD, including allergic inflammation, barrier disruption, and hyperplasia. Future studies will reveal that whether the observed net effect on KC is due to immune cell activation or a direct effect of Der p 2 peptides on KC in AD.

Data availability statement

The analyzed datasets for this study can be found in Supplementary Tables S6–S14. The raw count matrix (https://doi.org/10.6084/m9.figshare.23898333.v1) and phenotype file (https://doi.org/10.6084/m9.figshare.23898330.v1) presented in this study were deposited in Figshare.

Ethics statement

The studies involving humans were approved by Ethikkommission der Medizinischen Universität Wien. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

KP planned experiments, supervised the study, and organized participant appointments; CB, KP, SV, and RV developed patch test protocols; H-JH and RC prepared recombinant allergens and allergen peptides; RC verified the allergic status of patients via IgE ELISA; DoS took biopsies and blood; KP and MF designed sample processing pipelines for sequencing; KP and PW isolated cells from the skin; LS processed single cells for scRNA-seq; MW planned and supervised data analysis; MW and DaS analyzed data; WW conceived the study; WW and RV planned the initial study outline, KP interpreted the data and wrote the manuscript with critical input from all authors. All authors contributed to the article and approved the submitted version.

Funding

This project is part of the Danube Allergy Research Cluster and was funded by the Government of Lower Austria (Land Niederösterreich).

Acknowledgments

The authors would like to thank all the participants that made this study possible. The illustration in Figure 1A was created with BioRender.com using (academic licence).

Conflict of interest

RV has received research grants from Worg Pharmaceuticals, Hangzhou, China and HVD Biotech, Vienna, Austria and serves as consultant for Worg and Viravaxx AG, Vienna, Austria.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2023.1240289/full#supplementary-material

References

Akdis, C. A. (2021). Does the epithelial barrier hypothesis explain the increase in allergy, autoimmunity and other chronic conditions? *Nat. Rev. Immunol.* 21, 739–751. doi:10.1038/s41577-021-00538-7

Ali, N. J. A., Dias Gomes, M., Bauer, R., Brodesser, S., Niemann, C., and Iden, S. (2016). Essential role of polarity protein Par3 for epidermal homeostasis through regulation of barrier function, keratinocyte differentiation, and stem cell maintenance. *J. Investigative Dermatology* 136, 2406–2416. doi:10.1016/j.jid.2016.07.011

Beck, L. A., Cork, M. J., Amagai, M., De Benedetto, A., Kabashima, K., Hamilton, J. D., et al. (2022). Type 2 inflammation contributes to skin barrier dysfunction in atopic dermatitis. *JID Innov.* 2, 100131. doi:10.1016/j.xjidi.2022.100131

Bhattacharjee, O., Ayyangar, U., Kurbet, A. S., Ashok, D., and Raghavan, S. (2019). Unraveling the ECM-immune cell crosstalk in skin diseases. *Front. Cell Dev. Biol.* 7, 68. doi:10.3389/fcell.2019.00068

Blanpain, C., and Fuchs, E. (2009). Epidermal homeostasis: A balancing act of stem cells in the skin. *Nat. Rev. Mol. Cell Biol.* 10, 207–217. doi:10.1038/nrm2636

Candi, E., Schmidt, R., and Melino, G. (2005). The cornified envelope: A model of cell death in the skin. *Nat. Rev. Mol. Cell Biol.* 6, 328–340. doi:10.1038/nrm1619

Carter, W. G., Wayner, E. A., Bouchard, T. S., and Kaur, P. (1990). The role of integrins alpha 2 beta 1 and alpha 3 beta 1 in cell-cell and cell-substrate adhesion of human epidermal cells. *J. Cell Biol.* 110, 1387–1404. doi:10.1083/jcb.110.4.1387

Chen, E. Y., Tan, C. M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G. V., et al. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinforma.* 14, 128. doi:10.1186/1471-2105-14-128

Chen, K. W., Fuchs, G., Sonneck, K., Gieras, A., Swoboda, I., Douladiris, N., et al. (2008). Reduction of the *in vivo* allergenicity of Der p 2, the major house-dust mite allergen, by genetic engineering. *Mol. Immunol.* 45, 2486–2498. doi:10.1016/j.molimm.2008.01.006

Cheng, J. B., Sedgewick, A. J., Finnegan, A. I., Harirchian, P., Lee, J., Kwon, S., et al. (2018). Transcriptional programming of normal and inflamed human epidermis at single-cell resolution. *Cell Rep.* 25, 871–883. doi:10.1016/j.celrep.2018.09.006

Cohen, E., Johnson, C., Redmond, C. J., Nair, R. R., and Coulombe, P. A. (2022). Revisiting the significance of keratin expression in complex epithelia. *J. Cell Sci.* 135, jcs260594. doi:10.1242/jcs.260594

Dai, X., Sayama, K., Tohyama, M., Shirakata, Y., Hanakawa, Y., Tokumaru, S., et al. (2011). Mite allergen is a danger signal for the skin via activation of inflammasome in keratinocytes. *J. Allergy Clin. Immunol.* 127, 806–814. e1-4. doi:10.1016/j.jaci.2010.12.006

De Arcangelis, A., Hamade, H., Alpy, F., Normand, S., Bruyère, E., Lefebvre, O., et al. (2017). Hemidesmosome integrity protects the colon against colitis and colorectal cancer. *Gut* 66, 1748–1760. doi:10.1136/gutjnl-2015-310847

De Benedetto, A., Rafaels, N. M., Mcgirt, L. Y., Ivanov, A. I., Georas, S. N., Cheadle, C., et al. (2011). Tight junction defects in patients with atopic dermatitis. *J. Allergy Clin. Immunol.* 127, 773–786. e1-7. doi:10.1016/j.jaci.2010.10.018

Depianto, D., and Coulombe, P. A. (2004). Intermediate filaments and tissue repair. *Exp. Cell Res.* 301, 68–76. doi:10.1016/j.yexcr.2004.08.007

Eyerich, K., and Novak, N. (2013). Immunology of atopic eczema: overcoming the Th1/Th2 paradigm. *Allergy* 68, 974–982. doi:10.1111/all.12184

Fabregat, A., Sidiropoulos, K., Garapati, P., Gillespie, M., Hausmann, K., Haw, R., et al. (2015). The reactome pathway knowledgebase. *Nucleic Acids Res.* 44, D481–D487. doi:10.1093/nar/gkv1351

Garrod, D. R. (1993). Desmosomes and hemidesmosomes. *Curr. Opin. Cell Biol.* 5, 30–40. doi:10.1016/s0955-0674(05)80005-5

Gittler, J. K., Shemer, A., Suárez-Fariñas, M., Fuentes-Duculan, J., Gulewicz, K. J., Wang, C. Q., et al. (2012). Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *J. Allergy Clin. Immunol.* 130, 1344–1354. doi:10.1016/j.jaci.2012.07.012

Gläser, R., Meyer-Hoffert, U., Harder, J., Cordes, J., Wittersheim, M., Kobliakova, J., et al. (2009). The antimicrobial protein psoriasin (S100A7) is upregulated in atopic dermatitis and after experimental skin barrier disruption. *J. Investigative Dermatology* 129, 641–649. doi:10.1038/jid.2008.268

Haensel, D., Jin, S., Sun, P., Cinco, R., Dragan, M., Nguyen, Q., et al. (2020). Defining epidermal basal cell states during skin homeostasis and wound healing using single-cell transcriptomics. *Cell Rep.* 30, 3932–3947. doi:10.1016/j.celrep.2020.02.091

Hafemeister, C., and Satija, R. (2019). Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. *Genome Biol.* 20, 296. doi:10.1186/s13059-019-1874-1

Hamill, K. J., Hiroyasu, S., Colburn, Z. T., Ventrella, R. V., Hopkinson, S. B., Skalli, O., et al. (2015). Alpha actinin-1 regulates cell-matrix adhesion organization in keratinocytes: consequences for skin cell motility. *J. Invest. Dermatol* 135, 1043–1052. doi:10.1038/jid.2014.505

Hobbs, R. P., Lessard, J. C., and Coulombe, P. A. (2012). Keratin intermediate filament proteins - novel regulators of inflammation and immunity in skin. *J. Cell Sci.* 125, 5257–5258. doi:10.1242/jcs.122929

Huang, H. J., Curin, M., Banerjee, S., Chen, K. W., Garmatiuk, T., Resch-Marat, Y., et al. (2019). A hypoallergenic peptide mix containing T cell epitopes of the clinically relevant house dust mite allergens. *Allergy* 74, 2461–2478. doi:10.1111/all.13956

Huber, M., Casares-Arias, J., Fässler, R., Müller, D. J., and Strohmeyer, N. (2023). In mitosis integrins reduce adhesion to extracellular matrix and strengthen adhesion to adjacent cells. *Nat. Commun.* 14, 2143. doi:10.1038/s41467-023-37760-x

Jin, S., Guerrero-Juarez, C. F., Zhang, L., Chang, I., Ramos, R., Kuan, C.-H., et al. (2021). Inference and analysis of cell-cell communication using CellChat. *Nat. Commun.* 12, 1088. doi:10.1038/s41467-021-21246-9

Kosumi, H., Watanabe, M., Shinkuma, S., Nohara, T., Fujimura, Y., Tsukiyama, T., et al. (2022). Wnt/β-Catenin signaling stabilizes hemidesmosomes in keratinocytes. *J. Invest. Dermatol* 142, 1576–1586.e2. doi:10.1016/j.jid.2021.10.018

Kabashima, K., Honda, T., Ginhoux, F., and Egawa, G. (2019). The immunological anatomy of the skin. *Nat. Rev. Immunol.* 19, 19–30. doi:10.1038/s41577-018-0084-5

Kaplan, D. H., Igyarto, B. Z., and Gaspari, A. A. (2012). Early immune events in the induction of allergic contact dermatitis. *Nat. Rev. Immunol.* 12, 114–124. doi:10.1038/nri3150

Kawashima, H., Nishimata, S., Kashiwagi, Y., Numabe, H., Sasamoto, M., Iwatsubo, H., et al. (2000). Squamous cell carcinoma-related antigen in children with atopic dermatitis. *Pediatr. Int.* 42, 448–450. doi:10.1046/j.1442-200x.2000.01253.x

Korsunsky, I., Millard, N., Fan, J., Slowikowski, K., Zhang, F., Wei, K., et al. (2019). Fast, sensitive and accurate integration of single-cell data with Harmony. *Nat. Methods* 16, 1289–1296. doi:10.1038/s41592-019-0619-0

La Manno, G., Soldatov, R., Zeisel, A., Braun, E., Hochgerner, H., Petukhov, V., et al. (2018). RNA velocity of single cells. *Nature* 560, 494–498. doi:10.1038/s41586-018-0414-6

Langan, S. M., Irvine, A. D., and Weidinger, S. (2020). Atopic dermatitis. *Lancet* 396, 345–360. doi:10.1016/S0140-6736(20)31286-1

Lessard, J. C., Piña-Paz, S., Rotty, J. D., Hickerson, R. P., Kaspar, R. L., Balmain, A., et al. (2013). Keratin 16 regulates innate immunity in response to epidermal barrier breach. *Proc. Natl. Acad. Sci.* 110, 19537–19542. doi:10.1073/pnas.1309576110

Leung, D. Y. M., Berdyshev, E., and Goleva, E. (2020). Cutaneous barrier dysfunction in allergic diseases. *J. Allergy Clin. Immunol.* 145, 1485–1497. doi:10.1016/j.jaci.2020.02.021

Li, Y., Huang, H., Liu, B., Zhang, Y., Pan, X., Yu, X.-Y., et al. (2021). Inflammasomes as therapeutic targets in human diseases. *Signal Transduct. Target. Ther.* 6, 247. doi:10.1038/s41392-021-00650-z

Liedén, A., Ekelund, E., Kuo, I. C., Kockum, I., Huang, C. H., Mallbris, L., et al. (2009). Cornulin, a marker of late epidermal differentiation, is down-regulated in eczema. *Allergy* 64, 304–311. doi:10.1111/j.1398-9995.2008.01856.x

Lun, A. T. L., Riesenfeld, S., Andrews, T., Dao, T. P., Gomes, T., Marioni, J. C., et al. (2019). EmptyDrops: distinguishing cells from empty droplets in droplet-based single-cell RNA sequencing data. *Genome Biol.* 20, 63. doi:10.1186/s13059-019-1662-y

Lun, A. T., Mccarthy, D. J., and Marioni, J. C. (2016). A step-by-step workflow for low-level analysis of single-cell RNA-seq data with Bioconductor. *F1000Res.* 5, 2122. doi:10.12688/f1000research.9501.2

Malik, K., Ungar, B., Garcet, S., Dutt, R., Dickstein, D., Zheng, X., et al. (2017). Dust mite induces multiple polar T cell axes in human skin. *Clin. Exp. Allergy* 47, 1648–1660. doi:10.1111/cea.13040

Mertens, A. E. E., Rygiel, T. P., Olivo, C., Van Der Kammen, R., and Collard, J. G. (2005). The Rac activator Tiam1 controls tight junction biogenesis in keratinocytes through binding to and activation of the Par polarity complex. *J. Cell Biol.* 170, 1029–1037. doi:10.1083/jcb.200502129

Mitamura, Y., Ogulur, I., Pat, Y., Rinaldi, A. O., Ardicli, O., Cevhertas, L., et al. (2021). Dysregulation of the epithelial barrier by environmental and other exogenous factors. *Contact Dermat.* 85, 615–626. doi:10.1111/cod.13959

Mitsuishi, K., Nakamura, T., Sakata, Y., Yuyama, N., Arima, K., Sugita, Y., et al. (2005). The squamous cell carcinoma antigens as relevant biomarkers of atopic dermatitis. *Clin. Exp. Allergy* 35, 1327–1333. doi:10.1111/j.1365-2222.2005.02353.x

Miyai, M., Matsumoto, Y., Yamanishi, H., Yamamoto-Tanaka, M., Tsuboi, R., and Hibino, T. (2014). Keratinocyte-specific mesotrypsin contributes to the desquamation process via kallikrein activation and LEKTI degradation. *J. Invest. Dermatol* 134, 1665–1674. doi:10.1038/jid.2014.3

Moosbrugger-Martinz, V., Leprince, C., Méchin, M. C., Simon, M., Blunder, S., Gruber, R., et al. (2022). Revisiting the roles of filaggrin in atopic dermatitis. *Int. J. Mol. Sci.* 23, 5318. doi:10.3390/ijms23105318

Mukoyama, Y., Utani, A., Matsui, S., Zhou, S., Miyachi, Y., and Matsuyoshi, N. (2007). T-cadherin enhances cell-matrix adhesiveness by regulating beta1 integrin trafficking in cutaneous squamous carcinoma cells. *Genes cells.* 12, 787–796. doi:10.1111/j.1365-2443.2007.01092.x

Muroyama, A., and Lechler, T. (2012). Polarity and stratification of the epidermis. *Seminars Cell and Dev. Biol.* 23, 890–896. doi:10.1016/j.semcdb.2012.08.008

Nahidiazar, L., Kreft, M., Van Den Broek, B., Secades, P., Manders, E. M. M., Sonnenberg, A., et al. (2015). The molecular architecture of hemidesmosomes, as revealed with super-resolution microscopy. *J. Cell Sci.* 128, 3714–3719. doi:10.1242/jcs.171892

Pfisterer, K., Shaw, L. E., Symmank, D., and Weninger, W. (2021). The extracellular matrix in skin inflammation and infection. *Front. Cell Dev. Biol.* 9, 682414. doi:10.3389/fcell.2021.682414

Polkoff, K. M., Gupta, N. K., Green, A. J., Murphy, Y., Chung, J., Gleason, K. L., et al. (2022). LGR5 is a conserved marker of hair follicle stem cells in multiple species and is present early and throughout follicle morphogenesis. *Sci. Rep.* 12, 9104. doi:10.1038/s41598-022-13056-w

Reithofer, M., and Jahn-Schmid, B. (2017). Allergens with protease activity from house dust mites. *Int. J. Mol. Sci.* 18, 1368. doi:10.3390/ijms18071368

Reynolds, G., Vegh, P., Fletcher, J., Poyner, E. F. M., Stephenson, E., Goh, I., et al. (2021). Developmental cell programs are co-opted in inflammatory skin disease. *Science* 371, eaba6500. doi:10.1126/science.aba6500

Roth, W., Kumar, V., Beer, H.-D., Richter, M., Wohlenberg, C., Reuter, U., et al. (2012). Keratin 1 maintains skin integrity and participates in an inflammatory network in skin through interleukin-18. *J. Cell Sci.* 125, 5269–5279. doi:10.1242/jcs.116574

Rotty, J. D., and Coulombe, P. A. (2012). A wound-induced keratin inhibits Src activity during keratinocyte migration and tissue repair. *J. Cell Biol.* 197, 381–389. doi:10.1083/jcb.201107078

Satija, R., Farrell, J. A., Gennert, D., Schier, A. F., and Regev, A. (2015). Spatial reconstruction of single-cell gene expression data. *Nat. Biotechnol.* 33, 495–502. doi:10.1038/nbt.3192

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. methods* 9, 676–682. doi:10.1038/nmeth.2019

Serhan, N., Basso, L., Sibilano, R., Petitfils, C., Meixiong, J., Bonnart, C., et al. (2019). House dust mites activate nociceptor–mast cell clusters to drive type 2 skin inflammation. *Nat. Immunol.* 20, 1435–1443. doi:10.1038/s41590-019-0493-z

Shih, B. B., Nirmal, A. J., Headon, D. J., Akbar, A. N., Mabbott, N. A., and Freeman, T. C. (2017). Derivation of marker gene signatures from human skin and their use in the interpretation of the transcriptional changes associated with dermatological disorders. *J. Pathol.* 241, 600–613. doi:10.1002/path.4864

Sivaprasad, U., Kinker, K. G., Ericksen, M. B., Lindsey, M., Gibson, A. M., Bass, S. A., et al. (2015). SERPINB3/B4 contributes to early inflammation and barrier dysfunction in an experimental murine model of atopic dermatitis. *J. Invest. Dermatol* 135, 160–169. doi:10.1038/jid.2014.353

Smith, A. R., Knaysi, G., Wilson, J. M., and Wisniewski, J. A. (2017). The skin as a route of allergen exposure: part I. Immune components and mechanisms. *Curr. allergy asthma Rep.* 17, 6. doi:10.1007/s11882-017-0674-5

Snel, B., Lehmann, G., Bork, P., and Huynen, M. A. (2000). String: A web-server to retrieve and display the repeatedly occurring neighbourhood of a gene. *Nucleic Acids Res.* 28, 3442–3444. doi:10.1093/nar/28.18.3442

Stremnitzer, C., Manzano-Szalai, K., Starkl, P., Willensdorfer, A., Schrom, S., Singer, J., et al. (2014). Epicutaneously applied Der p 2 induces a strong TH 2-biased antibody response in C57BL/6 mice, independent of functional TLR4. *Allergy* 69, 741–751. doi:10.1111/all.12399

Suárez-Fariñas, M., Tintle, S. J., Shemer, A., Chiricozzi, A., Nograles, K., Cardinale, I., et al. (2011). Nonlesional atopic dermatitis skin is characterized by broad terminal differentiation defects and variable immune abnormalities. *J. Allergy Clin. Immunol.* 127, 954–964. e1-4. doi:10.1016/j.jaci.2010.12.1124

Tan, D. W., Jensen, K. B., Trotter, M. W., Connelly, J. T., Broad, S., and Watt, F. M. (2013). Single-cell gene expression profiling reveals functional heterogeneity of undifferentiated human epidermal cells. *Development* 140, 1433–1444. doi:10.1242/dev.087551

Trompette, A., Divanovic, S., Visintin, A., Blanchard, C., Hegde, R. S., Madan, R., et al. (2009). Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457, 585–588. doi:10.1038/nature07548

Tsakok, T., Woolf, R., Smith, C. H., Weidinger, S., and Flohr, C. (2019). Atopic dermatitis: the skin barrier and beyond. *Br. J. Dermatology* 180, 464–474. doi:10.1111/bjd.16934

Wang, S., Drummond, M. L., Guerrero-Juarez, C. F., Tarapore, E., Maclean, A. L., Stabell, A. R., et al. (2020). Single cell transcriptomics of human epidermis identifies basal stem cell transition states. *Nat. Commun.* 11, 4239. doi:10.1038/s41467-020-18075-7

Weidinger, S., and Novak, N. (2016). Atopic dermatitis. *Lancet* 387, 1109–1122. doi:10.1016/S0140-6736(15)00149-X

Yazici, D., Ogulur, I., Kucukkase, O., Li, M., Rinaldi, A. O., Pat, Y., et al. (2022). Epithelial barrier hypothesis and the development of allergic and autoimmune diseases. *Allergo J. Int.* 31, 91–102. doi:10.1007/s40629-022-00211-y

Yoshimoto, T., Tsutsui, H., Tominaga, K., Hoshino, K., Okamura, H., Akira, S., et al. (1999). IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. *Proc. Natl. Acad. Sci. U. S. A.* 96, 13962–13966. doi:10.1073/pnas.96.24.13962

Zeeuwen, P. L. (2004). Epidermal differentiation: the role of proteases and their inhibitors. *Eur. J. Cell Biol.* 83, 761–773. doi:10.1078/0171-9335-00388

Zhou, S., Matsuyoshi, N., Miyachi, Y., Liang, S.-B., Takeuchi, T., and Ohtsuki, Y. (2002). Expression of T-cadherin in basal keratinocytes of skin. *J. Investigative Dermatology* 118, 1080–1084. doi:10.1046/j.1523-1747.2002.01795.x

frontiers | Frontiers in Medicine

TYPE Review
PUBLISHED 25 August 2023
DOI 10.3389/fmed.2023.1228938

OPEN ACCESS

EDITED BY
Elena Ortiz Zapater,
University of Valencia, Spain

REVIEWED BY
Jose A. Uranga,
Rey Juan Carlos University, Spain
Ahmed Ayedur Rahman,
Harvard Medical School, United States

*CORRESPONDENCE
Jay V. Patankar
✉ jay.patankar@uk-erlangen.de

RECEIVED 25 May 2023
ACCEPTED 24 July 2023
PUBLISHED 25 August 2023

CITATION
Bubeck M, Becker C and Patankar JV (2023) Guardians of the gut: influence of the enteric nervous system on the intestinal epithelial barrier.
Front. Med. 10:1228938.
doi: 10.3389/fmed.2023.1228938

Guardians of the gut: influence of the enteric nervous system on the intestinal epithelial barrier

Marvin Bubeck[1,2], Christoph Becker[1,2] and Jay V. Patankar[1,2]*

[1]Department of Medicine 1, Universitätsklinikum Erlangen, Erlangen, Germany, [2]Deutsches Zentrum Immuntherapie (DZI), Erlangen, Germany

The intestinal mucosal surface forms one of the largest areas of the body, which is in direct contact with the environment. Co-ordinated sensory functions of immune, epithelial, and neuronal cells ensure the timely detection of noxious queues and potential pathogens and elicit proportional responses to mitigate the threats and maintain homeostasis. Such tuning and maintenance of the epithelial barrier is constantly ongoing during homeostasis and its derangement can become a gateway for systemic consequences. Although efforts in understanding the gatekeeping functions of immune cells have led the way, increasing number of studies point to a crucial role of the enteric nervous system in fine-tuning and maintaining this delicate homeostasis. The identification of immune regulatory functions of enteric neuropeptides and glial-derived factors is still in its infancy, but has already yielded several intriguing insights into their important contribution to the tight control of the mucosal barrier. In this review, we will first introduce the reader to the current understanding of the architecture of the enteric nervous system and the epithelial barrier. Next, we discuss the key discoveries and cellular pathways and mediators that have emerged as links between the enteric nervous, immune, and epithelial systems and how their coordinated actions defend against intestinal infectious and inflammatory diseases. Through this review, the readers will gain a sound understanding of the current neuro-immune-epithelial mechanisms ensuring intestinal barrier integrity and maintenance of intestinal homeostasis.

KEYWORDS

enteric nervous system, enteric glia, gut epithelial barrier, intrinsic primary afferent neurons, inflammatory bowel disease

1. Introduction

1.1. The enteric nervous system architecture, composition, and epithelial communication

The enteric nervous system (ENS) encompasses two interconnected layers that span the entire length of the intestines: the myenteric (Auerbach's) plexus and the submucosal (Meissner's) plexus (1). These layers are anatomically separated by the circular muscle layer, and the myenteric plexus plays a crucial role in coordinating the motor movement of the gut.

1.1.1. Overview of enteric neuron subtypes

Comprised of a network of large unmyelinated neurons and enteric glial cells, the myenteric plexus forms an extensive ganglionic structure. Within this network, excitatory

(PEMN/eMN) and inhibitory (PIMN/iMN) motor neurons innervate both the circular and longitudinal muscles and are connected by interneurons (PIN/IN). Sensory neurons (PSN) extend through the circular muscle layer, innervating the submucosal plexus as well as the epithelial layer. With the advent of single cell transcriptomic technologies four major studies have reported the single cell transcriptomes of the human and mouse ENS. Following clustering and annotation of neuronal function, combined with cluster-labelling gene expression patterns, there have emerged seven distinct subtypes. Although studies have found common cell clusters, the readers should note that the annotation of these clusters varies between studies. This highlights the lack of a consensus for an integrated nomenclature and annotation of common clusters emerging from multiple studies.

Additionally, there are intramuscular glial cells situated directly between the muscle fibers. The submucosal plexus, located beneath the circular muscle layer, primarily innervates the submucosal layer and the surrounding crypts of the enteric epithelium. It receives input from primary afferent neurons (IPAN) or secretomotor/vasodilator neurons (PSVN) and exhibits a more compact network with fewer cell bodies compared to the myenteric plexus. The main function of the submucosal plexus is to regulate secretory activity (1). Although the ENS operates autonomously, it maintains innervation connections with the central nervous system through the vagus nerve and prevertebral ganglia (2). It also interacts with stromal cells, interstitial cells of Cajal, and immune cells, including scattered single glial cells throughout the enteric tissue. Thus, there has been a growing interest in understanding the neuronal regulation of the immune system in the context of intestinal inflammatory disorders and for the advancement of cell-based therapies for aganglionic gut motility disorders (3, 4). To facilitate a better understanding of the currently recognized enteric neuronal subtypes, we have provided a comparative Table 1 as a reference guide for neuronal subtypes identified by single-cell transcriptomic studies.

1.1.2. Overview of enteric glial subtypes

Recent single cell transcriptomic studies have identified four (9) to seven (7) major enteric glial cell types, which provide support to the various types of neurons and interneurons, along with enteric mesothelial fibroblasts derived from the neural crest. During prenatal development, these glial cells originate from neuroblasts, with sacral neural crest cells contributing to the development of posterior intestinal enteric neurons and glial cells (10). Signaling molecules such as GDNF and NT-3 are essential for the development of the ENS in an age-dependent manner, with lower efficacy in inducing ENS cell development in older individuals (11). A brief summary of anato-morphological and transcriptomic composition of enteric glial cells can be found in Box 1 and Table 2, which shows the anato-morphological and single-cell transcriptomic classification of enteric glial subtypes.

BOX 1: Anato-morphological classification of mouse EGCs: elucidated by Boesmans et al. (12, 13).

- Type I: highly branched, irregular pattern, "astrocyte-like" EGC, in contact with multiple neurons. 70–80% express GFAP, S100B and SOX10 expression is conserved.
- Type II: fibrous interganglionic connections, contact to neural fibers, but no ensheathing. ~50% express GFAP, S100B and SOX10 expression is conserved.
- MP/SMP Type III: sit in extraganglionic regions, up to four major processes with secondary branching running along neuronal processes or small blood vessels. 20–30% express GFAP, S100B and SOX10 expression is conserved
- Type IV: bipolar morphology sitting within the circular and longitudinal smooth muscle layers along nerve fibers. Expression pattern: N/A.
- Mucosal glia: sit within the lamina propria of the mucosa, similar morphology as M/SMP associated Type III. GFAP Expression 20 to 30%, S100B and SOX10 expression is conserved.

Furthermore, we strongly recommend the readers to visit two outstanding and recent reviews to gain an appreciation of the breadth of the developments that have occurred in the ENS field (14–16). These two reviews collectively summarize the discoveries from the initial description of neuronal morphotypes by Dogiel to the recent -omics driven transcriptomic classification of neurons and glial cells of the ENS (14, 15).

1.2. The gut epithelial barrier components that maintain host-environment homeostasis

1.2.1. Intestinal epithelial cell composition

The intestinal epithelial barrier, found in both the large and small intestine, is a remarkably dynamic tissue that undergoes self-renewal every 4–7 days. This continuous renewal process is made possible by a cluster of stem cells located at the base of the intestinal crypts. Within this niche, intestinal epithelial cells (IECs) undergo proliferation and differentiation, giving rise to various specialized cell types as depicted in Figure 1.

The maintenance of the IEC stem cell niche has been shown to heavily rely on WNT signaling from enteric glial cells of the enteric nervous system (ENS), as demonstrated in the study conducted by Baghdadi et al. (17). As IECs proliferate, they gradually move upwards within the crypt-villus structure until they reach the apex, where they reach full maturation. Upon completion of their lifespan, these mature cells undergo controlled cell death, known as anoikis, and are subsequently shed from the intestinal barrier, making room for new cells.

Among the IEC stem cells within the intestinal crypts, Paneth cells play a significant role by producing antimicrobial peptides that protect against potential pathogens and influence the microbiota. Additionally, they contribute to the regulation of stem cells through the secretion of Wnt molecules and metabolic intermediates. Enterocytes, the most abundant cell type among the IECs, specialize in nutrient absorption and the release of enzymes into the lumen. Goblet cells, crucial for the barrier function, produce mucins that serve as a physical barrier against the intestinal microbiome. The thickness of the mucus layer increases from the small to the large intestine in response to higher microbial pressure. Enteroendocrine cells, which engage in local

Abbreviations: ENS, enteric nervous system; PEMN/eMN, peripheral excitatory motor neurons or motor neurons; PIMN/iMN, peripheral inhibitory motor neurons or inhibitory motor neurons; PIN/IN, peripheral interneurons or interneurons; PSN, peripheral sensory neurons; EGC, enteric glial cells; IEC, intestinal epithelial cells; ENC, enteric nerve cells; IPANs, intrinsic primary afferent neurons.

TABLE 1 Molecular taxonomy of the human and mouse ENCs based on single cell transcriptomic studies.

Wright et al. (5)	Drokhlyansky et al. (6)	Zeisel et al. (7)	Morarach et al. (8)	Neuron type	Key genes
Chat 1	PEMN 1, 3, 4, 6	ENT5	ENC1,7	Intrinsic sensory neurons, Interneurons	Chat, Slc18a3, Tac1, Calb2
Chat 1	PEMN 1, 3, 4, 6	ENT5	ENC1,7	Intrinsic sensory neurons, interneurons, mechanosensitive	Chat,Slc18a3, Tac1, Piezo1
Chat 2	PEMN 2, or PIN1, PIN2	(ENT6-7) No equivalent cluster	ENC2-4	Interneurons or excitatory motor neurons 1	Chat, Tac1, Penk
Chat 3 (Met)	PIN1, PIN2 or PEMN 2	(ENT6) No equivalent cluster	ENC4	Interneurons or excitatory motor neurons 2	Chat, Met, Penk, Tac1
Chat 4 (Vglut2)	PIN3 or PSN3	ENT7	ENC12 > ENC7	Interneuron	Slc18a3,Chat, Nos1, Vip, Calb1, Penk, Nefm, Slc17a6
Calcb	PSN1	ENT9	ENC6 > ENC5	Intrinsic sensory neuron	Calcb, Nefm, Scn11a, Calb2, Tacr1, Htr3a, Htr3b, P2rx2, Nmu, Grp, Avil
Nos 1	PIMN1-7	ENT2 > ENT1	ENC8-10	Inhibitory motor neurons 1	Nos1, Vip, Gal, Npy, Htr3a, P2rx2
Nos 2	PIMN1-7	ENT1,3 > ENT2	ENC8-10	Inhibitory motor neurons 2	Nos1, Vip, Gal, Npy
Adult colon	Adult Colon	Adult small intestine	Adult small intestine		

TABLE 2 Molecular taxonomy of mouse and human enteric glial cells.

Mouse EGC			
Ziesel et al. (7)	Drokhlyansky et al. (6)	Cell annotation	Genes enriched
Small intestine	Colon		
ENTG1	Glia1/2	Proliferating	*Gfra2, Frmd4a, Sox12*
ENTG2	n.a.	n.a.	n.a.
ENTG3	Glia2	n.a	*Tmem200c*
ENTG4	n.a.	n.a.	n.a.
ENTG5	Glia1/2	n.a.	*Slc18a2, Scn7a*
ENTG6	Glia1/2	n.a.	*Lbp, Slc18a2, Scn7a*
ENTG7	Glia2/3	n.a.	*Slc18a2, Fam184b, Lsamp, Otor*
ENMFB	Glia3	Enteric mesothelial fibroblasts	*Ntsr1, Pdpn*

Human EGC			
Elmentaite et al. (10)	Drokhlyansky et al. (6)	Cell annotation	Genes enriched
Developing colon	Colon		
Glia1(DHH+)	Glia2, Glia3, Glia 4, Glia5	n.a.	*DHH, RXRG, NTRK2, MBP, MPZ*
Glia2(ELN+)	Glia1, Glia2, and Glia3	n.a.	*TELN, TFAP2A, SOX8, and BMP8B, MPZ*
Glia3(BCAN+)	Glia1 and Glia6	n.a.	*BCAN, APOE, CALCA, HES5, FRZB*
Differentiating glia	Glia1, Glia2, Glia5	Differentiating glia	*NRXN1*

First two columns refer to specific studies to draw equivalence between clusters from different studies. Readers should note that the study by Elmentaite et al. investigates human developing colon post conception week 6–17.

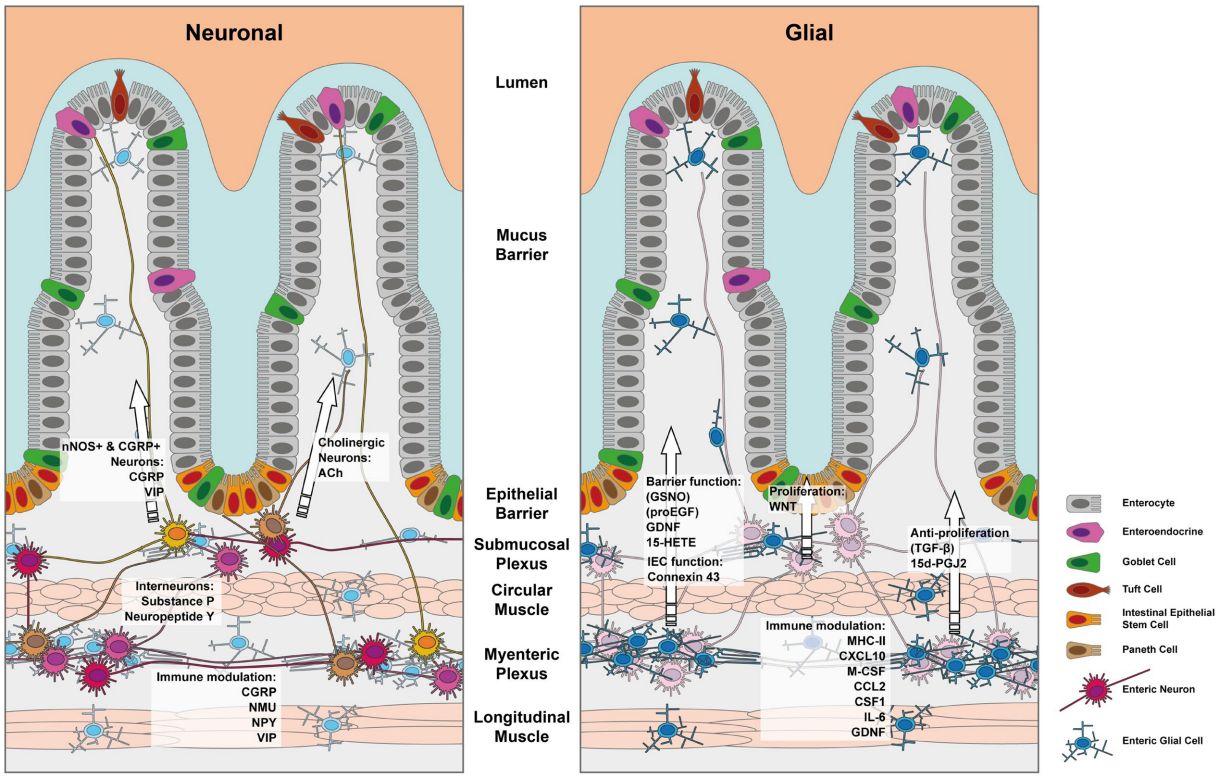


FIGURE 1
Depiction of the enteric nervous system and its components which influence the IEC barrier. The left panel depicts the effects mediated by enteric neurons and the right panel those exerted by enteric glia. Note that indirect effects via immune cells have been left out for simplicity.

signaling and chemoreception through hormones or peptides, directly interface with the ENS and the innate immune system to aid in microbial defense. They also serve as sources of important neuropeptides, including NPY, PYY, CCK, STT, GIP, GLP-1, GLP-2, among others. Lastly, Tuft cells, although rare, play a critical role in defense and chemosensation, particularly in response to parasitic infections. There is some evidence that Tuft cells may also interact with enteric neurons, however this is a hotly debated topic and several groups are trying to elucidate the existence of an IPAN-Tuft cell axis (18).

1.2.2. Overview of the intestinal epithelial barrier

The intestinal epithelial barrier relies on the formation of tight junctions (TJs) between individual cells. These TJs serve as the primary physical barrier that prevents the invasion of the microbiome into the host. They create a diffusion barrier for metabolites and maintain the polarity of the monolayer by acting as a fence for membrane components. The key proteins involved in TJ formation are claudins and occludin. Occludin, an integral membrane protein, consists of four transmembrane domains. Its N- and C-termini are located on the intracellular side of the membrane, resulting in one intracellular loop and two extracellular loops. These extracellular loops attach to neighboring cells through their respective occludin domains on the extracellular side. Claudins exhibit a similar structure, with one intracellular and two extracellular loops. However, they are more structurally and functionally diverse, with 23 genes identified in humans. In mammals, there are 27 known types of claudins, which exhibit high structural conservation but not genetic conservation. The composition of claudins in TJs varies, contributing to the specificity of ion diffusion. For example, in the blood–brain barrier, claudins create a more specific diffusion barrier, while in the intestinal barrier, they allow for less specific ion diffusion. Claudins are linked to the cellular cytoskeleton scaffold through proteins called zonula occludens (ZO). For a more detailed understanding of the structure and function of TJs and their associated proteins, we recommend referring to the reviews by Zihni et al. (19) and Otani and Furuse (20).

2. The enteric glia and the maintenance of the gut barrier

2.1. Direct modulation of epithelial barrier function by EGCs

2.1.1. Enteric glial-ablation and impact on gut barrier homeostasis

Research over the past two decades, has uncovered a wealth of information on the important role that enteric glial cells (EGCs) play in maintaining the integrity and function of the intestinal epithelial barrier. In particular, studies have shown that S-100β-immunoreactive EGCs form dense networks around the intestinal epithelial crypts, and that in co-culture settings; EGCs were able to inhibit the proliferation of the transformed IEC cell line Caco2. This effect was found to be due to EGC-derived TGF-β, as the addition of an anti-TGFβ antibody to

the co-cultures effectively nullified the anti-proliferative effect exerted by the EGCs on the Caco2 cells (21).

Interestingly, *in vivo* ablation of EGCs via an injection of ganciclovir in transgenic mice expressing the Herpes Simplex Virus type 1 (HSV)-thymidine kinase (tk) gene under the *Gfap* promoter was associated with an increase in the incorporation of tritiated thymidine into epithelial cells (21, 22). Other studies used the same model of EGC ablation and identified specific EGC-derived molecules that are involved in maintaining IEC barrier function, promoting epithelial cell proliferation and differentiation, and protecting IECs from pathogens. The authors showed that EGC-derived S-Nitrosoglutathione improves IEC barrier function by regulating the expression of tight junction-associated protein F-actin. Exogenous administration of S-Nitrosoglutathione rescued the intestinal inflammation and barrier dysfunction in mice where EGCs were ablated and prevented *Shigella flexneri* invasion of IECs (23, 24). In addition, EGC-derived S-Nitrosoglutathione was shown to protect against cytokine-induced barrier defects in an *ex vivo* co-culture model of IECs with EGCs, via the increased expression and localization of occludin and ZO-1 (25). Van Landeghem et al. identified proEGF as another EGC-secreted factor that promotes IEC healing using a combination of techniques involving EGC ablation via the HSV-tk system and *in vitro* assessment of the effects of the EGC-like cell line JUG2 on Caco2 monolayer proliferation in a scratch assay (26).

However, later studies that systematically analyzed the effect of EGC ablation independently of HSV-tk or the *Gfap* promoter identified off-target toxicity of the ganciclovir derivatives on adjacent cells as the main cause of the effects reported in this model, rather than EGC ablation as previously suggested (22, 27, 28). In addition, outstanding work on the classification of EGC subtypes has shown that only a subpopulation of EGCs expresses GFAP (9, 29). This implies that GFAP-driven recombination does not extend to the entire EGC population. Therefore, caution should be exercised in interpreting the conclusions drawn from studies using the HSV-tk-mediated EGC ablation strategy.

2.1.2. Enteric glia and glia-derived factors in the regulation of the epithelial barrier

Furthermore, other studies continued to identify various EGC-derived secreted factors, which affect the epithelial barrier. Some of these factors are graphically outlined in Figure 1. Enteric glial cell-derived GDNF was shown to ameliorate inflammation in a mouse model of barrier damage induced colitis. The authors showed that treatment of mice with recombinant adenoviruses to overexpress GDNF ameliorated dextran sulfate sodium (DSS)-induced colitis, improved colonic transit defects, and *in vitro* IEC healing responses (30–32). The role of GDNF in promoting intestinal barrier integrity was further confirmed *in vitro* using a co-culture model employing EGC and rat IEC cell lines (33). Mechanistically Meir et al. showed that GDNF binding to the RET receptor was important in the stabilization of the desmosomal protein desmoglein 2 in Caco2 cell membranes. Furthermore, the authors reported that inflammatory bowel disease and experimental colitis were associated with a reduction in the expression of GDNF and that restoring GDNF was sufficient to inhibit the inflammation-induced compromise in the epithelial barrier both *in vivo* and *in vitro* (34). In a separate study, Meir et al. used *Gfap-Cre* driven reporter system to FACS sort EGCs from the myenteric plexus of mice and showed that these cells indeed produced GDNF and the knockdown of GDNF in an EGC cell line, abrogated the IEC barrier promoting effects on Caco2 cells *in vitro* (35). However, it should be noted that recent single cell transcriptomic data from the human intestinal stromal cells have identified that apart from EGCs, a few other stromal cell types might also be a potential source of GDNF in the gut (36). Other members of the oxylipin family including prostaglandins can also influence the proliferation and differentiation of IECs. Indeed, Bach-Ngohou et al. reported that human submucosal plexus EGCs express lipocalin and EGC cell lines secrete the PPAR-γ ligand 15-deoxy-12, 14-prostaglandin J2 (15d-PGJ2) (37). The authors found that the EGC-derived 15d-PGJ2 exerted inhibitory effects on Caco2 cell proliferation but promoted their differentiation by upregulating the expression of E-cadherin and intestinal alkaline phosphatase (37).

Furthermore, the inhibition of inducible nitric oxide synthase (iNOS) was shown to enhance the IEC barrier protective effects exerted by EGCs in the context of LPS induced barrier disruption arguing that EGC-IEC NO signaling is detrimental under inflammatory contexts (38). Another study investigating the role of iNOS in regulating electrical stimulation evoked chloride and ion secretion the context of trinitrobenzene sulfonic acid– or DSS –induced colitis. The authors found that colitis dependent abrogation of ion secretion was reversed by the inhibition of iNOS, an effect that was mimicked by blocking EGC function with fluoroacetate. In all three colitis models that the authors tested, fluoroacetate mediated inhibition of EGC functions restored the impairment in electrogenic ion transport (39). Nonetheless, it should be noted that fluoroacetate is a non-specific metabolic poison and as such, the possibility of off-target toxicity to other cells cannot be excluded.

It is worth noting that one of the complications of studies that used isolated primary EGC cultures from the myenteric plexus was the question of culture purity. To address this issue, Soret et al. compared and characterized EGC cultures from human, mouse, and rat longitudinal muscle myenteric plexuses and found that approximately 80% of these cells were GFAP, S100β, and SOX10 immunoreactive EGCs (40). Additionally, the authors confirmed the previously described effects of EGCs on promoting IEC barrier and reducing IEC proliferation using the IEC transformed cell line Caco2 (40).

Although several reports involving the assessment of EGCs effects on the IEC barrier using *in vitro* testing of cell lines were indicative of a beneficial effect robust and direct evaluation of the roles of EGCs in maintaining the epithelial barrier were lacking. Important contributions were made over the last 5 years in our understanding of the effects of EGC on the gut barrier using cleaner *Cre* driver lines and a detailed assessment of barrier function *in vivo* during homeostatic and pathological conditions (27, 41, 42). These studies collectively challenged the paradigm that EGC are necessary for maintaining the IEC barrier. For, e.g., using the *Sox10-CreER*^{T2} driver line, which drives recombination in all EGCs, Grubišić et al. conditionally and inducibly knocked out connexin 43 in EGCs. Connexin 43 is crucial for EGC activity and upon its tamoxifen induced removal from IECs, caused an impairment in secretomotor functions by regulating electrogenic ion transport, but had no consequences on the IEC barrier (42). In addition, when Rao et al. specifically ablated, EGCs using transgenic diphtheria toxin subunit A (DTA) expression via the pan-EGC inducible driver *Plp1-CreER*^{T}, the authors failed to see any observable

defects in the barrier integrity and IEC proliferation (27). Based on these findings, one may infer that the EGCs are rather innocuous in mediating barrier changes, but in an interesting twist, Grubišić et al. identified a pathogenic role for the Sox10+ EGCs in barrier modulation. The authors showed that the *Sox10-CreER*^{T2} mediated knockout of the adenosine 2B receptor ADORA2B, in EGCs protected against DSS-induced colitis and normalized the mRNA expression and distribution of tight junction proteins (41). The view that EGCs do not influence the IEC barrier was also challenged recently in a study, which investigated distinct roles for the *Gfap*$^{+}$ and *Plp1*$^{+}$ EGC subsets in regulating the IEC barrier (17). The authors ablated EGCs using conditional and tamoxifen-inducible transgenic expression of the DTA in either *Plp1-CreER*^{T}, or *Gfap-CreER*^{T2} and performed single cell RNA-Seq to identify specific EGC subsets. The authors elegantly demonstrated the critical role of *Gfap*$^{+}$ pericryptal submucosal EGC, but not the Plp1+ EGC subset in regulating the proliferation of the IEC (17). By using the conditional inducible DTA expression approach, the authors also successfully overcame the previously reported off–target toxicity of the HSV-tk and ganciclovir method for EGC ablation. In addition, the authors also identified that the subset of *Gfap*$^{+}$ pericryptal submucosal EGCs are an important source of WNT signals driving the proliferation of the intestinal stem cells (17).

Collective evidence in the field demonstrates an important direct role of EGC subsets in regulating the IEC barrier by controlling proliferation and differentiation of IECs. Further evaluation of other EGC subsets and their influence on IECs will yield greater insights into the role of EGCs in barrier protection.

2.2. EGC-mediated immune cell modulation in the regulation of the intestinal barrier

2.2.1. Enteric glia - adaptive immune interactions and influence on the gut barrier

The discovery of EGCs took place over a century ago, but their contribution to regulating intestinal immunity has remained eclipsed due to their classification as cells that provide trophic and protective support to enteric neurons. The immune functions of EGCs were initially explored after the identification of MHC class II molecules expressed by EGCs in inflamed tissues of Crohn's disease patients. This raised the possibility of an inflammation-induced exogenous antigen presentation capability by EGCs to CD4+ T-cells in the gut (43, 44). Subsequent studies have shown that human GFAP+ EGCs cultured from non-involved margins of small bowel tumor resections, responded to exogenous enteroinvasive *Escherichia coli in vitro*, via upregulating the MHC-II mRNA and protein expression (45). Recently Chow et al. formally tested the hypothesis whether EGCs are capable of presenting exogenously phagocytosed antigens (46). The authors showed that a combination of IFNγ and LPS drove the expression of MHC-II in mouse GFAP+ EGCs at the mRNA and protein level. However, they could not detect any phagocytic activity in EGCs and concluded that the antigens presented by EGCs on the MHC-II are derived from autophagy (46). Furthermore, the authors showed that the EGC MHC-II molecules were involved in modulating T_H17 and T_{reg} T-cell subsets in the gut, affecting the regulation of the gut barrier and tolerance (46).

The functional significance of IFNγ signaling on enteric glial cells (EGCs) goes beyond just the upregulation of MHC-II. A recent study by Progatzky et al. has revealed an intriguing aspect of this signaling pathway in the context of helminth-induced intestinal inflammation (47). The authors demonstrated that the injury sustained by EGCs in mouse models and human gut inflammation is associated with an EGC-specific IFNγ transcriptional signature. Through single-cell transcriptomic analyses, they identified a subset of mouse and human EGCs, referred to as EGC2 that expresses high levels of GFAP and exhibits a transcriptional enrichment in the IFNγ response pathway. (47). Furthermore, the authors observed that EGCs produce CXCL10, which recruits CD8+ T-cells, as we have summarized in Figure 1. They also demonstrated that the EGC-specific ablation of the Ifngr2 gene leads to a worsening of infection parameters, a reduction in CD8+ T-cell numbers, and elevated histological damage scores. These findings underscore the crucial role of the IFNγ-EGC axis in the initiation of CD8+ T-cell mediated intestinal tissue repair (47).

2.2.2. Enteric glia - innate immune interactions and influence on the gut barrier

In a recent study by Grubišić et al., it was found that inflammation triggers the production of M-CSF from S100β + EGCs in both humans and mice. This subsequently modulates the proinflammatory phenotypic switch in muscularis macrophages (48). The authors discovered that the production of M-CSF from EGCs was dependent on the Connexin-43 protein and required signaling via TNF-alpha converting enzyme. Additionally, EGC Connexin-43 was found to be crucial for visceromotor responses, as a measure of visceral hypersensitivity, during chronicity of colitis (48). Although changes in gut barrier were not directly measured, the accumulation of proinflammatory macrophages can be detrimental to the barrier function in chronic colitis. This suggests that EGCs may function in perpetuating barrier damage. However, a recent study by Stakenborg et al. indicated a barrier-protective role of EGCs. The authors found that EGCs are involved in polarizing muscularis macrophages to an anti-inflammatory state (49). During early inflammation, EGCs recruit monocytes by producing CCL2, and during the resolution phase, EGC-derived CSF1 is required for the polarization of monocytes to two pro-resolving macrophage subsets, namely the Cd206^{+}, MhcII^{Hi}, and the Timp2^{+}, MhcII^{Lo} subsets (49). The apparent contradiction in pro- versus anti-inflammatory effects of EGCs on macrophages reported by these two studies may be due to the differences in the phase and type of inflammation investigated and the choice of promoters used to drive recombination in the EGCs. used by the two studies are different. Grubišić et al. investigated the inflammatory phase of DSS-colitis using the *Sox10-CreER*^{T2} line, whereas Stakenborg et al. describe the effects during the resolution phase of muscularis inflammation induced by surgical manipulation and used the *Plp1-CreER*^{T2} line.

An important study by Dora et al. investigating the macrophage populations in the avian and murine myenteric plexuses has indicated the presence of a myenteric plexus barrier modulated by unique macrophage populations (50). Their study revealed that the GFAP+ EGCs possess the ability to secrete extracellular matrix molecules that, along with the continuous layer of glial end feet, create a protective barrier around the healthy myenteric plexus of the gut. This barrier effectively blocks macromolecules larger than 4 kDa from entering the myenteric plexus. The authors further demonstrated that during

experimental colitis, this barrier is disrupted in a macrophage-dependent manner (50). The identification of the myenteric plexus barrier has contributed significantly to our understanding of the host organism's diverse mechanisms to separate self from the environment.

Previously, EGCs have been demonstrated to be a source of specific cytokines that are implicated in barrier repair and IEC proliferation, as opposed to mononuclear immune cells of the plexus (51, 52). Using purified GFAP+ EGC cultures from rat longitudinal-muscle myenteric plexuses, it has been shown that EGCs are a source of bioactive IL-6, and that the production of IL-6 from EGCs is dependent on IL1-β stimulation (51). In addition, a recent report by Schneider et al. showed functional IL1 signaling on EGCs regulated macrophage activation and enteric gliosis in a model of post-operative ileus (53). This indicates that EGCs can modulate the local immune milieu via cytokine signaling under specific inflammatory states. One of the most potent cytokines which promotes gut barrier function is IL-22. An important study by Ibiza et al. revealed the role of EGC-derived GDNF in the regulation of type 3 innate lymphoid cells (ILC3) in the gut. It was found that ILC3 express the high-affinity receptor RET for GDNF and responds to GDNF via the STAT3-mediated production of IL-22 (54). Knocking out the RET receptor in ILC3s or MYD88 in GFAP+ EGCs led to a reduction in IL-22 production and increased gut barrier damage and consequent inflammation. These findings highlight the important role of EGCs in modulating ILC3s and the intestinal barrier through microbial sensing functions (54).

While the mechanisms underlying how EGCs regulate intestinal immunity are still being explored, emerging studies have revealed the intricate regulatory involvement of EGCs in modulating the cell states or functional responses of multiple immune cells, including macrophages, T-cells, and ILCs that are critical in the maintenance of the intestinal barrier.

3. The enteric neurons in mucosal barrier function

3.1. Direct modulation of epithelial cells by enteric neurons

3.1.1. Epithelial innervation and modulation of fluid flux

Specific enteric neurons known as Dogiel type II multi-axon bearing neurons have long been considered as cells that directly regulate mucosal functions by innervating the mucosa. However, the exact functional significance of this anatomical finding has only recently become clearer. Direct neuron-epithelial innervations have been difficult to prove, but recent research by Bohórquez et al. has revealed the presence of neuropod extensions from the basolateral surfaces of intestinal and colonic enteroendocrine cells that allow them to directly synapse with mucosal efferent and afferent neuronal innervations (55, 56). These findings raise the intriguing possibility of a direct neuroepithelial circuit that allows the enteric nervous system (ENS) to sense and respond to environmental cues.

Previously, it was thought that cholinergic enteric neurons innervating IECs regulated fluid secretion from colonic IECs and granule release from ileal Paneth cells. Acetylcholine (ACh) was also shown to increase paracellular and transcellular permeability and induce chloride ion secretion across the IEC via muscarinic receptors (57, 58). The barrier protective role of ACh became apparent in a mouse model of severe burn-induced distal organ failure. Severe burns can lead to systemic shock, which has been associated with a compromised gut barrier. Constantini et al. measured the gut barrier function in male BALB/c mice exposed to a severe, 7-s steam burn over 30% of the body surface either with or without the administration of α-7 nicotinic acetylcholine receptor agonists (59). The authors reported a significant reduction in the appearance of 4 kDa fluorescently labelled dextran molecules, in the serum of mice that received the cholinergic agonist compared with those that received vehicle. In mice with a healthy gut barrier, the orally administered 4 kDa dextran is incapable of permeating through the gut into the blood stream (59). Recent discoveries have raised an interesting possibility of non-neuronal sources of ACh in the gut. Tuft cells, a specific type of IECs, can produce ACh, forming a non-neuronal cholinergic system in the gut upon sensing luminal components (60–62). However, relative contributions of the neuronal versus non-neuronal cholinergic systems in the regulation of IEC functions in homeostasis and disease remains to be evaluated.

3.1.2. Neuropeptides and cytokines from enteric neurons in epithelial barrier control

Recent research has revealed the direct effects of specific neuronal factors on the regulation of the IEC barrier via diverse mechanisms. For instance, an important function of nociceptive calcitonin gene-related peptide (CGRP) + enteric neurons that sense commensal microbiota was recently discovered (63). The authors showed that mouse and human goblet cells express the high affinity CGRP receptor, RAMP1 enabling a CGRP-mediated regulation of mucous secretion from goblet cells and protection against experimental colitis coupled with microbial sensing by these neurons (63). A subset of the enteric nociceptive CGRP+ sensory neurons also express vasoactive intestinal polypeptide (VIP). VIP has large effects on the intestinal barrier function by regulating multiple barrier functions. For example, VIP stimulates the release of mucous, induces electrolyte and fluid movement across the IECs, induces ZO-1 tight-junction mRNA and protein expression in IECs, and induces the expression of trefoil factor-3 leading to the stabilization of the mucous layer (64–67). Moreover, human nitrergic neurons, which express VIP, inhibit IEC proliferation and improve barrier integrity in *ex vivo* co-culture systems (68).

A subpopulation of enteric interneurons is Substance P (SP) + and innervate the peri-cryptal submucosal plexuses (6). Interestingly, exogenous SP supplementation protects against acute and chronic DSS-induced colitis and barrier breakdown via the upregulation of intestinal epithelial proliferation (69). Another neuropeptide which has strong direct effects on the IECs is neuropeptide Y (NPY). NPY acts on the IECs via the peptide YY receptors subtype 1, which is expressed highly on colonic absorptive enterocytes, goblet cells, and enteroendocrine cells through which NPY negatively regulates ion transport and secretion.

Apart from neuropeptide-mediated regulation of IEC function, recent research has revealed the role of neuronally derived interleukin-18 (IL-18) in regulating the IEC barrier by controlling the expression of antimicrobial peptides in IECs (70). When the authors knocked out IL-18 in epithelial and immune cells, the control of intestinal *S. typhimurium* remained unaffected. However, upon specific ablation of IL-18 from neuronal cells, bacterial killing and goblet cell

antimicrobial peptide expression was derailed (70). Interestingly, the IL-18 expressing neurons were VIP+, CHAT+, and nNOS+ and are most likely a population of peripheral sensory neurons (6). However, the conditions that trigger the expression and release of this neuronally-derived barrier protective factor is currently not known.

3.1.3. Enteric neurons as regulators of post-translational mechanisms of barrier protection

Fucosylation, a crucial post-translational modification of membrane glycoproteins and glycolipids, plays a vital role in the production, function, and integrity of intestinal mucous as well as in maintaining microbial homeostasis. Interestingly, a recent study by Lie et al. shed light on the function of VIP+ intestinal neurons in regulating IEC fucosylation, which in turn regulated microbial homeostasis. (71). The authors demonstrated that activation of VIP receptor 1 on IECs by enteric VIP+ neurons had a significant impact on α1,2-fucosylation on IECs by modulating the expression of fuscosyltransferase-2 and various glycoproteins and glycolipids. The absence of extrinsic vagal gut innervation or the chemogenetic perturbation of the ENS VIP+ neurons altered IEC fucosylation and the balance between opportunistic and commensal microbial communities (71). These findings support previous studies highlighting the crucial role of VIP in regulating intestinal barrier homeostasis, as germline VIP deficiency renders mice more susceptible to DSS- and 2,4-dinitrobenzenesulfonic acid-induced colitis, which can be rescued by exogenous VIP administration (72). Thus, it is evident that ENS-derived VIP directly regulates gut barrier function.

Enteric neurons have a profound impact on the intestinal epithelium by regulating mucous production, differentiation, and ion and fluid exchange. Future studies aimed at understanding IEC innervation and barrier integrity will reveal how dense axonal innervations and neuronally-derived factors impact the IEC barrier.

3.2. The enteric neurons modulate intestinal innate and adaptive immunity in maintaining the gut barrier

Recent studies have shed light on the complex interplay between various cellular components and neuropeptides involved in regulating barrier integrity. Macrophages in the muscularis have been found to possess unique characteristics and exhibit distinct polarization states regulated by catecholaminergic signaling. However, the impact of this polarization on barrier integrity remains unclear. Additionally, IPANs have emerged as potential sensors of tissue damage caused by microbial dynamics, with the activation of IPANs potentially mediated by intermediate cells such as Tuft IECs or myeloid dendritic cells. Furthermore, sensory neuropeptides like CGRP and NPY, as well as enteric neuropeptide Nmu, have been implicated in regulating immune responses and barrier defense. This section will discuss some of the key findings that link enteric neurons, immune cells, and the gut barrier.

3.2.1. Enteric neuron-macrophage axis in gut barrier protection

Gabanyi et al. in 2016 demonstrated that macrophages in the muscularis exhibit unique characteristics compared to those in the lamina propria and mucosa. These macrophages have a distinct tissue-protective phenotype which is regulated by norepinephrine signaling through the β2 adrenergic receptors in response to infection (73). The authors showed that infection elicited extrinsic sympathetic nerves to signal to these macrophages. However, the impact of this polarization on the IEC barrier integrity was not directly assessed. It would be interesting to note that taken together with the population of macrophages described as the intraplexial macrophages by Dora et al. (50) the role of enteric neuron – macrophage interaction in maintaining the gut barrier as well as the myenteric plexus barrier needs further exploration.

In studies exploring the gut sensory systems during pathogen intrusion, most focus has been on intermediary cells that respond to pathogen- or tissue-derived molecular patterns. However, it is possible that IPANs directly sense tissue damage caused by changes in microbial dynamics through alarmins like IL-25, given that there is some evidence from single cell transcriptomic studies indicating that both the IL-25 receptor chains are expressed by enteric neurons (6). The activation of IPANs in the context of infection may occur through intermediate cells such as Tuft IECs or myeloid dendritic cells via unknown mechanisms.

3.2.2. Enteric neuron-T-cell crosstalk in the regulation of barrier function

Although the governing mechanisms are unclear, functional neuro-immune effects have been identified. For instance, the sensory neuropeptide CGRP negatively regulates the proliferation of mouse naïve splenic T-cells and the production of IL-2 *ex vivo*, but whether similar effects are exerted on activated lamina propria T-cells in the context of inflammation has not been formally tested (74). Interestingly, a CGRP antagonist was found to worsen the severity of DSS-induced colitis, suggesting a barrier-protective role for CGRP (75). Similarly, in the context of Trichinella spirali infection, an IL-4-producing type 2 lymphocyte - CCK-producing enteric neuron axis is associated with enhanced barrier defence, but the exact mechanism of neuron activation during infection remains unknown (76). On the same lines, a subpopulation of CCK producing enteric neurons were recently transcriptomially classified as the mechanosensitive intestinofugal afferent neurons that co-transcribe the *Il4ra* and can induce intestinal smooth muscle contractions leading to worm expulsion and enhanced barrier defence. The exact mechanism of how these neurons is activated in the context of infection remains to be identified.

Specific mucosa innervating secretomotor neurons express the neuropeptide NPY, the transcript levels of which are induced by TNF treatment in primary enteric neuronal cultures (6, 77, 78). Furthermore, the ablation of NPY has been shown to ameliorate tissue injury and barrier disruption, which occurs due to DSS- or *S. typhimurium*- induced colitis (79). Contrary to these findings, in an adoptive T-cell transfer -induced model of colitis, Wheway et al. demonstrated a putative anti-inflammatory function of NPY on T-cells. When the authors transferred Y1 receptor deficient T-cells into leukopenic Rag1 knockout recipients, this caused aggressive colitis (80). Conversely, when the authors challenged Y1 deficient mice with DSS to induce colitis, or with methylated BSA to induce foot pad swelling, the levels inflammation indicators were lower in the knockout mice compared with the controls. Mechanistically, the authors could dissect distinct effects of NPY which strongly represses

T-cell activation but promotes antigen presentation (80). Therefore, over all action of NPY the gut seems to promote inflammation and reduce contractility and secretion, all of which are counterproductive for a healthy gut barrier. Further research is necessary to dissect the exact mechanism of NPY-mediated effects on the gut barrier in pathophysiological conditions.

3.2.3. Enteric neurons and the regulation of the gut barrier via the innate lymphoid cells

Regarding helminth control in the gut, neuromedin U (Nmu) has emerged as an important enteric neuropeptide expressed by a subset of IPANs. The receptor for *Nmu*, *Nmur1* is highly expressed on type 2 innate lymphoid cells (ILC2), which play a crucial role in the regulation of type 2 immunity in the gut. Infections by helminth pathogens like *Nippostrongylus brasiliensis* induce Nmu transcription, leading to elevated IL-13 release from ILC2s, increased mucous production from IECs, and smooth muscle contraction for worm expulsion and barrier protection (81, 82). Recent work has shown that CGRP counters the IL-33 and NMU mediated effector functions and proliferation of ILC2s (83, 84). The authors demonstrated that ILC2s are not only sensitive to, but are also capable of producing this sensory neuropeptide and its genetic ablation correlates with improved helminth clearance from the small intestine. Mechanistically, specific ILC2 subsets express CGRP and its CGRP receptors, which are responsible for limiting ILC2 proliferation and inhibiting IL-13 production, a barrier protective cytokine. Interestingly, CGRP uncouples the regulation of the ILC2 IL-5 and IL-13 production such that IL-5 production is enhanced, whereas IL-13 production is inhibited by CGRP (83, 84). This sensory neuropeptide – ILC2 regulatory axis also plays an important role in regulating anti-helminth responses in the lung, which is another barrier tissue (85).

Intestinal VIP+ neurons also regulate the type 3 innate lymphoid cells (ILC3) via the VIPR2 expressed on the ILC3. When the authors knocked out the VIP receptor 2 gene on RORγt+ ILC3, they observed an enhancement in the production of barrier protective cytokines such as IL-22. Moreover, the chemogenetic inhibition of the VIPergic neurons was associated with a heightened protection from pathology caused by the enteropathogenic bacterium *Citrobacter rodentium*. In addition, the barrier disruption, systemic infection, and organismal death observed by chemogenetic activation of the VIPergic intestinal neurons was reversed by exogenous administration of recombinant IL-22 showing that VIP mediated inhibition of ILC3s critically impacts IL-22 mediated barrier protection (86). In line with these findings, Vu et al. reported that the germline deficiency of the VIP gene or pharmacological antagonism of its receptors, protected mice from the adverse pathological and barrier damaging effects of DSS-induced colitis (87).

4. ENS and regulation of the gut barrier in IBD

Excessive diarrhea and arrhythmic gut motility are some of the indications for an altered ENS function during IBD. However, not much information is available on whether ENS alterations themselves can impact barrier function in IBD patients. Most of IBD research is being advanced through patient-derived mucosal biopsies, which fail to recapitulate the changes in the neurons and glia in the myenteric plexus embedded deep within the muscularis layers. At a histological level, myenteric plexitis, hyperplasia, and altered neuropeptide code are a common and well-documented finding in Crohn's disease and ulcerative colitis, especially in ileal manifestations of Crohn's disease with structuring fibro-stenosis (88–90). A positive evaluation of myenteric plexitis at the margins of the resection site is predictive of an earlier post-surgical recurrence and reflects functional consequences of such plexitis besides the obvious consequences on neuroinflammation and motility (91–96). Elevated levels of EGCs have been reported in patients with IBD, and elevated GFAP+ EGCs were encountered in the payer's patches of patients with ileal Crohn's disease (97). Interestingly, the EGC mediators S-nitroglutathione and GDNF worsened paracellular permeability in Crohn's disease, but not in non-IBD patients (97). However, the specificity of this finding using multiple glia markers such as S100B or PLP1 was not determined. Interestingly, while profiling the polyunsaturated fatty acids that may be secreted by EGCs, Pochard et al. found that human and rat EGCs produced the lipoxin precursor 15-HETE. They then showed that the levels of 15-HETE secreted by EGCs from Crohn's disease patients were lower than healthy controls EGCs. In addition, 15-HETE administration improved transepithelial permeability by increasing ZO-1 expression in an *in vitro* assay using Caco2 cell monolayers (98). Another interesting correlation is the simultaneous upregulation of TNF and NPY immunoreactivity in inflamed IBD patient samples (79, 99). Nevertheless, it must be noted that not only NPY but also other neuropeptide transcript signatures in bulk tissues may reflect cumulative expression from enteroendocrine cells and ENCs. Therefore, in contexts where there is no evidence of epithelial erosion, such results should be interpreted carefully and should be clearly ascribable via immunohistochemistry to nerve cells. On the same lines, a recent report showed that inflamed tissues from IBD patients have an elevated NPY immunoreactivity and that pharmacological inhibition of the NPY receptors was able to prevent the release of inflammatory cytokines from IBD patient tissue biopsies, as well as protect against experimental colitis and preserve barrier integrity (78, 100). Further studies which investigate the mechanisms of EGC and ENC mediated control of the intestinal barrier in IBD are warranted.

5. Conclusion

In conclusion, emerging evidence has highlighted the roles of the enteric glial and neuronal cells in coordinating the immune and epithelial barrier in the gut so as to detect and respond to potential threats while maintaining homeostasis. The maintenance of the gut barrier is crucial, as its disruption can have systemic consequences. The emerging understanding of immune regulatory functions mediated by enteric neuropeptides and glial-derived factors provides intriguing insights into their contributions to the tight control of the mucosal barrier. Figure 1 summarizes the neuronal and glial effectors covered in this review that impact the gut barrier. Continued research in this field holds great promise for developing targeted therapies to manage intestinal infectious and inflammatory diseases.

Author contributions

JP conceptualized, wrote, and edited the manuscript. MB participated in writing and was responsible for realizing the graphic visualizations. CB edited and proofread the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - TRR241 375876048 (A03, B05), SFB1181 (C05), and individual grants with project numbers 418055832 and 510624836. The project was also supported by the Interdisciplinary Centre for Clinical Research (IZKF: A76, A93, and ELAN P120).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Timmermans JP, Adriaensen D, Cornelissen W, Scheuermann DW. Structural organization and neuropeptide distribution in the mammalian enteric nervous system, with special attention to those components involved in mucosal reflexes. *Comp Biochem Phys A*. (1997) 118:331–40. doi: 10.1016/s0300-9629(96)00314-3

2. Li Y, Owyang C. Musings on the wanderer: what's new in our understanding of Vago-vagal reflexes? V. remodeling of vagus and enteric neural circuitry after vagal injury. *Am J Physiol Gastrointest Liver Physiol*. (2003) 285:G461–9. doi: 10.1152/ajpgi.00119.2003

3. Populin L, Stebbing MJ, Furness JB. Neuronal regulation of the gut immune system and neuromodulation for treating inflammatory bowel disease. *FASEB Bioadv*. (2021) 3:953–66. doi: 10.1096/fba.2021-00070

4. Furness JB, Lei E, Hunne B, Adams CD, Burns AJ, Wykosky J, et al. Development of the aganglionic colon following surgical rescue in a cell therapy model of Hirschsprung disease in rat. *Dis Model Mech*. (2023) 16:dmm050055. doi: 10.1242/dmm.050055

5. Wright CM, Schneider S, Smith-Edwards KM, Mafra F, Leembruggen AJL, Gonzalez MV, et al. scRNA-Seq reveals new enteric nervous system roles for GDNF, NRTN, and TBX3. *Cell Mol Gastroenterol Hepatol*. (2021) 11:1548–92 e1. doi: 10.1016/j.jcmgh.2020.12.014

6. Drokhlyansky E, Smillie CS, Van Wittenberghe N, Ericsson M, Griffin GK, Eraslan G, et al. The human and mouse enteric nervous system at single-cell resolution. *Cells*. (2020) 182:1606–22 e23. doi: 10.1016/j.cell.2020.08.003

7. Zeisel A, Hochgerner H, Lonnerberg P, Johnsson A, Memic F, van der Zwan J, et al. Molecular architecture of the mouse nervous system. *Cells*. (2018) 174:999–1014.e22. doi: 10.1016/j.cell.2018.06.021

8. Morarach K, Mikhailova A, Knoflach V, Memic F, Kumar R, Li W, et al. Diversification of molecularly defined myenteric neuron classes revealed by single-cell RNA sequencing. *Nat Neurosci*. (2021) 24:34–46. doi: 10.1038/s41593-020-00736-x

9. Boesmans W, Lasrado R, Vanden Berghe P, Pachnis V. Heterogeneity and phenotypic plasticity of glial cells in the mammalian enteric nervous system. *Glia*. (2015) 63:229–41. doi: 10.1002/glia.22746

10. Elmentaite R, Kumasaka N, Roberts K, Fleming A, Dann E, King HW, et al. Cells of the human intestinal tract mapped across space and time. *Nature*. (2021) 597:250–5. doi: 10.1038/s41586-021-03852-1

11. Chalazonitis A, Rothman TP, Chen J, Gershon MD. Age-dependent differences in the effects of GDNF and NT-3 on the development of neurons and glia from neural crest-derived precursors immunoselected from the fetal rat gut: expression of GFRalpha-1 in vitro and in vivo. *Dev Biol*. (1998) 204:385–406. doi: 10.1006/dbio.1998.9090

12. Boesmans W, Nash A, Tasnady KR, Yang W, Stamp LA, Hao MM. Development, diversity, and neurogenic capacity of enteric glia. *Front Cell Dev Biol*. (2021) 9:775102. doi: 10.3389/fcell.2021.775102

13. Hanani M, Reichenbach A. Morphology of horseradish peroxidase (HRP)-injected glial cells in the myenteric plexus of the guinea-pig. *Cell Tissue Res*. (1994) 278:153–60. doi: 10.1007/BF00305787

14. Dharshika C, Gulbransen BD. Enteric Neuromics: how high-throughput "omics" deepens our understanding of enteric nervous system genetic architecture. *Cell Mol Gastroenterol Hepatol*. (2023) 15:487–504. doi: 10.1016/j.jcmgh.2022.10.019

15. Timmermans JP. The autonomic nervous system from a morphofunctional perspective: historical overview and current concepts over the last two centuries highlighting contributions from Eastern Europe. *Anat Rec (Hoboken)*. (2023) 306:2222–9. doi: 10.1002/ar.25169

16. Sharkey KA, Mawe GM. The enteric nervous system. *Physiol Rev*. (2023) 103:1487–564. doi: 10.1152/physrev.00018.2022

17. Baghdadi MB, Ayyaz A, Coquenlorge S, Chu B, Kumar S, Streutker C, et al. Enteric glial cell heterogeneity regulates intestinal stem cell niches. *Cell Stem Cell*. (2022) 29:86–100 e6. doi: 10.1016/j.stem.2021.10.004

18. Cheng X, Voss U, Ekblad E. Tuft cells: distribution and connections with nerves and endocrine cells in mouse intestine. *Exp Cell Res*. (2018) 369:105–11. doi: 10.1016/j.yexcr.2018.05.011

19. Zihni C, Mills C, Matter K, Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. *Nat Rev Mol Cell Biol*. (2016) 17:564–80. doi: 10.1038/nrm.2016.80

20. Otani T, Furuse M. Tight junction structure and function revisited: (trends in cell biology 30, 805-817, 2020). *Trends Cell Biol*. (2020) 30:1014. doi: 10.1016/j.tcb.2020.10.001

21. Neunlist M, Aubert P, Bonnaud S, Van Landeghem L, Coron E, Wedel T, et al. Enteric glia inhibit intestinal epithelial cell proliferation partly through a TGF-beta1-dependent pathway. *Am J Physiol Gastrointest Liver Physiol*. (2007) 292:G231–41. doi: 10.1152/ajpgi.00276.2005

22. Bush TG, Savidge TC, Freeman TC, Cox HJ, Campbell EA, Mucke L, et al. Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. *Cells*. (1998) 93:189–201. doi: 10.1016/S0092-8674(00)81571-8

23. Savidge TC, Newman P, Pothoulakis C, Ruhl A, Neunlist M, Bourreille A, et al. Enteric glia regulate intestinal barrier function and inflammation via release of S-nitrosoglutathione. *Gastroenterology*. (2007) 132:1344–58. doi: 10.1053/j.gastro.2007.01.051

24. Flamant M, Aubert P, Rolli-Derkinderen M, Bourreille A, Neunlist MR, Mahe MM, et al. Enteric glia protect against *Shigella flexneri* invasion in intestinal epithelial cells: a role for S-nitrosoglutathione. *Gut*. (2011) 60:473–84. doi: 10.1136/gut.2010.229237

25. Cheadle GA, Costantini TW, Lopez N, Bansal V, Eliceiri BP, Coimbra R. Enteric glia cells attenuate cytomix-induced intestinal epithelial barrier breakdown. *PLoS One*. (2013) 8:e69042. doi: 10.1371/journal.pone.0069042

26. Van Landeghem L, Chevalier J, Mahe MM, Wedel T, Urvil P, Derkinderen P, et al. Enteric glia promote intestinal mucosal healing via activation of focal adhesion kinase and release of proEGF. *Am J Physiol Gastrointest Liver Physiol*. (2011) 300:G976–87. doi: 10.1152/ajpgi.00427.2010

27. Rao M, Rastelli D, Dong L, Chiu S, Setlik W, Gershon MD, et al. Enteric glia regulate gastrointestinal motility but are not required for maintenance of the epithelium in mice. *Gastroenterology*. (2017) 153:1068–81 e7. doi: 10.1053/j.gastro.2017.07.002

28. Yuan R, Bhattacharya N, Kenkel JA, Shen J, DiMaio MA, Bagchi S, et al. Enteric glia play a critical role in promoting the development of colorectal Cancer. *Front Oncol*. (2020) 10:595892. doi: 10.3389/fonc.2020.595892

29. Rao M, Nelms BD, Dong L, Salinas-Rios V, Rutlin M, Gershon MD, et al. Enteric glia express proteolipid protein 1 and are a transcriptionally unique population of glia in the mammalian nervous system. *Glia*. (2015) 63:2040–57. doi: 10.1002/glia.22876

30. Liu GX, Yang YX, Yan J, Zhang T, Zou YP, Huang XL, et al. Glial-derived neurotrophic factor reduces inflammation and improves delayed colonic transit in rat

models of dextran sulfate sodium-induced colitis. *Int Immunopharmacol*. (2014) 19:145–52. doi: 10.1016/j.intimp.2014.01.008

31. Xiao W, Wang W, Chen W, Sun L, Li X, Zhang C, et al. GDNF is involved in the barrier-inducing effect of enteric glial cells on intestinal epithelial cells under acute ischemia reperfusion stimulation. *Mol Neurobiol*. (2014) 50:274–89. doi: 10.1007/s12035-014-8730-9

32. Meir M, Flemming S, Burkard N, Bergauer L, Metzger M, Germer CT, et al. Glial cell line-derived neurotrophic factor promotes barrier maturation and wound healing in intestinal epithelial cells in vitro. *Am J Physiol Gastrointest Liver Physiol*. (2015) 309:G613–24. doi: 10.1152/ajpgi.00357.2014

33. Bauman BD, Meng J, Zhang L, Louiselle A, Zheng E, Banerjee S, et al. Enteric glial-mediated enhancement of intestinal barrier integrity is compromised by morphine. *J Surg Res*. (2017) 219:214–21. doi: 10.1016/j.jss.2017.05.099

34. Meir M, Burkard N, Ungewiss H, Diefenbacher M, Flemming S, Kannapin F, et al. Neurotrophic factor GDNF regulates intestinal barrier function in inflammatory bowel disease. *J Clin Invest*. (2019) 129:2824–40. doi: 10.1172/JCI120261

35. Meir M, Kannapin F, Diefenbacher M, Ghoreishi Y, Kollmann C, Flemming S, et al. Intestinal epithelial barrier maturation by enteric glial cells is GDNF-dependent. *Int J Mol Sci*. (2021) 22. doi: 10.3390/ijms22041887

36. Smillie CS, Biton M, Ordovas-Montanes J, Sullivan KM, Burgin G, Graham DB, et al. Intra- and inter-cellular rewiring of the human Colon during ulcerative colitis. *Cells*. (2019) 178:714–30 e22. doi: 10.1016/j.cell.2019.06.029

37. Bach-Ngohou K, Mahe MM, Aubert P, Abdo H, Boni S, Bourreille A, et al. Enteric glia modulate epithelial cell proliferation and differentiation through 15-deoxy-12,14-prostaglandin J2. *J Physiol*. (2010) 588:2533–44. doi: 10.1113/jphysiol.2010.188409

38. Xiao WD, Chen W, Sun LH, Wang WS, Zhou SW, Yang H. The protective effect of enteric glial cells on intestinal epithelial barrier function is enhanced by inhibiting inducible nitric oxide synthase activity under lipopolysaccharide stimulation. *Mol Cell Neurosci*. (2011) 46:527–34. doi: 10.1016/j.mcn.2010.12.007

39. MacEachern SJ, Patel BA, Keenan CM, Dicay M, Chapman K, McCafferty DM, et al. Inhibiting inducible nitric oxide synthase in enteric glia restores Electrogenic ion transport in mice with colitis. *Gastroenterology*. (2015) 149:445–55.e3. doi: 10.1053/j.gastro.2015.04.007

40. Soret R, Coquenlorge S, Cossais F, Meurette G, Rolli-Derkinderen M, Neunlist M. Characterization of human, mouse, and rat cultures of enteric glial cells and their effect on intestinal epithelial cells. *Neurogastroenterol Motil*. (2013) 25:e755–64. doi: 10.1111/nmo.12200

41. Grubisic V, Bali V, Fried DE, Eltzschig HK, Robson SC, Mazei-Robison MS, et al. Enteric glial adenosine 2B receptor signaling mediates persistent epithelial barrier dysfunction following acute DSS colitis. *Mucosal Immunol*. (2022) 15:964–76. doi: 10.1038/s41385-022-00550-7

42. Grubisic V, Gulbransen BD. Enteric glial activity regulates secretomotor function in the mouse colon but does not acutely affect gut permeability. *J Physiol*. (2017) 595:3409–24. doi: 10.1113/JP273492

43. Koretz K, Momburg F, Otto HF, Moller P. Sequential induction of MHC antigens on autochthonous cells of ileum affected by Crohn's disease. *Am J Pathol*. (1987) 129:493–502.

44. Geboes K, Rutgeerts P, Ectors N, Mebis J, Penninckx F, Vantrappen G, et al. Major histocompatibility class II expression on the small intestinal nervous system in Crohn's disease. *Gastroenterology*. (1992) 103:439–47. doi: 10.1016/0016-5085(92)90832-J

45. Turco F, Sarnelli G, Cirillo C, Palumbo I, De Giorgi F, D'Alessandro A, et al. Enteroglial-derived S100B protein integrates bacteria-induced toll-like receptor signalling in human enteric glial cells. *Gut*. (2014) 63:105–15. doi: 10.1136/gutjnl-2012-302090

46. Chow AK, Grubisic V, Gulbransen BD. Enteric glia regulate lymphocyte activation via autophagy-mediated MHC-II expression. *Cell Mol Gastroenterol Hepatol*. (2021) 12:1215–37. doi: 10.1016/j.jcmgh.2021.06.008

47. Progatzky F, Shapiro M, Chng SH, Garcia-Cassani B, Classon CH, Sevgi S, et al. Regulation of intestinal immunity and tissue repair by enteric glia. *Nature*. (2021) 599:125–30. doi: 10.1038/s41586-021-04006-z

48. Grubisic V, McClain JL, Fried DE, Grants I, Rajasekhar P, Csizmadia E, et al. Enteric glia modulate macrophage phenotype and visceral sensitivity following inflammation. *Cell Rep*. (2020) 32:108100. doi: 10.1016/j.celrep.2020.108100

49. Stakenborg M, Abdurahiman S, De Simone V, Goverse G, Stakenborg N, van Baarle L, et al. Enteric glial cells favor accumulation of anti-inflammatory macrophages during the resolution of muscularis inflammation. *Mucosal Immunol*. (2022) 15:1296–308. doi: 10.1038/s41385-022-00563-2

50. Dora D, Ferenczi S, Stavely R, Toth VE, Varga ZV, Kovacs T, et al. Evidence of a myenteric plexus barrier and its macrophage-dependent degradation during murine colitis: implications in enteric Neuroinflammation. *Cell Mol Gastroenterol Hepatol*. (2021) 12:1617–41. doi: 10.1016/j.jcmgh.2021.07.003

51. Ruhl A, Franzke S, Collins SM, Stremmel W. Interleukin-6 expression and regulation in rat enteric glial cells. *Am J Physiol Gastrointest Liver Physiol*. (2001) 280:G1163–71. doi: 10.1152/ajpgi.2001.280.6.G1163

52. Wang L, Srinivasan S, Theiss AL, Merlin D, Sitaraman SV. Interleukin-6 induces keratin expression in intestinal epithelial cells: potential role of keratin-8 in interleukin-6-induced barrier function alterations. *J Biol Chem*. (2007) 282:8219–27. doi: 10.1074/jbc.M604068200

53. Schneider R, Leven P, Mallesh S, Breßer M, Schneider L, Mazzotta E, et al. IL-1-dependent enteric gliosis guides intestinal inflammation and dysmotility and modulates macrophage function. *Commun Biol*. (2022). 5:811. doi: 10.1038/s42003-022-03772-4

54. Ibiza S, Garcia-Cassani B, Ribeiro H, Carvalho T, Almeida L, Marques R, et al. Glial-cell-derived neuroregulators control type 3 innate lymphoid cells and gut defence. *Nature*. (2016) 535:440–3. doi: 10.1038/nature18644

55. Bohorquez DV, Shahid RA, Erdmann A, Kreger AM, Wang Y, Calakos N, et al. Neuroepithelial circuit formed by innervation of sensory enteroendocrine cells. *J Clin Invest*. (2015) 125:782–6. doi: 10.1172/JCI78361

56. Bohorquez DV, Liddle RA. Axon-like basal processes in enteroendocrine cells: characteristics and potential targets. *Clin Transl Sci*. (2011) 4:387–91. doi: 10.1111/j.1752-8062.2011.00299.x

57. Cameron HL, Perdue MH. Muscarinic acetylcholine receptor activation increases transcellular transport of macromolecules across mouse and human intestinal epithelium in vitro. *Neurogastroenterol Motil*. (2007) 19:47–56. doi: 10.1111/j.1365-2982.2006.00845.x

58. Greenwood B, Mantle M. Mucin and protein release in the rabbit jejunum: effects of bethanechol and vagal nerve stimulation. *Gastroenterology*. (1992) 103:496–505. doi: 10.1016/0016-5085(92)90839-Q

59. Costantini TW, Krzyzaniak M, Cheadle GA, Putnam JG, Hageny AM, Lopez N, et al. Targeting alpha-7 nicotinic acetylcholine receptor in the enteric nervous system: a cholinergic agonist prevents gut barrier failure after severe burn injury. *Am J Pathol*. (2012) 181:478–86. doi: 10.1016/j.ajpath.2012.04.005

60. Yajima T, Inoue R, Matsumoto M, Yajima M. Non-neuronal release of ACh plays a key role in secretory response to luminal propionate in rat colon. *J Physiol*. (2011) 589:953–62. doi: 10.1113/jphysiol.2010.199976

61. Hirota CL, McKay DM. Cholinergic regulation of epithelial ion transport in the mammalian intestine. *Br J Pharmacol*. (2006) 149:463–79. doi: 10.1038/sj.bjp.0706889

62. Schutz B, Jurastow I, Bader S, Ringer C, von Engelhardt J, Chubanov V, et al. Chemical coding and chemosensory properties of cholinergic brush cells in the mouse gastrointestinal and biliary tract. *Front Physiol*. (2015) 6:87. doi: 10.3389/fphys.2015.00087

63. Yang D, Jacobson A, Meerschaert KA, Sifakis JJ, Wu M, Chen X, et al. Nociceptor neurons direct goblet cells via a CGRP-RAMP1 axis to drive mucus production and gut barrier protection. *Cells*. (2022) 185:4190–205 e25. doi: 10.1016/j.cell.2022.09.024

64. Neunlist M, Toumi F, Oreschkova T, Denis M, Leborgne J, Laboisse CL, et al. Human ENS regulates the intestinal epithelial barrier permeability and a tight junction-associated protein ZO-1 via VIPergic pathways. *Am J Physiol Gastrointest Liver Physiol*. (2003) 285:G1028–36. doi: 10.1152/ajpgi.00066.2003

65. Moro F, Levenez F, Durual S, Plaisancie P, Thim L, Giraud AS, et al. Secretion of the trefoil factor TFF3 from the isolated vascularly perfused rat colon. *Regul Pept*. (2001) 101:35–41. doi: 10.1016/s0167-0115(01)00257-9

66. Farack UM, Reiter J, Gross M, Moroder L, Wunsch E, Loeschke K. Influence of vasoactive intestinal peptide, secretin, and Ala4, Val5-secretin on the net movements of electrolytes, fluid, and mucus in the rat colon in vivo. *Scand J Gastroenterol Suppl*. (1987) 139:32–6. doi: 10.3109/00365528709089772

67. Schwartz CJ, Kimberg DV, Sheerin HE, Field M, Said SI. Vasoactive intestinal peptide stimulation of adenylate cyclase and active electrolyte secretion in intestinal mucosa. *J Clin Invest*. (1974) 54:536–44. doi: 10.1172/JCI107790

68. Toumi F, Neunlist M, Cassagnau E, Parois S, Laboisse CL, Galmiche JP, et al. Human submucosal neurones regulate intestinal epithelial cell proliferation: evidence from a novel co-culture model. *Neurogastroenterol Motil*. (2003) 15:239–42. doi: 10.1046/j.1365-2982.2003.00409.x

69. Hwang DY, Kim S, Hong HS. Substance-P ameliorates dextran sodium sulfate-induced intestinal damage by preserving tissue barrier function. *Tissue Eng Regen Med*. (2018) 15:63–73. doi: 10.1007/s13770-017-0085-7

70. Jarret A, Jackson R, Duizer C, Healy ME, Zhao J, Rone JM, et al. Enteric nervous system-derived IL-18 orchestrates mucosal barrier immunity. *Cells*. (2020) 180:813–4. doi: 10.1016/j.cell.2020.02.004

71. Lei C, Sun R, Xu G, Tan Y, Feng W, McClain CJ, et al. Enteric VIP-producing neurons maintain gut microbiota homeostasis through regulating epithelium fucosylation. *Cell Host Microbe*. (2022) 30:1417–34 e8. doi: 10.1016/j.chom.2022.09.001

72. Wu X, Conlin VS, Morampudi V, Ryz NR, Nasser Y, Bhinder G, et al. Vasoactive intestinal polypeptide promotes intestinal barrier homeostasis and protection against colitis in mice. *PLoS One*. (2015) 10:e0125225. doi: 10.1371/journal.pone.0125225

73. Gabanyi I, Muller PA, Feighery L, Oliveira TY, Costa-Pinto FA, Mucida D. Neuro-immune interactions drive tissue programming in intestinal macrophages. *Cells*. (2016) 164:378–91. doi: 10.1016/j.cell.2015.12.023

74. Boudard F, Bastide M. Inhibition of mouse T-cell proliferation by CGRP and VIP: effects of these neuropeptides on IL-2 production and cAMP synthesis. *J Neurosci Res*. (1991) 29:29–41. doi: 10.1002/jnr.490290104

75. Reinshagen M, Flamig G, Ernst S, Geerling I, Wong H, Walsh JH, et al. Calcitonin gene-related peptide mediates the protective effect of sensory nerves in a model of colonic injury. *J Pharmacol Exp Ther*. (1998) 286:657–61.

76. McDermott JR, Leslie FC, D'Amato M, Thompson DG, Grencis RK, McLaughlin JT. Immune control of food intake: enteroendocrine cells are regulated by CD4+ T lymphocytes during small intestinal inflammation. *Gut*. (2006) 55:492–7. doi: 10.1136/gut.2005.081752

77. Furness JB. The enteric nervous system and neurogastroenterology. *Nat Rev Gastroenterol Hepatol*. (2012) 9:286–94. doi: 10.1038/nrgastro.2012.32

78. Chandrasekharan B, Jeppsson S, Pienkowski S, Belsham DD, Sitaraman SV, Merlin D, et al. Tumor necrosis factor-neuropeptide Y cross talk regulates inflammation, epithelial barrier functions, and colonic motility. *Inflamm Bowel Dis*. (2013) 19:2535–46. doi: 10.1097/01.MIB.0000437042.59208.9f

79. Chandrasekharan B, Bala V, Kolachala VL, Vijay-Kumar M, Jones D, Gewirtz AT, et al. Targeted deletion of neuropeptide Y (NPY) modulates experimental colitis. *PLoS One*. (2008) 3:e3304. doi: 10.1371/journal.pone.0003304

80. Wheway J, Mackay CR, Newton RA, Sainsbury A, Boey D, Herzog H, et al. A fundamental bimodal role for neuropeptide Y1 receptor in the immune system. *J Exp Med*. (2005) 202:1527–38. doi: 10.1084/jem.20051971

81. Klose CSN, Mahlakoiv T, Moeller JB, Rankin LC, Flamar AL, Kabata H, et al. The neuropeptide neuromedin U stimulates innate lymphoid cells and type 2 inflammation. *Nature*. (2017) 549:282–6. doi: 10.1038/nature23676

82. Zhao A, McDermott J, Urban JF Jr, Gause W, Madden KB, Yeung KA, et al. Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves. *J Immunol*. (2003) 171:948–54. doi: 10.4049/jimmunol.171.2.948

83. Nagashima H, Mahlakoiv T, Shih HY, Davis FP, Meylan F, Huang Y, et al. Neuropeptide CGRP limits group 2 innate lymphoid cell responses and constrains type 2 inflammation. *Immunity*. (2019) 51:682–95 e6. doi: 10.1016/j.immuni.2019.06.009

84. Xu H, Ding J, Porter CBM, Wallrapp A, Tabaka M, Ma S, et al. Transcriptional atlas of intestinal immune cells reveals that neuropeptide alpha-CGRP modulates group 2 innate lymphoid cell responses. *Immunity*. (2019) 51:696–708 e9. doi: 10.1016/j.immuni.2019.09.004

85. Wallrapp A, Burkett PR, Riesenfeld SJ, Kim SJ, Christian E, Abdulnour RE, et al. Calcitonin gene-related peptide negatively regulates Alarmin-driven type 2 innate lymphoid cell responses. *Immunity*. (2019) 51:709–23 e6. doi: 10.1016/j.immuni.2019.09.005

86. Talbot J, Hahn P, Kroehling L, Nguyen H, Li D, Littman DR. Feeding-dependent VIP neuron-ILC3 circuit regulates the intestinal barrier. *Nature*. (2020) 579:575–80. doi: 10.1038/s41586-020-2039-9

87. Vu JP, Million M, Larauche M, Luong L, Norris J, Waschek JA, et al. Inhibition of vasoactive intestinal polypeptide (VIP) induces resistance to dextran sodium sulfate (DSS)-induced colitis in mice. *J Mol Neurosci*. (2014) 52:37–47. doi: 10.1007/s12031-013-0205-3

88. Neunlist M, Aubert P, Toquet C, Oreshkova T, Barouk J, Lehur PA, et al. Changes in chemical coding of myenteric neurones in ulcerative colitis. *Gut*. (2003) 52:84–90. doi: 10.1136/gut.52.1.84

89. Moynes DM, Lucas GH, Beyak MJ, Lomax AE. Effects of inflammation on the innervation of the colon. *Toxicol Pathol*. (2014) 42:111–7. doi: 10.1177/0192623313505929

90. Caruso ML, Di Pinto F, Ignazzi A, Coletta S, Valentini AM, Cavalcanti E, et al. Increased nerve twigs in small intestinal mucosa with programmed cell death-ligand 1 and somatostatin receptor type 2A expression in recurrent Crohn disease: a case report. *Medicine (Baltimore)*. (2018) 97:e13492. doi: 10.1097/MD.0000000000013492

91. Tandon P, Malhi G, Abdali D, Pogue E, Marshall JK, de Buck van Overstraeten A, et al. Active margins, plexitis, and granulomas increase postoperative Crohn's recurrence: systematic review and Meta-analysis. *Clin Gastroenterol Hepatol*. (2021) 19:451–62. doi: 10.1016/j.cgh.2020.08.014

92. Lakhan SE, Kirchgessner A. Neuroinflammation in inflammatory bowel disease. *J Neuroinflammation*. (2010) 7:37. doi: 10.1186/1742-2094-7-37

93. Nakao S, Itabashi M, Yamamoto T, Okamoto T. Predictive value of myenteric and submucosal plexitis for postoperative Crohn's disease recurrence. *J Anus Rectum Colon*. (2017) 1:56–64. doi: 10.23922/jarc.2016-006

94. Liu Q, Zhang X, Ko HM, Stocker D, Ellman J, Chen J, et al. Constrictive and hypertrophic strictures in Ileal Crohn's disease. *Clin Gastroenterol Hepatol*. (2022) 20:e1292–304. doi: 10.1016/j.cgh.2021.08.012

95. Ferrante M, de Hertogh G, Hlavaty T, D'Haens G, Penninckx F, D'Hoore A, et al. The value of myenteric plexitis to predict early postoperative Crohn's disease recurrence. *Gastroenterology*. (2006) 130:1595–606. doi: 10.1053/j.gastro.2006.02.025

96. Sokol H, Polin V, Lavergne-Slove A, Panis Y, Treton X, Dray X, et al. Plexitis as a predictive factor of early postoperative clinical recurrence in Crohn's disease. *Gut*. (2009) 58:1218–25. doi: 10.1136/gut.2009.177782

97. Biskou O, Meira De-Faria F, Walter SM, Winberg ME, Haapaniemi S, Myrelid P, et al. Increased numbers of enteric glial cells in the Peyer's patches and enhanced intestinal permeability by glial cell mediators in patients with Ileal Crohn's disease. *Cells*. (2022) 11. doi: 10.3390/cells11030335

98. Pochard C, Coquenlorge S, Jaulin J, Cenac N, Vergnolle N, Meurette G, et al. Defects in 15-HETE production and control of epithelial permeability by human enteric glial cells from patients with Crohn's disease. *Gastroenterology*. (2016) 150:168–80. doi: 10.1053/j.gastro.2015.09.038

99. El-Salhy M, Hausken T. The role of the neuropeptide Y (NPY) family in the pathophysiology of inflammatory bowel disease (IBD). *Neuropeptides*. (2016) 55:137–44. doi: 10.1016/j.npep.2015.09.005

100. Chandrasekharan B, Boyer D, Owens JA, Wolfarth AA, Saeedi BJ, Dhere T, et al. Intracolonic neuropeptide Y Y1 receptor inhibition attenuates intestinal inflammation in murine colitis and cytokine release in IBD biopsies. *Inflamm Bowel Dis*. (2022) 28:502–13. doi: 10.1093/ibd/izab243

frontiers | Frontiers in Cell and Developmental Biology

TYPE Review
PUBLISHED 19 September 2023
DOI 10.3389/fcell.2023.1249317

OPEN ACCESS

EDITED BY
Tadahiro Nagaoka,
Fujita Health University, Japan

REVIEWED BY
Deborah Lannigan,
Vanderbilt University, United States
Yasuko Ono,
Tokyo Metropolitan Institute of Medical Science, Japan
Cheguo Cai,
Wuhan University, China

*CORRESPONDENCE
Elena R. García-Trevijano,
elena.ruiz@uv.es

RECEIVED 28 June 2023
ACCEPTED 05 September 2023
PUBLISHED 19 September 2023

CITATION
García-Trevijano ER, Ortiz-Zapater E, Gimeno A, Viña JR and Zaragozá R (2023), Calpains, the proteases of two faces controlling the epithelial homeostasis in mammary gland.
Front. Cell Dev. Biol. 11:1249317.
doi: 10.3389/fcell.2023.1249317

Calpains, the proteases of two faces controlling the epithelial homeostasis in mammary gland

Elena R. García-Trevijano[1,2]*, Elena Ortiz-Zapater[1,2], Amparo Gimeno[3], Juan R. Viña[1,2] and Rosa Zaragozá[2,3]

[1]Department of Biochemistry and Molecular Biology, Universitat de Valencia, Valencia, Spain, [2]INLIVA Biomedical Research Institute, Valencia, Spain, [3]Department of Anatomy and Human Embryology, Universitat de Valencia, Valencia, Spain

Calpain-1 and calpain-2 are calcium-dependent Cys-proteases ubiquitously expressed in mammalian tissues with a processive, rather than degradative activity. They are crucial for physiological mammary gland homeostasis as well as for breast cancer progression. A growing number of evidences indicate that their pleiotropic functions depend on the cell type, tissue and biological context where they are expressed or dysregulated. This review considers these standpoints to cover the paradoxical role of calpain-1 and -2 in the mammary tissue either, under the physiological conditions of the postlactational mammary gland regression or the pathological context of breast cancer. The role of both calpains will be examined and discussed in both conditions, followed by a brief snapshot on the present and future challenges for calpains, the two-gateway proteases towards tissue homeostasis or tumor development.

KEYWORDS

breast cancer, involution, apoptosis, CAPN, adhesion, nucleus, differentiation

Introduction

Calpains are a family of calcium-dependent intracellular Cys-proteases involved in a number of different physiological and pathological processes. Since the first description of calpain in 1964, up-to-date 15 different genes have been described, encoding CAPN1 to 3 and 5 to 16, which are the large catalytic subunit of different isoforms. In addition, two small regulatory subunits with non-catalytic activity, *CAPNS1* and *CAPNS2* have been also identified. The different isoforms have been classified as classical and non-classical calpains, according to the presence or absence of a penta-EF (PEF) hand domain at the C-terminal domain of large subunits and the N-terminal domain of small regulatory subunits. The presence of PEF in classical calpains allows the heterodimerization of both, catalytic and regulatory subunits (for structural review see Sorimachi et al., 2011; Campbell and Davies, 2012; Briz and Baudry, 2017; Nian and Ma, 2021).

Although most of these isoforms (CAPN1, 2, 5, 7, 10 and 13–16) are ubiquitously distributed in mammalian tissues, classical calpain-1 and calpain-2 were the first to be identified and the isoforms best studied in different tissues and pathological conditions. These two isoforms share high sequence homology (Sorimachi et al., 2011) and are known as conventional calpains. Both, calpain-1 and calpain-2 (originally known as μ-calpain and m-calpain, respectively) are formed by heterodimers, consisting of a ~80 KDa large catalytic subunit (CAPN1 or CAPN2) and a common ~28 KDa small regulatory subunit CAPNS1 (also, CAPN4), which provides stability to the enzyme (Nian and Ma, 2021).

Conventional calpains are processing rather than degradative enzymes (Nian and Ma, 2021). Indeed, in contrast to other proteases calpains are intracellular proteases that exhibit a limited proteolytic activity on their substrates, acting as regulatory proteases. The end-products of calpains may have functions, protein-protein associations or subcellular distributions different from the corresponding unprocessed substrates (Ono and Sorimachi, 2012; Raimondi et al., 2016; Briz and Baudry, 2017; Miyazaki et al., 2021). However, the governing rules of substrate recognition by calpains are still elusive. Calpains do not recognize a specific sequence in the primary structure of their substrates or a post-translational modification (Sorimachi et al., 2011). Instead, the overall three-dimensional conformation or higher order structures in their substrates have been pointed as the main determinants for substrate recognition (Pariat et al., 2000; Ono and Sorimachi, 2012). Hundreds of substrates have been described as *in vitro* targets of calpain activity, which does not indicate they are necessarily processed by calpains *in vivo*. All in all, calpain-mediated cleavage has been observed in cytoskeleton proteins, membrane-associated proteins, receptors/channels, scaffolding/anchoring proteins, and protein kinases and phosphatases in a variety of tissues and cell types (Croall and Ersfeld, 2007; Ono and Sorimachi, 2012).

Earlier works on the role of calpain system were focused on the activity and *in vitro* regulation of conventional calpains. Since then, several mechanisms have been described to modulate (either activating or inhibiting) their enzymatic activity, such as Ca^{2+} concentration, phosphorylation by ERK1/2 or PKA, binding to phospholipids and acyl-CoA-binding protein, or even to its endogenous inhibitor, calpastatin (CAST) (Ono and Sorimachi, 2012; Nian and Ma, 2021). However, the identity of the calpains end-products and consequently, the isoform-specific functions of both proteases in most tissues is still poorly understood. Are they pro-apoptotic or pro-survival proteases? Do they promote cell proliferation or cell differentiation? Is an isoform limiting the activity of the other or they can compensate each other? Although it is generally accepted that they are not redundant enzymes and that the target-specificity of each isoform depends on their subcellular distribution (Shao et al., 2006; Raynaud et al., 2008; Leloup et al., 2010; Kosenko et al., 2011; Arnandis et al., 2012; Arnandis et al., 2014; Rodríguez-Fernández, 2019; Telechea-Fernández et al., 2018; Rodríguez-Fernández et al., 2021) or the signaling pathways in which they are involved (Wang et al., 2020), a number of reports indicate that the cell type and biological context need to be considered when trying to answer those questions.

Herein we will review the role of conventional calpains in the context of mammary tissue, either under the physiological conditions of the pregnancy/lactation cycle or the pathological breast cancer. Interestingly, these two biological processes share more key regulatory proteins than could be thought at first glance. In fact, much of the molecular signaling regulating the mammary gland homeostasis during the pregnancy/lactation cycle was first identified as oncogenic drivers of breast cancer.

Janus, the classical god of changes and transformations, is represented with two faces symbolizing the uncertainty of what is to come. Likewise, calpain-1 and calpain-2 could be considered the proteases of two faces controlling physiological mammary gland homeostasis, but also promoting breast tumor progression. In this review we will summarize the main findings related to the regulatory and sometimes contradictory role of conventional calpains in these two biological contexts of mammary gland, focused in cell adhesion, cell death and cell proliferation/differentiation. Unfortunately, although an increasing number of reports describe the important functions and effects of calpain activity in different tissues including mammary gland, the specific regulation of calpain distribution and activity to modulate these processes in mammary tissue remains unknown.

Expression of conventional calpains in the mammary tissue

The pregnancy/lactation cycle

The mammary gland is a complex and specialized tissue whose main function is to synthetize milk, providing nutrition and immunological protection to mammalian offspring (Ward and German, 2004). It is a compound tubule-alveolar gland embedded within an irregular connective tissue known as mammary fat pad (Figure 1A). The glandular epithelial compartment shows two different cell populations, epithelial and myoepithelial, lining ducts, and alveoli. The inner layer of epithelial cells are luminal secretory and ductal cells, undergoing functional differentiation during pregnancy to form the milk-producing secretory acini. The outer myoepithelial/basal cells encasing the luminal cells, are contractile and participate in the delivery of milk in response to oxytocin stimulation. This basal epithelium also harbors stem and progenitor cells, which form both luminal and myoepithelial cells/layer. Finally, the basement membrane separates this epithelial tissue from the surrounding stroma, mainly composed of adipocytes, fibroblasts, macrophages, and other immune and endothelial cells (Stewart et al., 2020; Watson and Khaled, 2020; Biswas et al., 2022) (Figure 1B).

Mammary gland is a unique organ mostly developed after birth, with just the primordia of the gland formed early in embryogenesis. This highly dynamic organ undergoes a series of physiological changes in morphology and function throughout life from menarche to menopause, during each menstrual and pregnancy/lactation cycles (Biswas et al., 2022). At menarche, a rudimental mammary gland is expanded to invade the subjacent mesenchymal tissue creating a more extensive ductal network (Watson and Khaled, 2020). At puberty, the increase in breast size is mainly caused by the accumulation of adipose tissue within the gland. Terminal functional differentiation is reached with the development of alveoli and the synthesis of specific milk proteins late in pregnancy and during lactation. At this point, the mammary gland consists almost entirely of secretory epithelium forming alveolar structures with lumens full of milk fat globules and milk. After weaning, at the end of lactation, there is an extensive regression of mammary tissue in a process known as involution (Wang and Scherer, 2019; Biswas et al., 2022) (Figure 1C).

The process of postlactational involution is finely orchestrated and takes place in two phases in mice. The first stage lasting for 48 h after weaning, is driven mainly by local factors due to milk accumulation within the lumen. This phase is reversible if the suckling stimuli is recovered. Although detectable by regular Western blot, the expression levels of conventional calpains during lactation and the early onset of this

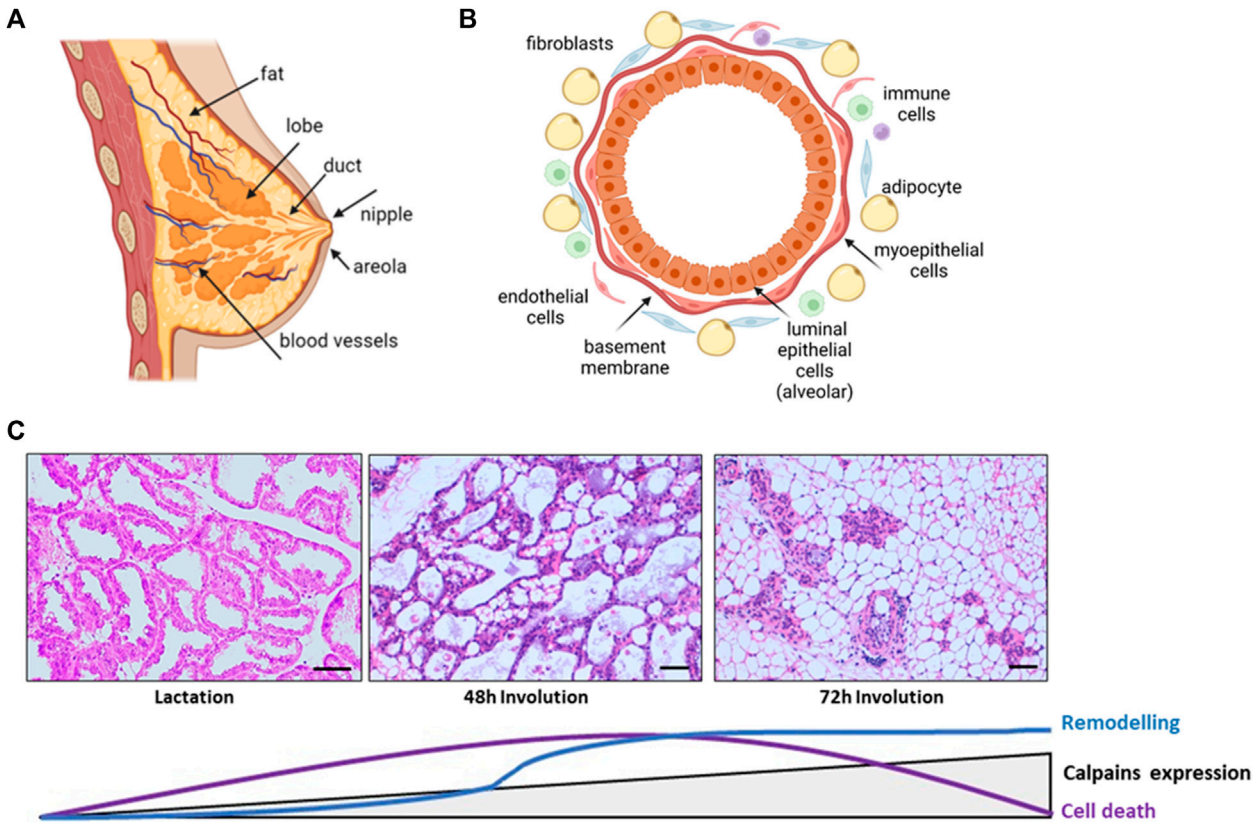


FIGURE 1
Schematic representation of the structure and morphological changes in the mammary tissue. **(A)** Normal breast anatomy **(B)** Alveolar structure of the gland. Polarized secretory epithelial cells form the lumen of the alveoli and are stretched by contractile myoepithelial cells and basement membrane. Surrounding these glandular structures, the mammary stroma is mainly formed by extracellular matrix, adipocytes, fibroblast, macrophages, and endothelial cells. **(C)** H&E stained sections of lactating and involuting mice mammary gland. The progression of epithelial cell death and remodeling is represented together with the levels of calpains expression. Lactating tissue shows the glandular structure, with several ducts and alveoli tightly closed together, surrounded by extracellular matrix, blood vessels and residual adipocytes. At 48 h weaning, alveoli start to collapse and detached epithelial cells are shed into the lumen. Collapse of alveoli progresses and at 72 h of involution the glandular structures are reduced and adipocytes repopulate the mammary fat pad. Scale bar: 250 μm.

phase remain low and constant. Upon weaning, there is a decrease of systemic lactogenic hormones, epithelial cells of the lobulo-alveolar compartment rapidly undergo cell death with increased caspase 3 activation, breakdown of tight junctions, shedding of alveolar dead cells into the lumen and stimulation of a pro-inflammatory environment. A controlled flow of macrophages and other immune cells to the mammary gland would clear dead milk-secreting cells; moreover, some of the survival secretory epithelial cells become phagocytes to remove dying cells from milk (Monks et al., 2005; Atabai et al., 2007). The transcription factor NFκB has been recognized as one of the most important nodes of regulation of mammary gland involution after lactation. This factor known to modulate a number of pro-inflammatory pathways, was shown to be activated in mammary gland and to bind to *CAPN*-1 and -2 gene promoters at 48 h after weaning (Torres et al., 2011). The expression levels of conventional calpains progressively increase from the first phase of involution through the second phase, reaching their higher levels at 72 h involution (Arnandis et al., 2012; Dang et al., 2015). Mammary tissue during this second and irreversible phase is characterized by the proteolytic degradation of the basement membrane and a massive remodeling of the glandular architecture with alveolar collapse, increased number of immune cells within the stroma, and epithelial cell replacement with re-differentiated adipocytes. In the end, mammary gland returns to a pre-pregnant state in preparation for subsequent pregnancies (Landskroner-Eiger et al., 2010; Arnandis et al., 2014; Howard and Lu, 2014; Zaragozá et al., 2015).

From these data and data elsewhere, it can be inferred that all these processes during mammary gland involution must be tightly coordinated by a complex regulatory network where several proteins will play crucial functions as nodes of regulation or as effectors for the final resolution of the biological response (Zaragozá et al., 2015; Biswas et al., 2022) The role of conventional calpains as important effectors for the appropriate physiological resolution of the pregnancy/lactation cycle seems unquestionable. Consequently, dysregulation of calpain-1 and -2 is expected to have important consequences for the homeostasis of this tissue.

Breast cancer

Altered regulation of conventional calpains have been described in breast tumors or breast cancer cell lines. Indeed, calpain activity

affects several signaling pathways related to tumorigenesis (Storr et al., 2011a; Potz et al., 2016; Chen et al., 2019; Nian and Ma, 2021), including cell death/survival pathways, adhesion/migration and invasion, cell cycle control or even the function of different oncoproteins such as HER2 (Pianetti et al., 2001; Kulkarni et al., 2010; Ho et al., 2012; MacLeod et al., 2018). In addition, several studies in HER2$^+$ breast cancer cell lines have correlated calpain expression with resistance to chemotherapeutics such as trastuzumab, doxorubicin and cisplatin (Kulkarni et al., 2010; Grieve et al., 2016; Al-Bahlani et al., 2017; MacLeod et al., 2018). Nevertheless, these studies were carried out in cultured cell lines not reflecting the biological conditions found in mammary tissue where calpain expression or activity could be differentially modulated.

Some clinical studies have tried to correlate the clinical outcome of breast cancer patients with calpain protein levels. High calpain-1 protein levels correlated with poor relapse-free survival of HER2+ breast cancer patients treated with trastuzumab following adjuvant chemotherapy (Storr et al., 2011b; Pu et al., 2016). No correlation was found between calpain-2 levels and clinicopathological variables in this group of patients. However, as stated by the authors this *in vivo* study cannot be compared to previous *in vitro* studies since calpain levels in the clinical study were examined before trastuzumab treatment, calpain activity in these patients was not measured and finally, the number of patients included in the study was limited. Therefore, the potential of calpain-1 as a specific biomarker for trastuzumab resistance could not be definitively established.

Additional reports in the literature about the correlation between expression of conventional calpains and relapse-free survival in breast cancer patients result confusing and contradictory at times. Indeed, translational studies in a large cohort of triple-negative and basal-like breast cancer patients found that calpain-1 was not associated with relapse-free survival and identified calpain-2 as the isoform associated with adverse outcomes (Storr et al., 2012). Other clinical study also showed that calpain-1 was overexpressed in breast triple-negative tumors with a significant correlation to lymph node status but not with the other clinicopathological variables, recurrence-free survival, or the overall survival of patients (Al-Bahlani SM et al., 2017). In contrast, high calpain-1 expression was associated with improved disease-free survival of all patients enrolled in a clinical study including different breast cancer subtypes, although improved rates of overall survival were found in the triple negative breast cancer subgroup (Rajković-Molek et al., 2020). In summary, some reports correlate high calpain-1 expression with adverse disease outcome, some with improve disease-free survival and some others found that the levels of calpain-1 did not correlate with any clinicopathological variable. Consequently, a definitive prognostic value for calpains levels in these patients could not be demonstrated (Chen et al., 2019).

It has been argued that discrepancies among reports are most probably caused by the type of analysis and classification of breast tumor subtypes, the limited number of patients in clinical studies and comparison of data obtained *in vitro* and *in vivo* (Storr et al., 2011a; Storr et al. 2011b; Storr et al. 2012; Rajković-Molek et al., 2020; Shapovalov et al., 2022). Calpain activity, protein levels and mRNA levels do not necessarily correlate (Rodriguez-Fernández, 2019; Shapovalov et al., 2022). Importantly, most of clinical reports only study levels of calpain expression, and no data about enzymatic activity or subcellular distribution of calpains in breast cancer patients have been shown (Sorimachi et al., 2011; Storr et al., 2015; Storr et al., 2016). In that sense, early studies showed that calpain-1 is differentially expressed in the peritumoural and intratumoral area of breast cancer patients. Interestingly, only peritumoral calpain-1 expression correlated with relapse-free survival (Storr et al., 2011b). Although the meaning of this observation is unclear, it suggests a complex mechanism of calpains regulation yet to be determined. In addition, calpain activity can be modulated by a number of factors and signaling pathways which could be influenced by the type of tumor and peritumoral area (Ono and Sorimachi, 2012; Nian and Ma, 2021).

Thus, while most authors suggest a pro-tumorigenic role for calpains in cancer progression, discrepancies among reports suggest that calpains may also have anti-tumorigenic roles in different tumor subtypes, phases during cancer progression or context found in the tumoral and peritumoral tissue. In agreement with this, it has been suggested that the effect of overexpression of a specific calpain isoform on breast cancer-survival might depend on the inflammatory context of breast tumors. While high calpain-2 and low CAST expression was associated with improved survival in patients with non-inflammatory breast cancer treated with neoadjuvant chemotherapy, high calpain-1 and high CAST expression in the inflammatory group was associated with improved breast cancer survival (Storr et al., 2016).

The influence of the pro-inflammatory environment on the role of each calpain-isoform is an important aspect to consider in the pregnancy associated breast cancer risk. It has been suggested that the pro-inflammatory environment of mammary gland involution could promote tumor progression (Lim et al., 2010; Torres et al., 2011; Rauner and Kuperwasser, 2021). In addition, the immune tolerance found in mammary gland after weaning has been proposed to also contribute to the neoplastic promotion in mammary tissue (Betts et al., 2018). Many of the regulatory nodes of mammary gland involution, such as STAT3, TGF-β or NFκB, have been identified as persistently activated oncogenes or pro-inflammatory factors favoring neoplasia transformation and metastasis; a notable observation, since as mentioned above, the expression of both calpains is modulated by NFκB. A recent study shows that forced weaning induces morphological changes in the murine mammary gland after short lactation, which were not evident in the long-lactation mice (Basree et al., 2019). Mammary gland from the short-lactation mice exhibited ductal hyperplasia and squamous metaplasia at 4 months after parturition, both preneoplastic conditions for breast cancer and accordingly, a breast cancer risk factor. Moreover, a prevalence study in women showed increased breast cancer risk during the 5 years following parturition (Meier-Abt and Bentires-Alj, 2014). The same study reports that pregnancy-associated increase in breast cancer risk becomes more pronounced with increasing age at first pregnancy.

In the near future, to decipher the potential relationship between calpains dysfunction and pregnancy-associated breast cancer risk, or the isoform-specific role of conventional calpains in breast cancer progression, the subcellular distribution, the cell type and the tissue context need to be considered.

TABLE 1 Direct targets of calpain activity in mammary gland.

Target	Isoform	Experimental model	References
Cell Adhesion and cytoskeleton pathways			
Cortactin	CAPN1	MDA-MB-231	Hoskin et al. (2015)
	CAPN2	MTLn3	Cortesio et al. (2008)
E-cadherin	CAPN2	Mouse mammary gland	Rodríguez-Fernández (2019)
	CAPN1	MCF-7, BT-474	Rodríguez-Fernández (2019)
	n.s.	MCF-7	Rios-Doria et al. (2003)
Ezrin	CAPN2	TS/A	Lee and Shyur (2012)
FAK	CAPN1	MDA-MB-231	Hoskin et al. (2015)
	CAPN2	MCF-7	Li et al. (2017)
	n.s	BT20, MDA-MB-231	Xu et al. (2010)
	CAPN2	TS/A	Lee and Shyur (2012)
	CAPN2	MCF-7, T47D	Libertini et al. (2005)
	CAPN1	MCF-7	Hou et al. (2012)
Fodrin	n.s.	MCF-7	Sareen et al. (2007)
	CAPN1	MDA-MB-231	Al-Bahlani et al. (2017)
α-Spectrin	n.s.	MCF-7	Rios-Doria et al., 2003, 2004
LIMK1	CAPN2	MDA-MB-231	Rodríguez-Fernández et al. (2021)
Paxillin	n.s	BT20, MDA-MB-231	Xu et al. (2010)
Pp60c-Src	n.s.	MDA-MB-231	Xu et al. (2010)
	n.s.	MDA-MB-435	Tan et al. (2005)
PTPμ (PTP1B)	CAPN2	MTLn3 cells	Cortesio et al. (2008)
Talin	n.s.	Mouse mammary gland	Rodríguez-Fernández (2019)
	CAPN1	MDA-MB-231, MDA-MB-468, MCF-7, BT-474	Rodríguez-Fernández (2019)
	CAPN1	MDA-MB-231	Hoskin et al. (2015)
	n.s.	MDA-MB-231	Xu et al. (2010)
Vimentin	n.s.	MDA-MB-231, Hs578T	Kim et al. (2017)
β-catenin	n.s.	MCF-7	Rios-Doria et al. (2004)
	n.s.	Mouse mammary gland	Rodríguez-Fernández (2019)
	CAPN1	MCF-7, BT-474	Rodríguez-Fernández (2019)
δ-catenin (p120)	n.s.	Mouse mammary gland	Rodríguez-Fernández (2019)
	CAPN1	MCF-7, BT-474	Rodríguez-Fernández (2019)
Epithelial cell death pathways			
Bax	n.s.	MCF-7	Sobhan et al. (2013)
	CAPN1	MCF-7	Gao and Dou (2000)
Bid	n.s.	MDA-MB-231, MCF-7	Mandic et al. (2002)
Bcl-2	n.s.	MCF-7	Pozo-Guisado et al. (2005)
Caspase-12	CAPN1	MDA-MB-231	Al-Bahlani et al. (2017)
Lamp2a	CAPN1	Mouse mammary gland	Arnandis et al. (2012)

(Continued on following page)

TABLE 1 (*Continued*) Direct targets of calpain activity in mammary gland.

Target	Isoform	Experimental model	References
VATB2	n.s.	Mouse mammary gland	Arnandis et al. (2012)
Nup62	CAPN1	Mouse mammary gland	Arnandis et al. (2014)
PARP	CAPN1	MCF-7	Tagliarino et al. (2003)
	n.s.	MCF-7 and T47D	Pink et al. (2000)
	n.s.	MCF-7	Cui et al. (2007)
p53	CAPN2	MCF-7, T47D	Libertini et al. (2005)
PP2A (B56)	n.s	MCF-7	Bertoli et al. (2009)
RelA	n.s.	MCF-7	Fei et al. (2013)
PMCA1a/b	n.s.	MCF-7	Sareen et al. (2007)
Proliferation/Differentiation pathways			
Cyclin E	CAPN2	MCF-7, T47D and xenografts	Libertini et al. (2005)
	CAPN1	MCF-7	Hou et al. (2012)
	n.s.	ZR-75	Wang et al. (2003)
Cyclin D1	n.s.	MDA-MB-231	Choi et al. (1997)
$p21^{waf1/cip1}$	n.s.	MCF-7	Khan et al. (2002)
Histone H3	CAPN1	Mouse mammary gland	Arnandis et al. (2014)

n.s. CAPN isoform was not specified. Name of breast cell lines is indicated.

Role of calpains within the mammary tissue

Calpains in the modulation of cell adhesion

Conventional calpains have been recognized as key proteases for the regulation of cell adhesion promoting either, epithelial cell clearance during mammary gland involution after lactation or cell migration and invasion during breast tumor progression and metastasis.

Adherens junctions (AJs) are cell-cell adhesion complexes crucial for tissue homeostasis and barrier function of the epithelia. At the cytoplasmic side, AJs are linked to the actin cytoskeleton, stabilizing the epithelium, establishing epithelial cell polarity, and facilitating the cell-to-cell communication needed to regulate cell proliferation and movement. Consequently, disruption of AJs is one of the hallmarks of cancer of epithelial origin including breast carcinoma (Bruner and Derksen, 2018). However, disruption of AJ is not necessarily a pathological condition, but a required mechanism for cell plasticity and tissue reorganization during development, cell proliferation or cell death.

One of the major players of cell-cell adhesion during mammary gland development is E-cadherin. E-cadherins are transmembrane receptors with extracellular regions mediating cell-cell adhesion and their intracellular tails interacting with anchor proteins clustered with several actin binding proteins. Both, calpain-1 and 2 are known to target directly or indirectly several proteins from this junctional network, going from E-cadherin, to different anchor proteins and actin binding proteins (Table 1). E-cadherin is broadly expressed in luminal epithelial cells in the mammary gland during all developmental stages, from early embryonic stages to pregnancy or lactation (Bruner and Derksen, 2018). E-cadherin disruption in mammary gland from conditional knockout mice triggers luminal cell apoptosis and cell clearance soon after parturition, preventing the terminal differentiation of milk-producing cells (Boussadia et al., 2002). From a physiological point of view, during mammary gland involution after lactation, disruption of epithelial cell adhesion is an important mechanism to remove undesired secretory cells and to remodel the tissue for the next pregnancy/lactation cycle. Both, calpain-1 and calpain-2 can proteolyze E-cadherin and other adhesion proteins from lactating mammary tissue *in vitro* (Rodríguez-Fernández, 2019). However, according to a cell type and biological context-dependent role of calpains, calpain-2 is the only isoform colocalizing with E-cadherin at epithelial cell membranes during post-lactating mammary gland involution (Figure 2, left). This finding further highlights the context-dependent role of each isoform which will be specifically regulated. Indeed, calpain-2/E-cadherin interaction barely detected at the peak of lactation, increases as the involution progresses. Mice treatment with calpeptin, the inhibitor of calpain activity, was reported to prevent the E-cadherin cleavage during mammary gland involution after lactation (Rodríguez-Fernández, 2019).

Calpain-mediated cleavage of E-cadherin has been shown to disrupt its interaction with its anchor proteins β-catenin and p120, promoting the disassembly of protein complexes crucial for cytoskeleton function (Rios-Doria et al., 2003; Bruner and Derksen, 2018). In addition, proteolysis of anchor proteins is also known to decrease the stability of E-cadherin at AJs (Davis et al., 2003). Interestingly, both β-catenin and p-120 are also targets of

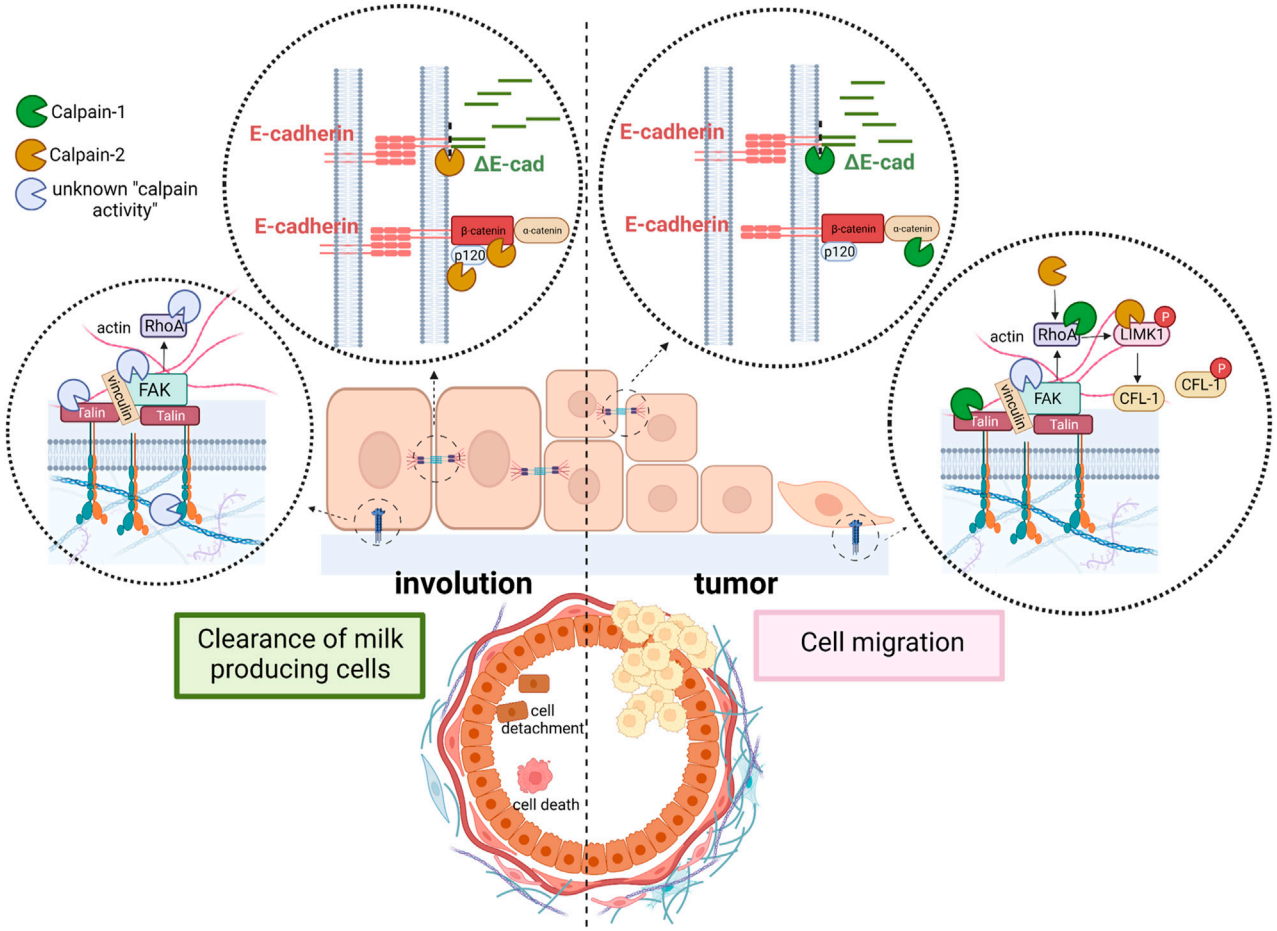


FIGURE 2
Schematic representation of the context-dependent role of conventional calpains in mammary tissue. Cell adhesion is disrupted during mammary gland involution to clear milk-secreting cells (left). While calpain-2 cleaves E-cadherin and other proteins from AJ, an unknown calpain isoform (most likely calpain-1) cleaves proteins from FA complex. Detached cells are shed into the lumen of alveoli. In contrast, in mammary tumors (right), calpain-1 is the isoform cleaving proteins from both, AJ and FA to induce cell migration and promote invasion and metastasis. In addition, calpain-2-mediated cleavage, and activation of LIMK1 induce phosphorylation and inactivation of the acting severing protein CFL-1, further contributing to cytoskeleton remodeling, cell spreading and mitosis.

calpain activity during mammary gland involution (Rodríguez-Fernández, 2019), an event which further assures the complete disassembly of AJs during mammary gland remodeling (Figure 2, left). However, a fast AJ reassembly should be guaranteed for the next pregnancy/lactation cycle.

Cleavage of E-cadherin induced by a number of stress stimuli, including high calcium concentration and its accumulation in cultured medium was long ago reported in mammary tumor cells (Wheelock et al., 1987). E-cadherin undergoes endocytosis when AJs are disrupted. It seems that the fate of truncated E-cadherin (ΔE-cadherin) is not necessarily degradation, but will rather depend on whether it is bound to AJs or is a free E-cadherin-complex (Bruner and Derksen, 2018). Calpain-2/ΔE-cadherin complex, analyzed by PLA assay in mammary gland during postlactating involution, was not degraded but instead accumulated in the cytoplasm of epithelial cells (Rodríguez-Fernández, 2019). The cytoplasmic accumulation of several forms of ΔE-cadherin as a result of calpain proteolytic activity was also demonstrated in breast and prostate carcinoma cell lines as well as in several types of adenocarcinoma (Rios-Doria et al., 2003). Although a soluble ΔE-cadherin product of calpain activity has been proposed to have prognosis value for breast cancer (Hofmann et al., 2013), the role of the cytosolic ΔE-cadherin accumulation in breast epithelial cells has not been completely elucidated.

Although mammary gland-specific depletion of E-cadherin did not develop tumors in knockout mice (Boussadia et al., 2002), several evidences indicate that inhibition of E-cadherin function is sufficient to induce invasion of cancer cells (Bruner and Derksen, 2018). It has been hypothesized that tumors will not develop unless the pro-tumorigenic event that induces the loss of E-cadherin is preceded or occurs concomitantly with the loss of protecting signals such as p53 or PTEN (Bruner and Derksen, 2018). Consistently, the combined loss or inactivation of E-cadherin/p53 or E-cadherin/PTEN in mammary gland leads to the development of invasive lobular carcinoma (Libertini et al., 2005; Annunziato et al., 2016). However, it seems that alternative mechanisms to disrupt E-cadherin function must contribute to the invasive phenotype; a fraction of patients with invasive lobular carcinoma retain

E-cadherin expression and 50% of patients showing loss of E-cadherin do not have *CDH1* inactivating mutations (Bruner and Derksen, 2018). According to a putative role of calpains in these alternative mechanisms, calpain-1 has been shown to disrupt cell-cell adhesion and promote cell migration in breast cancer cells (Rios-Doria et al., 2003; Rodríguez-Fernández, 2019) (Figure 2, right). In addition, both p53 and PTEN are well known targets of calpain activity in breast cancer cells as well as in other cell types (Kubbutat and Vousden, 1997; Libertini et al., 2005; Kulkarni et al., 2010). Interestingly, although calpain-1 has been described to proteolyze E-cadherin in breast cancer cell lines (Annunziato et al., 2016; Rodríguez-Fernández, 2019), it is not involved in the physiological cleavage of E-cadherin during mammary gland involution after weaning. The pro-tumoral environment might trigger the molecular switch from the physiological calpain-2/E-cadherin to the metastatic calpain-1/E-cadherin-cleavage observed in breast cancer cell lines. It is tempting to speculate that the fate of ΔE-cadherin might be conditioned by the calpain-isoform inducing its cleavage. After cleavage by calpain-2 in physiological conditions, ΔE-cadherin is internalized and stored in the cytoplasm. This mechanism would allow the remaining epithelial cells in the tissue to re-express cadherin at the cell surface and to re-establish AJs in a more efficient manner, a mechanism predicted to have important implications during mammary gland remodeling after lactation.

In addition to AJs, the presence of integrin receptors and the cytoplasmic proteins that form the focal adhesion (FA) complexes also contribute to the process of cell detachment or migration. Calpains are long ago known to be involved in FA turnover. An increasing number of reports identify different calpain targets either in integrin clusters, FAs, or downstream pathways (Bialkowska et al., 2000; Franco and Huttenlocher, 2005; Paavolainen and Peuhu, 2021) (Table 1).

Integrin β3, described to be the target of calpain activity is an essential integrin for lobulo-alveolar differentiation of mouse mammary gland. Moreover, integrin clusters seem to be dependent on calpain activity for their formation (Bialkowska et al., 2000). Talin is a key protein from FAs that directly connects integrins to the actin cytoskeleton and is the protein from FA complexes most frequently reported to be a calpain target in different tissues (Franco and Huttenlocher, 2005; Storr et al., 2011a; Chen et al., 2019; Nian and Ma, 2021). Mutation of talin at its calpain cleavage site skipped proteolysis in response to increased Ca^{2+} influx, but most importantly, it also attenuated the degradation of the other proteins from the FA complex which retained their interaction (Chang et al., 2017). During mammary gland involution talin-1 was shown to be the target of calpain activity (Figure 2, left), although the precise calpain isoform involved in such cleavage has not been identified yet (Rodríguez-Fernández, 2019). However, in either luminal or triple negative breast carcinoma cell lines talin-1 was proteolyzed only by calpain-1 (Rodríguez-Fernández, 2019) (Figure 2, right). Since calpain-2 protein levels are much higher than calpain-1 levels in triple negative breast cancer cell lines, the latter observation suggests that the isoform-specific role of calpains in FAs is not dependent on calpain levels but on the cell context regulation of its activity or subcellular distribution. In agreement with this, in other cell types such as fibroblast, calpain-2, but not calpain-1, is required for proteolysis of talin (Franco et al., 2004; Franco and Huttenlocher, 2005).

RhoA is another downstream effector of FA described to be the target of both calpains and crucial for the modulation of cell spreading and morphology (Figure 2, right). Although not specifically studied in mammary gland, the resultant effect of RhoA cleavage is again dependent on the cell type and context. Calpain-1 cleavage of the RhoA C-terminal domain inhibits integrin-induced actin filament assembly and cell spreading in endothelial cells (Kulkarni et al., 2002). The same effect was observed in cultured fibroblast where calpain-1 degrades a stable and functional N-terminal-RhoA fragment produced by serin proteases (Girouard et al., 2016). On the contrary, calpain-2 promotes mTOR/ROCK-RhoA pathway and actin polymerization through the cleavage and inactivation of PTEN in rat hippocampus (Briz and Baudry, 2017). Nevertheless, since RhoA activity is tightly regulated through several mechanisms (De Seze et al., 2023), is not surprising that calpain activity on different targets might either induce or block RhoA activity in specific cell types and conditions. In this sense, Piezo channels, functionally expressed in malignant breast cancer cell lines, mediate Ca^{2+}-influx to activate RhoA by a calpain-dependent mechanism regulating the formation and orientation of FAs (Pardo-Pastor et al., 2018). RhoA is known to induce the phosphorylation of the actin-severing protein cofilin-1 (CFL-1) through the activation of ROCK/RhoA/LIMK1 pathway (Briz and Baudry, 2017). However, recent data suggest a calpain-2-mediated cleavage of LIMK1 as a novel RhoA-independent mechanism for LIMK1 activation and CFL-1 phosphorylation in breast cancer cell lines (Rodríguez-Fernández et al., 2021) (Figure 2, right).

All in all, these data highlight the important role played by conventional calpains on cell adhesion disruption and actin dynamics. Depending on the cell type and context, cleavage of adhesion complexes or their downstream effectors by specific calpain isoforms will lead to a different outcome; while cell adhesion disruption will result in cell death during mammary gland involution, in breast cancer cells it will promote cell migration. Moreover, the role of calpains on cytoskeleton organization and cell adhesion might be extended to other biological and pathological processes modulated by actin-dynamics yet to be determined.

Calpains functions in epithelial cell death

Following milk stasis, mammary involution is the process by which senescent mammary cells are cleared, the lobuloalveolar structures regress and the gland returns to a pre-pregnant state. Among the signaling pathways regulating this programmed cell death both, STAT3 and NFκB are essential (Watson, 2006; Torres et al., 2011; Zaragozá et al., 2015; Watson and Khaled, 2020). As mentioned before, conventional calpains are NFκB target-genes upregulated early during the weaning process. Calpains will then propagate the response proteolyzing various substrates to promote cell apoptosis (Table 1); indeed, several caspases such as caspase-7, -9, -10 and -12 have been identified as calpain-targets (Storr et al., 2011a; Nian and Ma, 2021). Besides, activated calpains are involved in endoplasmic reticulum-mediated apoptosis (Storr et al., 2016),

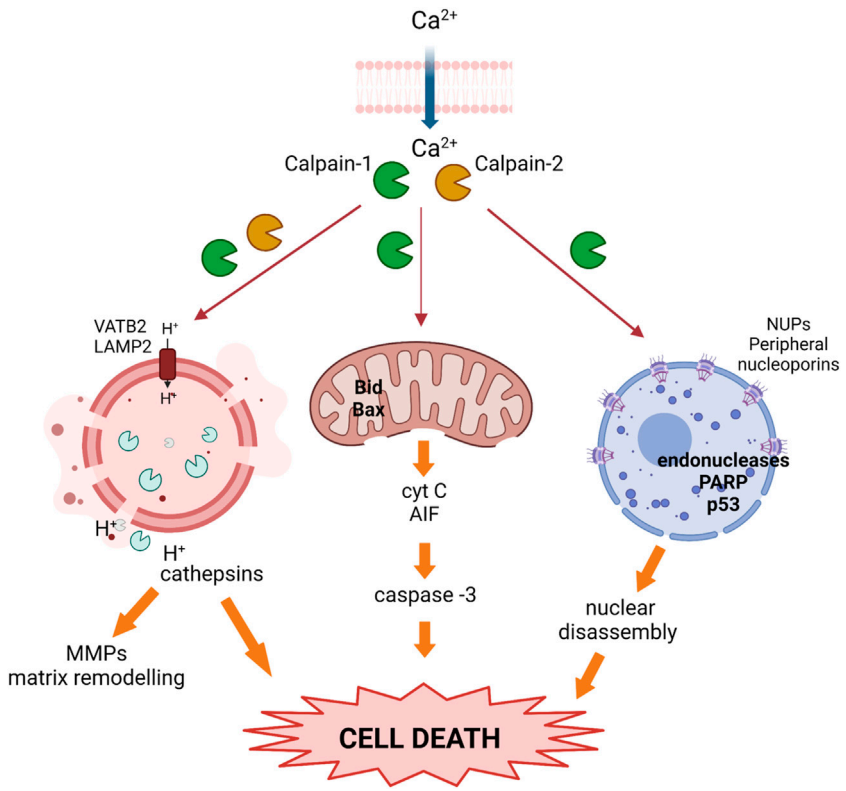


FIGURE 3
Activated calpains trigger cell death during the weaning process. Upon calcium overload, calpains become activated and translocate to different subcellular organelles where they cleave target proteins, inducing nuclear, lysosomal, and mitochondrial membrane destabilization, the release of cathepsins and pro-apoptotic proteins, and prompting cell death.

and in the mitochondrial apoptotic pathway through the cleavage of proteins from the Bcl-2 family (Storr et al., 2011a; Nian and Ma, 2021). Both conventional calpains are known to cleave the N-terminal domain of Bcl-2, Bax, and Bid proteins and these truncated forms translocate to the mitochondria where they induce mitochondrial permeabilization and the release of cytochrome c and apoptosis inducing factor (AIF) to the cytosol. This mitochondrial leakiness will lead to the activation of caspase-3 and initiate apoptotic execution. In fact, mitochondrial fractions incubated with either recombinant Bcl-2 or Bid showed only very low cytochrome c release whereas incubation of mitochondria with calpain-truncated Bcl-2 or Bid induced substantial or almost complete release of cytochrome c (Gil-Parrado et al., 2002). Similarly, Bax cleavage generates a potent proapoptotic 18 kDa fragment that does not interact with the antiapoptotic Bcl-2 protein and mediates cytochrome c release (Gao and Dou, 2000). It is noteworthy that calpain proteolysis of Bcl-2 transforms it from an anti- to a pro-apoptotic molecule whereas the proapoptotic proteins Bax and Bid become even more active in their calpain-truncated forms.

In the murine mammary gland, initial studies on the mechanism of cell death during involution focused on the activation of the intrinsic apoptotic pathway, characterized by mitochondrial outer membrane permeabilization, release of cytochrome c and other proapoptotic factors (Zaragozá et al., 2005; Watson, 2006; Kreuzaler et al., 2011; Wang and Scherer, 2019). It has been demonstrated that calpain-1 is involved in this mitochondrial proapoptotic pathway in either, physiological or pathological mammary gland (Arnandis et al., 2012; Sobhan et al., 2013; Al-Bahlani et al., 2017; Ciscato et al., 2020). Indeed, calpain-1 is present in mitochondrial fractions in both, lactating and involuting mammary gland; however, its protease activity increases as weaning progresses, presumably due to the cytosolic increase in calcium levels as a result of milk stasis, reaching its highest level at 72 h involution. Concomitant with calpain-1 activation at the mitochondria, there is cytochrome c release from mitochondrial fractions to the cytosolic compartment during involution, suggesting that calpain-1 is the major player in mitochondrial destabilization (Figure 3) (Arnandis et al., 2012). Similarly, in MCF-7 treated with the proapoptotic drug zerumbone, calpain activity is required for Bax activation preceding the mitochondrial permeabilization and caspase-dependent cell death (Sobhan et al., 2013). Other studies have shown that cisplatin-induced apoptosis of triple negative MB-231 breast cancer cells takes place through the calpain-1-mediated cleavage of caspase-12. Cisplatin treatment induced endoplasmic reticulum stress and structural changes in mitochondria in a concentration-dependent manner. In contrast, calpain-1 silencing or calpeptin treatment, attenuated cisplatin-induced apoptosis in these cells (Al-Bahlani et al., 2017). However, other studies in human and murine breast cancer cells, have suggested that mitochondrial permeabilization and mitochondrial calcium overload are a priming event needed for calpain activation and

induction of cell death (Sareen et al., 2007; Ciscato et al., 2020). Nonetheless, the role of calpain-1 on the mitochondrial death pathway described in mammary gland seems to be common to other tissues. Calpain-1 found in mitochondrial-enriched fractions from ischemic neurons (Cao et al., 2007) cleaves substrates such as the Na^{+}/Ca^{+2} exchanger NCX3 in the inner mitochondrial membrane, causing mitochondrial calcium overload and release of cytochrome c and apoptosis-inducing factor (Kar et al., 2009; Norberg et al., 2010; Chelko et al., 2021). Whatever the sequence of events that occur in the mitochondrial membrane, these studies pinpoint the importance of the subcellular location of calpains for their subsequent activation, substrate accessibility and final fate of the cell.

Apart from this mitochondrial apoptotic pathway, several findings in the involution process pointed out that there were other mechanisms in the mammary tissue early in involution that also led to programmed cell death of epithelial cells. Alteration of nuclear morphology is a common feature shared by different cell death programs. In this sense, calpains translocate to the nuclear membrane during mammary gland involution affecting nuclear pore complexes and, thus, nuclear membrane permeability (Arnandis et al., 2014) (Figure 3). Conventional calpains were present in nuclear fractions after 72 h involution; immunofluorescence or immunoprecipitation analysis showed that both proteases interacted with several nucleoporins that form the nuclear pore complex. Indeed, it was demonstrated that calpains cleave several peripheral nucleoporins during involution, affecting the structure of nuclear pore complexes with the subsequent impairment of nuclear transport selectivity. Calpain activity and location within the nucleus has already been described in other tissues, causing altered permeability of the nuclear membrane and cell death (Bano et al., 2010; Chang et al., 2015; Sheng et al., 2015). Alteration of the nuclear envelope may have a key role in the redistribution of death-inducing factors, in a positive amplification loop that would contribute to cell death and disassembly. Supporting the cell-context dependent role of calpains, their nuclear targets in transformed breast cancer cells are not at the nuclear pore complex but in the nucleoplasm. Induced apoptosis in MCF-7 or MD-468 breast cancer cells was shown to be mediated by calpain-1 translocation into the nucleus. Upon calpain-dependent endonuclease activation, PARP and p53 were proteolytically cleaved, leading to DNA fragmentation and apoptosis (Tagliarino et al., 2003; Cui et al., 2007). However, given the pleiotropic role of calpains, these proteases may have contradictory roles in the cell nucleus. Studies in non-transformed mammary MCF10A found that the calpain-2-mediated cleavage of nuclear Ku80 could be a mechanism of resistance to induced-DNA double-strand breaks (Baek et al., 2016). In contrast, calpain-2 played an important role in the nucleocytoplasmic trafficking of forkhead box protein P1 (FOXP1) via the PI3K-AKT pathway in breast cancer patients; cytoplasmic relocalization of FOXP1 correlated with reduced overall survival in breast invasive ductal carcinoma patients (Yu et al., 2018).

On the other hand, the relevance of calpain in the modulation of other cell death pathways during mammary gland involution have been also studied. Mitochondrial or nuclear permeabilization are not the only pathways to be modulated by calpain activity to induce cell death. Further studies on murine mammary gland involution showed that during the involution first phase, luminal alveolar cells also die via a lysosomal-mediated pathway (Kreuzaler et al., 2011; Arnandis et al., 2012). Lysosomal activity is essential to preserve cellular homeostasis in mammary gland, and lysosomal membrane permeabilization results in massive release of the lysosomal contents into the cytosol (Kreuzaler et al., 2011; Lloyd-Lewis et al., 2018). Therefore, the lysosomal-mediated cell death is triggered by disruption of lysosomal membrane stability. The role of calpains in the lysosomal-mediated death pathways during mammary gland involution has been studied. It has been demonstrated that calpain activity in lysosomal-enriched fractions increased by twofold after 24 h weaning and remained elevated thereafter, leading to lysosomal destabilization and the release of lysosomal proteases into the cytosolic compartment (Figure 3) (Arnandis et al., 2012). Lysosomal-membrane integrity is ensured by several membrane proteins such as HSP70, the glycoproteins LAMP1 and LAMP2 (Eskelinen, 2006) or the vacuolar-type H^{+}-ATPase (V-ATPase). Cleavage of these proteins will destabilize the lysosomal membrane and induce cell death. The identification of calpain targets in lysosomal fractions of involuting mammary gland revealed the mechanisms of the calpain-mediated destabilization of lysosomes. Indeed, it was observed that as involution progressed, calpain-1 and calpain-2 translocated from the cytosol to the lysosomal membrane where they degraded the cytosolic tail of LAMP2A and the subunit b of the vacuolar-type proton ATPase. Furthermore, calpain-1 silencing with siRNA prevented LAMP2A degradation in 72 h weaned mice (Arnandis et al., 2012).

The consequences of the calpain-mediated destabilization of lysosomes have been reported. Activation of STAT3 in mammary tissue during involution upregulates the expression of cathepsins B and L which are known lysosomal proteases (Kreuzaler et al., 2011). Calpain-mediated lysosomal destabilization triggers the release of cathepsins (Zaragozá et al., 2009; Margaryan et al., 2010). Since these cathepsins will act on downstream targets such as MMP-9, calpains will expand the signaling cascade that leads to epithelial cell death and mammary tissue remodeling through lysosomes-leakiness.

Lysosomal weakness that involves cathepsins release is a known pathway to be targeted in breast cancer cells (Ostenfeld et al., 2005) and, based on current knowledge, one could hypothesize that calpains are key mediators in this lysosomal cell death. VATB2, identified as a calpain-target, is crucial for lysosomal-mediated cell death. In breast and gastric cancer cell lines, inhibition of the V-ATPase causes lysosomal dysfunction and induces apoptosis (McHenry et al., 2009; Chen et al., 2022), sensitizing cancer cells to chemotherapy (Piao and Amaravadi, 2016; Dong et al., 2022). Nevertheless, the release of lysosomal content, such as cathepsins B and D, initiates a cascade of cell signaling events that may not always lead to cell death. Under specific circumstances cell fate can be the opposite and lysosomal leakage may be associated to cell survival, as it is the case for cancer cells, in which partial release of lysosomal cathepsins has a key role in tumor progression. Indeed, V-ATPases participate in the invasion and metastasis of tumor cells facilitating cathepsins activation and release; a process associated with cell invasion through matrix metalloproteinase activation (Jung et al., 2021).

All these studies in mammary gland involution and breast cancer emphasize the complexity of the calpain system. As inferred from the information previously given, calpains can be

both, proapoptotic or survival factors depending on cellular context, type of apoptotic stimuli and subcellular localization of the protease (Tan et al., 2006). Although several studies in breast cancer cells have shown that calpain activation and mitochondrial dysfunction are key mechanisms for the cytotoxicity of different pharmacological anticancer drugs (Tagliarino et al., 2003; Cui et al., 2007; Sareen et al., 2007; Sobhan et al., 2013; Al-Bahlani et al., 2017; Ciscato et al., 2020), calpain activity has also been implicated in the pro-survival activity of NFκB or p53 in cancer cells (Pianetti et al., 2001; Pozo-Guisado et al., 2005; Fei et al., 2013). Interestingly, these two calpain-targets are crucial regulatory nodes for mammary gland involution, (Zaragozá et al., 2009; Torres et al., 2011); indeed, this process is delayed in the absence of a functional p53 gene (Jerry et al., 1999; Jerry et al., 2002) or NFκB (Connelly et al., 2010). Once again, it has been remarked the multi-faceted role of calpains in diverse signaling pathways. The findings presented herein highlight the context-dependent and opposing pro-survival or pro-apoptotic roles of conventional calpains, though further research is needed to elucidate the precise mechanisms and the specific isoforms playing a particular role in each cellular context.

Role of calpains in proliferation/differentiation in mammary gland

Cell proliferation and differentiation take place in different cell types throughout the whole pregnancy/lactation/involution cycle. Some of the signaling pathways involved in the latter processes are also triggered and altered during breast tumorigenesis. Calpains are long known to be involved in the process of cell differentiation and proliferation. The important function of calpains in those processes was reported in early experiments where calpain inhibitors such as calpeptin and other thiol protease inhibitors were shown to restrict cell cycle progression or reduce the growth rate of transformed and non-transformed mammalian cells in response to a number of stimuli. Exogenous overexpression of CAST, or depletion of specific calpain isoforms facilitated the identification of calpain substrates as well as those signaling pathways modulated by calpains during cell proliferation. Calpain activity promotes the cell cycle progression through the modulation of key proteins for G1 restriction checkpoint, such as Cyclins E, D1, p21 (waf1/cip1), CDKs or RB. In addition, calpains also have an important function in other phases of cell cycle (Table 1) (Nian and Ma, 2021).

Unfortunately, the role of calpains in epithelial cell proliferation and differentiation during pregnancy or lactation has not been studied yet. However, 3D studies in MCF-10A non-tumoral breast cell line to mimic structures that resemble the acini of human breast, revealed that the architecture of acini derived from CAPNS1 depleted cells is altered (Raimondi et al., 2016). Although not identifying the specific isoform, the authors conclude that calpains may play an important role in the initiation of the differentiation process in this system. Nevertheless, during mammary gland involution after lactation most cells undergoing proliferation/differentiation are not epithelial but stromal cells. In fact, most of the non-lactating mammary gland mass consists of stromal adipose tissue (Landskroner-Eiger et al., 2010). During the second phase of involution, after epithelial cell death, the basement membrane and ECM break down and, dedifferentiated adipocytes proliferate and re-differentiate back into mature adipocytes to repopulate the mammary fat pad (Wang and Scherer, 2019). Calpain-1 was shown to be localized in the nucleus of dedifferentiated adipocytes during the second phase of involution (Arnandis et al., 2014). Conversely to its subcellular distribution in epithelial cells, nuclear calpain-1 was not found to be associated to the nuclear pore complex, but interacting with histone H3. This calpain-1/histone H3 interaction seems to be part of the differentiation program of pre-adipocytes repopulating the mammary fat pad during involution. Analysis of adipocyte fractions from involuting mammary gland as well as *in vitro* enzymatic assays showed that calpain-1 was the specific isoform cleaving the N-terminal tail of histone H3. Although the functional consequences of calpain-1-mediated cleavage of histone H3 are unknown, it is tempting to speculate that this cleavage might be an epigenetic signature for selected genes upon adipocyte differentiation. In this sense, differentiating preadipocytes have been reported to experience important epigenetic changes in the nuclear compartment affecting chromosome positioning (Kuroda et al., 2004), promoter interactions prior to adipogenic genes activation and expression (He et al., 2018) and chromatin remodeling (Salma et al., 2004). In agreement with this, a full colocalization of calpain-1 and the euchromatin/active marker H3K4me3 was observed in tissue sections from mammary gland involution (Arnandis et al., 2014). Interestingly, this colocalization was exclusively observed in stromal cells. In addition, it is well-established that adipogenic gene promoters are marked by early changes in histone modification patterns (Macchia et al., 2021) to keep an opened chromatin structure accessible to transcription factors. Accordingly, cleavage of the histone H3 tail might result in a decondensed chromatin structure in those adipogenic genes which need to be expressed during the differentiation process (Figure 4A). Calpain-1 was found to bind to the C/EBPα and leptin gene promoters, two adipogenic genes involved in terminal differentiation and the acquisition of the adipocyte phenotype, respectively. Consequently, the expression of these two genes was increased in mammary stroma during involution compared to the lactating mammary gland (Arnandis et al., 2014).

Interestingly, further reinforcing the idea of a context-dependent role of conventional calpains, observations from our group (Rodríguez-Fernández, 2019) indicate that both calpain isoforms interact with N-terminal tail of histone H3 during the differentiation of 3T3-L1 pre-adipocytes (Figure 4B). However, their distribution during preadipocyte mitotic clonal expansion is completely different. Calpain-1 was observed surrounding condensed metaphasic chromosomes, but not colocalizing with them, and at telophase, it was widely distributed into the cytosol. In contrast, calpain-2 was found either colocalizing with the prometaphasic chromosome rosette, surrounding chromosomes along the metaphase plate or concentrated in the whole nucleus during telophase. These data suggest that although not observed in mammary gland, both calpains might have different and important functions for adipocyte differentiation. In that sense, it has been proposed that calpain-2 limits the activity of calpain-1 (Shinkai-Ouchi et al., 2020) and consequently, the pattern of activation of each isoform might be sequential. However, this hypothesis has not been demonstrated *in vivo* and in addition, the inflammatory component from mammary gland involution, which is absent in

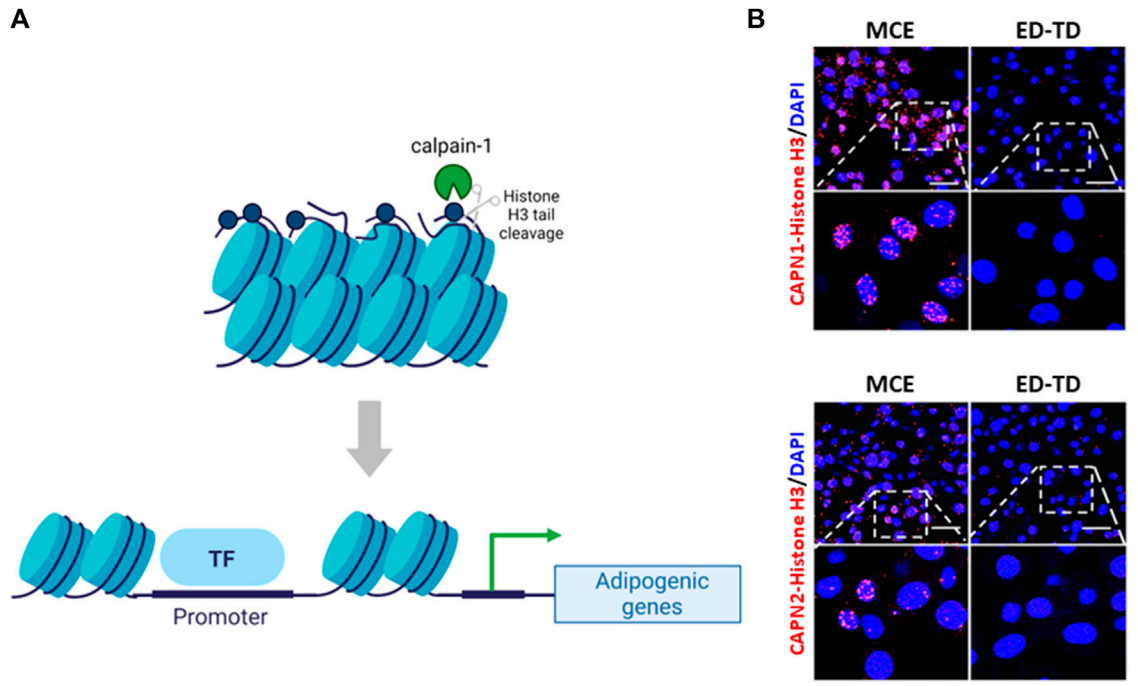


FIGURE 4
Calpains and chromatin interaction during pre-adipocyte differentiation. **(A)** Schematic representation of the epigenetic cleavage of Nt-histone H3 by calpain-1 in adipocytes. During mammary gland involution calpain-1 binds to and cleaves Nt-histone H3 on adipogenic gene promoters inducing chromatin relaxation and gene expression **(B)** Representative images of CAPN1 and CAPN2 interaction with Nt-histone H3 during the differentiation of 3T3-L1 cells are shown. CAPNs/Nt-histone H3 interaction was analyzed by PLA (red) during pre-adipocyte monoclonal expansion (MCE) and transition from early to terminal differentiation (ED-TD). Nuclei were counterstained with DAPI (blue). Scale bar, 21 μm. (Nt: N-terminal tail).

cultured cells, might condition the behavior of both calpains. Although this epigenetic mark was exclusively observed in adipocytes, the possibility of calpains also having a role in the nuclear compartment of mammary epithelial cells during cell proliferation or progenitor differentiation is yet to be explored.

The role of calpains in proliferation of breast cancer cells has been more extensively studied. Depletion of *CAPN2* or *CAPNS1* by knockdown experiments in breast carcinoma cell lines reduced tumor growth in mouse orthotopic xenografts, (Ho et al., 2012; Grieve et al., 2016). Ablation of the regulatory subunit *CAPNS1* in the mammary epithelium delays spontaneous tumor onset in a model of mammary HER2$^+$ tumorigenesis (MacLeod et al., 2018). Through the modulation of PP2A/Akt/FoxO3a pathway, *CAPN2* silencing induces the expression of cyclin-dependent p27Kip1 kinase inhibitor and reduces breast carcinoma cell proliferation (Ho et al., 2012). Furthermore, accumulation of nuclear calpain-2 has been associated to breast cancer cell proliferation. Nuclear calpain-2 has been observed in both, triple-negative and luminal breast cancer cell lines (Telechea-Fernández et al., 2018). *CAPN2* knockdown in triple-negative breast cancer cell lines causes a higher percentage of cells at G2/M, aberrant mitosis, fails in cytokinesis and consequently, an increased number of multinucleated cells (Rodríguez-Fernández et al., 2021). All these data suggest that while calpain-1, or both conventional calpains, participate in the differentiation program of adipocytes during mammary gland involution, calpain-2 preferentially accumulated in the nuclear compartment, seems to be the main isoform modulating cell proliferation in breast tumor cells.

However, the nuclear localization of calpain-2 has been described also in non-transformed epithelial cells. It has been reported that while in proliferating cells calpain-2 is mainly localized in the nucleus (König et al., 2003; Raynaud et al., 2004; Raynaud et al., 2008), in fully differentiated quiescent cells calpain-2 is restricted to the cytosol (Raynaud et al., 2004). High expression of *CAPN2* has been associated to its nuclear accumulation and active mitosis in ES cells as well as in 8-cell embryos (Raynaud et al., 2008). According to these data, calpain-2 might be expected to modulate cell proliferation and differentiation of epithelial cells in mammary gland during the pregnancy/lactation cycle. However, the effect of tissue and cell type in the physiological or pathological mammary gland needs to be considered. The physiological mammary stroma consists of adipocytes, fibroblasts, endothelial and inflammatory cells, as well as ECM, tightly regulated during each phase of the pregnancy/lactation cycle. The communication and interaction between the mammary epithelium and stroma drive the proper patterning and function of the normal mammary gland (Howard and Lu, 2014) and consequently, they will be determinant for the different functions of conventional calpains. Likewise, a malignant breast carcinoma includes more components than just epithelial tumor cells. Disruption of the above mentioned interactions or altered stroma composition in breast cancer (Landskroner-Eiger et al., 2010; Vizovisek et al., 2021) could alter the subcellular distribution and functions of conventional calpains in specific cell types. Although much progress has been made in understanding the function of conventional calpains on cell proliferation using breast cancer cells, these data highlight the need to consider the tissue

composition and particular microenvironment when trying to elucidate the specific role and regulation of each calpain isoform.

Challenges of calpains as therapeutic targets and concluding remarks

As we have extensively covered in this review, *in vitro* and *in vivo* experiments demonstrate that conventional calpains are involved in tumor progression and metastasis. Moreover, calpain inhibition has the potential to attenuate carcinogenesis and block metastasis of aggressive tumors and particularly, of breast cancer. Hence, targeting calpain-1 and calpain-2 was proposed as a novel therapeutic strategy for mammary tumors. However, there are not clinical trials involving inhibitors of conventional calpains for breast cancer treatment. One of the main reasons for failure in calpain-targeted chemotherapies is that calpains are a big family of proteases with more than 15 isoforms, and even when only limited to conventional calpains, the same isoform can have opposite effects in different tissues or cell types. Since it is difficult to dissect the isoform-specific role of both calpains for each particular cell context, the potential use of calpains as therapeutic targets is necessarily limited. In addition, when we consider that the role of each isoform might also depend on its subcellular location, targeting calpains seems an *a priori* unaffordable task.

Indeed, ablation of *CAPN2* in mice is embryonically lethal (Dutt et al., 2006) which further remarks the essential role of these proteases for the epithelial homeostasis. Consequently, the complete suppression of calpain activity can be harmful to the organism. However, conditional deletion of total or tissue-specific *CAPNS1*, which abrogates both conventional calpains, has been well tolerated and has been demonstrated to be a useful tool to unveil the essential role of calpains in maintaining tissue homeostasis (Grieve et al., 2016; MacLeod et al., 2018).

Recent advances in biomedicine and technology have further contributed to the design of new site-directed inhibitors of conventional calpains with promising effects on inhibition of the enzymatic activity. Nevertheless, many of these inhibitors, like the classical inhibitors ALLN or calpeptin, resulted not as specific as desired and the activity of other proteases such as cathepsins or caspases was also blocked (Shapovalov et al., 2022).

The main calpain inhibitors that have been disclosed over the last 10 years include different types of agents such as calpastatin-based peptidomimetics, thalassospiramide lipopeptides, disulfide analogs of α-mercaptoacrylic acids, allosteric modulators, azoloimidazolidenones and, macrocyclic/non-macrocyclic carboxamides (Donkor, 2020). All of them showing different characteristics, benefits, and disadvantages.

To name some of them, the peptidomimetics calpain inhibitors include agents based on the CAST structure. CAST is the only known endogenous inhibitor of conventional calpain (Kiss et al., 2008). Although other compounds can also inhibit other unconventional calpains like calpain-8 or -9, these agents inhibit mainly calpain-1 and -2 (Hata et al., 2016). Even though these peptides show higher specificity than others, they still show poor cell permeability and pharmacokinetic properties. As a result, a structure-guided design of isoform-specific inhibitors of calpains is yet to be accomplished.

The thalassospiramide are lipopeptides isolated from marine bacteria, found to inhibit human calpain-1 in the nanomolar range (Ross et al., 2013). These compounds have potential anti-inflammatory properties and exhibit low toxicity and good selectivity (Lu et al., 2015). Other compounds in this group include derivatives of the MG132, a tripeptide that inhibits both, 26S proteasome and calpain activity (Pehere et al., 2019). The detailed structure of the latter agents and the effectiveness of some of the inhibitors in preclinical animal models has been thoroughly discussed elsewhere (Donkor, 2020).

Having said that, there are some phase II/III and even preclinical studies with calpains inhibitors, but they have been tested in the context of other diseases, such as Alzheimer's disease, multiple sclerosis, spinal muscular atrophy, traumatic brain injury, acute myocardial infarction, ophthalmic diseases, or muscular dystrophy (Ono et al., 2016). As commented in this review, the calpain system has been predicted to be an important target for cancer treatment (Miyazaki et al., 2015). In that sense, calpain-1 activity was shown to be important in the treatment of other types of cancer such as myelodysplastic syndrome (Fang et al., 2016) colorectal cancer (Vaish and Sanyal, 2012) or melanoma (Del Bello et al., 2007). However, although the latter reports show the initial benefits of inhibiting conventional calpains, other studies in melanoma have suggested that calpain activity is required for the success of cisplatin-induced apoptosis of cancer cells (Moretti et al., 2014).

In summary, as we have highlighted throughout this review, conventional calpains can have different or even opposite functions in different cell types or biological contexts. It is noteworthy to mention that a scarce number of reports in the literature show *in vivo* models for the study of conventional calpains in physiological or pathological mammary gland. Even more, while a limited number of publications show studies in breast cancer cell lines, most of reports on the role of conventional calpains do not use mammary cells as experimental models. Consequently, the mechanisms of regulation of calpain activity in mammary tissue are still unknown. Indeed, it is not known how conventional calpains are regulated to specifically recognize a substrate among all the proteins known to be their targets in mammary tissue. Although the effect of calpains have been studied in breast cancer or mammary gland involution, the mechanisms of calpain activation have not been studied and only hypothetical and not demonstrated connections between regulatory factors and calpains can be found in the literature.

On the other hand, the subcellular compartmentalization of calpains, which limits their access to substrates, seems to be a key event for their functions. Thus, understanding the mechanisms underlying subcellular distribution of calpains will be crucial to decipher or inhibit their functions. A major challenge in targeting conventional calpains as a therapeutic approach for breast cancer would be to specifically abolish a calpain isoform within a cell compartment and cell type in mammary tissue. Hence, important questions to be answered are: How are conventional calpains differentially distributed into cell compartments in breast cancer cells? Might the subcellular localization of calpain isoforms, instead of the expression levels of calpains or calpastatin, have a prognosis value in breast cancer? In the meanwhile, post-lactation mammary gland involution seems to be the most useful model to answer those questions. Pregnancy associated breast cancer has been explained as

the progression of a pre-existing disease promoted by the microenvironment of post-lactating mammary gland (Macdonald, 2020). If involuting mammary gland mimics the microenvironment of a developing tumor, unraveling the multifaceted and isoform-specific roles of calpains in the context of mammary gland involution will lead us to gain insights into breast cancer development and the design of new calpain-targeting therapies.

Author contributions

ERG-T, EO-Z, JRV, and RZ, wrote the initial draft of the manuscript. EO-Z and AG prepared figures and table, respectively. ERG-T designed the structure of the article, supervised and edited the final version of manuscript. All authors contributed to the article and approved the submitted version.

Funding

Research was supported by the Consellería de Educación [Grant Number GVPROMETEO CIPROM/2021/051 (to ERG-T and JRV)].

Acknowledgments

We acknowledge the contribution of our PHD students and all the laboratory members to the research in the field of calpains. Figures were created with BioRender.com.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Al-Bahlani, S. M., Al-Bulushi, K. H., Al-Alawi, Z. M., Al-Abri, N. Y., Al-Hadidi, Z. R., and Al-Rawahi, S. S. (2017). Cisplatin induces apoptosis through the endoplasmic reticulum-mediated, calpain 1 pathway in triple-negative breast cancer cells. *Clin. Breast Cancer* 17, e103–e112. doi:10.1016/j.clbc.2016.12.001

Annunziato, S., Kas, S. M., Nethe, M., Yücel, H., Del Bravo, J., Pritchard, C., et al. (2016). Modeling invasive lobular breast carcinoma by CRISPR/Cas9-mediated somatic genome editing of the mammary gland. *Genes Dev.* 30, 1470–1480. doi:10.1101/gad.279190.116

Arnandis, T., Ferrer-Vicens, I., García-Trevijano, E. R., Miralles, V. J., García, C., Torres, L., et al. (2012). Calpains mediate epithelial-cell death during mammary gland involution: mitochondria and lysosomal destabilization. *Cell Death Differ.* 19, 1536–1548. doi:10.1038/cdd.2012.46

Arnandis, T., Ferrer-Vicens, I., Torres, L., García, C., Garcia-Trevijano, E. R., Zaragoza, R., et al. (2014). Differential functions of calpain 1 during epithelial cell death and adipocyte differentiation in mammary gland involution. *Biochem. J.* 459, 355–368. doi:10.1042/BJ20130847

Atabai, K., Sheppard, D., and Werb, Z. (2007). Roles of the innate immune system in mammary gland remodeling during involution. *J. Mammary Gland. Biol. Neoplasia* 12, 37–45. doi:10.1007/s10911-007-9036-6

Baek, K. H., Yu, H. V., Kim, E., Na, Y., and Kwon, Y. (2016). Calcium influx-mediated translocation of m-calpain induces Ku80 cleavage and enhances the Ku80-related DNA repair pathway. *Oncotarget* 7, 30831–30844. doi:10.18632/oncotarget.8791

Bano, D., Dinsdale, D., Cabrera-Socorro, A., Maida, S., Lambacher, N., Mccoll, B., et al. (2010). Alteration of the nuclear pore complex in Ca2+-mediated cell death. *Cell Death Differ.* 17, 119–133. doi:10.1038/cdd.2009.112

Basree, M. M., Shinde, N., Koivisto, C., Cuitino, M., Kladney, R., Zhang, J., et al. (2019). Abrupt involution induces inflammation, estrogenic signaling, and hyperplasia linking lack of breastfeeding with increased risk of breast cancer. *Breast Cancer Res.* 21, 80. doi:10.1186/s13058-019-1163-7

Bertoli, C., Copetti, T., Lam, E. W.-F., Demarchi, F., and Schneider, C. (2009). Calpain small-1 modulates Akt/FoxO3A signaling and apoptosis through PP2A. *Oncogene* 28, 721–733. doi:10.1038/onc.2008.425

Betts, C. B., Pennock, N. D., Caruso, B. P., Ruffell, B., Borges, V. F., and Schedin, P. (2018). Mucosal immunity in the female murine mammary gland. *J. Immunol.* 201, 734–746. doi:10.4049/jimmunol.1800023

Bialkowska, K., Kulkarni, S., Du, X., Goll, D. E., Saido, T. C., and Fox, J. E. B. (2000). Evidence that beta3 integrin-induced Rac activation involves the calpain-dependent formation of integrin clusters that are distinct from the focal complexes and focal adhesions that form as Rac and RhoA become active. *J. Cell Biol.* 151, 685–696. doi:10.1083/jcb.151.3.685

Biswas, S. K., Banerjee, S., Baker, G. W., Kuo, C.-Y., and Chowdhury, I. (2022). The mammary gland: basic structure and molecular signaling during development. *Int. J. Mol. Sci.* 23, 3883. doi:10.3390/ijms23073883

Boussadia, O., Kutsch, S., Hierholzer, A., Delmas, V., and Kemler, R. (2002). E-cadherin is a survival factor for the lactating mouse mammary gland. *Mech. Dev.* 115, 53–62. doi:10.1016/S0925-4773(02)00090-4

Briz, V., and Baudry, M. (2017). Calpains: master regulators of synaptic plasticity. *Neuroscientist* 23, 221–231. doi:10.1177/1073858416649178

Bruner, H. C., and Derksen, P. W. B. (2018). Loss of E-cadherin-dependent cell–cell adhesion and the development and progression of cancer. *Cold Spring Harb. Perspect. Biol.* 10, a029330. doi:10.1101/cshperspect.a029330

Campbell, R. L., and Davies, P. L. (2012). Structure–function relationships in calpains. *Biochem. J.* 447, 335–351. doi:10.1042/BJ20120921

Cao, G., Xing, J., Xiao, X., Liou, A. K. F., Gao, Y., Yin, X.-M., et al. (2007). Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. *J. Neurosci.* 27, 9278–9293. doi:10.1523/JNEUROSCI.2826-07.2007

Chang, H., Sheng, J.-J., Zhang, L., Yue, Z.-J., Jiao, B., Li, J.-S., et al. (2015). ROS-induced nuclear translocation of calpain-2 facilitates cardiomyocyte apoptosis in tail-suspended rats: mechanisms of cardiomyocyte apoptosis in rats. *J. Cell. Biochem.* 116, 2258–2269. doi:10.1002/jcb.25176

Chang, S.-J., Chen, Y.-C., Yang, C.-H., Huang, S.-C., Huang, H.-K., Li, C.-C., et al. (2017). Revealing the three dimensional architecture of focal adhesion components to explain Ca2+-mediated turnover of focal adhesions. *Biochimica Biophysica Acta General Subj.* 1861, 624–635. doi:10.1016/j.bbagen.2017.01.002

Chelko, S. P., Keceli, G., Carpi, A., Doti, N., Agrimi, J., Asimaki, A., et al. (2021). Exercise triggers CAPN1-mediated AIF truncation, inducing myocyte cell death in arrhythmogenic cardiomyopathy. *Sci. Transl. Med.* 13, eabf0891. doi:10.1126/scitranslmed.abf0891

Chen, F., Kang, R., Liu, J., and Tang, D. (2022). The V-ATPases in cancer and cell death. *Cancer Gene Ther.* 29, 1529–1541. doi:10.1038/s41417-022-00477-y

Chen, J., Wu, Y., Zhang, L., Fang, X., and Hu, X. (2019). Evidence for calpains in cancer metastasis. *J. Cell. Physiology* 234, 8233–8240. doi:10.1002/jcp.27649

Choi, Y. H., Lee, S. J., Nguyen, P., Jang, J. S., Lee, J., Wu, M.-L., et al. (1997). Regulation of cyclin D1 by calpain protease. *J. Biol. Chem.* 272, 28479–28484. doi:10.1074/jbc.272.45.28479

Ciscato, F., Filadi, R., Masgras, I., Pizzi, M., Marin, O., Damiano, N., et al. (2020). Hexokinase 2 displacement from mitochondria-associated membranes prompts Ca^{2+}-dependent death of cancer cells. *EMBO Rep.* 21, e49117. doi:10.15252/embr.201949117

Connelly, L., Barham, W., Pigg, R., Saint-Jean, L., Sherrill, T., Cheng, D.-S., et al. (2010). Activation of nuclear factor kappa B in mammary epithelium promotes milk loss during mammary development and infection. *J. Cell. Physiol.* 222, 73–81. doi:10.1002/jcp.21922

Cortesio, C. L., Chan, K. T., Perrin, B. J., Burton, N. O., Zhang, S., Zhang, Z.-Y., et al. (2008). Calpain 2 and PTP1B function in a novel pathway with Src to regulate

invadopodia dynamics and breast cancer cell invasion. *J. Cell Biol.* 180, 957–971. doi:10.1083/jcb.200708048

Croall, D. E., and Ersfeld, K. (2007). The calpains: modular designs and functional diversity. *Genome Biol.* 8, 218. doi:10.1186/gb-2007-8-6-218

Cui, Q., Yu, J., Wu, J., Tashiro, S., Onodera, S., Minami, M., et al. (2007). P53-mediated cell cycle arrest and apoptosis through a caspase-3-independent, but caspase-9-dependent pathway in oridonin-treated MCF-7 human breast cancer cells. *Acta Pharmacol. Sin.* 28, 1057–1066. doi:10.1111/j.1745-7254.2007.00588.x

Dang, H. V., Sakai, T., Pham, T. A., Tran, D. H., Yorita, K., Shishido, Y., et al. (2015). Nucling, a novel apoptosis-associated protein, controls mammary gland involution by regulating NF-κB and STAT3. *J. Biol. Chem.* 290, 24626–24635. doi:10.1074/jbc.M115.673848

Davis, M. A., Ireton, R. C., and Reynolds, A. B. (2003). A core function for p120-catenin in cadherin turnover. *J. Cell Biol.* 163, 525–534. doi:10.1083/jcb.200307111

De Seze, J., Gatin, J., and Coppey, M. (2023). RhoA regulation in space and time. *FEBS Lett.* 597, 836–849. doi:10.1002/1873-3468.14578

Del Bello, B., Moretti, D., Gamberucci, A., and Maellaro, E. (2007). Cross-talk between calpain and caspase-3/-7 in cisplatin-induced apoptosis of melanoma cells: a major role of calpain inhibition in cell death protection and p53 status. *Oncogene* 26, 2717–2726. doi:10.1038/sj.onc.1210079

Dong, Y., Zhu, G., Wang, S.-F., Keon, K. A., Rubinstein, J. L., Zeng, S.-X., et al. (2022). Toosendanin, a novel potent vacuolar-type H^+-translocating ATPase inhibitor, sensitizes cancer cells to chemotherapy by blocking protective autophagy. *Int. J. Biol. Sci.* 18, 2684–2702. doi:10.7150/ijbs.71041

Donkor, I. O. (2020). An update on the therapeutic potential of calpain inhibitors: a patent review. *Expert Opin. Ther. Pat.* 30, 659–675. doi:10.1080/13543776.2020.1797678

Dutt, P., Croall, D. E., Arthur, J. S. C., De Veyra, T., Williams, K., Elce, J. S., et al. (2006). m-Calpain is required for preimplantation embryonic development in mice. *BMC Dev. Biol.* 6, 3. doi:10.1186/1471-213X-6-3

Eskelinen, E.-L. (2006). Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. *Mol. Aspects Med.* 27, 495–502. doi:10.1016/j.mam.2006.08.005

Fang, J., Liu, X., Bolanos, L., Barker, B., Rigolino, C., Cortelezzi, A., et al. (2016). A calcium- and calpain-dependent pathway determines the response to lenalidomide in myelodysplastic syndromes. *Nat. Med.* 22, 727–734. doi:10.1038/nm.4127

Fei, B., Yu, S., and Geahlen, R. L. (2013). Modulation by Syk of Bcl-2, calcium and the calpain–calpastatin proteolytic system in human breast cancer cells. *Biochimica Biophysica Acta (BBA) - Mol. Cell Res.* 1833, 2153–2164. doi:10.1016/j.bbamcr.2013.05.010

Franco, S. J., and Huttenlocher, A. (2005). Regulating cell migration: calpains make the cut. *J. Cell Sci.* 118, 3829–3838. doi:10.1242/jcs.02562

Franco, S., Perrin, B., and Huttenlocher, A. (2004). Isoform specific function of calpain 2 in regulating membrane protrusion. *Exp. Cell Res.* 299, 179–187. doi:10.1016/j.yexcr.2004.05.021

Gao, G., and Dou, Q. P. (2000). N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. *J. Cell Biochem.* 80, 53–72. doi:10.1002/1097-4644(20010101)80:1<53::aid-jcb60>3.0.co;2-e

Gil-Parrado, S., Fernández-Montalván, A., Assfalg-Machleidt, I., Popp, O., Bestvater, F., Holloschi, A., et al. (2002). Ionomycin-activated calpain triggers apoptosis. A probable role for Bcl-2 family members. *J. Biol. Chem.* 277, 27217–27226. doi:10.1074/jbc.M202945200

Girouard, M.-P., Pool, M., Alchini, R., Rambaldi, I., and Fournier, A. E. (2016). RhoA proteolysis regulates the actin cytoskeleton in response to oxidative stress. *PLoS ONE* 11, e0168641. doi:10.1371/journal.pone.0168641

Grieve, S., Gao, Y., Hall, C., Hu, J., and Greer, P. A. (2016). Calpain genetic disruption and HSP90 inhibition combine to attenuate mammary tumorigenesis. *Mol. Cell. Biol.* 36, 2078–2088. doi:10.1128/MCB.01062-15

Hata, S., Kitamura, F., Yamaguchi, M., Shitara, H., Murakami, M., and Sorimachi, H. (2016). A gastrointestinal calpain complex, G-calpain, is a heterodimer of CAPN8 and CAPN9 calpain isoforms, which play catalytic and regulatory roles, respectively. *J. Biol. Chem.* 291, 27313–27322. doi:10.1074/jbc.M116.763912

He, M., Li, Y., Tang, Q., Li, D., Jin, L., Tian, S., et al. (2018). Genome-wide chromatin structure changes during adipogenesis and myogenesis. *Int. J. Biol. Sci.* 14, 1571–1585. doi:10.7150/ijbs.25328

Ho, W., Pikor, L., Gao, Y., Elliott, B. E., and Greer, P. A. (2012). Calpain 2 regulates akt-FoxO-p27Kip1 protein signaling pathway in mammary carcinoma. *J. Biol. Chem.* 287, 15458–15465. doi:10.1074/jbc.M112.349308

Hofmann, G., Balic, M., Dandachi, N., Resel, M., Schippinger, W., Regitnig, P., et al. (2013). The predictive value of serum soluble E-cadherin levels in breast cancer patients undergoing preoperative systemic chemotherapy. *Clin. Biochem.* 46, 1585–1589. doi:10.1016/j.clinbiochem.2013.06.010

Hoskin, V., Szeto, A., Ghaffari, A., Greer, P. A., Côté, G. P., and Elliott, B. E. (2015). Ezrin regulates focal adhesion and invadopodia dynamics by altering calpain activity to promote breast cancer cell invasion. *MBoC* 26, 3464–3479. doi:10.1091/mbc.E14-12-1584

Hou, J., Wang, X., Li, Y., Liu, X., Wang, Z., An, J., et al. (2012). 17beta-estradiol induces both up-regulation and processing of cyclin E in a calpain-dependent manner in MCF-7 breast cancer cells. *FEBS Lett.* 586, 892–896. doi:10.1016/j.febslet.2012.02.018

Howard, B. A., and Lu, P. (2014). Stromal regulation of embryonic and postnatal mammary epithelial development and differentiation. *Seminars Cell & Dev. Biol.* 25 (26), 43–51. doi:10.1016/j.semcdb.2014.01.004

Jerry, D. J., Dickinson, E. S., Roberts, A. L., and Said, T. K. (2002). Regulation of apoptosis during mammary involution by the p53 tumor suppressor gene. *J. Dairy Sci.* 85, 1103–1110. doi:10.3168/jds.S0022-0302(02)74171-4

Jerry, D. J., Pinkas, J., Kuperwasser, C., Dickinson, E. S., and Naber, S. P. (1999). Regulation of p53 and its targets during involution of the mammary gland. *J. Mammary Gland. Biol. Neoplasia* 4, 177–181. doi:10.1023/a:1018777224808

Jung, Y., Stratton, S. A., Lee, S. H., Kim, M., Jun, S., Zhang, J., et al. (2021). TMEM9-v-ATPase activates wnt/β-catenin signaling via APC lysosomal degradation for liver regeneration and tumorigenesis. *Hepatology* 73, 776–794. doi:10.1002/hep.31305

Kar, P., Chakraborti, T., Samanta, K., and Chakraborti, S. (2009). mu-Calpain mediated cleavage of the Na+/Ca2+ exchanger in isolated mitochondria under A23187 induced Ca2+ stimulation. *Archives Biochem. Biophysics* 482, 66–76. doi:10.1016/j.abb.2008.11.024

Khan, Q. A., Dipple, A., and Anderson, L. M. (2002). Protease inhibitor-induced stabilization of p21waf1/cip1 and cell-cycle arrest in chemical carcinogen-exposed mammary and lung cells. *Mol. Carcinog.* 33, 1–8. doi:10.1002/mc.10013

Kim, J. Y., Lee, N., Kim, Y.-J., Cho, Y., An, H., Oh, E., et al. (2017). Disulfiram induces anoikis and suppresses lung colonization in triple-negative breast cancer via calpain activation. *Cancer Lett.* 386, 151–160. doi:10.1016/j.canlet.2016.11.022

Kiss, R., Kovács, D., Tompa, P., and Perczel, A. (2008). Local structural preferences of calpastatin, the intrinsically unstructured protein inhibitor of calpain. *Biochemistry* 47, 6936–6945. doi:10.1021/bi800201a

Kosenko, E., Poghosyan, A., and Kaminsky, Y. (2011). Subcellular compartmentalization of proteolytic enzymes in brain regions and the effects of chronic β-amyloid treatment. *Brain Res.* 1369, 184–193. doi:10.1016/j.brainres.2010.10.078

Kreuzaler, P. A., Staniszewska, A. D., Li, W., Omidvar, N., Kedjouar, B., Turkson, J., et al. (2011). Stat3 controls lysosomal-mediated cell death *in vivo*. *Nat. Cell Biol.* 13, 303–309. doi:10.1038/ncb2171

Kubbutat, M. H. G., and Vousden, K. H. (1997). Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. *Mol. Cell. Biol.* 17, 460–468. doi:10.1128/MCB.17.1.460

Kulkarni, S., Goll, D. E., and Fox, J. E. B. (2002). Calpain cleaves RhoA generating a dominant-negative form that inhibits integrin-induced actin filament assembly and cell spreading. *J. Biol. Chem.* 277, 24435–24441. doi:10.1074/jbc.M203457200

Kulkarni, S., Reddy, K. B., Esteva, F. J., Moore, H. C. F., Budd, G. T., and Tubbs, R. R. (2010). Calpain regulates sensitivity to trastuzumab and survival in HER2-positive breast cancer. *Oncogene* 29, 1339–1350. doi:10.1038/onc.2009.422

Kuroda, M., Tanabe, H., Yoshida, K., Oikawa, K., Saito, A., Kiyuna, T., et al. (2004). Alteration of chromosome positioning during adipocyte differentiation. *J. Cell Sci.* 117, 5897–5903. doi:10.1242/jcs.01508

Landskroner-Eiger, S., Park, J., Israel, D., Pollard, J. W., and Scherer, P. E. (2010). Morphogenesis of the developing mammary gland: stage-dependent impact of adipocytes. *Dev. Biol.* 344, 968–978. doi:10.1016/j.ydbio.2010.06.019

Lee, W.-L., and Shyur, L.-F. (2012). Deoxyelephantopin impedes mammary adenocarcinoma cell motility by inhibiting calpain-mediated adhesion dynamics and inducing reactive oxygen species and aggresome formation. *Free Radic. Biol. Med.* 52, 1423–1436. doi:10.1016/j.freeradbiomed.2012.01.020

Leloup, L., Shao, H., Bae, Y. H., Deasy, B., Stolz, D., Roy, P., et al. (2010). m-calpain activation is regulated by its membrane localization and by its binding to phosphatidylinositol 4,5-bisphosphate*. *J. Biol. Chem.* 285, 33549–33566. doi:10.1074/jbc.M110.123604

Li, C.-L., Yang, D., Cao, X., Wang, F., Hong, D.-Y., Wang, J., et al. (2017). Fibronectin induces epithelial-mesenchymal transition in human breast cancer MCF-7 cells via activation of calpain. *Oncol. Lett.* 13, 3889–3895. doi:10.3892/ol.2017.5896

Libertini, S. J., Robinson, B. S., Dhillon, N. K., Glick, D., George, M., Dandekar, S., et al. (2005). Cyclin E both regulates and is regulated by calpain 2, a protease associated with metastatic breast cancer phenotype. *Cancer Res.* 65, 10700–10708. doi:10.1158/0008-5472.CAN-05-1666

Lim, E., Wu, D., Pal, B., Bouras, T., Asselin-Labat, M.-L., Vaillant, F., et al. (2010). Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. *Breast Cancer Res.* 12, R21. doi:10.1186/bcr2560

Lloyd-Lewis, B., Krueger, C. C., Sargeant, T. J., D'Angelo, M. E., Deery, M. J., Feret, R., et al. (2018). Stat3-mediated alterations in lysosomal membrane protein composition. *J. Biol. Chem.* 293, 4244–4261. doi:10.1074/jbc.RA118.001777

Lu, L., Meehan, M. J., Gu, S., Chen, Z., Zhang, W., Zhang, G., et al. (2015). Mechanism of action of thalassospiramides, A new class of calpain inhibitors. *Sci. Rep.* 5, 8783. doi:10.1038/srep08783

Macchia, P. E., Nettore, I. C., Franchini, F., Santana-Viera, L., and Ungaro, P. (2021). Epigenetic regulation of adipogenesis by histone-modifying enzymes. *Epigenomics* 13, 235–251. doi:10.2217/epi-2020-0304

Macdonald, H. R. (2020). Pregnancy associated breast cancer. *Breast J.* 26, 81–85. doi:10.1111/tbj.13714

MacLeod, J. A., Gao, Y., Hall, C., Muller, W. J., Gujral, T. S., and Greer, P. A. (2018). Genetic disruption of calpain-1 and calpain-2 attenuates tumorigenesis in mouse models of HER2+ breast cancer and sensitizes cancer cells to doxorubicin and lapatinib. *Oncotarget* 9, 33382–33395. doi:10.18632/oncotarget.26078

Mandic, A., Viktorsson, K., Strandberg, L., Heiden, T., Hansson, J., Linder, S., et al. (2002). Calpain-mediated Bid cleavage and calpain-independent bak modulation: two separate pathways in cisplatin-induced apoptosis. *Mol. Cell. Biol.* 22, 3003–3013. doi:10.1128/MCB.22.9.3003-3013.2002

Margaryan, N. V., Kirschmann, D. A., Lipavsky, A., Bailey, C. M., Hendrix, M. J. C., and Khalkhali-Ellis, Z. (2010). New insights into cathepsin D in mammary tissue development and remodeling. *Cancer Biol. Ther.* 10, 457–466. doi:10.4161/cbt.10.5.12534

McHenry, P., Wang, W. L. W., Devitt, E., Kluesner, N., Davisson, V. J., McKee, E., et al. (2009). Iejimalides A and B inhibit lysosomal vacuolar H^+-ATPase (V-ATPase) activity and induce S-phase arrest and apoptosis in MCF-7 cells. *J. Cell. Biochem.* 109, 634–642. doi:10.1002/jcb.22438

Meier-Abt, F., and Bentires-Alj, M. (2014). How pregnancy at early age protects against breast cancer. *Trends Mol. Med.* 20, 143–153. doi:10.1016/j.molmed.2013.11.002

Miyazaki, T., Akasu, R., and Miyazaki, A. (2021). Calpain-associated proteolytic regulation of the stromal microenvironment in cancer. *Curr. Pharm. Des.* 27, 3128–3138. doi:10.2174/1381612827666210311143053

Miyazaki, T., Taketomi, Y., Saito, Y., Hosono, T., Lei, X.-F., Kim-Kaneyama, J., et al. (2015). Calpastatin counteracts pathological angiogenesis by inhibiting suppressor of cytokine signaling 3 degradation in vascular endothelial cells. *Circ. Res.* 116, 1170–1181. doi:10.1161/CIRCRESAHA.116.305363

Monks, J., Rosner, D., Jon Geske, F., Lehman, L., Hanson, L., Neville, M. C., et al. (2005). Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release. *Cell Death Differ.* 12, 107–114. doi:10.1038/sj.cdd.4401517

Moretti, D., Del Bello, B., Allavena, G., and Maellaro, E. (2014). Calpains and cancer: friends or enemies? *Archives Biochem. Biophysics* 564, 26–36. doi:10.1016/j.abb.2014.09.018

Nian, H., and Ma, B. (2021). Calpain–calpastatin system and cancer progression. *Biol. Rev.* 96, 961–975. doi:10.1111/brv.12686

Norberg, E., Orrenius, S., and Zhivotovsky, B. (2010). Mitochondrial regulation of cell death: processing of apoptosis-inducing factor (AIF). *Biochem. Biophysical Res. Commun.* 396, 95–100. doi:10.1016/j.bbrc.2010.02.163

Ono, Y., Saido, T. C., and Sorimachi, H. (2016). Calpain research for drug discovery: challenges and potential. *Nat. Rev. Drug Discov.* 15, 854–876. doi:10.1038/nrd.2016.212

Ono, Y., and Sorimachi, H. (2012). Calpains — an elaborate proteolytic system. *Biochimica Biophysica Acta Proteins Proteomics* 1824, 224–236. doi:10.1016/j.bbapap.2011.08.005

Ostenfeld, M. S., Fehrenbacher, N., Høyer-Hansen, M., Thomsen, C., Farkas, T., and Jäättelä, M. (2005). Effective tumor cell death by σ-2 receptor ligand siramesine involves lysosomal leakage and oxidative stress. *Cancer Res.* 65, 8975–8983. doi:10.1158/0008-5472.CAN-05-0269

Paavolainen, O., and Peuhu, E. (2021). Integrin-mediated adhesion and mechanosensing in the mammary gland. *Seminars Cell & Dev. Biol.* 114, 113–125. doi:10.1016/j.semcdb.2020.10.010

Pardo-Pastor, C., Rubio-Moscardo, F., Vogel-González, M., Serra, S. A., Afthinos, A., Mrkonjic, S., et al. (2018). Piezo2 channel regulates RhoA and actin cytoskeleton to promote cell mechanobiological responses. *Proc. Natl. Acad. Sci. U.S.A.* 115, 1925–1930. doi:10.1073/pnas.1718177115

Pariat, M., Salvat, C., Bébien, M., Brockly, F., Altieri, E., Carillo, S., et al. (2000). The sensitivity of c-Jun and c-Fos proteins to calpains depends on conformational determinants of the monomers and not on formation of dimers. *Biochem. J.* 345 (1), 129–138. doi:10.1042/bj3450129

Pehere, A. D., Nguyen, S., Garlick, S. K., Wilson, D. W., Hudson, I., Sykes, M. J., et al. (2019). Tripeptide analogues of MG132 as protease inhibitors. *Bioorg. Med. Chem.* 27, 436–441. doi:10.1016/j.bmc.2018.12.022

Pianetti, S., Arsura, M., Romieu-Mourez, R., Coffey, R. J., and Sonenshein, G. E. (2001). Her-2/neu overexpression induces NF-kappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN. *Oncogene* 20, 1287–1299. doi:10.1038/sj.onc.1204257

Piao, S., and Amaravadi, R. K. (2016). Targeting the lysosome in cancer: targeting the lysosome in cancer. *Ann. N.Y. Acad. Sci.* 1371, 45–54. doi:10.1111/nyas.12953

Pink, J. J., Wuerzberger-Davis, S., Tagliarino, C., Planchon, S. M., Yang, X., Froelich, C. J., et al. (2000). Activation of a cysteine protease in MCF-7 and T47D breast cancer cells during β-lapachone-mediated apoptosis. *Exp. Cell Res.* 255, 144–155. doi:10.1006/excr.1999.4790

Potz, B. A., Abid, M. R., and Sellke, F. W. (2016). Role of calpain in pathogenesis of human disease processes. *J. Nat. Sci.* 2, e218.

Pozo-Guisado, E., Merino, J. M., Mulero-Navarro, S., Lorenzo-Benayas, M. J., Centeno, F., Alvarez-Barrientos, A., et al. (2005). Resveratrol-induced apoptosis in MCF-7 human breast cancer cells involves a caspase-independent mechanism with downregulation of Bcl-2 and NF-kappaB. *Int. J. Cancer* 115, 74–84. doi:10.1002/ijc.20856

Pu, X., Storr, S. J., Ahmad, N. S., Chan, S. Y., Moseley, P. M., Televantou, D., et al. (2016). Calpain-1 is associated with adverse relapse free survival in breast cancer: a confirmatory study. *Histopathology* 68, 1021–1029. doi:10.1111/his.12896

Raimondi, M., Marcassa, E., Cataldo, F., Arnandis, T., Mendoza-Maldonado, R., Bestagno, M., et al. (2016). Calpain restrains the stem cells compartment in breast cancer. *Cell Cycle* 15, 106–116. doi:10.1080/15384101.2015.1121325

Rajković-Molek, K., Mustać, E., Avirović, M., Georgev, P., Demaria, M., Aničić, J., et al. (2020). The expression of calpain-1 and androgen receptor in breast cancer and their correlation with clinicopathological characteristics: an immunohistochemical retrospective study. *Pathology - Res. Pract.* 216, 153068. doi:10.1016/j.prp.2020.153068

Rauner, G., and Kuperwasser, C. (2021). Microenvironmental control of cell fate decisions in mammary gland development and cancer. *Dev. Cell* 56, 1875–1883. doi:10.1016/j.devcel.2021.06.016

Raynaud, F., Carnac, G., Marcilhac, A., and Benyamin, Y. (2004). m-Calpain implication in cell cycle during muscle precursor cell activation. *Exp. Cell Res.* 298, 48–57. doi:10.1016/j.yexcr.2004.03.053

Raynaud, F., Marcilhac, A., Chebli, K., Benyamin, Y., and Rossel, M. (2008). Calpain 2 expression pattern and sub-cellular localization during mouse embryogenesis. *Int. J. Dev. Biol.* 52, 383–388. doi:10.1387/ijdb.072448fr

Rios-Doria, J., Day, K. C., Kuefer, R., Rashid, M. G., Chinnaiyan, A. M., Rubin, M. A., et al. (2003). The role of calpain in the proteolytic cleavage of E-cadherin in prostate and mammary epithelial cells. *J. Biol. Chem.* 278, 1372–1379. doi:10.1074/jbc.M208772200

Rios-Doria, J., Kuefer, R., Ethier, S. P., and Day, M. L. (2004). Cleavage of β-catenin by calpain in prostate and mammary tumor cells. *Cancer Res.* 64, 7237–7240. doi:10.1158/0008-5472.CAN-04-1048

Rodríguez-Fernández, L., Company, S., Zaragozá, R., Viña, J. R., and García-Trevijano, E. R. (2021). Cleavage and activation of LIM kinase 1 as a novel mechanism for calpain 2-mediated regulation of nuclear dynamics. *Sci. Rep.* 11, 16339. doi:10.1038/s41598-021-95797-8

Rodríguez-Fernández, L. (2019). Subcellular distribution of calpain-1 and calpain-2 as a key event for calpain-mediated functions in physiological and neoplastic mammary models. http://hdl.handle.net/10550/69799.

Ross, A. C., Xu, Y., Lu, L., Kersten, R. D., Shao, Z., Al-Suwailem, A. M., et al. (2013). Biosynthetic multitasking facilitates thalassospiramide structural diversity in marine bacteria. *J. Am. Chem. Soc.* 135, 1155–1162. doi:10.1021/ja3119674

Salma, N., Xiao, H., Mueller, E., and Imbalzano, A. N. (2004). Temporal recruitment of transcription factors and SWI/SNF chromatin-remodeling enzymes during adipogenic induction of the peroxisome proliferator-activated receptor γ nuclear hormone receptor. *Mol. Cell. Biol.* 24, 4651–4663. doi:10.1128/MCB.24.11.4651-4663.2004

Sareen, D., Darjatmoko, S. R., Albert, D. M., and Polans, A. S. (2007). Mitochondria, calcium, and calpain are key mediators of resveratrol-induced apoptosis in breast cancer. *Mol. Pharmacol.* 72, 1466–1475. doi:10.1124/mol.107.039040

Shao, H., Chou, J., Baty, C. J., Burke, N. A., Watkins, S. C., Stolz, D. B., et al. (2006). Spatial localization of m-calpain to the plasma membrane by phosphoinositide biphosphate binding during epidermal growth factor receptor-mediated activation. *Mol. Cell. Biol.* 26, 5481–5496. doi:10.1128/MCB.02243-05

Shapovalov, I., Harper, D., and Greer, P. A. (2022). Calpain as a therapeutic target in cancer. *Expert Opin. Ther. Targets* 26, 217–231. doi:10.1080/14728222.2022.2047178

Sheng, J.-J., Chang, H., and Yu, Z.-B. (2015). Nuclear translocation of calpain-2 mediates apoptosis of hypertrophied cardiomyocytes in transverse aortic constriction rat: mechanisms of apoptosis in hypertrophied cardiomyocytes. *J. Cell. Physiol.* 230, 2743–2754. doi:10.1002/jcp.24999

Shinkai-Ouchi, F., Shindo, M., Doi, N., Hata, S., and Ono, Y. (2020). Calpain-2 participates in the process of calpain-1 inactivation. *Biosci. Rep.* 40, BSR20200552. doi:10.1042/BSR20200552

Sobhan, P. K., Seervi, M., Deb, L., Varghese, S., Soman, A., Joseph, J., et al. (2013). Calpain and reactive oxygen species targets bax for mitochondrial permeabilisation and caspase activation in zerumbone induced apoptosis. *PLoS ONE* 8, e59350. doi:10.1371/journal.pone.0059350

Sorimachi, H., Hata, S., and Ono, Y. (2011). Impact of genetic insights into calpain biology. *J. Biochem.* 150, 23–37. doi:10.1093/jb/mvr070

Stewart, T. A., Hughes, K., Stevenson, A. J., Marino, N., Ju, A. L., Morehead, M., et al. (2020). Mammary mechanobiology: investigating roles for mechanically-activated ion channels in lactation and involution. *J. Cell Sci. jcs* 134, 248849. doi:10.1242/jcs.248849

Storr, S. J., Carragher, N. O., Frame, M. C., Parr, T., and Martin, S. G. (2011a). The calpain system and cancer. *Nat. Rev. Cancer* 11, 364–374. doi:10.1038/nrc3050

Storr, S. J., Lee, K. W., Woolston, C. M., Safuan, S., Green, A. R., Macmillan, R. D., et al. (2012). Calpain system protein expression in basal-like and triple-negative invasive breast cancer. *Ann. Oncol.* 23, 2289–2296. doi:10.1093/annonc/mds176

Storr, S. J., Thompson, N., Pu, X., Zhang, Y., and Martin, S. G. (2015). Calpain in breast cancer: role in disease progression and treatment response. *Pathobiology* 82, 133–141. doi:10.1159/000430464

Storr, S. J., Woolston, C. M., Barros, F. F. T., Green, A. R., Shehata, M., Chan, S. Y., et al. (2011b). Calpain-1 expression is associated with relapse-free survival in breast cancer patients treated with trastuzumab following adjuvant chemotherapy. *Int. J. Cancer* 129, 1773–1780. doi:10.1002/ijc.25832

Storr, S. J., Zhang, S., Perren, T., Lansdown, M., Fatayer, H., Sharma, N., et al. (2016). The calpain system is associated with survival of breast cancer patients with large but operable inflammatory and non-inflammatory tumours treated with neoadjuvant chemotherapy. *Oncotarget* 7, 47927–47937. doi:10.18632/oncotarget.10066

Tagliarino, C., Pink, J. J., Reinicke, K., Simmers, S. M., and Boothman, D. A. (2003). Mu-calpain activation in beta-lapachone-mediated apoptosis. *Cancer Biol. Ther.* 2, 141–152. doi:10.4161/cbt.2.2.237

Tan, M., Li, P., Klos, K. S., Lu, J., Lan, K.-H., Nagata, Y., et al. (2005). ErbB2 promotes src synthesis and stability: novel mechanisms of src activation that confer breast cancer metastasis. *Cancer Res.* 65, 1858–1867. doi:10.1158/0008-5472.CAN-04-2353

Tan, Y., Wu, C., De Veyra, T., and Greer, P. A. (2006). Ubiquitous calpains promote both apoptosis and survival signals in response to different cell death stimuli. *J. Biol. Chem.* 281, 17689–17698. doi:10.1074/jbc.M601978200

Telechea-Fernández, M., Rodríguez-Fernández, L., García, C., Zaragozá, R., Viña, J. R., Cervantes, A., et al. (2018). New localization and function of calpain-2 in nucleoli of colorectal cancer cells in ribosomal biogenesis: effect of KRAS status. *Oncotarget* 9, 9100–9113. doi:10.18632/oncotarget.23888

Torres, L., Serna, E., Bosch, A., Zaragozá, R., García, C., Miralles, V. J., et al. (2011). NF-κB as node for signal amplification during weaning. *Cell Physiol. Biochem.* 28, 833–846. doi:10.1159/000335797

Vaish, V., and Sanyal, S. N. (2012). Role of sulindac and celecoxib in chemoprevention of colorectal cancer via intrinsic pathway of apoptosis: exploring NHE-1, intracellular calcium homeostasis and calpain 9. *Biomed. Pharmacother.* 66, 116–130. doi:10.1016/j.biopha.2011.11.019

Vizovisek, M., Ristanovic, D., Menghini, S., Christiansen, M. G., and Schuerle, S. (2021). The tumor proteolytic landscape: A challenging frontier in cancer diagnosis and therapy. *IJMS* 22, 2514. doi:10.3390/ijms22052514

Wang, Q. A., and Scherer, P. E. (2019). Remodeling of murine mammary adipose tissue during pregnancy, lactation, and involution. *J. Mammary Gland. Biol. Neoplasia* 24, 207–212. doi:10.1007/s10911-019-09434-2

Wang, X. D., Rosales, J. L., Magliocco, A., Gnanakumar, R., and Lee, K.-Y. (2003). Cyclin E in breast tumors is cleaved into its low molecular weight forms by calpain. *Oncogene* 22, 769–774. doi:10.1038/sj.onc.1206166

Wang, Y., Liu, Y., Bi, X., and Baudry, M. (2020). Calpain-1 and calpain-2 in the brain: new evidence for a critical role of calpain-2 in neuronal death. *Cells* 9, 2698. doi:10.3390/cells9122698

Ward, R. E., and German, J. B. (2004). Understanding milk's bioactive components: A goal for the genomics toolbox. *J. Nutr.* 134, 962S–967S. doi:10.1093/jn/134.4.962S

Watson, C. J. (2006). Involution: apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ. *Breast Cancer Res.* 8, 203. doi:10.1186/bcr1401

Watson, C. J., and Khaled, W. T. (2020). Mammary development in the embryo and adult: new insights into the journey of morphogenesis and commitment. *Development* 147, dev169862. doi:10.1242/dev.169862

Wheelock, M. J., Buck, C. A., Bechtol, K. B., and Damsky, C. H. (1987). Soluble 80-kd fragment of cell-CAM 120/80 disrupts cell-cell adhesion. *J. Cell. Biochem.* 34, 187–202. doi:10.1002/jcb.240340305

Xu, Y., Bismar, T. A., Su, J., Xu, B., Kristiansen, G., Varga, Z., et al. (2010). Filamin A regulates focal adhesion disassembly and suppresses breast cancer cell migration and invasion. *J. Exp. Med.* 207, 2421–2437. doi:10.1084/jem.20100433

Yu, B.-H., Li, B.-Z., Zhou, X.-Y., Shi, D.-R., and Yang, W.-T. (2018). Cytoplasmic FOXP1 expression is correlated with ER and calpain II expression and predicts a poor outcome in breast cancer. *Diagn Pathol.* 13, 36. doi:10.1186/s13000-018-0715-y

Zaragozá, R., García-Trevijano, E. R., Lluch, A., Ribas, G., and Viña, J. R. (2015). Involvement of Different networks in mammary gland involution after the pregnancy/lactation cycle: implications in breast cancer. *IUBMB Life* 67, 227–238. doi:10.1002/iub.1365

Zaragozá, R., Miralles, V. J., Rus, A. D., García, C., Carmena, R., García-Trevijano, E. R., et al. (2005). Weaning induces NOS-2 expression through NF-kappaB modulation in the lactating mammary gland: importance of GSH. *Biochem. J.* 391, 581–588. doi:10.1042/BJ20050507

Zaragozá, R., Torres, L., García, C., Eroles, P., Corrales, F., Bosch, A., et al. (2009). Nitration of cathepsin D enhances its proteolytic activity during mammary gland remodelling after lactation. *Biochem. J.* 419, 279–288. doi:10.1042/BJ20081746

frontiers | Frontiers in Cell and Developmental Biology

TYPE Review
PUBLISHED 26 September 2023
DOI 10.3389/fcell.2023.1258843

OPEN ACCESS

EDITED BY
Rocío López Posadas,
University Hospital Erlangen, Germany

REVIEWED BY
Kristina Koop,
University Hospital Erlangen, Germany
Andrew G. Clark,
University of Stuttgart, Germany

*CORRESPONDENCE
Dolores Ortiz-Masià,
m.dolores.ortiz@uv.es

RECEIVED 14 July 2023
ACCEPTED 14 September 2023
PUBLISHED 26 September 2023

CITATION
Macias-Ceja DC, Mendoza-Ballesteros MT, Ortega-Albiach M, Barrachina MD and Ortiz-Masià D (2023), Role of the epithelial barrier in intestinal fibrosis associated with inflammatory bowel disease: relevance of the epithelial-to mesenchymal transition.
Front. Cell Dev. Biol. 11:1258843.
doi: 10.3389/fcell.2023.1258843

Role of the epithelial barrier in intestinal fibrosis associated with inflammatory bowel disease: relevance of the epithelial-to mesenchymal transition

Dulce C. Macias-Ceja[1], M. Teresa Mendoza-Ballesteros[2], María Ortega-Albiach[3], M. Dolores Barrachina[1] and Dolores Ortiz-Masià[1,4]*

[1]Departamento de Farmacología and CIBEREHD, Facultad de Medicina, Universidad de Valencia, Valencia, Spain, [2]Instituto de Educación Superior Isabel de Villena, Conselleria de Educación, Cultura y Deporte, Valencia, Spain, [3]INCLIVA Biomedical Research Institute, Valencia, Spain, [4]Departamento de Medicina, Facultad de Medicina, Universidad de Valencia, Valencia, Spain

In inflammatory bowel disease (IBD), chronic inflammation in the gastrointestinal tract can lead to tissue damage and remodelling, which can ultimately result in fibrosis. Prolonged injury and inflammation can trigger the activation of fibroblasts and extracellular matrix (ECM) components. As fibrosis progresses, the tissue becomes increasingly stiff and less functional, which can lead to complications such as intestinal strictures, obstructive symptoms, and eventually, organ dysfunction. Epithelial cells play a key role in fibrosis, as they secrete cytokines and growth factors that promote fibroblast activation and ECM deposition. Additionally, epithelial cells can undergo a process called epithelial-mesenchymal transition, in which they acquire a more mesenchymal-like phenotype and contribute directly to fibroblast activation and ECM deposition. Overall, the interactions between epithelial cells, immune cells, and fibroblasts play a critical role in the development and progression of fibrosis in IBD. Understanding these complex interactions may provide new targets for therapeutic interventions to prevent or treat fibrosis in IBD. In this review, we have collected and discussed the recent literature highlighting the contribution of epithelial cells to the pathogenesis of the fibrotic complications of IBD, including evidence of EMT, the epigenetic control of the EMT, the potential influence of the intestinal microbiome in EMT, and the possible therapeutic strategies to target EMT. Finally we discuss the pro-fibrotic interactions epithelial-immune cells and epithelial-fibroblasts cells.

KEYWORDS

fibrosis, epithelial cells, intestinal fibrosis, inflammatory bowel disease, crohn, colitis, epithelial mesenchymal transition

1 Introduction

Inflammatory bowel disease (IBD) is a complex condition influenced by a combination of genetic, environmental, and immunological factors. Environmental factors, such as changes in diet, increased hygiene practices, and alterations in the gut microbiota, are believed to play a role in the development of IBD. The two main types of IBD are Crohn's

disease (CD) and ulcerative colitis (UC). CD is characterized by inflammation that can occur anywhere in the gastrointestinal (GI) tract, although it affects most commonly the small intestine and the right colon. The inflammation in CD involves multiple layers of the bowel wall. On the other hand, UC is limited to the colon and the rectum. Both, CD and UC, are chronic conditions characterised by periods of active disease and periods of remission, and the eventual development of intestinal fibrosis. This inevitable progression towards fibrosis suggests that fibrosis becomes inflammation-independent and auto-propagative (Santacroce et al., 2022; Park et al., 2023). The course and extent of fibrosis show significant variability between individual patients, indicating a genetic component (Jarmakiewicz-Czaja et al., 2023; Macias-Ceja et al., 2023). In CD, approximately 50% of patients develop fibrotic strictures or penetrating lesions (Cosnes et al., 2011) and it is estimated that up to 70% of patients will eventually require surgery at some point during their disease course (Yoo et al., 2020). Despite undergoing surgical interventions, it is not uncommon for patients to experience post-operative recurrence of fibrosis, particularly at the site of an ileocolonic anastomosis. This recurrence can lead to the development of re-stricturing disease, potentially necessitating additional surgeries (Gklavas et al., 2017). The incidence of intestinal strictures in CD has not significantly changed, as current anti-inflammatory therapies neither prevent nor reverse the established fibrosis/strictures, indicating that control of inflammation does not essentially affect the fibrotic course.

Intestinal fibrosis involves the accumulation of extracellular matrix (ECM) components in the intestinal wall, and this process is driven by activated cells of mesenchymal source, including fibroblasts, myofibroblasts, and smooth muscle cells. The deposition of ECM differs between UC and CD: in UC, fibrosis is primarily restricted to the superficial layers of the intestine (mucosal and submucosal layers) (Gordon et al., 2014), while fibrosis in CD occurs mainly in the ileocecal valve and can affect the entire thickness of the bowel wall due to the transmural nature of the inflammation (Yoo et al., 2020). Currently, one of the main goals of IBD treatment is to induce wound healing. Mucosal healing is a biological process activated by inflammation that is capable, depending on the equilibrium between production/degradation of the ECM component, of either restoring the integrity of the damaged epithelial barrier with reconstitution of normal intestinal function or triggering fibrosis (D'Haens et al., 2022; Otte et al., 2023).

Various factors contribute to the development and progression of fibrosis in IBD. Soluble molecules, including growth factors and cytokines, play a significant role, with transforming growth factor-beta 1 (TGFβ1) being considered a key player. These molecules are released by activated immune and nonimmune cells and contribute to the activation of fibroblasts and myofibroblasts, leading to excessive ECM production and fibrotic remodelling. In addition to soluble molecules, other mechanisms involved in intestinal fibrosis include G protein-coupled receptors, the gut microbiota and epithelial-to-mesenchymal transition (EMT) or endothelial-to-mesenchymal transition (EndoEMT), which are processes where epithelial or endothelial cells acquire a mesenchymal phenotype and contribute to fibrosis (D'Alessio et al., 2022).

Since fibrosis can progress once established, regardless of whether inflammation is suppressed or not, antifibrotic drugs are now targeting mechanisms that are independent of inflammation (Solitano et al., 2023). Science has focused on various aspects, including the inflammation-independent mechanisms behind the gut fibrotic process (Zhao et al., 2020), or environmental (Amamou et al., 2022) and genetic risk factors (Macias-Ceja et al., 2023). This has led to a deeper exploration of aspects such as aberrant wound healing, dysregulated extracellular matrix production, and activation of specific cell types (such as fibroblasts) that promote fibrogenesis (Yoo et al., 2020). The traditional opinion that intestinal fibrosis is an irreversible process is changing in light of an improved understanding of the cellular and molecular mechanisms that underline the pathogenesis of fibrosis.

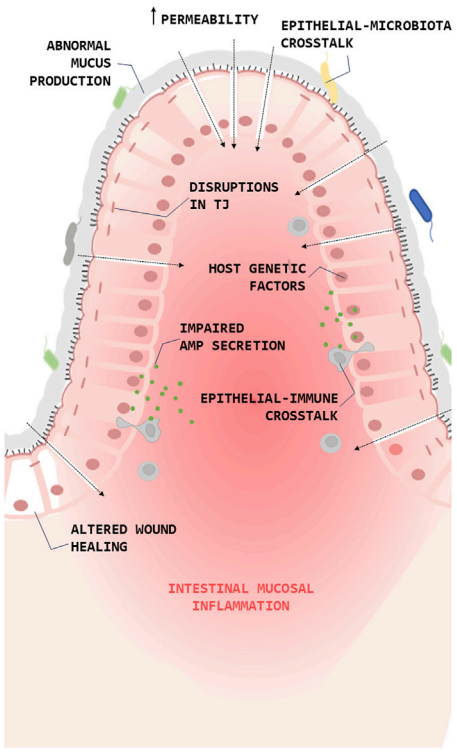


FIGURE 1
Role of the epithelial barrier in the pathophysiology of Inflammatory Bowel Diseases (IBDs). Simplified illustration of the role of epithelial barrier in the pathophysiology of IBD. The illustration shows the main processes in which epithelial cells are involved in the pathogenesis of IBD. Antimicrobial peptide (AMP); Tight junction (TJ).

Epithelial cells are involved in the fibrotic process at both the cellular and molecular level. They can secrete cytokines and growth factors, such as TGFβ and platelet-derived growth factor (PDGF), that promote the activation of fibroblasts or extend the pool of mesenchymal cells through the EMT process. The interactions between epithelial cells, immune cells, and fibroblasts form a complex network that promotes the development and progression of fibrosis in IBD. In this review, we have collected and discussed the recent literature highlighting the contribution of epithelial cells to the pathogenesis of the fibrotic complications of IBD, including evidence of EMT, the epigenetic control of EMT, the potential influence of the intestinal microbiome in EMT, and the

possible therapeutic strategies to target EMT. Finally, we discuss the pro-fibrotic interactions between epithelial cells, immune cells, and fibroblasts.

2 A brief outline of the epithelial barrier role in the pathophysiology of IBD

Under normal and homeostasis conditions, the intestinal epithelial barrier comprises a thick mucosal layer that is associated with specialized intestinal epithelial cells (IECs) linked together by tight junctions (TJs) and resident microbiota, collectively forming a healthy layer. Intestinal stem cells, located in the base of the crypt, divide and differentiate to give rise to five different types of IECs (enterocytes, Paneth cells, goblet cells (GCs), enteroendocrine cells and microfold cells) maintaining the integrity of the intestinal epithelium.

It is known that the loss of barrier integrity and the increase in overall barrier permeability are fundamental processes in the IBD pathophysiology (barrier loss activates immunoregulatory processes) (Kotla and Rochev, 2023). The barrier loss can be triggered by various factors, including disruptions in the tight junction, abnormal mucus production, impaired antimicrobial peptide (AMPs) secretion, altered wound healing, or environmental and genetic factors (Figure 1). In IBD, the high levels of proinflammatory (T helper type 1 (Th1)) cytokines (Tumor necrosis factor alpha (TNFα), Interleukin (IL)1β, and IL6) change the composition of the TJs decreasing transepithelial electrical resistance and amplify mucosal inflammation (Lee, 2015). Along this line, clinical studies have shown a decrease in the expression and redistribution of the junctional complexes in both UC (Blair et al., 2006) and CD (Zeissig et al., 2007) patients.

Over the years, a series of studies showed that mucin expression/secretion by GCs (the primary secretory cells of the GI trac) is mediated by cytokines (both Th1 and Th2 cytokines), inflammasome related proteins (autophagy dysregulation), gut microbiota and the diet (Melhem et al., 2021). For instance, Western diets (high fat/high sugar) lead to endoplasmic reticulum stress and oxidative stress in GCs reducing the production/secretion of mucins (Gulhane et al., 2016) and alters microbial communities, improving the colonization of *E. coli* (Martinez-Medina et al., 2014) or favouring an overgrowth of pro-inflammatory bacteria, such as *Proteobacteria* (Agus et al., 2016). This, together with the fact that the products derived from bacteria can regulate the production and secretion of mucin, thus promoting the loss of the integrity of the epithelial barrier (Figure 1). Lastly, epithelial repair is known to be altered in IBD and this is reflected in the creation of aberrant intestinal anastomosis after a bowel resection, giving rise to the recurrence of the disease in the same place (Kelm and Anger, 2022).

Mucosal healing is a complex process that encompasses the migration/proliferation of IECs as well as regulation by gut microbial peptides, and growth factors (Alam and Neish, 2018), that can be altered by genetic and epigenetic factors. Genome-wide association studies (GWAS) have indicated risk alleles in IBD patients in genes involved in intestinal cell restitution (ERRFI1, PTGER4 or HNF4), in cell polarity (PARD3) or in intercellular junctions (MYO9B, MAGI2, GNAI2, LAMB1 or CDH1) (McCole, 2014). At a epigenetic level, long non coding RNA (ncRNA) CCAT1 and FBXL19-AS1 (Ma et al., 2019; Zhao et al., 2022), circular CDKN2B-AS1 and SMAD4 (Rankin et al., 2019; Zhao et al., 2023) and microRNAs (miR) miR-21, miR23a, miR-182-5p (Shi et al., 2013; Yang et al., 2013; Felwick et al., 2020; Xu et al., 2022) overexpression can increase the degradation of the epithelial barrier while miR-195-5p reduces the permeability (Scalavino et al., 2022).

Currently, the goal of IBD therapy (gold standard) in long remission is the epithelial repair and mucosal healing (Colombel et al., 2020; D'Arcangelo and Aloi, 2020). However, there are no approved therapies targeting the epithelium. There are indeed various approaches being explored in the field of epithelial research that have the potential to lead to new therapies, such as the use of epithelial stem cells, growth factors or cytokines, and modifications of the intestinal microbiota (Liu et al., 2021). It is worth noting that these approaches are still under active research and development. However, they represent exciting paths for potential therapeutic interventions in IBD.

3 EMT role in intestinal fibrosis

EMT, first described in 1995 (Hay, 1995), is a reversible process in which the characteristics of epithelial cells are modified until reaching the characteristics of mesenchymal cells, passing through intermediate characteristics between both cell types. In the literature, there are three types of EMT described: the ones associated with embryogenesis/development (type-1 EMT); the ones involved in wound healing (type-2 EMT); and the ones associated with cancer progression (type-3 EMT) (Marconi et al., 2021). Several studies have reported that damaged epithelial cells may act as crucial sources of fibroblasts and contribute to organ fibrosis through type-2 EMT in different fibrotic tissues (Tennakoon et al., 2015; Rout-Pitt et al., 2018; Ortiz-Masiá et al., 2020a; Macias-Ceja et al., 2022; Hadpech and Thongboonkerd, 2023) where specialized epithelial cells give rise to myofibroblasts with profibrotic and pro-inflammatory activity, which expresses α smooth muscle actin (α-SMA) and VIMENTIN but does not express epithelial markers, such as E-CADHERIN (CDH1), ZONULAE OCCLUDENTES (ZOS) or claudins. Various transcriptional factors regulate the process, such as SNAIL Family Transcriptional Repressor (SNAIL1/2), ZINC-FINGER E-BOX-BINDING (ZEB1/2), SLUG or TWIST transcription factors (TWIST1/2) (Xu et al., 2019).

Numerous studies support the role of EMT in the pathogenesis of intestinal fibrosis. In this section we will review the contribution of EMT to the pathogenesis of the fibrotic complications of IBD. Specifically, we review the evidence of EMT in patients, the molecular mechanisms involved, and the role of epigenetic and genetic. Finally, we discuss the role EMT as a therapeutic target in IBD.

3.1 Evidence of EMT in IBD patients and *in vivo* models

In IBD, EMT was observed for the first time in 2008 in the intestinal fistulae of CD patients (Bataille et al., 2008). From

TABLE 1 Reports of EMT in inflammatory bowel diseases (IBDs). Alpha Smooth Muscle Actin (α-SMA); E-cadherin (CDH1); Crohn's disease (CD); N-cadherin (CDH2); Epithelial growth factor (EGF); Fibroblast activation protein (FAP); Fibroblast growth factors (FGF); Fibronectin (FN); Matrix metalloproteinase (MMP); Transforming growth factor β (TGFβ); Tumour necrosis factor (TNF); Tumour necrosis factor receptor (TNFR); Zinc-finger E-box-binding (ZEB).

Intestinal fibrotic samples	EMT histological localization/EMT markers	References
CD N = 15 resections	**Entero-cutaneous fistula specimens** (+) CYTOKERATIN 8/20, β6-INTEGRIN, nuclear B-CATENIN, TGFβ1/2 (−) VIMENTIN, CDH1	Bataille et al. (2008)
IBD N = ¿? resections	**Intestinal crypts** ↑ α-SMA/↓ CDH1 cells	Flier et al. (2010)
CD N = 7 resections	**Entero-cutaneous fistula specimens** (+) SNAIL, FGF1/2/4/7 (−) EGF/TWIST	Scharl et al. (2011)
	Fibrotic lesions (+) SLUG, TNF/TNFR1	
IBD N = 22 biopsies	**Inflamed mucosa** (+) N-CADHERIN, ↑VIMENTIN/↓CDH1 cells	Chen et al. (2013)
CD N = 18 biopsies/resections	**Fibrotic lesions** (+) nuclear B-CATENIN, SLUG, FAP, TGFβ1	Scharl et al. (2015)
IBD N = 20 resections	**Inflamed mucosa** (+) SLUG/SNAIL	Zidar et al. (2016)
CD N = 26 resections	**Fibrotic lesions** ↑VIMENTIN ↓CDH1	Xu X et al. (2017)
IBD pediatric N = 44 biopsies	**Inflamed mucosa** ↑ SNAIL/↓CDH1	Pierdomenico et al. (2018)
IBD N = 10 biopsies	**Inflamed mucosa** ↑ SNAIL, ZEB2, VIMENTIN, MMP9, ↓CDH1	Boros et al. (2018)
CD N = 31biopsies	**Inflamed mucosa** ↑FSP1, VIMENTIN, nuclear B-CATENIN, ↓CDH1	He et al. (2018)
CD N = 57 resections	**Fibrotic lesions/Entero-cutaneous fistula specimens/Intestinal crypts** ↑VIMENTIN/↓ CDH1cells ↑ SNAIL/SLUG, CDH2, DESMIN, ZEB1	Ortiz-Masià et al. (2020b)
IBD N = 16 resections	**Inflamed mucosa** ↑ SNAIL/SLUG	Ortiz-Masiá et al. (2020a)
IBD N = 32 resections	**Fibrotic lesions** ↑FSP1 and α-SMA/↓ CDH1cells (+) nuclear B-CATENIN	Wenxiu et al. (2021)
CD N = 30 biopsies	**Fibrotic lesions** (+) CDH2,VIMENTIN, TIMP1, FN	Wang et al. (2022)
IBD N = 133 biopsies	**Inflamed mucosa** ↑ SNAIL, CDH2 ↓CDH1	Ghorbaninejad et al. (2022)
IBD N = 5 biopsies	**Inflamed mucosa** ↑VIMENTIN/↓ CDH1 cells, nuclear B-CATENIN	Pompili et al. (2023)

2008 to 2023, several studies have revealed the presence of EMT markers in CD and UC patients (Table 1). In CD, the presence of EMT markers has been demonstrated in all disease phenotypes [Montreal classification (Satsangi et al., 2006)]: in transitional cells from entero-cutaneous surrounding fistulae, in fibrotic areas from fistulae, in stenotic tissues and in inflamed mucosa. In relation with type-3 EMT, SLUG expression has been related with tumor progression in CD (Scharl et al., 2014). Regarding UC, the literature about type-2 EMT in intestinal fibrosis is limited. The intestinal samples analysed in most studies do not specify the presence of fibrosis or are performed on inflamed tissue (Table 1). Penetrating or stricturing complications are more common in CD (Thia et al., 2010) than in UC (Yamagata et al., 2011). However, the core problem in UC is the risk of dysplasia/cancer (CD, 2.4%; UC, 10.0%) (Fumery et al., 2015). This could be an explanation about why studies in patients with UC are more directed towards type-3 EMT (Saito et al., 2011; Wang et al., 2012; Tahara et al., 2014; Zhao et al., 2015).

Animal models have played a crucial role in advancing our understanding of intestinal fibrosis. Murine models of intestinal fibrosis include chemical induction (trinitrobenzene sulfonic acid (TNBS) or dextran sodium sulfate (DSS)), genetic manipulation (IL-10KO), ionizing radiation, or surgical techniques (hetero transplantation of small bowel) (Li et al., 2021). A very interesting animal model that has helped to gain insight into the EMT process in intestinal fibrosis are the VillinCre; R26Rosa-lox-STOP-lox-LacZ double transgenic mice, which have made it possible to track mesenchymal cells derived from epithelial cells (Flier et al., 2010). The presence of EMT markers in fibrotic mouse models has been widely demonstrated by Lovisa's review (Lovisa et al., 2019). Two interesting findings from animal models are that one-third of the fibroblasts are derived from epithelial cells in the TNBS model (Flier et al., 2010), or that the cells that enter in EMT do not move and remain in their original anatomical location in the DSS model, favouring fibroblasts transdifferentiation through the release of profibrotic mediators (Zeng et al., 2022).

3.2 EMT in intestinal fibrosis: molecular mechanism

Type-2 EMT is particularly observed in CD. Although the factors that drive type-2 EMT in IBD are not yet fully understood, various signalling pathways (TGFβ/SMAD, WNT, NOTCH, hypoxia-inducible factor-1α (HIF1α) and Hedgehog pathways) and molecules (growth factors, cytokines, proteases, oxidative stress, and hormones) have been implicated. In this section, we review the main pathways and molecules involved in the EMT-modulation of intestinal fibrosis associated with IBD (Table 2) (Figure 2).

TABLE 2 Molecular mechanism implicated in upregulation of type-2 EMT in IBD samples, and *in vivo* and *in vitro* IBD related models. The symbol "/" indicates treatment. Advanced oxidation protein products (AOPPs); Carbohydrate sulfotransferase 15 (CHST15); Crohn's disease (CD); Bone morphogenic protein-7 (BMP7); Dextran sodium sulfate (DSS); Dickkopf-homolog-1 (DKK1); Intestinal epithelial cell (IEC); Interferon (IFN); lipopolysaccharide (LPS); Parathyroid hormone-like hormone (PTHLH); parathyroid hormone receptor 1 (PTH1R); protein kinase A (PKA); Runt-related transcription factor 2 (Runx2); Sonic Hedgehog (SHH); Trinitrobenzene sulfonic acid (TNBS); Tumour necrosis factor-like ligand 1A (TL1A); Transitional cells lining the fistula tract (TC); Transforming growth factor (TGF); Interleukin (IL); Tumour necrosis factor (TNF); Toll-like receptor 4 (TLR4); Ulcerative colitis (UC); Zinc-finger E-box-binding (ZEB).

Protein	Type of study		EMT	Molecular mechanism in EMT	References
TGFβ	*In vitro*	HT29 cells/TGFβ	↑	IL13, SNAIL1	Scharl et al. (2013)
IL13	*In vitro*	HT29 cells/IL13	↑	SLUG	Scharl et al. (2013)
DKK1	*In vitro* Human	HT29 cells/TGFβ1 CD: ↑DKK1 in TC	↑	IL13	Frei et al. (2013)
TL1A	*In vitro In vivo* Human	HT29 cells/TL1A/BMP7 DSS TL1A overexpression IBD: ↑TL1A	↑	TGFβ/Smad3	Wenxiu et al. (2021)
IL22	*In vitro I n In vivo*	Caco-2, HT29 and T84 cells/IL22, TGFβ1, IFNγ, TNFα Toxoplasma model IL22$^{-/-}$	↑	ERK	Delbue et al. (2021)
IL17A	*In vitro In vivo*	IEC6/IL17A Mouse intestine	↑	SNAIL	Zhang et al. (2018)
IFNγ	*In vitro* Human	HT29 cells cocultured with IFNγ -U937 macrophages CD: ↑IFNγ and IFNγ receptor	↑	WNT/FZD4	Macias-Ceja et al. (2022)
SHH	*In vitro* Human	Caco2 cells coculture with LPS-RAW264.7cells/HPI-1 or GANT-61 (HH inhibitors) IBD: ↑SHH activity	↑	SHH	Ghorbaninejad et al. (2022)
WNT2b/ FZD4	*In vitro Human*	HT29 cells/WNT2b CD Biopsies/WNT2b CD: ↑WNT2B/ FZD4	↑	FZD4	Ortiz-Masiá et al. (2020a)
SUCNR1	*In vitro In vivo* Human	HT29/TGFβ Hetero transplantation SUCNR1$^{-/-}$CD B3: ↑Succinate, SUCNR1	↑	WNT	Ortiz-Masiá et al. (2020a)
AXL	*In vitro In vivo* Human	HT29 cells/TNFα TNBS IBD: ↑AXL	↑	ZEB/SNAIL	Boros et al. (2017)
TLR4	*In vitro In vivo*	HCT116 cells/LPS DSS TLR4$^{-/-}$	↑	Cytokine expression	Jun et al. (2020)
AOPPs	*In vitro In vivo* Human	IEC6/AOPPs Rats/AOPPs, apocynin CD: ↑AOPPs	↑	PKC δ- NFκB	Xu X et al. (2017)
ZNF281	*In vitro In vivo* Human	HT29, IBD Biopsies/IFNγ,TNFα DSS IBD: ↑ZNF281	↑	SNAIL	Pierdomenico et al. (2018)
CHST15	*In vitro In vivo*	HCT116/TGFβ DSS CHST15 siRNA	↑	BMP7	Suzuki et al. (2016)
PTHLH	*In vivo* Human	TNBS overexpression PTH1R CD: ↑PTHLH and PTH1R	↑	PKA-Runx2	He et al. (2018)

3.2.1 Cytokines and intestinal type-2 EMT

To begin with, TGFβ is the most important trigger of EMT (Yun et al., 2019). In the *canonical* pathway TGFβ induce the activation of the Small mothers against decapentaplegic (SMAD)2/3/4 complex which regulates the transcription of pro-EMT transcription factors (such SNAIL, ZEB or TWIST). *Non-canonical* (non-SMAD) TGFβ signalling mediates the induction of EMT through the activation of several kinases (ERK1/2, Akt, NFκB, TAK1, p38, JNK, ILK). Several studies indicate that aberrant signalling of TGFβ and its pathways lead to profibrotic EMT in IBD (Johnson et al., 2013; Di Gregorio et al., 2020). *In vitro* studies have shown that TGFβ induces the expression of IL13 via Dickkopf-homolog-1 (a WNT signalling antagonist) in IEC, and both cytokines exert a synergic effect on EMT activation (Frei et al., 2013; Scharl et al., 2013). In fact, the fourth European Crohn's and Colitis Organisation (ECCO) guidelines state that the TGFβ/SMAD pathway activated by IL13 is a central process in the formation of intestinal fibrosis (Latella et al., 2014). Elevated secretion of IL13 is also associated with the expression of TNF-like cytokine 1A (TL1A) in *vivo* models (Giuffrida et al., 2019), a factor that is also capable of promoting EMT via TGFβ/SMAD (Wenxiu et al., 2021), which endorses the role of IL13 in fibrosis. Indeed, TL1A expression is upregulated in both UC and CD patients (Arimura et al., 2014).

Other cytokines involved in triggering EMT in intestinal fibrosis in *vivo* models include IL22 (Delbue et al., 2021) and IL17A (H.-J. Zhang et al., 2018). IL17A is a pro-inflammatory cytokine, mainly produced and secreted by Th17 cells, which contribute to the fibrotic process in multiple organs (Ramani and Biswas, 2019). On the other hand, the role of IL22 (a member of the IL-10 family) is more controversial and seems to depend on the cell type or type of inflammatory trigger (Keir et al., 2020). For instance, in spite of high levels of IL22 seen in IBD, epithelial barrier dysfunction persists (Pelczar et al., 2016) and IL22 trigger EMT via ERK in some preclinical models (Delbue et al., 2021). Interferon gamma (IFNγ) is another cytokine implicated in the activation of EMT in IBD, which acting on macrophages activate WNT signalling pathway (Macias-Ceja et al., 2022).

To sum up, the cytokines released during chronic inflammation can create an environment conducive to EMT, triggering fibrotic

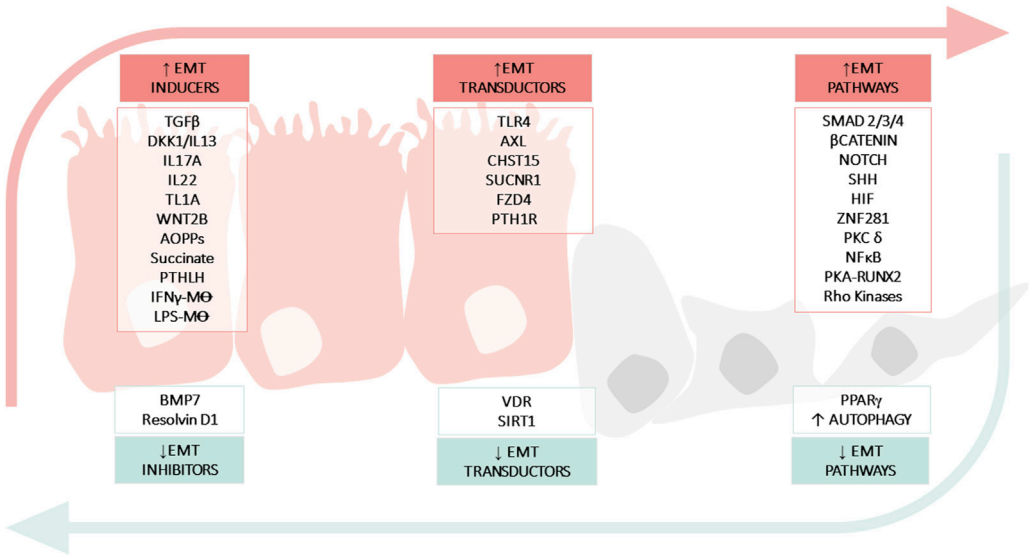


FIGURE 2
Molecular mechanisms implicated in epithelial mesenchymal transition (EMT) in IBD. The figure shows: soluble molecules, cells or hormones related with the induction (INDUCERS) or inhibition (INHIBITORS) of the EMT process; receptors or enzymes implicated in the EMT process (TRANSDUCTORS) and pathways related with EMT (PATHWAYS). Advanced oxidation protein products (AOPPs); Bone morphogenic protein-7 (BMP7); Carbohydrate sulfotransferase 15 (CHST15); Crohn's Disease (CD); Dickkopf-homolog-1 (DKK1); Hypoxia inducible factor (HIF); Inflammatory Bowel disease (IBD); Interferon (IFN); Interleukin (IL); Lipopolysaccharide (LPS); Macrophage (MΘ); Nuclear Factor kB (NFκB); Toll-like receptor 4 (TLR4); Parathyroid hormone-like hormone (PTHLH); Parathyroid hormone receptor 1 (PTH1R); Protein kinase (PK); Peroxisome proliferator-activated receptor (PPARγ); Runt-related transcription factor 2 (Runx2); Silent information regulator 1 (SIRT1); Sonic Hedgehog (SHH); Transforming growth factor (TGF); Tumour necrosis factor-like ligand 1A (TL1A); Ulcerative Colitis (UC); Vitamin D receptor (VDR); Zinc-finger E-box-binding (ZEB).

processes and therefore the progression of the disease. In IBD fibrotic context, the main pro-EMT cytokines described are Th1, Th2 or Th17 (TGFβ1, TL1A, IL17A, IL13, IL22 and IFNγ), where TGFβ1 is the best characterized pro-EMT agent, capable of triggering the induction of other pro-EMT cytokines. In addition, cytokines can exert their pro-EMT role both directly on epithelial cells but also indirectly through macrophages, further amplifying their pro-fibrotic effect. So, the intricate interplay between cytokines, EMT, and fibrosis highlights the complexity of cellular processes and the importance of maintaining proper balance for healthy tissue repair and function.

3.2.2 Immune system and intestinal type-2 EMT

Macrophages play an important role in intestinal fibrosis since they are capable of generating a profibrotic environment, triggering EMT or fibroblast activation, and perpetuating the disease (Lis-López et al., 2021). In this line, Lipopolysaccharide (LPS)-treated macrophages induce EMT through Sonic Hedgehog (SHH) signalling (Ghorbaninejad et al., 2022), while IFNγ -treated macrophages (Macias-Ceja et al., 2022) trigger EMT via the WNT/FZD4 pathway. SHH and WNT signalling are evolutionary conserved signalling pathways which play a regulatory role in gut development and homeostasis and are both related with tumor progression and fibrosis (Castellone and Laukkanen, 2017). SHH protein is highly expressed in IEC and is involved in the regulation of epithelial cell turnover. In the inflamed tissues of IBD patients, SHH signalling components are overexpressed and *in vitro* assays have shown that inhibition of epithelial SHH signalling exerts a dual protective effect against inflammation and EMT (Ghorbaninejad et al., 2022). On the other hand, the WNT signalling pathway plays a vital role in homeostasis and repair, and has also been related to intestinal fibrosis (Lewis et al., 2022) and penetrating behaviour in CD (McGregor et al., 2023). In relation to the modulation of intestinal EMT, it has been described that IEC cocultured with IFNγ -treated macrophages (Macias-Ceja et al., 2022) or WNT2b (Ortiz-Masiá et al., 2020a) trigger EMT via the FZD4 receptor. These *in vitro* assays have been endorsed by the fact that IFNγ, the IFNγ receptor and the WNT2b/FZD4 pathway are overexpressed in CD patients with stenotic and/or penetrating behaviour (Ortiz-Masiá et al., 2020a; Macias-Ceja et al., 2022).

Other proteins involved in immune responses in fibrotic conditions are Toll-like receptors (TLRs) and TAM receptors. Within the family of TAM receptors (pleiotropic negative regulators of the immune system), the AXL receptor has been specifically implicated in the regulation of cell motility and EMT in IBD. In both *in vivo* models and in IBD tissue, inflammation has been shown to trigger AXL overexpression in epithelial cells and macrophages which is accompanied by an increase in the EMT markers (VIMENTIN, ZEB2 and SNAIL) (Boros et al., 2017; Boros et al., 2018). However, the TLR4 receptor (a facilitator of inflammatory responses through maturation of innate immunity) also triggers intestinal EMT in *vivo* and *in vitro* models (Jun et al., 2020). The actions of both receptors are mediated through profibrotic NFκB signalling, which may partly explain that both trigger EMT (Lemke and Rothlin, 2008; Jeong and Lee, 2011).

In summary, macrophages and immune receptors (such TLRs and TAM receptors) in IBD exert their pro-fibrotic role through the activation of pro-EMT pathways related with development and homeostasis regeneration (WNT or SHH pathways) and NFκB signaling, respectively. These results support the close relationship between inflammation and fibrosis in IBD, such that imbalance between immune responses and tissue repair processes potentially promotes fibrosis.

3.2.3 Oxidative stress and intestinal type-2 EMT

Oxidative stress is a hallmark of IBD and there is a well-established link between ROS production, oxidative stress, and the activation of pro-fibrotic growth factors and cytokines, suggesting the existence of feedback as well as feed-forward cycle in intestinal fibrosis (Latella, 2018). In relation to EMT-type 2, oxidative stress is considered a stimulus in lung fibrosis (Cheresh et al., 2013). In IBD, the accumulation of advanced oxidation protein products (AOPPs) promote inflammation and fibrosis formation by activating cellular oxidative stress (Balmus et al., 2016). AOPPs correlate with the expression the EMT markers in intestinal fibrosis, and *in vitro* and *in vivo* administration of AOPPs induces EMT via the protein kinase C δ isoform (PKC δ) that triggers NFκB pathway (Xu X et al., 2017).

Another molecule implicated in oxidative stress is succinate. Succinate levels and its receptor SUCNR1 are increased in CD patients and correlate with EMT markers. (Macias-Ceja et al., 2019; Ortiz-Masiá et al., 2020a) (Table 2). Succinate is an important metabolite at the cross-road of several metabolic pathways, also involved in the formation and elimination of reactive oxygen species (ROS), and succinate accumulation contributes to oxidative stress and mitochondrial ROS production (Zhang et al., 2021). SUCNR1 is activated by succinate when this metabolite is secreted to the extracellular milieu after accumulation inside cells suffering metabolic alterations provoked by inflammatory mediators. In IBD models, succinate and SUCNR1 are capable of triggering EMT through the WNT pathway *in vitro* and *in vivo* (in a heterotopic intestinal transplant model of fibrosis in SUCNR1−/− mice) (Ortiz-Masiá et al., 2020a) (Table 2).

3.2.4 Other molecules related with intestinal type-2 EMT

Other novel molecules that have been linked to intestinal EMT are the transcription factor ZNF281 (Pierdomenico et al., 2018), the enzyme carbohydrate sulfotransferase 15 (CHST15) (Suzuki et al., 2016) and the parathyroid hormone–like hormone (PTHLH) (He et al., 2018). The novel factor ZNF281is overexpressed in IBD patients and required for the induction of SNAIL-dependent EMT. CHST15 is an enzyme biosynthesizing chondroitin sulphate E which binds to various proinflammatory and profibrotic mediators and is known to create local fibrotic lesions. In fact, STNM01, a synthetic double-stranded RNA oligonucleotide directed against CHST15, is currently in a Phase 1 Clinical Study (safety) in CD patients (Suzuki et al., 2017). Finally, PTHLH is a multifunctional peptide implicated in fibrosis formation (Ardura et al., 2010), and induces EMT in IEC of CD patients by modulating protein kinase A (He et al., 2018).

3.2.5 Molecular mechanisms implicated in the downregulation of type-2 EMT in IBD

At the other end of the spectrum of the molecular mechanisms involved, there are the molecules or pathways that favour mesenchymal epithelial transition (MET) or prevent EMT (Table 3) (Figure 2). For instance, bone morphogenic protein 7 (BMP7) is a member of the TGFβ family and prevents TGFβ-induced EMT *in vivo* and *in vitro* due to its ability to counteract the profibrotic effect of TGFβ (Flier et al., 2010). Other molecules that appear to downregulate EMT are the peroxisome proliferator-activated receptor (PPAR)γ and SIRT1 (a class III lysine deacetylase) as their ablation has been shown to exacerbate EMT in *vivo* models of intestinal fibrosis (Di Gregorio et al., 2017; Chen et al., 2021). PPARγ is a well-known inhibitor of TGFβ-induced EMT by antagonizing SMAD3 function (Reka et al., 2010), and PPARγ activators seem to reverse intestinal fibrosis (Di Gregorio et al., 2017; Xu S et al., 2017). SIRT1 is an enzyme that plays a crucial role in aging and chronic diseases. It functions by deacetylating several transcription factors, thereby regulating various pathways. One such pathway in which SIRT1 has been implicated is intestinal fibrosis-associated EMT (Chen et al., 2021), in which deacetyl SMAD4 and subsequently block the signalling TGFβ (Simic et al., 2013). Finally, it has been shown that the vitamin D receptor (VDR) inhibits EMT modulating the mitochondrial respiratory chain. VDR deficiency causes mitochondrial dysfunction in the intestinal epithelium and promotes fibrosis by upregulating the EMT pathway. In fact, low levels of VDR have been detected in patients with CD (Yu et al., 2021).

Dysregulated autophagy is a hallmark of IBD (Shao et al., 2021), and while its role in intestinal fibrosis is controversial (Macias-Ceja et al., 2023), several studies support that autophagy stimulation may be an antifibrotic strategy (Cosin-Roger et al., 2019; Zeng et al., 2022). It has been reported that autophagy activation can suppress EMT by crosstalking with various signaling pathways (e.g., WNTs, NF-kB, TGF-β, NOTCH and Fibrinogen-like protein 1 (FGL-1) signaling pathways) (H.-T. Chen et al., 2019; Gao et al., 2023; Hill et al., 2019). Indeed, in lung fibrosis, autophagy inhibition-induced EMT of alveolar epithelial cells contributes to fibrosis not only by affecting the epithelial phenotype but also via aberrant epithelial–fibroblast crosstalk (Hill et al., 2019). In intestinal fibrosis, Zeng's work showed that autophagy stimulation inhibited EMT in a DSS model, ameliorating intestinal fibrosis (Zeng et al., 2022) (Table 6).

EMT and its converse, MET, are integral stages of many physiologic processes (e.g., wound healing) and as such, are tightly coordinated. In wound healing, EMT as a response to injury can be beneficial. However, if the wound healing process is exaggerated, it may lead to fibrosis. Carrying this idea over to IBD, intestinal epithelial cells are chronically immersed in a pro-EMT factor-rich environment that disrupts the EMT/MET imbalance. In the previous sections, numerous pro-EMT factors have been described that are increased in tissues from patients with IBD, such as cytokines (IL13, TGFβ, TL1A, or IFNγ), pathways involved in development and homeostasis regeneration (WNT or SHH pathways), among others (Table 2). But in addition, there are processes such as the inhibition of autophagy that would also contribute to the imbalance, favoring and further perpetuating intestinal fibrosis in IBD.

TABLE 3 Molecular mechanisms implicated in the downregulation of type-2 EMT in IBD samples, and *in vivo* and *in vitro* IBD related models. The symbol "/" indicates treatment. Bone morphogenic protein-7 (BMP7); Dextran sodium sulfate (DSS); Glycogen synthase kinase (GSK); Intestinal epithelial cell (IEC); Peroxisome proliferator-activated receptor (PPARγ); Silent information regulator 1 (SIRT1); Transforming growth factor (TGF); Trinitrobenzene sulfonic acid (TNBS); Vitamin D (VD); Vitamin D receptor (VDR).

Protein	Type of study		EMT	Molecular mechanism in EMT	References
BMP7	*In vivo*	TNBS VillinCre; R26Rosa-lox-STOP-lox-LacZ mice (trace IECs)	↓	BMP7 is an inhibitor of TGFβ	Flier et al. (2010)
GSK3β/ PPARγ	*In vivo*	DSS/GW9662 (PPARγ inhibitor) DSS/GED-0507-34 Levo (PPARγ agonist)	↓	GSK3β activate PPARγ signaling	Di Gregorio et al. (2017)
SIRT1	*In vitro In vivo*	IEC6/TGFβ TNBS SIRT1$^{-/-}$	↓	Blocks TGFβ through SMAD4 and KDM4-DBC1axis	Simic et al. (2013), Chen et al. (2021)
VDR	*In vitro In vivo* Human	HT29, CCDA18Co cells/VD TNBS VDR$^{-/-}$CD: ↓VDR	↓	Epithelial mitochondria-mediated EMT	Yu et al. (2021)

3.3 Epigenetic factors in intestinal EMT

Epigenetic modifications, which include DNA methylation, or ncRNA molecules, can play a crucial role in regulating EMT. ncRNAs have been proved to participate in the fibrotic diseases of multiple organs (e.g., liver diseases, myocardial fibrosis, and renal fibrosis). The ncRNAs involved in fibrotic diseases mainly consist of microRNAs (miRNAs), long noncoding RNAs, and circular RNAs (circRNAs). NcRNAs modulate the function of mesenchymal cells, inflammatory cascades, ECM, and microbiota via mechanisms of endogenous RNA competition, RNA transcription regulation, protein sponges, and translation regulation (Zhou et al., 2021). The role of microRNAs in the intestinal EMT has been extensively studied (Boros et al., 2017; Boros and Nagy, 2019). Most of the works analyse the role of miRNAs in type-3 EMT, that is, in the progression of colorectal cancer in IBD. However, miR-200b has been shown to be effective in preventing EMT and in alleviating intestinal fibrosis. miR-200b functions by targeting the 3′untranslated region (UTR) of ZEB1 and ZEB2 mRNAs, leading to translational repression (Chen et al., 2012; 2013; Zidar et al., 2016) (Table 4). Indeed, a downregulation of the miR-200 family has been described in patients with IBD (Zidar et al., 2016). Other microRNAs with a potential role in the type-2 EMT associated to IBD are miR-199a, miR-34a, miR-155-5p, miR-146a-3p, and miR-213p (Table 4). In the inflamed tissue of patients with IBD, it has been described that miR-199a and miR-34a expression is reduced and is accompanied by a high expression of AXL tyrosine kinase receptor (Boros et al., 2018). It is interesting that, in a similar way, both miRNA downregulate AXL in lung, colorectal, and breast cancer models (Mudduluru et al., 2011). More recently, miR-155-5p, miR-146a-3p and miR-213p expression have been shown to be inversely correlated with E-cadherin gene expression in tissue biopsies from CD patients (Guz et al., 2020), but further investigations are necessary to establish their specific mechanisms. Finally, a recent study has shown that Circ_0001666, a circRNA, controls EMT by regulating the stability of BMP7 mRNA through its interaction with Serine/arginine-rich splicing factor 1 (SRSF1), thus promoting fibrosis in pediatric CD. Indeed, the expression of circ_0001666 is upregulated in CD pediatric tissues (Table 4) (Li et al., 2023).

In relation to DNA methylation, several studies suggest a link between EMT and UC progression/prognosis (type-3 EMT), specifically in the context of epigenetic modifications of EMT-related genes (Saito et al., 2011; Wang et al., 2012; Tahara et al., 2014; Zhao et al., 2015). The findings suggest that hypermethylation of CDH1, CDH13, NEUROG1, CDX1, and miR-1247 are associated with inflammatory rectal samples compared to non-inflammatory mucosa in control samples. Furthermore, this hypermethylation is correlated with a more severe clinical phenotype in UC patients.

3.4 Microbiome as inductor of intestinal EMT

Several studies indicate that gut microbiota plays crucial roles in fibrosis. In several animal models, microbes initiate or perpetuate gut fibrosis (Rieder, 2013). In CD fibroblasts, there is an increased expression of several TLRs that can be activated by perceiving microbial components and promote transdifferentiation (Zorzi et al., 2015). However, there is little direct evidence so far on the possible involvement of EMT in microbiome-induced intestinal fibrosis, and the studies are indirect and involve TGFβ changes. In this line, antibiotic treatment significantly inhibits TGFβ1 or the injection of faecal material or extracts from anaerobic bacteria into the bowel wall induced fibrosis and increased levels of TGFβ1 (Mourelle et al., 1998).

Some enteric pathogens have been shown to be able to modulate EMT in IECs (Table 5), such us *Helicobacter pylori* (Yin et al., 2010; Ouyang et al., 2021), *Citrobacter rodentium* (Chandrakesan et al., 2014), *Escherichia coli* (Cane et al., 2010) or *Clostridium butyricum* (Zhang et al., 2023), however these infections are not related with intestinal fibrosis.

3.5 EMT as a therapeutic target in intestinal fibrosis associated with IBD

Although organ fibrosis was considered an irreversible process, it is now known to be a dynamic process with the potential for reversibility and restoration of near-normal tissue architecture and organ function. Several approaches (antioxidants, inhibition of fibrotic signalling pathways, stem cell therapies, modulation of fibrogenic cells or anti-inflammatory targets) have shown anti-fibrotic effects in animal models of organ fibrosis (Horowitz and Thannickal, 2019; Lurje et al., 2023), and some of them are currently approved for human use in certain fibrotic diseases (Bocchino et al., 2023). Given the potential role of EMT in IBD-associated fibrosis, several strategies targeting EMT have been explored as potential therapeutic approaches for IBD. In this section, we review the main

TABLE 4 Genes involved in intestinal EMT and their non-coding RNA (ncRNA) regulators in IBD. The symbol "/" indicates treatment. Bone morphogenic protein-7 (BMP7), E-cadherin (CDH1), N-cadherin (CDH2), Crohn's disease (CD); Dextran sodium sulfate (DSS); Epithelial Growth factor (EGF); Fibroblast growth factor (FGF); Intestinal epithelial cell (IEC); Serine/arginine-rich splicing factor 1 (SRSF1); Transforming growth factor (TGF); Trinitrobenzene sulfonic acid (TNBS); Zinc-finger E-box-binding (ZEB).

ncRNA	Target	Type of study		Effect in EMT		References
miR-200	ZEB1/ SMAD2	*In vitro* Human	IEC6/TGFβ1 IBD: ↓ miR-200b	↓	↑CDH1 ↓Vimentin	Chen et al. (2012), Chen et al. (2013), Zidar et al. (2016)
miR-199a and miR-34a	AXL	Human	IBD: ↓ miR-199a and miR-34a	↓	↓ZEB2, SNAIL1	Mudduluru et al. (2011), Cho et al. (2016), Boros et al. (2017), Boros et al. (2018)
miR-155-5p, miR-146a-3p, miR-213p	CDH1	Human	CD: ↑miR-146a3p, miR-155-5p and miR-213p	↑	↓CDH1	Guz et al. (2020)
Circ_0001666	SRSF1/ BMP7	*In vitro* Human	IEC/TGFβ1 CD: ↑ Circ_0001666	↑	↓CDH1 ↑Vimentin, SNAIL, CDH2	Li et al. (2023)

products tested in *vivo* and *in vitro* models of intestinal fibrosis, whose main mechanism is to modulate intestinal EMT (Table 6).

The use of natural products and derivatives, including those derived from traditional Chinese medicine, has gained attention in the field of fibrosis research. Many natural products have been investigated, in *vivo* models of IBD, for their potential anti-fibrotic effects by targeting EMT pathways. Most products have TGF-mediated EMT as their primary target: both *canonical* (curcumin (Xu S et al., 2017), silibin (Kim et al., 2017), *Abelmoschus manihot* (Yang et al., 2018), Wu-Mei-Wan (Wu et al., 2020), halofuginone (Duan et al., 2020), and Atractylenolide III (Huang et al., 2022)) and *non-canonica*l downstream pathways (Wu-Mei-Wan (Wu et al., 2020), *Forsythia koreana* (T.-W. Kim et al., 2019), HLJ2 (Song et al., 2020) and Artemisinin (Huai et al., 2021)).

Several studies support that autophagy stimulation may be an antifibrotic strategy (Cosin-Roger et al., 2019; Zeng et al., 2022). In this sense, Xue-Jie-San, a traditional Chinese herb, protects against EMT-mediated fibrosis through the stimulation of autophagy, blocking the NOTCH1 and FGL1 signalling pathways (Gao et al., 2023). In fact, NOTCH signalling is a profibrotic pathway that has been little studied in IBD-related intestinal fibrosis (Marti-Chafer et al., 2023). Other molecule that prevents intestinal EMT by stimulating epithelial autophagy is resolvin D1, an omega-3 polyunsaturated fatty acid (Zeng et al., 2022).

There is growing evidence suggesting that the interactions between the gut microbiota and the host can influence EMT and contribute to the development of intestinal fibrosis (Table 5). Modifying the microbiota through dietary interventions has emerged as a potential strategy to influence EMT and attenuate fibrosis in various intestinal fibrotic models (Yang et al., 2017a; Zhou et al., 2018; Chung et al., 2021). Similarly, cell therapy as a control mechanism for EMT has also been analysed in intestinal fibrosis. Indeed, TNBS models have shown that: mesenchymal stem cell (MSC) exert anti-fibrogenic activity by regulating the inflammatory environment, inhibiting the TGFβ/SMAD signalling pathway and ameliorating EMT (Lian et al., 2018). Likewise, the delivery of miR-200b through bone marrow MSC-derived microvesicles inhibits EMT and ameliorate fibrosis (Yang et al., 2017b).

Finally, there are other synthetic molecules tested in preclinical models that inhibit intestinal EMT which have a promising future: the recombinant human BMP7 (rhBMP7), GED-0507-34 Levo, AMA0825 or A83-01. In preclinical studies, rhBMP7 has demonstrated the ability to inhibit EMT and attenuate fibrosis in various organs (Weiskirchen and Meurer, 2013), including the intestine (Flier et al., 2010). It exerts its anti-fibrotic effects by antagonizing TGFβ-induced EMT and promoting tissue repair and regeneration. GED-0507-34 Levo is an orally active synthetic compound and a selective agonist of PPARγ that has been shown to inhibit EMT, reduce inflammation, and ameliorate fibrosis in a DSS model (Di Gregorio et al., 2017; Pompili et al., 2023). In fact, GED-0507-34 is in a Phase 2 clinical trial in subjects with active, mild-to-moderate UC (ClinicalTrials.gov Identifier: NCT02808390). AMA0825, a Rho kinase inhibitor, is a synthetic small molecule that has been studied in intestinal fibrosis due to its potential effects on EMT and autophagy (Holvoet et al., 2017). Finally, A83-01, a new type I receptor ALK5 kinase inhibitor molecule, that in an *in vitro* assay blocks TGFβ-induced EMT (Gao et al., 2023).

In summary, there have been multiple trials focused on targeting EMT to manage intestinal fibrosis in the context of IBD. While many of these trials are still in the preclinical stages, some have progressed to clinical trials, such as the trial involving GED-0507-34.

4 Other roles of epithelial cells in intestinal fibrosis: lessons from other fibrotic tissues

Epithelial cell injury and death are common events in inflammatory diseases, such us UC and CD, but they have been only recently recognized as drivers of fibrosis. For instance, an increasing number of studies have linked necroptosis (a form of programmed necrosis) to inflammation and fibrosis in renal, liver, heart or lung fibrosis (Liu et al., 2022). Cell products released by cells undergoing necrosis (passive, programmed, or after apoptosis) are called damage-associated molecular patterns (DAMPs), that can directly activate profibrotic responses of immune cells or nonimmune cells (epithelial cells, endothelial cells, and fibroblasts) triggering fibrosis (Liu et al., 2022). In addition, epithelial cells contain a myriad of intracellular substances normally not recognized by the immune system but, during cell necrosis, they are passively released in the surrounding microenvironment and trigger inflammation. These responses may represent a novel fibrotic pathogenic component of IBD since epithelial damage is a typical feature of both UC and CD.

DAMPs are classified into molecules that perform noninflammatory functions or alarmins. The noninflammatory

TABLE 5 Microbiome as inductor of intestinal EMT in IBD. Hypoxia inducible factor (HIF); vascular endothelial growth factor (VEGF); m^{6}A methyltransferase (METTL3).

Microbiome	Effect in EMT		References
Helicobacter pylori	↑	AKT/GSK3β signaling	Yin et al. (2010), Ouyang et al. (2021)
Citrobacter rodentium	↑	WNT/NOTCH signaling	Chandrakesan et al. (2014)
Escherichia coli	↑	HIF1α/IL8/VEGF/TWIST1	Cane et al. (2010)
Clostridium butyricum	↓	METTL3	Zhang et al. (2023)

TABLE 6 EMT as a therapeutic target in intestinal fibrosis associated to IBD. Activating Protein-1 (AP1); Crohn's disease (CD); Dextran sodium sulfate (DSS); Hypoxia inducible factor (HIF); Intestinal epithelial cell (IEC); Ionizing radiation (IR); Nuclear Factor kB (NFκB); Peroxisome proliferator-activated receptor (PPARγ); Recombinant human bone morphogenic protein-7 (rhBMP7); Signal transducer and activator of transcription (STAT); Transforming growth factor (TGF); Trinitrobenzene sulfonic acid (TNBS); Zinc-finger E-box-binding (Zeb).

Product	Target	Type of study		References
Exopolysaccharide *Bacillus subtilis*	NFKb, STAT3 Immune cell infiltration	*In vivo*	DSS	Chung et al. (2021)
Curcumin	PPARγ activator	*In vivo*	TNBS	Xu S et al. (2017)
Silibinin	TGFβ1	*In vivo/vitro*	IR, CD	Kim et al. (2017)
Abelmoschus manihot	TGFβ1	*In vitro*	IEC6	Yang et al. (2018)
Halofuginone	TGFβ/Smad	*In vitro*	IPEC-J2 cells	Duan et al. (2020)
Atractylenolide III	TGFβ1	*In vitro*	IEC6	Huang et al. (2022)
Wu-Mei-Wan	NFκB, STAT3 TGFβ/Smad Wnt/β-catenin	*In vivo*	TNBS	Wu et al. (2020)
Forsythia koreana	AP1, NFκB, and STAT1/3 macrophages	*In vivo/vitro*	DSS, RAW264.7 cells	Kim et al. (2019)
HLJ2 (berberine)	NFκB	*In vivo*	DSS	Song et al. (2020)
Artemisinin	ERK/MYD88 signaling M2 macrophages	*In vivo/vitro*	DSS, CD, RAW264.7 cells	Huai et al. (2021)
Xue-Jie-San	Autophagy stimulation	*In vivo*	TNBS	Gao et al. (2023)
Resolvin D1	Autophagy stimulation	*In vivo*	DSS	Zeng et al. (2022)
Mesenchymal cells	TGFβ/SMAD	*In vivo*	TNBS	Lian et al. (2018)
miR-200b	Zeb1/2	*In vivo/vitro*	TNBS, IEC6	Yang et al. (2017b)
A83-01	TGFβ1	*In vitro*	Caco2	Ghorbaninejad et al. (2023)
rhBMP7	TGFβ1	*In vivo*	TNBS	Flier et al. (2010)
GED-0507-34 Levo	PPARγ activator	*In vivo*	DSS	Di Gregorio et al. (2017), Pompili et al. (2023)
Saccharomyces boulardii	HIF1/2	*In vivo*	DSS	Zhou et al. (2018)
AMA0825	Rho Kinases	*In vivo*	DSS	Holvoet et al. (2017)
Xue-Jie-San	Autophagy stimulation	*In vivo*	TNBS	Gao et al. (2023)

DAMPS in living cells (such as high-mobility group box 1, HMGB1) can acquire immunomodulatory properties when released, secreted, modified, or exposed on the cell surface during cellular stress, damage, or injury. On the other hand, alarmins alert the immune system and trigger a sterile inflammatory response (such as IL1α, S100A8, and IL33) (Kaczmarek et al., 2013). IBD tissue releases calprotectin (S100A12, S100A8/S100A9 complexes) and HMGB1 which serve as faecal biomarkers of intestinal inflammation (Nanini et al., 2018). The role of several necroptotic DAMPs and their receptors have been described in the main fibrotic diseases, except in intestinal fibrosis associated to IBD (Liu et al., 2022), where further studies are needed. In this line, Scarpa and collaborators have reported that epithelial cell-derived DAMPS (IL1α) elicit a potent proinflammatory cytokine response from human intestinal fibroblasts. Fibroblasts would act as first responders to products of IECs necrosis due to their anatomical proximity (Scarpa et al., 2015). Necroptotic DAMP receptors have also been reported to promote fibrosis. In IBD, genetic knockout TLR4 (a well-known necroptotic receptor) can alleviate systemic inflammation and tissue fibrosis in intestine, via cytokine expression and EMT (Jun et al., 2020). In the same line of the role of epithelial cells as sources of profibrotic ligands, accumulated data regarding

pulmonary fibrosis show that EMT transdifferentiation does not occur completely Rather, the EMT cells act as sources of soluble ligands that favour the transdifferentiation of fibroblasts (Hill et al., 2019; Yue et al., 2022). This phenomenon has also been observed in IBD by Zeng and collaborations, who found that the co-culture of EMT cells with intestinal fibroblast induced fibroblast activation (Zeng et al., 2022).

On the other hand, there is the epithelial-immune crosstalk described in both pulmonary and cutaneous fibrosis (Planté-Bordeneuve et al., 2021; Rosenblum and Naik, 2022). Interactions between epithelium and the immune system involve a tight regulation to prevent inappropriate reactions. Recent data regarding pulmonary fibrosis suggest a two-way process, so that epithelial cells' biology and their crosstalk with immune cells and microbes may trigger aberrant pro-fibrotic signalling (Planté-Bordeneuve et al., 2021). Intestinal epithelium and immunity have been implicated in the pathogenesis and disease course of IBD. However, consequences of their abnormal interplay in fibrosis remain unknown.

5 Conclusion and remarks

Intestinal fibrosis associated with IBD is a complex condition that has been the focus of ongoing research in the last decade, especially in CD. The role of epithelial cells in the pathogenesis of intestinal fibrosis has been widely studied and currently, one of the main cellular mechanisms involved in intestinal fibrosis is the epithelial-mesenchymal transition. Given the potential role of EMT in IBD-associated fibrosis and the lack of pharmacological therapies for this condition, several novel strategies targeting EMT have been explored. It is important to note that these therapies hold promise, but more research is needed to determine their efficacy, safety, and long-term outcomes in the IBD setting.

In relation to future perspectives, more in-depth studies are required on the role of the microbiota and epigenetics in EMT-mediated intestinal fibrosis since the available works are focused either on the oncological progression of the disease or not directly related with intestinal fibrosis. Similarly, outside the epithelial transition, evidence for the involvement of epithelial-immune or epithelial-mesenchymal crosstalk in IBD fibrosis is limited. Determining the exact contribution of these mechanisms is challenging, as they are at the crossroads of multiple regulatory networks. Nonetheless, in-depth understanding of the epithelial contribution to the fibrotic paradigm will help to design more specific and effective anti-fibrotic therapies.

Author contributions

DM: Writing–original draft. MM: Writing–original draft. MO: Writing–original draft. MB: Funding acquisition, Validation, Writing–review and editing. DO: Conceptualization, Supervision, Validation, Writing–original draft, Writing–review and editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was funded by grants from CIBEREHD, Instituto de Salud Carlos III (CB06/04/0071), Ministerio de Ciencia e Innovación (PID 2019-108996RB-I00), and Conselleria de Innovación, Universidades, Ciencia y Sociedad Digital, Generalitat Valenciana (CIPROM 2021/044).

Acknowledgments

We thank Brian Normanly and Luis Alfonso Espinosa Ortega for their English language editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Agus, A., Denizot, J., Thévenot, J., Martinez-Medina, M., Massier, S., Sauvanet, P., et al. (2016). Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive *E. coli* infection and intestinal inflammation. *Sci. Rep.* 6 (1), 19032. Article 1. doi:10.1038/srep19032

Alam, A., and Neish, A. (2018). Role of gut microbiota in intestinal wound healing and barrier function. *Tissue Barriers* 6 (3), 1539595. doi:10.1080/21688370.2018.1539595

Amamou, A., O'Mahony, C., Leboutte, M., Savoye, G., Ghosh, S., and Marion-Letellier, R. (2022). Gut microbiota, macrophages and diet: an intriguing new triangle in intestinal fibrosis. *Microorganisms* 10 (3), 490. doi:10.3390/microorganisms10030490

Ardura, J. A., Rayego-Mateos, S., Rámila, D., Ruiz-Ortega, M., and Esbrit, P. (2010). Parathyroid hormone-related protein promotes epithelial-mesenchymal transition. *J. Am. Soc. Nephrol.* 21 (2), 237–248. Scopus. doi:10.1681/ASN.2009050462

Arimura, Y., Isshiki, H., Onodera, K., Nagaishi, K., Yamashita, K., Sonoda, T., et al. (2014). Characteristics of Japanese inflammatory bowel disease susceptibility loci. *J. Gastroenterology* 49 (8), 1217–1230. doi:10.1007/s00535-013-0866-2

Balmus, I. M., Ciobica, A., Trifan, A., and Stanciu, C. (2016). The implications of oxidative stress and antioxidant therapies in inflammatory bowel disease: clinical aspects and animal models. *Saudi J. Gastroenterology Official J. Saudi Gastroenterology Assoc.* 22 (1), 3–17. doi:10.4103/1319-3767.173753

Bataille, F., Rohrmeier, C., Bates, R., Weber, A., Rieder, F., Brenmoehl, J., et al. (2008). Evidence for a role of epithelial mesenchymal transition during pathogenesis of fistulae in Crohn's disease. *Inflamm. Bowel Dis.* 14 (11), 1514–1527. doi:10.1002/ibd.20590

Blair, S. A., Kane, S. V., Clayburgh, D. R., and Turner, J. R. (2006). Epithelial myosin light chain kinase expression and activity are upregulated in inflammatory bowel

disease. *Laboratory Investigation; a J. Tech. Methods Pathology* 86 (2), 191–201. doi:10.1038/labinvest.3700373

Bocchino, M., Bruzzese, D., Scioscia, G., Capitelli, L., Tondo, P., Rea, G., et al. (2023). Disease stage-related survival in idiopathic pulmonary fibrosis patients treated with nintedanib and pirfenidone: an exploratory study. *Respir. Med. Res.* 84, 101013. doi:10.1016/j.resmer.2023.101013

Boros, É., Csatári, M., Varga, C., Bálint, B., and Nagy, I. (2017). Specific gene- and MicroRNA-expression pattern contributes to the epithelial to mesenchymal transition in a rat model of experimental colitis. *Mediat. Inflamm.* 2017, e5257378. doi:10.1155/2017/5257378

Boros, É., Kellermayer, Z., Balogh, P., Strifler, G., Vörös, A., Sarlós, P., et al. (2018). Elevated expression of *AXL* may contribute to the epithelial-to-mesenchymal transition in inflammatory bowel disease patients. *Mediat. Inflamm.* 2018, e3241406. doi:10.1155/2018/3241406

Boros, É., and Nagy, I. (2019). The role of MicroRNAs upon epithelial-to-mesenchymal transition in inflammatory bowel disease. *Cells* 8 (11), 1461. doi:10.3390/cells8111461

Cane, G., Ginouvès, A., Marchetti, S., Buscà, R., Pouysségur, J., Berra, E., et al. (2010). HIF-1alpha mediates the induction of IL-8 and VEGF expression on infection with Afa/Dr diffusely adhering *E. coli* and promotes EMT-like behaviour. *Cell. Microbiol.* 12 (5), 640–653. doi:10.1111/j.1462-5822.2009.01422.x

Castellone, M. D., and Laukkanen, M. O. (2017). TGF-beta1, WNT, and SHH signaling in tumor progression and in fibrotic diseases. *Front. Biosci. (Scholar Ed.* 9 (1), 31–45. doi:10.2741/s470

Chandrakesan, P., Roy, B., Jakkula, L. U. M. R., Ahmed, I., Ramamoorthy, P., Tawfik, O., et al. (2014). Utility of a bacterial infection model to study epithelial-mesenchymal transition, mesenchymal-epithelial transition or tumorigenesis. *Oncogene* 33 (20), 2639–2654. doi:10.1038/onc.2013.210

Chen, B., Dong, W., Shao, T., Miao, X., Guo, Y., Liu, X., et al. (2021). A KDM4-DBC1-SIRT1 Axis contributes to TGF-b induced mesenchymal transition of intestinal epithelial cells. *Front. Cell Dev. Biol.* 9, 697614. doi:10.3389/fcell.2021.697614

Chen, H.-T., Liu, H., Mao, M.-J., Tan, Y., Mo, X.-Q., Meng, X.-J., et al. (2019). Crosstalk between autophagy and epithelial-mesenchymal transition and its application in cancer therapy. *Mol. Cancer* 18 (1), 101. doi:10.1186/s12943-019-1030-2

Chen, Y., Ge, W., Xu, L., Qu, C., Zhu, M., Zhang, W., et al. (2012). MiR-200b is involved in intestinal fibrosis of Crohn's disease. *Int. J. Mol. Med.* 29 (4), 601–606. doi:10.3892/ijmm.2012.894

Chen, Y., Xiao, Y., Ge, W., Zhou, K., Wen, J., Yan, W., et al. (2013). MiR-200b inhibits TGF-β1-induced epithelial-mesenchymal transition and promotes growth of intestinal epithelial cells. *Cell Death Dis.* 4 (3), e541. doi:10.1038/cddis.2013.22

Cheresh, P., Kim, S.-J., Tulasiram, S., and Kamp, D. W. (2013). Oxidative stress and pulmonary fibrosis. *Biochimica Biophysica Acta (BBA) - Mol. Basis Dis.* 1832 (7), 1028–1040. doi:10.1016/j.bbadis.2012.11.021

Cho, C.-Y., Huang, J.-S., Shiah, S.-G., Chung, S.-Y., Lay, J.-D., Yang, Y.-Y., et al. (2016). Negative feedback regulation of AXL by miR-34a modulates apoptosis in lung cancer cells. *RNA* 22 (2), 303–315. doi:10.1261/rna.052571.115

Chung, K.-S., Shin, J.-S., Lee, J.-H., Park, S.-E., Han, H.-S., Rhee, Y. K., et al. (2021). Protective effect of exopolysaccharide fraction from Bacillus subtilis against dextran sulfate sodium-induced colitis through maintenance of intestinal barrier and suppression of inflammatory responses. *Int. J. Biol. Macromol.* 178, 363–372. doi:10.1016/j.ijbiomac.2021.02.186

Colombel, J.-F., D'haens, G., Lee, W.-J., Petersson, J., and Panaccione, R. (2020). Outcomes and strategies to support a treat-to-target approach in inflammatory bowel disease: A systematic review. *J. Crohn's Colitis* 14 (2), 254–266. doi:10.1093/ecco-jcc/jjz131

Cosin-Roger, J., Canet, F., Macias-Ceja, D. C., Gisbert-Ferrándiz, L., Ortiz-Masiá, D., Esplugues, J. V., et al. (2019). Autophagy stimulation as a potential strategy against intestinal fibrosis. *Cells* 8 (9), 1078. Article 9. doi:10.3390/cells8091078

Cosnes, J., Gower-Rousseau, C., Seksik, P., and Cortot, A. (2011). Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 140 (6), 1785–1794. doi:10.1053/j.gastro.2011.01.055

D'Alessio, S., Ungaro, F., Noviello, D., Lovisa, S., Peyrin-Biroulet, L., and Danese, S. (2022). Revisiting fibrosis in inflammatory bowel disease: the gut thickens. *Nat. Rev. Gastroenterology Hepatology* 19 (3), 169–184. Article 3. doi:10.1038/s41575-021-00543-0

D'Arcangelo, G., and Aloi, M. (2020). Treat-to-Target in pediatric inflammatory bowel disease: what does the evidence say? *Pediatr. Drugs* 22 (5), 463–472. doi:10.1007/s40272-020-00406-2

Delbue, D., Lebenheim, L., Cardoso-Silva, D., Dony, V., Krug, S. M., Richter, J. F., et al. (2021). Reprogramming intestinal epithelial cell polarity by interleukin-22. *Front. Med.* 8, 656047. doi:10.3389/fmed.2021.656047

D'Haens, G., Rieder, F., Feagan, B. G., Higgins, P. D. R., Panés, J., Maaser, C., et al. (2022). Challenges in the pathophysiology, diagnosis, and management of intestinal fibrosis in inflammatory bowel disease. *Gastroenterology* 162 (1), 26–31. doi:10.1053/j.gastro.2019.05.072

Di Gregorio, J., Robuffo, I., Spalletta, S., Giambuzzi, G., De Iuliis, V., Toniato, E., et al. (2020). The epithelial-to-mesenchymal transition as a possible therapeutic target in fibrotic disorders. *Front. Cell Dev. Biol.* 8, 607483. doi:10.3389/fcell.2020.607483

Di Gregorio, J., Sferra, R., Speca, S., Vetuschi, A., Dubuquoy, C., Desreumaux, P., et al. (2017). Role of glycogen synthase kinase-3β and PPAR-γ on epithelial-to-mesenchymal transition in DSS-induced colorectal fibrosis. *PloS One* 12 (2), e0171093. doi:10.1371/journal.pone.0171093

Duan, M., Wei, X., Cheng, Z., Liu, D., Fotina, H., Xia, X., et al. (2020). Involvement of eIF2α in halofuginone-driven inhibition of TGF-β1-induced EMT. *J. Biosci.* 45, 71. doi:10.1007/s12038-020-00042-5

Felwick, R. K., Dingley, G. J. R., Martinez-Nunez, R., Sanchez-Elsner, T., Cummings, J. R. F., and Collins, J. E. (2020). MicroRNA23a overexpression in Crohn's disease targets Tumour necrosis factor alpha inhibitor protein 3, increasing sensitivity to TNF and modifying the epithelial barrier. *J. Crohn's Colitis* 14 (3), 381–392. doi:10.1093/ecco-jcc/jjz145

Flier, S. N., Tanjore, H., Kokkotou, E. G., Sugimoto, H., Zeisberg, M., and Kalluri, R. (2010). Identification of epithelial to mesenchymal transition as a novel source of fibroblasts in intestinal fibrosis. *J. Biol. Chem.* 285 (26), 20202–20212. doi:10.1074/jbc.M110.102012

Frei, S. M., Hemsley, C., Pesch, T., Lang, S., Weber, A., Jehle, E., et al. (2013). The role for dickkopf-homolog-1 in the pathogenesis of Crohn's disease-associated fistulae. *PLOS ONE* 8 (11), e78882. doi:10.1371/journal.pone.0078882

Fumery, M., Pineton De Chambrun, G., Stefanescu, C., Buisson, A., Bressenot, A., Beaugerie, L., et al. (2015). Detection of dysplasia or cancer in 3.5% of patients with inflammatory bowel disease and colonic strictures. *Clin. Gastroenterology Hepatology* 13 (10), 1770–1775. doi:10.1016/j.cgh.2015.04.185

Gao, Y., Lu, L.-J., Zhang, Z.-Z., Yang, X., Du, J., Wen, K., et al. (2023). Xue-Jie-San prevents the early development of colitis-associated intestinal fibrosis by blocking Notch1 and FGL1 signaling pathways. *J. Ethnopharmacol.* 315, 116678. doi:10.1016/j.jep.2023.116678

Ghorbaninejad, M., Abdollahpour-Alitappeh, M., Shahrokh, S., Fayazzadeh, S., Asadzadeh-Aghdaei, H., and Meyfour, A. (2023). TGF-β receptor I inhibitor may restrict the induction of EMT in inflamed intestinal epithelial cells. *Exp. Biol. Med.* 248, 665–676. doi:10.1177/15353702231151959

Ghorbaninejad, M., Meyfour, A., Maleknia, S., Shahrokh, S., Abdollahpour-Alitappeh, M., and Asadzadeh-Aghdaei, H. (2022). Inhibition of epithelial SHH signaling exerts a dual protective effect against inflammation and epithelial-mesenchymal transition in inflammatory bowel disease. *Toxicol. Vitro Int. J. Publ. Assoc. BIBRA* 82, 105382. doi:10.1016/j.tiv.2022.105382

Giuffrida, P., Caprioli, F., Facciotti, F., and Di Sabatino, A. (2019). The role of interleukin-13 in chronic inflammatory intestinal disorders. *Autoimmun. Rev.* 18 (5), 549–555. doi:10.1016/j.autrev.2019.03.012

Gklavas, A., Dellaportas, D., and Papaconstantinou, I. (2017). Risk factors for postoperative recurrence of Crohn's disease with emphasis on surgical predictors. *Ann. Gastroenterology* 30 (6), 598–612. doi:10.20524/aog.2017.0195

Gordon, I. O., Agrawal, N., Goldblum, J. R., Fiocchi, C., and Rieder, F. (2014). Fibrosis in ulcerative colitis: mechanisms, features, and consequences of a neglected problem. *Inflamm. Bowel Dis.* 20 (11), 2198–2206. doi:10.1097/MIB.0000000000000080

Gulhane, M., Murray, L., Lourie, R., Tong, H., Sheng, Y. H., Wang, R., et al. (2016). High fat diets induce colonic epithelial cell stress and inflammation that is reversed by IL-22. *Sci. Rep.* 6, 28990. doi:10.1038/srep28990

Guz, M., Dworzański, T., Jeleniewicz, W., Cybulski, M., Kozicka, J., Stepulak, A., et al. (2020). Elevated miRNA inversely correlates with E-cadherin gene expression in tissue biopsies from Crohn disease patients in contrast to ulcerative colitis patients. *BioMed Res. Int.* 2020, 4250329. doi:10.1155/2020/4250329

Hadpech, S., and Thongboonkerd, V. (2023). Epithelial-mesenchymal plasticity in kidney fibrosis. *Genes. (New York, N.Y. 2000)*, e23529. doi:10.1002/dvg.23529

Hay, E. D. (1995). An overview of epithelio-mesenchymal transformation. *Acta Anat.* 154 (1), 8–20. doi:10.1159/000147748

He, S., Xue, M., Liu, C., Xie, F., and Bai, L. (2018). Parathyroid hormone-like hormone induces epithelial-to-mesenchymal transition of intestinal epithelial cells by activating the runt-related transcription factor 2. *Am. J. Pathology* 188 (6), 1374–1388. doi:10.1016/j.ajpath.2018.03.003

Hill, C., Li, J., Liu, D., Conforti, F., Brereton, C. J., Yao, L., et al. (2019). Autophagy inhibition-mediated epithelial-mesenchymal transition augments local myofibroblast differentiation in pulmonary fibrosis. *Cell Death Dis.* 10 (8), 591. doi:10.1038/s41419-019-1820-x

Holvoet, T., Devriese, S., Castermans, K., Boland, S., Leysen, D., Vandewynckel, Y.-P., et al. (2017). Treatment of intestinal fibrosis in experimental inflammatory bowel disease by the pleiotropic actions of a local Rho kinase inhibitor. *Gastroenterology* 153 (4), 1054–1067. doi:10.1053/j.gastro.2017.06.013

Horowitz, J. C., and Thannickal, V. J. (2019). Mechanisms for the resolution of organ fibrosis. *Physiology* 34 (1), 43–55. doi:10.1152/physiol.00033.2018

Huai, M., Zeng, J., and Ge, W. (2021). Artemisinin ameliorates intestinal inflammation by skewing macrophages to the M2 phenotype and inhibiting

epithelial-mesenchymal transition. *Int. Immunopharmacol.* 91, 107284. doi:10.1016/j.intimp.2020.107284

Huang, M., Jiang, W., Luo, C., Yang, M., and Ren, Y. (2022). Atractylenolide III inhibits epithelial-mesenchymal transition in small intestine epithelial cells by activating the AMPK signaling pathway. *Mol. Med. Rep.* 25 (3), 98. doi:10.3892/mmr.2022.12614

Jarmakiewicz-Czaja, S., Sokal, A., Ferenc, K., Motyka, E., Helma, K., and Filip, R. (2023). The role of genetic and epigenetic regulation in intestinal fibrosis in inflammatory bowel disease: A descending process or a programmed consequence? *Genes* 14 (6), 1167. Article 6. doi:10.3390/genes14061167

Jeong, E., and Lee, J. Y. (2011). Intrinsic and extrinsic regulation of innate immune receptors. *Yonsei Med. J.* 52 (3), 379–392. doi:10.3349/ymj.2011.52.3.379

Johnson, L. A., Rodansky, E. S., Sauder, K. L., Horowitz, J. C., Mih, J. D., Tschumperlin, D. J., et al. (2013). Matrix stiffness corresponding to strictured bowel induces a fibrogenic response in human colonic fibroblasts. *Inflamm. Bowel Dis.* 19 (5), 891–903. doi:10.1097/MIB.0b013e3182813297

Jun, Y. K., Kwon, S. H., Yoon, H. T., Park, H., Soh, H., Lee, H. J., et al. (2020). Toll-like receptor 4 regulates intestinal fibrosis via cytokine expression and epithelial-mesenchymal transition. *Sci. Rep.* 10 (1), 19867. doi:10.1038/s41598-020-76880-y

Kaczmarek, A., Vandenabeele, P., and Krysko, D. V. (2013). Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity* 38 (2), 209–223. doi:10.1016/j.immuni.2013.02.003

Keir, M., Yi, T., Lu, T., and Ghilardi, N. (2020). The role of IL-22 in intestinal health and disease. *J. Exp. Med.* 217 (3), e20192195. doi:10.1084/jem.20192195

Kelm, M., and Anger, F. (2022). Mucosa and microbiota—the role of intrinsic parameters on intestinal wound healing. *Front. Surg.* 9, 905049. doi:10.3389/fsurg.2022.905049

Kim, J. S., Han, N.-K., Kim, S.-H., and Lee, H.-J. (2017). Silibinin attenuates radiation-induced intestinal fibrosis and reverses epithelial-to-mesenchymal transition. *Oncotarget* 8 (41), 69386–69397. doi:10.18632/oncotarget.20624

Kim, T.-W., Shin, J.-S., Chung, K.-S., Lee, Y.-G., Baek, N.-I., and Lee, K.-T. (2019). Anti-inflammatory mechanisms of koreanaside A, a lignan isolated from the flower of Forsythia koreana, against LPS-induced macrophage activation and DSS-induced colitis mice: the crucial role of AP-1, NF-κB, and JAK/STAT signaling. *Cells* 8 (10), 1163. doi:10.3390/cells8101163

Kotla, N. G., and Rochev, Y. (2023). IBD disease-modifying therapies: insights from emerging therapeutics. *Trends Mol. Med.* 29 (3), 241–253. doi:10.1016/j.molmed.2023.01.001

Latella, G. (2018). Redox imbalance in intestinal fibrosis: beware of the tgfβ-1, ROS, and Nrf2 connection. *Dig. Dis. Sci.* 63 (2), 312–320. doi:10.1007/s10620-017-4887-1

Latella, G., Rogler, G., Bamias, G., Breynaert, C., Florholmen, J., Pellino, G., et al. (2014). Results of the 4th scientific workshop of the ECCO (I): pathophysiology of intestinal fibrosis in IBD. *J. Crohn's Colitis* 8 (10), 1147–1165. doi:10.1016/j.crohns.2014.03.008

Lee, S. H. (2015). Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. *Intestinal Res.* 13 (1), 11–18. doi:10.5217/ir.2015.13.1.11

Lemke, G., and Rothlin, C. V. (2008). Immunobiology of the TAM receptors. *Nat. Rev. Immunol.* 8 (5), 327–336. Article 5. doi:10.1038/nri2303

Lewis, A., Sánchez, S., Berti, G., Pan-Castillo, B., Nijhuis, A., Mehta, S., et al. (2022). Small-molecule Wnt inhibitors are a potential novel therapy for intestinal fibrosis in Crohns disease. *Clin. Sci. Lond. Engl. 1979)* 136 (19), 1405–1423. doi:10.1042/CS20210889

Li, J., Dejanovic, D., Zangara, M. T., Chandra, J., McDonald, C., and Rieder, F. (2021). Mouse models of intestinal fibrosis. *Methods Mol. Biol. Clift. N.J.)* 2299, 385–403. doi:10.1007/978-1-0716-1382-5_26

Li, J., Xu, J.-Z., Dou, B., Huang, T.-F., Chen, J., Wang, T.-M., et al. (2023). Circ_0001666 upregulation promotes intestinal epithelial cell fibrosis in pediatric Crohn's disease via the SRSF1/BMP7 axis. *Kaohsiung J. Med. Sci.* doi:10.1002/kjm2.12734

Lian, L., Huang, Q., Zhang, L., Qin, H., He, X., He, X., et al. (2018). Anti-fibrogenic potential of mesenchymal stromal cells in treating fibrosis in Crohn's disease. *Dig. Dis. Sci.* 63 (7), 1821–1834. doi:10.1007/s10620-018-5082-8

Lis-López, L., Bauset, C., Seco-Cervera, M., and Cosín-Roger, J. (2021). Is the macrophage phenotype determinant for fibrosis development? *Biomedicines* 9 (12), 1747. doi:10.3390/biomedicines9121747

Liu, C. Y., Cham, C. M., and Chang, E. B. (2021). Epithelial wound healing in inflammatory bowel diseases: the next therapeutic frontier. *Transl. Res.* 236, 35–51. doi:10.1016/j.trsl.2021.06.001

Liu, X., Lu, F., and Chen, X. (2022). Examination of the role of necroptotic damage-associated molecular patterns in tissue fibrosis. *Front. Immunol.* 13, 886374. doi:10.3389/fimmu.2022.886374

Lovisa, S., Genovese, G., and Danese, S. (2019). Role of epithelial-to-mesenchymal transition in inflammatory bowel disease. *J. Crohn's Colitis* 13 (5), 659–668. doi:10.1093/ecco-jcc/jjy201

Lurje, I., Gaisa, N. T., Weiskirchen, R., and Tacke, F. (2023). Mechanisms of organ fibrosis: emerging concepts and implications for novel treatment strategies. *Mol. Aspects Med.* 92, 101191. doi:10.1016/j.mam.2023.101191

Ma, D., Cao, Y., Wang, Z., He, J., Chen, H., Xiong, H., et al. (2019). CCAT1 lncRNA promotes inflammatory bowel disease malignancy by destroying intestinal barrier via downregulating miR-185-3p. *Inflamm. Bowel Dis.* 25 (5), 862–874. doi:10.1093/ibd/izy381

Macias-Ceja, D. C., Barrachina, M. D., and Ortiz-Masià, D. (2023). Autophagy in intestinal fibrosis: relevance in inflammatory bowel disease. *Front. Pharmacol.* 14, 1170436. doi:10.3389/fphar.2023.1170436

Macias-Ceja, D. C., Coll, S., Bauset, C., Seco-Cervera, M., Gisbert-Ferrándiz, L., Navarro, F., et al. (2022). Ifnγ-treated macrophages induce EMT through the WNT pathway: relevance in Crohn's disease. *Biomedicines* 10 (5), 1093. doi:10.3390/biomedicines10051093

Macias-Ceja, D. C., Ortiz-Masiá, D., Salvador, P., Gisbert-Ferrándiz, L., Hernández, C., Hausmann, M., et al. (2019). Succinate receptor mediates intestinal inflammation and fibrosis. *Mucosal Immunol.* 12 (1), 178–187. doi:10.1038/s41385-018-0087-3

Marconi, G. D., Fonticoli, L., Rajan, T. S., Pierdomenico, S. D., Trubiani, O., Pizzicannella, J., et al. (2021). Epithelial-mesenchymal transition (EMT): the type-2 EMT in wound healing, tissue regeneration and organ fibrosis. *Cells* 10 (7), 1587. doi:10.3390/cells10071587

Marti-Chafer, S., Jimenez-Mallen, E., Mendoza, T., Macias-Ceja, D. C., Bauset, C., Seco-Cervera, M., et al. (2023). P038 notch pathway in fibrosis: A new anti-fibrotic therapy in Crohn's disease? *J. Crohn's Colitis* 17 (1), i205. doi:10.1093/ecco-jcc/jjac190.0168

Martinez-Medina, M., Denizot, J., Dreux, N., Robin, F., Billard, E., Bonnet, R., et al. (2014). Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. *Gut* 63 (1), 116–124. doi:10.1136/gutjnl-2012-304119

McCole, D. F. (2014). IBD candidate genes and intestinal barrier regulation. *Inflamm. Bowel Dis.* 20 (10), 1829–1849. doi:10.1097/MIB.0000000000000090

McGregor, C. G. C., Tandon, R., and Simmons, A. (2023). Pathogenesis of fistulating Crohn's disease: A review. *Cell. Mol. Gastroenterology Hepatology* 15 (1), 1–11. doi:10.1016/j.jcmgh.2022.09.011

Melhem, H., Regan-Komito, D., and Niess, J. H. (2021). Mucins dynamics in physiological and pathological conditions. *Int. J. Mol. Sci.* 22 (24), 13642. Article 24. doi:10.3390/ijms222413642

Mourelle, M., Salas, A., Guarner, F., Crespo, E., García-Lafuente, A., Malagelada, J. R., et al. (1998). Stimulation of transforming growth factor beta1 by enteric bacteria in the pathogenesis of rat intestinal fibrosis. *Gastroenterology* 114 (3), 519–526. doi:10.1016/s0016-5085(98)70535-9

Mudduluru, G., Ceppi, P., Kumarswamy, R., Scagliotti, G. V., Papotti, M., and Allgayer, H. (2011). Regulation of Axl receptor tyrosine kinase expression by miR-34a and miR-199a/b in solid cancer. *Oncogene* 30 (25), 2888–2899. Article 25. doi:10.1038/onc.2011.13

Nanini, H. F., Bernardazzi, C., Castro, F., and de Souza, H. S. P. (2018). Damage-associated molecular patterns in inflammatory bowel disease: from biomarkers to therapeutic targets. *World J. Gastroenterology* 24 (41), 4622–4634. doi:10.3748/wjg.v24.i41.4622

Ortiz-Masiá, D., Gisbert-Ferrándiz, L., Bauset, C., Coll, S., Mamie, C., Scharl, M., et al. (2020a). Succinate activates EMT in intestinal epithelial cells through SUCNR1: A novel protagonist in fistula development. *Cells* 9 (5), 1104. doi:10.3390/cells9051104

Ortiz-Masià, D., Salvador, P., Macias-Ceja, D. C., Gisbert-Ferrándiz, L., Esplugues, J. V., Manyé, J., et al. (2020b). WNT2b activates epithelial-mesenchymal transition through FZD4: relevance in penetrating Crohn´s disease. *J. Crohn's Colitis* 14 (2), 230–239. doi:10.1093/ecco-jcc/jjz134

Otte, M. L., Tamang, R. L., Papapanagiotou, J., Ahmad, R., Dhawan, P., and Singh, A. B. (2023). Mucosal healing and inflammatory bowel disease: therapeutic implications and new targets. *World J. Gastroenterology* 29 (7), 1157–1172. doi:10.3748/wjg.v29.i7.1157

Ouyang, Y., Liu, G., Xu, W., Yang, Z., Li, N., Xie, C., et al. (2021). *Helicobacter pylori* induces epithelial-mesenchymal transition in gastric carcinogenesis via the AKT/GSK3β signaling pathway. *Oncol. Lett.* 21 (2), 165. doi:10.3892/ol.2021.12426

Park, J. M., Kim, J., Lee, Y. J., Bae, S. U., and Lee, H. W. (2023). Inflammatory bowel disease–associated intestinal fibrosis. *J. Pathology Transl. Med.* 57 (1), 60–66. doi:10.4132/jptm.2022.11.02

Pelczar, P., Witkowski, M., Perez, L. G., Kempski, J., Hammel, A. G., Brockmann, L., et al. (2016). A pathogenic role for T cell-derived IL-22BP in inflammatory bowel disease. *Sci. (New York, N.Y.)* 354 (6310), 358–362. doi:10.1126/science.aah5903

Pierdomenico, M., Palone, F., Cesi, V., Vitali, R., Mancuso, A. B., Cucchiara, S., et al. (2018). Transcription factor ZNF281: A novel player in intestinal inflammation and fibrosis. *Front. Immunol.* 9, 2907. doi:10.3389/fimmu.2018.02907

Planté-Bordeneuve, T., Pilette, C., and Froidure, A. (2021). The epithelial-immune crosstalk in pulmonary fibrosis. *Front. Immunol.* 12, 631235. doi:10.3389/fimmu.2021.631235

Pompili, S., Vetuschi, A., Latella, G., Smakaj, A., Sferra, R., and Cappariello, A. (2023). PPAR-gamma orchestrates EMT, AGE, and cellular senescence pathways in colonic epithelium and restrains the progression of IBDs. *Int. J. Mol. Sci.* 24 (10), 8952. Article 10. doi:10.3390/ijms24108952

Ramani, K., and Biswas, P. S. (2019). Interleukin-17: friend or foe in organ fibrosis. *Cytokine* 120, 282–288. doi:10.1016/j.cyto.2018.11.003

Rankin, C. R., Lokhandwala, Z. A., Huang, R., Pekow, J., Pothoulakis, C., and Padua, D. (2019). Linear and circular CDKN2B-AS1 expression is associated with Inflammatory Bowel Disease and participates in intestinal barrier formation. *Life Sci.* 231, 116571. doi:10.1016/j.lfs.2019.116571

Reka, A. K., Kurapati, H., Narala, V. R., Bommer, G., Chen, J., Standiford, T. J., et al. (2010). Peroxisome proliferator-activated receptor-gamma activation inhibits tumor metastasis by antagonizing Smad3-mediated epithelial-mesenchymal transition. *Mol. Cancer Ther.* 9 (12), 3221–3232. doi:10.1158/1535-7163.MCT-10-0570

Rieder, F. (2013). The gut microbiome in intestinal fibrosis: environmental protector or provocateur? *Sci. Transl. Med.* 5 (190), 190ps10. doi:10.1126/scitranslmed.3004731

Rosenblum, D., and Naik, S. (2022). Epithelial-immune crosstalk in health and disease. *Curr. Opin. Genet. Dev.* 74, 101910. doi:10.1016/j.gde.2022.101910

Rout-Pitt, N., Farrow, N., Parsons, D., and Donnelley, M. (2018). Epithelial mesenchymal transition (EMT): A universal process in lung diseases with implications for cystic fibrosis pathophysiology. *Respir. Res.* 19 (1), 136. doi:10.1186/s12931-018-0834-8

Saito, S., Kato, J., Hiraoka, S., Horii, J., Suzuki, H., Higashi, R., et al. (2011). DNA methylation of colon mucosa in ulcerative colitis patients: correlation with inflammatory status. *Inflamm. Bowel Dis.* 17 (9), 1955–1965. doi:10.1002/ibd.21573

Santacroce, G., Lenti, M. V., and Di Sabatino, A. (2022). Therapeutic targeting of intestinal fibrosis in Crohn's disease. *Cells* 11 (3), 429. Article 3. doi:10.3390/cells11030429

Satsangi, J., Silverberg, M. S., Vermeire, S., and Colombel, J.-F. (2006). The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut* 55 (6), 749–753. doi:10.1136/gut.2005.082909

Scalavino, V., Piccinno, E., Bianco, G., Schena, N., Armentano, R., Giannelli, G., et al. (2022). The increase of miR-195-5p reduces intestinal permeability in ulcerative colitis, modulating tight junctions' expression. *Int. J. Mol. Sci.* 23 (10), 5840. doi:10.3390/ijms23105840

Scarpa, M., Kessler, S., Sadler, T., West, G., Homer, C., McDonald, C., et al. (2015). The epithelial danger signal IL-1α is a potent activator of fibroblasts and reactivator of intestinal inflammation. *Am. J. Pathology* 185 (6), 1624–1637. doi:10.1016/j.ajpath.2015.02.018

Scharl, M., Frei, P., Frei, S. M., Biedermann, L., Weber, A., and Rogler, G. (2014). Epithelial-to-mesenchymal transition in a fistula-associated anal adenocarcinoma in a patient with long-standing Crohn's disease. *Eur. J. Gastroenterology Hepatology* 26 (1), 114–118. doi:10.1097/MEG.0b013e32836371a2

Scharl, M., Frei, S., Pesch, T., Kellermeier, S., Arikkat, J., Frei, P., et al. (2013). Interleukin-13 and transforming growth factor β synergise in the pathogenesis of human intestinal fistulae. *Gut* 62 (1), 63–72. doi:10.1136/gutjnl-2011-300498

Scharl, M., Huber, N., Lang, S., Fürst, A., Jehle, E., and Rogler, G. (2015). Hallmarks of epithelial to mesenchymal transition are detectable in Crohn's disease associated intestinal fibrosis. *Clin. Transl. Med.* 4, 1. doi:10.1186/s40169-015-0046-5

Scharl, M., Weber, A., Fürst, A., Farkas, S., Jehle, E., Pesch, T., et al. (2011). Potential role for SNAIL family transcription factors in the etiology of Crohn's disease-associated fistulae. *Inflamm. Bowel Dis.* 17 (9), 1907–1916. doi:10.1002/ibd.21555

Shao, B.-Z., Yao, Y., Zhai, J.-S., Zhu, J.-H., Li, J.-P., and Wu, K. (2021). The role of autophagy in inflammatory bowel disease. *Front. Physiology* 12, 621132. doi:10.3389/fphys.2021.621132

Shi, C., Liang, Y., Yang, J., Xia, Y., Chen, H., Han, H., et al. (2013). MicroRNA-21 knockout improve the survival rate in DSS induced fatal colitis through protecting against inflammation and tissue injury. *PloS One* 8 (6), e66814. doi:10.1371/journal.pone.0066814

Simic, P., Williams, E. O., Bell, E. L., Gong, J. J., Bonkowski, M., and Guarente, L. (2013). SIRT1 suppresses the epithelial-to-mesenchymal transition in cancer metastasis and organ fibrosis. *Cell Rep.* 3 (4), 1175–1186. doi:10.1016/j.celrep.2013.03.019

Solitano, V., Dal Buono, A., Gabbiadini, R., Wozny, M., Repici, A., Spinelli, A., et al. (2023). Fibro-stenosing Crohn's disease: what is new and what is next? *J. Clin. Med.* 12 (9), 3052. doi:10.3390/jcm12093052

Song, H., Tang, X., Li, X., Wang, Y., Deng, A., Wang, W., et al. (2020). HLJ2 effectively ameliorates colitis-associated cancer via inhibition of NF-κB and epithelial-mesenchymal transition. *Drug Des. Dev. Ther.* 14, 4291–4302. doi:10.2147/DDDT.S262806

Suzuki, K., Arumugam, S., Yokoyama, J., Kawauchi, Y., Honda, Y., Sato, H., et al. (2016). Pivotal role of carbohydrate sulfotransferase 15 in fibrosis and mucosal healing in mouse colitis. *PLOS ONE* 11 (7), e0158967. doi:10.1371/journal.pone.0158967

Suzuki, K., Yokoyama, J., Kawauchi, Y., Honda, Y., Sato, H., Aoyagi, Y., et al. (2017). Phase 1 clinical study of siRNA targeting carbohydrate sulphotransferase 15 in Crohn's disease patients with active mucosal lesions. *J. Crohn's Colitis* 11 (2), 221–228. doi:10.1093/ecco-jcc/jjw143

Tahara, T., Shibata, T., Okubo, M., Ishizuka, T., Nakamura, M., Nagasaka, M., et al. (2014). DNA methylation status of epithelial-mesenchymal transition (EMT)—related genes is associated with severe clinical phenotypes in ulcerative colitis (UC). *PloS One* 9 (10), e107947. doi:10.1371/journal.pone.0107947

Tennakoon, A. H., Izawa, T., Kuwamura, M., and Yamate, J. (2015). Pathogenesis of type 2 epithelial to mesenchymal transition (EMT) in renal and hepatic fibrosis. *J. Clin. Med.* 5 (1), 4. doi:10.3390/jcm5010004

Thia, K. T., Sandborn, W. J., Harmsen, W. S., Zinsmeister, A. R., and Loftus, E. V. (2010). Risk factors associated with progression to intestinal complications of Crohn's disease in a population-based cohort. *Gastroenterology* 139 (4), 1147–1155. doi:10.1053/j.gastro.2010.06.070

Wang, Z., Yuan, X., Jiao, N., Zhu, H., Zhang, Y., and Tong, J. (2012). CDH13 and FLBN3 gene methylation are associated with poor prognosis in colorectal cancer. *Pathology Oncol. Res. POR* 18 (2), 263–270. doi:10.1007/s12253-011-9437-0

Wang, Z., Zhou, H., Cheng, F., Zhang, Z., and Long, S. (2022). MiR-21 regulates epithelial-mesenchymal transition in intestinal fibrosis of Crohn's disease by targeting PTEN/mTOR. *Dig. Liver Dis.* 54 (10), 1358–1366. doi:10.1016/j.dld.2022.04.007

Weiskirchen, R., and Meurer, S. K. (2013). BMP-7 counteracting TGF-beta1 activities in organ fibrosis. *Front. Biosci. (Landmark Ed.* 18 (4), 1407–1434. doi:10.2741/4189

Wenxiu, J., Mingyue, Y., Fei, H., Yuxin, L., Mengyao, W., Chenyang, L., et al. (2021). Effect and mechanism of TL1A expression on epithelial-mesenchymal transition during chronic colitis-related intestinal fibrosis. *Mediat. Inflamm.* 2021, 5927064. doi:10.1155/2021/5927064

Wu, F., Shao, Q., Hu, M., Zhao, Y., Dong, R., Fang, K., et al. (2020). Wu-Mei-Wan ameliorates chronic colitis-associated intestinal fibrosis through inhibiting fibroblast activation. *J. Ethnopharmacol.* 252, 112580. doi:10.1016/j.jep.2020.112580

Xu, R., Won, J.-Y., Kim, C.-H., Kim, D.-E., and Yim, H. (2019). Roles of the phosphorylation of transcriptional factors in epithelial-mesenchymal transition. *J. Oncol.* 2019, e5810465. doi:10.1155/2019/5810465

Xu, S., Jiang, B., Wang, H., Shen, C., Chen, H., and Zeng, L. (2017). Curcumin suppresses intestinal fibrosis by inhibition of ppary-mediated epithelial-mesenchymal transition. *Evidence-Based Complementary Altern. Med. ECAM* 2017, 7876064. doi:10.1155/2017/7876064

Xu, X., Sun, S., Xie, F., Ma, J., Tang, J., He, S., et al. (2017). Advanced oxidation protein products induce epithelial-mesenchymal transition of intestinal epithelial cells via a PKC δ-mediated, redox-dependent signaling pathway. *Antioxidants Redox Signal.* 27 (1), 37–56. doi:10.1089/ars.2015.6611

Xu, Y., Yang, J., Chen, X., Deng, J., Gong, H., Li, F., et al. (2022). MicroRNA-182-5p aggravates ulcerative colitis by inactivating the Wnt/β-catenin signaling pathway through DNMT3A-mediated SMARCA5 methylation. *Genomics* 114 (3), 110360. doi:10.1016/j.ygeno.2022.110360

Yamagata, M., Mikami, T., Tsuruta, T., Yokoyama, K., Sada, M., Kobayashi, K., et al. (2011). Submucosal fibrosis and basic-fibroblast growth factor-positive neutrophils correlate with colonic stenosis in cases of ulcerative colitis. *Gastroenterologia* 84 (1), 12–21. doi:10.1159/000320773

Yang, B.-L., Zhu, P., Li, Y.-R., Xu, M.-M., Wang, H., Qiao, L.-C., et al. (2018). Total flavone of Abelmoschus manihot suppresses epithelial-mesenchymal transition via interfering transforming growth factor-β1 signaling in Crohn's disease intestinal fibrosis. *World J. Gastroenterology* 24 (30), 3414–3425. doi:10.3748/wjg.v24.i30.3414

Yang, J., Ding, C., Dai, X., Lv, T., Xie, T., Zhang, T., et al. (2017a). Soluble dietary fiber ameliorates radiation-induced intestinal epithelial-to-mesenchymal transition and fibrosis. *JPEN. J. Parenter. Enter. Nutr.* 41 (8), 1399–1410. doi:10.1177/0148607116671101

Yang, J., Zhou, C.-Z., Zhu, R., Fan, H., Liu, X.-X., Duan, X.-Y., et al. (2017b). MiR-200b-containing microvesicles attenuate experimental colitis associated intestinal fibrosis by inhibiting epithelial-mesenchymal transition. *J. Gastroenterology Hepatology* 32 (12), 1966–1974. doi:10.1111/jgh.13797

Yang, Y., Ma, Y., Shi, C., Chen, H., Zhang, H., Chen, N., et al. (2013). Overexpression of miR-21 in patients with ulcerative colitis impairs intestinal epithelial barrier function through targeting the Rho GTPase RhoB. *Biochem. Biophysical Res. Commun.* 434 (4), 746–752. doi:10.1016/j.bbrc.2013.03.122

Yin, Y., Grabowska, A. M., Clarke, P. A., Whelband, E., Robinson, K., Argent, R. H., et al. (2010). *Helicobacter pylori* potentiates epithelial:mesenchymal transition in gastric cancer: links to soluble HB-EGF, gastrin and matrix metalloproteinase-7. *Gut* 59 (8), 1037–1045. doi:10.1136/gut.2009.199794

Yoo, J. H., Holubar, S., and Rieder, F. (2020). Fibrostenotic strictures in Crohn's disease. *Intestinal Res.* 18 (4), 379–401. doi:10.5217/ir.2019.09148

Yu, M., Wu, H., Wang, J., Chen, X., Pan, J., Liu, P., et al. (2021). Vitamin D receptor inhibits EMT via regulation of the epithelial mitochondrial function in intestinal fibrosis. *J. Biol. Chem.* 296, 100531. doi:10.1016/j.jbc.2021.100531

Yue, Y.-L., Zhang, M.-Y., Liu, J.-Y., Fang, L.-J., and Qu, Y.-Q. (2022). The role of autophagy in idiopathic pulmonary fibrosis: from mechanisms to therapies. *Ther. Adv. Respir. Dis.* 16, 17534666221140972. doi:10.1177/17534666221140972

Yun, S.-M., Kim, S.-H., and Kim, E.-H. (2019). The molecular mechanism of transforming growth factor-β signaling for intestinal fibrosis: A mini-review. *Front. Pharmacol.* 10, 162. doi:10.3389/fphar.2019.00162

Zeng, C., Liu, X., Xiong, D., Zou, K., and Bai, T. (2022). Resolvin D1 prevents epithelial-to-mesenchymal transition and reduces collagen deposition by stimulating autophagy in intestinal fibrosis. *Dig. Dis. Sci.* 67 (10), 4749–4759. doi:10.1007/s10620-021-07356-w

Zeissig, S., Bürgel, N., Günzel, D., Richter, J., Mankertz, J., Wahnschaffe, U., et al. (2007). Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut* 56 (1), 61–72. doi:10.1136/gut.2006.094375

Zhang, H.-J., Zhang, Y.-N., Zhou, H., Guan, L., Li, Y., and Sun, M.-J. (2018). IL-17A promotes initiation and development of intestinal fibrosis through EMT. *Dig. Dis. Sci.* 63 (11), 2898–2909. doi:10.1007/s10620-018-5234-x

Zhang, K., Dong, Y., Li, M., Zhang, W., Ding, Y., Wang, X., et al. (2023). Clostridium butyricum inhibits epithelial-mesenchymal transition of intestinal carcinogenesis through downregulating METTL3. *Cancer Sci.* 114, 3114–3127. doi:10.1111/cas.15839

Zhang, Y., Cui, Y., Cheng, Y., Zhu, W., Zhang, M., Li, S., et al. (2021). Succinate accumulation contributes to oxidative stress and iron accumulation in pentylenetetrazol-induced epileptogenesis and kainic acid-induced seizure. *Neurochem. Int.* 149, 105123. doi:10.1016/j.neuint.2021.105123

Zhao, J. F., Ling, F. M., Li, J. R., Chen, Y. D., Huang, L., and Zhu, L. R. (2020). Role of non-inflammatory factors in intestinal fibrosis. *J. Dig. Dis.* 21 (6), 315–318. doi:10.1111/1751-2980.12883

Zhao, J., Lin, Z., Ying, P., Zhao, Z., Yang, H., Qian, J., et al. (2023). CircSMAD4 promotes experimental colitis and impairs intestinal barrier functions by targeting janus kinase 2 through sponging miR-135a-5p. *J. Crohn's Colitis* 17 (4), 593–613. doi:10.1093/ecco-jcc/jjac154

Zhao, X., Cui, D.-J., Yang, L.-C., Yuan, W.-Q., and Yan, F. (2022). Long noncoding RNA FBXL19-AS1-mediated ulcerative colitis-associated intestinal epithelial barrier defect. *Tissue Eng. Regen. Med.* 19 (5), 1077–1088. doi:10.1007/s13770-022-00479-9

Zhao, X., Fan, J., Zhi, F., Li, A., Li, C., Berger, A. E., et al. (2015). Mobilization of epithelial mesenchymal transition genes distinguishes active from inactive lesional tissue in patients with ulcerative colitis. *Hum. Mol. Genet.* 24 (16), 4615–4624. doi:10.1093/hmg/ddv192

Zhou, H., Zhang, H.-J., Guan, L., Zhang, Y.-N., Li, Y., and Sun, M.-J. (2018). Mechanism and therapeutic effects of Saccharomyces boulardii on experimental colitis in mice. *Mol. Med. Rep.* 18 (6), 5652–5662. doi:10.3892/mmr.2018.9612

Zhou, L.-Y., Lin, S.-N., Rieder, F., Chen, M.-H., Zhang, S.-H., and Mao, R. (2021). Noncoding RNAs as promising diagnostic biomarkers and therapeutic targets in intestinal fibrosis of Crohn's disease: the path from bench to bedside. *Inflamm. Bowel Dis.* 27 (7), 971–982. doi:10.1093/ibd/izaa321

Zidar, N., Boštjančič, E., Jerala, M., Kojc, N., Drobne, D., Štabuc, B., et al. (2016). Down-regulation of microRNAs of the miR-200 family and up-regulation of snail and slug in inflammatory bowel diseases—hallmark of epithelial–mesenchymal transition. *J. Cell. Mol. Med.* 20 (10), 1813–1820. doi:10.1111/jcmm.12869

Zorzi, F., Calabrese, E., and Monteleone, G. (2015). Pathogenic aspects and therapeutic avenues of intestinal fibrosis in Crohn's disease. *Clin. Sci. Lond. Engl. 1979)* 129 (12), 1107–1113. doi:10.1042/CS20150472

frontiers | Frontiers in Medicine

TYPE Brief Research Report
PUBLISHED 10 October 2023
DOI 10.3389/fmed.2023.1221449

OPEN ACCESS

EDITED BY
Elena Ortiz Zapater,
University of Valencia, Spain

REVIEWED BY
Hiroshi Nakase,
Sapporo Medical University, Japan
Anurag Kumar Singh,
Martin Luther University of Halle-Wittenberg, Germany

*CORRESPONDENCE
Markus F. Neurath
markus.neurath@uk-erlangen.de

RECEIVED 12 May 2023
ACCEPTED 09 August 2023
PUBLISHED 10 October 2023

CITATION
Rath T, Atreya R, Bodenschatz J, Uter W, Geppert CI, Vitali F, Zundler S, Waldner MJ, Hartmann A and Neurath MF (2023) Healing of the epithelial barrier in the ileum is superior to endoscopic and histologic remission for predicting major adverse outcomes in ulcerative colitis.
Front. Med. 10:1221449.
doi: 10.3389/fmed.2023.1221449

Healing of the epithelial barrier in the ileum is superior to endoscopic and histologic remission for predicting major adverse outcomes in ulcerative colitis

Timo Rath[1], Raja Atreya[1], Julia Bodenschatz[1], Wolfgang Uter[2], Carol I. Geppert[3], Francesco Vitali[1], Sebastian Zundler[1], Maximilian J. Waldner[1], Arndt Hartmann[3] and Markus F. Neurath[1,4]*

[1]Department of Gastroenterology, Ludwig Demling Endoscopy Center of Excellence, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nuernberg, Erlangen, Germany, [2]Institute for Medical Informatics, Biometry and Epidemiology, Friedrich-Alexander University Erlangen-Nuernberg, Erlangen, Germany, [3]Institute for Pathology, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nuernberg, Erlangen, Germany, [4]Deutsches Zentrum für Immuntherapie DZI, Friedrich-Alexander University Erlangen-Nuernberg, Erlangen, Germany

Background: Achieving endoscopic remission is a key therapeutic goal in patients with ulcerative colitis (UC) that is associated with favorable long-term disease outcomes. Here, we prospectively compared the predictive value of endoscopic and/or histologic remission against ileal barrier healing for predicting long-term disease behavior in a large cohort of UC patients in clinical remission.

Methods: At baseline, UC patients in clinical remission underwent ileocolonoscopy with assessment of ileal barrier function by confocal endomicroscopy. Endoscopic and histologic disease activity and ileal barrier healing were scored using validated scores. During subsequent follow-up (FU), patients were closely monitored for clinical disease activity and occurrence of major adverse outcomes (MAO) defined as the following: disease relapse; UC-related hospitalization; UC-related surgery; necessity for initiation or dose escalation of systemic steroids, immunosuppressants, small molecules or biological therapy.

Results: Of the 73 UC patients included, 67% experienced MAO during a mean FU of 25 months. The probability of MAO-free survival was significantly higher in UC patients with endoscopic and/or histologic remission compared to patients with endoscopically and/or histologically active disease. Ileal barrier healing on endomicroscopy was highly accurate for predicting the further course of UC and outcompeted endoscopic and histologic remission for predicting MAO-free survival.

Conclusion: Ileal barrier healing in clinically remittent UC patients can accurately predict future MAO development and is superior in its predictive capabilities than endoscopic and histologic remission. Ileal barrier healing therefore represents a novel and superior surrogate parameter for stratification of UC patients according to their risk for development of complicated disease behavior.

Clinical trial registration: https://classic.clinicaltrials.gov/ct2/show/NCT05157750, identifier NCT05157750.

KEYWORDS

inflammatory bowel diseases, endoscopy, histology, intestinal barrier, ulcerative colitis

Introduction

Ulcerative colitis (UC) patients that achieve endoscopic remission have a more favorable course of disease with decreased flaring of disease and increased rates of steroid free clinical remission and colectomy-free survival as compared to UC patients without endoscopic remission. Therefore, achieving endoscopic remission in patients with ulcerative colitis is a major treatment goal that is advocated by several guidelines for clinical practice and for trial endpoints (1–5). In addition to endoscopic remission, histologic remission is another emerging endpoint in patients with ulcerative colitis that is associated, as consolidated by several meta-analyses (6–8), with better disease outcome compared to clinical remission and/or endoscopic healing. However, assessing histological remission UC is complex with currently 26 different histopathological scores out of which only two are validated (9). Furthermore, although acknowledged as a sensitive measure of inflammation, the STRIDE working group does not recommend histologic remssion as a formal treatment target in UC (4).

Just recently, we compared the value of endoscopic remission and histologic remission against the integrity of the intestinal barrier for predicting long-term disease behavior in clinically remittent IBD patients for predicting major adverse outcomes (MAO). In this ERIca trial (Erlangen Remission in IBD), a large cohort of IBD patients in clinical remission were prospectively included and closely monitored during long term follow for more than 2 years and this study provided first evidence that assessing the integrity of the intestinal barrier with confocal laser endomicroscopy (CLE) can not only accurately predict disease behavior but also that intestinal barrier healing is superior compared to endoscopic and histologic remission for predicting MAOs (10). However, in the ERIca trial, we only analyzed colonic barrier function for predicting disease behavior in UC. Therefore, we now aimed to extend these observations and to explore whether assessment of ileal barrier function in patients with ulcerative colitis can predict the occurrence of major adverse outcomes in clinically remitted patients with ulcerative colitis.

Study design and participants

This study was an extended analysis of data from the ERIca trial which was conducted at the Ludwig Demling Endoscopy Center of Excellence and the IBD outpatient department at the University Hospital of Erlangen as a prospective observational study (10). The study was approved by the local ethics committee as well as the Institutional Review Board of the Medical Faculty of the Friedrich-Alexander University Erlangen-Nuremberg. After written informed consent was obtained, patients with an established diagnosis of UC for at least 12 months and which presented in clinical remission were enrolled. Exclusion criteria were as follows: poor bowel preparation, total colectomy, concomitant beta blocker therapy, known allergy to fluorescein or a planned change in IBD-related pharmacotherapy. Clinical disease activity was assessed along the Mayo clinical disease activity score (MCS) prior to study inclusion (11). After ileocolonoscopy with confocal laser endomicroscopy, close meshed followed up in our IBD outpatient department every 4 to 8 weeks for patients under biological therapy and every 8 weeks for patients under conventional therapy was performed. At each visit, clinical disease activity using the MCS along with routine laboratory parameters and current and past medications were assessed. Furthermore, major adverse outcomes (MAO), defined as the following, were recorded at each visit: (i) disease relapse; (ii) UC-related hospitalization, (iii) UC-related surgery, (iv) necessity for initiation or dose escalation of systemic steroids, immunosuppressants, small molecules or biological therapy.

Colonoscopy and confocal laser endomicroscopy

Bowel preparation was performed with low-volume PEG-based bowel lavage in a split dose regimen in all patients scheduled for ileocolonoscopy. In case the patients were scheduled for sigmoidoscopy only, the patients received dihydrogen dihydrate enema prior to sigmoidoscopy. According to consensus statements, endoscopic remission and/or healing during WLE were defined in the following way (3, 12): Endoscopic remission, Mayo Endoscopy Score (MES) $\leq$1; Endoscopic healing, MES = 0 (13, 14). Representative endoscopic images of patients with and without endoscopic remission are shown in Supplementary Figure S1.

Confocal Laser Endomicroscopy was performed as previously described (10). After reaching the terminal ileum, 5 mL Fluorescein 10% were intravenously injected as a contrast agent. Afterwards, the CLE probe was positioned under endoscopic guidance onto the mucosa of the terminal ileum, low-powered blue laser light of a wavelength of 488 nm was activated for tissue illumination by the hit of a foot pedal and a CLE video of approximately 2 min was recorded with an image acquisition rate of 8 frames per second. All CLE images for each patient were stored on an external hard drive and were independently reviewed for presence of ileal barrier dysfunction by three expert readers (T.R., J.B., F.V.) blinded to the clinical results of the patients.

Barrier dysfunction in the terminal ileum was assessed using the semi-quantitative Watson score into three grades as previously described (10, 15–20): (I) *intact epithelial barrier* with no fluorescein leakage, (II) *functional barrier defect* with shedding of single epithelial cells and fluorescein leakage into the intestinal lumen, (III) *structural barrier defect* with shedding of multiple epithelial cells, exposure of the lamina propria to the lumen and fluorescein leakage into the lumen. The different grades of ileal barrier (dys)function as assessed by CLE are shown in Supplementary Figure S2.

Histologic analysis

From each patient, samples for histopathology were obtained at the sites where CLE imaging was performed. In addition, in case macroscopic inflammation was present during WLE, these areas were

Abbreviations: CD, Crohn's disease; CLE, Confocal Laser Endomicroscopy; FU, Follow up; IBD, Inflammatory Bowel Diseases; IO-IBD, International Organization for the Study of Inflammatory Bowel Disease; MES, Mayo Endoscopy Score; MAO, Major adverse outcome; MCS, Mayo Clinical Score; MH, Mucosal healing; NHI, Nancy Histological Index; RHI, Robarts Histopathology Index; STRIDE, Selected Therapeutic Targets in Inflammatory Bowel Disease; UC, Ulcerative Colitis.

also biopsied matching those areas that were also examined by CLE. All samples were scored by an experienced GI pathologist (A.H.) blinded to clinical and endoscopic patient data. For histopathological scoring in UC, Robarts Histopathology Index (RHI) [24] as well as Nancy histological index (NHI) [25] were used as validated histology scores. Histologic disease remission was defined as a RHI ≤ 3 without lamina propria or epithelial neutrophils or a NHI ≤ 1. Representative histolopathologic images are shown in Supplementary Figure S3.

Endpoints, sample size and statistical analysis

The primary endpoint of this study was to compare the predictive values of ileal barrier healing, endoscopic remission and histologic remission for predicting occurrence of MAO in UC patients. Statistical analyzes were performed using the R statistical software package, version 4.0.x.[1] All statistical tests were considered explorative without alpha adjustment. Moreover, Kaplan–Meier analysis was performed to examine the time to the occurrence of MAOs (or censoring at end of follow-up).

Results

Study inclusion and clinical patient characteristics

Between 2017 and 2019, a total of 81 UC patients were included in the study. From these 81 patients, 73 patients had valid and complete information regarding the occurrence of MAOs during follow-up and data on endoscopic remission and healing, histologic remission and barrier function in the terminal ileum were available for all patients. Clinical, endoscopic and histological characteristics of the UC patient cohort are summarized in Table 1.

From these 73 UC patients included in the final analysis, 41 (56.2%) patients had endoscopic remission on WLE, as defined by a MES ≤ 1, at study inclusion (Table 1). Histologic remission, as defined by RHI and NHI, was observed in 56.2 and 53.4% patients, respectively, during baseline endoscopy. In 34 UC patients (44.4%), the combination between endoscopic and histologic remission (as assessed by the RHI), was present. In contrast, barrier healing in the ileum was observed in only 22 UC patients in the terminal ileum (30.1%) during baseline endoscopy. Detailed clinical, endoscopic and histologic characteristics in UC patients with and without ileal barrier healing are comparatively displayed in Supplementary Table S1. In additional studies, we determined levels of serum zonulin, as a marker that has been used in a variety of studies to assess integrity of the intestinal barrier, and noted that serum zonulin levels did not significantly differ between UC patients with intact ileal barrier as compared to those with ileal barrier dysfunction (Supplementary Figure S4).

TABLE 1 Clinical, endoscopic and histologic characteristics of the UC patient cohort.

Ulcerative colitis (n = 73)	
Clinical characteristics	
Age (y)	
Mean, range	38.3 (18–69)
Sex (m/f)	36/37
BMI	
Mean, range	25.6 (17.2–39.2)
Disease duration (y)	
Mean ± SD	9 ± 7.6
Extent of disease, *n* (%)	
Proctitis	5 (6.8)
Leftsided colitis	34 (46.6)
Pancolitis	34 (46.6)
Extraintestinal manifestations, *n* (%)	18 (24.7)
Primary sclerosing cholangitis, *n* (%)	2 (2.7)
Medication, n (%)	
5-ASA derivates	
Mesalazin	12 (16.4)
Corticosteroids	
Budesonide (with colonic delivery)	2 (2.7)
Prednisolone (n)	2 (2.7)
Mean dose (mg) ± SD	10 ± 4
Immunomodulator	
6-Mercaptopurin	1 (1.4)
Azathioprin	3 (4.1)
Biological therapy	
Anti-TNF	28 (38.4)
Vedolizumab	11 (15.1)
Tofacitinib	3 (4.1)
Ustekinumab	2 (2.7)
Combination therapy	5 (6.8)
No medication	4 (5.5)
Laboratory parameters, mean ± SD	
Leukocyte count (10^9/L)	7.9 ± 3.3
C-reactive Protein (mg/L)	5.0 ± 8.3
Hematocrit (%)	41.5 ± 4.1
Endoscopic and histopathologic data	
Mayo endoscopic score, *n* (%)	
≤ 1	41 (56.2)
> 1	32 (43.8)
Barrier function, *n* (%)	
Ileum	
Barrier healing present	22 (30.1)
Histopathology scoring, *n* (%)	
RHI ≤ 3	41 (56.2)

(Continued)

1 www.r-project.org

TABLE 1 (Continued)

Ulcerative colitis ($n = 73$)	
RHI > 3	32 (43.8)
Nancy <1	39 (53.4)
Nancy ≥1	34 (46.6)
Follow up (FU)	
Mean ± SD (months)	26 ± 12
Patients without MAO during FU, *n* (%)	24 (32.9)

RHI, Robarts Histology Index; Nancy, Nancy Histological Index; MAO, major adverse outcomes: disease flare; UC-related hospitalization; UC-related surgery; necessity for initiation or escalation of systemic steroids, immunosuppressants, small molecules or biological therapy.

TABLE 2 Major adverse outcome (MAO) rates in patients with ulcerative colitis.

Parameter	MAO rate
Endoscopic remission	46.3% (19/41)
Endoscopic healing	31.3% (5/16)
RHI histologic remission	48.8% (20/41)
NHI histologic remission	46.2% (18/39)
Endoscopic remission + RHI histologic remission	41.2% (14/34)
Barrier healing ileum	9.1% (2/22)

MAO, major clinical events (disease flare; IBD-related hospitalization; IBD-related surgery; necessity for initiation or escalation of systemic steroids, immunosuppressants, small molecules or biological therapy) Endoscopic remission, MES ≤ 1; Endoscopic healing, MES = 0; RHI Histologic remission, RHI ≤ 3 without lamina propria or epithelial neutrophils; NHI Histologic remission, NHI ≤ 1.

Follow up and occurrence of major adverse outcomes in UC patients

Mean follow up in UC patients was 26 months (Table 1). In 24 UC patients, no MAOs occurred in the course of follow up, while in the remaining 49 patients it was, with a mean time lag for MAO occurrence of 3.2 months (SD ± 2.5 months, range 1–10 months) from baseline endoscopy.

The MAO rates in patients with endoscopic and histologic remission and in patients with barrier healing are summarized in Table 2. As shown in Table 2, of the 41 patients with endoscopic remission at study inclusion, 19 developed MAOs during FU, leading to a MAO rate for endoscopic remission of 46.3%. Time to event analysis using Kaplan–Meier estimates showed that UC patients with endoscopic remission had a significantly higher probability of remaining free of MAOs during FU compared to those patients with endoscopically active disease ($p < 0.0001$, Figure 1A). When applying a more stringent endoscopic definition considering only patients with a MES = 0 (i.e., endoscopic healing), a total of 16 UC patients exhibited endoscopic healing. Of these, 5 experienced MAO during the course of follow-up, leading to a MAO rate in patients with MES = 0 of 31.3% (Table 2). Correspondingly, the probability for MAO-free survival during FU was significantly higher in UC patients with endoscopic healing as compared to those with a MES > 0 ($p = 0.007$, Figure 1B).

From the 41 UC patients with histologic remission as defined by the RHI, 20 developed MAO during follow-up (RHI MAO-rate: 48.8%) while in 18 out of 39 patients with histologic remission as defined by the NHI, MAO occurred during the course of follow-up (NHI MAO-rate: 46.2%). On Kaplan–Meier analysis, patients with histologic remission along the RHI and the NHI were significantly more likely to remain MAO-free during FU as compared to UC patients with histologically active disease (both $p < 0.0001$, Figure 2). From those 34 patients with combined histologic (as defined by the RHI) and endoscopic remission, 14 experienced MAOs during study follow-up (MAO rate: 41.2%) and likewise, those patients with combined endoscopic and histologic remission had a significantly better course of disease in terms of remaining free of MAO on Kaplan–Meier estimates ($p < 0.0001$, Figure 3).

Of the 22 UC patients with barrier healing in the terminal ileum, only 2 patients developed MAO during FU, hence MAO rate in patients with ileal barrier healing was 9.1% (Table 2). Consistent with this, UC patients with barrier healing in the terminal ileum had a significantly more favorable course of disease as shown by Kaplan–Meier analysis ($p < 0.0001$, Figure 4).

Diagnostic performances of endoscopic healing, histologic healing and barrier healing for the prediction of the course of disease

Based on the low MAO rates in UC patients with intact ileal barrier and the high probabilities for remaining without MAOs during follow up, we further set off to directly compare the diagnostic performances of endoscopic and histologic remission as established parameters against ileal barrier healing for the prediction of long-term disease outcome.

Endoscopic remission (MES ≤ 1), had an overall accuracy of 71.2% for predicting the further course of disease with positive and negative predictive values of 53.7 and 93.8%, respectively (Table 3). Considering only patients with endoscopic healing (MES = 0), the accuracy for predicting MAO-free course of disease was increased with an accuracy of 75.3% and positive and negative predictive values of 68.8 and 77.1%, respectively (Table 3).

Histologic remission, as defined by the RHI and the NHI, exhibited an accuracy of 68.5 and 71.2%, respectively, with comparable positive and negative predictive values of the two histopathology scores for predicting the occurrence of MAO (Table 3).

Using the combination of endoscopic remission (as defined by a MES ≤ 1) and histologic remission as assessed by the RHI, overall accuracy for predicting the occurrence of major clinical events was increased to 75.3% with a positive and negative prediction of 58.8 and 89.7%, respectively (Table 3).

In contrast, the diagnostic performance of ileal barrier integrity as a new surrogate parameter for the prediction of long-term disease behavior was increased compared to the aforementioned parameters. In this regard, barrier healing in the terminal ileum had an overall accuracy of 91.8% with a positive and negative prediction of 90.9 and 92.2% (Table 3) and was therefore clearly superior in its predictive capabilities compared to the other parameters.

Discussion

Increased intestinal permeability in IBD patients was first noted already more than 30 years ago and found to predict clinical relapse in

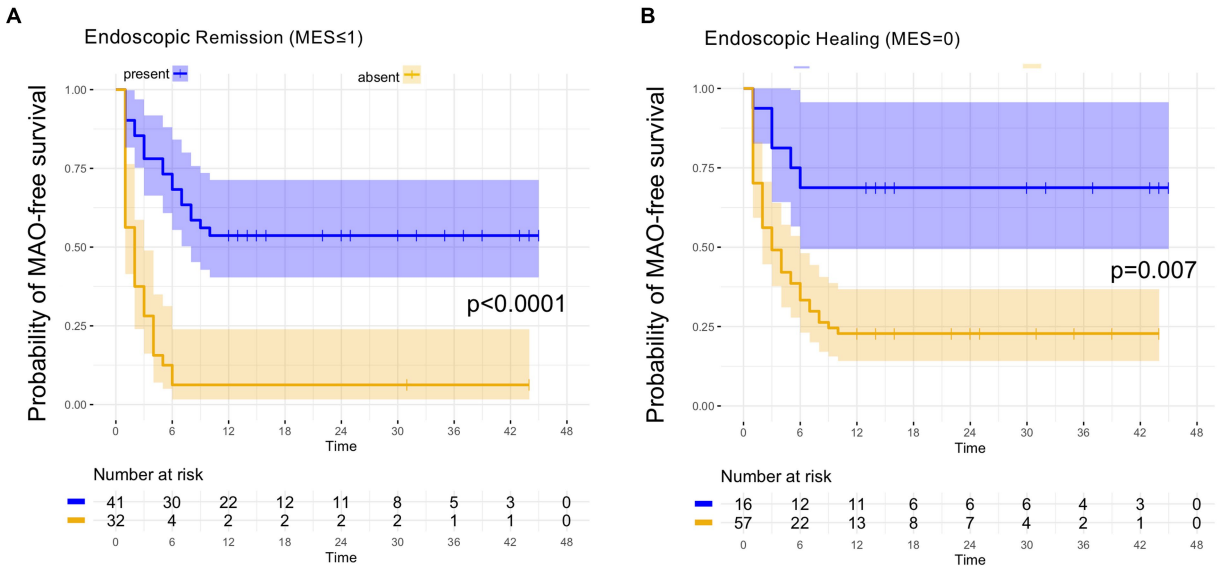


FIGURE 1
Kaplan–Meier analyzes for the occurrence of major adverse outcomes in UC patients with endoscopic remission and endoscopic healing. **(A)** In UC patients with endoscopic remission, as defined by an MES ≤ 1, the probability of remaining free of major adverse outcomes (MAO) during FU was significantly higher compared to patients with endoscopically active disease (MES > 1). **(B)** UC patients with endoscopic healing, as defined by an MES = 0, exhibited a significantly higher likelihood of remaining without MAO during FU as compared to UC patients with a MES > 0.

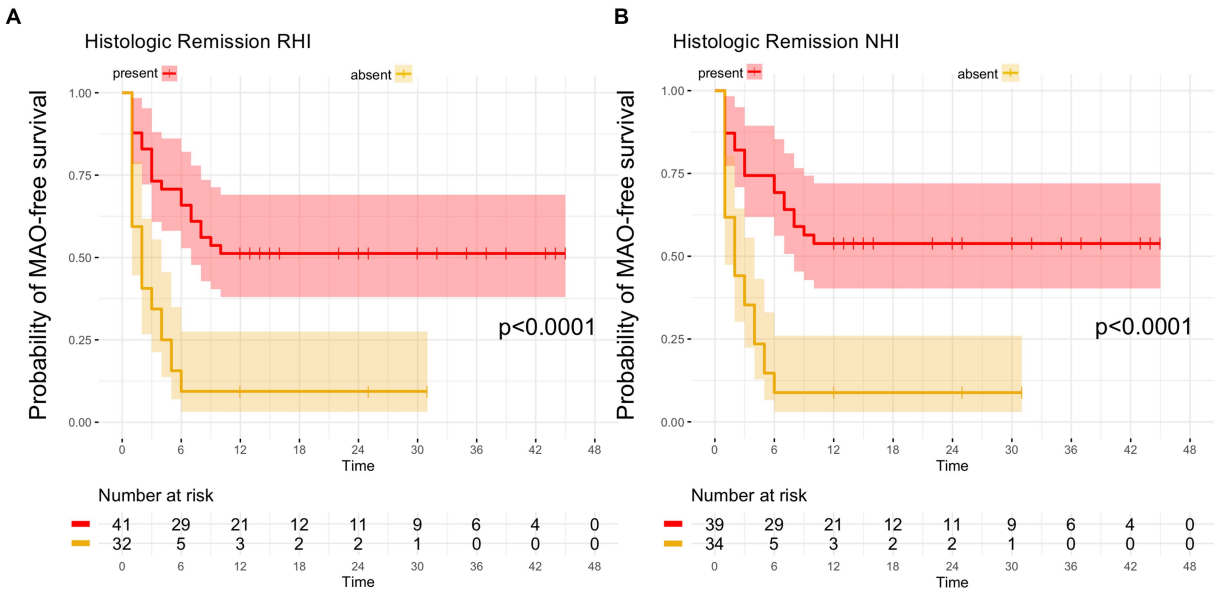


FIGURE 2
Kaplan–Meier analyzes for the occurrence of major adverse outcomes in UC patients with histologic remission. **(A)** In UC patients with histologic remission, defined by a Robarts Histology Index (RHI) ≤ 3, the probability of remaining free of major adverse outcomes (MAO) during FU was significantly higher compared to patients with histologically active disease (RHI > 3). **(B)** UC patients with histologic remission, as determined by a Nancy Histology Index (NHI) ≤1, exhibited a significantly higher likelihood of remaining without MAO during FU as compared to UC patients with an NHI > 1.

CD patients in remission (21, 22) and even earlier evidence on relatives of CD patients already suggested that increased intestinal permeability is not secondary to clinically manifest intestinal inflammation but rather constitutes a primary defect that is etiologically involved in disease pathogenesis (23).

In addition to this almost historic evidence, a just recently published study assessed intestinal permeability by the lactulose-mannitol-ratio (LMR) in over 1,400 asymptomatic first-degree relatives of CD patients. Importantly, as observed during long term follow up, increased LMR as a marker of increased intestinal

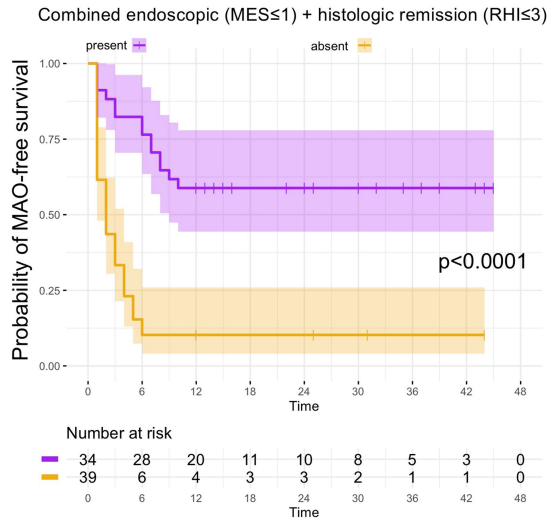


FIGURE 3
Kaplan–Meier analyzes for the occurrence of major adverse outcomes in UC patients with combined endoscopic and histologic remission. In UC patients, in which the combination between endoscopic and histologic remission was present, the probability of MAO-free survival was significantly higher compared to UC patients without combined endoscopic and histologic remission.

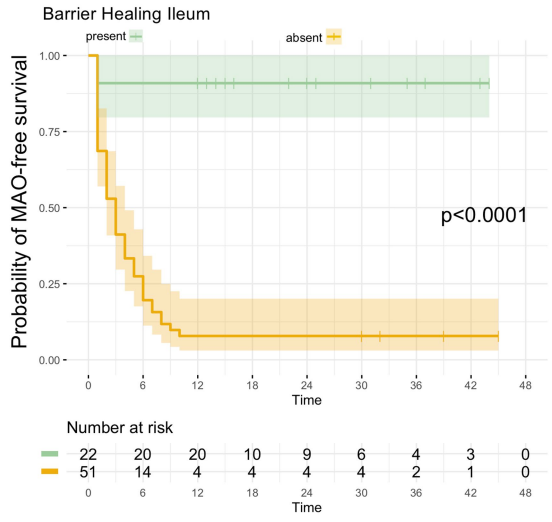


FIGURE 4
Kaplan–Meier analyzes for the occurrence of major adverse outcomes in UC patients with ileal barrier healing. UC patients with ileal barrier healing exhibited a significantly higher likelihood of remaining free of MAO during FU as compared to UC patients in which ileal barrier dysfunction was present during baseline endoscopy.

permeability acted as an independent risk factor for developing Crohn's disease in first degree relatives in the future conferring a 3-fold risk increase (24).

Several studies have already used CLE for dynamic visualization and assessment of intestinal barrier integrity. Published already a decade ago, Kiesslich and co-workers were able to show that in CD and UC patients in clinical remission increased cell shedding with fluorescein leakage in the ileum, as visualized with CLE, is associated with subsequent disease relapse within 12 months after endomicroscopic examination. Importantly, in this study a novel scoring system for semiquantitative grading system (the "Watson-Score") of ileal barrier dysfunction was devised that exhibited a specificity >90% for predicting subsequent disease flare in clinically remittent IBD patients. Using this score, these results were subsequently corroborated in an independent cohort of IBD patients by Karstensen and co-workers. In this study, a Watson-Score of 2 or 3, representative of functional or structural ileal barrier dysfunction, exhibited a sensitivity of 89% for predicting disease relapse within the next 12 months in clinically remittent CD patients (16). Another prospective study on 110 IBD patients with endoscopic mucosal healing was able to establish an association between impaired intestinal permeability, as assessed by quantitative grading of barrier dysfunction by CLE, and persistence of clinical symptoms. Importantly, increases in intestinal permeability in the ileum directly correlated with severity of diarrhea in both, UC and CD patients and led the authors to speculate that resolution of mucosal permeability beyond mucosal healing might improve outcomes of patients with IDB (15).

Just recently, we reported the results of our ERIca trial in which we compared the value of endoscopic and histologic remission against intestinal barrier healing for predicting the further course of disease in a large cohort of clinically remitted IBD patients (10). As shown in this trial, in CD patients barrier healing in the ileum and colon by far outcompeted endoscopic and histologic remission in forecasting the further course of disease during close-meshed multiannual follow-up. In UC patients, we observed that barrier healing in the colon was also associated with decreased risk of development of major adverse outcomes with superior predictive performance compared with endoscopic and histologic remission. However, in the ERIca trial, we did not analyze barrier function in the ileum for predicting disease behavior in UC patients.

Therefore, against the background of published reports on the relevance of ileal barrier function in UC (15, 17), we now aimed to extend the observations of the ERIca trial and explored whether assessment of ileal barrier function in patients with UC can likewise be used to predict the occurrence of major adverse outcomes in clinically remitted UC patients. Our results show that ileal barrier healing is indeed related with favorable disease outcome: of the 73 UC patients included, 22 patients exhibited ileal barrier healing and of these 22 patients with barrier healing, only 2 patients developed major adverse outcomes during a mean follow-up period of 26 months. Consistent with this, time-to-event analysis using Kaplan Meier estimates showed that ileal barrier healing was associated with a significantly more favorable course of disease over a mean follow-up period of 26 months in clinically remittent UC patients. In addition to that, our data clearly indicate that ileal barrier is superior to endoscopic or histologic remission, or the combination of the later. As such, the MAO rate was by far lower for ileal barrier healing as compared to endoscopic or histologic remission and the diagnostic accuracy of ileal barrier healing for forecasting the further course of disease outcompeted those of endoscopic or histologic remission or their combination.

TABLE 3 Diagnostic performances of endoscopic remission, histologic remission and ileal barrier healing for predicting major adverse outcomes in UC patients.

Parameter	Accuracy (95% CI-Interval)	Sensitivity (95% CI-Interval)	Specificity (95% CI-Interval)	PPV (95% CI-Interval)	NPV (95% CI-Interval)
Endoscopic remission (MES ≤ 1)	71.2% (59.5–81.2%)	91.7% (73–99%)	61.2% (46.2–74.8%)	53.7% (44.4–62.7%)	93.8% (79.6–98.3%)
Endoscopic healing (MES = 0)	75.3% (63.9–84.7%)	45.8% (25.6–67.2%)	89.8% (77.8–96.6%)	68.8% (46.3–84.9%)	77.1% (69.8–83.2%)
Robarts histologic remission[a]	68.5% (56.6–78.9%)	87.5% (67.6–97.3%)	59.2% (44.2–73%)	51.2% (42.1–60.3%)	90.6% (76.6–96.6%)
Nancy histologic remission[b]	71.2% (59.5–81.2%)	87.5% (67.6–97.3%)	63.3% (48.3–76.6%)	53.9% (44–63.5%)	91.2% (77.8–96.8%)
Endoscopic remission (MES ≤ 1) + Robarts histologic remission[a]	75.3% (63.9–84.7%)	83.3% (62.6–95.3%)	71.4% (56.7–83.4%)	58.8% (47–69.7%)	89.7% (77.9–95.6%)
Barrier healing ileum	91.8% (83–96.2%)	83.3% (62.6–95.3%)	95.9% (86–99.5%)	90.9% (71.8–97.5%)	92.2% (82.7–96.6%)

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.
[a]Histologic remission according to the Robarts' histology index.
[b]Histologic remission according to the Nancy histology index.

In parallel to these clinical data strengthening the relevance of impaired function in IBD patients, basic science has identified impairments in tight junctions and epithelial resistance in both, UC and CD (25–27). In their togetherness, impaired barrier function leading to increased intestinal permeability is increasingly recognized a key etiologic factors in the development of IBD (28). Based on our observation that ileal barrier healing is highly predictive for more favorable disease outcome in UC, which has been commonly defined as a disease confined to the colon and the rectum, the following aspects are worth considering: (i) although traditionally regarded as two distinct diseases with clear distinction between UC and CD, emerging evidence suggests that IBD is more and more perceived as a continuous spectrum. As such, already a decade ago whole genome gene expression meta-analysis in IBD demonstrated a lack of major differences between Crohn's disease and ulcerative colitis (29) and aggregated genetic risk scores representing the cumulative burden of mutations in known IBD risk loci introduced the concept of a disease spectrum along the disease location axis (30) and (ii) the affection of the terminal ileum in patients with UC is increasingly recognized as a further disease manifestation that is different from clinical evident backwash ileitis. As such the existence of ulcerations in the terminal ileum without co-existing evidence of backwash ileitis in UC patients have been described with varying frequencies (31) and a recent review proposed that ileal inflammation in UC represents a primary manifestation of UC which has been referred to as "UC-associated ileitis" (iii) we and others have previously identified macroscopically intact ileum as a site of increased intestinal permeability not only in CD but also in UC (15, 17). Although, these studies, by the nature of their methodology, do not provide a mechanistic explanation of increased permeability in macroscopically unaffected ileal mucosa, they strengthen the concept that the ileum is critically involved in disease etiology and disease behavior in UC patients.

Recently, Hiyama analyzed whether the phenotypic appearance of Peyer's Patches in the terminal ileum, evaluated under narrow-band imaging and magnification endoscopy, is associated with clinical disease behavior. As such, this multicenter study on 105 UC patients in clinical remission was able to demonstrate that the presence of a "Villi Index Low" type was a significant factor for predicting sustained clinical remission (32).

In summary, in this additional analysis of our ERIca trial on the relevance of barrier function in IBD patients, we were able to show that ileal barrier healing is a novel parameter that is highly predictive of the further course of disease in clinically remittent UC with superior predictive capabilities compared to endoscopic and histologic remission. With this, CLE-based assessment of ileal barrier function during routine ileocolonoscopy might be a helpful tool in clinical practice for stratification of UC patients according to their risk for development of complicated disease behavior.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by ethics committee and nstitutional Review Board of the Medical Faculty of the Friedrich-Alexander University Erlangen-Nuremberg. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

TR and MN designed the trial. TR, SZ, MW, and JB analyzed study data and images. WU performed statistical analyzes. AH performed pathologic analyzes. RA, TR, and MN discussed and interpreted findings. All authors contributed to the article and approved the submitted version.

Funding

RA and MN were supported by the DFG SFB 1181 and TRR241.

Conflict of interest

RA has served as a speaker, or consultant, or received research grants from AbbVie, Amgen, Arena Pharmaceuticals, Biogen, Boehringer Ingelheim, Bristol-Myers Squibb, Cellgene, Celltrion Healthcare, DrFalk Pharma, Galapagos, Gilead, InDex Pharmaceuticals, Janssen-Cilag, Lilly, MSD Sharp & Dohme, Novartis, Pandion Therapeutics, Pfizer, Roche Pharma, Samsung Bioepis, Takeda Pharma, Viatris. MN served as advisor or speaker for Pentax, Roche Pharma, Takeda Pharma, Pfizer, MSD, PPM, Janssen, Gilead, DrFalkPharma, Boehringer Ingelheim, Amgen and AbbVie. TR served as a speaker for Pentax, AbbVie, Olympus, Medtronic, Takeda Pharma, Lilly, Janssen-Cilag, Galapagos.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1221449/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Endoscopic disease activity under high definition white light endoscopy. Endoscopic disease activity was assessed along the Mayo Endoscopy Score (MES). According to consensus statement, endoscopic remission was defined as a MES≤1 (upper row) while endoscopically active disease was defined as a MES>1 (lower row).

SUPPLEMENTARY FIGURE S2
Ileal barrier (dys)function under CLE. Watson °I is defined by an intact epithelial barrier without sites of fluorescein leakage (left image). A functional defect of the ileal barrier (Watson °II) is characterized by single cells that lost epithelial integrity, leading to the formation of an epithelial gap which is accompanied by the efflux of fluorescein into the intestinal lumen (middle image). A structural barrier defect (Watson °III) is defined by shedding of multiple neighboring, leading to the formation of microerosions, and the efflux of fluorescein through the site of epithelial damage into the lumen (right image). White arrows: sites of ileal barrier defect.

SUPPLEMENTARY FIGURE S3
Histopathologic disease activity. For histopathological scoring in UC, Robarts Histopathology Index (RHI) and Nancy Histopathology Index (NHI) as validated histopathological scores were used. Histologic disease remission was defined as a RHI≤3 without lamina propria or epithelial neutrophils or a NHI≤1. Representative histopathology images under 20-fold and 40-fold magnification are shown.

SUPPLEMENTARY FIGURE S4
Serum zonulin levels in UC patients with and without ileal barrier dysfunction.

References

1. Le Berre C, Peyrin-Biroulet Lgroup S-Is. Selecting end points for disease-modification trials in inflammatory bowel disease: the SPIRIT consensus from the IOIBD. *Gastroenterology*. (2021) 160:1452–60 e21. doi: 10.1053/j.gastro.2020.10.065

2. Peyrin-Biroulet L, Ferrante M, Magro F, Campbell S, Franchimont D, Fidder H, et al. Results from the 2nd scientific workshop of the ECCO. I: impact of mucosal healing on the course of inflammatory bowel disease. *J Crohns Colitis*. (2011) 5:477–83. doi: 10.1016/j.crohns.2011.06.009

3. Peyrin-Biroulet L, Sandborn W, Sands BE, Reinisch W, Bemelman W, Bryant RV, et al. Selecting therapeutic targets in inflammatory bowel disease (STRIDE): determining therapeutic goals for treat-to-target. *Am J Gastroenterol*. (2015) 110:1324–38. doi: 10.1038/ajg.2015.233

4. Turner D, Ricciuto A, Lewis A, D'Amico F, Dhaliwal J, Griffiths AM, et al. STRIDE-II: an update on the selecting therapeutic targets in inflammatory bowel disease (STRIDE) initiative of the International Organization for the Study of IBD (IOIBD): determining therapeutic goals for treat-to-target strategies in IBD. *Gastroenterology*. (2021) 160:1570–83. doi: 10.1053/j.gastro.2020.12.031

5. Walsh AJ, Bryant RV, Travis SP. Current best practice for disease activity assessment in IBD. *Nat Rev Gastroenterol Hepatol*. (2016) 13:567–79. doi: 10.1038/nrgastro.2016.128

6. Gupta A, Yu A, Peyrin-Biroulet L, Ananthakrishnan AN. Treat to target: the role of histologic healing in inflammatory bowel diseases: a systematic review and Meta-analysis. *Clin Gastroenterol Hepatol*. (2021) 19:1800–13 e4. doi: 10.1016/j.cgh.2020.09.046

7. Park S, Abdi T, Gentry M, Laine L. Histological disease activity as a predictor of clinical relapse among patients with ulcerative colitis: systematic review and Meta-analysis. *Am J Gastroenterol*. (2016) 111:1692–701. doi: 10.1038/ajg.2016.418

8. Yoon H, Jangi S, Dulai PS, Boland BS, Prokop LJ, Jairath V, et al. Incremental benefit of achieving endoscopic and histologic remission in patients with ulcerative colitis: a systematic review and Meta-analysis. *Gastroenterology*. (2020) 159:1262–75 e7. doi: 10.1053/j.gastro.2020.06.043

9. Rath T, Atreya R, Neurath MF. Is histological healing a feasible endpoint in ulcerative colitis? *Expert Rev Gastroenterol Hepatol*. (2021) 15:665–74. doi: 10.1080/17474124.2021.1880892

10. Rath T, Atreya R, Bodenschatz J, Uter W, Geppert CE, Vitali F, et al. Intestinal barrier healing is superior to endoscopic and histologic remission for predicting major adverse outcomes in inflammatory bowel disease: the prospective ERIca trial. *Gastroenterology*. (2023) 164:241–55. doi: 10.1053/j.gastro.2022.10.014

11. Peyrin-Biroulet L, Panes J, Sandborn WJ, Vermeire S, Danese S, Feagan BG, et al. Defining disease severity in inflammatory bowel diseases: current and future directions. *Clin Gastroenterol Hepatol*. (2016) 14:348–54 e17. doi: 10.1016/j.cgh.2015.06.001

12. Bryant RV, Winer S, Travis SP, Riddell RH. Systematic review: histological remission in inflammatory bowel disease. Is 'complete' remission the new treatment paradigm? An IOIBD initiative. *J Crohns Colitis*. (2014) 8:1582–97. doi: 10.1016/j.crohns.2014.08.011

13. Daperno M, D'Haens G, Van Assche G, Baert F, Bulois P, Maunoury V, et al. Development and validation of a new, simplified endoscopic activity score for Crohn's disease: the SES-CD. *Gastrointest Endosc*. (2004) 60:505–12. doi: 10.1016/S0016-5107(04)01878-4

14. Vuitton L, Marteau P, Sandborn WJ, Levesque BG, Feagan B, Vermeire S, et al. IOIBD technical review on endoscopic indices for Crohn's disease clinical trials. *Gut*. (2016) 65:1447–55. doi: 10.1136/gutjnl-2015-309903

15. Chang J, Leong RW, Wasinger VC, Ip M, Yang M, Phan TG. Impaired intestinal permeability contributes to ongoing bowel symptoms in patients with inflammatory bowel disease and mucosal healing. *Gastroenterology*. (2017) 153:723–31 e1. doi: 10.1053/j.gastro.2017.05.056

16. Karstensen JG, Saftoiu A, Brynskov J, Hendel J, Klausen P, Cartana T, et al. Confocal laser endomicroscopy: a novel method for prediction of relapse in Crohn's disease. *Endoscopy*. (2016) 48:364–72. doi: 10.1055/s-0034-1393314

17. Kiesslich R, Duckworth CA, Moussata D, Gloeckner A, Lim LG, Goetz M, et al. Local barrier dysfunction identified by confocal laser endomicroscopy predicts relapse in inflammatory bowel disease. *Gut*. (2012) 61:1146–53. doi: 10.1136/gutjnl-2011-300695

18. Lim LG, Neumann J, Hansen T, Goetz M, Hoffman A, Neurath MF, et al. Confocal endomicroscopy identifies loss of local barrier function in the duodenum of patients with Crohn's disease and ulcerative colitis. *Inflamm Bowel Dis*. (2014) 20:892–900. doi: 10.1097/MIB.0000000000000027

19. Karstensen JG, Saftoiu A, Brynskov J, Hendel J, Ciocalteu A, Klausen P, et al. Confocal laser endomicroscopy in ulcerative colitis: a longitudinal study of endomicroscopic changes and response to medical therapy (with videos). *Gastrointest Endosc*. (2016) 84:279–86 e1. doi: 10.1016/j.gie.2016.01.069

20. Rath T, Dieterich W, Katscher-Murad C, Neurath MF, Zopf Y. Cross-sectional imaging of intestinal barrier dysfunction by confocal laser endomicroscopy can identify patients with food allergy in vivo with high sensitivity. *Sci Rep*. (2021) 11:12777. doi: 10.1038/s41598-021-92262-4

21. D'Inca R, Di Leo V, Corrao G, Martines D, D'Odorico A, Mestriner C, et al. Intestinal permeability test as a predictor of clinical course in Crohn's disease. *Am J Gastroenterol*. (1999) 94:2956–60. doi: 10.1111/j.1572-0241.1999.01444.x

22. Wyatt J, Vogelsang H, Hubl W, Waldhoer T, Lochs H. Intestinal permeability and the prediction of relapse in Crohn's disease. *Lancet*. (1993) 341:1437–9. doi: 10.1016/0140-6736(93)90882-H

23. Hollander D, Vadheim CM, Brettholz E, Petersen GM, Delahunty T, Rotter JI. Increased intestinal permeability in patients with Crohn's disease and their relatives. A possible etiologic factor. *Ann Intern Med*. (1986) 105:883–5. doi: 10.7326/0003-4819-105-6-883

24. Turpin W, Lee SH, Raygoza Garay JA, Madsen KL, Meddings JB, Bedrani L, et al. Increased intestinal permeability is associated with later development of Crohn's disease. *Gastroenterology*. (2020) 159:2092–100 e5. doi: 10.1053/j.gastro.2020.08.005

25. Gitter AH, Wullstein F, Fromm M, Schulzke JD. Epithelial barrier defects in ulcerative colitis: characterization and quantification by electrophysiological imaging. *Gastroenterology*. (2001) 121:1320–8. doi: 10.1053/gast.2001.29694

26. Schmitz H, Barmeyer C, Fromm M, Runkel N, Foss HD, Bentzel CJ, et al. Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. *Gastroenterology*. (1999) 116:301–9. doi: 10.1016/S0016-5085(99)70126-5

27. Zeissig S, Burgel N, Gunzel D, Richter J, Mankertz J, Wahnschaffe U, et al. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut*. (2007) 56:61–72. doi: 10.1136/gut.2006.094375

28. Mehandru S, Colombel JF. The intestinal barrier, an arbitrator turned provocateur in IBD. *Nat Rev Gastroenterol Hepatol*. (2021) 18:83–4. doi: 10.1038/s41575-020-00399-w

29. Granlund A, Flatberg A, Ostvik AE, Drozdov I, Gustafsson BI, Kidd M, et al. Whole genome gene expression meta-analysis of inflammatory bowel disease colon mucosa demonstrates lack of major differences between Crohn's disease and ulcerative colitis. *PLoS One*. (2013) 8:e56818. doi: 10.1371/journal.pone.0056818

30. Cleynen I, Boucher G, Jostins L, Schumm LP, Zeissig S, Ahmad T, et al. Inherited determinants of Crohn's disease and ulcerative colitis phenotypes: a genetic association study. *Lancet*. (2016) 387:156–67. doi: 10.1016/S0140-6736(15)00465-1

31. Lim HJ, Do Kim H, Soh JS, Kim SY, Jung YJ, Lim H, et al. Discrete terminal ileal ulcers in patients diagnosed with ulcerative colitis: clinical significance and natural course. *BMC Gastroenterol*. (2021) 21:285. doi: 10.1186/s12876-021-01866-7

32. Hiyama S, Iijima H, Sakakibara Y, Yamada T, Mukai A, Otake Y, et al. Endoscopic alterations in Peyer's patches in patients with ulcerative colitis: a prospective, multicenter study. *J Gastroenterol Hepatol*. (2020) 35:1143–9. doi: 10.1111/jgh.14933

frontiers | Frontiers in Cell and Developmental Biology

TYPE Original Research
PUBLISHED 19 October 2023
DOI 10.3389/fcell.2023.1241008

OPEN ACCESS

EDITED BY
Chih-Yang Wang,
Taipei Medical University, Taiwan

REVIEWED BY
Feng Yuan,
The University of Iowa, United States
Dinesh Devadoss,
Florida International University,
United States

*CORRESPONDENCE
Rocio T. Martinez-Nunez,
rocio.martinez_nunez@kcl.ac.uk

†PRESENT ADDRESS
Jennifer Rynne,
Centre for Inflammation Research,
Institute for Regeneration and Repair,
University of Edinburgh, Edinburgh,
United Kingdom
Elena Ortiz-Zapater,
Department of Biochemistry and
Molecular Biology, Faculty of Medicine-
IIS INCLIVA, University of Valencia,
Valencia, Spain

‡These authors have contributed equally
to this work and share first authorship

RECEIVED 15 June 2023
ACCEPTED 25 September 2023
PUBLISHED 19 October 2023

CITATION
Rynne J, Ortiz-Zapater E, Bagley DC,
Zanin O, Doherty G, Kanabar V, Ward J,
Jackson DJ, Parsons M, Rosenblatt J,
Adcock IM and Martinez-Nunez RT
(2023), The RNA binding proteins
ZFP36L1 and *ZFP36L2* are dysregulated in
airway epithelium in human and a murine
model of asthma.
Front. Cell Dev. Biol. 11:1241008.
doi: 10.3389/fcell.2023.1241008

The RNA binding proteins *ZFP36L1* and *ZFP36L2* are dysregulated in airway epithelium in human and a murine model of asthma

Jennifer Rynne[1†‡], Elena Ortiz-Zapater[2,3†‡], Dustin C. Bagley[2‡], Onofrio Zanin[1], George Doherty[1], Varsha Kanabar[3], Jon Ward[4], David J. Jackson[3], Maddy Parsons[2], Jody Rosenblatt[2], Ian M. Adcock[5] and Rocio T. Martinez-Nunez[1*]

[1]Department of Infectious Diseases, School of Immunology and Microbial Sciences, King's College London, London, United Kingdom, [2]The Randall Centre for Cell and Molecular Biophysics, School of Basic and Medical Biosciences, King's College London, London, United Kingdom, [3]Peter Gorer Department of Immunobiology, School of Immunology and Microbial Sciences, King's College London, London, United Kingdom, [4]Histochemistry Research Unit, University of Southampton, Southampton, United Kingdom, [5]National Heart and Lung Institute and Data Science Institute, Imperial College London, London, United Kingdom

Introduction: Asthma is the most common chronic inflammatory disease of the airways. The airway epithelium is a key driver of the disease, and numerous studies have established genome-wide differences in mRNA expression between health and asthma. However, the underlying molecular mechanisms for such differences remain poorly understood. The human TTP family is comprised of ZFP36, ZFP36L1 and ZFP36L2, and has essential roles in immune regulation by determining the stability and translation of myriad mRNAs encoding for inflammatory mediators. We investigated the expression and possible role of the tristetraprolin (TTP) family of RNA binding proteins (RBPs), poorly understood in asthma.

Methods: We analysed the levels of *ZFP36*, *ZFP36L1* and *ZFP36L2* mRNA in several publicly available asthma datasets, including single cell RNA-sequencing. We also interrogated the expression of known targets of these RBPs in asthma. We assessed the lung mRNA expression and cellular localization of Zfp36l1 and Zfp36l2 in precision cut lung slices in murine asthma models. Finally, we determined the expression in airway epithelium of ZFP36L1 and ZFP36L2 in human bronchial biopsies and performed rescue experiments in primary bronchial epithelium from patients with severe asthma.

Results: We found *ZFP36L1* and *ZFP36L2* mRNA levels significantly downregulated in the airway epithelium of patients with very severe asthma in different cohorts (5 healthy vs. 8 severe asthma; 36 moderate asthma vs. 37 severe asthma on inhaled steroids vs. 26 severe asthma on oral corticoids). Integrating several datasets allowed us to infer that mRNAs potentially targeted by these RBPs are increased in severe asthma. Zfp36l1 was downregulated in the lung of a mouse model of asthma, and immunostaining of *ex vivo* lung slices with a dual antibody demonstrated that Zfp36l1/l2 nuclear localization was increased in the airway epithelium of an acute asthma mouse model, which was further enhanced in a chronic model. Immunostaining of human bronchial biopsies showed that airway epithelial cell staining of ZFP36L1 was decreased in severe asthma as compared with mild, while ZFP36L2 was upregulated. Restoring the levels of ZFP36L1 and

ZFP36L2 in primary bronchial epithelial cells from patients with severe asthma decreased the mRNA expression of *IL6*, *IL8* and *CSF2*.

Discussion: We propose that the dysregulation of ZFP36L1/L2 levels as well as their subcellular mislocalization contributes to changes in mRNA expression and cytoplasmic fate in asthma.

KEYWORDS

airway and lung cell biology, RNA binding protein, asthma, post-transcriptional control, tristetraprolin, ZFP36L1, ZFP36L2

1 Introduction

Asthma is a common chronic respiratory disease affecting between 1%–29% of the population in different countries (Asher et al., 2021; Mortimer et al., 2022; GINA, 2023). It is characterised by variable symptoms of wheeze, chest tightness, shortness of breath and variable expiratory airflow limitation. Asthma is often defined as an inflammatory disease, implying that asthma has a major immune-related component. However, it is well established that structural cells including smooth muscle and airway epithelium play a major role in the disease.

Airway epithelial cells lie at the interface between the lung and the external environment, primarily acting as a protective barrier but also as immune modulators (Holgate, 2008; Frey et al., 2020). The importance of airway epithelial cells is well described in the pathophysiology of asthma. Patients with asthma present altered barrier function, mucus overproduction by goblet cells, epithelial cell damage and impaired epithelial repair. All these features contribute to airway remodelling, which is a broad term to define the airway structural changes that are always present in asthma (Holgate et al., 2015). Multiple genome-wide approaches have been implemented to further our understanding of asthma and the role and profile of the bronchial epithelium in patients with this disease. These include RNA expression analysis (Kuo et al., 2017; Hekking et al., 2018), breathomics (Rufo et al., 2016), metabolomics (Kelly et al., 2017) or sputum proteomics (Schofield et al., 2019). However, there is poor insight into the underlying mechanisms regulating gene expression or driving their phenotype at the molecular level.

From transcription to translation into protein, RNA undergoes multiple steps such as splicing, transport and stability, encompassed under 'post transcriptional regulation'. Post transcriptional regulation is mainly undertaken by microRNAs and RNA binding proteins (RBPs). Most omics approaches overlook these regulatory mechanisms and consider that mRNA expression is synonymous with corresponding protein levels. However, not all RNAs encode for proteins and there is little mRNA-to-protein correlation in many coding genes (Liu et al., 2016; Brion et al., 2020). To further our understanding of mRNA cytoplasmic fate, we developed subcellular fractionation and RNA-sequencing (Frac-seq) (Sterne-Weiler et al., 2013). Frac-seq enables analysing mRNA steady levels (transcriptional) and those of transcripts bound to the translation machinery (ribosomes), the latter a better proxy for protein levels (King and Gerber, 2016). Our previous work showed the disconnection between steady and ribosome-bound mRNA levels in bronchial epithelial cells from patients with asthma (Martinez-Nunez et al., 2018). We identified a network of six microRNAs that accounted for roughly 50% of the changes we observed in mRNA dysregulation (Martinez-Nunez et al., 2018). Thus, the remaining 50% levels must be mostly driven by RBPs. There is some evidence of RBP levels being dysregulated in airway epithelium in other respiratory diseases such as chronic obstructive pulmonary disease (Ricciardi et al., 2018), however, their role in airway epithelium in asthma remains poorly understood.

The tristetraprolin (TTP) family of RBPs, consisting of ZFP36, ZFP36L1 and ZFP36L2 in humans, has been implicated in the regulation of immune responses (Makita et al., 2021), but little is known about their role in epithelium or asthma. These RBPs inhibit mRNA expression by binding to AU-rich elements (ARE) present in the 3′UnTranslated Region (UTR) of their target RNAs (Blackshear, 2002; Cassandri et al., 2017). TTP is known to modulate the effect of glucocorticoids (Ishmael et al., 2008), and we have recently discovered that ZFP36L1/L2 modulate the effect of glucocorticoids and the expression levels of mRNAs encoding epithelial-related functions (Rynne et al., 2022). Considering that glucocorticoids are the mainstay treatment of patients with asthma (Chung et al., 2014), we hypothesised that the TTP family may be dysregulated in asthma and set out to investigate their expression and potential roles in airway epithelium of human samples and asthma murine models.

2 Results

2.1 *ZFP36L1* and *ZFP36L2* mRNA levels are downregulated in bronchial epithelial cells from patients with severe asthma

We initially mined our previous Frac-seq dataset to determine the levels of all TTP members. Patient details were reported previously (Martinez-Nunez et al., 2018). Changes in mRNA levels were determined by comparing total (Total) and polyribosome bound (Polyribosome) mRNA in health vs. asthma. Polyribosome fractions excluded the monosomal (80 S or one ribosome) fraction. We observed significantly decreased binding of *ZFP36L1* and *ZFP36L2* mRNAs to polyribosomes in primary bronchial epithelial cells from severe asthma patients compared to age- and sex-matched healthy controls (both $p < 0.05$, Figure 1A). Comparing total mRNA from healthy controls to severe asthma patients showed no difference in the expression of *ZFP36*, *ZFP36L1* or *ZFP36L2* mRNA (Figure 1A).

These data prompted us to further interrogate the levels and role of these RBPs in asthma in a larger cohort. We analysed datasets from U-BIOPRED, the largest European consortium of

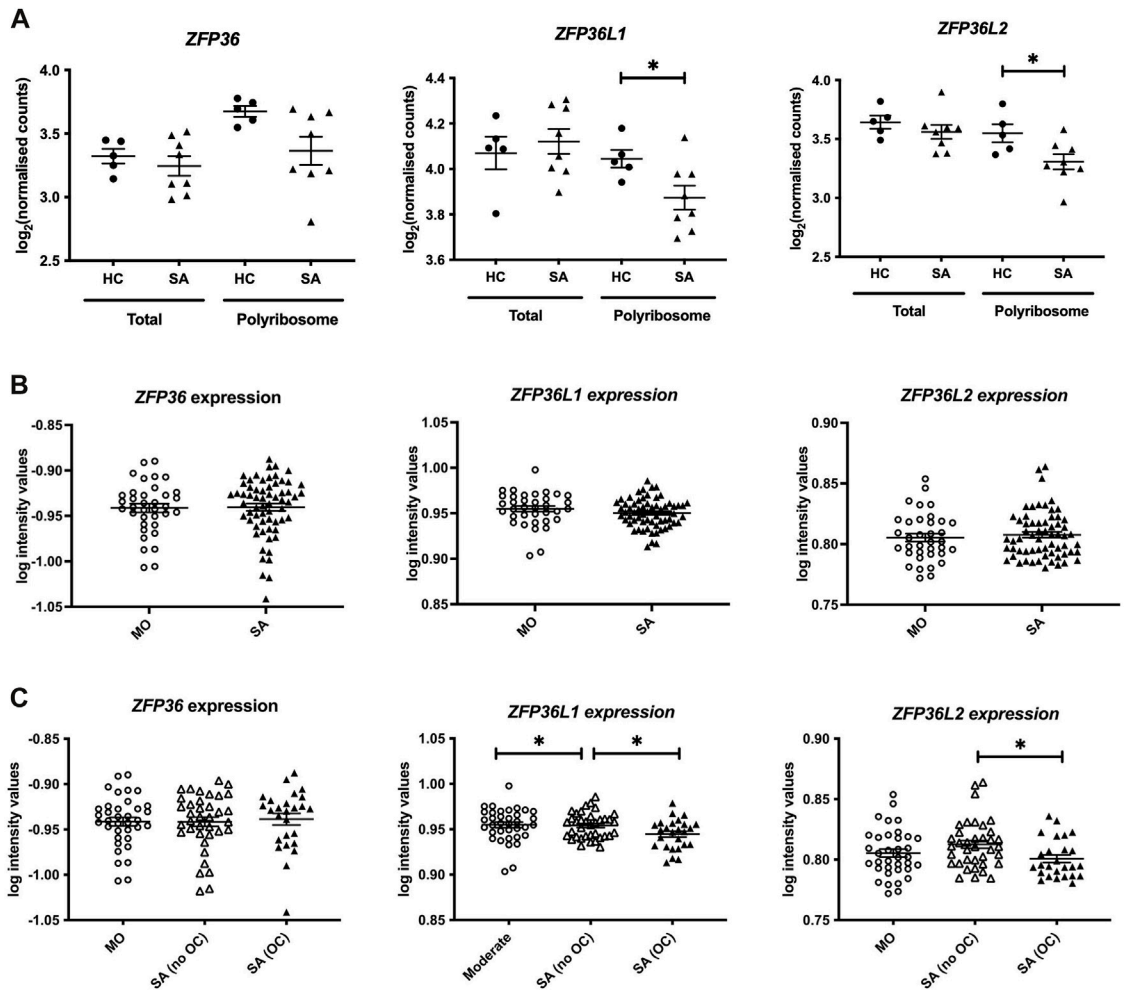


FIGURE 1
Analysis of the expression of the TTP family in human bronchial epithelial samples. **(A)** Frac-seq data showing the log counts for *ZFP36*, *ZFP36L1* and *ZFP36L2* in bronchial epithelial cells from severe asthma (SA, n = 8) vs. healthy control (HC, n = 5) donors from (Martinez-Nunez et al., 2018) in total mRNA (Total) or polyribosome-bound mRNA (Polyribosome). **(B)** Analysis of GSE76227 dataset from U-BIOPRED showing the log intensity values of microarrays in bronchial brushings from patients with moderate (MO) and severe asthma (SA). **(C)** Analysis of GSE76227 dataset from U-BIOPRED showing the log intensity values of microarrays in bronchial brushing from patients with moderate (MO, n = 36), severe asthma on oral corticoids (SA (OC), n = 26) and severe asthma on inhaled GCs (SA (no OC)), n = 37). Statistical significance was assessed by two-tailed t-tests on log transformed data. *$p < 0.05$.

severe asthma patients. The U-BIOPRED dataset (GSE76227) consists of transcriptomic arrays from bronchial brushings, which are enriched in bronchial epithelium (Perotin et al., 2019). The authors compared moderate asthma patients on inhaled glucocorticoids (MO), representing patients with disease control, to severe asthma patients (SA), without differentiating between corticosteroid use. SA patients taking oral corticosteroids represent patients with inadequate control of disease. Figure 1B depicts graphs displaying the log intensity values from the microarray probes showing that there was no difference in *ZFP36*, *ZFP36L1* or *ZFP36L2* mRNA expression. Upon stratification of severe asthma patients into those on inhaled glucocorticoids (SA-no OC) or taking oral glucocorticoids (SA-OC), we observed downregulation of *ZFP36L1* mRNA expression in SA-no OC patients compared to MO patients ($p < 0.05$), and a further downregulation in SA-OC *versus* SA-no OC patients ($p < 0.05$, Figure 1C). We also found that the expression of *ZFP36L2* mRNA was downregulated in SA-OC patients compared to SA-no OC (Figure 1C). We did not observe differential expression of *ZFP36* mRNA levels.

2.2 ZFP36L1 and ZFP36L2 modulate genome-wide expression changes in bronchial epithelium in asthma

We further explored the potential role of *ZFP36L1* and *ZFP36L2* in bronchial epithelial cells in asthma. Our Frac-seq dataset consisted of mainly basal epithelial cells, while bronchial brushings from U-BIOPRED will contain a mixture of different airway epithelial cells. We interrogated the Lung Cell Atlas datasets comparing very mild patients with asthma not on inhaled corticosteroid therapy vs. healthy controls (Vieira Braga et al., 2019). In health, *ZFP36L1* mRNA was expressed quite broadly in most epithelial cell types while *ZFP36L2*

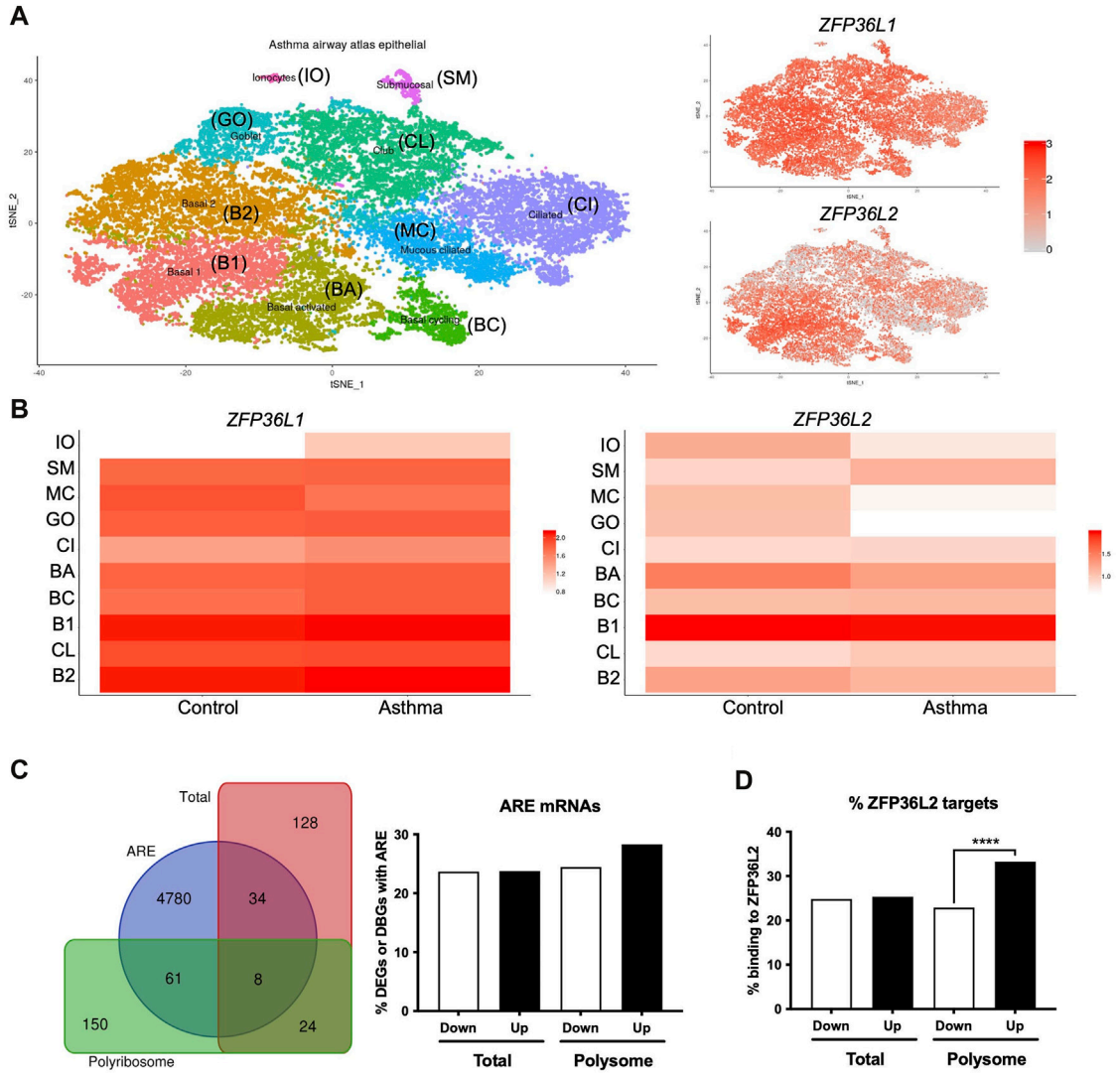


FIGURE 2
Airway epithelial cell-specific patterns of *ZFP36L1* and *ZFP36L2* mRNA expression and ZFP36L2 targets' behaviour in severe asthma. **(A)** t-Distributed Stochastic Neighbor Embedding (t-SNE) plots representing the different airway epithelial cell types present in very mild asthma as per (Vieira Braga et al., 2019) (left), with *ZFP36L1* and *ZFP36L2* mRNA expression patterns represented on the tSNE's on the right. **(B)** Relative expression *ZFP36L1* and *ZFP36L2* mRNA levels between health and asthma per airway epithelial cell type. IO: Ionocytes, SM: Submucosal, MC: Mucous ciliated, GO: Goblet, CI: Ciliated, BA: Basal activated, BC: Basal Cycling, B1: Basal 1, CL: Club, B2: Basal 2. **(C)** Left: Venn diagram representing the overlap of differentially expressed genes (DEGs in Total, red) or differentially bound genes (DBGs in Polyribosome, green) containing AU-rich element (ARE, blue) (Bakheet et al., 2018) when comparing health vs. severe asthma. Right: bar plot showing the proportion and direction of changes (Up or Downregulated) of ARE-containing mRNAs in DEGs and DBGs when comparing health vs. severe asthma. **(D)** Bar plot showing the proportion of ZFP36L2 targets as per (Zhang et al., 2013) present per subcellular fraction in our Frac-seq health vs. severe asthma dataset. Statistics were done employing a two-sided Chi-square test. ****: $p < 0.0001$.

mRNAs appeared more present in Basal 1 and Basal 2 cells (Supplementary Figure S1). In asthma samples, *ZFP36L1* mRNA was decreased in mucous ciliated cells while it appeared to increase in ionocytes, ciliated and basal cycling cells (Figures 2A,B). *ZFP36L2* mRNA showed a trend towards downregulation in most airway cell types, with only submucosal and Club cells showing an increase in expression. Thus, single cell data support the concept that RBP mRNA expression is decreased in specific epithelial cell subsets in asthma.

We next analyzed if mRNAs containing AREs (Bakheet et al., 2018) were dysregulated in our Frac-seq dataset comparing health vs. severe asthma (Martinez-Nunez et al., 2018). ARE-containing transcripts are targets of ZFP36L1 and ZFP36L2. Although we observed that a proportion of differentially expressed genes (DEGs) in total mRNA and differentially bound genes to polyribosome (DBGs) contain ARE in their 3′UTR (Figure 2C), these did not appear to be significantly enriched amongst DEGs or DBGs. To determine if ZFP36L2, specifically, may target DEGs and DBGs, we extracted known targets to be bound by ZFP36L2 from (Zhang et al., 2013) and analyzed their presence in our Frac-seq DEGs and DBGs. Figure 2D shows that direct targets of ZFP36L2 were predominantly upregulated in polyribosome-bound genes in severe asthma. This enrichment was absent in total DEGs between healthy controls and severe asthma patients.

Considering that patients with severe asthma are treated with high doses of inhaled and/or oral corticosteroids, we also

10.3389/fcell.2023.1241008

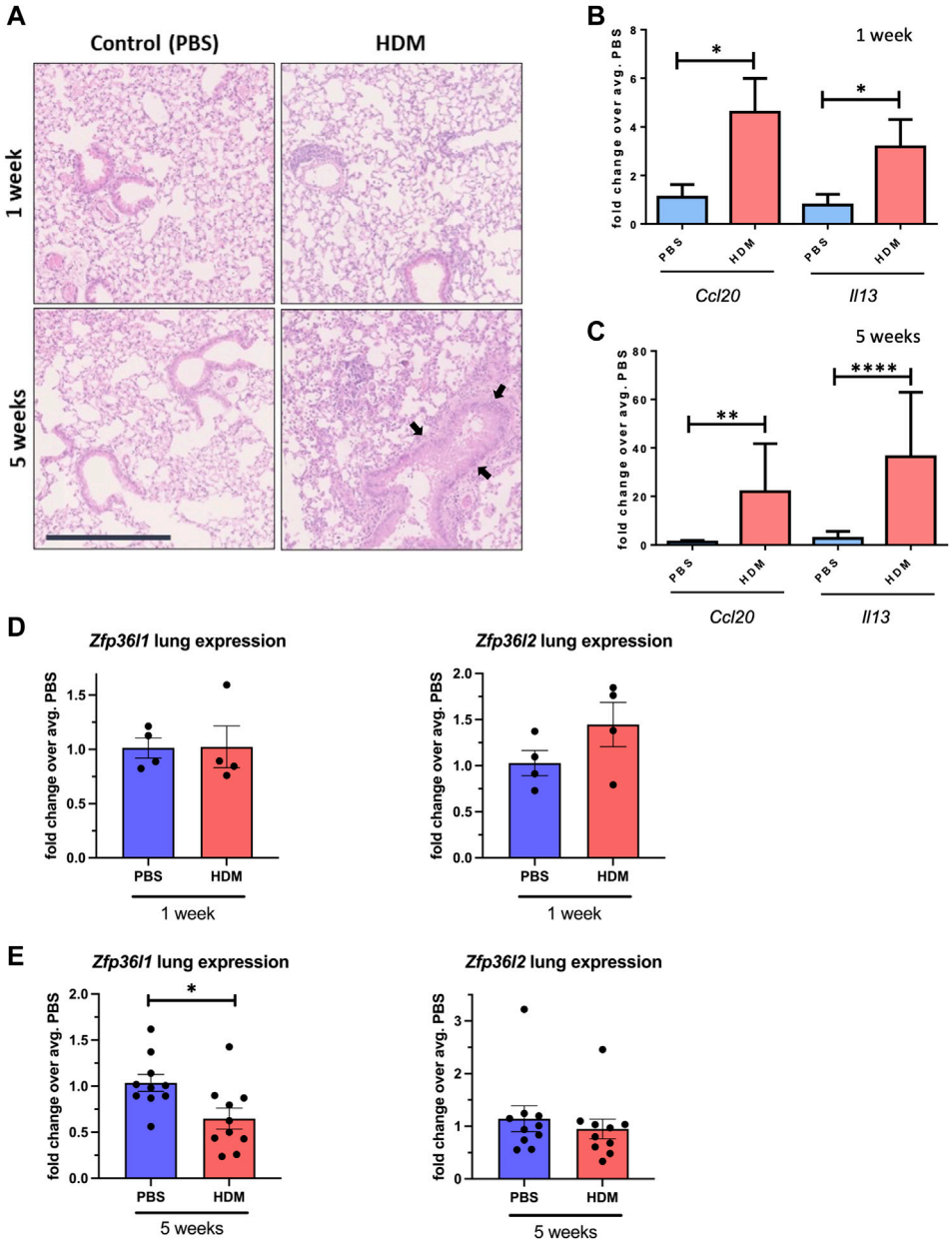


FIGURE 3
Zfp36l1 and *Zfp36l2* mRNA expression in the lungs different mouse models of asthma. **(A)** Representative images of Hematoxilyn and Eosin (H and E) staining of FFPE lung sections from mice treated with PBS as a control or with HDM for different times as indicated in the figure. Scale bars 125 μm. **(B)**. The expression of two cytokines (*Ccl20* and *Il13*) in whole lung mRNA was analyzed by quantitative qPCR for 1 week and in **(C)** for 5 weeks. Bar graphs represent mean fold change (±SEM) over the average PBS-treated control (n = 3 mice per group at PBS/HDM for 1 week and n = 5 mice per group in 3 independent experiments for the 5 weeks treatment). **(D)** Expression of both *Zfp36l1* and *Zfp26l2* in whole lung mRNA analysed by quantitative PCR for 1 week and in **(E)** for 5 weeks. Bar graphs represent mean fold change (±SEM) over the average PBS-treated control (n = 3 mice per group at PBS/HDM for 1 week and n = 5 mice per group in 3 independent experiments for the 5 weeks treatment). Statistical significance was assessed by multiple two-tailed parametric t-tests on log transformed data. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** and $p \leq 0.0001$****. Black arrows point towards increased epithelium height.

investigated if the changes seen in the DEGs and DBGs between health and SA were due to glucocorticoid (GC) exposure. We cross referenced our health vs. severe asthma Frac-seq dataset with our recent Frac-seq data investigating post-transcriptional changes induced by GCs in primary bronchial epithelial cells (Rynne et al., 2022). To that end, we compared upregulated and downregulated DEGs and DBGs between health and severe asthma and upregulated and downregulated DEGs and DBGs upon GC exposure. We only found 5 DEGs and 1 DBG between healthy controls and severe asthma that followed the same trend (up- or downregulated in a fraction-dependent manner) upon GC treatment). We also investigated the expression of these mRNAs in the U-BIOPRED dataset and found only 3 were present, namely, *DUSP1*, *SRM*, and *PTK2B*. These genes were not altered between

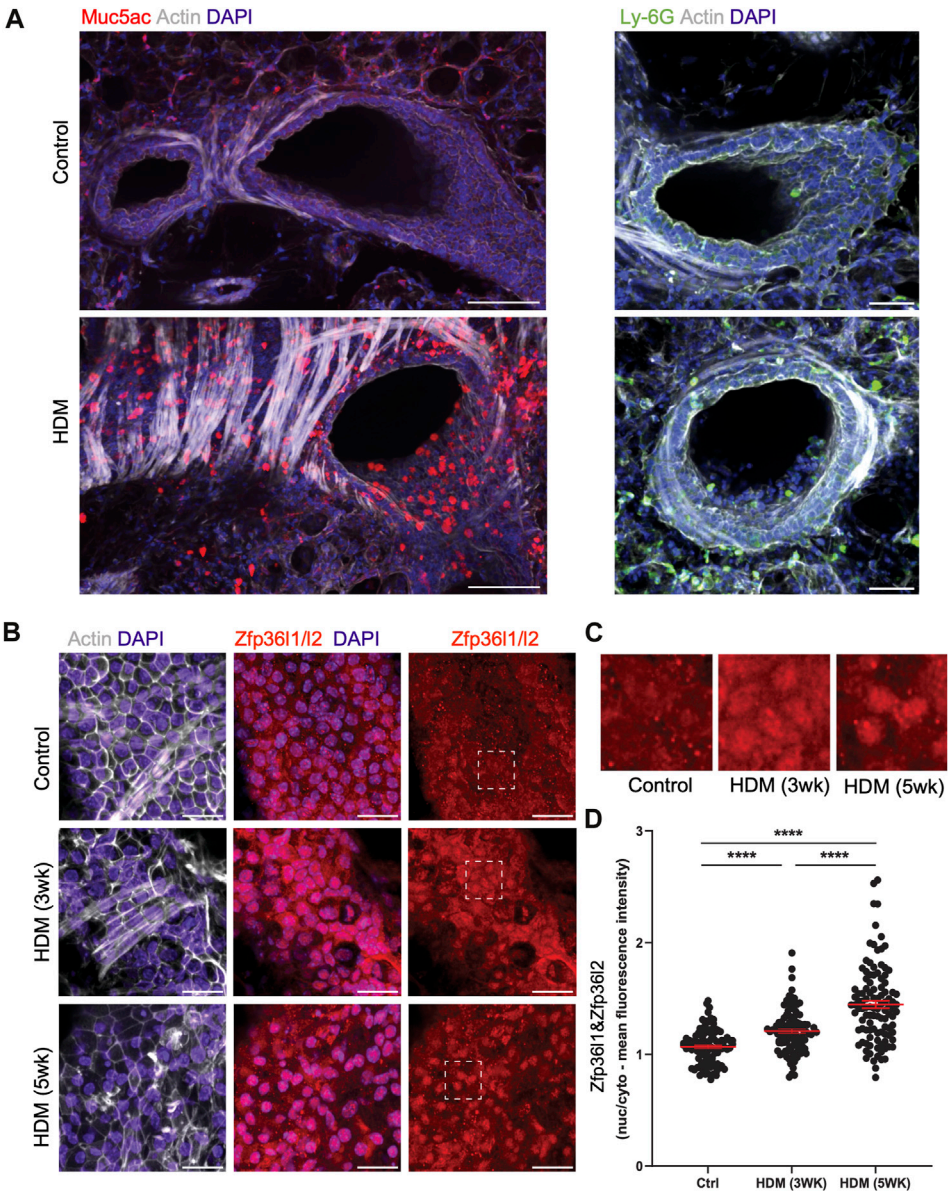


FIGURE 4
Intracellular mislocalization of Zfp36l1/Zfp36l2 in airway epithelial cells of mice with asthma-like characteristics. **(A)** Representative spinning disc confocal images of *ex vivo* lung slices from healthy controls and HDM-treated (3 weeks) mice were fixed and immune-stained for asthmatic markers of mucus (Muc5ac) and neutrophils (Ly-6G) to demonstrate an asthma phenotype (scale bars are 100 and 50 microns, respectively). **(B)** Confocal projections of healthy and HDM-treated airway epithelial cells immune-stained for Zfp36l1/l2, actin and nuclei (scale bar 25 microns), with insets highlighting Zfp36l1/Zfp36l2 intracellular localization in epithelial cells in health (Control), HDM after 3 weeks of exposure (HDM 3 weeks) and after 5 weeks of exposure (HDM 5 weeks). **(C)**: Insets highlighting its nuclear accumulation of Zfp36l1/l2 in epithelial cells in HDM. **(D)** Quantification of nuclear and cytoplasmic mean fluorescence intensity of Zfp36l1/Zfp36l2 in control and HDM-treated airways (3 weeks and 5 weeks of exposure), using regions of interest (ROIs) defined by the DAPI (nuclei) and actin (cytoplasm) channels. Mann-Whitney test was performed on 100 cells from 3 mice per group. ****: $p < 0.0001$. Ctrl: Control. HDM: House dust mite.

moderate and severe asthma patients, or between severe asthma patients stratified by OCS usage (Supplementary Figure S2). These data suggest that decreased RBP expression in severe asthma is unlikely to be due simply to oral corticosteroid use.

In summary, our analysis inferred that *ZFP36L1* and *ZFP36L2* encoding mRNAs are present in distinct airway epithelial cells and that ZFP36L2 preferentially targets mRNAs that are bound to polyribosomes in bronchial epithelial cells from patients with severe asthma.

2.3 *Zfp36l1* mRNA levels are downregulated in the lungs of mice with asthma-like characteristics

After having analysed the levels of *ZFP36L1* and *ZFP36L2* in omics mRNA datasets and inferring their role in ARE regulation and epithelial cell biology, we interrogated their expression levels in an *in vivo* model of asthma. The House Dust Mite (HDM) model is an established model of allergen-induced inflammation and one of the

most used *in vivo* models of asthma (McMillan and Lloyd, 2004). C57BL/6J mice were administered intranasally 25 µL (1 mg/mL) protein weight solution dissolved in PBS of HDM extract (Citeq) or equal volumes of PBS five times a week for 1 week or 5 weeks, to produce acute or chronic inflammation. This model has already been published elsewhere (Johnson et al., 2004; Ortiz-Zapater et al., 2022) but we tested the effect of the HDM at different times using both H&E staining from formalin-fixed paraffin embedded (FFPE) sections of mice from the different groups (Figure 3A). Supplementary Figure S3 shows a diagram of the different lung reagions employed in each experiment. We can observe immune infiltration in the section of HDM mice, increased after chronic exposure of HDM. After 5 weeks, the increase in the epithelium height is also evident (see arrows in Figure 3A). Total RNA from whole lung tissue from the different four groups was also analysed for the expression of two asthma-associated inflammatory cytokines, *Ccl20* and *Il13*. As expected, there was a clear increase in the expression of both transcripts after 1 week of HDM treatment (Figure 3B) and 5 weeks of HDM treatment (Figure 3C). We then assessed the mRNA levels of both *Zfp36l1* and *Zfp36l2* after both acute and chronic HDM treatment. As shown in Figure 3D, the expression of both *Zfp36l1* and *Zfp36l2* was not modified between PBS and acutely HDM treated mice. However, the mRNA expression of *Zfp36l1* in total lung tissue was significantly downregulated after chronic HDM exposure (Figure 3E, left panel). This was not seen for *Zfp36l2*, although there was lower expression compared to PBS mice in around half of the mice (Figure 3E, right panel).

2.4 Zfp36l1/l2 are mis-localized in the airways of mice with asthma-like lung characteristics

To further investigate our findings *in vivo*, we assessed the localization of Zfp36l1 and Zfp36l2 in the airways of HDM exposed mice. We employed mice exposed to HDM over three and 5 weeks; these mice produced a robust T2-driven inflammatory response and asthma-like phenotype including airway hyperresponsiveness, mucus hyperproduction, and immune cell infiltration by 3 weeks. PBS- and HDM-treated mice were sacrificed and lungs harvested and processed to obtain precision-cut *ex vivo* lung slices (PCLS) that were fixed and analyzed by immunofluorescence and confocal imaging. Indeed, in healthy airways and bronchioles there was little to no Muc5ac (a pathological mucin highly upregulated in asthma) expression in the epithelial monolayer, nor immune cell infiltrate near airways in the surrounding alveolar space (Figure 4A, top left panels). Unsurprisingly, HDM-treated airways had drastically increased expression of Muc5ac in the epithelium and significant immune cell infiltration near airways as shown by Ly-6G + neutrophils (immune cell type notoriously increased in many patients with asthma) (Figure 4A, top right panels). We found that Zfp36l1/Zfp36l2 (using a dual-staining antibody for both Zfp36l1 and Zfp36l2 proteins) were expressed in airway epithelial cells and nearly evenly distributed between the nucleus and cytoplasm in healthy mice. However, in airways exposed to HDM, Zfp36l1/Zfp36l2 became robustly recruited into the nucleus of airway epithelial cells (Figures 4B,C). Notably, nuclear accumulation of Zfp36l1/l2 at 5-week HDM treatment was even more dramatic, demonstrating a positive correlation between nuclear Zfp36l1/l2 and asthma severity (Figures 4B,C). To confirm this, measurements of fluorescence intensity were made for nuclear and cytoplasmic Zfp36l1/Zfp36l2 and ratios calculated from healthy and HDM treated airways at three and 5 weeks, demonstrating a dramatic recruitment of Zfp36l1/l2 to the nucleus with HDM treatment that increased over time and disease severity (Figure 4D).

2.5 ZFP36L1 and ZFP36L2 immunostaining of bronchial biopsies differs with severity in human asthma and rescuing ZFP36L1/L2 in severe asthma decreases mRNA expression of pro-inflammatory mediators

To further investigate the dysregulation of both *ZFP36L1* and *ZFP36L2* mRNA levels in human (Figure 1) and mouse asthma (Figure 3; Figure 4) we performed immunostaining of ZFP36L1 and ZFP36L2 in bronchial biopsies from patients with different asthma severities and non-asthma controls. Our results show that immunostaining with both ZFP36L1 and ZFP36L2 was predominant in bronchial epithelium (Figures 5A,B) while also present in other cell types. Analysis of the positively stained airway epithelial cells showed that patients with mild asthma had increased staining of ZFP36L1 in epithelial cells as compared with healthy controls and severe asthma (Figure 5C). Patients with severe asthma showed low *ZFP36L1* staining, significantly decreased as compared with mild but not healthy controls. *ZFP36L2* staining was increased in patients with severe asthma as compared to both healthy controls and mild patients (Figure 5D), but there was no difference in the *ZFP36L2* staining between healthy controls and mild asthmatic patients. To determine the effects of restoring *ZFP36L1* and *ZFP36L2* levels, we employed a vector to overexpress Zfp36l1 and siRNAs to deplete *ZFP36L2* in primary bronchial epithelial cells from patients with severe asthma. Transfection efficiency showed increased *ZFP36L1* and decreased *ZFP36L2* mRNA levels (Figure 5E) and led to decreased levels of pro-inflammatory *IL6*, *IL8* and *CSF2* mRNAs.

Taken together, our data show that *ZFP36L1* and *ZFP36L2* are expressed in airway epithelial cells and that their levels and subcellular localization are modified in asthma, in a severity-dependent manner. Restoring *ZFP36L1* and *ZFP36L2* levels in primary bronchial epithelial cells from patients with severe asthma led to decreased expression of mRNAs encoding pro-inflammatory factors. *ZFP36L1* and *ZFP36L2* alter post-transcriptional gene expression in bronchial epithelial cells in severe asthma, granting further investigation into their role in chronic airway inflammation.

3 Discussion

Our data demonstrate that *ZFP36L1* and *ZFP36L2* levels and subcellular localization are dysregulated in primary samples from both human and murine-like asthma. We have also performed

10.3389/fcell.2023.1241008

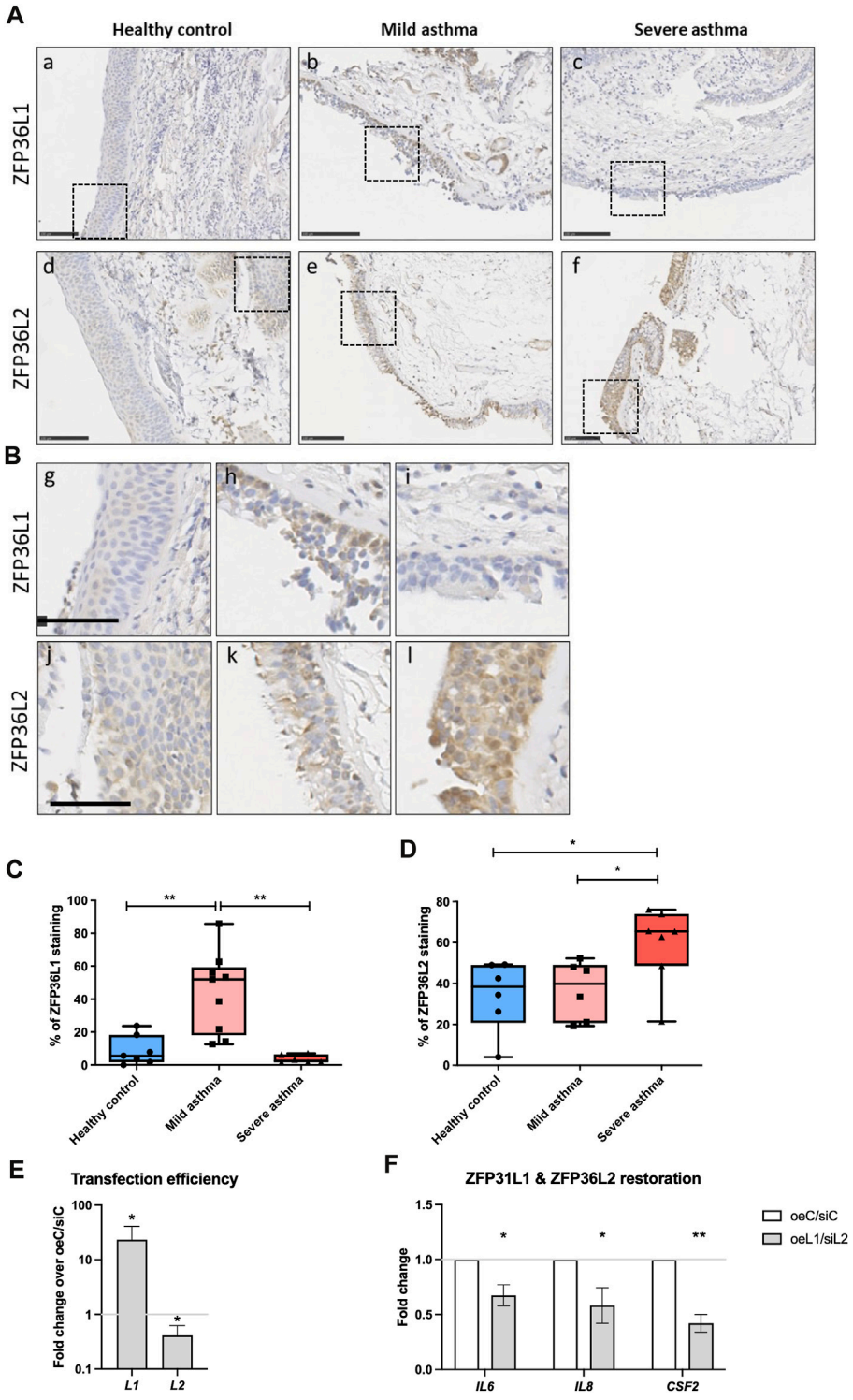


FIGURE 5
Immunohistochemistry staining of *ZFP36L1* and *ZFP36L2* in from bronchial biopsies from healthy controls and patients with mild and severe asthma. **(A)** Representative images of sections from patients classified as healthy controls **(A, D)**, mild asthmatics **(B, E)** or patients with severe asthma **(C, F)**. Staining was performed for *ZFP36L1* and *ZFP36L2* as indicated in the figure. Scale bar represents 100 µm. **(B)** Insets of the different sections shown in **(A)**. The scale bar represents 50 µm. **(C)** and **(D)**. Quantification of ZFP36L1 and ZFP36L2 staining in FFPE sections from each experimental group. In **(C)** Healthy controls n = 7, Mild asthmatics n = 9 and severe asthmatics n = 6. In **D**, Healthy controls n = 6, mild asthmatics n = 6 and severe asthmatics n = 7. Statistical significance was assessed by multiple two-tailed t-tests on log-transformed data. **(E)**: transfection efficiency of primary bronchial epithelial cells from patients with severe asthma (2 donors, 1 donor at two different passages, n = 3). Co-transfection of an overexpression vector for Zfp36l1 and siRNAs against *ZFP36L2* led to upregulation of *ZFP36L1* and downregulation of *ZFP36L2* mRNAs. **(F)**: *IL6*, *IL8* and *CSF2* mRNA expression was decreased upon upregulation of *ZFP36L1* and downregulation of *ZFP36L2*. One-tailed *t*-test of log transformed data. *: $p < 0.05$, **: $p < 0.01$. oeC, overexpression control; oeL1, overexpression plasmid for Zfp36l1; siC, siRNA control; siL2, siRNA targeting *ZFP36L2*.

in silico analysis of publicly available datasets of Zfp36l2 targets and observed that these are particularly enriched in genes that present increased binding to polyribosomes in severe asthma human primary bronchial epithelium. Modulating *ZFP36L1* and *ZFP36L2* in primary bronchial epithelial cells from patients with asthma decreased the expression of known pro-inflammatory mediators. Together, our data strongly suggest that *ZFP36L1* and *ZFP36L2* drive changes in gene expression in primary epithelial cells in asthma.

Airway epithelial cells contribute to disease pathobiology in asthma through immune modulation, defective barrier function, and airway remodelling. All asthma phenotypes present epithelial cell damage (Holgate, 2008; Xiao et al., 2011; Hardyman et al., 2013; Calven et al., 2020), and airway structural changes can be present before the onset of inflammation (Pohunek et al., 2005; Barbato et al., 2006). These observations are contrary to the old paradigm of repeated inflammation driving epithelial damage and deficient repair resulting in airway remodelling (Bush, 2008). Thus, epithelial changes appear to occur early on and be central to disease progression. It is possible that early epithelial cell gene expression reprogramming occurs leading to these cells behaving differently in asthma. This is indeed supported by studies that have determined a differential epigenetic signature in primary airway epithelium in asthma (McErlean et al., 2020). It is also possible that relatively small changes in master-regulators, such as RBPs, cause a sustained effect that leads to a different profile and cellular behaviour.

RBPs can modulate mRNA transcript levels and fate by binding to their targets and controlling most post-transcriptional steps, including alternative splicing, export, stability, storage, decay or translation into protein. The role of post-transcriptional gene expression regulation in inflammation is well established (Piccirillo et al., 2014; Makita et al., 2021) and defective RNA-RBP interactions can contribute to dysregulated immune responses (Liu and Cao, 2023). Additionally, RBPs have been found dysregulated in the epithelium of patients with chronic obstructive pulmonary disease (Ricciardi et al., 2018). There is no literature, to our knowledge, about the role of RBPs in asthma, where research in post-transcriptional regulation has focused on microRNAs (Solberg et al., 2012).

Amongst RBPs, the TTP family has well-established roles in modulating the immune system (Galloway et al., 2016; Vogel et al., 2016; Salerno et al., 2018), as well as the response to glucocorticoids (Ishmael et al., 2008; Rynne et al., 2022). Our data in Figure 1 showed a decreased expression of *ZFP36L1* and *ZFP36L2* mRNA in airway epithelial cells from patients with severe asthma, particularly of those that are undergoing oral corticosteroid treatment. We also found decreased of *Zfp36l1* and *Zfp36l2* mRNA in chronic asthma-like inflammation (akin to human severe asthma) in mice (Figure 3). At the protein level, in human bronchial biopsies (Figure 5), ZFP36L1 showed an increase in expression in mild patients, i.e., not taking glucocorticoids, as compared to healthy controls, and a decrease in patients with severe asthma (Figure 5). Contrary to mRNA expression, ZFP36L2 protein appeared upregulated in patients with severe asthma, i.e., on high doses of inhaled and/or oral glucocorticoids. These data highlight the importance of performing validations at the protein level, and the poor correlation existing between mRNA and protein levels (Gygi et al., 1999). It is also important to highlight that immunohistochemistry is a good methodology to indicate where a protein is expressed, but it is semi-quantitative. Further analysis of protein levels in specific airway epithelial types employing other methodologies such as fluorescent western blotting could offer further insight into total protein levels.

Mechanistically, it possible that expression levels of *ZFP36L1* and *ZFP36L2* are driven by different mechanisms in, particularly, severe/chronic asthma. Our data on murine models of acute and chronic asthma-like inflammation (Figure 3) showed decreased of *Zfp36l1* mRNA in the lung of chronic asthma-like inflammation (akin to human severe asthma), although these data correspond to whole lung. More detailed time courses of HDM exposure and analysis of specific cell types may unravel the dynamics of expression of these RBPs. Zfp36l1/l2 nuclear localization was increased in the airways of mice with asthma-like characteristics (Figure 4). *ZFP36L1* and predominantly *ZFP36L2* are increased in bronchial epithelial cells exposed to glucocorticoids, while their nuclear localization is also enhanced upon glucocorticoid exposure (Rynne et al., 2022). Noteworthy, our antibody staining for immunofluorescence (in both (Rynne et al., 2022) and Figure 4 PCLS) did not allow us to distinguish between ZFP36L1/Zfp36l1 and ZFP36L2/Zfp36l2, which is a caveat in our study. Further determination of the localization of each protein separately is warranted. In bronchial biopsies, we observed a trend towards increased nuclear localization for *ZFP36L2* in patients with severe asthma (Supplementary Figure S4) while *ZFP36L1* appeared more localised in the cytosol. Together, these data point towards chronic inflammation driving changes in *ZFP36L1* expression in asthma, while glucocorticoids may be the main drivers of *ZFP36L2* protein expression in severe asthma. In either case, it appears that there is an increase in nuclear localization for these proteins in asthma. While this may infer an increase in their nuclear role, our integration of Zfp36l2 targets showed that ZFP36L2 exerts its effects mainly by modulating polyribosome association of mRNAs in severe asthma (Figure 2C). Thus, it is possible that the changes exerted by ZFP36L2 in the cytosol are driven by is nuclear kidnapping.

ZFP36L1 and *ZFP36L2* encoding mRNAs also contain ARE in their 3′UTRs and can therefore regulate the expression of one another. Although our ARE analysis did not show an enrichment in ARE-containing mRNAs in patients with severe asthma, ARE-containing mRNAs accounted for 20%–28% of the genes differentially expressed and differentially bound to polyribosomes between health and severe asthma (Figure 2C). Our co-transfections also demonstrated that restoring the levels of *ZFP36L1* and *ZFP36L2* decreased the levels of *IL6*, *IL8* and *CSF2* (Figures 5E,F) mRNAs, which are known to contain AREs. However, *TNFA* and *VEGF* were not modulated (Supplementary Figure S5), suggesting a more complex interplay of these RBPs. Further investigation into the specific dynamics of these two RBPs in primary epithelium will enable elucidating their potential influence on each other's expression and/or targets. Determining the levels of *ZFP36L1* and *ZFP36L2* in bronchial biopsies and those of their direct targets employing methodologies such as spatial omics would enable dissecting their complementary, separate or synergistic roles in a cell-type dependent manner. It is also possible that these proteins exert distinct effects in an airway cell-type manner. *ZFP36L1* and

ZFP36L2 mRNAs appeared differentially expressed in individual airway epithelial cell types, with these expression patterns varying between health and asthma (Figures 2A,B). This granularity is lost in bulk RNA-sequencing and these data suggest that these RBPs are indeed not redundant.

In summary, we provide, to our knowledge, the first evidence that RBPs are dysregulated in airway epithelial cells in human asthma and murine models of asthma in a severity-dependent manner. ZFP36L1/L2 appear to be modulated post-transcriptionally and post-translationally, with their subcellular localization influenced by chronic inflammation and possibly glucocorticoids. Recent evidence shows that ZFP36L1/L2 modulate epithelial-encoding mRNAs (Rynne et al., 2022). We propose RBPs as novel modulators of inflammation and epithelial structure in asthma and their further investigation in chronic airway disease.

4 Materials and methods

4.1 Human samples

Our Frac-seq cohort (n = 5 healthy and n = 8 severe asthma) and those utilized in biopsy staining were part of the Wessex severe asthma cohort (Azim et al., 2019), in which patients with severe asthma were defined as those fulfilling the European Respiratory Society/American Thoracic Society (ERS/ATS) criteria for severe asthma (Chung et al., 2014) and thus treated with high doses of inhaled and/or oral corticosteroids. Bronchial biopsies from Figure 5 were also part of the same cohort. Primary bronchial epithelial cells from Figures 5E,F and Supplementary Figure S5 were collected under REC 23/YH/0018. For all the rest of data we are using previously collected data that is publicly available. REC Numbers 06/Q0505/12 and 05/Q1702/165.

4.2 *In vivo* experiments: house dust mite sensitization

All the research in this manuscript complies with ethical regulations. The use of animals for this study was approved by the Ethical Review Committee at King's College London and the Home Office, United Kingdom. All animals were housed in the Biological Support Unit (BSU) located in New Hunt's House at King's College London. All experiments were carried out under project license number P9672569A and personal license number I0F9CA46A. For House Dust Mite (HDM) immune-sensitised protocol, 6–8 weeks female C57BL/6 mice, were anaesthetised with isofluorane and administered either 25 µg (total protein) of HDM extract (Citeq Biologics; 1 mg/mL protein weight solution dissolved in PBS) or 25 µL of PBS intranasally 5 times per week for 5 consecutive weeks. Control mice received 25 µL of PBS. Mice were culled 24 h after the final HDM or PBS dose. Mice were sacrificed in a CO_2 gas chamber for lung dissection.

4.3 RNA extraction, reverse transcription and quantitative PCR (RT-qPCR)

RNA was isolated from cells or lung tissues using the RNeasy Qiagen kit (Qiagen; cat no 74004) following the manufacturer's instructions. In the case of isolating RNA from lung tissue, lung lobes were chopped and stored in TRIzol. The tissue was then disaggregated using a Bullet Blender. RNA was used for cDNA synthesis using the LunaScript RT SuperMix Kit (NEB; cat no. E3010). cDNA synthesis reaction, consisting of 4 µL of 5X LunaScript RT SuperMix, 1 µg of RNA and Nuclease-free water, was prepared. Using a thermal cycler, the reaction was initialised by a primer annealing step of 25 °C for 2 min, followed by a cDNA synthesis step of 55 °C for 10 min, and a heat inactivation step of 95 °C for 1 min. Quantitative real-time PCR (qPCR) was performed using a QuantStudio 5 (Applied Biosystems/ThermoFisher) thermal cycler, the reaction was first heated to 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s. This was followed by an extension time of 30 s at 60 °C. We employed TaqMan assays (ThermoFisher Scientific).

4.4 Tissue processing and analysis of immunohistochemistry

Immunohistochemistry staining of *ZFP36L1* and *ZFP36L2* in lung tissue from bronchial biopsies in non-asthmatic patients, mild asthma, and severe asthma was carried out by Dr Jon Ward at the University of Southampton. Briefly, 10 µm thick sections paraffin-embedded sections were melted at 95°C for 2 h and de-waxed by dipping slides in xylene 2 × 10 min, 100% EtOh 2 × 5 min, 70% EtOH 1 × 5 min and 50% EtOH 1 × 5 min. Antigen retrieval was carried using sodium citrate buffer (0.0874 M sodium citrate, 0.0126 M citric acid pH 6) and incubating the slides for 20 min in a pressure cooker at 95°C. Endogenous peroxidase activity was blocked via 10 min incubation in hydrogen peroxide (3% in TBS) for DAB staining. Tissues were then washed 3 × with TBS and non-specific binding was blocked via incubation with TBS-1%BSA-1% FBS blocking solution for 1 h at room temperature. Primary antibody was added to the tissues, and these were left at 4°C overnight. After 3 × washes with TBS, DAB staining was visualised by adding DAB developing solution for up to 20 min (Dako). Tissues were then counterstained using haematoxylin for 1 s. Finally, tissues were dehydrated with graded alcohols and xylene before being mounted with DPX. The slides were stained for ZFP36L1 (abx124297, Abbexa, 1:3000 dilution) and for *ZFP36L2* (PA5-30644, Invitrogen, 1:1500 dilution). Immunohistochemistry analysis was done employing Qupath (https://qupath.github.io/) for quantitative analysis. Positive epithelial cells were counted and expressed as a percentage of the total epithelium per slide. Mild asthma patients had controlled asthma, while severe asthma samples were obtained from the Wessex Severe Asthma Cohort and were classified as having inadequately controlled disease and fulfilled the ERS/ATS criteria for severe asthma.

4.5 Precision-cut *ex vivo* lung slices

Ex vivo lung slices were obtained from mice, within 48hrs of their last allergen priming challenge, adapted from the protocol of (Akram et al., 2019). Briefly, mice were humanely killed by CO_2 inhalation followed by cervical dislocation. The lungs were inflated with 2% low melting agarose (Fisher–BP1360) prepared in HBSS+ (Gibco–14,025) before lungs, along with the heart and trachea, were excised, washed in PBS, and the lobes separated. Individual lobes were then embedded in 4% low melting agarose and solidified on ice. 200micron thick slices were cut on a Leica VT1200S vibratome and washed and incubated in DMEM/F-12 medium supplemented with 10% foetal bovine serum (FBS) and antibiotics.

4.6 Immunofluorescence and imaging of fixed PCLS

PFA fixed *ex vivo* lung slices were incubated for 1 hour at room temperature in blocking solution: PBS containing 0.1% triton X-100, 0.1% sodium azide, and 2% bovine albumin (BSA), before incubating overnight a 4°C at 1:100 in blocking solution for all primary antibodies used: mouse anti-Muc5ac (Abcam ab3649), rat anti-Ly-6G (Abcam ab2557), rabbit anti-Zfp36l1/l2 (Cell Signaling Technologies). *Ex vivo* lung slices were washed 3 × 30 min in PBS+0.5% Triton X-100) before incubating overnight at 4°C overnight with: 1:100 Alexa Fluor 488 goat anti-rabbit (Thermo Scientific - A11008) or anti-mouse (A32723) IgG, Alexa Fluor 568 goat anti-rabbit (A11011) or anti-mouse (A11004) IgG, or Alexa Fluor 647 goat anit-rabbit (A32733) or anti-mouse (A21235) IgG +1:250 Alexa Fluor 488, 568, or 647 Phalloidin (Thermo Scientific–A12379, A12380, A22287, respectively). Slices were washed 3 × 30 min in PBS+0.5% Triton X-100, stained with 1: 1000 DAPI in PBS for 20 min, mounted in ProLong Gold (Invitrogen P36930), and imaged on a Nikon Eclipse Ti2 spinning disc confocal microscope with a 20X or ×40 objective.

4.7 Transfection

Primary bronchial epithelial cells were cultured on collagen-coated 24 well plates and transfected employing Interferin (Polyplus) with an over-expression vector for Zfp36l1 (courtesy of Prof Mayr, Memorial Sloan Kettering Cancer Center, United States) and siRNAs against *ZFP36L2*. RNA was extracted using TRIzol following manufacturer's instructions. RT was performed employing H-minus RT (ThermoFisher Scientific) and qPCR performed using Luna Universal Probe qPCR Master Mix (New England Biolabs) using TaqMan primers (ThermoFisher Scientific).

4.8 Statistical analyses

GraphPad Prism and R studio software was used for the generation of graphs and analysis of data. All packages are available at CRAN. Two group analyses were done employing one or two-tailed tests. Enrichment analysis was performed using binomial tests. Differential proportions in Figure 2 were done employing a Chi-Square test. In all cases, $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***. RNA-seq analysis was evaluated using the DESeq2 package for differential gene expression, for details please see (Rynne et al., 2022). Microarray analysis was performed using the limma package.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE213495 https://www.ncbi.nlm.nih.gov/geo/, GSE76227.

Ethics statement

The studies involving humans were approved Primary bronchial epithelial cells from Figures 5E,F were collected under REC 23/YH/0018. For all the rest of data we are using previously collected data that is publicly available. Biopsies belonging to the Wessex Severe Asthma Cohort were collected under REC 14/WM/1226 and 05/Q1702/165. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The use of animals for this study was approved by the Ethical Review Committee at King's College London and the Home Office, United Kingdom. All animals were housed in the Biological Support Unit (BSU) located in New Hunt's House at King's College London. All experiments were carried out under project license number P9672569A and personal license number I0F9CA46A. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JR, EO-Z, and DB acquired, analyzed and interpreted the data; OZ performed experiments and analyzed the data; GD and VK contributed experimentally; DJ, MP, and JR contributed intellectually and/or financially; IA provided critical intellectual input; RM-N conceived and designed the work and analyzed data. JW performed the titrations and immunostainings of human bronchial biopsies. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by King's Health Partners (Challenge Fund to RM-N); AAIR Charity to RM-N; Asthma United Kingdom Centre Grant (G1000758) to RM-N; Huo Family Foundation Grant to RM-N; Wellcome Trust (221908/Z/20/Z) to JR; Medical Research Council United Kingdom (MR/S009191/1 and R151002 to MP); GSK (RM-N) and AstraZeneca (DJ and RM-N). For the purpose of open access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We thank all the participants of the Wessex Severe Asthma Cohort (Immunohistochemistry Unit, University of Southampton) for his work immunostaining human bronchial biopsies.

Conflict of interest

RM-N has received consultancy fees from Roche outside the scope of this work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2023.1241008/full#supplementary-material

References

Akram, K. M., Yates, L. L., Mongey, R., Rothery, S., Gaboriau, D. C. A., Sanderson, J., et al. (2019). Live imaging of alveologenesis in precision-cut lung slices reveals dynamic epithelial cell behaviour. *Nat. Commun.* 10 (1), 1178. doi:10.1038/s41467-019-09067-3

Asher, M. I., Rutter, C. E., Bissell, K., Chiang, C. Y., El Sony, A., Ellwood, E., et al. (2021). Worldwide trends in the burden of asthma symptoms in school-aged children: global asthma network phase I cross-sectional study. *Lancet* 398 (10311), 1569–1580. doi:10.1016/S0140-6736(21)01450-1

Azim, A., Mistry, H., Freeman, A., Barber, C., Newell, C., Gove, K., et al. (2019). Protocol for the Wessex AsThma CoHort of difficult asthma (WATCH): a pragmatic real-life longitudinal study of difficult asthma in the clinic. *BMC Pulm. Med.* 19 (1), 99. doi:10.1186/s12890-019-0862-2

Bakheet, T., Hitti, E., and Khabar, K. S. A. (2018). ARED-plus: an updated and expanded database of AU-rich element-containing mRNAs and pre-mRNAs. *Nucleic Acids Res.* 46 (D1), D218–D20. doi:10.1093/nar/gkx975

Barbato, A., Turato, G., Baraldo, S., Bazzan, E., Calabrese, F., Panizzolo, C., et al. (2006). Epithelial damage and angiogenesis in the airways of children with asthma. *Am. J. Respir. Crit. Care Med.* 174 (9), 975–981. doi:10.1164/rccm.200602-189OC

Blackshear, P. J. (2002). Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover. *Biochem. Soc. Trans.* 30 (6), 945–952. doi:10.1042/bst0300945

Brion, C., Lutz, S. M., and Albert, F. W. (2020). Simultaneous quantification of mRNA and protein in single cells reveals post-transcriptional effects of genetic variation. *Elife* 9, e60645. doi:10.7554/eLife.60645

Bush, A. (2008). How early do airway inflammation and remodeling occur? *Allergol. Int.* 57 (1), 11–19. doi:10.2332/allergolint.R-07-155

Calven, J., Ax, E., and Radinger, M. (2020). The airway epithelium-A central player in asthma pathogenesis. *Int. J. Mol. Sci.* 21 (23), 8907. doi:10.3390/ijms21238907

Cassandri, M., Smirnov, A., Novelli, F., Pitolli, C., Agostini, M., Malewicz, M., et al. (2017). Zinc-finger proteins in health and disease. *Cell Death Discov.* 3, 17071. doi:10.1038/cddiscovery.2017.71

Chung, K. F., Wenzel, S. E., Brozek, J. L., Bush, A., Castro, M., Sterk, P. J., et al. (2014). International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur. Respir. J.* 43 (2), 343–373. doi:10.1183/09031936.00202013

Frey, A., Lunding, L. P., Ehlers, J. C., Weckmann, M., Zissler, U. M., and Wegmann, M. (2020). More than just a barrier: the immune functions of the airway epithelium in asthma pathogenesis. *Front. Immunol.* 11, 761. doi:10.3389/fimmu.2020.00761

Galloway, A., Saveliev, A., Lukasiak, S., Hodson, D. J., Bolland, D., Balmanno, K., et al. (2016). RNA-binding proteins ZFP36L1 and ZFP36L2 promote cell quiescence. *Science* 352 (6284), 453–459. doi:10.1126/science.aad5978

GINA (2023). *GINA 2023 - global strategy for asthma management and prevention*.

Gygi, S. P., Rochon, Y., Franza, B. R., and Aebersold, R. (1999). Correlation between protein and mRNA abundance in yeast. *Mol. Cell Biol.* 19 (3), 1720–1730. doi:10.1128/mcb.19.3.1720

Hardyman, M. A., Wilkinson, E., Martin, E., Jayasekera, N. P., Blume, C., Swindle, E. J., et al. (2013). TNF-alpha-mediated bronchial barrier disruption and regulation by src-family kinase activation. *J. Allergy Clin. Immunol.* 132 (3), 665–675. doi:10.1016/j.jaci.2013.03.005

Hekking, P. P., Loza, M. J., Pavlidis, S., de Meulder, B., Lefaudeux, D., Baribaud, F., et al. (2018). Pathway discovery using transcriptomic profiles in adult-onset severe asthma. *J. Allergy Clin. Immunol.* 141 (4), 1280–1290. doi:10.1016/j.jaci.2017.06.037

Holgate, S. T. (2008). The airway epithelium is central to the pathogenesis of asthma. *Allergol. Int.* 57 (1), 1–10. doi:10.2332/allergolint.R-07-154

Holgate, S. T., Wenzel, S., Postma, D. S., Weiss, S. T., Renz, H., and Sly, P. D. (2015). *Asthma. Nat. Rev. Dis. Prim.* 1 (1), 15025. doi:10.1038/nrdp.2015.25

Ishmael, F. T., Fang, X., Galdiero, M. R., Atasoy, U., Rigby, W. F., Gorospe, M., et al. (2008). Role of the RNA-binding protein tristetraprolin in glucocorticoid-mediated gene regulation. *J. Immunol.* 180 (12), 8342–8353. doi:10.4049/jimmunol.180.12.8342

Johnson, J. R., Wiley, R. E., Fattouh, R., Swirski, F. K., Gajewska, B. U., Coyle, A. J., et al. (2004). Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am. J. Respir. Crit. Care Med.* 169 (3), 378–385. doi:10.1164/rccm.200308-1094OC

Kelly, R. S., Dahlin, A., McGeachie, M. J., Qiu, W., Sordillo, J., Wan, E. S., et al. (2017). Asthma metabolomics and the potential for integrative omics in research and the clinic. *Chest* 151 (2), 262–277. doi:10.1016/j.chest.2016.10.008

King, H. A., and Gerber, A. P. (2016). Translatome profiling: methods for genome-scale analysis of mRNA translation. *Brief. Funct. Genomics* 15 (1), 22–31. doi:10.1093/bfgp/elu045

Kuo, C. S., Pavlidis, S., Loza, M., Baribaud, F., Rowe, A., Pandis, I., et al. (2017). A transcriptome-driven analysis of epithelial brushings and bronchial biopsies to define asthma phenotypes in U-biopred. *Am. J. Respir. Crit. Care Med.* 195 (4), 443–455. doi:10.1164/rccm.201512-2452OC

Liu, J., and Cao, X. (2023). RBP-RNA interactions in the control of autoimmunity and autoinflammation. *Cell Res.* 33 (2), 97–115. doi:10.1038/s41422-022-00752-5

Liu, Y., Beyer, A., and Aebersold, R. (2016). On the dependency of cellular protein levels on mRNA abundance. *Cell* 165 (3), 535–550. doi:10.1016/j.cell.2016.03.014

Makita, S., Takatori, H., and Nakajima, H. (2021). Post-transcriptional regulation of immune responses and inflammatory diseases by RNA-binding ZFP36 family proteins. *Front. Immunol.* 12, 711633. doi:10.3389/fimmu.2021.711633

Martinez-Nunez, R. T., Rupani, H., Plate, M., Niranjan, M., Chambers, R. C., Howarth, P. H., et al. (2018). Genome-wide posttranscriptional dysregulation by MicroRNAs in human asthma as revealed by frac-seq. *J. Immunol.* 201 (1), 251–263. doi:10.4049/jimmunol.1701798

McErlean, P., Kelly, A., Dhariwal, J., Kirtland, M., Watson, J., Ranz, I., et al. (2020). Profiling of H3K27Ac reveals the influence of asthma on the epigenome of the airway epithelium. *Front. Genet.* 11, 585746. doi:10.3389/fgene.2020.585746

McMillan, S. J., and Lloyd, C. M. (2004). Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clin. Exp. Allergy* 34 (3), 497–507. doi:10.1111/j.1365-2222.2004.01895.x

Mortimer, K., Lesosky, M., Garcia-Marcos, L., Asher, M. I., Pearce, N., Ellwood, E., et al. (2022). The burden of asthma, hay fever and eczema in adults in 17 countries: GAN phase I study. *Eur. Respir. J.* 60 (3), 2102865. doi:10.1183/13993003.02865-2021

Ortiz-Zapater, E., Bagley, D. C., Hernandez, V. L., Roberts, L. B., Maguire, T. J. A., Voss, F., et al. (2022). Epithelial coxsackievirus adenovirus receptor promotes house dust mite-induced lung inflammation. *Nat. Commun.* 13 (1), 6407. doi:10.1038/s41467-022-33882-w

Perotin, J. M., Schofield, J. P. R., Wilson, S. J., Ward, J., Brandsma, J., Strazzeri, F., et al. (2019). Epithelial dysregulation in obese severe asthmatics with gastro-oesophageal reflux. *Eur. Respir. J.* 53 (6), 1900453. doi:10.1183/13993003.00453-2019

Piccirillo, C. A., Bjur, E., Topisirovic, I., Sonenberg, N., and Larsson, O. (2014). Translational control of immune responses: from transcripts to translatomes. *Nat. Immunol.* 15 (6), 503–511. doi:10.1038/ni.2891

Pohunek, P., Warner, J. O., Turzikova, J., Kudrmann, J., and Roche, W. R. (2005). Markers of eosinophilic inflammation and tissue re-modelling in children before

clinically diagnosed bronchial asthma. *Pediatr. Allergy Immunol.* 16 (1), 43–51. doi:10.1111/j.1399-3038.2005.00239.x

Ricciardi, L., Col, J. D., Casolari, P., Memoli, D., Conti, V., Vatrella, A., et al. (2018). Differential expression of RNA-binding proteins in bronchial epithelium of stable COPD patients. *Int. J. Chron. Obstruct Pulmon Dis.* 13, 3173–3190. doi:10.2147/COPD.S166284

Rufo, J. C., Madureira, J., Fernandes, E. O., and Moreira, A. (2016). Volatile organic compounds in asthma diagnosis: a systematic review and meta-analysis. *Allergy* 71 (2), 175–188. doi:10.1111/all.12793

Rynne, J., Ortiz-Zapater, E., Khooshemehri, P., Ponde, N. O., Bucca, G., Hesketh, A., et al. (2022). *The RNA binding proteins ZFP36L1 and ZFP36L2 modulate transcriptional and post-transcriptional genome-wide effects of glucocorticoids*. bioRxiv.

Salerno, F., Engels, S., van den Biggelaar, M., van Alphen Fpj, Guislain, A., Zhao, W., et al. (2018). Translational repression of pre-formed cytokine-encoding mRNA prevents chronic activation of memory T cells. *Nat. Immunol.* 19 (8), 828–837. doi:10.1038/s41590-018-0155-6

Schofield, J. P. R., Burg, D., Nicholas, B., Strazzeri, F., Brandsma, J., Staykova, D., et al. (2019). Stratification of asthma phenotypes by airway proteomic signatures. *J. Allergy Clin. Immunol.* 144 (1), 70–82. doi:10.1016/j.jaci.2019.03.013

Solberg, O. D., Ostrin, E. J., Love, M. I., Peng, J. C., Bhakta, N. R., Hou, L., et al. (2012). Airway epithelial miRNA expression is altered in asthma. *Am. J. Respir. Crit. Care Med.* 186 (10), 965–974. doi:10.1164/rccm.201201-0027OC

Sterne-Weiler, T., Martinez-Nunez, R. T., Howard, J. M., Cvitovik, I., Katzman, S., Tariq, M. A., et al. (2013). Frac-seq reveals isoform-specific recruitment to polyribosomes. *Genome Res.* 23 (10), 1615–1623. doi:10.1101/gr.148585.112

Vieira Braga, F. A., Kar, G., Berg, M., Carpaij, O. A., Polanski, K., Simon, L. M., et al. (2019). A cellular census of human lungs identifies novel cell states in health and in asthma. *Nat. Med.* 25 (7), 1153–1163. doi:10.1038/s41591-019-0468-5

Vogel, K. U., Bell, L. S., Galloway, A., Ahlfors, H., and Turner, M. (2016). The RNA-binding proteins Zfp36l1 and Zfp36l2 enforce the thymic beta-selection checkpoint by limiting DNA damage response signaling and cell cycle progression. *J. Immunol.* 197 (7), 2673–2685. doi:10.4049/jimmunol.1600854

Xiao, C., Puddicombe, S. M., Field, S., Haywood, J., Broughton-Head, V., Puxeddu, I., et al. (2011). Defective epithelial barrier function in asthma. *J. Allergy Clin. Immunol.* 128(3), 549–556. doi:10.1016/j.jaci.2011.05.038

Zhang, L., Prak, L., Rayon-Estrada, V., Thiru, P., Flygare, J., Lim, B., et al. (2013). ZFP36L2 is required for self-renewal of early burst-forming unit erythroid progenitors. *Nature* 499 (7456), 92–96. doi:10.1038/nature12215

frontiers | Frontiers in Cell and Developmental Biology

TYPE Original Research
PUBLISHED 23 October 2023
DOI 10.3389/fcell.2023.1147625

OPEN ACCESS

EDITED BY
Rocío López Posadas,
University Hospital Erlangen, Germany

REVIEWED BY
Laralynne Przybyla,
University of California, San Francisco, United States
Sei Kuriyama,
Akita University, Japan

*CORRESPONDENCE
Shin-Ichi Ohnuma,
s.ohnuma@ucl.ac.uk

‡PRESENT ADDRESS
Hongorzul Davaapil, Wellcome—MRC Cambridge Stem Cell Institute, Jeffrey Cheah Biomedical Centre, Puddicombe Way, Cambridge Biomedical Campus, United Kingdom; Vasiliki Papadaki, Institute of Fundamental Biological Research, Biomedical Sciences Research Center "Alexander Fleming", Athens, Greece; Takumi Tashima, Daiichi Sankyo Co., Ltd., Tokyo, Japan.

†These authors have contributed equally to this work

RECEIVED 19 January 2023
ACCEPTED 13 July 2023
PUBLISHED 23 October 2023

CITATION
Davaapil H, Hopkins J, Bonnin N, Papadaki V, Leung A, Kosuge H, Tashima T, Nakakido M, Sekido R, Tsumoto K, Sagoo MS and Ohnuma S-I (2023), PRELP secreted from mural cells protects the function of blood brain barrier through regulation of endothelial cell-cell integrity.
Front. Cell Dev. Biol. 11:1147625.
doi: 10.3389/fcell.2023.1147625

PRELP secreted from mural cells protects the function of blood brain barrier through regulation of endothelial cell-cell integrity

Hongorzul Davaapil[1†‡], Jack Hopkins[1†], Nadia Bonnin[1], Vasiliki Papadaki[1‡], Alex Leung[1], Hirofumi Kosuge[2], Takumi Tashima[2‡], Makoto Nakakido[2], Ryohei Sekido[1], Kouhei Tsumoto[2], Mandeep S. Sagoo[1,3,4] and Shin-Ichi Ohnuma[1*]

[1]UCL Institute of Ophthalmology, UCL, London, Untited Kingdom, [2]Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [3]NIHR Biomedical Research Centre for Ophthalmology, Moorfields Eye Hospital, London, Untited Kingdom, [4]Retinoblastoma Genetic Screening Unit, Barts Health NHS Trust, Royal London Hospital, London, Untited Kingdom

Introduction: Proline/arginine-rich end leucine-rich repeat protein (PRELP), is a small secreted proteoglycan expressed by pericytes and vascular smooth muscle cells surrounding the brain vasculature of adult mouse.

Methods: We utilised a *Prelp* knockout (*Prelp*$^{-/-}$) mouse model to interrogate vasculature integrity in the brain alongside performing in vitro assays to characterise PRELP application to endothelial cells lines. Our findings were supplemented with RNA expression profiling to elucidate the mechanism of how PRELP maintains neurovasculature function.

Results: *Prelp*$^{-/-}$ mice presented with neuroinflammation and reducedneurovasculature integrity, resulting in IgG and dextran leakage in the cerebellum and cortex. Histological analysis of *Prelp*$^{-/-}$ mice revealed reducedcell-cell integrity of the blood brain barrier, capillary attachment of pericytes andastrocyte end-feet. RNA-sequencing analysis found that cell-cell adhesion andinflammation are affected in *Prelp*$^{-/-}$ mice and gene ontology analysis as well as gene set enrichment analysis demonstrated that inflammation related processes and adhesion related processes such as epithelial-mesenchymal transition and apical junctions were significantly affected, suggesting PRELP is a regulator of cell-cell adhesion. Immunofluorescence analysis showed that adhesion junction protein expression levels of cadherin, claudin-5, and ZO-1, was suppressed in *Prelp*$^{-/-}$ mice neurovasculature. Additionally, *in vitro* studies revealed that PRELP application to endothelial cells enhances cell-cell integrity, induces mesenchymal-endothelial transition and inhibits TGF-β mediated damage to cell-cell adhesion.

Discussion: Our study indicates that PRELP is a novel endogenous secreted regulator of neurovasculature integrity and that PRELP application may be a potential treatment for diseases associated with neurovascular damage.

KEYWORDS

PRELP, vasculature, neuroinflammation, integrity, adhesion, EndMT, BBB

1 Introduction

The blood-brain barrier (BBB) is a tight functional barrier composed of capillary endothelial cells, astrocytes, pericytes, and neurons, which prevents neurotoxic plasma components, blood cells, and pathogens from entering the brain (Weiss et al., 2009; Sweeney et al., 2019). Defects in the integrity of the BBB results in the accumulation of toxic molecules leaked from the vasculature in the brain. This leakage causes central nervous system (CNS) diseases such as Alzheimer's disease, Huntington's disease, and stroke (Weiss et al., 2009; Sweeney et al., 2019). The BBB is characterized by strong cell-cell adhesions through adherens and tight junctions (Alahmari, 2021). These dynamic structures are governed by diverse proteins secreted from the neuro vascular components, which trigger downstream signalling events involved in cytoskeletal reorganization, and endothelial permeability (Gaengel et al., 2009). The endothelial-to-mesenchymal transition (EndMT) has been recognized as a major biological event that controls vascular leakage (Cho et al., 2018; Man et al., 2019; Piera-Velazquez and Jimenez, 2019).

Recently, we have reported that two secreted proteoglycans, proline/arginine-rich end and leucine-rich protein (PRELP) and osteomodulin (OMD) function as inhibitors of bladder cancer initiation by inhibiting epithelial-mesenchymal transition (EMT) and by activating cell-cell adhesion of bladder epithelial cells (Papadaki et al., 2020). Intriguingly, *PRELP* expression was regulated by epigenetically through acetylation of lysine residue 5 of histone H2B in the *PRELP* gene promoter region in bladder cancer (Shozu et al., 2022). In addition, we demonstrated that Prelp was expressed in mouse retina and loss of Prelp contributed to retinoblastoma cell progression by reducing cell-cell adhesion and facilitated EMT (Hopkins et al., 2022). We further investigated the roles in other tumors and identified that PRELP showed a tumor suppressive role by regulated PI3K-AKT signalling pathway in high-grade ovarian cancer (Dozen et al., 2022).

PRELP is a class II member of the small leucine rich proteoglycan (SLRP) family (Dellett et al., 2012; Iozzo and Schaefer, 2015). SLRP family members bind various extracellular proteins such as TGF-β, BMP, EGF, IGF, Wnt, and collagens and can regulate multiple signalling pathways in context dependent manners (Morris et al., 2007; Dellett et al., 2012; Chacon-Solano et al., 2022; Lopez and Bonassar, 2022). They are involved in various biological processes such as cancer, inflammation, and development (Birke et al., 2014; Luehders et al., 2015; Papadaki et al., 2020). In this paper, we report that PRELP is selectively expressed in mural cells around the neurovasculature and contributes to the regulation of BBB integrity. PRELP depletion in mouse brain caused blood leakage, indicating that PRELP is responsible for BBB integrity. Our results suggest that PRELP could be used as a new strategy to inhibit neurovascular leakage or protect BBB dysfunction against neurological disorders.

2 Materials and methods

For Antibodies, reagents, data accession number, software, and primes, all antibodies, reagents, accession number, software and algorithms, and primes used in this study were listed in Supplementary Tables S1, S2.

2.1 *Omd*$^{-/-}$ and *Prelp*$^{-/-}$ mice

Mouse lines were generated by Takeda Pharmaceutical Company and wild type and heterozygote founders were imported to our animal facility. All animal procedures were performed in accordance to the Animals (Scientific procedures) Act 1986 of the United Kingdom Government and housed in compliance with the Home Office Code of Practice. Mice were kept in individually ventilated cages (IVCs), in a 12 h light: dark cycle and were fed a complete pelleted mouse diet and with constant access to water.

Briefly, *Omd*^{flox} or *Prelp*^{flox} ES cells were generated from C57BL/6J ES cells by homologous recombination. Targeting vectors were constructed by insertion of the first LoxP sequence upstream of exon two, containing the initiation codon on the *Omd* or *Prelp* locus. A second LoxP sequence, neomycin resistant unit, and LacZ unit was inserted downstream of exon three. Cre expression plasmid was electroporated into the recombinant flox ES cells to generate ES cells harboring the knockout allele. The resulting cells were injected into ICR tetraploid blastocysts to generate chimeric male mice which were backcrossed to C57BL/6J females. Single knockout mice (*Omd*$^{LacZ/LacZ}$ and *Prelp*$^{LacZ/LacZ}$) were generated by cross breeding within the colony.

Genotyping: Genotyping PCR reactions were performed as follows. Mouse ear punches were mixed with 180 µL DirectPCR Lysis Reagent (Viagen Biotech) and 0.4 mg/mL Proteinase K (Sigma) solution before rocking at 55°C overnight. The samples were then incubated at 85°C for 45 min before centrifugation and collection of the resulting lysate. OMD samples were genotyped using an Invitrogen kit. A master mix was prepared according to the manufacturers protocol using the primers described in Supplementary Table S2 and added to 5 µL genomic DNA. Samples were incubated at 95°C for 3 min prior to 35 cycles consisting of 30 s at 95°C, 90 s at 61°C and 90 s at 72°C. The final extension was 10 min at 72°C. This produced amplicons of different lengths: 298 bp in wild type mice with OMD-A and OMD-B2 primers; 541 bp in knockout mice with OMD-B2 and LacZ-5756 primers; or both amplicons present in heterozygotic mice. PRELP samples were genotyped using a Multiplex PCR kit (Qiagen). A master mix was prepared according to the manufacturers protocol using the primers described in Supplementary Table S2 and added to 2.5 µL genomic DNA. Samples were incubated at 95°C for 15 min prior to 40 cycles consisting of 30 s at 94°C, 90 s at 63°C and 90 s at 72°C. The final extension was 10 min at 72°C. Amplicons were 846 bp for wild type mice with PRELP-A and PRELP-B primers, 634 bp for knockout mice with PRELP-C and LacZ-B primers, or both amplicons present in heterozygotic mice.

2.2 Tissue processing and staining

Mouse brains were isolated and fixed in 4% PFA for 24 h before paraffin embedding and sectioning. Tissue for paraffin sectioning was processed in the Institute's Pathology department using an automated machine (Leica ASP300S). Samples were sectioned into 5 µm slices on superfrost slides treated with poly-L-lysine, dried and stored at room temperature. Histological staining was performed in an automated system in the Pathology department. H&E, von Kossa, alcian blue, congo red and MSB, were performed in the Pathology department following department's specific protocol for each stain. Methylene blue

and basic fuchsin staining was performed on semi-thin sections of xenografted tumors. For immunostaining, slides were dewaxed for 10 min in Histoclear and rehydrated in an ethanol-water graded series. Antigen retrieval was performed by boiling the samples for 15 min in citrate (pH 6.0) or Tris-EDTA buffer (pH 9.0) depending on the antibody. Sections were blocked for 1 h in 10% goat serum in PBS and were incubated overnight with primary antibodies at 4°C. Detection was performed by incubation with anti-rabbit or anti-mouse Alexa Fluor 488 secondary antibodies, for 1 h at room temperature (1:500 dilution, Life technologies).

2.3 β-Galactosidase analysis

Mouse brains were isolated from adult mice and were fixed in 4% PFA at 4°C briefly for 2 h with gentle agitation. Afterwards they were washed in PBS and left at 30% sucrose at 4°C overnight before subsequently frozen in OCT. Cryosections 10 µm thick were washed twice in PBS +2 mM $MgCl_2$ for 20 min and were stained overnight in X-gal at 37°C. Sections were washed in PBS and then either counterstained and mounted in Nuclear Fast Red (Vector Laboratories) or followed by IHC. For IHC, briefly, samples were immediately blocked for 1 h with 10% goat serum and were incubated with the primary antibodies overnight. Secondary staining was completed with anti-rabbit or anti mouse Alexa Fluo-488 antibodies, counterstained in DAPI and mounted.

2.4 Immunocytochemisty

Coverslips with cell monolayers were washed in PBS and fixed with either 4% PFA or ice-cold methanol for 10 min. Samples were then washed and incubated for 1 h in blocking buffer. After blocking and incubation with primary antibodies overnight at 4°C, the samples were incubated with secondary Alexa Fluor 488 (1:500, Life Technologies) in blocking buffer for 1 h at room temperature, counterstained with Hoechst solution (Invitrogen) and mounted. Slides were imaged using a Zeiss LSM710 at ×10 and ×40 magnification. After laser intensity settings were optimized, images were processed on ImageJ and a standard threshold to remove background noise across all samples.

2.5 Microscope settings and image analysis

A preliminary analysis determined the thickness of samples, and the microscopic settings were adjusted accordingly to enable the detection of structures. In this setting, Hamamatsu ORCA-ER Digital Camera (Hamamatsu, Japan) and µManager software (Edelstein et al., 2010) were used to obtain fluorescent images on an Axioskop 2 Plus (Zeiss, Germany) from sections. A ×40 differential interference contrast objective with an aperture of 0.95 and 0.25 working distance and 10X ocular lens were used to obtain each region of interest. Imaging parameters of laser intensity and exposure time were optimized and uniformly set in the same experiments. Images were then processed, despeckled, background subtracted and applied with a median filter at 2px in order to remove all background noise. Huang auto threshold was then applied, and the threshold was saved as an ROI. This ROI was then used as the "outline" of immunostaining to measure, and regions of interest measured and quantified with FIJI software.

2.6 Vascular capillaries leakage studies

25 mg/mL 70 kDa Dextran-Texas Red was injected into mice by intravenously. After circulation for 3 h, mice were culled and brain tissues harvested. Perfusion was performed through the heart before collecting tissues. Dye excess was washed out through fixation and washing in PBS before cryoprotection. Sample preparation was done as described above. Vascular permeability was then visualized with fluorescence microscopy. Vessels were traced on ImageJ and interior staining removed. The resulting external staining was then quantified by methods mentioned above.

2.7 Expression profiling of meningeal vessels in RNA-seq analysis

2.7.1 Sample preparation

Four wild-type mice, three *Omd*$^{-/-}$ and three *Prelp*$^{-/-}$ knockout mice meningeal samples were used for RNA-seq analysis. RNA was extracted via ARCTURUS PicoPure RNA Isolation kit for mouse samples or PureLinkTM RNA Mini Kit for HUVECs. In brief, meningeal vessels were excised and homogenized using a rotor-stator homogenizer. After centrifugation at 3,000 × g for 2 min and extracted in accordance with manufacturer's instructions RNA was quantified and qualified by Agilent's 2200 TapeStation, measuring RNA concentration and agarose gel electrophoresis.

2.7.2 Library preparation

Samples were processed using the KAPA mRNA HyperPrep Kit (Roche KK8580) according to manufacturer's instructions.

Briefly, mRNA was isolated from total RNA using Oligo dT beads to pull down poly-adenylated transcripts. The purified mRNA was fragmented using chemical hydrolysis (heat and divalent metal cation) and primed with random hexamers. Strand-specific first strand cDNA was generated using Reverse Transcriptase in the presence of Actinomycin D. This allows for RNA dependent synthesis while preventing spurious DNA-dependent synthesis. The second cDNA strand was synthesized using dUTP in place of dTTP, to mark the second strand. The resultant cDNA is then "A-tailed" at the 3' end to prevent self-ligation and adapter dimerization. Full length xGen adaptors (IDT), containing two unique 8 bp sample specific indexes, a unique molecular identifier (N8) and a T overhang are ligated to the A-Tailed cDNA. Successfully ligated cDNA molecules were then enriched with limited cycle PCR (50 ng of starting material, 15 PCR cycles). The high fidelity polymerase employed in the PCR is unable to extend through uracil. This means only the first strand cDNA is amplified for sequencing, making the library strand specific (first-strand).

2.7.3 Sequencing

Libraries to be multiplexed in the same run are pooled in equimolar quantities, calculated from Qubit and Bioanalyser fragment analysis.

Samples were sequenced on the NextSeq 500 instrument (Illumina, San Diego, US) using a 75 bp single read run with a corresponding 16 bp UMI read.

2.7.4 Data analysis

Run data were demultiplexed and converted to fastq files using Illumina's bcl2fastq Conversion Software v2.19. Fastq files were then aligned to the *Mus musculus* genome GRCm38 or *Homo sapiens* genome GRCh38 using RNA-STAR 2.5.2b then UMI deduplicated using Je-suite (1.2.1). Reads per transcript were counted using FeatureCounts and differential expression was estimated using Galaxy. Log2 fold change and *p* values of pairwise differential expression between wild type samples and knockout samples or PRELP treated and untreated samples were then analysed using Qiagen's Ingenuity Pathway Analysis (version 48207413).

2.8 Measurement of microglial morphological change

Microglial morphological change was measured using the previously reported protocol with minor modifications (Morrison and Filosa, 2013). Briefly, images of Iba-1 staining were processed to remove noise and any background staining. A threshold was applied to produce a binary image and skeletonized which was analysed using Analyse Skeleton plugin. The image was then analysed, and the sum of all branch lengths were extracted and used for further quantification. All images were processed using the same parameters. Branch lengths were normalized by number of microglia per area and the resulting value was expressed as branch length per microglial density.

2.9 Brain integrity studies

Transepithelial/transendothelial electrical resistance (TEER) were used to assess the brain integrity using an EVOM Volt-Ohmeter (World Precision Instruments). STX2 probes were disinfected and air-dried before being inserted into upper and bottom chambers. Resistance was measured for empty wells containing media before determined across monolayers. Values were calculated by multiplying raw values by transwell growth area once the base value for empty wells had been subtracted. 70,000 cells per 24-well transwel insert were seed and allow them to grow for 4 days in control and PRELP conditioned media. TEER measurements were completed 96 h after PRELP treatment.

For the membrane permeability, permeability assays were completed using an *in vitro* vascular permeability assay kit (Merck). In brief, inserts were hydrated and seeded with 0.5×10^5 cells/insert in media and incubated until a monolayer formed. PRELP conditioned media was applied and incubated for 24 h. FITC-Dextran was added to each insert and incubated for 20 min in the dark before the insert was removed from the well. Media in the well was mixed and transferred to black 96-well opaque plate to measure fluorescence intensity at 485 nm and 535 nm excitation and emission respectively.

2.10 Experimental design and statistical analysis

Experiments were performed at least in three independent replicates. All data shown as the mean ± SEM. All data was tested for normal distribution before a Student's T-test was used to calculate significance. A two-tailed student-t test was used for statistical analysis. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. NS, not significant. Imaging data were analysed by NIH software ImageJ (Ljosa et al., 2012). N shown in the figure legends indicates the number of animals used in the experiments. For image analyses, five fields were randomly imaged per animal.

2.11 Data availability

The data that support the findings of this study are available from the Gene Expression Omnibus (GEO) (GSE199122) and from the corresponding author upon reasonable request.

3 Results

3.1 PRELP is selectively expressed in vascular smooth muscle cells (vSMCs) and pericytes around brain vasculature

PRELP expression in the CNS was examined by X-gal staining of $Prelp^{+/LacZ}$ mouse brain. The mouse expresses the lac-Z gene under the control of endogenous PRELP transcription elements (Papadaki et al., 2020). At embryonic stages, we observed expression in the cortical hem of the hippocampal allocortex (Figure 1A, arrowheads) and at sites of bone formation around the CNS (Figure 1B). However, we did not observe any other strong PRELP expression in the head (Figure 1C).

At the adult stages, a unique X-gal staining was observed in mural cells around the CNS vessels, covering both the dorsal and ventral part of the brain, as well as around the optic nerves, the space between the two hemispheres (superior sagittal sinus) and the central canal of the spinal cord (Figures 1D–G). Enlarged images revealed X-gal staining (Figures 1H, I), reminiscent of pericytes which encircle capillaries and vascular smooth muscle cells (vSMCs) around large arterial and venous vessels (Chen et al., 2017; Vanlandewijck et al., 2018). Staining with PRELP antibodies (α-PRELP #15) identified secreted PRELP protein largely localized around large arterioles/venules (Figure 1J) and capillaries (Figure 1K). In addition to vascular mural cells, we observed strong staining at ependymal layers of the ventricle walls (Figure 1L), the choroid plexus (Figure 1M), and non-pigmented layer of ciliary body of the retina (Figure 1N).

The analysis of *PRELP* expression in pericytes and vSMCs, but not in endothelial cells has been confirmed by published single cell mRNA expression profiling data (Zeisel et al., 2015; He et al., 2016; Vanlandewijck et al., 2018) (Figure 2A). To confirm these previously published results in this study, we performed double staining to examine where PRELP was expressed. X-gal staining did not overlap with endothelial marker PECAM-1 positive cells (Figures 2B–D).

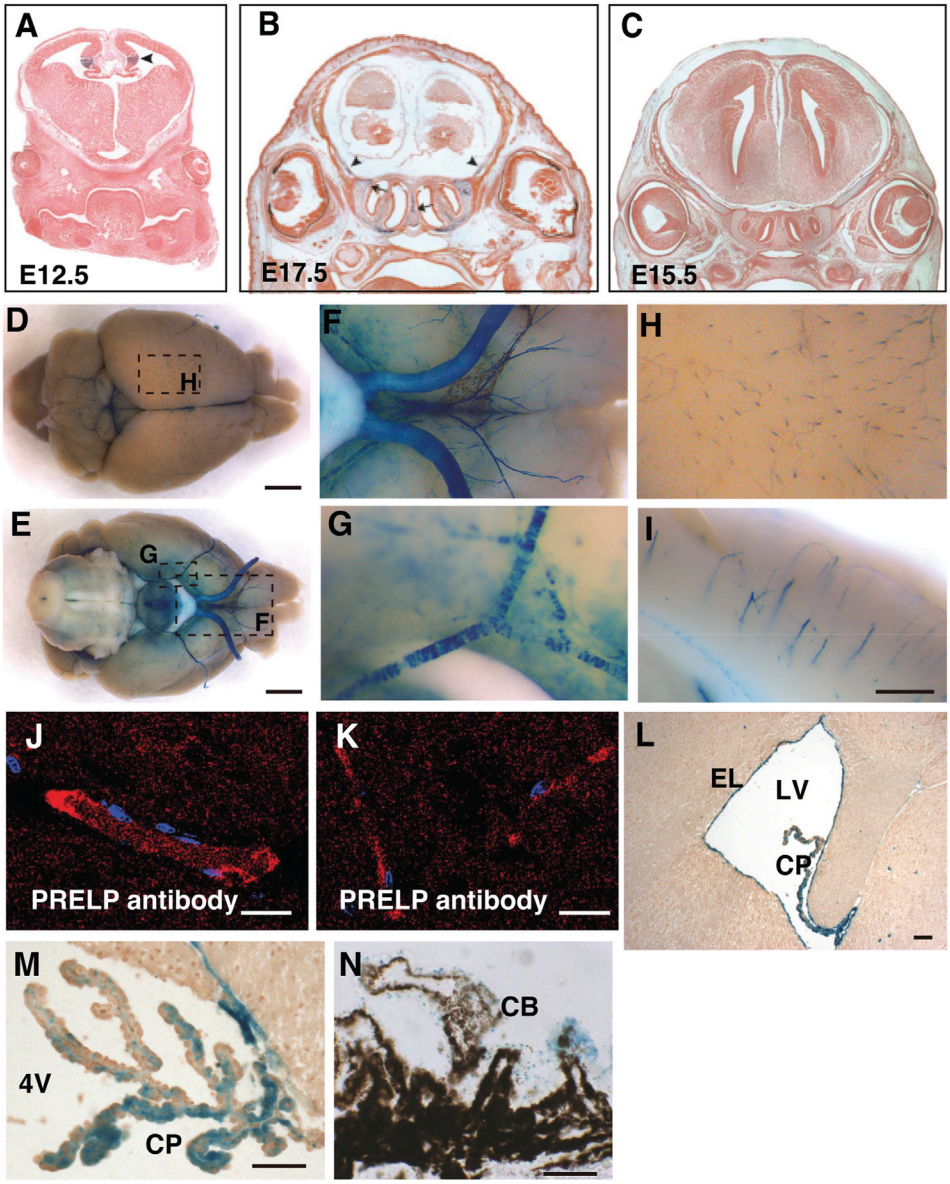


FIGURE 1
PRELP is expressed mural cells around brain vasculatures and ependymal cells in mouse brain. **(A–C)** Embryonic PRELP expression of head. Sections of *Prelp*$^{+/LacZ}$ embryos were stained for X-gal. **(A)** Embryonic day 12.5 (E12.5). Arrowhead indicate cortical hem. **(B)** Embryonic day 17.5 (E17.5). Arrow indicate the nasal septum and arrowhead indicate the sites of bone formation **(C)** Embryonic day 15.5 (E15.5). **(D–I)** Whole-mount X-gal staining of adult *Prelp*$^{+/LacZ}$ brains. **(D–E)** Dorsal and ventral views. X-gal staining is observed in the blood vessels. Scale bar: 2 mm. **(F)** Magnified from **(E)** around the ventral-posterior area. **(G)**, Magnified from **(E)** around ventral lateral. **(H)** Magnified from **(D)** around cerebrum. **(I)** Sagittal section of X-gal stained brain around cerebellum. Scale bar: 500 µm. **(J–K)** Anti–PRELP antibody staining of rat brain. Staining is visible in large arterioles/venules **(J)** and **(K)** capillaries Scale bar: 20 µm. **(L)** Section around lateral ventricle (LV). Ependymal layer (EL). Choroid plexus (CP). Scale bar: 50 µm. **(M)** Section around 4th ventricle (4V). Scale bar: 50 µm. **(N)**, Section of retina around ciliary body (CB). Scale bar: 50 µm.

We observed that X-gal staining was co-localised with α-SMA, marking vSMCs (Figures 2E–G). There was no detectable staining of X-gal with astrocyte marker GFAP on capillaries (Figures 2H–J) although single cell analysis indicated gene expression of *PRELP* in astrocyte subpopulations (Figure 2A). Double staining with pericyte marker, NG2, showed that co-staining was limited to the pericyte processes around the vasculature (arrowhead) and not the pericyte cell body (Figure 2K–M) (arrow in Figure 2M). This suggests that the receptor gene may be expressed by pericytes—localisation with the X-gal staining (Figures 2N–P), indicating PRELP was not expressed in the endothelial cells and expressed in vSMCs and in pericytes at specific locations, rather than astrocytes surrounding vascular capillaries.

In addition to the *Prelp*$^{-/-}$ mice, we examined the vasculature in *Omd*$^{-/-}$ mice. OMD is also a class II SLRP family, highly conserved with PRELP. We previously demonstrated that OMD and PRELP are both expressed in umbrella bladder epithelial cells and involved in

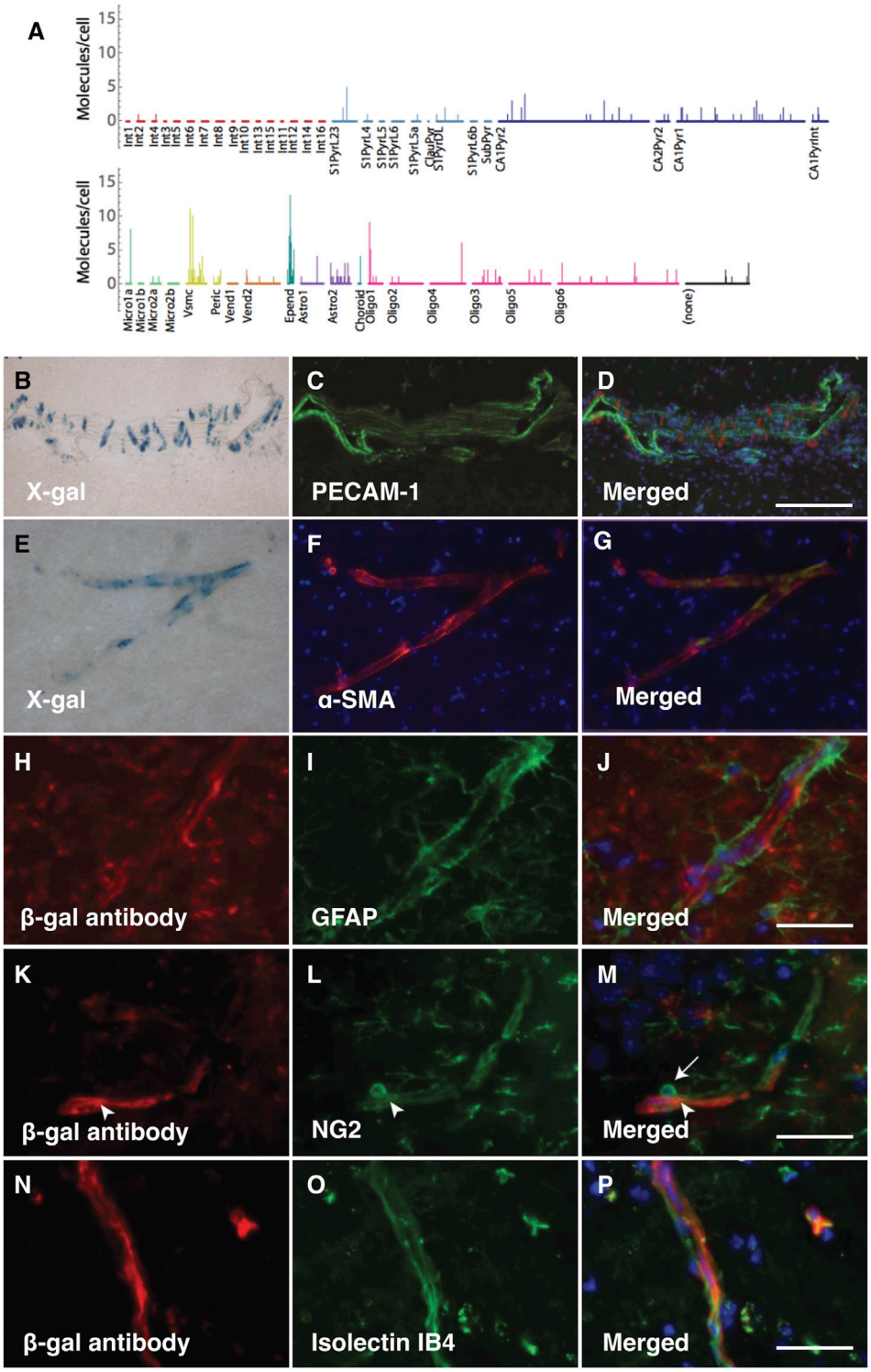


FIGURE 2
PRELP is expressed in vSMCs around vascular vessels and pericyte around capillaries. **(A)** Single cell analysis data of PRELP expression in cortical and hippocampal cells were obtained from previously published studies (Zeisel et al., 2015). Figure was generated by tool provided by the paper (Zeisel et al., 2015). **(B–D)** Double staining images with X-gal **(B)** and PECAM-1 (endothelial cell marker) antibody **(C)** of *Prelp*$^{-/+}$ large vascular. **(D)** Merged image. **(E–F)** Double staining with X-gal **(E)** and α-smooth muscle actin (α-SMA) antibody ((**F**); vSMC marker) of *Prelp*$^{-/+}$ intermediate vasculature. **(G)** Merged image. **(H–J)** Double staining images with β-galactosidase (β-gal) antibody **(H)** and GFPA antibody [**(I)**; astrocyte marker) of *Prelp*$^{-/+}$ brain capillaries. **(J)** Merged image. Scale bar: 25 μm **(J,M,P)**. **(K–M)** Double staining images with β-gal antibody **(K)** and NG2 antibody **(L)**; pericyte marker) of *Prelp*$^{-/+}$ brain capillaries. **(M)** Merged image. **(N–P)** Double staining images with β-gal antibody **(N)** and Isolectin IB4 **(O)**; endothelial cell marker) of *Prelp*$^{-/+}$ brain capillaries. **(P)** Merged image. Pericyte bodies are marked by arrows. Pericyte processes are marked by arrowheads.

10.3389/fcell.2023.1147625

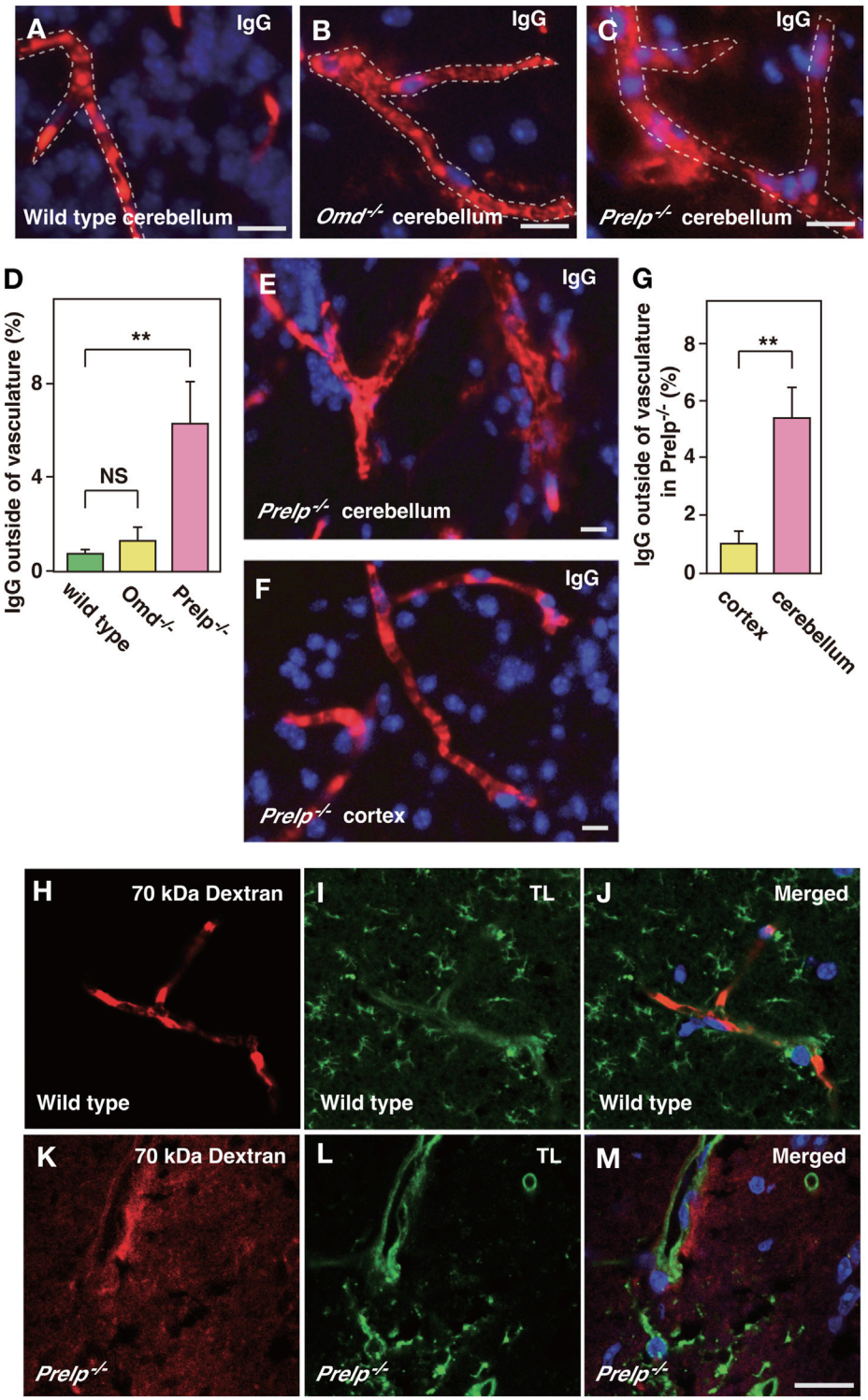


FIGURE 3
PRELP deletion results in leakage from the BBB. **(A–D)** Assessment of BBB integrity in the cerebellum by IgG staining in wild-type **(A)**, *Omd*$^{-/-}$ **(B)** and *Prelp*$^{-/-}$ **(C)**. Immunofluorescence was performed using anti-mouse IgG conjugated with Alexa Fluor A594 fluorophore. DAPI was used as a nuclear stain. Scale bar: 10 μm. **(D)** IgG signal outside blood vessels was quantified (n = 4). **(E–G)** BBB disruption in *Prelp*$^{-/-}$ is more apparent in the cerebellum. IgG and DAPI staining of *Prelp*$^{-/-}$ cerebellum **(E)** and cortex **(F)**. Scale bar: 10 μm. **(G)**, Quantified result (n = 3). **(H–M)** 70 kDa Dextran injection confirms BBB leakage in *Prelp*$^{-/-}$ cerebellum. Wild-type **(H–J)** and *Prekp*$^{-/-}$ **(K–M)** mice were injected with 70 kDa Dextran-Texas Red **(H,K)**. Tissues were processed and stained with tomato lectin (TL, vascular vessel and microglia marker) **(I,L)**. Merged image **(J,M)**. Scale bar: 20 μm.

bladder cancer initiation in a partially redundant manner (Papadaki et al., 2020). *Omd* expression was observed across the cerebrum, but not the cerebellum, optic nerves or optic chiasm (unpublished result). *Omd* was also strongly expressed in neurons and weakly expressed in cells around vasculature (unpublished result). Thus, besides wild-type controls, we also examined $Omd^{-/-}$ mice as the second control.

3.2 Cell-cell adhesion weakened in $Prelp^{-/-}$ mice results in leakage from vascular capillaries in mouse brain

vSMCs and pericytes have important roles in controlling endothelial cell-cell integrity (Armulik et al., 2010; Hayes et al., 2022), therefore, we examined the effect of PRELP deletion on leakage from neural capillaries and the BBB. Firstly, the status of vascular integrity or BBB was assessed by immunoglobulin G (IgG) staining using one-year-old mice. IgG is a 160 kDa protein, which exclusively localizes in blood plasma. The intact BBB prevents IgG from passing through the vasculature and coming into contact with neural tissues. Using anti-mouse IgG-Alexa Fluor 594, we stained for plasma IgG in wild-type, $Omd^{-/-}$ and $Prelp^{-/-}$ mouse cerebellum (Figures 3A–C). In wild type, IgG staining was retained within the vasculature and along the walls of the blood vessels in a regular striated pattern perpendicular to the length of the vessel. In the $Omd^{-/-}$ mice brain, diffused staining outside the vasculature was not observed (Figure 3B). However, in the $Prelp^{-/-}$ brain, IgG was often found to be highly diffused outside vasculature. (Figures 3C, D). This suggests that PRELP, but not OMD, is responsible for the regulation of neurovasculature integrity. Disruption of the $Prelp^{-/-}$ BBB was most intense in the cerebellum, compared with the cortex (Figures 3E–G). This may reflect the higher levels of PRELP expression in the cerebellum. To confirm the BBB damage in $Prelp^{-/-}$, we performed another leakage assay through injection of Texas Red conjugated 70 kDa-Dextran. Consistent with the IgG staining result, Dextran was restricted to the vasculature in wild type animals (Figures 3H–J). In comparison, we detected areas of the posterior brain where the dye was detected outside of the vasculature in $Prelp^{-/-}$ (Figures 3K–M).

One of the proposed mechanisms of vascular leakage is activation of EndMT of vascular endothelial cells (Sweeney et al., 2019), which is associated with a reduction in adherens and tight junctions. Recently, we demonstrated that PRELP has the ability to activate mesenchymal-to-epithelial transition (MET), resulting in the enhancement in bladder epithelial cell-cell and retinoblastoma cells (Papadaki et al., 2020; Hopkins et al., 2022). As the EndMT/MEndT mechanism is largely conserved with EMT/MET, loss of PRELP may cause vascular leakage through activation of EndMT (Saito, 2013; Hong et al., 2018).

We previously demonstrated the cell-cell junction dysfunction in response to the OMD and PRELP expression levels in bladder cancer cells using electron microscope analysis along with immunofluorescence analysis (Papadaki et al., 2020). Therefore, we performed immunostaining against an adherens junction marker (VE-cadherin) and tight junction markers (Claudin-5 and ZO-1). In $Prelp^{-/-}$, we observed uneven, inconsistent VE-cadherin staining in contrast to the uniform staining in wild type and $Omd^{-/-}$ mice (Figures 4A–I). Quantification of VE-cadherin signal revealed that there was significant reduction of VE-cadherin in $Prelp^{-/-}$ (Figure 4J). For claudin-5, we observed uniform membrane staining in the wild type and $Omd^{-/-}$ neurovasculature, although in $Prelp^{-/-}$ mice presented significantly weaker expression (Figures 4K–T). ZO-1 formed a stripe-type staining pattern around the membrane in wild type mice, which was punctuated in $Prelp^{-/-}$ mice (Figures 4U–CC). ZO-1 staining intensity of $Prelp^{-/-}$ was also significantly reduced compared to controls (Figure 4DD). These observations indicate weakened cell-cell contacts between VSMCs in $Prelp^{-/-}$ mice.

Next, we examined neurovascular unit (NVU) components in the $Prelp^{-/-}$ mouse. The basement membrane (BM) is a relatively thick layer of secreted proteoglycans, laminins, collagens and perlecan which underlies endothelial cells. These components are organised by SLRP proteins, with most SLRPs able to bind collagen (Tashima et al., 2018) and PRELP specifically shown to interact with perlecan (Bengtsson et al., 2002). Laminin staining in wildtype and $Omd^{-/-}$ is intense, clearly surrounding blood vessels (Supplementary Figures S1A–F). This intensity is lost in $Prelp^{-/-}$ mice, especially at sites with intense IgG leakage (Supplementary Figures S1G–J). Perlecan staining was also reduced in $Prelp^{-/-}$ (Supplementary Figures S1K–T). However, we did not observe a significant reduction of collagen IV staining in $Prekp^{-/-}$ compared with wild-type mice (Supplementary Figures S1U–DD), suggesting collagen IV expression was maintained independent of IgG leakage in the $Prelp^{-/-}$ vasculature (Supplementary Figures S1AA–CC, arrows).

Next, we examined the effect of PRELP on the distribution of pericyte and astrocyte perivascular end-feet. Aquaporin 4 (AQP4) is a water channel found on astrocyte end-feet (Haj-Yasein et al., 2011). Double staining with AQP4 and IgG in mouse cerebellum revealed that there was a significant decrease in AQP4 signal around the vasculature in $Prelp^{-/-}$, whereas no difference was observed in $Omd^{-/-}$ (Supplementary Figures S1EE–NN). We then examined the effect on pericytes. Pericytes were distinguished from endothelial cells by their nuclear morphology and the staining pattern of PDGFR-β. The point of association between endothelial cells with pericytes was diminished (Supplementary Figures S1OO–XX), suggesting pericyte detachment from capillaries (Supplementary Figures S1UU, arrow).

3.3 mRNA expression profiling and the effect of PRELP on neuroinflammation in knockout mice

The meninges contain two sites highly expressing PRELP: the meningeal vessels and cells directly contacting with cerebrospinal fluid (Figure 5A), similar to ependymal cells and choroid plexus (Figures 1L, M). To elucidate PRELP mediated biological events and their molecular mechanisms, we performed mRNA expression profiling of meninges on wild-type and $Prelp^{-/-}$ mice as the meninges is easily dissected with less contamination of neural tissues. However, a major disadvantage of studying the meningeal vessels is a lack of astrocytes, although as PRELP is not expressed in astrocytes surrounding neurocapillaries, this may not be important (Figures 2H–J).

10.3389/fcell.2023.1147625

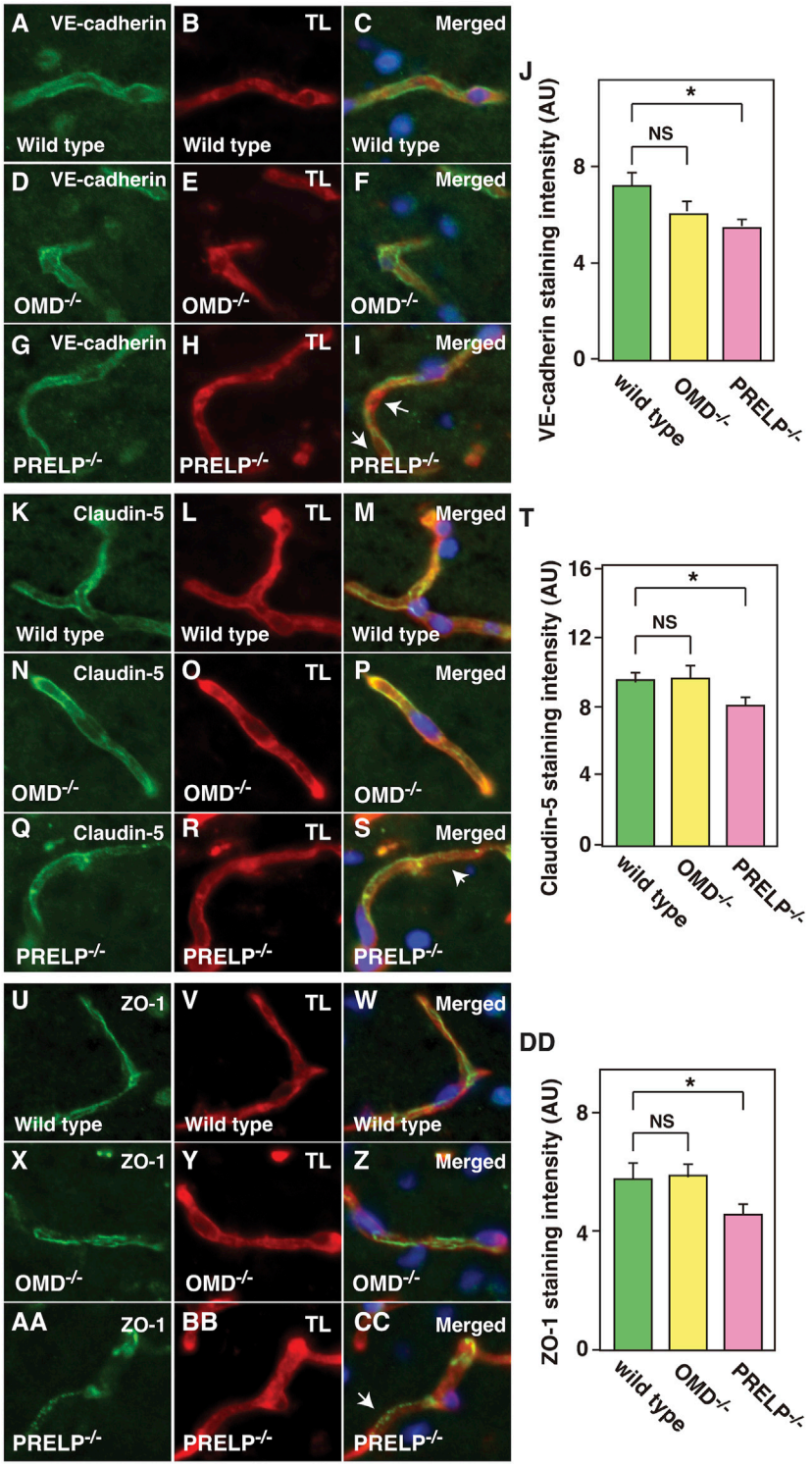
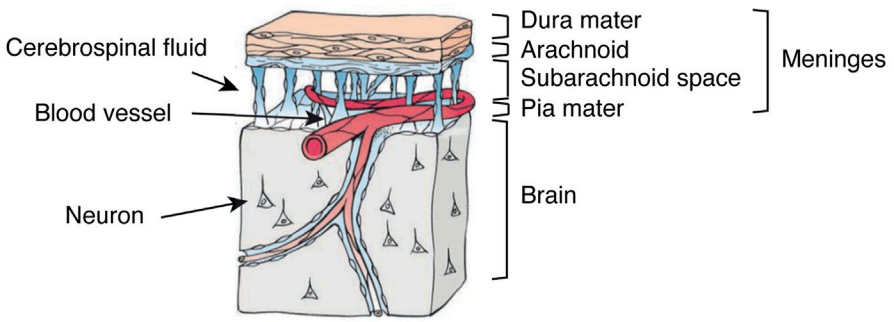


FIGURE 4
Cell-cell adhesion of cerebellum vasculature is downregulated in *Prelp*$^{-/-}$ mice. **(A–J)** Reduced VE-cadherin coverage of *Prelp*$^{-/-}$ vessels. Sections of wild-type **(A–C)**, *Omd*$^{-/-}$ **(D–F)** and *Prelp*$^{-/-}$ **(G–I)** cerebellums were stained with VE-cadherin **(A,D,G)** and TL **(B,E,H)**. Staining of *Prelp*$^{-/-}$ vessels was found to be uneven, inconsistent (arrows). Scale bar 15 µm. **(J)** VE-cadherin staining intensity was quantified (n = 3). **(K–T)** Weaker claudin-5 staining in *Prelp*$^{-/-}$ vessels. Claudin-5 and TL staining was performed in wild-type **(K–M)**, *Omd*$^{-/-}$ **(N–P)** and *Prelp*$^{-/-}$ **(Q–S)** cerebellum. Staining in *Prelp*$^{-/-}$ vessels was found to be more discontinuous (arrow). **(T)** Quantification of staining intensity (n = 3). **(U–DD)** ZO-1 staining is reduced in *Prelp*$^{-/-}$. Wild-type **(U–W)**, *Omd*$^{-/-}$ **(X–Z)** and *Prelp*$^{-/-}$ **(AA–CC)** sections were stained with ZO-1 and TL. Punctated staining along vessels were found (arrow). **(DD)** Quantification of staining intensity (n = 3). TL; Tomato lectin.

10.3389/fcell.2023.1147625

A



Adopted from M.H.Ross and W.Pawlina, Histology: a text and atlas, Lippincott Williams & Wilkins, 2011

B Top 10 GO Biological Processes

GO Biological Process	GO number	Input	Expected	Fold enrich	p-value
Macrophage activation	GO:0042116	8	1.43	5.59	1.15E-04
Immune system process	GO:0002376	17	6.34	2.68	2.65E-04
Cell-cell adhesion	GO:0016337	9	2.29	3.92	5.84E-04
Sufur compound metabolic process	GO:0006790	7	1.42	4.94	6.38E-04
Extracellular transport	GO:0006858	4	0.42	9.54	9.07E-04
Intracellular protein transport	GO:0006886	22	10.48	2.1	9.82E-04
Lipid transport	GO:0006869	6	1.18	5.07	1.35E-03
Protein transport	GO:0015031	22	10.76	2.04	1.37E-03
Blood circulation	GO:0008015	7	1.64	4.27	1.47E-03
Cell-matrix adhesion	GO:0007160	5	0.81	6.14	1.48E-03

C Top 10 GO Cellular component over-representation analysis

GO Cellular component	GO number	Input	Expected	Fold enrich	p-value
Extracellular matrix	GO:0031012	21	6.29	3.34	2.65E-03
Proteinaceous extracellular matrix	GO:0005578	17	4.3	3.95	2.94E-03
Extracellular region part	GO:0044421	79	48.57	1.63	5.65E-03
Extracellular exosome	GO:0070062	57	31.42	1.81	8.53E-03
Extracellular vesicle	GO:1903561	57	31.6	1.80	1.02E-02
Extracellular organelle	GO:0043230	57	31.69	1.80	1.10E-02
Cell projection	GO:0042995	49	26.67	1.84	3.17E-02
Cell projection part	GO:0044463	31	13.75	2.25	3.23E-02
Plasma membranebounded cell projection part	GO:0120038	31	13.75	2.25	3.23E-02
Plasma membranebounded cell projection	GO:0120025	46	24.54	1.87	3.60E-02

D GSEA of genes up-regulated in PRELP$^{-/-}$

Gene Set Name	Input	p-value	FDR
Interferon Gamma Response	22	4.24E-16	2.12E-14
Interferon Alpha Response	16	6.93E-15	1.73E-13
Complement	18	6.37E-12	7.96E-11
Myc Targets	18	6.37E-12	7.96E-11
mTORC1 signalling	16	5.47E-10	5.47E-11
Inflammatory Response	15	4.59E-09	3.82E-08
Xenobiotic Metabolism	14	3.58E-08	2.56E-07
UV Response up	12	1.72E-07	9.53E-07
Apoptosis	12	1.72E-07	9.53E-07
Estrogen Response Late	13	2.60E-07	1.30E-06

E GSEA of genes down-regulated in PRELP$^{-/-}$

Gene Set Name	Input	p-value	FDR
Myogenesis	9	7.02E-05	3.51E-03
Hem Metabolism	8	3.91E-04	9.77E-03
UV response down	6	1.68E-03	2.41E-02
Epithelial Mesenchymal Transition	7	1.97E-03	2.41E-02
Bile Acid Metabolism	5	3.02E-03	2.85E-02
Hedgehog Signalling	3	3.75E-03	2.65E-02
Cholesterol Homeostasis	4	3.99E-03	2,85E-02
Apical Junction	6	8.33E-03	4.16E-02
Xenobiotic Metabolism	6	8.33E-03	4.16E-02

FIGURE 5
Expression profiling analysis of wild type and *Prelp*$^{-/-}$ mouse meninges. **(A)** Schematic draw of meninges. **(B)** Top 10 GO Biological Processes. **(C)** Top 10 Cellular components over-representative analysis. **(D)** GSEA of genes upregulated in *Prelp*$^{-/-}$. **(E)** GSEA of genes downregulated in *Prelp*$^{-/-}$.

RNA for expression profiling was obtained from isolated meninges from wildtype (n = 3) and *Prelp*$^{-/-}$ (n = 3) mice. We identified 288 statistically differentially expressed genes (p < 0.01), of which 87 genes encode extracellular proteins. Ontological analysis was performed using the tools provided by GO consortium to produce four sets of analyses; GO Biological Processes (Figure 5B), GO Cellular components (Figure 5C), Gene set enrichment analysis (GSEA) of genes

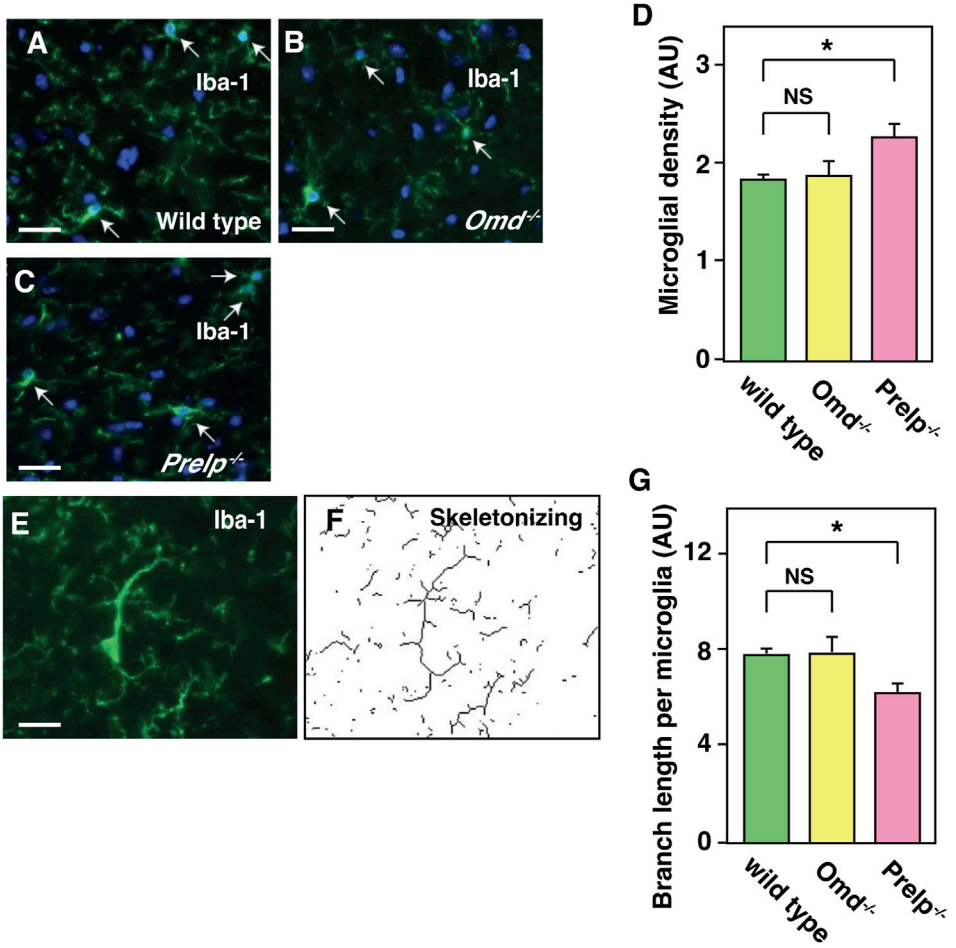


FIGURE 6
Microglia is activated in *Prelp*$^{-/-}$ mouse cerebellum. **(A–G)** Iba-1 staining of wild-type **(A)**, *Omd*$^{-/-}$ **(B)**, and *Prelp*$^{-/-}$ **(C)** cerebellum sections. Microglia were manually counted based on Iba-1 and nuclear DAPI staining (arrows). Scale bar 25 μm. **(D)** Microglial density was quantified. **(E-F)**, Skeletonizing images of Iba-1 staining to quantify microglial branch length. **(G)**, Quantification of branch length per microglial density in wild-type, *Omd*$^{-/-}$ and *Prelp*$^{-/-}$ brains (n = 3).

upregulated in *Prelp*$^{-/-}$ (Figure 5D), and GSEA of genes downregulated in *Prelp*$^{-/-}$ (Figure 5E).

The top 10 GO Biological Processes showed that two inflammation related processes of "Macrophage activation" and "Immune system process" were the most strongly affected followed by two adhesion related process; "Cell-cell adhesion" and "Cell-matrix adhesion". "Blood circulation" was also significantly affected. In the case of GO Cellular components, the majority of affected cellular components were related to the extracellular matrix. This is probably a reflection of the extracellular localization of PRELP. In addition, eight categories were associated with change in cellular morphology, including "Cell projection part" and "Plasma membrane bounded cell projection". As changes to cell-cell adhesion is a major biological event that induces alterations in cell morphology, these results suggest PRELP is a regulator of cell-cell adhesion.

To further elucidate PRELP function, we utilized GSEA. We analysed upregulated and downregulated genes separately (Figures 5D, E). Interestingly, gene sets related to inflammation such as "Interferon γ response", "Interferon α response" and "Complement" were strongly affected in *Prelp*$^{-/-}$ meninges. Cell-cell adhesion related categories, "EMT" and "Apical Junction" were significantly affected (Figure 5E), which suggests that the functional role of PRELP as regulator of partial EMT may be conserved across tissues (Papadaki et al., 2020; Hopkins et al., 2022). In summary, ontological analysis proposes two main biological roles of PRELP within the meninges: cell-cell adhesion and inflammation.

Expression profiling data and vascular analyses suggest that, in the *Prelp*$^{-/-}$ brain, vascular leakage may trigger inflammation. We therefore examined the status of microglia and astrocytes in the mouse cerebellum by the labelling with Iba-1 and GFAP antibodies, respectively. Firstly, the number of microglia cell bodies was counted. Irrespective of morphological change (Figures 6A–C), quantification revealed that there was an increase in the number of Iba-1 positive microglia in *Prelp*$^{-/-}$ sections (Figure 6D). Microglial functional responses in accordance with the protocol established by Morrison (Morrison and Filosa, 2013) was used with some minor modifications to examine inflammation and the sum of the branch lengths was used for quantification (Figures 6E, F).

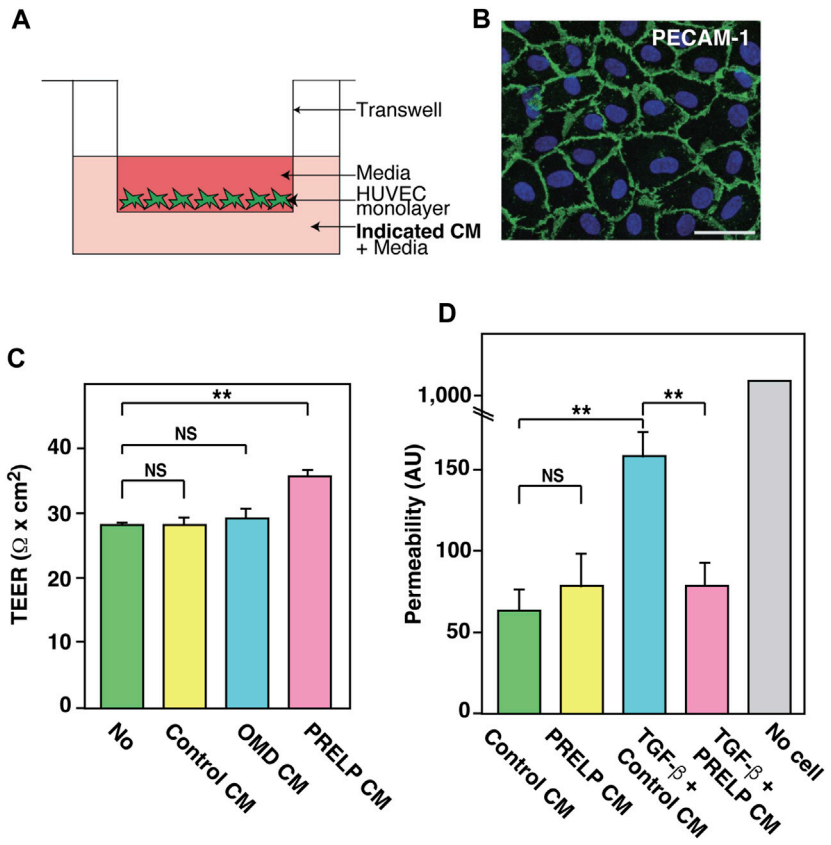


FIGURE 7
Effect of PRELP on leakage from endothelial cell monolayer. **(A,B)** Effect of PRELP on HUVEC monolayer TEER. **(A)** Schematic drawing of the assay. **(B)** Confirmation of HUVECs monolayer confluency by PECAM-1 staining. **(C)** TEER measurement. **(D)** Permeability assay was performed using HUVEC monolayer.

Quantification of branch length per microglial density revealed that there was a statistically significant decrease in *Prelp*$^{-/-}$ mice (Figure 6G), indicating increased microglial response and supporting the findings in our expression profiling (Figure 5B) as microglia are known as a neural type of macrophages (Masuda et al., 2020). Furthermore, we investigated the effect of PRELP on the morphology and the number of astrocytes using antibody to GFAP. However, there were no differences in astrocyte number, morphology, and staining intensity between wild-type, *Omd*$^{-/-}$, and *Prelp*$^{-/-}$ mice (Supplementary Figures S2A–E). While leakage of fluids from vasculature or ependymal layer can affect water content in the brain causing hydrocephalus (Karimy et al., 2017), we did not observe differences of water content between the wild type and *Prelp*$^{-/-}$ brain (Supplementary Figures S2F).

3.4 Application of PRELP protein enhances endothelial cell-cell integrity by affecting EMT-related events

To determine the role of PRELP protein in consolidating BBB integrity and elucidate its mechanism, we performed *in vitro* experiments using either PRELP conditional medium (PRELP CM) or purified recombinant PRELP protein, produced in Mimic Sf9 insect cells (Supplementary Figure S3) (Kosuge et al., 2021). All PRELP proteins showed a phenotypic effect in our assays as shown below.

To ensure that PRELP is not secreted from the vasculature, we examined the expression of PRELP in HUVECs and found extremely low expression. This is consistent with RNA-seq results in other papers that showed no or very low expression in mouse brain endothelial cells (Vanlandewijck et al., 2018). Thus, we examined the effect of PRELP on a transepithelial/transendothelial electrical resistance (TEER) using a simple Human umbilical vein endothelial cells (HUVECs) monolayer (Figure 7A). After confirming HUVECs formed a monolayer via PECAM1 immunostaining (Figure 7B), we found that application of PRELP CM significantly increased TEER (Figure 7C), indicating that PRELP can enhance endothelial cell-cell integrity. Furthermore, we also examined the effect of PRELP on permeability using fluorophore-tagged 70 kDa dextran. Under these conditions, PRELP CM did not reduce permeability compared to the control but was effective at preventing TGF-β-mediated permeability, suggesting that PRELP can inhibit TGF-β signalling (Figure 7D).

EMT exists along a spectrum of different states. These partial EMT states, pEMT, are important for understanding human diseases such as cancer (Lamouille et al., 2014; Nieto et al., 2016; Aiello et al., 2018;

Brabletz et al., 2018). Recently, we reported that PRELP regulates cell-cell adhesion of bladder umbrella epithelial cells and retinoblastoma cells through pEMT (Papadaki et al., 2020; Hopkins et al., 2022). Similar mechanisms, endothelial-mesenchymal transition (EndMT) has been demonstrated in vascular cells (Lamouille et al., 2014; Nieto et al., 2016; Kovacic et al., 2019). EndMT is important for regulating vascular leakage. Our expression profiling analysis of meninges showed that EMT was strongly affected by PRELP deletion (Figure 5E), suggesting that the vascular leakage in *PRELP*$^{-/-}$ mice might be caused by partial EndMT. TGF-β is a potent mediator of EMT and PRELP-mediated inhibition through the application of PRELP CM in TEER and permeability assay may increase cell adhesion via pMEndT (Figures 7C, D).

To elucidate the mechanism of PRELP action, we applied purified recombinant PRELP protein to HUVEC monolayers. HUVECs exhibit pEndMT states which contributes to cell-cell permeability (Guo et al., 2015). mRNA expression profiling was performed on HUVEC cultures incubated with PRELP for 48 h. Using Ingenuity Pathway Analysis (IPA) software, we performed ontological analysis to identify 1,903 significantly affected genes and 220 significantly affected canonical pathways. These pathways were largely classified into three categories; EndMT/cell adhesion (Supplementary Figure S4A), cancer (Supplementary Figure S4B), inflammation (Supplementary Figure S4C) and EMT related Signalling pathways. EndMT/cell adhesion events included "Regulation of the EMT pathway", "Hepatic Fibrosis Signalling Pathway", "Epithelial adherens junction signalling", and "Integrin Signalling" (Supplementary Figure S4A). As EMT is strongly implicated in cancer-related pathways, we found many associated pathways such as "Molecular Mechanism of Cancer", and "Bladder Cancer Signalling" which were also affected (Supplementary Figure S4B, (Brabletz et al., 2018). Furthermore, several interleukins related proinflammation pathways were negatively affected (IL-8, IL-3, IL-7, IL-6, and IL-4) (Supplementary Figure S4C), suggesting that PRELP may have an anti-inflammatory role as we discussed in the previous section (Effect of PRELP on neuroinflammation) and activated EMT through TGF-β, Met and Wnt signalling was observed in the "Regulation of the EMT pathway" (Supplementary Figure S5).

3.5 PRELP activates cell-cell adhesion of HUVEC cell culture and reverses TGF-β mediated pEndMT

The membrane localization of β-catenin, an intracellular protein directly associated with cadherin molecules, was enhanced by PRELP (Supplementary Figure S6A–F). These data indicate that PRELP enhances adherens junction formation and/or stability. We examined the effect of PRELP on tight junctions using ZO-1 (Supplementary Figures S6G, H) and claudin-5 (Supplementary Figures S6K, L) staining but could not detect tight junction formation in our conditions.

TGF-β is the strongest activator of EndMT. As observed in many other biological systems, TGF-β has complex and dual roles in vascular biology. This includes a dual role as an activator and an inhibitor of BBB function in context dependent manners (Li et al., 2011; Diniz et al., 2019). Application of TGF-β to HUVECs has been reported to cause damage to endothelial cell-cell adhesion through activation of pEndMT (Guo et al., 2015). Using this system, we examined the effect of PRELP on TGF-β mediated pEndMT. As shown in Supplementary Figure S6 , 20 ng/mL TGF-β resulted in the increase of β-catenin membrane staining (Supplementary Figure S6C). PRELP application reversed all TGF-β mediated effects (Supplementary Figures S6D, J, N) suggesting that PRELP can rescue TGF-β mediated vascular damage in association with inhibition of pEndMT and all of these may be associated with activation of pEndMT (Guo et al., 2015).

4 Discussion

4.1 PRELP is a novel regulator of pEndMT in vascular homeostasis

Our *in vitro* studies show that PRELP activates EndMT and enhances cell-cell adhesion of endothelial cells which may occur in a TGF-β-dependent manner. Conversely, the *in vivo* phenotype in *Prelp*$^{-/-}$ mice also demonstrated pEndMT activation and reduced cell-cell adhesion in the cerebellum. Furthermore, involvement of PRELP mediated regulation of EndMT in both *in vivo* and *in vitro* was confirmed by expression profiling of PRELP-treated HUVECs and *Prelp*$^{-/-}$ meninges. As we previously mentioned in the result section in Figure 2A, the analysis of *PRELP* expression in pericytes and vSMCs, but not in endothelial cells has been confirmed by published single cell mRNA expression profiling data (Zeisel et al., 2015; He et al., 2016; Vanlandewijck et al., 2018). Our RNA-seq result shows that there are very low expression levels of PRELP in HUVECs. One paper demonstrated that the proteoglycan agrin, which is widely expressed in neurons and microvascular basal lamina in the rodent and avian central nervous system (Donahue et al., 1999) regulated the junction proteins of VE-cadherin, β-catenin, and ZO-1, and stabilized junctional localization of VE-cadherin *in vivo* (Steiner et al., 2014). This indicates that proteoglycans, including PRELP can maintain BBB function by regulating and stabilizing junction protein expression without being express in endothelial cells.

Recently we showed that PRELP activates bladder epithelial cell-cell adhesion by activation of MET. This was mediated via direct inhibition of TGF-β and/or EGF mediated pEMT (Papadaki et al., 2020).

Indeed, an independent study demonstrated that PRELP can antagonize TFG-β (Chacon-Solano et al., 2022). This activity is important for maintenance of the blood-urine barrier (Kreft et al., 2010). In addition to the BBB, the blood-CSF barrier, where cell-cell adhesions between choroid plexus and ventricle ependymal cells, plays an important role in separating the brain from non-brain tissues (Liddelow, 2015). These observations indicate that PRELP may have a conserved function to maintain biological barriers by regulating either pEndMT or pEMT.

4.2 The mechanism of PRELP deletion mediated leakage from BBB

In the *Prelp*$^{-/-}$ mouse brain, NVU components, BM proteins, pericytes and astrocyte endo-feet, were downregulated. Downregulation of BBB components has been frequently reported to cause leakage of the BBB. For example, mice lacking laminin α2 or laminin γ1 display significant abnormalities to brain vasculature integrity (Menezes et al., 2014; Yao et al., 2014; Gautam et al., 2016). Ablation of PDGF-β results in reduction of pericyte coverage and subsequent decreased vascular density and increased vascular permeability and vessel diameter (Bjarnegard et al., 2004). Interestingly, deletion of CD146, an EMT inducer in pericytes, results in reduced coverage of pericytes around vasculature (Zeng et al., 2012; Chen et al., 2017), suggesting that EMT/EndMT might be involved in interaction between pericytes and endothelial cells. We observed a decrease in the intensity of AQP4 staining in *Prelp*$^{-/-}$, which is often found in other BBB breakdown model mice (Menezes et al., 2014; Gautam et al., 2016).

These observations suggest that in addition to the direct effect of PRELP-mediated regulation of cell-cell adhesion between endothelial cells, PRELP may also indirectly control BBB integrity through regulation of the NVU components.

4.3 The mechanism of PRELP deletion mediated neuroinflammation

Our expression profiling analysis of meninges and immunohistochemical analysis of microglia indicated the presence of neuroinflammation in the *Prelp*$^{-/-}$ brain. This is likely to be an indirect effect, since blood proteins leaking into the brain tissue cause neuroinflammation and can perpetuate to neurodegenerative disorders (Weiss et al., 2009; Sweeney et al., 2019). Indeed, our expression profiling of HUVECs demonstrated that PRELP application inhibited proinflammatory interleukins including IL-8, IL-3, IL-7, IL-6, and IL-4. Moreover, PRELP has previously been reported to bind to C9 complement to prevent the formation of the membrane attack complex (Happonen et al., 2012) and acts as a potent inhibitor of complement-mediated damage in mouse eyes (Birke et al., 2014). Indeed, "Complement" pathway was also significantly affected in *Prelp*$^{-/-}$ meninges. Furthermore, the importance of EMT/EndMT in inflammation has been recognized (Lopez-Novoa and Nieto, 2009; Cho et al., 2018). Together these observations suggest that PRELP may regulate to neural inflammation as an anti-inflammatory factor.

Severe neural inflammation can lead to alterations in the BBB (Gaillard et al., 2003). However, our results provide evidence that inflammation in *Prelp*$^{-/-}$ mice was relatively mild and may not be sufficient to cause the BBB damage observed. We did not observe activation of astrocytes in *Prelp*$^{-/-}$ brain, common inflammation markers or abnormal behavior of mice and there was no change in water content (unpublished data). A cumulation of factors, including PRELP, may therefore be required to generate damaging levels of neuroinflammation.

In conclusion, our results indicate that PRELP, a secreted novel regulator of pEndMT, enhances BBB integrity, maintains vasculature homeostasis in the brain and might be a potential treatment for neural diseases associated with BBB leakage and neuroinflammation.

5 Limitation of the study

There are some limitations in analysing the effect of glycosylated proteins. First, proteoglycans including PRELP have different formulas based on varied amounts of post-translational sugar chain modifications which can modifications vary among species. Although two sources of PRELP proteins showed almost identical phenotypes, sugar modification may affect activity.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE199122.

Ethics statement

The animal study was reviewed and approved by the local UCL Animal Welfare and Ethical Review Body (AWERB) followed by the approval from Home Office (Home Office project licence: P6CCB6E4D).

Author contributions

HD, JH, NB, VP, AL, HK, TT, MN, and RS, conceived and performed the experiments and analysed the data. KT, MS, and S-IO supervised the experiments. HD, JH, NB, KT, MS, and S-IO wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work is supported by Fight for Sight to S-IO (5081/5082 and 1582/1583), JH (5081/5082), and RS (1582/1583), CHECT to S-IO and MS (23 July 2018 and 13 October 2021), Santen to S-IO and AL (5 April 2018), MRC to S-IO and HD (MR/K501268/1), MEC to S-IO and MS (R150036A/GR000113). MS is funded by Royal Blind and the Royal College of Surgeons of Edinburgh.

Acknowledgments

We thank to Prof Karl Matter, Drs, Kevin Broad and Joanna Nightingale for reagents, technical advice and helpful comments. Also, we acknowledge Steve Bolsover, John Greenwood, and Christiana Ruhrberg for critical reading of the manuscript. We also thank to Wahaj Alnefale for his support.

Conflict of interest

S-IO was the inventor of a patent on Inhibition Of Vascular Leakage. The patent number is: P217903GB.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2023.1147625/full#supplementary-material

References

Aiello, N. M., Maddipati, R., Norgard, R. J., Balli, D., Li, J., Yuan, S., et al. (2018). EMT subtype influences epithelial plasticity and mode of cell migration. *Dev. Cell* 45, 681–695.e4. doi:10.1016/j.devcel.2018.05.027

Alahmari, A. (2021). Blood-brain barrier overview: Structural and functional correlation. *Neural Plast.* 2021, 6564585. doi:10.1155/2021/6564585

Armulik, A., Genove, G., Mae, M., Nisancioglu, M. H., Wallgard, E., Niaudet, C., et al. (2010). Pericytes regulate the blood-brain barrier. *Nature* 468, 557–561. doi:10.1038/nature09522

Bengtsson, E., Morgelin, M., Sasaki, T., Timpl, R., Heinegard, D., and Aspberg, A. (2002). The leucine-rich repeat protein PRELP binds perlecan and collagens and may function as a basement membrane anchor. *J. Biol. Chem.* 277, 15061–15068. doi:10.1074/jbc.M108285200

Birke, M. T., Lipo, E., Adhi, M., Birke, K., and Kumar-Singh, R. (2014). AAV-mediated expression of human PRELP inhibits complement activation, choroidal neovascularization and deposition of membrane attack complex in mice. *Gene Ther.* 21, 507–513. doi:10.1038/gt.2014.24

Bjarnegard, M., Enge, M., Norlin, J., Gustafsdottir, S., Fredriksson, S., Abramsson, A., et al. (2004). Endothelium-specific ablation of PDGFB leads to pericyte loss and glomerular, cardiac and placental abnormalities. *Development* 131, 1847–1857. doi:10.1242/dev.01080

Brabletz, T., Kalluri, R., Nieto, M. A., and Weinberg, R. A. (2018). EMT in cancer. *Nat. Rev. Cancer.* 18, 128–134. doi:10.1038/nrc.2017.118

Chacon-Solano, E., Leon, C., Carretero, M., Garcia, M., Sanchez-Dominguez, R., Quero, F., et al. (2022). Mechanistic interrogation of mutation-independent disease modulators of RDEB identifies the small leucine-rich proteoglycan PRELP as a TGF-beta antagonist and inhibitor of fibrosis. *Matrix Biol.* 111, 189–206. doi:10.1016/j.matbio.2022.06.007

Chen, J., Luo, Y., Hui, H., Cai, T., Huang, H., Yang, F., et al. (2017). CD146 coordinates brain endothelial cell-pericyte communication for blood-brain barrier development. *Proc. Natl. Acad. Sci. U. S. A.* 114, E7622–E7631. doi:10.1073/pnas.1710848114

Cho, J. G., Lee, A., Chang, W., Lee, M. S., and Kim, J. (2018). Endothelial to mesenchymal transition represents a key link in the interaction between inflammation and endothelial dysfunction. *Front. Immunol.* 9, 294. doi:10.3389/fimmu.2018.00294

Dellett, M., Hu, W., Papadaki, V., and Ohnuma, S. (2012). Small leucine rich proteoglycan family regulates multiple signalling pathways in neural development and maintenance. *Dev. Growth Differ.* 54, 327–340. doi:10.1111/j.1440-169X.2012.01339.x

Diniz, L. P., Matias, I., Siqueira, M., Stipursky, J., and Gomes, F. C. A. (2019). Astrocytes and the TGF-β1 pathway in the healthy and diseased brain: A double-edged sword. *Mol. Neurobiol.* 56, 4653–4679. doi:10.1007/s12035-018-1396-y

Donahue, J. E., Berzin, T. M., Rafii, M. S., Glass, D. J., Yancopoulos, G. D., Fallon, J. R., et al. (1999). Agrin in alzheimer's disease: Altered solubility and abnormal distribution within microvasculature and brain parenchyma. *Proc. Natl. Acad. Sci. U. S. A.* 96, 6468–6472. doi:10.1073/pnas.96.11.6468

Dozen, A., Shozu, K., Shinkai, N., Ikawa, N., Aoyama, R., Machino, H., et al. (2022). Tumor suppressive role of the PRELP gene in ovarian clear cell carcinoma. *J. Personalized Med.* 12, 1999. doi:10.3390/jpm12121999

Edelstein, A., Amodaj, N., Hoover, K., Vale, R., and Stuurman, N. (2010). Computer control of microscopes using microManager. *Curr. Protoc. Mol. Biol.* 92, 14.20.1–14.20.17. doi:10.1002/0471142727.mb1420s92

Gaengel, K., Genove, G., Armulik, A., and Betsholtz, C. (2009). Endothelial-mural cell signaling in vascular development and angiogenesis. *Arterioscler. Thromb. Vasc. Biol.* 29, 630–638. doi:10.1161/ATVBAHA.107.161521

Gaillard, P. J., de Boer, A. B., and Breimer, D. D. (2003). Pharmacological investigations on lipopolysaccharide-induced permeability changes in the blood-brain barrier *in vitro*. *Microvasc. Res.* 65, 24–31. doi:10.1016/s0026-2862(02)00009-2

Gautam, J., Zhang, X., and Yao, Y. (2016). The role of pericytic laminin in blood brain barrier integrity maintenance. *Sci. Rep.* 6, 36450. doi:10.1038/srep36450

Guo, Y., Li, P., Bledsoe, G., Yang, Z. R., Chao, L., and Chao, J. (2015). Kallistatin inhibits TGF-beta-induced endothelial-mesenchymal transition by differential regulation of microRNA-21 and eNOS expression. *Exp. Cell Res.* 337, 103–110. doi:10.1016/j.yexcr.2015.06.021

Haj-Yasein, N. N., Vindedal, G. F., Eilert-Olsen, M., Gundersen, G. A., Skare, O., Laake, P., et al. (2011). Glial-conditional deletion of aquaporin-4 (Aqp4) reduces blood-brain water uptake and confers barrier function on perivascular astrocyte endfeet. *Proc. Natl. Acad. Sci. U. S. A.* 108, 17815–17820. doi:10.1073/pnas.1110655108

Happonen, K. E., Furst, C. M., Saxne, T., Heinegard, D., and Blom, A. M. (2012). PRELP protein inhibits the formation of the complement membrane attack complex. *J. Biol. Chem.* 287, 8092–8100. doi:10.1074/jbc.M111.291476

Hayes, G., Pinto, J., Sparks, S. N., Wang, C., Suri, S., and Bulte, D. P. (2022). Vascular smooth muscle cell dysfunction in neurodegeneration. *Front. Neurosci.* 16, 1010164. doi:10.3389/fnins.2022.1010164

He, L., Vanlandewijck, M., Raschperger, E., Andaloussi Mae, M., Jung, B., Lebouvier, T., et al. (2016). Analysis of the brain mural cell transcriptome. *Sci. Rep.* 6, 35108. doi:10.1038/srep35108

Hong, L., Du, X., Li, W., Mao, Y., Sun, L., and Li, X. (2018). EndMT: A promising and controversial field. *Eur. J. Cell Biol.* 97, 493–500. doi:10.1016/j.ejcb.2018.07.005

Hopkins, J., Asada, K., Leung, A., Papadaki, V., Davaapil, H., Morrison, M., et al. (2022). PRELP regulates cell-cell adhesion and EMT and inhibits retinoblastoma progression. *Cancers (Basel)* 14, 4926. doi:10.3390/cancers14194926

Iozzo, R. V., and Schaefer, L. (2015). Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. *Matrix Biol.* 42, 11–55. doi:10.1016/j.matbio.2015.02.003

Karimy, J. K., Zhang, J., Kurland, D. B., Theriault, B. C., Duran, D., Stokum, J. A., et al. (2017). Inflammation-dependent cerebrospinal fluid hypersecretion by the choroid plexus epithelium in posthemorrhagic hydrocephalus. *Nat. Med.* 23, 997–1003. doi:10.1038/nm.4361

Kosuge, H., Nakakido, M., Nagatoishi, S., Fukuda, T., Bando, Y., Ohnuma, S. I., et al. (2021). Proteomic identification and validation of novel interactions of the putative tumor suppressor PRELP with membrane proteins including IGFI-R and p75NTR. *J. Biol. Chem.* 296, 100278. doi:10.1016/j.jbc.2021.100278

Kovacic, J. C., Dimmeler, S., Harvey, R. P., Finkel, T., Aikawa, E., Krenning, G., et al. (2019). Endothelial to mesenchymal transition in cardiovascular disease: JACC state-of-the-art Review. *J. Am. Coll. Cardiol.* 73, 190–209. doi:10.1016/j.jacc.2018.09.089

Kreft, M. E., Hudoklin, S., Jezernik, K., and Romih, R. (2010). Formation and maintenance of blood-urine barrier in urothelium. *Protoplasma* 246, 3–14. doi:10.1007/s00709-010-0112-1

Lamouille, S., Xu, J., and Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. *Nat. Rev. Mol. Cell Biol.* 15, 178–196. doi:10.1038/nrm3758

Li, F., Lan, Y., Wang, Y., Wang, J., Yang, G., Meng, F., et al. (2011). Endothelial Smad4 maintains cerebrovascular integrity by activating N-cadherin through cooperation with Notch. *Dev. Cell.* 20, 291–302. doi:10.1016/j.devcel.2011.01.011

Liddelow, S. A. (2015). Development of the choroid plexus and blood-CSF barrier. *Front. Neurosci.* 9, 32. doi:10.3389/fnins.2015.00032

Ljosa, V., Sokolnicki, K. L., and Carpenter, A. E. (2012). Annotated high-throughput microscopy image sets for validation. *Nat. Methods* 9, 637. doi:10.1038/nmeth.2083

Lopez, S. G., and Bonassar, L. J. (2022). The role of SLRPs and large aggregating proteoglycans in collagen fibrillogenesis, extracellular matrix assembly, and mechanical function of fibrocartilage. *Connect. Tissue Res.* 63, 269–286. doi:10.1080/03008207.2021.1903887

Lopez-Novoa, J. M., and Nieto, M. A. (2009). Inflammation and EMT: An alliance towards organ fibrosis and cancer progression. *EMBO Mol. Med.* 1, 303–314. doi:10.1002/emmm.200900043

Luehders, K., Sasai, N., Davaapil, H., Kurosawa-Yoshida, M., Hiura, H., Brah, T., et al. (2015). The small leucine-rich repeat secreted protein Asporin induces eyes in Xenopus embryos through the IGF signalling pathway. *Development* 142, 3351–3361. doi:10.1242/dev.124438

Man, S., Sanchez Duffhues, G., Ten Dijke, P., and Baker, D. (2019). The therapeutic potential of targeting the endothelial-to-mesenchymal transition. *Angiogenesis.* 22, 3–13. doi:10.1007/s10456-018-9639-0

Masuda, T., Sankowski, R., Staszewski, O., and Prinz, M. (2020). Microglia heterogeneity in the single-cell era. *Cell Rep.* 30, 1271–1281. doi:10.1016/j.celrep.2020.01.010

Menezes, M. J., McClenahan, F. K., Leiton, C. V., Aranmolate, A., Shan, X., and Colognato, H. (2014). The extracellular matrix protein laminin α2 regulates the maturation and function of the blood-brain barrier. *J. Neurosci.* 34, 15260–15280. doi:10.1523/JNEUROSCI.3678-13.2014

Morris, S. A., Almeida, A. D., Tanaka, H., Ohta, K., and Ohnuma, S. (2007). Tsukushi modulates Xnr2, FGF and BMP signaling: Regulation of Xenopus germ layer formation. *PLoS One* 2, e1004. doi:10.1371/journal.pone.0001004

Morrison, H. W., and Filosa, J. A. (2013). A quantitative spatiotemporal analysis of microglia morphology during ischemic stroke and reperfusion. *J. Neuroinflammation* 10, 4. doi:10.1186/1742-2094-10-4

Nieto, M. A., Huang, R. Y., Jackson, R. A., and Thiery, J. P. (2016). Emt: 2016. *Cell* 166, 21–45. doi:10.1016/j.cell.2016.06.028

Papadaki, V., Asada, K., Watson, J. K., Tamura, T., Leung, A., Hopkins, J., et al. (2020). Two secreted proteoglycans, activators of urothelial cell-cell adhesion, negatively contribute to bladder cancer initiation and progression. *Cancers (Basel).* 12, 3362. doi:10.3390/cancers12113362

Piera-Velazquez, S., and Jimenez, S. A. (2019). Endothelial to mesenchymal transition: Role in physiology and in the pathogenesis of human diseases. *Physiol. Rev.* 99, 1281–1324. doi:10.1152/physrev.00021.2018

Saito, A. (2013). EMT and EndMT: Regulated in similar ways? *J. Biochem.* 153, 493–495. doi:10.1093/jb/mvt032

Shozu, K., Kaneko, S., Shinkai, N., Dozen, A., Kosuge, H., Nakakido, M., et al. (2022). Repression of the PRELP gene is relieved by histone deacetylase inhibitors through acetylation of histone H2B lysine 5 in bladder cancer. *Clin. Epigenetics.* 14, 147. doi:10.1186/s13148-022-01370-z

Steiner, E., Enzmann, G. U., Lyck, R., Lin, S., Ruegg, M. A., Kroger, S., et al. (2014). The heparan sulfate proteoglycan agrin contributes to barrier properties of mouse brain endothelial cells by stabilizing adherens junctions. *Cell Tissue Res.* 358, 465–479. doi:10.1007/s00441-014-1969-7

Sweeney, M. D., Zhao, Z., Montagne, A., Nelson, A. R., and Zlokovic, B. V. (2019). Blood-brain barrier: From physiology to disease and back. *Physiol. Rev.* 99, 21–78. doi:10.1152/physrev.00050.2017

Tashima, T., Nagatoishi, S., Caaveiro, J. M. M., Nakakido, M., Sagara, H., and Kusano-Arai, O. (2018). Molecular basis for governing the morphology of type-I collagen fibrils by Osteomodulin. *Commun. Biol.* 1, 33. doi:10.1038/s42003-018-0038-2

Vanlandewijck, M., He, L., Mae, M. A., Andrae, J., Ando, K., Del Gaudio, F., et al. (2018). A molecular atlas of cell types and zonation in the brain vasculature. *Nature* 554, 475–480. doi:10.1038/nature25739

Weiss, N., Miller, F., Cazaubon, S., and Couraud, P. O. (2009). The blood-brain barrier in brain homeostasis and neurological diseases. *Biochim. Biophys. Acta.* 1788, 842–857. doi:10.1016/j.bbamem.2008.10.022

Yao, Y., Chen, Z. L., Norris, E. H., and Strickland, S. (2014). Astrocytic laminin regulates pericyte differentiation and maintains blood brain barrier integrity. *Nat. Commun.* 5, 3413. doi:10.1038/ncomms4413

Zeisel, A., Munoz-Manchado, A. B., Codeluppi, S., Lonnerberg, P., La Manno, G., Jureus, A., et al. (2015). Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. *Science* 347, 1138–1142. Epub 2015/02/24. doi:10.1126/science.aaa1934

Zeng, Q., Li, W., Lu, D., Wu, Z., Duan, H., Luo, Y., et al. (2012). CD146, an epithelial-mesenchymal transition inducer, is associated with triple-negative breast cancer. *Proc. Natl. Acad. Sci. U. S. A.* 109, 1127–1132. doi:10.1073/pnas.1111053108

frontiers | Frontiers in Cell and Developmental Biology

TYPE Review
PUBLISHED 11 March 2024
DOI 10.3389/fcell.2024.1258859

OPEN ACCESS

EDITED BY
Ramani Ramchandran,
Medical College of Wisconsin, United States

REVIEWED BY
Chiwei Xu,
The Rockefeller University, United States
Rafael Luís Luporini,
Federal University of São Carlos, Brazil

*CORRESPONDENCE
Rocío López-Posadas,
rocio.lopez-posadas@uk-erlangen.de
Elena Ortiz-Zapater,
elena.ortiz-zapater@uv.es

RECEIVED 14 July 2023
ACCEPTED 22 February 2024
PUBLISHED 11 March 2024

CITATION
López-Posadas R, Bagley DC, Pardo-Pastor C and Ortiz-Zapater E (2024), The epithelium takes the stage in asthma and inflammatory bowel diseases.
Front. Cell Dev. Biol. 12:1258859.
doi: 10.3389/fcell.2024.1258859

The epithelium takes the stage in asthma and inflammatory bowel diseases

Rocío López-Posadas[1,2]*, Dustin C. Bagley[3], Carlos Pardo-Pastor[3] and Elena Ortiz-Zapater[4,5]*

[1]Department of Medicine 1, University Hospital of Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany, [2]Deutsches Zentrum für Immuntherapie, Friedrich-Alexander-Universtiy Eralngen-Nürnberg, Erlangen, Germany, [3]Randall Centre for Cell and Molecular Biophysics, New Hunt's House, School of Basic and Medical Sciences, Faculty of Life Sciences and Medicine, King's College London, London, United Kingdom, [4]Department of Biochemistry and Molecular Biology, Universitat de Valencia, Valencia, Spain, [5]Instituto Investigación Hospital Clínico-INCLIVA, Valencia, Spain

The epithelium is a dynamic barrier and the damage to this epithelial layer governs a variety of complex mechanisms involving not only epithelial cells but all resident tissue constituents, including immune and stroma cells. Traditionally, diseases characterized by a damaged epithelium have been considered "immunological diseases," and research efforts aimed at preventing and treating these diseases have primarily focused on immuno-centric therapeutic strategies, that often fail to halt or reverse the natural progression of the disease. In this review, we intend to focus on specific mechanisms driven by the epithelium that ensure barrier function. We will bring asthma and Inflammatory Bowel Diseases into the spotlight, as we believe that these two diseases serve as pertinent examples of epithelium derived pathologies. Finally, we will argue how targeting the epithelium is emerging as a novel therapeutic strategy that holds promise for addressing these chronic diseases.

KEYWORDS

epithelium, barrier, mucus, asthma, IBD, therapeutics

1 Introduction

The epithelium has an essential role in development, physiology, and mucosal immunity. Its primary function is to act as a dynamic barrier, not only providing physical protection but also central to maintaining homeostasis and avoiding disease. Remarkably, despite experiencing high rates of cellular death and division, the epithelium maintains barrier function, underscoring the tissue's need for precise spatial and temporal regulation. Healthy epithelial monolayers effectively shield against toxins, viruses, pollutants, pathogens, and a long list of insults and attacks. Notably, when the integrity of the monolayer is compromised, a range of disorders follow, many of which remain classified as inflammatory disease, such as asthma and Inflammatory Bowel Disease (IBD) that we discuss herein.

Epithelial barrier damage triggers manifold and complex, inter-connected mechanisms involving not only epithelial cells, but also other resident cells within the mucosa, including immune and stroma cells. Traditionally, the immune cell population has been viewed as the "police" of the barrier, and many diseases known to have damaged epithelium and dysfunctional barriers have long been regarded by the scientific community as "immunological diseases". Subsequently, studies aimed at understanding, preventing, and treating these diseases have heavily relied on immune-centric therapeutic strategies

that even though, effective at symptom management, cannot stop, nor revert, the disease's natural progression. For example, targeting inflammation in asthma has been successful in managing major symptoms resulting in decreased exacerbation, hospitalization, and mortality (Rupani et al., 2021). However, it has been clearly demonstrated that these treatments do not impede the relentless progression of the disease, suggesting we are missing an underlying aetiology. Indeed, epithelial damage is seen in every type of asthma and is correlated with disease severity (Holgate, 2007; Lambrecht and Hammad, 2012; Calven et al., 2020; Porsbjerg et al., 2023). We can observe a similar situation in IBD, where past clinical practice has been restricted to symptom control using unspecific immunosuppressive drugs. But in the last years, the concept of mucosal healing has revolutionized the medical management of IBD patients, which goes beyond the symptom control towards the resolution of inflammation and ultimately complete healing (Rath et al., 2021; Neurath and Vieth, 2023). Thus, endoscopic and histological remission are nowadays considered as key therapeutic goals and prognostic parameters. More recent studies also argue for the importance of intestinal barrier healing in this context (Rath et al., 2023), highlighting again the role of epithelial function in the disease pathogenesis. In fact, several observations in the last 20–30 years support the causative role of epithelial alterations in IBD pathogenesis. For instance, there is a familial background in the increased intestinal permeability in IBD patients and their relatives (Munkholm et al., 1994; Soderholm et al., 1999; Irvine and Marshall, 2000), and the occurrence of epithelial leakage has been shown to be reliable for the prediction of IBD flares (Kiesslich et al., 2012), while does not correlate to inflammation severity (Benjamin et al., 2008). The lack of response to current therapy in chronic diseases, such as asthma or IBD, and the low safety profile of immunosuppressive drugs implies the need of alternative therapies. In fact, strategies targeting epithelial restoration emerge as attractive candidates and deserve further investigations.

In this review, we aim to discuss specific epithelial-driven mechanisms that ensure barrier function. These include 1) mechanisms related to the architecture and structure of the epithelium that regulate epithelial paracellular permeability, 2) the existence of a mucus layer that is able to eliminate particles and impact on the microbiota and 3) secretion of chemo/cytokines or antimicrobial substances (Ganesan et al., 2013; Mookherjee et al., 2020). We will discuss in detail the epithelium in the lungs and in the gut with the goal of understanding the different mechanisms named above and how those are dysregulated in respiratory and intestinal diseases, putting both asthma and IBD in the focus. We will also argue how targeting the epithelium is emerging as a new therapeutic strategy that could provide solution for these two chronic diseases and others.

2 Structure of the epithelial layer in the lung and in the gut

2.1 Cell types in the lung and gut epithelium

Epithelia are formed by a continuous layer of interconnected cells encapsulating organs and lining cavities. Epithelial cells are anchored to the *basal lamina* or basement membrane, a thin layer of extracellular matrix that provides structural support and signalling cues and sits on top of the underlying stromal tissue, which provides nutritional support and contains nerve terminals and immune cells that exchange signals with the epithelial sheet, capable of actively orchestrating and maintaining adaptive responses in health and disease (Lambrecht and Hammad, 2012).

For decades, researchers relied on microscopy-based morphological criteria to define different epithelial cell types that, combined with tissue architecture, determine the balance between different epithelial functions: protective, absorptive, and/or secretory. As an example, airway ciliated cells were first described in 1837, followed in 1852 by description of cells lacking cilia, loaded with granules, with a narrow stem connected to the basement membrane by a circular structure (goblet cells) and two cell types lacking access to the airway lumen: spherical cells, adjacent to the basement membrane (basal cells) and two layers of elongated cells (intermediate cells) (Figure 1). Remarkably, these early studies already were able to appreciate cell type similitudes between different tissues and proposed basal cells were precursors of the other airway epithelial cell types (for comprehensive historical perspective of airway cell type discoveries, see (Widdicombe, 2019).

Later, development of molecular markers and transgenics offered functional criteria to further define these cell types, genealogies, and functions, and how all these depend on tissue architecture. The gut is an example with clear spatial segregation of division, differentiation, tissue-specific functions, and death. Intestinal stem cells residing at the bottom of the crypts give rise to transient-amplifying cells (Duckworth, 2021). These are pluripotent cells that sequentially differentiate into absorptive (enterocytes) and secretory lineages. The latter gives rise to different cell subtypes achieving pleiotropic functions: i) antimicrobial peptide-producing paneth cells, not present in the colon; ii) mucus secreting goblet cells; iii) enteroendocrine cells releasing hormones, and chemosensory tuft cells (Fre et al., 2005). Epithelial cell differentiation is linked to migration upwards from the crypt to the villus or surface epithelium; except for paneth cells in the small intestine, which remain at the crypt bottom in close connection with stem cells (Garabedian et al., 1997). Cell migration and compartmentalization of crypts and villus is regulated by the activation and/or gradient between different pathways (Wnt, EGF, Notch or BMP), in most cases due to the contribution of pericryptal cells and the sub-epithelial microenvironment (Reynolds et al., 2014; Chen et al., 2019). Finally, differentiated cells at the villus tip will be extruded into the lumen where they finally die, to allow the renewal of the epithelial layer or epithelial turnover (Watson et al., 2009) (Figure 2).

In recent years, single cell and spatial transcriptomics have redefined and expanded cell types in virtually all tissues analysed, highlighting commonalities and tissue-specific features, echoing Waymouth Reid's conclusion that "it is extremely probable that several varieties of such [secreting] structures exist" and greatly contributing to the description of cellular complexity of the intestinal and respiratory epithelia. Single-cell RNA sequencing (sc-RNA-Seq) has confirmed the suggested variability in terms of cell composition and heterogeneity between organs and regions, e.g., small intestine vs. colon, crypt vs. villi, different airway regions, like trachea, airways or alveoli (McKinley et al., 2017; Beumer et al.,

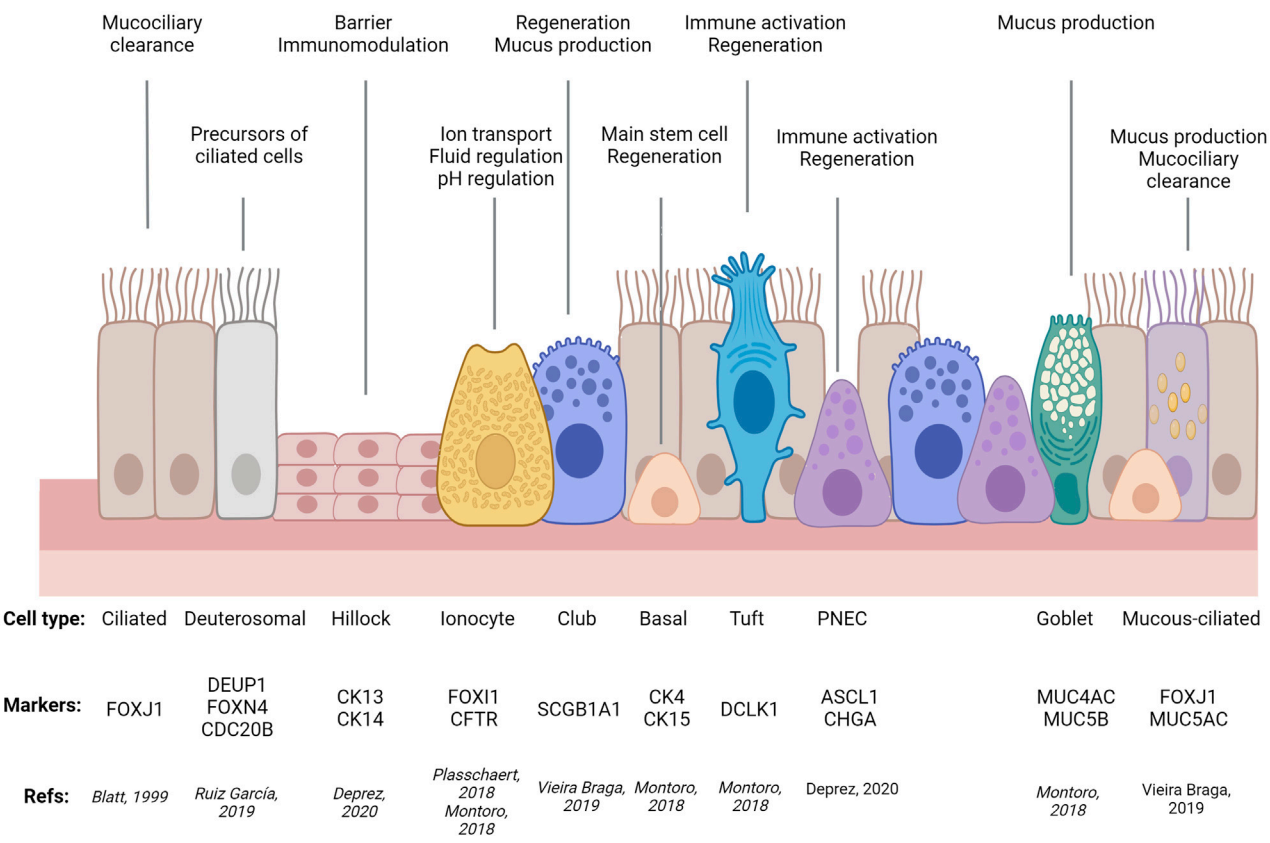


FIGURE 1
Simplified diagram of airway epithelial cell types, their molecular markers, and main functions. PNEC: Pulmonary Neuro-Endocrine Cell. Adapted from (Ortiz-Zapater et al., 2022a) using Biorender.com.

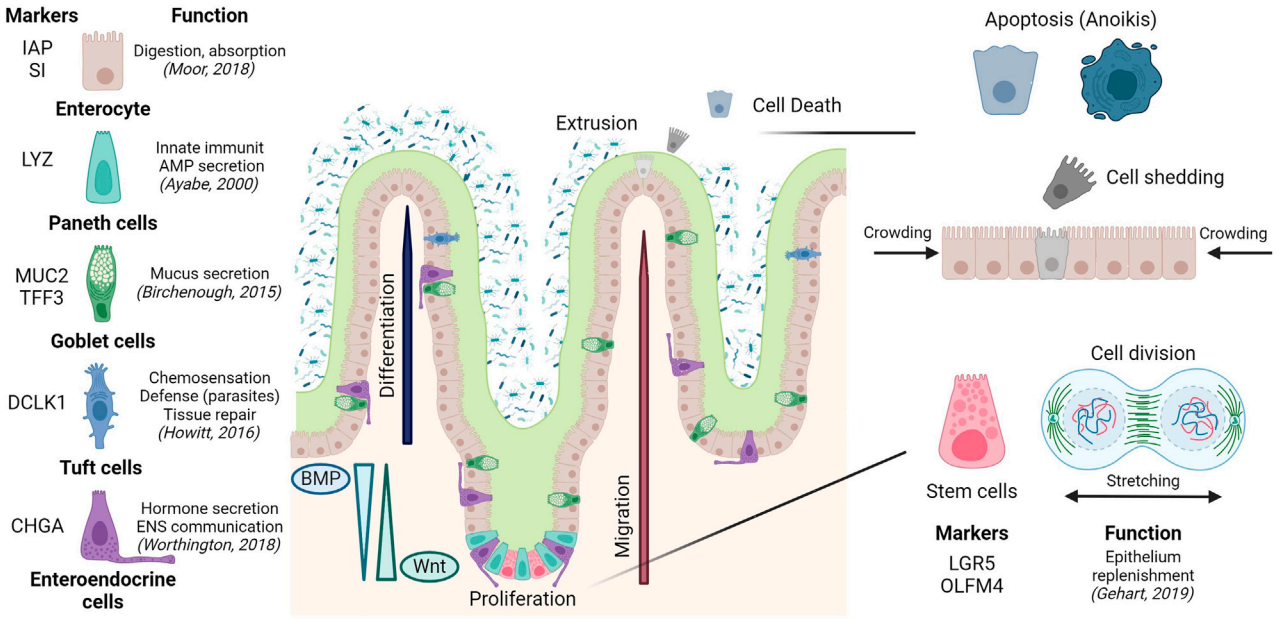


FIGURE 2
Epithelial composition and architecture in the small intestine, including epithelial turnover along the crypt-villus axis. Created with Biorender.com.

2018; Montoro et al., 2018; Moor et al., 2018; Plasschaert et al., 2018; Burclaff et al., 2022).

Additionally, sc-RNA-Seq has identified previously uncharacterised rare types of tissue-specific cells (e.g., lung ionocytes (Montoro et al., 2018; Plasschaert et al., 2018) and ones shared by different epithelia (e.g., tuft cells in airways, gastrointestinal tract, and other tissues (Elmentaite et al., 2021). Importantly, these techniques have demonstrated that the transcriptional profile between different cell subtypes, and thus our distinction between secretory and absorptive (gut) or ciliated types (airways), is not as clear as previously thought. Examples of this are colonic deep secretory cells contributing to the stem cell niche but with classical markers of differentiated goblet cells (Parikh et al., 2019) or mucous-ciliated and suprabasal cells in the airways (Elmentaite et al., 2021). Moreover, these techniques enable tracking of cells transitioning between states (trajectories), identifying new regulatory roles for Sox4, Foxm1, Mxd3, Batf2 in enterocytes (Haber et al., 2017) or Foxi1 in airway ionocytes (Montoro et al., 2018); as well as segregated populations within a given trajectory, such as tuft-2 cells displaying immunological functions (Haber et al., 2017). By enabling comparison between airway states (development, homeostasis, disease) these techniques have shed light on disease mechanisms like a general upregulation of secretory gene expression in all asthmatic airway epithelial types, in addition to a novel intermediate mucous-ciliated cell state expressing markers of both classic cell types that, with goblet cell hyperplasia, contributes to mucous hyperplasia in asthma. In the gut, the same approach has also identified defective mucus maturation in goblet cell as a potential driver of IBD and colorectal cancer, in addition to a new pH-sensing absorptive cell type, pericryptal stromal signalling, lymphocyte imbalance, and platelet aggregation as key contributors to barrier dysfunction in IBD (Regev et al., 2017; Beumer et al., 2018; Kinchen et al., 2018; Huang et al., 2019a; Parikh et al., 2019; Vieira Braga et al., 2019; Deprez et al., 2020; Jackson et al., 2020; Travaglini et al., 2020; Beumer and Clevers, 2021; Elmentaite et al., 2021; Haniffa et al., 2021; Tang et al., 2022).

All this demonstrates how recent advances in genomics, cell lineage tracing, and sc-RNA-Seq have revealed not only the need to redefine the meaning of cell identity, but also have uncovered new cell types involved in epithelial homeostasis and disease.

2.2 No cell is an island: how to build a monolayer from a single cell

Cell-cell junctions weave single epithelial cells into a functioning and dynamic monolayer that acts as a polarized barrier while selectively allowing transepithelial movement of water, ions, and macromolecules. Physiological transepithelial transport is classified as transcellular (mediated by transporters in apical and basolateral membranes) and paracellular transport (mainly determined by tight junctions). In the later, pore and leak pathways act in an interdependent manner (Weber et al., 2010). All these aspects have been nicely reviewed recently (Horowitz et al., 2023). Conversely, in damaged epithelia, transport becomes unrestricted and unselective, even allowing passage of bacteria from the lumen to the underlying tissue.

According to their location, composition, and function, epithelial intercellular junctions are classified as tight junctions (TJs), adherens junctions (AJs) or desmosomes, but all have common features like transmembrane components that physically link neighbour cells, in complex with cytoplasmic scaffolding and adaptor proteins linking the junctions to the cytoskeleton, which confers them mechanosensitivity (Garcia et al., 2018; Beutel et al., 2019; Pannekoek et al., 2019; Angulo-Urarte et al., 2020; Haas et al., 2022). TJs are formed by homotypic claudin and occludin contacts at the apex of lateral membranes between contacting cells. TJs form a regulable belt around a cell, separating the apical and basolateral membrane domains, while also sealing the paracellular pathway to control water and solute diffusion. The cytoplasmic side of TJs binds to adaptor proteins (ZO-1, -2, -3, cingulin) that interact with microtubules and the cytoskeleton. AJs are formed by the calcium-dependent extracellular trans binding of cadherins and force-dependent cytoplasmic binding to actin and microtubules via catenins. AJs are essential for cell-cell adhesion and epithelial mechanical responses, detailed later. Desmosomes are strong intercellular junctions based on cadherins desmoglein and desmocolin, bound to intermediate filaments via catenins plakoglobin and plakophilin. Moreover, junctions act also as signalling hubs, in close interconnection with Rho GTPases (Citi et al., 2014). Small GTPases are frequently found inactive, bound to GDP. After GDP-GTP replacement by Guanine Exchange Factors (GEFs), GTPases are recruited and interact with effector proteins, regulating essential cell functions controlling cell-cell adhesion and barrier function like mechanotransduction, vesicle trafficking, or junctional component dynamics (Braga, 2018). In summary, junctional integrity is essential for epithelial function, and its disruption is a key aspect of diseases like asthma and IBD.

In fact, mechanotransduction between epithelial cells determines tissue homeostasis at different levels. External forces (breathing, circulation flow, peristaltic movements), GTPases, and cytoskeletal contractility control long-term biological outcomes at cell (identity, proliferation, migration, extrusion) and tissue levels (folding, compartmentalization) in development, differentiation, homeostasis, and repair at the cell (identity, proliferation, migration and extrusion) and tissue levels (folding, compartmentalization) (Mahoney et al., 2014; Zhao et al., 2014; Goodwin and Nelson, 2021; Alvarez and Smutny, 2022; Perez-Gonzalez et al., 2022; He et al., 2023). Architecture of the gut epithelium represents a good example in this context; thus, myosin contractility initiates crypt invagination, the GTPase Rac1 controls crypt-villus compartmentalisation, and mechanical tension drives homeostatic intestinal cell migration from crypts to villus (Sumigray et al., 2018; Yui et al., 2018; Krndija et al., 2019; Perez-Gonzalez et al., 2021; Yang et al., 2021; Perez-Gonzalez et al., 2022).

As mentioned, mechanical forces also regulate cell identities, frequently via the transcriptional regulator YAP and its interplay with other signalling pathways, with remarkable tissue-specific features. In the gut, stiffening decreases stemness and promotes YAP-dependent gut stem cell differentiation into goblet cells (He et al., 2023); whereas in the lung, YAP is essential to maintain tissue organization and prevent stem cell loss and excessive goblet cell differentiation and mucin hypersecretion during homeostasis (Mahoney et al., 2014; Zhao et al., 2014; Hicks-Berthet et al., 2021). These differences could be partly explained by YAP being essential in all regenerative scenarios and lung homeostasis, but not

TABLE 1 Examples of mucus-related diseases and alterations.

Alteration in which mucus-related components?	Disease	References
GUT (Main mucin: MUC2)		
Alteration of the mucin O-glyocosylation profile	IBD and colorectal cancer	Etienne-Mesmin et al. (2019)
Increase of mucin degradating bacteria such as Ruminococcus family	Ulcerative colitis	Hansson, (2019)
loss of mucus viscoelastic properties and consequently a loss of protective function	Crohn's disease	Cornick et al. (2015)
LUNG (Main mucins: MUC5B and MUC5AC)		
Alterations in the CTFR channel in the Goblet cells, mucin hyperconcentration and corresponding impaired mucus clearance	Cystic fibrosis	Gustafsson et al. (2012), Henderson et al. (2014), Hill et al. (2018)
Trapped mucus in the epithelium	Asthma, COPD	Fahy et al. (1993)
Increased ratio of MUC5AC to MU5B	Pediatric asthma	Welsh et al. (2017)
Elevated sputum production with both MUC5AC and MUC5B	Non-CF bronchiectasis	Ramsey et al. (2020)
Reduced MUC5B	Pulmonary alveolar proteinosis	Takeyama K et al. (2015)

in gut homeostasis (Camargo et al., 2007; Barry et al., 2013; Zhao et al., 2014; Yui et al., 2018; Hicks-Berthet et al., 2021).

Mechanical forces also regulate cell numbers in shorter time scales. Cell stretching signals through E-cadherin and Piezo1 to increase nuclear levels of YAP and β-catenin and CDK1 activity, driving cell cycle re-entry (Streichan et al., 2014; Benham-Pyle et al., 2015; Gudipaty et al., 2017; Uroz et al., 2018). Conversely, crowding or compression arrests cell cycle and restores homeostatic cell numbers via cell extrusion, an evolutionarily conserved mechanism where a supracellular actomyosin cable formed around the unwanted cell ratchets in and down, resulting in seamless cell eviction without compromising barrier function (Rosenblatt et al., 2001; Eisenhoffer et al., 2012; McClatchey and Yap, 2012; Puliafito et al., 2012). In that sense, extrusion also works as an innate defence mechanism against external aggression, with healthy cells collectively squeezing cells infected by bacteria or viruses, thus limiting pathogen spreading in the monolayer and ensuring epithelial barrier function (Bastounis et al., 2021; Hippee et al., 2021; Lin et al., 2021; Moshiri et al., 2023).

3 The mucus and the secretome: let's keep it wet and clean!

Epithelial barrier function is not limited to a single sheet of interconnected epithelial cells; a layer of mucus coats the apical side of these cells and acts as a first barrier coating internal surfaces of organs. In turn, epithelial cells not only act to form a barrier. Instead, they communicate with other cell types, including immune or stromal cells, via secreted molecules, what can be defined as the "epithelium secretome". For a detailed description of the evolution of the cell secretome, we recommend (Sanchez-Guzman et al., 2021).

3.1 The mucus

Both the gut and the respiratory epithelium luminal surface are protected by mucus, a selective barrier to particles and molecules that is built around a family of polymeric glycoproteins called mucins. Mucus that coats the epithelium is a complex hydrogel biopolymer barrier, present not only in the airways and the gastrointestinal tract, but also in the reproductive tract and eyes (Lieleg and Ribbeck, 2011). During homeostasis, the protective mucus layer is produced by the goblet cells that are equipped with specific biological machinery for the secretion of mucins. Notably, some respiratory diseases are characterised by changes in goblet cells function (like asthma or COPD, see Table 1) and we will discuss later the importance of mucus production dysregulation in the pathology of asthma and IBD.

In the gut, mucus offers moisturising and lubricant properties, protecting the epithelial cells from dehydration and mechanical stress during the passage of luminal content and peristalsis forces (Johansson et al., 2013). It also operates as a surface cleaner, removing debris and bacteria, through binding, collecting, and flushing them away via intestinal flow. The small intestine has a single layer of mucus; while in the stomach and colon, the mucus layer is composed by an inner layer, attached to the epithelium, and an outer layer that interacts with luminal components. The inner layer is impermeable to bacteria and renewed by globlet cells every hour. The outer mucus layer is less dense and is the habitat for commensal bacterial (Hansson, 2019). Notably, in the small intestine, mucus leaves pores that allow the bacteria to penetrate, which is not the case in the large intestine, where the mucus layer is thick and completely avoids the contact with bacteria and the epithelial cells (Paone and Cani, 2020). In the gut, the main mucin is MUC2, which composes the skeleton of the mucus layer. In addition to MUC2, the IgG Fc-binding protein, FCGBP and the intestinal trefoil factor, TFF3 act synergistically to enhance the mucus barrier and exert antibacterial effects, while the metalloenzyme CLCA1 is involved mainly in the stratification and expansion of mucus. Moreover, ZG16, RELMβ, Lypd8, sIgA, and AMP exert bacteriostatic or bactericidal effects under different conditions (Song et al., 2023).

In the airway, mucus is composed of water, different proportions of polymerizing mucin glycoproteins MUC5B and MUC5AC in proximal *versus* distal regions (Meldrum and Chotirmall, 2021), a

range of antimicrobial molecules (defensins, lysozyme, etc.), cellular debris including DNA, and protective factors (trefoil factors) (Thornton et al., 2008). The protective response is driven by microbial sensors in the goblet cells that initiate secretion of mucus, to entrap invading microbes and remove bacteria away through mucociliary clearance (Abdullah et al., 2018). The ciliated cells, which line the surface epithelium of the airways, provide the force necessary for mucociliary clearance by the coordinated beating of their cilia, which confers an escalator motion to bring unwanted material to the mouth to be coughed out. These highly specialized cells are therefore critical to the health and function of the pulmonary system, and often preferential destroyed in favour of mucus producing cells in pathologies like asthma, with mucus hyper-production and -secretion remaining a massive obstacle in asthma treatment.

Many diseases arise from an imbalance between mucus production and elimination. The role of mucus and mucins in diseases of the intestinal and respiratory tracts is excellently reviewed by Hansson and others (Hansson, 2019) and we will describe later the specific importance of mucus regulation in asthma and IBD. We have included in Table 1 other diseases showing specific alteration linked to respiratory or intestinal diseases and the link to different respiratory and digestive diseases. See also (Meldrum and Chotirmall, 2021) for additional information.

It is now clear that the maturation and function of the mucus layer are strongly influenced by the microbiota (Schroeder, 2019). In fact, the consideration of the microbiota as a continuous element of homeostatic regulation of the epithelium has undoubtedly made physicians and researchers to confirm the relationship between the microbes and the epithelial barrier, and to adopt a more holistic view of the disease (Runge and Rosshart, 2021).

On of the main factors that influences the presence of a specific microbiota is the composition of the mucosal layer. Indeed, the mucin glycosylation profile influences the composition of mucus-associated bacteria, selecting specific species (Bergstrom and Xia, 2013). The composition of the mucus not only controls bacteria adhesion, but mucin glycans can also serve as nutrients for specific microorganisms, depending on their glycan-degrading enzyme's content, highlighting an example of how the host controls the microbiota within the mucus layer (Paone and Cani, 2020). Finally, bacteria can use host glycans to form new polymers used in the creation of their capsule, promoting evasion from the immune system (Martens et al., 2009).

Factors like age, diet, drugs, or disease affect microbiota composition too, even compromising its barrier function. For example, during pulmonary infection, microbial dysbiosis leads to invasion by opportunistic pathogens. These communities disrupt tissue compartments within the airway lumen, including mucus and causing progressive, localized, and chronic infection, particularly in pulmonary diseases (Montassier et al., 2023). Moreover, in asthma, exacerbations are classically induced by infections, like the ones produced by *P.* aeruginosa, which disrupts pulmonary mucins significantly contributing to disease progression (Meldrum and Chotirmall, 2021). Although the association between IBD and dysbiosis is accepted, whether alterations of the microbiota represent a cause or consequence of the disease is still a matter of discussion (Palm et al., 2014; Forbes et al., 2016; Schaubeck et al., 2016; Becker et al. 2015).

3.2 The secretome: secreted molecules in the gut and lung

Epithelial cells produce and secrete several molecules that contribute to epithelial integrity and elimination of microorganisms and contaminants, as well as intercellular communication. These molecules, collectively known as "the secretome" support epithelial homeostasis by controlling important cellular processes like proliferation, different mechanisms of cell death, safeguarding of epithelial tight junctions, maintenance of a healthy microbiota, and of course, communication with other cell types, like immune or stromal cells. We will cover some of the key players in the epithelium secretome in the gut and lung and we have summarised their main function in Figure 3.

3.2.1 Cationic host defense peptides (CHDP)

CHDP are one of the major components of the inmate immunity both in the lungs and in the gut. Also known as antimicrobial peptides, CHDP are amphipathic peptides that combat infections through their direct microbicidal properties and/or by influencing the host's immune responses. There are two main classes of CHDP in vertebrates, defensins and cathelicidins, produced as prepropeptides later cleaved to yield mature active peptides (Mookherjee et al., 2020). The last 25 years have seen an increasing interest in using CHDP as therapeutical targets, with potential clinical uses for asthma (Piyadasa et al., 2018) or colitis (Ho et al., 2013) treatment.

Defensins are key effector molecules in host defense against infection due to their broad-spectrum, and they contribute specially to the defense in the skin, lung, and gut. Defensins form producing destructive pores in the membrane of pathogens, and are also involved in inflammation, modulation of immune responses, wound repair, and disease (Weber, 2014). Epithelial cells are the main cellular sources, but they are also produced by neutrophils and other immune cells (Hiemstra, 2006). The main defensins produced by the epithelial cells in the respiratory tract and the gut are the β-defensins, with human β-defensin 2 mutations associated to asthma and atopy in children (Borchers et al., 2021) and their inhibition suppressing features of asthma in murine models (Pinkerton et al., 2021). Defensins produced by paneth cells in the small intestine contribute to tissue homeostasis by directly affecting the microbiota composition, but also by regulating the function of immune cells. In fact, reduced α- and increased β-defensins expression, as well as imbalance between the different mocules in therm of expression have been detected in the gut of IBD patients (Wehkamp et al., 2005; Elphick et al., 2008; Simms et al., 2008). In addition, a gene cluster polymorphism with low gene copy number of β-defensin-2 shows a predisposition for colonic CD (Fellermann et al., 2006).

Cathelicidins are also produced by epithelial cells of the respiratory and gastrointestinal tracts, but also by keratinocytes and neutrophils (Mookherjee et al., 2020). Cathelicidins have been studied in asthma in relation with viral-induced exacerbations, as its level could be used as a predictor marker (Arikoglu et al., 2017). In the gut, the cathelicidin LL37 has been shown to have a protective role and it has been postulated as a biomarker of pediatric IBD (Krawiec and Pac-Kozuchowska, 2021).

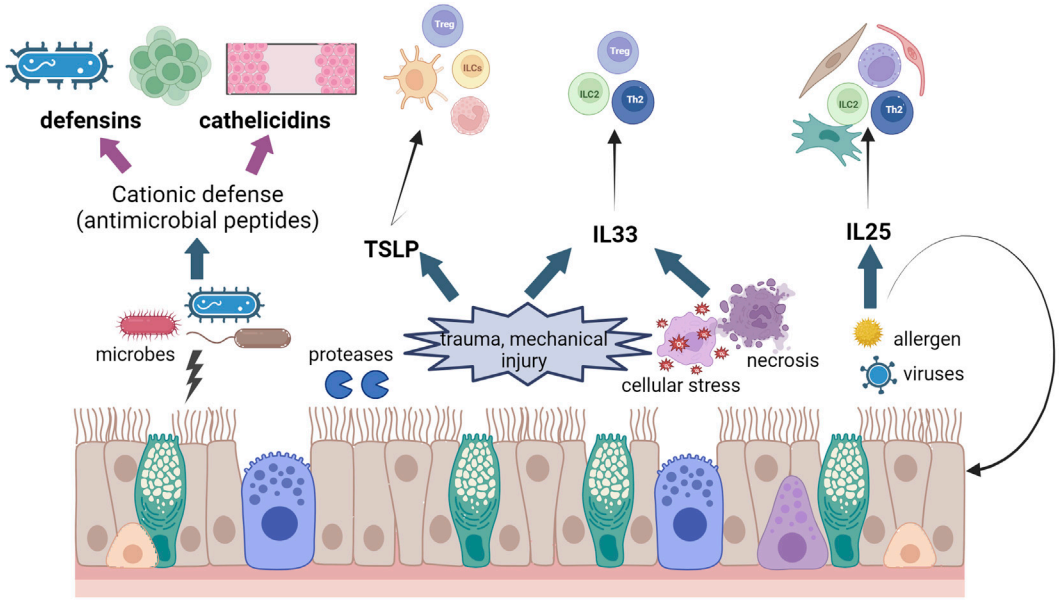


FIGURE 3
Schematic diagram of effects of epithelial damage in asthma and IBD. We have illustrated the release of CHDP and cytokines from the epithelium after different insults and the different cell populations that these molecules activate. Created using Biorender.com.

TABLE 2 Summary of characteristic of main epithelial cytokines.

Alarmin	Produced by	Produced because of...	Main targets	Related disease in the respiratory or gut epithelium	Receptor and signaling pathway
TSLP	Epithelial, stromal, dentritic cells, mast cells and basophils	Infection, inflammation, trauma, mechanical injury or proteases such as trypsin and papain (Allakhverdi et al., 2007)	Dendritic cells, Tregs, basophils and innate lymphoids cells (ILCs) (Roan et al., 2019)	Asthma, allergic rhinoconjunctivitis, nasal polyposis, COPD, esophagitis, gastrointestinal allergy, ulcerative colitis and Chron's Disease	Heterodimer receptor, TSLPR/IL-7Ra, recruitment of JAK1 and JAK2 and activation of STAT5 that is translocated to the nucleus
IL33	Epithelial, endothelial, smooth muscle cells, fibroblasts, platelets and mast cells	Cellular stress, injury or necrosis	ILC2s, memory Th2 cells and Tregs (Salimi et al., 2013; Halim et al., 2014; Vasanthakumar et al., 2015)	Asthma, COPD, gastrointestinal allergy, ulcerative colitis, Chron's Disease	Heterodimer receptor, formed by ST2 and IL-1RAP and activation of MYD88. This can activate both the NF-kB or the AP-1 pathway
IL25	Lung epithelial cells, endothelial cells, fibroblasts, alveolar macrophages, mast cells, basophils, eosinophils, chemosensory cells in the nasal mucosa	Allergen and viruses	T cells, ILC2s, Natural Killer Cells (NK), fibroblasts, epithelial, mesenchymal or endothelial cells (Stock et al., 2009; Saenz et al., 2010; Huang et al., 2015; Roan et al., 2019)	Asthma, atopic disease	Heterodimer receptor composed of IL17RA and IL17 RB. Binding recruits the adaptor proteins, such as ACT1 and TRAF6, and then activates NF-kB, MAPK-ERK and JNK.

3.2.2 Cytokines

TSLP (thymic stromal lymphopoietin), interleukin 33 (IL33) and interleukin 25 (IL25) are three typical epithelial cytokines that contribute to epithelial homeostasis and alert the immune system to external insults in order to regulate tissue restoration and repair (Ham et al., 2022; Mahapatro et al. 2021; Roan et al. 2019). These three "alarmin" cytokines are specifically potent in activating type 2 innate lymphoid cells (ILC2s) and therefore their roles have been widely studied in allergic inflammation and exacerbations, as well as parasite infections in the gut (Hammad and Lambrecht, 2015; Topczewska et al., 2023). Amplification or intensification of their secretion signals lead to different inflammatory diseases that we have tried to summarise in Table 2.

TLSP is a member of the IL2 cytokine family mainly produced by epithelial cells in the lungs, but also by other cells types like intestinal tuft-2 cells, an example of finding made possible by sc-

RNASeq techniques (see Table 2 and (Kashyap et al., 2011; Roan et al., 2019). Basal TSLP secretion is increased by several stimuli, although the existence of two isoforms of TSLP, long and short, may indicate status-dependent expression and secretion in homeostasis and disease. This has been studied in mice but its conservation in humans and the functional consequences of the variants remain unknown (Fornasa et al., 2015). Several publications have shown that a TSLP/ILC axis may play a pivotal role in steroid-resistant allergic airway inflammation (Kabata et al., 2013; Liu et al., 2018), very important in the treatment of asthma. IL33 is enriched in the barrier surfaces of the skin, lung, and intestine. Epithelial, and endothelial, cells express IL33 constitutively in the nucleus. Although some studies suggest that IL33 could have a role as a transcription factor (Ali et al., 2011), the nuclear localization is better explained as a mechanism to fine the release of this cytokine (Travers et al., 2018). IL33 can be present as a full-length protein, but proteolytic cleavage by other cell types or by molecules as caspases can produce both its activation or inactivation (Luthi et al., 2009; Lefrancais et al., 2014; Clancy et al., 2018). TSLP and IL33 have been suggested as protective molecules in IBD (Taylor et al., 2009). UC patients show reduced expression of TSLP (Tahaghoghi-Hajghorbani et al., 2019) and controversial data are available concerning IL33 in CD and UC (Seidelin et al., 2010; Tahaghoghi-Hajghorbani et al., 2019). Finally, IL25 can be secreted by specific subtypes of epithelial cells (Kohanski et al., 2018), but also other cell types, such as mastocytes and macrophages (Ikeda et al., 2003; Kang et al., 2005). In the gut, tuft cells are the main source of this cytokine (von Moltke et al., 2016). IL25 is secreted as a disulfide-linked homodimer. The activity of IL25 can be regulated by the matrix metalloproteinase, MMP7, which can cleave IL25 (Goswami et al., 2009); and also by splicing mechanisms. Although these three cytokines share target cells and have been implied in promoting type 2 inflammation, it could be interesting to understand what the interplay is among the three of them is, and whether pattern of expression of these epithelia cell-derived cytokines may distinguish distinct allergic endotypes or phenotypes.

There are other cytokines produced by the barrier epithelium that we cannot cover in this review. For example, cigarette smoke, another important insult for the barrier, and also other inhaled irritants promote expression and release of inflammatory mediators such as tumor necrosis factor (TNFα), IL1β, CXCL8 or the granulocyte-macrophage colony-stimulating factor, GM-CSF (Gao et al., 2015). The attenuation of GM-CSF signalling has been seen to decrease allergic inflammation in different mice models (Sheih et al., 2017). IL18 has been shown to be critical in driving the pathological breakdown of barrier integrity (Nowarski et al., 2015). On the other hand, IL-1α, produced by keratinocytes, can drive chronic skin inflammation (Archer et al., 2019).

3.2.3 TGFβ

Finally, another important molecule for the communication between epithelial cells and stromal cells in the context of the extracellular matrix (ECM) remodelling that can occur after dysfunction of the epithelial barrier is the transforming growth factor β (TGFβ). The role of TGFβ has been extensively studied in the epithelium, where it enhances epithelial barrier dysfunction, cell differentiation or epithelial to mesenchymal transition (Kahata et al., 2018). TGFβ is secreted in an inactive form bound to the latency-associated peptide (LAP) and its activation requires conformational changes leading to the protein cleavage of LAP (Bauche and Marie, 2017). In its canonical pathway, TGFβ, in a dimeric form, binds to a tetrameric complex composed of TGFβ receptor I and II. The activated receptor phosphorylates Smad2/3 transcription factors, triggering their translocation to the nucleus (Meng et al., 2016) to regulate the transcription of several genes like collagens (I and IV) or fibronectin, components of the ECM (Huang et al., 2020). We believe that it is important to highlight the role of TGFβ as the main character of fibrosis, understanding fibrosis as an excessive way of healing a wound after putting at risk the epithelial barrier. This has been for example, demonstrated in mice models of asthma where disruption of the barrier produces an increase in TGFβ production and consequent remodelling (Ortiz-Zapater et al., 2022a) or in the gut (Yun et al., 2019), just to name some. Moreover, TGFβ is one of the main communication molecules between the epithelium, immune cells and, especially in the context of ECM and remodelling, fibroblasts. In fact, TGFβ is the main molecule implicated in the differentiation/activation of myofibroblasts (see among many others (Ortiz-Zapater et al., 2022b), the main cell type producing ECM seen in many chronic pathological diseases including asthma and IBD. In that sense, there are numerous studies demonstrating the importance of TGFβ in the asthmatic inflammation and remodelling (Halwani et al., 2011; Al-Alawi et al., 2014) and more recently, it has postulated that the study of TGFβ polymorphisms, in combination with clinical factors, could predict asthma diagnosis with high sensitivity (Panek et al., 2022). In IBD, TGFβ has been studied due to its effect from and towards the epithelium, but also related to the immune response, and curiously, acting directly on the intestinal microbiota (Ihara et al., 2017).

4 Asthma

In this review, we have described so far molecules and mechanisms involved in epithelial homeostasis. In the next two sections, we will describe in detail both asthma and IBD as two example diseases where different epithelial driven alterations lead to epithelial barrier dysfunction, highlighting work done in this field and focusing on the epithelium as the potential therapeutic target, alone and in combination with established treatments.

4.1 Asthma, the attack, and inflammation

In the second century, Aretaeus of Cappadocia described asthma as *aazein*: a short-drawn breath or panting, a death rattle. Aretaeus went on to describe the defining characteristic of all asthmatics, the attack: "...they open the mouth since no house is sufficient for their respiration, they breathily standing, as if desiring to draw in all the air which they possibly can inhale ... ", and if the symptoms abate, he concludes, "the asthmatic escapes death, but in the intervals between severe attacks or even when they are walking on ground level, they bear in mind the symptoms of the disease (Karamanou and Androutsos, 2011)." The haunting trauma of an asthma attack is echoed in Henry Salter's account, "...not only is asthma not an uncommon disease, but it is one of the direst suffering; the horrors of

the asthmatic paroxysm far exceed any acute bodily pain". The language defining asthma has remained abstract and scantily more informative through the centuries. And it was not till 2017, when the Global Initiative for Asthma (GINA) defined asthma as, "a heterogenous disease usually characterised by chronic inflammation. It is defined by a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness, and cough that vary over time and in intensity, together with variable expiratory airflow limitations."

Today, asthma affects more than 300 million people globally, at a staggering financial cost and a burden to quality of life and remains one of the most common, non-communicable diseases (Papi et al., 2018; Pavord et al., 2018; Porsbjerg et al., 2023). Significant advances have been made in asthma care, as hospital admissions and deaths due to asthma are on the decline since the 1990s. The majority of asthma sufferers present with a type 2 inflammatory response and profile characterised by hyper-production of IL4, IL5, and IL13, increased blood eosinophils and fractional exhaled nitric oxide (FeNO) (Porsbjerg et al., 2023). However, current treatments only manage symptoms and have little-to-no effect on the natural progression of this disease. Even the diagnosis and term itself is umbrella, widely understood by clinicians that asthma could represent manifold pulmonary diseases. The use of inhaled corticosteroids became aggressively prescribed in the late 1980s, which resulted in fewer exacerbations and better control of patient symptoms and mortality. This biased physicians and researchers to approach asthma as a chronic inflammatory disorder, where disease symptoms are to be managed but not cured. This "inflammatory-centric" approach was implemented with wilful disregard that asthma attacks, or airway hyperresponsiveness, the sentinel event of all asthmatics, can occur in individuals without inflammation. Further, the degree of inflammation and the types of inflammation effecting asthmatics (eosinophilic, non-eosinophilic, high-type 2, low-type 2, etc.) is well documented to be highly variable (Hammad and Lambrecht, 2021; Porsbjerg et al., 2023). To this, commissions have been gathered to address the problem of asthma, focusing on the outdated thinking and antiquated research practices governing its treatment and prevention (Papi et al., 2018; Pavord et al., 2018; Porsbjerg et al., 2023). Recently, the Lancet compiled a commission to redefine asthma with the aim, ". . .to identify entrenched areas of asthma management and treatment in which progress has stalled and to challenge current principles . . . "We believe that the most important cause of this stagnation is a continued reliance on outdated and unhelpful disease labels, treatment and research frameworks, and monitoring strategies, which have reached the stage of unchallenged veneration and have subsequently stifled new thinking (Pavord et al., 2018)."

4.2 Epithelial dysregulation and damage in all asthma

It has long been speculated that epithelial loss or damage in asthma studies is due to artefacts from the harvesting and processing protocols while obtaining and analysing tissue samples (e.g., bronchial brushings and biopsies). However, an ever growing number of studies are revealing the loss, damage, and dysregulation of the epithelium in all asthmatics (Payne et al., 2003; Pohunek et al., 2005; van Rijt et al., 2011; Papi et al., 2018; Hammad and Lambrecht, 2021). Loss of the superficial epithelial layer, preferential destruction of ciliated cells, and over expression and activation of EGFR with increases in growth factors, including TGFβ (Hoshino et al., 1998; Shahana et al., 2005; Boxall et al., 2006; Holgate, 2007), are found in the majority of asthma suffers; even occurring in mild, early, and non-fatal asthma. Discussed earlier, damaged epithelium releases a number of soluble mediators promoting remodelling and inflammation (e.g., TSLP, IL25, and IL33), and are not only highly expressed in asthmatic airways, but represent genetic loci identified in a number of genome-wide association (GWA) studies correlating with asthma susceptibility (Cookson, 2004; Allakhverdi et al., 2007; Grotenboer et al., 2013; Moheimani et al., 2016). As an example, Steven Holgate's group demonstrated that asthmatic children have damaged epithelium with increased expression of EGFR, that was significantly correlated with basement membrane thickness (an important pathological feature of adult asthma), by excessive deposition of collagen III, seen in the absence of eosinophilic inflammation (Fedorov et al., 2005). Moreover, using bronchial biopsies from healthy and asthmatic cohorts, Barbato et al., showed loss of epithelium, increase in angiogenesis, and basement membrane thickening in asthmatic children prior to a mounted inflammatory state (Barbato et al., 2006). In an even earlier study, Marguet and co-workers found increased numbers of epithelial cells in the bronchoalveolar lavage fluid from asthmatic children compared to health controls (Marguet et al., 1999), further suggesting that epithelial loss and damage-not present at birth-is occurring before-or-at disease conception, and likely initiating and sustaining the adaptive response characteristic of most asthmatics.

4.3 Barrier dysfunction and asthma

As reviewed before, the epithelium can act as a barrier through the cooperative action of cell junctions with the cytoskeletal apparatus, essential for barrier function and downstream signalling. Dysregulation of the junctions themselves can orchestrate pro-inflammatory signalling pathways, fuelling an inflammatory cascade and feed-forward mechanisms initiated by the wounded barrier. A consequence of barrier damage, is the release of pro-inflammatory factors (e.g. alarmins), known to elicit a type-2 response resulting in increased IL4 and IL13 in airways that are now appreciated to also perpetuate junction dysfunction by downregulation of claudins, occludin, JAM proteins and ZO-1 (Ahdieh et al., 2001; Ortiz-Zapater et al., 2022a). House dust mite (HDM) extract, one of the major causes of asthma (and asthma exacerbations) in children, contains proteases that are known to cleave junctional proteins including occludin and ZO-1, directly participating in barrier dysfunction. Notably, Tan et al., demonstrated that three chronic HDM experimental asthma mouse models, with distinct inflammatory profiles (eosinophilic, neutrophilic, and mixed granulocytic), all had decreased expression of claudin-5, -8, -18, and -23, ZO-1, and occludin, further suggesting that a dysfunctional epithelium is activating and maintaining inflammatory pathologies rather than

inflammation as the initial source of epithelial wounding (Tan et al., 2019). This has been recapitulated in human bronchial epithelial cells in air-liquid interface (ALI) culture systems, and bronchial brushings from asthmatic patients. Downregulation of E-cadherin alone resulted in an EGFR-dependent, type 2-biased inflammatory response, and claudin-18 deficiency was demonstrated to promote barrier dysfunction in asthmatic mice and human epithelial cells (Heijink et al., 2007). An ultra-structural analysis of bronchial biopsies of both allergic and non-allergic asthmatics showed junctions and desmosomes damaged, as well as the destruction of ciliated cells in favour of goblet cell hyperplasia and impaired wound healing, with increased basement membrane thickening (Shahana et al., 2005).

The destruction of barrier proteins results in the activation of signalling pathways promoting asthmatic inflammation while directly inhibiting barrier function through the decreased expression of junctional proteins providing a viscous feed-forward cycle of wounding, repair, inflammation, and re-wounding. This highlights the need for therapeutics that are targeted to maintain barrier proteins and function in chronic disease, such as asthma and IBD.

4.4 Mechanics effecting epithelium and asthma

There is an established notion that chronic inflammation results in airway hyper-responsiveness, and numerous studies have demonstrated that high doses of oral and inhaled corticosteroids are unable to stop, nor reverse, asthma exacerbations (Childhood Asthma Management Program Research Group et al., 2000; Kips et al., 2000; Guilbert et al., 2006; Porsbjerg et al., 2023). Ultimately, bronchoconstriction is the result of airway remodelling and as we have discussed above, when the epithelium is damaged and junctional proteins disrupted, downstream signalling occurs to respond to assaults; this is true of mechanical forces applied to monolayers. Unique to the lung (and the heart) is that at birth its movements, required for respiration, will not cease until death, causing the lung to be under constant, and constantly changing mechanical forces. Indeed, these forces are required for healthy lung development *in utero* and after birth, and regulated repair responses (Liu et al., 2016; Li et al., 2018). As earlier discussed, mechanical forces govern epithelial numbers within a monolayer. When crowded regions experience compression, unwanted cells are removed by extrusion to regain homeostatic densities, relieving mechanical stresses (Bagley et al., 2023; Eisenhoffer et al., 2012). Airway epithelium during bronchoconstriction will experience dramatic compressive forces, likely causing excessive cell extrusion, damaging the epithelium, while losing barrier function, and promoting further inflammation (Bagley et al., 2023). Importantly, the mechanically-activated protein YAP1 is well-characterized in airway homeostasis and disease, required for proper airway branching (Lin et al., 2017), maintenance, size regulation, and identity of epithelial cells. Mechanical forces are deeply integrated and unavoidably required for all biological aspects needed for lung development, homeostasis, and pathology, and these mechanically-activated epithelial pathways represent a novel, druggable target in wound repair and disease.

The compressive forces applied to the epithelium during an asthma attack is estimated to be about 30 cm H_2O, at least an order of magnitude greater than the forces felt during normal respiration (Park et al., 2015). Stealing a line from Chris Grainge's review on airway mechanical compression, "Bronchoconstriction is not only a symptom of asthma but is also a disease modifier" (Veerati et al., 2020). It has now been demonstrated that compressive forces, *in vitro* and *vivo*, lead to expression of genes known to elicit pathological responses in lung disease, including early growth response-1 (EGFR-1), platelet-derived growth factor (PDGF), and TGFβ. Stimulation of repair response pathways through EGFR activation and down-stream signalling, leads to the release of growth factors (e.g., TGFβ) and ECM components (collagens) involved in airway remodelling and disease progression (Ressler et al., 2000). Incubating fibroblasts with conditioned medium from compressed airway epithelial cells resulted in increased collagen deposition, all in the absence of an inflammatory component (Tschumperlin et al., 2003). Park et al. nicely demonstrated that repeated compressive forces alone, over a relatively short time period, could elicit mucus production, e.g., Muc5AC, in normal human bronchiole epithelial cells that was dependent upon EGFR and TGFβ2 (Park and Tschumperlin, 2009). This work was confirmed in humans: volunteers underwent methacholine challenges (only three times over 4 days) to induce bronchoconstriction that lead to increases in TGFβ, collagen, and mucus production in airway epithelial cells, also in the absence of an inflammatory response (Grainge et al., 2011). This is important as mucus hyper-production and secretion remains an intractable problem in many pulmonary disorders, including asthma. Indeed, in a study of 93 fatal asthma cases, near all had mucus obstructions in their airways, where half had more than 80% of airways occluded with mucus plugs (Aegerter and Lambrecht, 2023). Now we appreciate that this mucus problem is not simply a result of goblet cell hyperplasia but also, the expression of mucus in *bona fide* ciliated cells, which is important if we are to develop effective and targeted therapeutics currently missing in today's clinics. Finally, wounding epithelium itself can induce airway smooth muscle constriction, actively participating in the airway compression-remodelling response. Elegant work by Steven George's group used *ex vivo* lung slices from rats and laser ablation to destroy signal airway epithelial cells that resulted in a 70% reduction in airway lumens within seconds of cell wounding, followed by further airway smooth muscle contractions over minutes, again in the absence of an inflammatory response by inflammation (Zhou et al., 2012).

4.5 The epithelium as a druggable target in asthma

Currently, asthma therapy is big business with annual revenues in the billions, which is on the rise, as all these medications can do is manage symptoms of this common disease, not capable of stopping or reversing its progression. This sentiment is not new, and indeed clinical trials targeting the epithelial-derived alarmins, released by wounded epithelial barriers, have shown promising results. The monoclonal antibody inhibiting TSLP, Tezepelumab, has been demonstrated to significantly supress all three type-2 clinical

biomarkers for asthmatics: peripheral blood eosinophils and total IgE (Schleich et al., 2024); while the anti-IL25 and IL33 drugs, Brodalumab and Itepekimab, respectively, were less successful (Chan et al., 2022). Promising work by Wawrzyniak and others in primary human cells were able to reconstitute barrier function, damaged by IL4 and IL13 exposure, from asthmatic patients by inhibiting histone deacetylases (upregulated in asthma), resulting in junctional protein synthesis (Wawrzyniak et al., 2017). Lastly, as we have discussed, mucus is a problem in many pulmonary diseases, and asthma is no exception, with little treatment options available. However, using a mouse model of IL13-induced mucus hyperplasia and primary cells from asthmatics, inhibiting the heat shock protein 90 (HSP90), upregulated in asthma with geldanamycin, blocked, and even reverted, mucus hyperproduction and goblet cell hyperplasia (Pezzulo et al., 2019). In fact, there are ongoing clinical trials for HSP90 inhibitors for various disease (Kitson and Moody, 2013).

To expand upon current asthma treatments and experimental approaches, we need pathophysiologically relevant platforms that allow for efficient and effective drug discovery and development. In the 1990s, Martin Sanders began iconoclastic work in the use of precision cut lung slices (PCLSs) to study lung physiology and pathology (Martin et al., 1996) that has snowballed over the last three decades, as more researchers are being introduced to the power of this *ex vivo* system in basic cell biology and translational studies (Davies et al., 2015; Alsafadi et al., 2017; Huang et al., 2019b; Lam et al., 2023). PCLSs are thin sections of live tissue containing all resident cell-types, while maintaining proper tissue architecture, preserving cell-to-matrix relationships, within complex, interconnected cellular hierarchies, which make up all tissues and organs. *Ex vivo* lung slices have been successfully used in studies from, mice, rats, pigs, sheep, non-human primates, and humans (Alsafadi et al., 2020). They have been used to study airway and arteriole contraction (Martin et al., 1996), tumour biology within intact tissue (Davies et al., 2015), viral infection (Rosales Gerpe et al., 2018), HDM-induced asthma (Ortiz-Zapater et al., 2022a), and fibrosis (Alsafadi et al., 2017), with seemingly endless potential in novel therapeutic development (Liu et al., 2021; Lam et al., 2023). Importantly, the use of *ex vivo* tissue slices reduces the ethical burden for *in vivo* models, because dozens of slices can be obtained from a single lung, decreasing the number of animals needed, and allowing for multiple treatments assessed in a lone animal. And PCLSs are amenable to many live and fixed imaging techniques (including watching an asthma attack in real time), as well as genetic, biochemical, and molecular biology analyses. An important limitation to PCLSs is that viability decreases in culture conditions over time (usually 7–14 days). Therefore, *ex vivo* modelling of chronic diseases or assessing treatments to reverse established pathologies can be limited, requiring the development of better culturing conditions to overcome this problem. Regardless, the power of PCLSs to bridge disease characterization in animal models, and humans, with translational research and positive clinical outcomes is undeniable.

5 Inflammatory bowel disease (IBD)

Medical reports from the 17th and 18th century described cases of patients dying after prolonged episodes of diarrhoea, abdominal pain and fever. Later, the first cases of Crohn's Disease (CD) and Ulcerative Colitis (UC) were described in Great Britain, in 1859 and 1875, respectively. The pathology of UC was firstly described as affecting the mucosa and submucosal of the rectum and extending to the whole colon, featuring a marked infiltration of inflammatory cells, vascular congestion, goblet cell depletion and crypt abscesses (Kirsner and Palmer, 1951). In the case of CD, Warren mentioned, "A progressive sclerosing granulomatous lymphangitis, probably a reaction to an irritative lipid substance in the bowel content." (Warren and Sommers, 1948), and etiologically associated with microorganisms, abdominal trauma, or impaired vascular/lymphatic circulation. Currently, IBD is used as an over-reaching term to name chronic and relapsing inflammation of the gastrointestinal tract; being CD and UC the most common clinical manifestations. The first epidemiologic approach to study IBD in 1955 initially suggested the impact of the life-style (Melrose, 1955). Ulterior population studies pointed to key epidemiological features of IBD, such as the ethnicity contribution, environment, as well as the familial background. Increasing incidence during the 20th century has been largely seen and presently it is well accepted that IBD has a worldwide distribution, with 6.8 million people being affected in 2017 (GBD, 2017 Inflammatory Bowel Disease Collaborators, 2020).

Most IBD research between the 19th and the 20th centuries was aimed at a differential diagnostic and the development of a therapy in order of improve the life quality of these patients, until the introduction of biological drugs, mainly anti-TNF antibodies. While in the 21st century, researchers focused on the identification of causative pathological mechanisms, which has spotlighted different players leading to the complex breakdown of gut mucosa homeostasis. Currently, IBD is considered a multifactorial disease, which occurs because of an interplay between genetics, environmental and immunological factors, resulting in an uncontrolled immune response against the intestinal microbiome. The complex nature of the disease pathogenesis implies a clear limitation for the development of curative pharmacological treatments, but also highlight the importance of considering alternative approaches. To this, clinicians are beginning to exploit epithelial features for the diagnosis or treatment of IBD patients that have shown promising results, supporting further investigations to understand the causative role of epithelial dysregulation in IBD.

5.1 Barrier dysfunction in IBD

In order to maintain tissue homeostasis, the intestinal epithelium acts as a physical and immunological barrier separating the lumen, which contains the microbiota, and the host. "Epithelial leakage", a common feature in IBD, allows for the invasion of luminal components, which can activate immune cells located at the sub-epithelial space contributing to intestinal inflammation (Martini et al., 2017). Abnormalities in epithelial barrier function can be reflected by an increased permeability, which has been observed in small bowel and colon in CD patients (Jenkins et al., 1988), and has been correlated to the degree of inflammation (Jenkins et al., 1987; Sanderson et al., 1987; Suenaert et al., 2002; Turpin et al., 2020). Moreover, it has been shown that increased intestinal permeability in IBD patients in

remission can predict the occurrence of relapse or flares (Wyatt et al., 1993; Irvine and Marshall, 2000; Tibble et al., 2000; Vivinus-Nebot et al., 2014). Together, the occurrence of epithelial barrier dysfunction before the outbreak of the inflammatory response supports the hypothesis of epithelial defects as etiological factors in IBD pathogenesis. GWAS studies identified genes linked to altered barrier function to be associated to IBD; including genetic variants of CARD15/NOD2 gene, resulting in severe forms of CD (D'Inca et al., 2006; Buhner et al., 2006). In fact, several genes relevant to epithelial barrier function have been categorized as IBD loci, such as HFN4, CDH1 and LAMB1 in UC (Consortium et al., 2009). In agreement, several animal models demonstrate that epithelia permeability precedes the development of intestinal inflammation, for example, the IL10 KO (Madsen et al., 1999), and the SAMP/YitFc mouse (Olson et al., 2006), as well as the mouse strain deficient for the xenobiotic transporter mdr1a (Resta-Lenert et al., 2005). The etiological role of epithelial leakage in inflammation is further supported by IBD-like phenotypes in patients suffering from monogenic diseases. For instance, the very-early onset IBD called Tufting enteropathy is caused by mutations in EpCAM, leading to cell-cell contact disruption (Sivagnanam et al., 2008). Although these and other data support the epithelial contribution to the onset and progression of IBD, the low IBD-like phenotype penetrance of these monogenic diseases indicates the existence of functional redundancy between different proteins/pathways within the enterocyte, and/or the requirement for non-epithelial factors for the onset of intestinal inflammation. This idea was indeed confirmed by other mouse models targeting TJ proteins, showing that a leaky barrier is not sufficient to trigger intestinal inflammation, such as JAM-a deficient animals (Khounlotham et al., 2012), or transgenics mice with expression of claudin-2 in IECs (Ahmad et al., 2014).

5.2 Epithelial alteration in IBD

As mentioned above, increased epithelial permeability is a hallmark of patients suffering from IBD (Teshima et al., 2012). In 2007, Zeissig *et al.* described upregulation of the pore-formin Claudin-2 and downregulation and/or redistribution of claudin-5, -8 and occludin as the main alterations affecting the apical junctional complex (AJC), and thereby contributing to impaired barrier function in CD (Zeissig et al., 2007). In UC, claudin-2 is also upregulated, while the barrier forming claudin-4 and -7 are downregulated (Oshima et al., 2008), as well as occludin (Heller et al., 2005). In IBD or immune-driven colitis the upregulation of claudin-2 can be attributed, at least partially, to the increased levels of several proinflammatory cyotkines, such as IL13 (Heller et al., 2005). Conversely, Myosin Light Chain Kinase (MLCK) activation causing phosphorylation of MLC and occludin endocytosis contribute to permeability mediated by the leak pathway (Clayburgh et al., 2005; Marchiando et al., 2010; Van Itallie et al., 2010). Previously mentioned, occludin is downregulated in IBD patients (Heller et al., 2005; Kuo et al., 2019), which can be triggered by cytokines such as TNF (Su et al., 2013) or LIGHT (Schwarz et al., 2007). Additionally, the tricellular TJ proteins tricellulin (Krug et al., 2009; Saito et al., 2021) and angulin-1 (Sugawara et al., 2021) also contribute to the leak pathway permeability. Recent studies also pointed to a downregulation of tricellulin expression in UC patients (Krug et al., 2018). Indeed, *in vitro* studies have shown that the pro-inflammatory milieu in the inflamed gut of IBD patients can lead to alterations on several proteins within the AJC, as upon stimulation with IL1β (Al-Sadi et al., 2008), IL6 (Suzuki et al., 2011), IL4 and IL13 (Ceponis et al., 2000), TNF-α (Ma et al., 2004) or IFN-γ (Madara and Stafford, 1989; Wang et al., 2005).

Regulated cytoskeleton function is crucial for TJ assembly and epithelial barrier function, In fact, transcriptional regulation of ACF7, a cytoskeleton crosslinking protein, is observed in UC patients (Ma et al., 2017). Accordingly, mice with an epithelial-specific knockout of non-muscle MyosinIIA suffer from increased intestinal permeability, low scale mucosal inflammation, and increased susceptibility to experimental colitis (Naydenov et al., 2016). Cell stress can also induce changes in actin dynamics and affect actin-binding proteins, such as Villin-1 and Gelsolin, which in turn control survival of Intestinal Epithelial Cells (IECs) and barrier function (Roy et al., 2018). Recent *in vivo* studies demonstrated that prenylation of Rac1 and RhoA, tightly associated to the cytoskeleton, significantly contribute to epithelial barrier function in the gut, and this correlated with alterations of its expression and/or subcellular localization in the intestinal epithelium of IBD patients (Lopez-Posadas et al., 2016; Martinez-Sanchez et al., 2022).

Beyond structural defects, IBD is associated with changes in the epithelial secretome. IBD has been associated to defects of goblet cell differentiation, supporting the key role of the mucus in the intestine (Gersemann et al., 2009). Indeed, the composition of gut mucus is altered in IBD, which is depicted by reduced TFF3, expression diminished levels mucin2 and reduced mucus sulfatation (Tytgat et al., 1996). Focusing on alarmins, TSLP and IL33 have been suggested as protective molecules in IBD (Taylor et al., 2009). Although UC patients show reduced expression of TSLP (Tahaghoghi-Hajghorbani et al., 2019); controversial data are available concerning IL33 in CD and UC (Seidelin et al., 2010; Tahaghoghi-Hajghorbani et al., 2019).

5.3 Leaky epithelium as a diagnostic tool

The increasing acceptance of the causative role of epithelial-derived mechanisms in IBD pathogenesis is also reflected by the current effort to exploit this in the clinic, both for treatment and diagnosis of chronic inflammatory diseases. Traditional sugar permeability assays (Meddings and Gibbons, 1998; Teshima and Meddings, 2008) are giving way to molecular imaging, such as confocal-laser endomicroscopy (CLE) using a tracer dye to assess intestinal permeability. This technique permits the identification of epithelial gaps (Kiesslich et al., 2007), even correlating to the occurrence of relapses (Kiesslich et al., 2012) and the identification of subclinical lesions in IBD (Lim et al., 2014; Zaidi et al., 2016). Using this CLE, a recent cross-sectional diagnostic study demonstrated the superiority of barrier healing (*versus* endoscopic/histologic remission) for the prediction of adverse outcomes in CD and UC, validating epithelial leakage as a prognostic marker of the disease (Rath et al., 2023). In order to overcome safety limitations of CLE, a multimodal imaging label-free imaging technique has been used to assess intestinal permeability in UC patients (Quansah et al., 2023). Despite these advanced imaging

techniques, there is a clear need for the identification and validation of non-invasive methods for the diagnosis of "leaky gut". Although several biological markers have been suggested in this context [plasma/serum citrulline, FABP-2, alpha-GST or zonulin; urine claudin-3; or faecal defensins (Bischoff et al., 2014)], none of them has been efficacious in disease prognosis or progression. We believe that the use of CLE (or alternative imaging methods) alone, or in combination with other standard methods (endoscopy/histology), and the identification of biomarkers for impaired intestinal permeability, will allow us to define the functional state of epithelial integrity and contribute to the prediction of IBD flares.

5.4 Epithelium as a druggable target in IBD

Currently, the clinical management of IBD strives to control symptoms and mucosal healing (Neurath and Travis, 2012). However, the lack of response to therapy and the low safety profile of immunosuppressive drugs implies the need of alternative therapies, and epithelial restoration emerges as a key component to achieve mucosal healing in IBD, with the final objective of achieving sustained clinical remission, reduced rate of surgery and lower incidence of long term complications. Thus, new knowledge of epithelial dysfunction would likely impact IBD clinical management.

Therapeutic strategies based on promoting the integrity of TJs might have a beneficial effect in IBD. For instance, the zonulin inhibitor AT-1001 (lazarotide) impairs TJ disassembly due to cytoskeleton rearrangement and ameliorates experimental colitis in mice (Arrieta et al., 2009; Sturgeon et al., 2017). Inhibition of the pore function from TJ can limit increased paracellular permeability, which can be achieved for example, by inhibiting casein kinase 2 (Raleigh et al., 2011), indeed providing a certain protection against experimental colitis (Raju et al., 2020). On the other hand, the group of JR. Turner has extensively characterized the MLCK-dependent signaling transduction regulating the leak pathway, culminating in the identification of the small molecule divertin (Graham et al., 2019). Divertin blocks the MLCK1 recruitment via the IgG3 domain to the perijunctional actomyosin ring inhibiting occludin endocytosis and promoting barrier function without altering MLCK enzymatic activity (He et al., 2008; Graham et al., 2019). Strikingly, divertin showed a similar therapeutic effect as anti-TNF in immune-mediated mouse experimental intestinal inflammation (Graham et al., 2019).

The accumulated evidence about the causative role of barrier function in IBD implies the need of assessing the impact of current treatments on the intestinal epithelium, and the potential link to success/lack of response in specific patients. One case in this context is the barrier repair observed upon anti-TNF treatment in CD patients (D'Haens et al., 1999; Rutgeerts et al., 2012; Kierkus et al., 2012), which has been mechanistically linked to the inhibition of IEC apoptosis and Notch pathway modulation (Kawamoto et al., 2019). Additionally, mesalamine treatment improved mucosal healing in clinical trials including mild-to-moderate UC patients (Lichtenstein et al., 2011; Bokemeyer et al., 2012; Probert et al., 2014). In this case, epithelial wound healing can also be promoted by increasing epithelial cell migration and proliferation (Baumgart et al., 2005) and impaired cytokine-driven paracellular permeability (Khare et al., 2019). The beneficial effects of corticosteroids was initially thought to be through the regulation of inflammatory factors (Wild et al., 2003). However, it was recently shown that the exposure of intestinal organoids derived from CD patients to prednisolone rescued the modulated expression/distribution of E-cadherin, ILDR-1, Claudin-2, MLCK and phospho-STAT1 upon cytokine treatment (Xu et al., 2021). Elegant work by Zuo *et al.* described the capacity of tacrolimus to interact with FKBP8, which in turn impairs their interaction with MLCK1 for its recruitment to the acto-myosin ring for the induction of epithelial barrier function (Zuo et al., 2023). Together, this shows the potential contribution of classical immunosuppressive drugs and biologicals to epithelial restoration in the context of IBD.

Many experts have demonstrated that there is a way to confer benefit to the host by administration of probiotics. Probiotics are live organisms that can shape the commensal microbiota and the composition of the mucus. Thus, bacteria such as *Bacillus subtilis* (Li et al., 2020b; Ahl et al., 2016), or *Lactobacillus spp* (Bron et al., 2017) or the *Lactobacillus reuteri* alter mucin production. In fact, *Lactobacillus* showed a protective effect, increasing the mucus layer thickness (Ahl et al., 2016). There are also many different studies reporting the beneficial effects of the supplementation with *A. muciniphila* (Wu et al., 2017; van der Lugt et al., 2019). Moreover, bacteria-derived metabolites altering the mucus composition, such as indoleacrylic acid, have shown protective effects in experimental colitis (Wlodarska et al., 2017). Thus, probiotics and their impact on the mucus layer emerge as interesting mechanisms to impact on intestinal epithelial integrity.

Despite attractive strategies, there is still no pharmacological treatment for epithelial restoration in IBD. This is partially because the limitations for primary intestinal epithelial cultures until the development of intestinal organoids, which impeded the segregation of epithelial intrinsic mechanisms. Organoids are multicellular culture systems embedding in an ECM-like matrix mimicking the 3D architecture of the intestinal epithelium, which are valuable surrogates for intestinal tissue. Importantly, the cellular complexity and plasticity of the intestinal epithelium can also be mimicked in intestinal organoids (Basak et al., 2017; Treveil et al., 2020; Martinez-Silgado et al., 2023), and they can be used for genetic manipulation, biobanking (van de Wetering et al., 2015), and translational studies, since they conserve genetic and epigenetics of the original tissue if derived from ASCs (Dotti et al., 2017). The use of organoids has made possible the validation of molecular signatures linked epithelial alterations in disease (Bigorgne et al., 2014), as well as the identification of new targets in epithelial cell biology with a potential direct application in IBD (Bayrer et al., 2018; Glal et al., 2018; Deuring et al., 2019; Li et al., 2020a). Functionally, permeability assays can be applied to intestinal organoid cultures (Bardenbacher et al., 2020; Rallabandi et al., 2020). For example, the restoration of permeability and remission upon low dose naltrexone organoids studies showed the restoration (Lie et al., 2018), or the cytokine-mediated induction of impaired barrier function in human-derived material (Gleeson et al., 2020). Moreover, the use of organoids has made it possible to study epithelial crosstalk with other players within the intestinal tissue, such as the microbiota (Leber et al., 2018; Roodsant et al., 2020) and immune cells. Thus, co-cultures of mononuclear phagocytes and organoids demonstrated that this intercellular communication is involved in epithelial cell differentiation and can be targetable in IBD (Ihara

et al., 2018). Moreover, organoid culture has opened the path to the development of stem cell transplantation therapy to treat refractory ulcers in IBD patients (Yui et al., 2012). Technical development in the field has also allowed to overcome inherent limitations: the use of microinjection into the organoid lumen to study host-microbiota interactions (Saxena et al., 2016); or the inverted polarity of apical-out organoids mimicking the open intestinal lumen (Co et al., 2021). Further, the development of gut-on-a-chip models including non-epithelial cells within the gut tissue, such as the enteric nervous system, the endothelium and immune mediators will for sure have an enormous impact on biomedical research (Shin and Kim, 2022). Altogether, organoids are nowadays an indispensable tool for the development of new therapies in IBD in general, as nicely reviewed by Yoo and Donowitz (Yoo and Donowitz, 2019).

6 Concluding remarks

In this review, our aim is to highlight a body of past and present research demonstrating the epithelium's supremacy in orchestrating all the necessary molecular players and signalling pathways needed to initiate and sustain inflammatory disorders. There is now abundant data to this, clearly demanding a response from researchers, clinicians, and pharmaceutical industries. Using technologies like PCLSs and organoids focusing on the epithelium and its intrinsic pathways and responses will likely produce needed new therapies in chronic inflammatory disorders. Combing these new and evolving epithelial-centric drug targeting strategies with current anti-inflammation treatments could have a powerful impact on the presently situation we find ourselves with chronic disease prevention and progression, especially in asthma and IBD.

Author contributions

RL-P: Conceptualization, Funding acquisition, Investigation, Software, Writing–review and editing. DCB: Conceptualization, Funding acquisition, Investigation, Writing–review and editing. CP-P: Conceptualization, Funding acquisition, Investigation, Software, Writing–review and editing. EO-Z: Conceptualization, Funding acquisition, Investigation, Software, Supervision, Writing–review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. RLP is funded by the DFG (Deutsches Forchungsgemeinschaft; Project numbers 461063481, 375876048 and 505539112). DCB and CP-P are funded by the Wellcome Investigator Award 221908/z/20/Zs. CP-P is the recipient of a Long-Term Fellowship (LT000654/2019-L) from the Human Frontier Science Program organization and a Marie Skłodowska-Curie Fellowship (898067) from the European Union's Horizon 2020 research and innovation program. EO-Z is funded by the UV-LaFe 2022 Program and the Valencian Respiratory Society (2023).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Abdullah, L. H., Coakley, R., Webster, M. J., Zhu, Y., Tarran, R., Radicioni, G., et al. (2018). Mucin production and hydration responses to mucopurulent materials in normal versus cystic fibrosis airway epithelia. *Am. J. Respir. Crit. Care Med.* 197, 481–491. doi:10.1164/rccm.201706-1139OC

Aegerter, H., and Lambrecht, B. N. (2023). The pathology of asthma: what is obstructing our view? *Annu. Rev. Pathol.* 18, 387–409. doi:10.1146/annurev-pathol-042220-015902

Ahdieh, M., Vandenbos, T., and Youakim, A. (2001). Lung epithelial barrier function and wound healing are decreased by IL-4 and IL-13 and enhanced by IFN-gamma. *Am. J. Physiol. Cell Physiol.* 281, C2029–C2038. doi:10.1152/ajpcell.2001.281.6.C2029

Ahl, D., Liu, H., Schreiber, O., Roos, S., Phillipson, M., and Holm, L. (2016). Lactobacillus reuteri increases mucus thickness and ameliorates dextran sulphate sodium-induced colitis in mice. *Acta Physiol. (Oxf)* 217, 300–310. doi:10.1111/apha.12695

Ahmad, R., Chaturvedi, R., Olivares-Villagomez, D., Habib, T., Asim, M., Shivesh, P., et al. (2014). Targeted colonic claudin-2 expression renders resistance to epithelial injury, induces immune suppression, and protects from colitis. *Mucosal Immunol.* 7, 1340–1353. doi:10.1038/mi.2014.21

Al-Alawi, M., Hassan, T., and Chotirmall, S. H. (2014). Transforming growth factor β and severe asthma: a perfect storm. *Respir. Med.* 108, 1409–1423. doi:10.1016/j.rmed.2014.08.008

Ali, S., Mohs, A., Thomas, M., Klare, J., Ross, R., Schmitz, M. L., et al. (2011). The dual function cytokine IL-33 interacts with the transcription factor NF-κB to dampen NF-κB-stimulated gene transcription. *J. Immunol.* 187, 1609–1616. doi:10.4049/jimmunol.1003080

Allakhverdi, Z., Comeau, M. R., Jessup, H. K., Yoon, B. R., Brewer, A., Chartier, S., et al. (2007). Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. *J. Exp. Med.* 204, 253–258. doi:10.1084/jem.20062211

Al-Sadi, R., Ye, D., Dokladny, K., and Ma, T. Y. (2008). Mechanism of IL-1beta-induced increase in intestinal epithelial tight junction permeability. *J. Immunol.* 180, 5653–5661. doi:10.4049/jimmunol.180.8.5653

Alsafadi, H. N., Staab-Weijnitz, C. A., Lehmann, M., Lindner, M., Peschel, B., Konigshoff, M., et al. (2017). An *ex vivo* model to induce early fibrosis-like changes in human precision-cut lung slices. *Am. J. Physiol. Lung Cell Mol. Physiol.* 312, L896–L902. doi:10.1152/ajplung.00084.2017

Alsafadi, H. N., Uhl, F. E., Pineda, R. H., Bailey, K. E., Rojas, M., Wagner, D. E., et al. (2020). Applications and approaches for three-dimensional precision-cut lung slices. Disease modeling and drug discovery. *Am. J. Respir. Cell Mol. Biol.* 62, 681–691. doi:10.1165/rcmb.2019-0276TR

Alvarez, Y., and Smutny, M. (2022). Emerging role of mechanical forces in cell fate acquisition. *Front. Cell Dev. Biol.* 10, 864522. doi:10.3389/fcell.2022.864522

Angulo-Urarte, A., Van Der Wal, T., and Huveneers, S. (2020). Cell-cell junctions as sensors and transducers of mechanical forces. *Biochim. Biophys. Acta Biomembr.* 1862, 183316. doi:10.1016/j.bbamem.2020.183316

Archer, N. K., Jo, J. H., Lee, S. K., Kim, D., Smith, B., Ortines, R. V., et al. (2019). Injury, dysbiosis, and filaggrin deficiency drive skin inflammation through keratinocyte IL-1α release. *J. Allergy Clin. Immunol.* 143, 1426–1443 e6. doi:10.1016/j.jaci.2018.08.042

Arikoglu, T., Akyilmaz, E., Yildirim, D. D., Batmaz, S. B., Ulger, S. T., Aslan, G., et al. (2017). The relation of innate and adaptive immunity with viral-induced acute asthma attacks: focusing on IP-10 and cathelicidin. *Allergol. Immunopathol. Madr.* 45, 160–168. doi:10.1016/j.aller.2016.07.003

Arrieta, M. C., Madsen, K., Doyle, J., and Meddings, J. (2009). Reducing small intestinal permeability attenuates colitis in the IL10 gene-deficient mouse. *Gut* 58, 41–48. doi:10.1136/gut.2008.150888

Bagley, D. C., Russell, T., Ortiz-Zapater, E., Fox, K., Redd, P. F., Joseph, M., et al. (2023). Bronchoconstriction damages airway epithelia by excess crowding-induced extrusion. *bioRxiv*, 2023.08.04.551943. 2023.08.04.551943. doi:10.1101/2023.08.04.551943

Barbato, A., Turato, G., Baraldo, S., Bazzan, E., Calabrese, F., Panizzolo, C., et al. (2006). Epithelial damage and angiogenesis in the airways of children with asthma. *Am. J. Respir. Crit. Care Med.* 174, 975–981. doi:10.1164/rccm.200602-189OC

Bardenbacher, M., Ruder, B., Britzen-Laurent, N., Naschberger, E., Becker, C., Palmisano, R., et al. (2020). Investigating intestinal barrier breakdown in living organoids. *J. Vis. Exp.* doi:10.3791/60546

Barry, E. R., Morikawa, T., Butler, B. L., Shrestha, K., De La Rosa, R., Yan, K. S., et al. (2013). Restriction of intestinal stem cell expansion and the regenerative response by YAP. *Nature* 493, 106–110. doi:10.1038/nature11693

Basak, O., Beumer, J., Wiebrands, K., Seno, H., Van Oudenaarden, A., and Clevers, H. (2017). Induced quiescence of Lgr5+ stem cells in intestinal organoids enables differentiation of hormone-producing enteroendocrine cells. *Cell Stem Cell* 20, 177–190. doi:10.1016/j.stem.2016.11.001

Bastounis, E. E., Serrano-Alcalde, F., Radhakrishnan, P., Engstrom, P., Gomez-Benito, M. J., Oswald, M. S., et al. (2021). Mechanical competition triggered by innate immune signaling drives the collective extrusion of bacterially infected epithelial cells. *Dev. Cell* 56, 443–460 e11. doi:10.1016/j.devcel.2021.01.012

Bauche, D., and Marie, J. C. (2017). Transforming growth factor β: a master regulator of the gut microbiota and immune cell interactions. *Clin. Transl. Immunol.* 6, e136. doi:10.1038/cti.2017.9

Baumgart, D. C., Vierziger, K., Sturm, A., Wiedenmann, B., and Dignass, A. U. (2005). Mesalamine promotes intestinal epithelial wound healing *in vitro* through a TGF-beta-independent mechanism. *Scand. J. Gastroenterol.* 40, 958–964. doi:10.1080/00365520510015854

Bayrer, J. R., Wang, H., Nattiv, R., Suzawa, M., Escusa, H. S., Fletterick, R. J., et al. (2018). LRH-1 mitigates intestinal inflammatory disease by maintaining epithelial homeostasis and cell survival. *Nat. Commun.* 9, 4055. doi:10.1038/s41467-018-06137-w

Becker, C., Neurath, M. F., and Wirtz, S. (2015). The intestinal microbiota in inflammatory bowel disease. *ILAR J.* 56, 192–204. doi:10.1093/ilar/ilv030

Benham-Pyle, B. W., Pruitt, B. L., and Nelson, W. J. (2015). Cell adhesion. Mechanical strain induces E-cadherin-dependent Yap1 and β-catenin activation to drive cell cycle entry. *Science* 348, 1024–1027. doi:10.1126/science.aaa4559

Benjamin, J., Makharia, G. K., Ahuja, V., Kalaivani, M., and Joshi, Y. K. (2008). Intestinal permeability and its association with the patient and disease characteristics in Crohn's disease. *World J. Gastroenterol.* 14, 1399–1405. doi:10.3748/wjg.14.1399

Bergstrom, K. S., and Xia, L. (2013). Mucin-type O-glycans and their roles in intestinal homeostasis. *Glycobiology* 23, 1026–1037. doi:10.1093/glycob/cwt045

Beumer, J., Artegiani, B., Post, Y., Reimann, F., Gribble, F., Nguyen, T. N., et al. (2018). Enteroendocrine cells switch hormone expression along the crypt-to-villus BMP signalling gradient. *Nat. Cell Biol.* 20, 909–916. doi:10.1038/s41556-018-0143-y

Beumer, J., and Clevers, H. (2021). Cell fate specification and differentiation in the adult mammalian intestine. *Nat. Rev. Mol. Cell Biol.* 22, 39–53. doi:10.1038/s41580-020-0278-0

Beutel, O., Maraspini, R., Pombo-Garcia, K., Martin-Lemaitre, C., and Honigmann, A. (2019). Phase separation of zonula occludens proteins drives formation of tight junctions. *Cell* 179, 923–936. doi:10.1016/j.cell.2019.10.011

Bigorgne, A. E., Farin, H. F., Lemoine, R., Mahlaoui, N., Lambert, N., Gil, M., et al. (2014). TTC7A mutations disrupt intestinal epithelial apicobasal polarity. *J. Clin. Invest.* 124, 328–337. doi:10.1172/JCI71471

Bischoff, S. C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J. D., Serino, M., et al. (2014). Intestinal permeability--a new target for disease prevention and therapy. *BMC Gastroenterol.* 14, 189. doi:10.1186/s12876-014-0189-7

Bokemeyer, B., Hommes, D., Gill, I., Broberg, P., and Dignass, A. (2012). Mesalazine in left-sided ulcerative colitis: efficacy analyses from the PODIUM trial on maintenance of remission and mucosal healing. *J. Crohns Colitis* 6, 476–482. doi:10.1016/j.crohns.2011.10.006

Borchers, N. S., Santos-Valente, E., Toncheva, A. A., Wehkamp, J., Franke, A., Gaertner, V. D., et al. (2021). Human β-defensin 2 mutations are associated with asthma and atopy in children and its application prevents atopic asthma in a mouse model. *Front. Immunol.* 12, 636061. doi:10.3389/fimmu.2021.636061

Boxall, C., Holgate, S. T., and Davies, D. E. (2006). The contribution of transforming growth factor-beta and epidermal growth factor signalling to airway remodelling in chronic asthma. *Eur. Respir. J.* 27, 208–229. doi:10.1183/09031936.06.00130004

Braga, V. (2018). Signaling by small GTPases at cell-cell junctions: protein interactions building control and networks. *Cold Spring Harb. Perspect. Biol.* 10, a028746. doi:10.1101/cshperspect.a028746

Bron, P. A., Kleerebezem, M., Brummer, R. J., Cani, P. D., Mercenier, A., Macdonald, T. T., et al. (2017). Can probiotics modulate human disease by impacting intestinal barrier function? *Br. J. Nutr.* 117, 93–107. doi:10.1017/S0007114516004037

Buhner, S., Buning, C., Genschel, J., Kling, K., Herrmann, D., Dignass, A., et al. (2006). Genetic basis for increased intestinal permeability in families with Crohn's disease: role of CARD15 3020insC mutation? *Gut* 55, 342–347. doi:10.1136/gut.2005.065557

Burclaff, J., Bliton, R. J., Breau, K. A., Ok, M. T., Gomez-Martinez, I., Ranek, J. S., et al. (2022). A proximal-to-distal survey of healthy adult human small intestine and colon epithelium by single-cell transcriptomics. *Cell Mol. Gastroenterol. Hepatol.* 13, 1554–1589. doi:10.1016/j.jcmgh.2022.02.007

Calven, J., Ax, E., and Radinger, M. (2020). The airway epithelium-A central player in asthma pathogenesis. *Int. J. Mol. Sci.* 21, 8907. doi:10.3390/ijms21238907

Camargo, F. D., Gokhale, S., Johnnidis, J. B., Fu, D., Bell, G. W., Jaenisch, R., et al. (2007). YAP1 increases organ size and expands undifferentiated progenitor cells. *Curr. Biol.* 17, 2054–2060. doi:10.1016/j.cub.2007.10.039

Ceponis, P. J., Botelho, F., Richards, C. D., and Mckay, D. M. (2000). Interleukins 4 and 13 increase intestinal epithelial permeability by a phosphatidylinositol 3-kinase pathway. Lack of evidence for STAT 6 involvement. *J. Biol. Chem.* 275, 29132–29137. doi:10.1074/jbc.M003516200

Chan, R., Stewart, K., Misirovs, R., and Lipworth, B. J. (2022). Targeting downstream type 2 cytokines or upstream epithelial alarmins for severe asthma. *J. Allergy Clin. Immunol. Pract.* 10, 1497–1505. doi:10.1016/j.jaip.2022.01.040

Chen, L., Brenner, D. A., and Kisseleva, T. (2019). Combatting fibrosis: exosome-based therapies in the regression of liver fibrosis. *Hepatol. Commun.* 3, 180–192. doi:10.1002/hep4.1290

Childhood Asthma Management Program Research Group, Szefler, S., Weiss, S., Tonascia, J., Adkinson, N. F., Bender, B., et al. (2000). Long-term effects of budesonide or nedocromil in children with asthma. *N. Engl. J. Med.* 343, 1054–1063. doi:10.1056/NEJM200010123431501

Citi, S., Guerrera, D., Spadaro, D., and Shah, J. (2014). Epithelial junctions and Rho family GTPases: the zonular signalosome. *Small GTPases* 5, 1–15. doi:10.4161/21541248.2014.973760

Clancy, D. M., Sullivan, G. P., Moran, H. B. T., Henry, C. M., Reeves, E. P., Mcelvaney, N. G., et al. (2018). Extracellular neutrophil proteases are efficient regulators of IL-1, IL-33, and IL-36 cytokine activity but poor effectors of microbial killing. *Cell Rep.* 22, 2937–2950. doi:10.1016/j.celrep.2018.02.062

Clayburgh, D. R., Barrett, T. A., Tang, Y., Meddings, J. B., Van Eldik, L. J., Watterson, D. M., et al. (2005). Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea *in vivo*. *J. Clin. Invest.* 115, 2702–2715. doi:10.1172/JCI24970

Co, J. Y., Margalef-Catala, M., Monack, D. M., and Amieva, M. R. (2021). Controlling the polarity of human gastrointestinal organoids to investigate epithelial biology and infectious diseases. *Nat. Protoc.* 16, 5171–5192. doi:10.1038/s41596-021-00607-0

Consortium, U. I. G., Barrett, J. C., Lee, J. C., Lees, C. W., Prescott, N. J., Anderson, C. A., et al. (2009). Genome-wide association study of ulcerative colitis identifies three new susceptibility loci, including the HNF4A region. *Nat. Genet.* 41, 1330–1334. doi:10.1038/ng.483

Cookson, W. (2004). The immunogenetics of asthma and eczema: a new focus on the epithelium. *Nat. Rev. Immunol.* 4, 978–988. doi:10.1038/nri1500

Cornick, S., Tawiah, A., and Chadee, K. (2015). Roles and regulation of the mucus barrier in the gut. *Tissue Barriers* 3, e982426. doi:10.4161/21688370.2014.982426

Davies, E. J., Dong, M., Gutekunst, M., Narhi, K., Van Zoggel, H. J., Blom, S., et al. (2015). Capturing complex tumour biology *in vitro*: histological and molecular characterisation of precision cut slices. *Sci. Rep.* 5, 17187. doi:10.1038/srep17187

Deprez, M., Zaragosi, L. E., Truchi, M., Becavin, C., Ruiz Garcia, S., Arguel, M. J., et al. (2020). A single-cell atlas of the human healthy airways. *Am. J. Respir. Crit. Care Med.* 202, 1636–1645. doi:10.1164/rccm.201911-2199OC

Deuring, J. J., Li, M., Cao, W., Chen, S., Wang, W., De Haar, C., et al. (2019). Pregnane X receptor activation constrains mucosal NF-κB activity in active inflammatory bowel disease. *PLoS One* 14, e0221924. doi:10.1371/journal.pone.0221924

D'Haens, G., Van Deventer, S., Van Hogezand, R., Chalmers, D., Kothe, C., Baert, F., et al. (1999). Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: a European multicenter trial. *Gastroenterology* 116, 1029–1034. doi:10.1016/s0016-5085(99)70005-3

D'Inca, R., Annese, V., Di Leo, V., Latiano, A., Quaino, V., Abazia, C., et al. (2006). Increased intestinal permeability and NOD2 variants in familial and sporadic Crohn's

disease. *Aliment. Pharmacol. Ther.* 23, 1455–1461. doi:10.1111/j.1365-2036.2006.02916.x

Dotti, I., Mora-Buch, R., Ferrer-Picon, E., Planell, N., Jung, P., Masamunt, M. C., et al. (2017). Alterations in the epithelial stem cell compartment could contribute to permanent changes in the mucosa of patients with ulcerative colitis. *Gut* 66, 2069–2079. doi:10.1136/gutjnl-2016-312609

Duckworth, C. A. (2021). Identifying key regulators of the intestinal stem cell niche. *Biochem. Soc. Trans.* 49, 2163–2176. doi:10.1042/BST20210223

Eisenhoffer, G. T., Loftus, P. D., Yoshigi, M., Otsuna, H., Chien, C. B., Morcos, P. A., et al. (2012). Crowding induces live cell extrusion to maintain homeostatic cell numbers in epithelia. *Nature* 484, 546–549. doi:10.1038/nature10999

Elmentaite, R., Kumasaka, N., Roberts, K., Fleming, A., Dann, E., King, H. W., et al. (2021). Cells of the human intestinal tract mapped across space and time. *Nature* 597, 250–255. doi:10.1038/s41586-021-03852-1

Elphick, D., Liddell, S., and Mahida, Y. R. (2008). Impaired luminal processing of human defensin-5 in Crohn's disease: persistence in a complex with chymotrypsinogen and trypsin. *Am. J. Pathol.* 172, 702–713. doi:10.2353/ajpath.2008.070755

Etienne-Mesmin, L., Chassaing, B., Desvaux, M., De Paepe, K., Gresse, R., Sauvaitre, T., et al. (2019). Experimental models to study intestinal microbes–mucus interactions in health and disease. *FEMS Microbiol. Rev.* 43, 457–489. doi:10.1093/femsre/fuz013

Fahy, J. V., Steiger, D. J., Liu, J., Basbaum, C. B., Finkbeiner, W. E., and Boushey, H. A. (1993). Markers of mucus secretion and DNA levels in induced sputum from asthmatic and from healthy subjects. *Am. Rev. Respir. Dis.* 147, 1132–1137. doi:10.1164/ajrccm/147.5.1132

Fedorov, I. A., Wilson, S. J., Davies, D. E., and Holgate, S. T. (2005). Epithelial stress and structural remodelling in childhood asthma. *Thorax* 60, 389–394. doi:10.1136/thx.2004.030262

Fellermann, K., Stange, D. E., Schaeffeler, E., Schmalzl, H., Wehkamp, J., Bevins, C. L., et al. (2006). A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn disease of the colon. *Am. J. Hum. Genet.* 79, 439–448. doi:10.1086/505915

Forbes, J. D., Van Domselaar, G., and Bernstein, C. N. (2016). Microbiome survey of the inflamed and noninflamed gut at different compartments within the gastrointestinal tract of inflammatory bowel disease patients. *Inflamm. Bowel Dis.* 22, 817–825. doi:10.1097/MIB.0000000000000684

Fornasa, G., Tsilingiri, K., Caprioli, F., Botti, F., Mapelli, M., Meller, S., et al. (2015). Dichotomy of short and long thymic stromal lymphopoietin isoforms in inflammatory disorders of the bowel and skin. *J. Allergy Clin. Immunol.* 136, 413–422. doi:10.1016/j.jaci.2015.04.011

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., and Artavanis-Tsakonas, S. (2005). Notch signals control the fate of immature progenitor cells in the intestine. *Nature* 435, 964–968. doi:10.1038/nature03589

Ganesan, S., Comstock, A. T., and Sajjan, U. S. (2013). Barrier function of airway tract epithelium. *Tissue Barriers* 1, e24997. doi:10.4161/tisb.24997

Gao, W., Li, L., Wang, Y., Zhang, S., Adcock, I. M., Barnes, P. J., et al. (2015). Bronchial epithelial cells: the key effector cells in the pathogenesis of chronic obstructive pulmonary disease? *Respirology* 20, 722–729. doi:10.1111/resp.12542

Garabedian, E. M., Roberts, L. J., Mcnevin, M. S., and Gordon, J. I. (1997). Examining the role of Paneth cells in the small intestine by lineage ablation in transgenic mice. *J. Biol. Chem.* 272, 23729–23740. doi:10.1074/jbc.272.38.23729

Garcia, M. A., Nelson, W. J., and Chavez, N. (2018). Cell-cell junctions organize structural and signaling networks. *Cold Spring Harb. Perspect. Biol.* 10, a029181. doi:10.1101/cshperspect.a029181

GBD 2017 Inflammatory Bowel Disease Collaborators (2020). The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Gastroenterol. Hepatol.* 5, 17–30. doi:10.1016/S2468-1253(19)30333-4

Gersemann, M., Becker, S., Kubler, I., Koslowski, M., Wang, G., Herrlinger, K. R., et al. (2009). Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. *Differentiation* 77, 84–94. doi:10.1016/j.diff.2008.09.008

Glal, D., Sudhakar, J. N., Lu, H. H., Liu, M. C., Chiang, H. Y., Liu, Y. C., et al. (2018). ATF3 sustains IL-22-induced STAT3 phosphorylation to maintain mucosal immunity through inhibiting phosphatases. *Front. Immunol.* 9, 2522. doi:10.3389/fimmu.2018.02522

Gleeson, J. P., Estrada, H. Q., Yamashita, M., Svendsen, C. N., Targan, S. R., and Barrett, R. J. (2020). Development of physiologically responsive human iPSC-derived intestinal epithelium to study barrier dysfunction in IBD. *Int. J. Mol. Sci.* 21, 1438. doi:10.3390/ijms21041438

Goodwin, K., and Nelson, C. M. (2021). Mechanics of development. *Dev. Cell* 56, 240–250. doi:10.1016/j.devcel.2020.11.025

Goswami, S., Angkasekwinai, P., Shan, M., Greenlee, K. J., Barranco, W. T., Polikepahad, S., et al. (2009). Divergent functions for airway epithelial matrix metalloproteinase 7 and retinoic acid in experimental asthma. *Nat. Immunol.* 10, 496–503. doi:10.1038/ni.1719

Graham, W. V., He, W., Marchiando, A. M., Zha, J., Singh, G., Li, H. S., et al. (2019). Intracellular MLCK1 diversion reverses barrier loss to restore mucosal homeostasis. *Nat. Med.* 25, 690–700. doi:10.1038/s41591-019-0393-7

Grainge, C. L., Lau, L. C., Ward, J. A., Dulay, V., Lahiff, G., Wilson, S., et al. (2011). Effect of bronchoconstriction on airway remodeling in asthma. *N. Engl. J. Med.* 364, 2006–2015. doi:10.1056/NEJMoa1014350

Grotenboer, N. S., Ketelaar, M. E., Koppelman, G. H., and Nawijn, M. C. (2013). Decoding asthma: translating genetic variation in IL33 and IL1RL1 into disease pathophysiology. *J. Allergy Clin. Immunol.* 131, 856–865. doi:10.1016/j.jaci.2012.11.028

Gudipaty, S. A., Lindblom, J., Loftus, P. D., Redd, M. J., Edes, K., Davey, C. F., et al. (2017). Mechanical stretch triggers rapid epithelial cell division through Piezo1. *Nature* 543, 118–121. doi:10.1038/nature21407

Guilbert, T. W., Morgan, W. J., Zeiger, R. S., Mauger, D. T., Boehmer, S. J., Szefler, S. J., et al. (2006). Long-term inhaled corticosteroids in preschool children at high risk for asthma. *N. Engl. J. Med.* 354, 1985–1997. doi:10.1056/NEJMoa051378

Gustafsson, J. K., Ermund, A., Ambort, D., Johansson, M. E., Nilsson, H. E., Thorell, K., et al. (2012). Bicarbonate and functional CFTR channel are required for proper mucin secretion and link cystic fibrosis with its mucus phenotype. *J. Exp. Med.* 209, 1263–1272. doi:10.1084/jem.20120562

Haas, A. J., Zihni, C., Krug, S. M., Maraspini, R., Otani, T., Furuse, M., et al. (2022). ZO-1 guides tight junction assembly and epithelial morphogenesis via cytoskeletal tension-dependent and -independent functions. *Cells* 11, 3775. doi:10.3390/cells11233775

Haber, A. L., Biton, M., Rogel, N., Herbst, R. H., Shekhar, K., Smillie, C., et al. (2017). A single-cell survey of the small intestinal epithelium. *Nature* 551, 333–339. doi:10.1038/nature24489

Halim, T. Y., Steer, C. A., Matha, L., Gold, M. J., Martinez-Gonzalez, I., Mcnagny, K. M., et al. (2014). Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 40, 425–435. doi:10.1016/j.immuni.2014.01.011

Halwani, R., Al-Muhsen, S., Al-Jahdali, H., and Hamid, Q. (2011). Role of transforming growth factor-β in airway remodeling in asthma. *Am. J. Respir. Cell Mol. Biol.* 44, 127–133. doi:10.1165/rcmb.2010-0027TR

Ham, J., Shin, J. W., Ko, B. C., and Kim, H. Y. (2022). Targeting the epithelium-derived innate cytokines: from bench to bedside. *Immune Netw.* 22, e11. doi:10.4110/in.2022.22.e11

Hammad, H., and Lambrecht, B. N. (2015). Barrier epithelial cells and the control of type 2 immunity. *Immunity* 43, 29–40. doi:10.1016/j.immuni.2015.07.007

Hammad, H., and Lambrecht, B. N. (2021). The basic immunology of asthma. *Cell* 184, 1469–1485. doi:10.1016/j.cell.2021.02.016

Haniffa, M., Taylor, D., Linnarsson, S., Aronow, B. J., Bader, G. D., Barker, R. A., et al. (2021). A roadmap for the human developmental cell atlas. *Nature* 597, 196–205. doi:10.1038/s41586-021-03620-1

Hansson, G. C. (2019). Mucus and mucins in diseases of the intestinal and respiratory tracts. *J. Intern Med.* 285, 479–490. doi:10.1111/joim.12910

He, W. Q., Peng, Y. J., Zhang, W. C., Lv, N., Tang, J., Chen, C., et al. (2008). Myosin light chain kinase is central to smooth muscle contraction and required for gastrointestinal motility in mice. *Gastroenterology* 135, 610–620. doi:10.1053/j.gastro.2008.05.032

Heijink, I. H., Kies, P. M., Kauffman, H. F., Postma, D. S., Van Oosterhout, A. J., and Vellenga, E. (2007). Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity. *J. Immunol.* 178, 7678–7685. doi:10.4049/jimmunol.178.12.7678

Heller, F., Florian, P., Bojarski, C., Richter, J., Christ, M., Hillenbrand, B., et al. (2005). Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology* 129, 550–564. doi:10.1016/j.gastro.2005.05.002

Henderson, A. G., Ehre, C., Button, B., Abdullah, L. H., Cai, L. H., Leigh, M. W., et al. (2014). Cystic fibrosis airway secretions exhibit mucin hyperconcentration and increased osmotic pressure. *J. Clin. Invest.* 124, 3047–3060. doi:10.1172/JCI73469

He, S., Lei, P., Kang, W., Cheung, P., Xu, T., Mana, M., et al. (2023). Stiffness restricts the stemness of the intestinal stem cells and skews their differentiation toward goblet cells. *Gastroenterology* 164, 1137–1151 e15. doi:10.1053/j.gastro.2023.02.030

Hicks-Berthet, J., Ning, B., Federico, A., Tilston-Lunel, A., Matschulat, A., Ai, X., et al. (2021). Yap/Taz inhibit goblet cell fate to maintain lung epithelial homeostasis. *Cell Rep.* 36, 109347. doi:10.1016/j.celrep.2021.109347

Hiemstra, P. (2006). *DEFENSINS. Encyclopedia of respiratory medicine*. Elsevier. Internet 2006 cited 2023 May 31.

Hill, D. B., Long, R. F., Kissner, W. J., Atieh, E., Garbarine, I. C., Markovetz, M. R., et al. (2018). Pathological mucus and impaired mucus clearance in cystic fibrosis patients result from increased concentration, not altered pH. *Eur. Respir. J.* 52, 1801297. doi:10.1183/13993003.01297-2018

Hippee, C. E., Singh, B. K., Thurman, A. L., Cooney, A. L., Pezzulo, A. A., Cattaneo, R., et al. (2021). Measles virus exits human airway epithelia within dislodged metabolically active infectious centers. *PLoS Pathog.* 17, e1009458. doi:10.1371/journal.ppat.1009458

Holgate, S. T. (2007). Epithelium dysfunction in asthma. *J. Allergy Clin. Immunol.* 120, 1233–1244. quiz 1245-6. doi:10.1016/j.jaci.2007.10.025

Horowitz, A., Chanez-Paredes, S. D., Haest, X., and Turner, J. R. (2023). Paracellular permeability and tight junction regulation in gut health and disease. *Nat. Rev. Gastroenterol. Hepatol.* 20, 417–432. doi:10.1038/s41575-023-00766-3

Ho, S., Pothoulakis, C., and Koon, H. W. (2013). Antimicrobial peptides and colitis. *Curr. Pharm. Des.* 19, 40–47. doi:10.2174/13816128130108

Hoshino, M., Nakamura, Y., and Sim, J. J. (1998). Expression of growth factors and remodelling of the airway wall in bronchial asthma. *Thorax* 53, 21–27. doi:10.1136/thx.53.1.21

Huang, B., Chen, Z., Geng, L., Wang, J., Liang, H., Cao, Y., et al. (2019a). Mucosal profiling of pediatric-onset colitis and IBD reveals common pathogenics and therapeutic pathways. *Cell* 179, 1160–1176. doi:10.1016/j.cell.2019.10.027

Huang, S., Chen, B., Humeres, C., Alex, L., Hanna, A., and Frangogiannis, N. G. (2020). The role of Smad2 and Smad3 in regulating homeostatic functions of fibroblasts *in vitro* and in adult mice. *Biochim. Biophys. Acta Mol. Cell Res.* 1867, 118703. doi:10.1016/j.bbamcr.2020.118703

Huang, X., Li, L., Ammar, R., Zhang, Y., Wang, Y., Ravi, K., et al. (2019b). Molecular characterization of a precision-cut rat lung slice model for the evaluation of antifibrotic drugs. *Am. J. Physiol. Lung Cell Mol. Physiol.* 316, L348–L357. doi:10.1152/ajplung.00339.2018

Huang, Y., Guo, L., Qiu, J., Chen, X., Hu-Li, J., Siebenlist, U., et al. (2015). IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat. Immunol.* 16, 161–169. doi:10.1038/ni.3078

Ihara, S., Hirata, Y., Hikiba, Y., Yamashita, A., Tsuboi, M., Hata, M., et al. (2018). Adhesive interactions between mononuclear phagocytes and intestinal epithelium perturb normal epithelial differentiation and serve as a therapeutic target in inflammatory bowel disease. *J. Crohns Colitis* 12, 1219–1231. doi:10.1093/ecco-jcc/jjy088

Ihara, S., Hirata, Y., and Koike, K. (2017). TGF-β in inflammatory bowel disease: a key regulator of immune cells, epithelium, and the intestinal microbiota. *J. Gastroenterol.* 52, 777–787. doi:10.1007/s00535-017-1350-1

Ikeda, K., Nakajima, H., Suzuki, K., Kagami, S., Hirose, K., Suto, A., et al. (2003). Mast cells produce interleukin-25 upon Fc epsilon RI-mediated activation. *Blood* 101, 3594–3596. doi:10.1182/blood-2002-09-2817

Irvine, E. J., and Marshall, J. K. (2000). Increased intestinal permeability precedes the onset of Crohn's disease in a subject with familial risk. *Gastroenterology* 119, 1740–1744. doi:10.1053/gast.2000.20231

Jackson, N. D., Everman, J. L., Chioccioli, M., Feriani, L., Goldfarbmuren, K. C., Sajuthi, S. P., et al. (2020). Single-cell and population transcriptomics reveal pan-epithelial remodeling in type 2-high asthma. *Cell Rep.* 32, 107872. doi:10.1016/j.celrep.2020.107872

Jenkins, R. T., Jones, D. B., Goodacre, R. L., Collins, S. M., Coates, G., Hunt, R. H., et al. (1987). Reversibility of increased intestinal permeability to 51Cr-EDTA in patients with gastrointestinal inflammatory diseases. *Am. J. Gastroenterol.* 82, 1159–1164.

Jenkins, R. T., Ramage, J. K., Jones, D. B., Collins, S. M., Goodacre, R. L., and Hunt, R. H. (1988). Small bowel and colonic permeability to 51Cr-EDTA in patients with active inflammatory bowel disease. *Clin. Invest. Med.* 11, 151–155.

Johansson, M. E., Sjovall, H., and Hansson, G. C. (2013). The gastrointestinal mucus system in health and disease. *Nat. Rev. Gastroenterol. Hepatol.* 10, 352–361. doi:10.1038/nrgastro.2013.35

Kabata, H., Moro, K., Fukunaga, K., Suzuki, Y., Miyata, J., Masaki, K., et al. (2013). Thymic stromal lymphopoietin induces corticosteroid resistance in natural helper cells during airway inflammation. *Nat. Commun.* 4, 2675. doi:10.1038/ncomms3675

Kahata, K., Dadras, M. S., and Moustakas, A. (2018). TGF-B family signaling in epithelial differentiation and epithelial-mesenchymal transition. *Cold Spring Harb. Perspect. Biol.* 10, a022194. doi:10.1101/cshperspect.a022194

Kang, C. M., Jang, A. S., Ahn, M. H., Shin, J. A., Kim, J. H., Choi, Y. S., et al. (2005). Interleukin-25 and interleukin-13 production by alveolar macrophages in response to particles. *Am. J. Respir. Cell Mol. Biol.* 33, 290–296. doi:10.1165/rcmb.2005-0003OC

Karamanou, M., and Androutsos, G. (2011). Aretaeus of Cappadocia and the first clinical description of asthma. *Am. J. Respir. Crit. Care Med.* 184, 1420–1421. doi:10.1164/ajrccm.184.12.1420b

Kashyap, M., Rochman, Y., Spolski, R., Samsel, L., and Leonard, W. J. (2011). Thymic stromal lymphopoietin is produced by dendritic cells. *J. Immunol.* 187, 1207–1211. doi:10.4049/jimmunol.1100355

Kawamoto, A., Nagata, S., Anzai, S., Takahashi, J., Kawai, M., Hama, M., et al. (2019). Ubiquitin D is upregulated by synergy of Notch signalling and TNF-α in the inflamed intestinal epithelia of IBD patients. *J. Crohns Colitis* 13, 495–509. doi:10.1093/ecco-jcc/jjy180

Khare, V., Krnjic, A., Frick, A., Gmainer, C., Asboth, M., Jimenez, K., et al. (2019). Mesalamine and azathioprine modulate junctional complexes and restore epithelial barrier function in intestinal inflammation. *Sci. Rep.* 9, 2842. doi:10.1038/s41598-019-39401-0

Khounlotham, M., Kim, W., Peatman, E., Nava, P., Medina-Contreras, O., Addis, C., et al. (2012). Compromised intestinal epithelial barrier induces adaptive immune compensation that protects from colitis. *Immunity* 37, 563–573. doi:10.1016/j.immuni.2012.06.017

Kierkus, J., Dadalski, M., Szymanska, E., Oracz, G., Wegner, A., Gorczewska, M., et al. (2012). The impact of infliximab induction therapy on mucosal healing and clinical remission in Polish pediatric patients with moderate-to-severe Crohn's disease. *Eur. J. Gastroenterol. Hepatol.* 24, 495–500. doi:10.1097/MEG.0b013e32835159f2

Kiesslich, R., Duckworth, C. A., Moussata, D., Gloeckner, A., Lim, L. G., Goetz, M., et al. (2012). Local barrier dysfunction identified by confocal laser endomicroscopy predicts relapse in inflammatory bowel disease. *Gut* 61, 1146–1153. doi:10.1136/gutjnl-2011-300695

Kiesslich, R., Goetz, M., Angus, E. M., Hu, Q., Guan, Y., Potten, C., et al. (2007). Identification of epithelial gaps in human small and large intestine by confocal endomicroscopy. *Gastroenterology* 133, 1769–1778. doi:10.1053/j.gastro.2007.09.011

Kinchen, J., Chen, H. H., Parikh, K., Antanaviciute, A., Jagielowicz, M., Fawkner-Corbett, D., et al. (2018). Structural remodeling of the human colonic mesenchyme in inflammatory bowel disease. *Cell* 175, 372–386. doi:10.1016/j.cell.2018.08.067

Kips, J. C., O'Connor, B. J., Inman, M. D., Svensson, K., Pauwels, R. A., and O'Byrne, P. M. (2000). A long-term study of the antiinflammatory effect of low-dose budesonide plus formoterol versus high-dose budesonide in asthma. *Am. J. Respir. Crit. Care Med.* 161, 996–1001. doi:10.1164/ajrccm.161.3.9812056

Kirsner, J. B., and Palmer, W. L. (1951). Effect of corticotropin (ACTH) in chronic ulcerative colitis; observations in forty patients. *J. Am. Med. Assoc.* 147, 541–549. doi:10.1001/jama.1951.03670230007003

Kitson, R. R., and Moody, C. J. (2013). Learning from nature: advances in geldanamycin- and radicicol-based inhibitors of Hsp90. *J. Org. Chem.* 78, 5117–5141. doi:10.1021/jo4002849

Kohanski, M. A., Workman, A. D., Patel, N. N., Hung, L. Y., Shtraks, J. P., Chen, B., et al. (2018). Solitary chemosensory cells are a primary epithelial source of IL-25 in patients with chronic rhinosinusitis with nasal polyps. *J. Allergy Clin. Immunol.* 142, 460–469. doi:10.1016/j.jaci.2018.03.019

Krawiec, P., and Pac-Kozuchowska, E. (2021). Cathelicidin - a novel potential marker of pediatric inflammatory bowel disease. *J. Inflamm. Res.* 14, 163–174. doi:10.2147/JIR.S288742

Krndija, D., El Marjou, F., Guirao, B., Richon, S., Leroy, O., Bellaiche, Y., et al. (2019). Active cell migration is critical for steady-state epithelial turnover in the gut. *Science* 365, 705–710. doi:10.1126/science.aau3429

Krug, S. M., Amasheh, S., Richter, J. F., Milatz, S., Gunzel, D., Westphal, J. K., et al. (2009). Tricellulin forms a barrier to macromolecules in tricellular tight junctions without affecting ion permeability. *Mol. Biol. Cell* 20, 3713–3724. doi:10.1091/mbc.e09-01-0080

Krug, S. M., Bojarski, C., Fromm, A., Lee, I. M., Dames, P., Richter, J. F., et al. (2018). Tricellulin is regulated via interleukin-13-receptor α2, affects macromolecule uptake, and is decreased in ulcerative colitis. *Mucosal Immunol.* 11, 345–356. doi:10.1038/mi.2017.52

Kuo, W. T., Shen, L., Zuo, L., Shashikanth, N., Ong, M., Wu, L., et al. (2019). Inflammation-induced occludin downregulation limits epithelial apoptosis by suppressing caspase-3 expression. *Gastroenterology* 157, 1323–1337. doi:10.1053/j.gastro.2019.07.058

Lambrecht, B. N., and Hammad, H. (2012). The airway epithelium in asthma. *Nat. Med.* 18, 684–692. doi:10.1038/nm.2737

Lam, M., Lamanna, E., Organ, L., Donovan, C., and Bourke, J. E. (2023). Perspectives on precision cut lung slices-powerful tools for investigation of mechanisms and therapeutic targets in lung diseases. *Front. Pharmacol.* 14, 1162889. doi:10.3389/fphar.2023.1162889

Leber, A., Hontecillas, R., Tubau-Juni, N., Zoccoli-Rodriguez, V., Abedi, V., and Bassaganya-Riera, J. (2018). NLRX1 modulates immunometabolic mechanisms controlling the host-gut microbiota interactions during inflammatory bowel disease. *Front. Immunol.* 9, 363. doi:10.3389/fimmu.2018.00363

Lefrancais, E., Duval, A., Mirey, E., Roga, S., Espinosa, E., Cayrol, C., et al. (2014). Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proc. Natl. Acad. Sci. U. S. A.* 111, 15502–15507. doi:10.1073/pnas.1410700111

Li, C., Zhou, Y., Rychahou, P., Weiss, H. L., Lee, E. Y., Perry, C. L., et al. (2020a). SIRT2 contributes to the regulation of intestinal cell proliferation and differentiation. *Cell Mol. Gastroenterol. Hepatol.* 10, 43–57. doi:10.1016/j.jcmgh.2020.01.004

Lichtenstein, G. R., Ramsey, D., and Rubin, D. T. (2011). Randomised clinical trial: delayed-release oral mesalazine 4.8 g/day vs. 2.4 g/day in endoscopic mucosal healing--ASCEND I and II combined analysis. *Aliment. Pharmacol. Ther.* 33, 672–678. doi:10.1111/j.1365-2036.2010.04575.x

Lieleg, O., and Ribbeck, K. (2011). Biological hydrogels as selective diffusion barriers. *Trends Cell Biol.* 21, 543–551. doi:10.1016/j.tcb.2011.06.002

Lie, M., Van Der Giessen, J., Fuhler, G. M., De Lima, A., Peppelenbosch, M. P., Van Der Ent, C., et al. (2018). Low dose Naltrexone for induction of remission in inflammatory bowel disease patients. *J. Transl. Med.* 16, 55. doi:10.1186/s12967-018-1427-5

Li, J., Wang, Z., Chu, Q., Jiang, K., Li, J., and Tang, N. (2018). The strength of mechanical forces determines the differentiation of alveolar epithelial cells. *Dev. Cell* 44, 297–312. doi:10.1016/j.devcel.2018.01.008

Lim, L. G., Neumann, J., Hansen, T., Goetz, M., Hoffman, A., Neurath, M. F., et al. (2014). Confocal endomicroscopy identifies loss of local barrier function in the duodenum of patients with Crohn's disease and ulcerative colitis. *Inflamm. Bowel Dis.* 20, 892–900. doi:10.1097/MIB.0000000000000027

Lin, W. W., Tsay, A. J., Lalime, E. N., Pekosz, A., and Griffin, D. E. (2021). Primary differentiated respiratory epithelial cells respond to apical measles virus infection by shedding multinucleated giant cells. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2013264118. doi:10.1073/pnas.2013264118

Lin, C., Yao, E., Zhang, K., Jiang, X., Croll, S., Thompson-Peer, K., et al. (2017). YAP is essential for mechanical force production and epithelial cell proliferation during lung branching morphogenesis. *Elife* 6, e21130. doi:10.7554/eLife.21130

Liu, G., Saren, L., Douglasson, H., Zhou, X. H., Aberg, P. M., Ollerstam, A., et al. (2021). Precision cut lung slices: an *ex vivo* model for assessing the impact of immunomodulatory therapeutics on lung immune responses. *Arch. Toxicol.* 95, 2871–2877. doi:10.1007/s00204-021-03096-y

Liu, S., Verma, M., Michalec, L., Liu, W., Sripada, A., Rollins, D., et al. (2018). Steroid resistance of airway type 2 innate lymphoid cells from patients with severe asthma: the role of thymic stromal lymphopoietin. *J. Allergy Clin. Immunol.* 141, 257–268. doi:10.1016/j.jaci.2017.03.032

Liu, Z., Wu, H., Jiang, K., Wang, Y., Zhang, W., Chu, Q., et al. (2016). MAPK-mediated YAP activation controls mechanical-tension-induced pulmonary alveolar regeneration. *Cell Rep.* 16, 1810–1819. doi:10.1016/j.celrep.2016.07.020

Li, Y., Zhang, T., Guo, C., Geng, M., Gai, S., Qi, W., et al. (2020b). Bacillus subtilis RZ001 improves intestinal integrity and alleviates colitis by inhibiting the Notch signalling pathway and activating ATOH-1. *Pathog. Dis.* 78, ftaa016. doi:10.1093/femspd/ftaa016

Lopez-Posadas, R., Becker, C., Gunther, C., Tenzer, S., Amann, K., Billmeier, U., et al. (2016). Rho-A prenylation and signaling link epithelial homeostasis to intestinal inflammation. *J. Clin. Invest.* 126, 611–626. doi:10.1172/JCI80997

Luthi, A. U., Cullen, S. P., Mcneela, E. A., Duriez, P. J., Afonina, I. S., Sheridan, C., et al. (2009). Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* 31, 84–98. doi:10.1016/j.immuni.2009.05.007

Ma, T. Y., Iwamoto, G. K., Hoa, N. T., Akotia, V., Pedram, A., Boivin, M. A., et al. (2004). TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. *Am. J. Physiol. Gastrointest. Liver Physiol.* 286, G367–G376. doi:10.1152/ajpgi.00173.2003

Madara, J. L., and Stafford, J. (1989). Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers. *J. Clin. Invest.* 83, 724–727. doi:10.1172/JCI113938

Madsen, K. L., Malfair, D., Gray, D., Doyle, J. S., Jewell, L. D., and Fedorak, R. N. (1999). Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. *Inflamm. Bowel Dis.* 5, 262–270. doi:10.1097/00054725-199911000-00004

Mahapatro, M., Erkert, L., and Becker, C. (2021). Cytokine-mediated crosstalk between immune cells and epithelial cells in the gut. *Cells* 10, 111. doi:10.3390/cells10010111

Mahoney, J. E., Mori, M., Szymaniak, A. D., Varelas, X., and Cardoso, W. V. (2014). The hippo pathway effector Yap controls patterning and differentiation of airway epithelial progenitors. *Dev. Cell* 30, 137–150. doi:10.1016/j.devcel.2014.06.003

Marchiando, A. M., Shen, L., Graham, W. V., Weber, C. R., Schwarz, B. T., Austin, J. R., et al. (2010). Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation *in vivo*. *J. Cell Biol.* 189, 111–126. doi:10.1083/jcb.200902153

Marguet, C., Jouen-Boedes, F., Dean, T. P., and Warner, J. O. (1999). Bronchoalveolar cell profiles in children with asthma, infantile wheeze, chronic cough, or cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 159, 1533–1540. doi:10.1164/ajrccm.159.5.9805028

Martens, E. C., Roth, R., Heuser, J. E., and Gordon, J. I. (2009). Coordinate regulation of glycan degradation and polysaccharide capsule biosynthesis by a prominent human gut symbiont. *J. Biol. Chem.* 284, 18445–18457. doi:10.1074/jbc.M109.008094

Martin, C., Uhlig, S., and Ullrich, V. (1996). Videomicroscopy of methacholine-induced contraction of individual airways in precision-cut lung slices. *Eur. Respir. J.* 9, 2479–2487. doi:10.1183/09031936.96.09122479

Martinez-Sanchez, L. D. C., Ngo, P. A., Pradhan, R., Becker, L. S., Boehringer, D., Soteriou, D., et al. (2022). Epithelial RAC1-dependent cytoskeleton dynamics controls cell mechanics, cell shedding and barrier integrity in intestinal inflammation. *Gut* 72, 275–294. doi:10.1136/gutjnl-2021-325520

Martinez-Silgado, A., Beumer, J., and Clevers, H. (2023). Directed differentiation of murine and human small intestinal organoids toward all mature lineages. *Methods Mol. Biol.* 2650, 107–122. doi:10.1007/978-1-0716-3076-1_9

Martini, E., Krug, S. M., Siegmund, B., Neurath, M. F., and Becker, C. (2017). Mend your fences: the epithelial barrier and its relationship with mucosal immunity in inflammatory bowel disease. *Cell Mol. Gastroenterol. Hepatol.* 4, 33–46. doi:10.1016/j.jcmgh.2017.03.007

Ma, Y., Yue, J., Zhang, Y., Shi, C., Odenwald, M., Liang, W. G., et al. (2017). ACF7 regulates inflammatory colitis and intestinal wound response by orchestrating tight junction dynamics. *Nat. Commun.* 8, 15375. doi:10.1038/ncomms15375

Mcclatchey, A. I., and Yap, A. S. (2012). Contact inhibition (of proliferation) redux. *Curr. Opin. Cell Biol.* 24, 685–694. doi:10.1016/j.ceb.2012.06.009

Mckinley, E. T., Sui, Y., Al-Kofahi, Y., Millis, B. A., Tyska, M. J., Roland, J. T., et al. (2017). Optimized multiplex immunofluorescence single-cell analysis reveals tuft cell heterogeneity. *JCI Insight* 2, e93487. doi:10.1172/jci.insight.93487

Meddings, J. B., and Gibbons, I. (1998). Discrimination of site-specific alterations in gastrointestinal permeability in the rat. *Gastroenterology* 114, 83–92. doi:10.1016/s0016-5085(98)70636-5

Meldrum, O. W., and Chotirmall, S. H. (2021). Mucus, microbiomes and pulmonary disease. *Biomedicines* 9, 675. doi:10.3390/biomedicines9060675

Melrose, A. G. (1955). The geographical incidence of chronic ulcerative colitis in Britain. *Gastroenterology* 29, 1055–1060. doi:10.1016/s0016-5085(19)35927-x

Meng, X. M., Nikolic-Paterson, D. J., and Lan, H. Y. (2016). TGF-β: the master regulator of fibrosis. *Nat. Rev. Nephrol.* 12, 325–338. doi:10.1038/nrneph.2016.48

Moheimani, F., Hsu, A. C., Reid, A. T., Williams, T., Kicic, A., Stick, S. M., et al. (2016). The genetic and epigenetic landscapes of the epithelium in asthma. *Respir. Res.* 17, 119. doi:10.1186/s12931-016-0434-4

Montassier, E., Kitsios, G. D., Radder, J. E., Le Bastard, Q., Kelly, B. J., Panzer, A., et al. (2023). Robust airway microbiome signatures in acute respiratory failure and hospital-acquired pneumonia. *Nat. Med.* 29, 2793–2804. doi:10.1038/s41591-023-02617-9

Montoro, D. T., Haber, A. L., Biton, M., Vinarsky, V., Lin, B., Birket, S. E., et al. (2018). A revised airway epithelial hierarchy includes CFTR-expressing ionocytes. *Nature* 560, 319–324. doi:10.1038/s41586-018-0393-7

Mookherjee, N., Anderson, M. A., Haagsman, H. P., and Davidson, D. J. (2020). Antimicrobial host defence peptides: functions and clinical potential. *Nat. Rev. Drug Discov.* 19, 311–332. doi:10.1038/s41573-019-0058-8

Moor, A. E., Harnik, Y., Ben-Moshe, S., Massasa, E. E., Rozenberg, M., Eilam, R., et al. (2018). Spatial reconstruction of single enterocytes uncovers broad zonation along the intestinal villus Axis. *Cell* 175, 1156–1167. doi:10.1016/j.cell.2018.08.063

Moshiri, J., Craven, A. R., Mixon, S. B., Amieva, M. R., and Kirkegaard, K. (2023). Mechanosensitive extrusion of Enterovirus A71-infected cells from colonic organoids. *Nat. Microbiol.* 8, 629–639. doi:10.1038/s41564-023-01339-5

Munkholm, P., Langholz, E., Hollander, D., Thornberg, K., Orholm, M., Katz, K. D., et al. (1994). Intestinal permeability in patients with Crohn's disease and ulcerative colitis and their first degree relatives. *Gut* 35, 68–72. doi:10.1136/gut.35.1.68

Naydenov, N. G., Feygin, A., Wang, D., Kuemmerle, J. F., Harris, G., Conti, M. A., et al. (2016). Nonmuscle myosin IIA regulates intestinal epithelial barrier *in vivo* and plays a protective role during experimental colitis. *Sci. Rep.* 6, 24161. doi:10.1038/srep24161

Neurath, M. F., and Travis, S. P. (2012). Mucosal healing in inflammatory bowel diseases: a systematic review. *Gut* 61, 1619–1635. doi:10.1136/gutjnl-2012-302830

Neurath, M. F., and Vieth, M. (2023). Different levels of healing in inflammatory bowel diseases: mucosal, histological, transmural, barrier and complete healing. *Gut* 72, 2164–2183. doi:10.1136/gutjnl-2023-329964

Nowarski, R., Jackson, R., Gagliani, N., De Zoete, M. R., Palm, N. W., Bailis, W., et al. (2015). Epithelial IL-18 equilibrium controls barrier function in colitis. *Cell* 163, 1444–1456. doi:10.1016/j.cell.2015.10.072

Olson, T. S., Reuter, B. K., Scott, K. G., Morris, M. A., Wang, X. M., Hancock, L. N., et al. (2006). The primary defect in experimental ileitis originates from a nonhematopoietic source. *J. Exp. Med.* 203, 541–552. doi:10.1084/jem.20050407

Ortiz-Zapater, E., Bagley, D. C., Hernandez, V. L., Roberts, L. B., Maguire, T. J. A., Voss, F., et al. (2022a). Epithelial coxsackievirus adenovirus receptor promotes house dust mite-induced lung inflammation. *Nat. Commun.* 13, 6407. doi:10.1038/s41467-022-33882-w

Ortiz-Zapater, E., Signes-Costa, J., Montero, P., and Roger, I. (2022b). Lung fibrosis and fibrosis in the lungs: is it all about myofibroblasts? *Biomedicines* 10, 1423. doi:10.3390/biomedicines10061423

Oshima, T., Miwa, H., and Joh, T. (2008). Changes in the expression of claudins in active ulcerative colitis. *J. Gastroenterol. Hepatol.* 23 (Suppl. 2), S146–S150. doi:10.1111/j.1440-1746.2008.05405.x

Palm, N. W., De Zoete, M. R., Cullen, T. W., Barry, N. A., Stefanowski, J., Hao, L., et al. (2014). Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell* 158, 1000–1010. doi:10.1016/j.cell.2014.08.006

Panek, M., Stawiski, K., Kaszkowiak, M., and Kuna, P. (2022). Cytokine TGFβ gene polymorphism in asthma: TGF-related SNP analysis enhances the prediction of disease diagnosis (A case-control study with multivariable data-mining model development). *Front. Immunol.* 13, 746360. doi:10.3389/fimmu.2022.746360

Pannekoek, W. J., De Rooij, J., and Gloerich, M. (2019). Force transduction by cadherin adhesions in morphogenesis. *F1000Res* 8, F1000 Faculty Rev-1044. doi:10.12688/f1000research.18779.1

Paone, P., and Cani, P. D. (2020). Mucus barrier, mucins and gut microbiota: the expected slimy partners? *Gut* 69, 2232–2243. doi:10.1136/gutjnl-2020-322260

Papi, A., Brightling, C., Pedersen, S. E., and Reddel, H. K. (2018). Asthma. *Lancet* 391, 783–800. doi:10.1016/S0140-6736(17)33311-1

Parikh, K., Antanaviciute, A., Fawkner-Corbett, D., Jagielowicz, M., Aulicino, A., Lagerholm, C., et al. (2019). Colonic epithelial cell diversity in health and inflammatory bowel disease. *Nature* 567, 49–55. doi:10.1038/s41586-019-0992-y

Park, J. A., Fredberg, J. J., and Drazen, J. M. (2015). Putting the squeeze on airway epithelia. *Physiol. (Bethesda)* 30, 293–303. doi:10.1152/physiol.00004.2015

Park, J. A., and Tschumperlin, D. J. (2009). Chronic intermittent mechanical stress increases MUC5AC protein expression. *Am. J. Respir. Cell Mol. Biol.* 41, 459–466. doi:10.1165/rcmb.2008-0195OC

Pavord, I. D., Beasley, R., Agusti, A., Anderson, G. P., Bel, E., Brusselle, G., et al. (2018). After asthma: redefining airways diseases. *Lancet* 391, 350–400. doi:10.1016/S0140-6736(17)30879-6

Payne, D. N., Rogers, A. V., Adelroth, E., Bandi, V., Guntupalli, K. K., Bush, A., et al. (2003). Early thickening of the reticular basement membrane in children with difficult asthma. *Am. J. Respir. Crit. Care Med.* 167, 78–82. doi:10.1164/rccm.200205-414OC

Perez-Gonzalez, C., Ceada, G., Greco, F., Matejcic, M., Gomez-Gonzalez, M., Castro, N., et al. (2021). Mechanical compartmentalization of the intestinal organoid enables crypt folding and collective cell migration. *Nat. Cell Biol.* 23, 745–757. doi:10.1038/s41556-021-00699-6

Perez-Gonzalez, C., Ceada, G., Matejcic, M., and Trepat, X. (2022). Digesting the mechanobiology of the intestinal epithelium. *Curr. Opin. Genet. Dev.* 72, 82–90. doi:10.1016/j.gde.2021.10.005

Pezzulo, A. A., Tudas, R. A., Stewart, C. G., Buonfiglio, L. G. V., Lindsay, B. D., Taft, P. J., et al. (2019). HSP90 inhibitor geldanamycin reverts IL-13- and IL-17-induced airway goblet cell metaplasia. *J. Clin. Invest.* 129, 744–758. doi:10.1172/JCI123524

Pinkerton, J. W., Kim, R. Y., Koeninger, L., Armbruster, N. S., Hansbro, N. G., Brown, A. C., et al. (2021). Human β-defensin-2 suppresses key features of asthma in murine models of allergic airways disease. *Clin. Exp. Allergy* 51, 120–131. doi:10.1111/cea.13766

Piyadasa, H., Hemshekhar, M., Altieri, A., Basu, S., Van Der Does, A. M., Halayko, A. J., et al. (2018). Immunomodulatory innate defence regulator (IDR) peptide alleviates airway inflammation and hyper-responsiveness. *Thorax* 73, 908–917. doi:10.1136/thoraxjnl-2017-210739

Plasschaert, L. W., Zilionis, R., Choo-Wing, R., Savova, V., Knehr, J., Roma, G., et al. (2018). A single-cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte. *Nature* 560, 377–381. doi:10.1038/s41586-018-0394-6

Pohunek, P., Warner, J. O., Turzikova, J., Kudrmann, J., and Roche, W. R. (2005). Markers of eosinophilic inflammation and tissue re-modelling in children before clinically diagnosed bronchial asthma. *Pediatr. Allergy Immunol.* 16, 43–51. doi:10.1111/j.1399-3038.2005.00239.x

Porsbjerg, C., Melen, E., Lehtimaki, L., and Shaw, D. (2023). Asthma. *Lancet* 401, 858–873. doi:10.1016/S0140-6736(22)02125-0

Probert, C. S., Dignass, A. U., Lindgren, S., Oudkerk Pool, M., and Marteau, P. (2014). Combined oral and rectal mesalazine for the treatment of mild-to-moderately active ulcerative colitis: rapid symptom resolution and improvements in quality of life. *J. Crohns Colitis* 8, 200–207. doi:10.1016/j.crohns.2013.08.007

Puliafito, A., Hufnagel, L., Neveu, P., Streichan, S., Sigal, A., Fygenson, D. K., et al. (2012). Collective and single cell behavior in epithelial contact inhibition. *Proc. Natl. Acad. Sci. U. S. A.* 109, 739–744. doi:10.1073/pnas.1007809109

Quansah, E., Gardey, E., Ramoji, A., Meyer-Zedler, T., Goehrig, B., Heutelbeck, A., et al. (2023). Intestinal epithelial barrier integrity investigated by label-free techniques in ulcerative colitis patients. *Sci. Rep.* 13, 2681. doi:10.1038/s41598-023-29649-y

Raju, P., Shashikanth, N., Tsai, P. Y., Pongkorpsakol, P., Chanez-Paredes, S., Steinhagen, P. R., et al. (2020). Inactivation of paracellular cation-selective claudin-2 channels attenuates immune-mediated experimental colitis in mice. *J. Clin. Invest.* 130, 5197–5208. doi:10.1172/JCI138697

Raleigh, D. R., Boe, D. M., Yu, D., Weber, C. R., Marchiando, A. M., Bradford, E. M., et al. (2011). Occludin S408 phosphorylation regulates tight junction protein interactions and barrier function. *J. Cell Biol.* 193, 565–582. doi:10.1083/jcb.201010065

Rallabandi, H. R., Yang, H., Oh, K. B., Lee, H. C., Byun, S. J., and Lee, B. R. (2020). Evaluation of intestinal epithelial barrier function in inflammatory bowel diseases using murine intestinal organoids. *Tissue Eng. Regen. Med.* 17, 641–650. doi:10.1007/s13770-020-00278-0

Ramsey, K. A., Chen, A. C. H., Radicioni, G., Lourie, R., Martin, M., Broomfield, A., et al. (2020). Airway mucus hyperconcentration in non-cystic fibrosis bronchiectasis. *Am. J. Respir. Crit. Care Med.* 201, 661–670. doi:10.1164/rccm.201906-1219OC

Rath, T., Atreya, R., Bodenschatz, J., Uter, W., Geppert, C. E., Vitali, F., et al. (2023). Intestinal barrier healing is superior to endoscopic and histologic remission for predicting major adverse outcomes in inflammatory bowel disease: the prospective ERIca trial. *Gastroenterology* 164, 241–255. doi:10.1053/j.gastro.2022.10.014

Rath, T., Atreya, R., and Neurath, M. F. (2021). Is histological healing a feasible endpoint in ulcerative colitis? *Expert Rev. Gastroenterol. Hepatol.* 15, 665–674. doi:10.1080/17474124.2021.1880892

Regev, A., Teichmann, S. A., Lander, E. S., Amit, I., Benoist, C., Birney, E., et al. (2017). The human cell atlas. *Elife* 6, e27041. doi:10.7554/eLife.27041

Ressler, B., Lee, R. T., Randell, S. H., Drazen, J. M., and Kamm, R. D. (2000). Molecular responses of rat tracheal epithelial cells to transmembrane pressure. *Am. J. Physiol. Lung Cell Mol. Physiol.* 278, L1264–L1272. doi:10.1152/ajplung.2000.278.6.L1264

Resta-Lenert, S., Smitham, J., and Barrett, K. E. (2005). Epithelial dysfunction associated with the development of colitis in conventionally housed mdr1a-/- mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 289, G153–G162. doi:10.1152/ajpgi.00395.2004

Reynolds, A., Wharton, N., Parris, A., Mitchell, E., Sobolewski, A., Kam, C., et al. (2014). Canonical Wnt signals combined with suppressed TGFβ/BMP pathways promote renewal of the native human colonic epithelium. *Gut* 63, 610–621. doi:10.1136/gutjnl-2012-304067

Roan, F., Obata-Ninomiya, K., and Ziegler, S. F. (2019). Epithelial cell-derived cytokines: more than just signaling the alarm. *J. Clin. Invest.* 129, 1441–1451. doi:10.1172/JCI124606

Roodsant, T., Navis, M., Aknouch, I., Renes, I. B., Van Elburg, R. M., Pajkrt, D., et al. (2020). A human 2D primary organoid-derived epithelial monolayer model to study host-pathogen interaction in the small intestine. *Front. Cell Infect. Microbiol.* 10, 272. doi:10.3389/fcimb.2020.00272

Rosales Gerpe, M. C., Van Vloten, J. P., Santry, L. A., De Jong, J., Mould, R. C., Pelin, A., et al. (2018). Use of precision-cut lung slices as an *ex vivo* tool for evaluating viruses and viral vectors for gene and oncolytic therapy. *Mol. Ther. Methods Clin. Dev.* 10, 245–256. doi:10.1016/j.omtm.2018.07.010

Rosenblatt, J., Raff, M. C., and Cramer, L. P. (2001). An epithelial cell destined for apoptosis signals its neighbors to extrude it by an actin- and myosin-dependent mechanism. *Curr. Biol.* 11, 1847–1857. doi:10.1016/s0960-9822(01)00587-5

Roy, S., Esmaeilniakooshkghazi, A., Patnaik, S., Wang, Y., George, S. P., Ahrorov, A., et al. (2018). Villin-1 and Gelsolin regulate changes in actin dynamics that affect cell survival signaling pathways and intestinal inflammation. *Gastroenterology* 154, 1405–1420. doi:10.1053/j.gastro.2017.12.016

Runge, S., and Rosshart, S. P. (2021). The mammalian metaorganism: a holistic view on how microbes of all kingdoms and niches shape local and systemic immunity. *Front. Immunol.* 12, 702378. doi:10.3389/fimmu.2021.702378

Rupani, H., Fong, W. C. G., Kyyaly, A., and Kurukulaaratchy, R. J. (2021). Recent insights into the management of inflammation in asthma. *J. Inflamm. Res.* 14, 4371–4397. doi:10.2147/JIR.S295038

Rutgeerts, P., Van Assche, G., Sandborn, W. J., Wolf, D. C., Geboes, K., Colombel, J. F., et al. (2012). Adalimumab induces and maintains mucosal healing in patients with Crohn's disease: data from the EXTEND trial. *Gastroenterology* 142, 1102–1111 e2. doi:10.1053/j.gastro.2012.01.035

Saenz, S. A., Siracusa, M. C., Perrigoue, J. G., Spencer, S. P., Urban, J. F., JR., Tocker, J. E., et al. (2010). IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature* 464, 1362–1366. doi:10.1038/nature08901

Saito, A. C., Higashi, T., Fukazawa, Y., Otani, T., Tauchi, M., Higashi, A. Y., et al. (2021). Occludin and tricellulin facilitate formation of anastomosing tight-junction strand network to improve barrier function. *Mol. Biol. Cell* 32, 722–738. doi:10.1091/mbc.E20-07-0464

Salimi, M., Barlow, J. L., Saunders, S. P., Xue, L., Gutowska-Owsiak, D., Wang, X., et al. (2013). A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J. Exp. Med.* 210, 2939–2950. doi:10.1084/jem.20130351

Sanchez-Guzman, D., Boland, S., Brookes, O., Mc Cord, C., Lai Kuen, R., Sirri, V., et al. (2021). Long-term evolution of the epithelial cell secretome in preclinical 3D models of the human bronchial epithelium. *Sci. Rep.* 11, 6621. doi:10.1038/s41598-021-86037-0

Sanderson, I. R., Boulton, P., Menzies, I., and Walker-Smith, J. A. (1987). Improvement of abnormal lactulose/rhamnose permeability in active Crohn's disease of the small bowel by an elemental diet. *Gut* 28, 1073–1076. doi:10.1136/gut.28.9.1073

Saxena, K., Blutt, S. E., Ettayebi, K., Zeng, X. L., Broughman, J. R., Crawford, S. E., et al. (2016). Human intestinal enteroids: a new model to study human rotavirus infection, host restriction, and pathophysiology. *J. Virol.* 90, 43–56. doi:10.1128/JVI.01930-15

Schaubeck, M., Clavel, T., Calasan, J., Lagkouvardos, I., Haange, S. B., Jehmlich, N., et al. (2016). Dysbiotic gut microbiota causes transmissible Crohn's disease-like ileitis independent of failure in antimicrobial defence. *Gut* 65, 225–237. doi:10.1136/gutjnl-2015-309333

Schleich, F., Sabbe, M., Moermans, C., and Louis, R. (2024). Tezepelumab (Tezspire®): new biological treatment of severe asthma. *Rev. Med. Liege* 79, 60–64.

Schroeder, B. O. (2019). Fight them or feed them: how the intestinal mucus layer manages the gut microbiota. *Gastroenterol. Rep. (Oxf)* 7, 3–12. doi:10.1093/gastro/goy052

Schwarz, B. T., Wang, F., Shen, L., Clayburgh, D. R., Su, L., Wang, Y., et al. (2007). LIGHT signals directly to intestinal epithelia to cause barrier dysfunction via cytoskeletal and endocytic mechanisms. *Gastroenterology* 132, 2383–2394. doi:10.1053/j.gastro.2007.02.052

Seidelin, J. B., Bjerrum, J. T., Coskun, M., Widjaya, B., Vainer, B., and Nielsen, O. H. (2010). IL-33 is upregulated in colonocytes of ulcerative colitis. *Immunol. Lett.* 128, 80–85. doi:10.1016/j.imlet.2009.11.001

Shahana, S., Bjornsson, E., Ludviksdottir, D., Janson, C., Nettelbladt, O., Venge, P., et al. (2005). Ultrastructure of bronchial biopsies from patients with allergic and non-allergic asthma. *Respir. Med.* 99, 429–443. doi:10.1016/j.rmed.2004.08.013

Sheih, A., Parks, W. C., and Ziegler, S. F. (2017). GM-CSF produced by the airway epithelium is required for sensitization to cockroach allergen. *Mucosal Immunol.* 10, 705–715. doi:10.1038/mi.2016.90

Shin, W., and Kim, H. J. (2022). 3D *in vitro* morphogenesis of human intestinal epithelium in a gut-on-a-chip or a hybrid chip with a cell culture insert. *Nat. Protoc.* 17, 910–939. doi:10.1038/s41596-021-00674-3

Simms, L. A., Doecke, J. D., Walsh, M. D., Huang, N., Fowler, E. V., and Radford-Smith, G. L. (2008). Reduced alpha-defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease. *Gut* 57, 903–910. doi:10.1136/gut.2007.142588

Sivagnanam, M., Mueller, J. L., Lee, H., Chen, Z., Nelson, S. F., Turner, D., et al. (2008). Identification of EpCAM as the gene for congenital tufting enteropathy. *Gastroenterology* 135, 429–437. doi:10.1053/j.gastro.2008.05.036

Soderholm, J. D., Olaison, G., Lindberg, E., Hannestad, U., Vindels, A., Tysk, C., et al. (1999). Different intestinal permeability patterns in relatives and spouses of patients with Crohn's disease: an inherited defect in mucosal defence? *Gut* 44, 96–100. doi:10.1136/gut.44.1.96

Song, C., Chai, Z., Chen, S., Zhang, H., Zhang, X., and Zhou, Y. (2023). Intestinal mucus components and secretion mechanisms: what we do and do not know. *Exp. Mol. Med.* 55, 681–691. doi:10.1038/s12276-023-00960-y

Stock, P., Lombardi, V., Kohlrautz, V., and Akbari, O. (2009). Induction of airway hyperreactivity by IL-25 is dependent on a subset of invariant NKT cells expressing IL-17RB. *J. Immunol.* 182, 5116–5122. doi:10.4049/jimmunol.0804213

Streichan, S. J., Hoerner, C. R., Schneidt, T., Holzer, D., and Hufnagel, L. (2014). Spatial constraints control cell proliferation in tissues. *Proc. Natl. Acad. Sci. U. S. A.* 111, 5586–5591. doi:10.1073/pnas.1323016111

Sturgeon, C., Lan, J., and Fasano, A. (2017). Zonulin transgenic mice show altered gut permeability and increased morbidity/mortality in the DSS colitis model. *Ann. N. Y. Acad. Sci.* 1397, 130–142. doi:10.1111/nyas.13343

Suenaert, P., Bulteel, V., Lemmens, L., Noman, M., Geypens, B., Van Assche, G., et al. (2002). Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease. *Am. J. Gastroenterol.* 97, 2000–2004. doi:10.1111/j.1572-0241.2002.05914.x

Sugawara, T., Furuse, K., Otani, T., Wakayama, T., and Furuse, M. (2021). Angulin-1 seals tricellular contacts independently of tricellulin and claudins. *J. Cell Biol.* 220, e202005062. doi:10.1083/jcb.202005062

Su, L., Nalle, S. C., Shen, L., Turner, E. S., Singh, G., Breskin, L. A., et al. (2013). TNFR2 activates MLCK-dependent tight junction dysregulation to cause apoptosis-mediated barrier loss and experimental colitis. *Gastroenterology* 145, 407–415. doi:10.1053/j.gastro.2013.04.011

Sumigray, K. D., Terwilliger, M., and Lechler, T. (2018). Morphogenesis and compartmentalization of the intestinal crypt. *Dev. Cell* 45, 183–197. doi:10.1016/j.devcel.2018.03.024

Suzuki, T., Yoshinaga, N., and Tanabe, S. (2011). Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. *J. Biol. Chem.* 286, 31263–31271. doi:10.1074/jbc.M111.238147

Tahaghoghi-Hajghorbani, S., Ajami, A., Ghorbanalipoor, S., Hosseini-Khah, Z., Taghiloo, S., Khaje-Enayati, P., et al. (2019). Protective effect of TSLP and IL-33 cytokines in ulcerative colitis. *Auto. Immun. Highlights* 10, 1. doi:10.1186/s13317-019-0110-z

Takeyama, K., Kondo, M., Akaba, T., and Tamaoki, J. (2015). Profile of airway mucins in bronchoalveolar lavage fluid of patients with pulmonary alveolar proteinosis. (European Respiratory Journal, 46, PA3870. doi:10.1183/13993003.congress-2015.PA3870

Tan, H. T., Hagner, S., Ruchti, F., Radzikowska, U., Tan, G., Altunbulakli, C., et al. (2019). Tight junction, mucin, and inflammasome-related molecules are differentially expressed in eosinophilic, mixed, and neutrophilic experimental asthma in mice. *Allergy* 74, 294–307. doi:10.1111/all.13619

Tang, W., Li, M., Teng, F., Cui, J., Dong, J., and Wang, W. (2022). Single-cell RNA-sequencing in asthma research. *Front. Immunol.* 13, 988573. doi:10.3389/fimmu.2022.988573

Taylor, B. C., Zaph, C., Troy, A. E., Du, Y., Guild, K. J., Comeau, M. R., et al. (2009). TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J. Exp. Med.* 206, 655–667. doi:10.1084/jem.20081499

Teshima, C. W., Dieleman, L. A., and Meddings, J. B. (2012). Abnormal intestinal permeability in Crohn's disease pathogenesis. *Ann. N. Y. Acad. Sci.* 1258, 159–165. doi:10.1111/j.1749-6632.2012.06612.x

Teshima, C. W., and Meddings, J. B. (2008). The measurement and clinical significance of intestinal permeability. *Curr. Gastroenterol. Rep.* 10, 443–449. doi:10.1007/s11894-008-0083-y

Thornton, D. J., Rousseau, K., and Mcguckin, M. A. (2008). Structure and function of the polymeric mucins in airways mucus. *Annu. Rev. Physiol.* 70, 459–486. doi:10.1146/annurev.physiol.70.113006.100702

Tibble, J. A., Sigthorsson, G., Bridger, S., Fagerhol, M. K., and Bjarnason, I. (2000). Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. *Gastroenterology* 119, 15–22. doi:10.1053/gast.2000.8523

Topczewska, P. M., Rompe, Z. A., Jakob, M. O., Stamm, A., Leclère, P. S., Preusser, A., et al. (2023). ILC2 require cell-intrinsic ST2 signals to promote type 2 immune responses. *Front. Immunol.* 14, 1130933. doi:10.3389/fimmu.2023.1130933

Travaglini, K. J., Nabhan, A. N., Penland, L., Sinha, R., Gillich, A., Sit, R. V., et al. (2020). A molecular cell atlas of the human lung from single-cell RNA sequencing. *Nature* 587, 619–625. doi:10.1038/s41586-020-2922-4

Travers, J., Rochman, M., Miracle, C. E., Habel, J. E., Brusilovsky, M., Caldwell, J. M., et al. (2018). Chromatin regulates IL-33 release and extracellular cytokine activity. *Nat. Commun.* 9, 3244. doi:10.1038/s41467-018-05485-x

Treveil, A., Sudhakar, P., Matthews, Z. J., Wrzesinski, T., Jones, E. J., Brooks, J., et al. (2020). Regulatory network analysis of Paneth cell and goblet cell enriched gut organoids using transcriptomics approaches. *Mol. Omics* 16, 39–58. doi:10.1039/c9mo00130a

Tschumperlin, D. J., Shively, J. D., Kikuchi, T., and Drazen, J. M. (2003). Mechanical stress triggers selective release of fibrotic mediators from bronchial epithelium. *Am. J. Respir. Cell Mol. Biol.* 28, 142–149. doi:10.1165/rcmb.2002-0121OC

Turpin, W., Lee, S. H., Raygoza Garay, J. A., Madsen, K. L., Meddings, J. B., Bedrani, L., et al. (2020). Increased intestinal permeability is associated with later development of crohn's disease. *Gastroenterology* 159, 2092–2100 e5. doi:10.1053/j.gastro.2020.08.005

Tytgat, K. M., Van Der Wal, J. W., Einerhand, A. W., Buller, H. A., and Dekker, J. (1996). Quantitative analysis of MUC2 synthesis in ulcerative colitis. *Biochem. Biophys. Res. Commun.* 224, 397–405. doi:10.1006/bbrc.1996.1039

Uroz, M., Wistorf, S., Serra-Picamal, X., Conte, V., Sales-Pardo, M., Roca-Cusachs, P., et al. (2018). Regulation of cell cycle progression by cell-cell and cell-matrix forces. *Nat. Cell Biol.* 20, 646–654. doi:10.1038/s41556-018-0107-2

Van Der Lugt, B., Van Beek, A. A., Aalvink, S., Meijer, B., Sovran, B., Vermeij, W. P., et al. (2019). Akkermansia muciniphila ameliorates the age-related decline in colonic mucus thickness and attenuates immune activation in accelerated aging Ercc1 -/Δ7 mice. *Immun. Ageing* 16, 6. doi:10.1186/s12979-019-0145-z

Van De Wetering, M., Francies, H. E., Francis, J. M., Bounova, G., Iorio, F., Pronk, A., et al. (2015). Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell* 161, 933–945. doi:10.1016/j.cell.2015.03.053

Van Itallie, C. M., Fanning, A. S., Holmes, J., and Anderson, J. M. (2010). Occludin is required for cytokine-induced regulation of tight junction barriers. *J. Cell Sci.* 123, 2844–2852. doi:10.1242/jcs.065581

Van Rijt, L. S., Vos, N., Willart, M., Muskens, F., Tak, P. P., Van Der Horst, C., et al. (2011). Persistent activation of dendritic cells after resolution of allergic airway inflammation breaks tolerance to inhaled allergens in mice. *Am. J. Respir. Crit. Care Med.* 184, 303–311. doi:10.1164/rccm.201101-0019OC

Vasanthakumar, A., Moro, K., Xin, A., Liao, Y., Gloury, R., Kawamoto, S., et al. (2015). The transcriptional regulators IRF4, BATF and IL-33 orchestrate development and maintenance of adipose tissue-resident regulatory T cells. *Nat. Immunol.* 16, 276–285. doi:10.1038/ni.3085

Veerati, P. C., Mitchel, J. A., Reid, A. T., Knight, D. A., Bartlett, N. W., Park, J. A., et al. (2020). Airway mechanical compression: its role in asthma pathogenesis and progression. *Eur. Respir. Rev.* 29, 190123. doi:10.1183/16000617.0123-2019

Vieira Braga, F. A., Kar, G., Berg, M., Carpaij, O. A., Polanski, K., Simon, L. M., et al. (2019). A cellular census of human lungs identifies novel cell states in health and in asthma. *Nat. Med.* 25, 1153–1163. doi:10.1038/s41591-019-0468-5

Vivinus-Nebot, M., Frin-Mathy, G., Bzioueche, H., Dainese, R., Bernard, G., Anty, R., et al. (2014). Functional bowel symptoms in quiescent inflammatory bowel diseases: role of epithelial barrier disruption and low-grade inflammation. *Gut* 63, 744–752. doi:10.1136/gutjnl-2012-304066

Von Moltke, J., Ji, M., Liang, H. E., and Locksley, R. M. (2016). Tuft-cell-derived IL-25 regulates an intestinal ILC2-epithelial response circuit. *Nature* 529, 221–225. doi:10.1038/nature16161

Wang, F., Graham, W. V., Wang, Y., Witkowski, E. D., Schwarz, B. T., and Turner, J. R. (2005). Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. *Am. J. Pathol.* 166, 409–419. doi:10.1016/s0002-9440(10)62264-x

Warren, S., and Sommers, S. C. (1948). Cicatrizing enteritis as a pathologic entity; analysis of 120 cases. *Am. J. Pathol.* 24, 475–501.

Watson, A. J. M., Duckworth, C. A., Guan, Y., and Montrose, M. H. (2009). Mechanisms of epithelial cell shedding in the Mammalian intestine and

maintenance of barrier function. *Ann. N. Y. Acad. Sci.* 1165, 135–142. doi:10.1111/j.1749-6632.2009.04027.x

Wawrzyniak, P., Wawrzyniak, M., Wanke, K., Sokolowska, M., Bendelja, K., Ruckert, B., et al. (2017). Regulation of bronchial epithelial barrier integrity by type 2 cytokines and histone deacetylases in asthmatic patients. *J. Allergy Clin. Immunol.* 139, 93–103. doi:10.1016/j.jaci.2016.03.050

Weber, C. R., Raleigh, D. R., Su, L., Shen, L., Sullivan, E. A., Wang, Y., et al. (2010). Epithelial myosin light chain kinase activation induces mucosal interleukin-13 expression to alter tight junction ion selectivity. *J. Biol. Chem.* 285, 12037–12046. doi:10.1074/jbc.M109.064808

Weber, F. (2014). *Antiviral innate immunity: introduction☆*. Reference Module in biomedical sciences. Elsevier. 2014 cited 2023 May 31. doi:10.1016/B978-0-12-801238-3.01886-9

Wehkamp, J., Salzman, N. H., Porter, E., Nuding, S., Weichenthal, M., Petras, R. E., et al. (2005). Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc. Natl. Acad. Sci. U. S. A.* 102, 18129–18134. doi:10.1073/pnas.0505256102

Welsh, K. G., Rousseau, K., Fisher, G., Bonser, L. R., Bradding, P., Brightling, C. E., et al. (2017). MUC5AC and a glycosylated variant of MUC5B alter mucin composition in children with acute asthma. *Chest* 152, 771–779. doi:10.1016/j.chest.2017.07.001

Widdicombe, J. H. (2019). Early studies on the surface epithelium of mammalian airways. *Am. J. Physiol. Lung Cell Mol. Physiol.* 317, L486–L495. doi:10.1152/ajplung.00240.2019

Wild, G. E., Waschke, K. A., Bitton, A., and Thomson, A. B. (2003). The mechanisms of prednisone inhibition of inflammation in Crohn's disease involve changes in intestinal permeability, mucosal TNFalpha production and nuclear factor kappa B expression. *Aliment. Pharmacol. Ther.* 18, 309–317. doi:10.1046/j.1365-2036.2003.01611.x

Wlodarska, M., Luo, C., Kolde, R., D'Hennezel, E., Annand, J. W., Heim, C. E., et al. (2017). Indoleacrylic acid produced by commensal peptostreptococcus species suppresses inflammation. *Cell Host Microbe* 22, 25–37. doi:10.1016/j.chom.2017.06.007

Wu, W., Lv, L., Shi, D., Ye, J., Fang, D., Guo, F., et al. (2017). Protective effect of akkermansia muciniphila against immune-mediated liver injury in a mouse model. *Front. Microbiol.* 8, 1804. doi:10.3389/fmicb.2017.01804

Wyatt, J., Vogelsang, H., Hubl, W., Waldhoer, T., and Lochs, H. (1993). Intestinal permeability and the prediction of relapse in Crohn's disease. *Lancet* 341, 1437–1439. doi:10.1016/0140-6736(93)90882-h

Xu, P., Elizalde, M., Masclee, A., Pierik, M., and Jonkers, D. (2021). Corticosteroid enhances epithelial barrier function in intestinal organoids derived from patients with Crohn's disease. *J. Mol. Med. Berl.* 99, 805–815. doi:10.1007/s00109-021-02045-7

Yang, Q., Xue, S. L., Chan, C. J., Rempfler, M., Vischi, D., Maurer-Gutierrez, F., et al. (2021). Cell fate coordinates mechano-osmotic forces in intestinal crypt formation. *Nat. Cell Biol.* 23, 733–744. doi:10.1038/s41556-021-00700-2

Yoo, J. H., and Donowitz, M. (2019). Intestinal enteroids/organoids: a novel platform for drug discovery in inflammatory bowel diseases. *World J. Gastroenterol.* 25, 4125–4147. doi:10.3748/wjg.v25.i30.4125

Yui, S., Azzolin, L., Maimets, M., Pedersen, M. T., Fordham, R. P., Hansen, S. L., et al. (2018). YAP/TAZ-Dependent reprogramming of colonic epithelium links ECM remodeling to tissue regeneration. *Cell Stem Cell* 22, 35–49. doi:10.1016/j.stem.2017.11.001

Yui, S., Nakamura, T., Sato, T., Nemoto, Y., Mizutani, T., Zheng, X., et al. (2012). Functional engraftment of colon epithelium expanded *in vitro* from a single adult Lgr5$^+$ stem cell. *Nat. Med.* 18, 618–623. doi:10.1038/nm.2695

Yun, S. M., Kim, S. H., and Kim, E. H. (2019). The molecular mechanism of transforming growth factor-β signaling for intestinal fibrosis: a mini-review. *Front. Pharmacol.* 10, 162. doi:10.3389/fphar.2019.00162

Zaidi, D., Bording-Jorgensen, M., Huynh, H. Q., Carroll, M. W., Turcotte, J. F., Sergi, C., et al. (2016). Increased epithelial gap density in the noninflamed duodenum of children with inflammatory bowel diseases. *J. Pediatr. Gastroenterol. Nutr.* 63, 644–650. doi:10.1097/MPG.0000000000001182

Zeissig, S., Burgel, N., Gunzel, D., Richter, J., Mankertz, J., Wahnschaffe, U., et al. (2007). Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut* 56, 61–72. doi:10.1136/gut.2006.094375

Zhao, R., Fallon, T. R., Saladi, S. V., Pardo-Saganta, A., Villoria, J., Mou, H., et al. (2014). Yap tunes airway epithelial size and architecture by regulating the identity, maintenance, and self-renewal of stem cells. *Dev. Cell* 30, 151–165. doi:10.1016/j.devcel.2014.06.004

Zhou, J., Alvarez-Elizondo, M. B., Botvinick, E., and George, S. C. (2012). Local small airway epithelial injury induces global smooth muscle contraction and airway constriction. *J. Appl. Physiol. (1985)* 112, 627–637. doi:10.1152/japplphysiol.00739.2011

Zuo, L., Kuo, W. T., Cao, F., Chanez-Paredes, S. D., Zeve, D., Mannam, P., et al. (2023). Tacrolimus-binding protein FKBP8 directs myosin light chain kinase-dependent barrier regulation and is a potential therapeutic target in Crohn's disease. *Gut* 72, 870–881. doi:10.1136/gutjnl-2021-326534

Frontiers in
Cell and Developmental Biology

Explores the fundamental biological processes of life, covering intracellular and extracellular dynamics.

The world's most cited developmental biology journal, advancing our understanding of the fundamental processes of life. It explores a wide spectrum of cell and developmental biology, covering intracellular and extracellular dynamics.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact