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PhD. Thesis

DIETARY TRPM8 LIGANDS IN INFLAMMATION-TRIGGERED GUT DISEASES

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ABSTRACT

Background: The interplay between diet, host genetics, microbiota, and immune system has a key role in the development and progression of gut chronic inflammatory conditions, such as inflammatory bowel diseases (IBDs) and colitis associated colorectal cancer (CAC). Therefore, the search for dietary components that may further enhance immune function in sub-clinical situations or prevent specific immune-related chronic diseases may lead to new therapeutic strategies. The transient receptor potential (TRP) cation channel, subfamily melastatin (M), member 8 (TRPM8), is an ion-gated receptor mainly permeable to calcium. Here, we explored the possible contribution of TRPM8 to intestinal inflammation and inflammation-triggered colon tumorigenesis.

Materials and Methods: A set of natural compounds was tested for TRPM8 affinity by using bioinformatic tools, and the flavonoid luteolin was identified as a novel and selective TRPM8 antagonist. *In vitro*, the experiments were conducted on bone marrow derived macrophages (BMDMs) obtained from wild type (WT), *Trpm8*$^{-/-}$ or IL-10$^{fl/fl}$ LysMCre mice femurs and tibias. TRPM8 expression was evaluated in BMDMs by immunofluorescence. Intracellular calcium measurements were conducted in cells loaded with Ca^{2+}-sensitive fluorescent dye FLUO-4AM. Pro-inflammatory mediators [i.e., interleukin (IL) -6, IL-1β and tumor necrosis factor-α (TNF α)] were quantified in supernatant collected from BMDM cultures, using commercial ELISA kits. Seahorse extracellular flux assay, NMR and GC-MS metabolomics analysis were performed to analyze the metabolic reprogramming of macrophages. In a different set of experiments, BMDMs were pre-treated with luteolin, before assessing calcium transients, cytokine production, and metabolic profile as previously stated. Luteolin was also orally administered in the dextran sodium sulfate (DSS) model of colitis *in vivo*.

Further, to evaluate the TRPM8 involvement in colon carcinogenesis, its expression and correlation with the survival rate was analyzed in patients with CRC patients. To identify the key pathways and genes related to TRPM8 high expression, Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were conducted. Then, the functional role of TRPM8 in inflammation-triggered colon cancer was assessed in the AOM/DSS model of CAC in WT and *Trpm8*$^{-/-}$ mice.

Results: TRPM8 mediates LPS- evoked Ca^{2+} influx in macrophages leading to their activation. TRPM8 is selectively blocked by the dietary flavonoid luteolin (IC50: 3.006 μM), which induced a pro-tolerogenic phenotype in pro-inflammatory BMDMs. Accordingly, genetic deletion of *Trpm8* in BMDMs caused a deficit in the activation of pro-inflammatory metabolic and transcriptional reprogramming, leading to reduced production of key pro-inflammatory

mediators. We proved that TRPM8 anti-inflammatory effect was dependent on lactate which in turn induces downstream epigenetic modifications to regulate IL-10 gene expression. *In vivo*, oral administration of luteolin ameliorated intestinal inflammation through an impairment in the innate immune response.

In CRC patients, TRPM8 is overexpressed in colon tumor specimens, and specifically in CD326+ tumor cell fraction. In such patients TRPM8 high expression was related to lower survival rate, Wnt–Frizzled signaling hyperactivation and APC (adenomatous polyposis coli) down-regulation. In a colitis-associated model of colon cancer, the genetic deletion of TRPM8 reduced tumor development.

Conclusions: Overall, this PhD thesis unmasks the potential of targeting TRPM8 through specific nutrient interventions to regulate immune function in sub-clinical scenarios or to treat inflammatory diseases, primarily driven by chronic immune responses, such as IBD or CAC. Moreover, human data provide valuable insights to propose TRPM8 as a prognostic marker with a negative predictive value for CRC patient survival.

Acronym	Definition
AHR	Aryl Hydrocarbon Receptor
AMPK	AMP-Activated Protein Kinase
ANOVA	Analysis of Variances
AOM	Azoxymethane
BFT	*B. Fragilis* Toxin
BMDM	Bone Marrow-Derived Macrophages
BSA	Bovine Serum Albumin
CAC	Colitis-Associated Colon Cancer
CD	Chron's Disease
CDED	Crohn's Disease Exclusion Diet
CGRP	Calcitonin Gene Related Peptide
CIMP	CpG Island Methylator Phenotype
CMC	Carboxymethyl Cellulose
CMS	Consensus Molecular Subtype
CRC	Colorectal Cancer
DAPI	4′,6-diamidino-2-phenylindole
DDIT4	DNA-Damage-Inducible Transcript 4
DEG	Differentially Expressed Genes
DMEM	Dulbecco's Modified Eagle Medium
DNBS	Dinitrobenzene Sulfonic Acid
DSS	Dextran Sodium Sulphate
ECAR	Extracellular Acidification Rate
EDTA	Ethylenediamine Tetraacetic Acid
EEN	Exclusive Enteral Nutrition
EGF	Endothelial Growth Factor
ESPEN	European Society for Clinical Nutrition and Metabolism
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
FN	*Fusobacterium nucleatum*
GSA	Gene Set Analysis
GSEA	Gene Set Enrichment Analysis
GWAS	Genome-Wide Association Studies
HBSS	Hank's Balanced Salt Solution
HCEC	Healthy Colonic Epithelial Cells
HIF	Hypoxia-Inducible Factor
IBD	Inflammatory Bowel Disease
IBS	Inflammatory Bowel Syndrome
IDH1	Isocitrate Dehydrogenase 1
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IL10R	Interleukin 10 Receptor
iNOS	Inducible Nitric Oxide Synthase
IOIBD	International Organization For the Study of Inflammatory Bowel Disease
KLA	Lysine Lactylation
LDH	Lactate Dehydrogenase

LPS	Lipopolysaccharides
MAPK	Mitogen-Activated Protein Kinase
MEICS	Murine Endoscopic Index of Colitis Severity
MEM	Minimum Essential Medium
MSI	Microsatellite Instability
NSAID	Non-Steroidal Anti-Inflammatory Drugs
PAMPs	Pathogen-Associated Molecular Pattern Molecules
PBS	Phosphate Buffer Saline
PCA	Principal Component Analysis
PEN	Partial Enteral Nutrition
PFI	Progression-Free Interval
PIP2	Phosphatidylinositol 4,5-Biphosphate
PKM2	Phosphofructokinase 2
PKS	Polyketide Synthetase
PPP	Pentose Phosphate Pathway
PUFAs	Polyunsaturated Fatty Acids
RTC	Randomized Control Trials
SCFA	Short-Chain Fatty Acids
sCRC	Sporadic Colorectal Cancer
SNP	Single Nucleotide Polymorfism
SP	P-Substance
SPF	Specific-Pathogen Free
TAM	Tumor-Associated Macrophages
TCA	Tricarboxylic Acid Cycle
TCGA	The Cancer Genome Atlas
TCR	T-cell Receptor
TGF	Transforming Growth Factor
TLR	Toll-Like Receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	Tumor Necrosis Factor
Trp	Tryptophan
TRP	Transient Receptor Potential
TRPA	Transient Receptor Potential Anankyrin
TRPC	Transient Receptor Potential Canonical
TRPM	Transient Receptor Potential Melastatin
TRPML	Transient Receptor Potential Mucolipin
TRPN	Transient Receptor Potential NO-mechano-potential
TRPP	Transient Receptor Potential Polycystin
TRPV	Transient Receptor Potential Vanilloid
UC	Ulcerative Colitis
UI	Uncertainity Interval
VEGF	Vascular Endothelial Growth Factor
VHS	Visceral Hypersensitivity
WT	Wild-Type

INTRODUCTION

Chapter I. Gastrointestinal pathologies triggered by inflammation

1.1 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) is a chronic and debilitating inflammatory disorder affecting the gastrointestinal tract, characterized by a progressive and unpredictable disease course (Baumgart and Carding 2007, Ng, Shi et al. 2017). IBD encompasses two main distinctive entities, i.e., ulcerative colitis (UC) and Crohn's disease (CD). While in UC the inflammation is restricted to the colon mucosa , CD is a more severe condition that involves transmurally any segment of the digestive tract from mouth to anus, although involvement of the terminal ileum is most common (Xavier and Podolsky 2007, Le Berre, Honap et al. 2023). Histopathological features include the presence of a significant number of neutrophils in both lamina propria and crypts and a conspicuous reduction in goblet cell mucin content. Crohn's disease is characterized by aggregation of macrophages, often giving rise to non-caseating granulomas (Figure 1) (Xavier and Podolsky 2007, Lamb, Kennedy et al. 2019). In the context of Crohn's disease, ulcers progress to transmural fissures, and over time, the inflammatory process contributes to the development of abscesses, fistulas, or formation of fibrotic stenosis. This progression may lead to the potential occurrence of secondary occlusion (Cosnes, Cattan et al. 2002, Lamb, Kennedy et al. 2019).

Furthermore, between 10% and 15% of colitis patients cannot be classified as UC or CD after applying clinical, endoscopic, radiologic, and histologic criteria, defining an intermediate condition known as unclassified or indeterminate colitis (Satsangi, Silverberg et al. 2006, Lamb, Kennedy et al. 2019). In addition to the gastrointestinal symptoms, which encompass symptoms like bloody diarrhea in UC, abdominal pain, fever, alterations in bowel habits, or perianal complications in CD, a significant subset of IBD patients suffer extraintestinal manifestations. These manifestations affect organs beyond the gastrointestinal tract, including joints, oral cavity, eyes, skin, liver, as well as the central and peripheral nervous systems (Peyrin-Biroulet, Loftus et al. 2011, Rogler, Singh et al. 2021). The extraintestinal manifestations associated with IBD might arise from nutritional and metabolic complications, a prothrombotic disposition, medication-related adverse effects; however, their primary mechanism is immunological. Not rarely, IBD may coexist with other chronic inflammatory and autoimmune diseases such as psoriasis or type I diabetes (Peyrin-Biroulet, Loftus et al. 2011, Rogler, Singh et al. 2021).

The onset of IBD commonly manifests during the second and third decades of life (Figure 2), with a substantial proportion of afflicted individuals transitioning towards a pattern of relapsing

and chronic disease (Chang 2020). The familiarity of IBD has long been acknowledged, wherein the first-degree relatives of affected individuals exhibit a relative risk that is quintuple or even more pronounced (Borowitz 2022). The hereditary element appears to exert a more potent influence in the context of CD in comparison to UC (Borowitz 2022).

There has been a global rise in the incidence of IBD over the last few decades. In Europe alone, up to 3 million people are affected by IBD, equating to approximately 0.2-0.3% of the entire European population (Ng, Shi et al. 2017, Mak, Zhao et al. 2020). It is of interest that in several countries with historically low rates of IBD, a pattern of rising incidence in the past one to two decades is documented with a Ceiling Incidence Range for CD in the range 23.8–29.3 per 100000, and for UC in the range 23.1–57.9 per 100000 (Figure 3) (Collaborators 2020). The changing epidemiology of IBD across time and geography suggests that environmental factors have a major role in inducing or modifying disease expression. The emergence of IBD in developing nations over the past 25 years suggests that this epidemiological evolution is related to westernization of lifestyle and industrialization, associated with changes in diet, antibiotic use, hygiene status, microbial exposures and pollution, which have been implicated as potential environmental risk factors for IBD (Mak, Zhao et al. 2020).

Due to its chronic nature and onset in young adulthood, annual direct IBD healthcare costs in Europe are estimated at €4.6-5.6 billion (Burisch, Jess et al. 2013, Kaplan 2015), without considering the major losses associated with a substantial decline in patients' quality of life (Reinisch, Sandborn et al. 2007). Although IBD patients share common clinical characteristics, the natural disease course is heterogeneous. Disease behavior can remain indolent or progress towards severe complications with various degrees of mucosal damage and tissue remodeling, which favor the development of ulcers, strictures and fistulae potentially associated with stromal cell activation (Rieder, Zimmermann et al. 2013, Rieder, Fiocchi et al. 2017). Overall, the evolution towards surgical resection occurs in 70% of cases, with a subsequent need for a second surgery in up to 30% of cases (Cosnes, Bourrier et al. 2012, Rieder, Fiocchi et al. 2017).

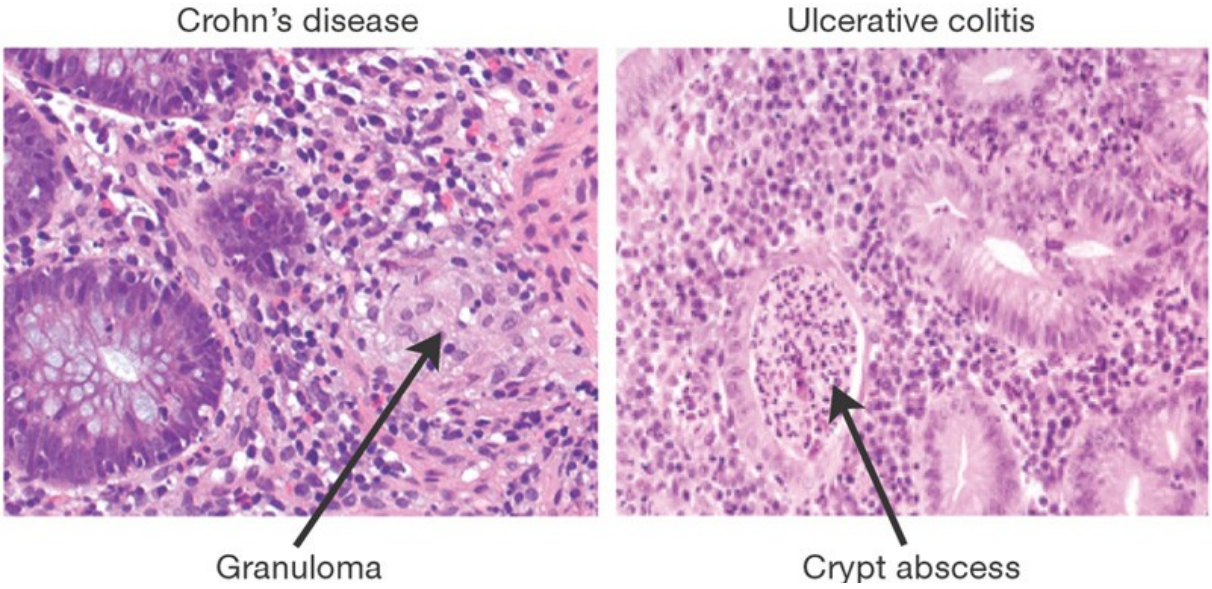


Figure 1. Histologic hallmarks of IBD (from Xavier and Podolsky, 2007). Crohn's diseasebiopsy from a terminal ileum exhibiting active disease in the left figure. The diagram depicts a distinct granuloma consisting of epithelioid cells, large cells, and compact macrophages. There is a noticeable infiltration of lymphoid, plasma, and other inflammatory cells surrounding the nodule, but there isn't any necrosis. Colonic mucosal biopsy from an ulcerative colitis patient in the right figure. The patient is undergoing active disease. Transmigrated neutrophils make up the crypt abscess, and the surrounding epithelium displays signs of recent mucosal damage.

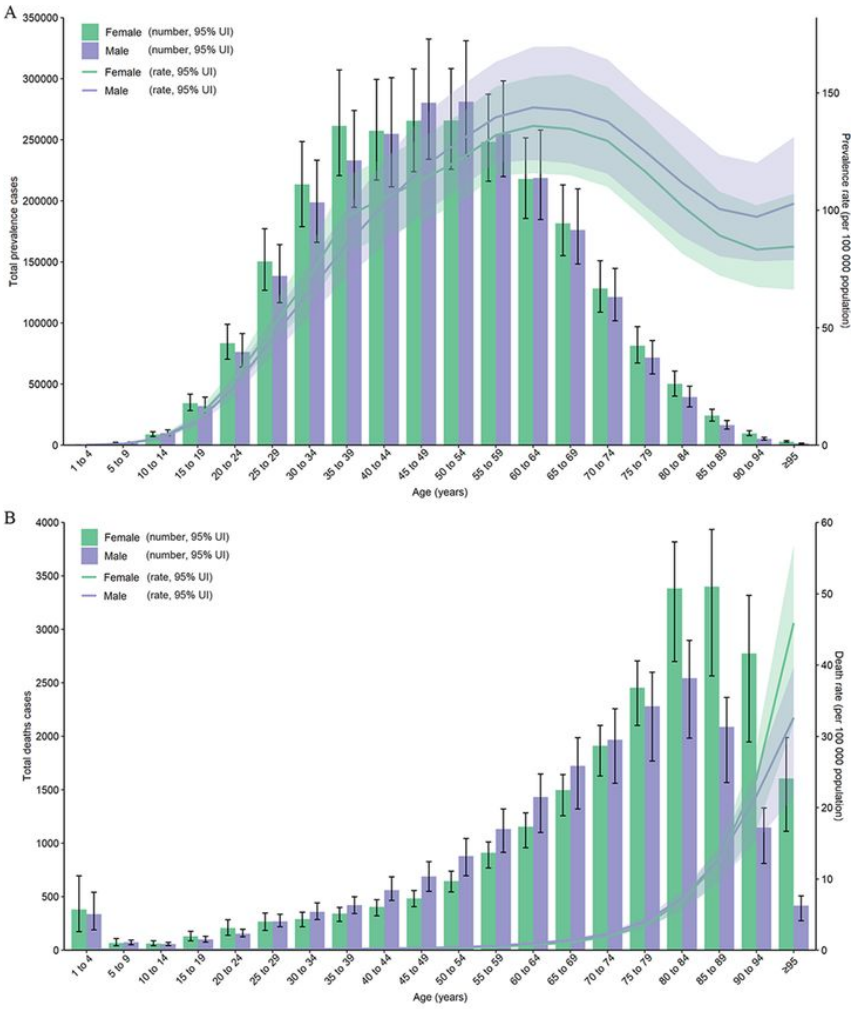


Figure 2. Age patterns by sex of the total number and age-standardized rates of prevalent cases (A) and deaths (B) due to IBD at the global level in 2019 (from Wang et al., 2023). Error bars indicate the 95% uncertainty interval (UI) for the number of cases. Shading indicates the 95% UI for the rates.

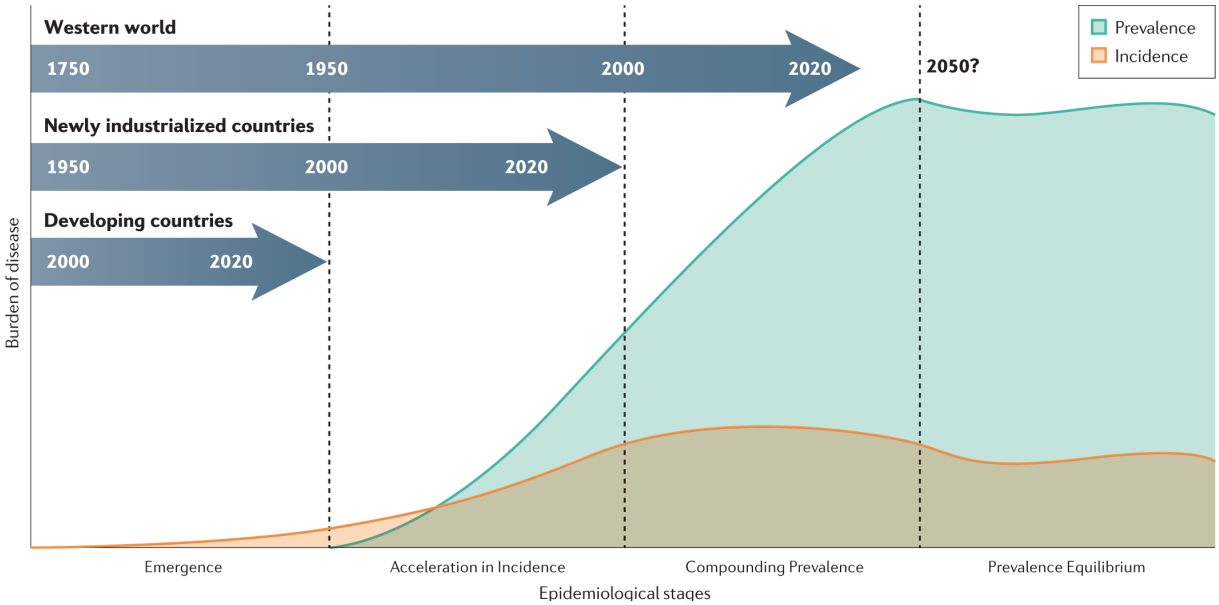


Figure 3. Epidemiological stages of IBD evolution (from Kaplan and Windsor, 2021). The stages of the progression of inflammatory bowel disease (IBD) are represented by the shifting patterns in incidence (orange) and prevalence (blue). In developing nations there are sporadic reports of IBD cases. The newly industrialized nations are experiencing a rising incidence and comparatively low prevalence of the disease. Western nations are currently experiencing a period in which prevalence is sharply increasing while incidence remains constant. When the population with IBD ages and the incidence of the disease increases, the slope of the prevalence increase will level out and enter a stage of equilibrium.

1.2 Pathogenesis of IBD

The barrier that separates the internal gut environment from the external one must be uncomplicated and permeable in order to allow the primary role of the intestinal system, i.e., to extract essential nutrients and water from the diet to support life. This barrier is actually formed by a sole layer of epithelial cells spanning the entire length of the intestine. While this arrangement facilitates the absorption of nutrients and water, it also exposes the body to potential invasion by harmful microorganisms. Therefore, an extensive immune network within the intestine ensures a delicate balance between tolerance to harmless substances and a strong defense against harmful agents (Figure 4). Disruptions to this state can result in the development of diseases, including IBDs (Turner 2009, Chen, Cui et al. 2021). In IBD patients, intestinal inflammation is caused by changes in how resident microbes and the mucosa interact. Such changes can be due to the impact of environmental elements and/or individual-specific factors (Figure 5) (Knights, Lassen et al. 2013, Ramos and Papadakis 2019).

Individual-specific factors are influenced by genetic traits present at multiple susceptibility loci, impacting functions like barrier integrity as well as innate and adaptive immune responses (Figure 6). Genome-wide searches for IBD susceptibility loci identified *NOD2, IBD5, IL23R* and *ATG16L1* as susceptibility genes in CD (Genomes Project, Abecasis et al. 2012, Mirkov, Verstockt et al. 2017). Furthermore, such studies have revealed and confirmed also several intergenic segments linked to IBD. Such intergenic regions evidence the involvement of novel genes and pathways that include those expressed directly within these regions, as well as those that undergo remote regulation, influencing the disease phenotype (Knights, Lassen et al. 2013, Braga-Neto, Gaballa et al. 2020).

The gut microbiome is also susceptible to the influence of host genetics. Approximately one-third of fecal bacterial taxa are heritable (Turpin, Espin-Garcia et al. 2016). A recent cohort study identified 58 single-nucleotide polymorphisms (SNPs) as associated with the relative abundance of 33 taxa. Alterations in taxonomic composition and function of microbiota contribute to the development of intestinal inflammation in IBD (Turpin, Espin-Garcia et al. 2016).

On the other hand, various crucial biological functions in the host involved in the regulation of the response to gut bacteria can be compromised by harmful genetic mutations in their constituent genes, such as NOD2 (Figure 7). This gene plays a pivotal role in triggering the immune system response to certain peptidoglycans produced by bacteria (Trindade and Chen 2020). Moreover, bacterial proteases, including those produced by commensal bacteria, contribute to intestinal inflammation (Steck, Mueller et al. 2012). For instance, the gut

commensal *Enterococcus faecalis* generates a metalloproteinase called gelatinase, which disrupts the epithelial barrier and exacerbates inflammation in mice. This disruption is only evident when the host possesses a genetic predisposition to inflammation, such as through deficiencies in IL10 or NOD2, thus linking the genetic risk of IBD to heightened susceptibility to by-products of commensal bacteria (Steck, Mueller et al. 2012). Finally, the potential correlation between MUC19 and the risk of IBD suggests the existence of an extra mechanism influencing host-microbe interactions. Bacterial species such as *Akkermansia muciniphila* possess the capability to destroy the mucus layer and penetrate within the mucosa (Derrien, van Passel et al. 2010). Thus, hereditary changes in the structure or production of the mucus layer could potentially impact the composition of luminal bacteria.

However, while IBD-associated susceptibility genes and pathways hold great potential to develop new targets and therapies, focusing on these risk factors alone can lead to an incomplete comprehension of the disease pathogenesis (Turpin, Goethel et al. 2018). This is partly because IBD-associated genetic variations are found in many individuals who never develop the disease, and there's a suggestion that traditional loss-of-function variants play a relatively minor role in IBD pathogenesis (Jostins, Ripke et al. 2012).

In addition to the genetic factors that contribute to IBD pathophysiology, environmental factors appear to play a significant role in both the onset and progression of this disease. Antibiotic use, childbirth mode, breastfeeding, air pollution, non-steroidal anti-inflammatory drugs (NSAID) use, hypoxia or high altitude, diet and urban environments have been shown to modify the composition of gut microbiota, affect the function of the mucosal barrier and the immune system (Figure 8) (Ananthakrishnan, Bernstein et al. 2018).

Given the widespread use of antibiotics in both developing and developed nations, it is plausible to view it as a significant predisposing factor in the pathogenesis of IBD (Ungaro, Bernstein et al. 2014). Also, the improper or excessive use of antibiotics, as well as their utilization in livestock, may exacerbate the pathology (Agrawal, Poulsen et al. 2023, Faye, Allin et al. 2023).

Both newborns delivery mode and breastfeeding exert strong influence on gut microbiota composition (Azad, Konya et al. 2016). However, an association between mode of birth and IBD has not been demonstrated in epidemiological studies, while a strong inverse association between breastfeeding and both CD and UC has been shown in numerous studies (Bruce, Black et al. 2014, Bernstein, Banerjee et al. 2016). Moreover, exposure to human milk during the development of the infant immune system may also provide a form of tolerance to dietary and microbial antigens (Azad, Konya et al. 2016).

A higher prevalence of IBD in urban areas has been demonstrated in some epidemiological studies (Benchimol, Kaplan et al. 2017). The impact of urbanization on the development of IBD may be influenced by a range of changes associated with urban development. These changes encompass shifts in lifestyle and behavior, increased exposure to environmental pollutants, and alterations in dietary habits (Kaplan, Hubbard et al. 2010, Benchimol, Kaplan et al. 2017).

Among all the environmental factors, diet is widely believed to play a crucial role in the onset and progression of IBD (Lewis and Abreu 2017, Khalili, Chan et al. 2018). While the precise pathophysiological mechanisms remain elusive, several plausible explanations have been suggested. Diet has a key role in defining the composition of the gut microbiota, the immune tolerance, and the mucosal barrier function, sustaining the hypothesis that dietary modification alters the risk of IBD (Khalili, Chan et al. 2018).

The inclusion of environmental factors introduces a significant level of complexity to the understanding of IBD pathogenesis, highlighting the essential concept that complex diseases necessitate intricate therapeutic strategies that extend beyond the existing approach of single-agent treatments.

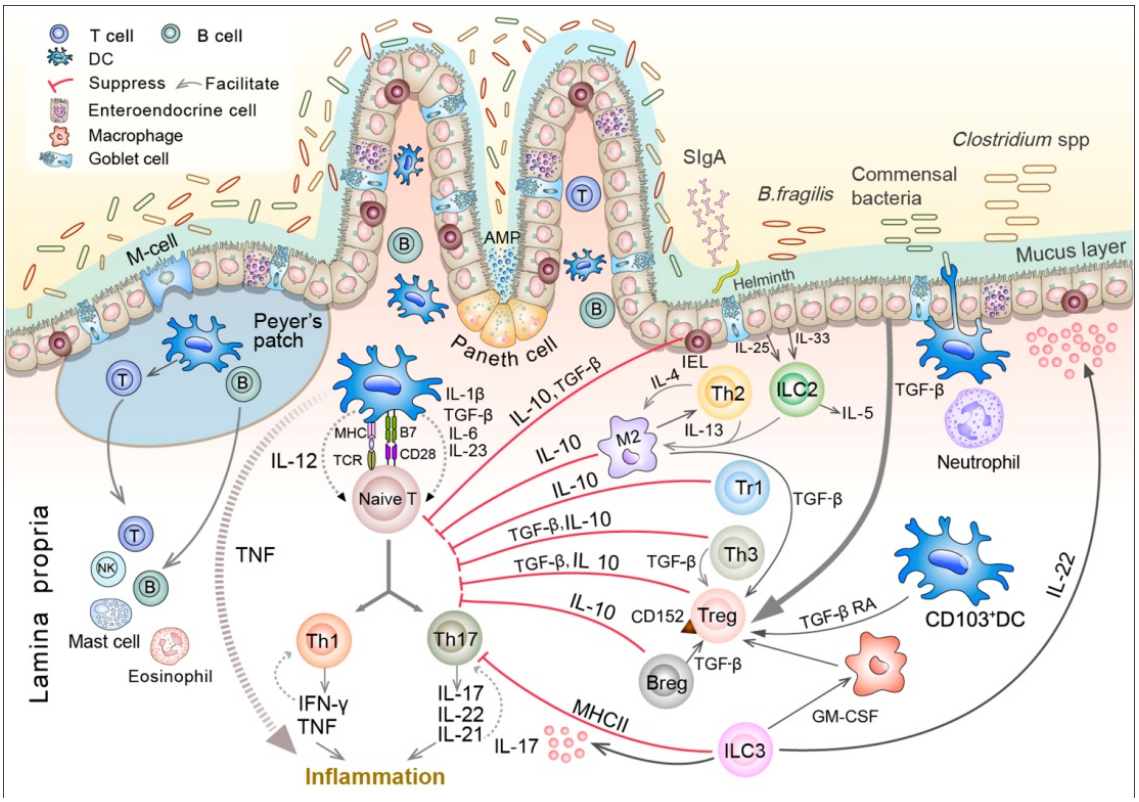
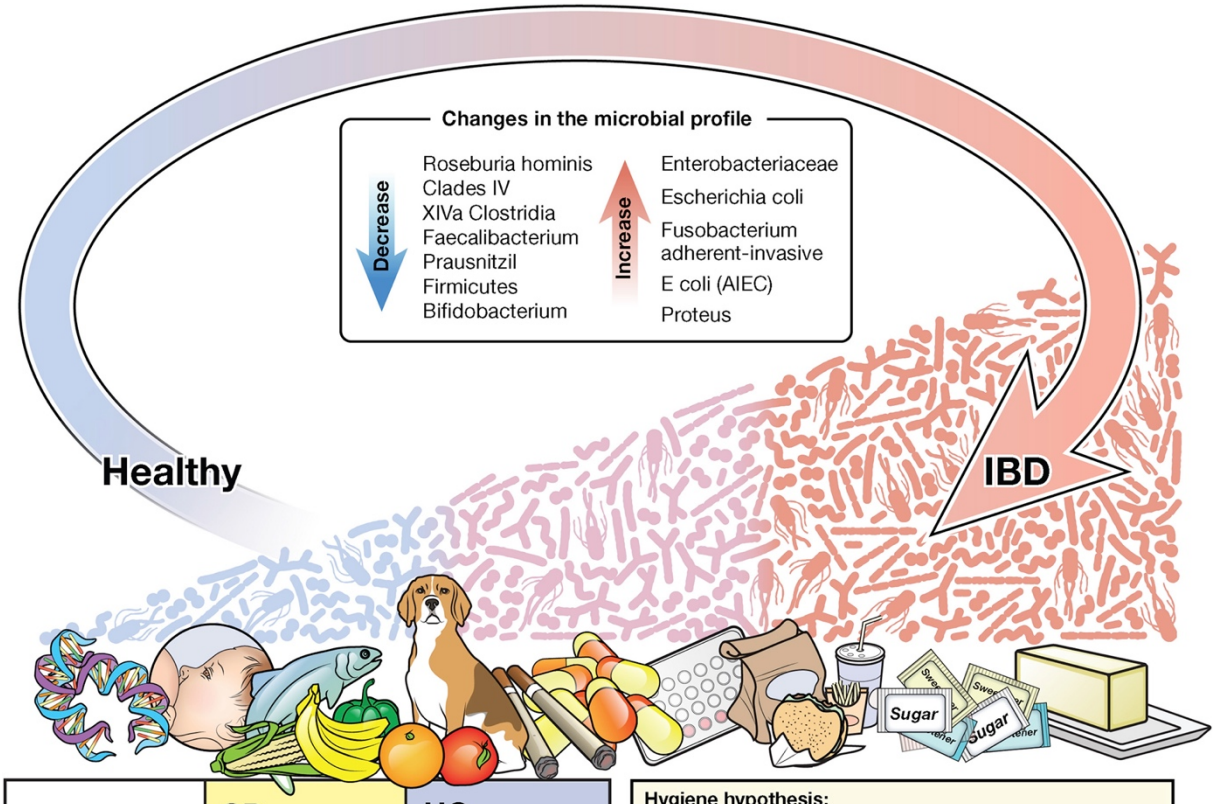


Figure 4. Intestinal barrier composition (from Sun et al., 2015). Schematic representation of the location of immune regulatory cells and their signaling cascades in the lamina propria of gut. The luminal microbiome lies adjacent to the intestinal epithelium. Breach of the tolerance leads to the development of inflammatory bowel diseases (IBD), inflammation, and tumor.



	CD	UC
Smoking	Risk in Caucasians and Middle Eastern migrants	Protective in Caucasians and Asians
Antibiotic use in childhood	Risk in Caucasians, protective in Asians/ Middle Eastern migrants	Risk in Caucasians, protective in Asians/ Middle Eastern migrants
Breastfeeding	Protective in Asians and most studies in Caucasians	Protective in Asians and most studies in Caucasians
Oral contraceptives	Risk in Caucasians	Inconclusive
Appendectomy	Risk in Caucasians	Protective in Caucasians
Low levels of vitamin D	Risk in Caucasians	Risk in Caucasians
Tea or coffee consumption	Protective in Asians	Protective in Asians

Hygiene hypothesis:

Having pets in childhood, living on a farm, larger family size, and drinking unpasteurized milk were inversely associated with the risk of CD and UC

Changing diet:

Introduction of packaged food, fast food chains, increased use of antibiotics, increased fat (monounsaturated and polyunsaturated fatty acids) consumption and sugar intake, less dietary fibers is associated with risk of IBD

Dietary chemicals:

Food additives – saccharin, sucralose, carboxymethycellulose and polysorbate-80, common emulsifiers (including polysorbates, sorbate esters, lecithin), might increase risk of IBD (data are derived from animal models)

Over 200 IBD risk loci (37 specific for Crohn's disease and 32 for ulcerative colitis) have been discovered. However, modest fraction of predicted heritability can be explained by known genes or loci.

Figure 5. The genetic, environmental, and microbial determinants of IBD (from Kaplan and Ng, 2017)**.** Several environmental risk factors have been identified as associated with IBD. These factors include cigarette smoking, antibiotic use during childhood, breastfeeding, oral contraceptives, appendectomy, low levels of vitamin D, and tea or coffee consumption. Moreover, genetic variables, together with particular dietary components and metabolites, influence the gut microbiota and hence the risk of inflammation.

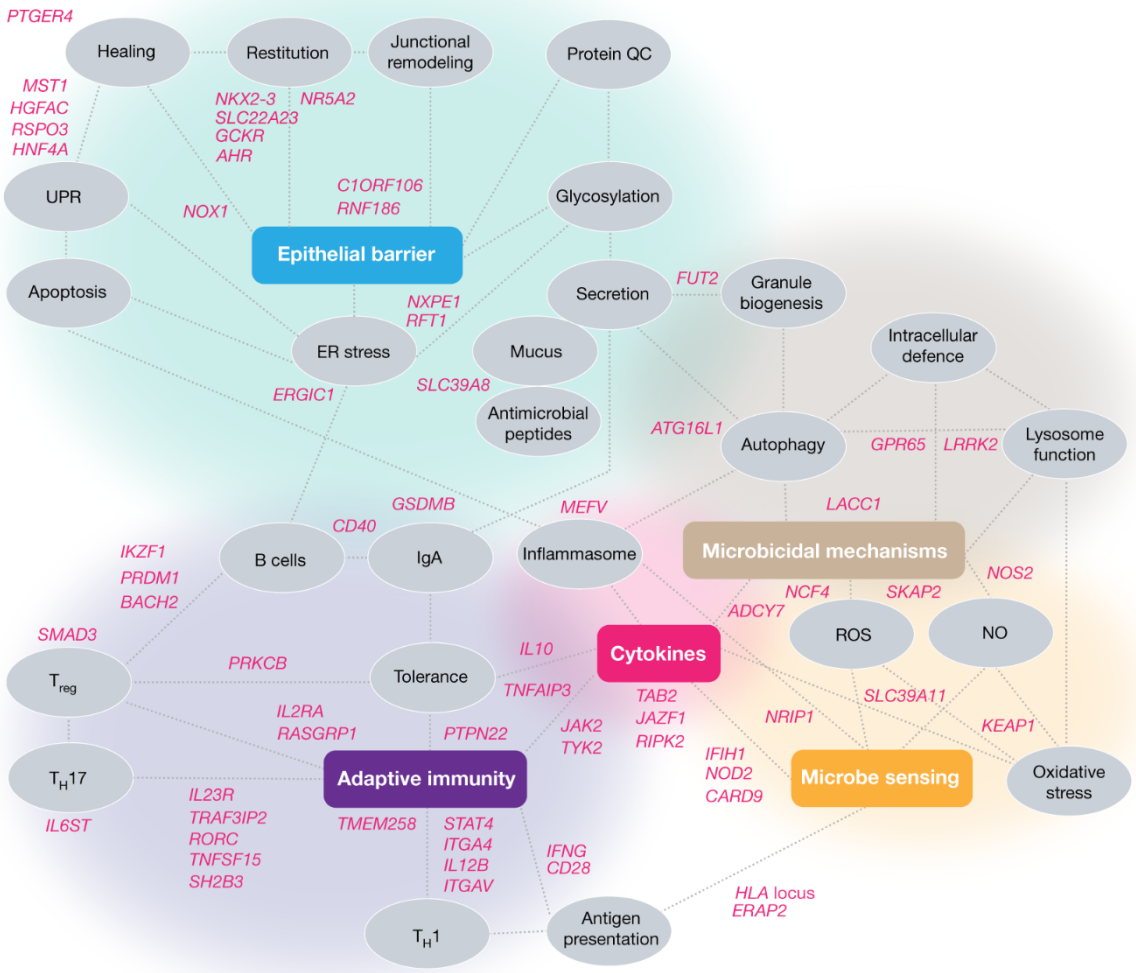


Figure 6. IBD risk genes and pathways (from Graham and Xavier, 2020). IBD genes (red text) have been implicated in key biological functions (grey circles) that are controlled by interconnected molecular pathways (coloured rectangles). Nodes are connected by lines that show how common genes overlap in their molecular regulation. Certain IBD risk genes control different biological processes based on the activities that are specific to the cell type.

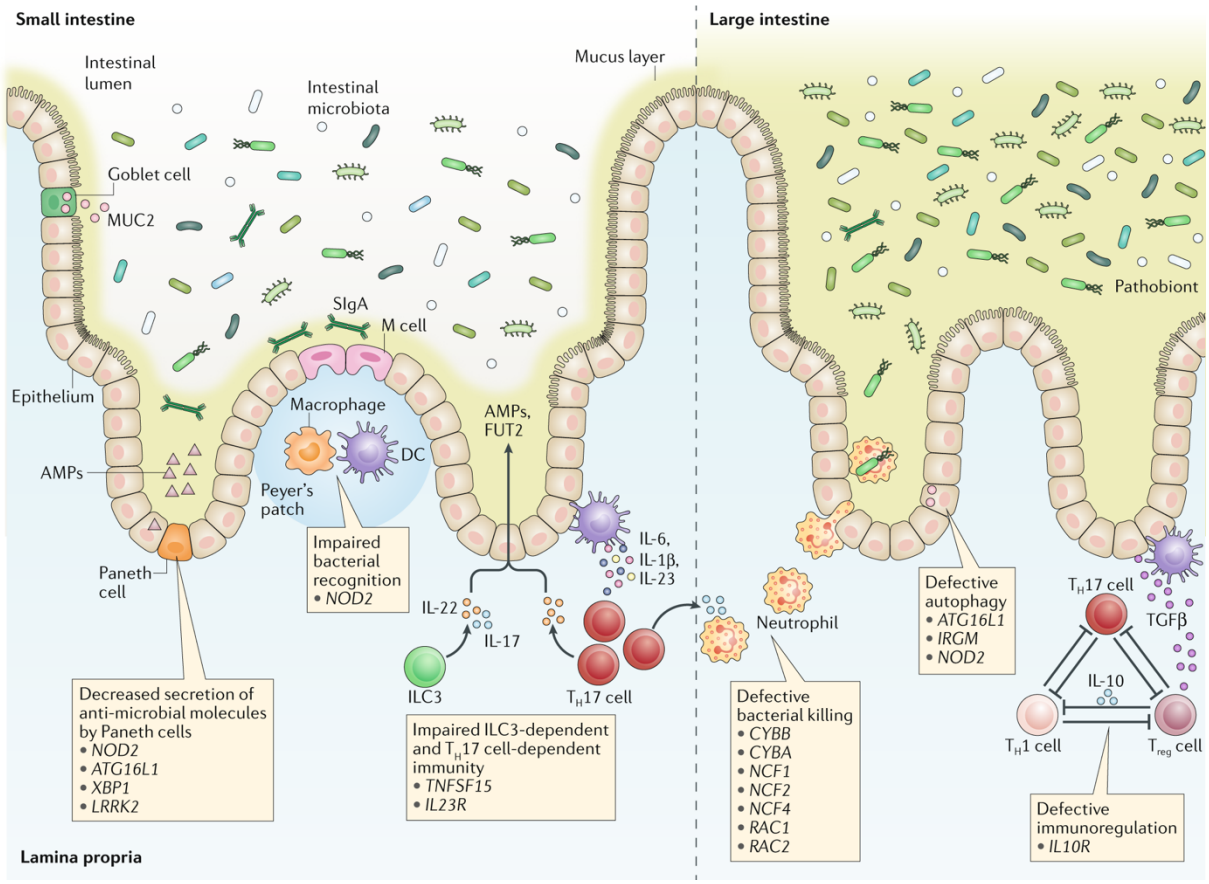


Figure 7. Breakdown of mucosal firewalls in IBD (from Caruso et al., 2020). Mutations in susceptibility genes for inflammatory bowel disease can impair the mucosal strategies that are used by the host to prevent harmful microorganisms from gaining access to the intestinal lamina propria.

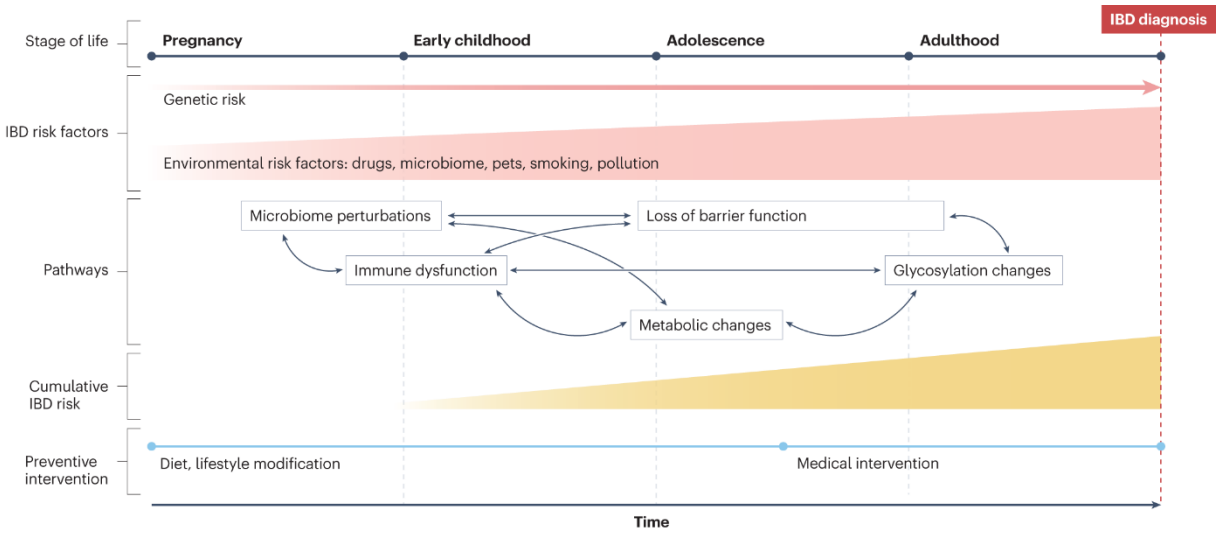


Figure 8. IBD window of susceptibility (from Rudbaek et al., 2023). In a background of genetic risk, progressive and cumulative exposure to relevant environmental risk factors (e.g., infections, antibiotics, pets, smoking and pollution) might contribute to IBD risk over the course of an individual's life.

1.3 Role of macrophages in intestinal inflammation

The dynamic equilibrium between microorganisms, especially the ones considered as commensal flora, and the host immune responses at the mucosal barrier plays a crucial role in maintaining gut homeostasis (Thaiss, Zmora et al. 2016). In particular, the exposure to microbiota and/or its derivatives clearly influences monocyte and macrophage activity in the intestine (Chen, Nair et al. 2022).

Analysis examining the hereditary aspects of IBD and the involvement of immune, stromal, and epithelial cells indicate that no single cell type can be attributed as unique cause of IBD development (Ramos and Papadakis 2019, Chang 2020). Nonetheless, an increasing body of evidences substantiates the idea that monocytes and the monocytes-derived macrophages play a central role in the pathology of IBD (Delfini, Stakenborg et al. 2022). First of all, mononuclear phagocytes, encompassing monocytes and macrophages, are amongst the most numerous leukocytes in the gut (Mulder, Patel et al. 2021). Macrophages form a dense network of cells throughout the gastrointestinal tract, and in patients with active IBD they massively accumulate, forming structures such as granulomas mostly observed in CD and associated with an increased risk of intestinal resection (Heresbach, Alexandre et al. 2005, Xavier and Podolsky 2007).

Our comprehension of macrophage immunobiology largely stems from preclinical models of intestinal inflammation (Kiesler, Fuss et al. 2015). In these models, alterations in the composition of macrophage compartment are a common feature, regardless of the different agent used to trigger inflammation [i.e., dextran sodium sulphate (DSS), 2,4,6-trinitrobenzene sulfonic acid (TNBS), dinitrobenzene sulfonic acid (DNBS)] (Bain, Bravo-Blas et al. 2014, Jones, Bain et al. 2018).

Homeostatic macrophages, often defined by their high expression of CX3CR1, are massively outnumbered by $CCR2^{+}Ly6C^{hi}$ classic monocytes. In the colonic mucosa, newly arrived inflammatory monocytes differentiate *in situ* into IL10-producing anti-inflammatory $CX3CR1^{hi}Ly6C^{-}MHCII^{+}$ macrophages through a series of intermediaries in a process referred to as the "monocyte waterfall" (Bain, Scott et al. 2013).

The likely evolutionary role of rapid intestinal monocyte recruitment is to combat infective pathogens following a self-limiting process that leads to the rapid resolution of inflammation (Delfini, Stakenborg et al. 2022). For instance, in physiological conditions the recruitment of monocytes subsequently leads to the accumulation of alternatively activated macrophages with pro-resolving capacity (Zigmond, Varol et al. 2012, Domanska, Majid et al. 2022). However, in the context of IBD, this phenomenon seems to be dysregulated, and the monocyte differentiation process is disrupted, leading to the accumulation of TLR-responsive

intermediate CX3CR1^{int}Ly6C^{+}MHCII^{+} monocytes that produce inflammatory mediators (including TNF, IL-1β, IL-6) (Figure 9) (Zigmond, Varol et al. 2012, Bain, Scott et al. 2013, Martin, Chang et al. 2019).

Whether this dysregulation reflects differential monocyte maturation in the inflamed intestine or in the nature of monocytes before they arrive in the gut is still not clear.

Circulating monocytes from patients with CD are characterized by excessive secretion of pro-inflammatory cytokines, abnormal morphological maturation, prolonged intracellular bacterial survival and differences in surface marker expression (Dige, Magnusson et al. 2016). Interestingly, the composition of macrophage compartment in these patients influences also the response to the therapy. For example, patients with IBD responding to anti-TNFα therapy show an accumulation of anti-inflammatory macrophages in the colon (Vos, Wildenberg et al. 2011, Vos, Wildenberg et al. 2012).

This evidence is consistent with the idea that genetic alterations can prevent the differentiation of inflammatory monocytes into pro-resolving macrophages, leading to the development of IBD. This hypothesis is supported by GWAS analysis concluding that many IBD susceptibility loci are associated with monocyte and macrophage function (Baillie, Arner et al. 2017). For instance, loss-of-function mutations in *NOD2* result in aberrant monocyte and macrophage activation, which can also induce a pathogenic differentiation of fibroblasts, increasing the expression of collagen and other pro-fibrotic mediators (Nayar, Morrison et al. 2021). Furthermore, macrophages carrying risk variants for autophagy (i.e., ATG16L1, IRGM), possess defects in antigen processing and clearance, showing that the macrophage ability to sense intracellular microbial products is crucially impaired in the pathogenesis of IBD (Lapaquette, Glasser et al. 2010, Lapaquette, Bringer et al. 2012).

Besides genetic predisposition, different environmental cues are thought to drive the imprinting of macrophage immune phenotype during monocyte *in situ* differentiation (Ananthakrishnan, Bernstein et al. 2018). During intestinal inflammation, the accumulated myeloid cell populations follow an activation process driven by pathogen-associated molecular patterns (PAMPs), such as bacterial lipopolysaccharides (LPS) or T/cell derived interferon (IFN) γ (Nakanishi, Sato et al. 2018, Castro-Dopico, Fleming et al. 2020). This process induces a potent pro-inflammatory signaling, leading to an increased expression of specific genes and/or secretion of inflammatory mediators (Medzhitov and Horng 2009). These effects strongly depend on the elevation of intracellular Ca^{2+} concentration and subsequent activation of intracellular signaling (Murakami, Ockinger et al. 2012, Rada, Park et al. 2014).

Moreover, an emerging mediator of gut macrophage function is the microbiota. Monocyte recruitment in the gut is diminished in germ-free mice and in specific pathogen-free (SPF) mice that have been subjected to broad-spectrum antibiotics, as indicated by several studies (Bain, Bravo-Blas et al. 2014, Shaw, Houston et al. 2018, Chen, Nair et al. 2022). This suggests that the pace at which macrophages are replenished is partially guided by the microbiota, resulting in changes of macrophage subsets (Shaw, Houston et al. 2018, Kang, Alvarado et al. 2020) and the organization of macrophage distribution (Honda, Surewaard et al. 2020).

Finally, IL-10 receptor (IL-10R) signaling has a fundamental role in determining the regulatory phenotype of intestinal macrophages in both mice and humans. IL-10, through the induction of the mechanistic target of rapamycin inhibitor DNA-damage-inducible transcript 4 (DDIT4), blocks the LPS-induced metabolic rewiring of macrophages, thus inhibiting glucose uptake and glycolysis and promoting oxidative phosphorylation (Figure 10) (Ip, Hoshi et al. 2017). As a matter of fact, over the last ten years, it has become more evident that the cellular metabolic profile plays a crucial role in regulating macrophage activation (Figure 11) (O'Neill, Kishton et al. 2016). The primary immunometabolic pathways in macrophages are glycolysis, tricarboxylic acid (TCA) cycle, pentose phosphate pathway, fatty acid synthesis and amino acid metabolism and their relative activation depends on external stimuli (O'Neill, Kishton et al. 2016). For instance, when macrophages are stimulated by LPS, they exhibit a decrease in oxidative phosphorylation while showing an increase in glycolysis flux through the pentose phosphate pathway (PPP), fatty acid synthesis, and a truncated TCA cycle (O'Neill, Kishton et al. 2016). This shift is accompanied by the accumulation of immunoregulatory metabolites like succinate, which is also found in high levels in the stool of patients with IBD (Tannahill, Curtis et al. 2013). In the context of an inflamed gut, the intestinal microenvironment experiences severe hypoxia, leading to a transition from oxidative phosphorylation to glycolysis, driven by hypoxia-inducible factor (HIF)-1α. This shift is facilitated by the upregulation of key glycolytic enzymes such as phosphofructokinase 2 (PKM2) and may potentially worsen colitis in mice (Kim, Lee et al. 2018, Macias-Ceja, Ortiz-Masia et al. 2019). Consistently with high glycolytic flux, pro-inflammatory macrophages show an enhanced PPP (Tannahill, Curtis et al. 2013). This plays a crucial role in generating NADPH, a cofactor essential for the activity of LPS-induced inducible nitric oxide synthase (iNOS), responsible of the production of nitric oxide (NO) (Clementi, Brown et al. 1998). NO- dependent nytrosilation of iron-sulfur-containing complexes in the electron transfer chain inhibits mitochondrial respiration and oxidative phosphorylation (Clementi, Brown et al. 1998, O'Neill, Kishton et al. 2016). Lastly, activation of macrophages by IFNγ/LPS gives rise to a truncated TCA cycle leading to the accumulation

of succinate and citrate (O'Neill, Kishton et al. 2016). The increase of citrate levels is the result of transcriptional downregulation of isocitrate dehydrogenase 1 (IDH1). The accumulated citrate serves as a precursor for the synthesis of the macrophage-specific metabolite itaconic acid, which in turn suppresses succinate dehydrogenase (SDH), leading to succinate accumulation (Lampropoulou, Sergushichev et al. 2016). When TCA cycle is inhibited, pyruvate does not enter in mitochondria for oxidation and is directly reduced to lactate in a process dependent on lactate dehydrogenase (LDH) (Fantin, St-Pierre et al. 2006). Lactate can be converted into lactyl-CoA and is involved in the lactylation of histones lysine (Kla) and non-histone proteins. Many studies have shown the accumulation of histone Kla on gene promoters in cells stimulated by hypoxia, IFN-γ, LPS, or bacterial attack to produce lactate, thereby directly regulating gene expression (Figure 12) (Zhang, Tang et al. 2019, Irizarry-Caro, McDaniel et al. 2020). In particular, Kla presence is significantly higher on the promoters of pro-resolving genes during inflammation, indicating that it likely serves as a "lactate clock" that facilitates the transition from an inflammatory phenotype to a pro-resolving one in macrophages (Zhang, Tang et al. 2019). This shift typically takes place in the later stages of inflammation and may be associated with processes like wound healing (Figure 13) (Zhang, Tang et al. 2019). Overall, rewiring the metabolic pathway of activated macrophages can serve as therapeutic approaches to treat inflammatory diseases and microbial infection.

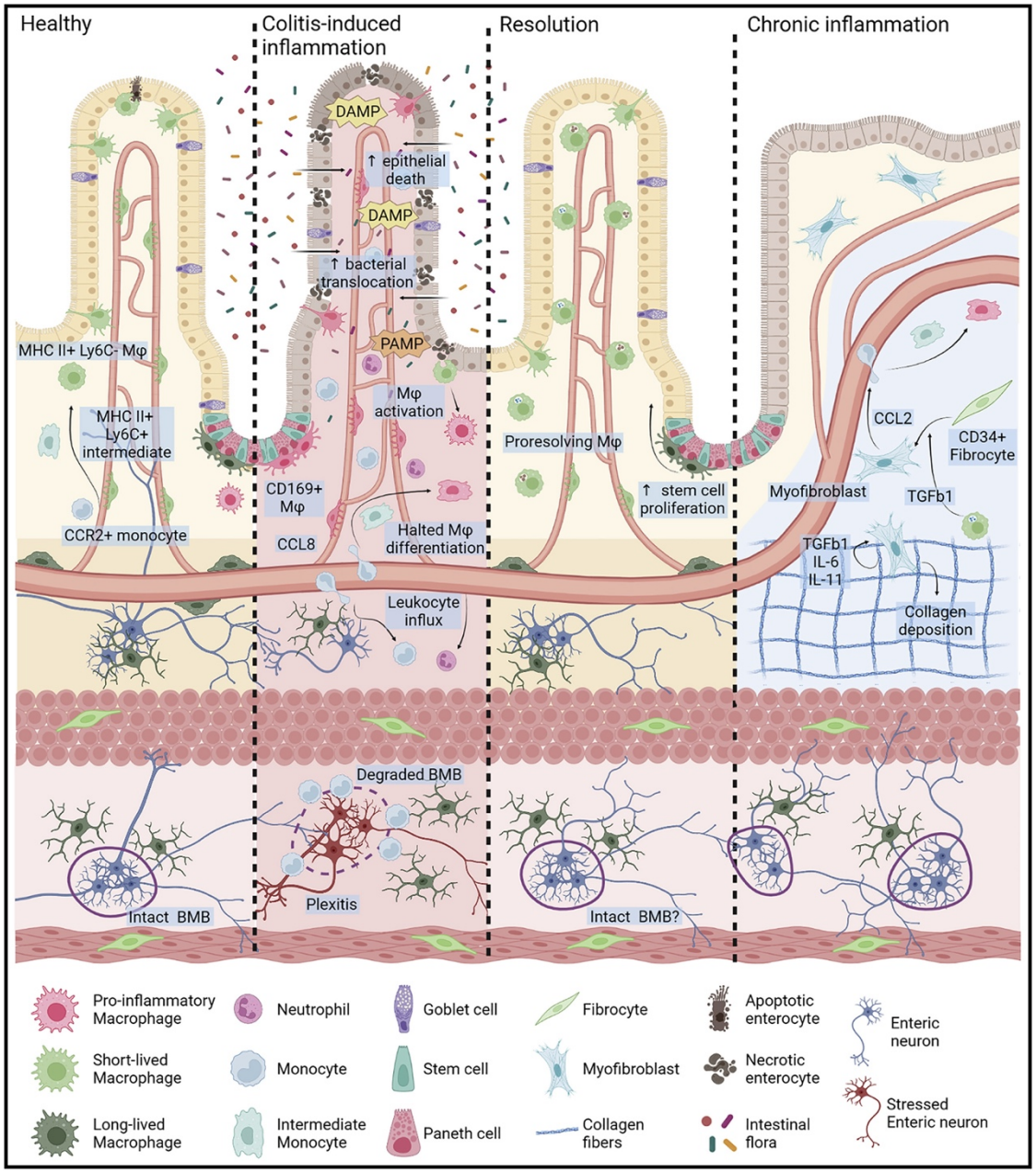


Figure 9. Macrophages in acute and chronic colitis (from Delfini et al., 2022). While recruited CCR2+ monocytes at steady state differentiate into mature MHCII+ Ly6C− macrophages, during colitis flares the differentiation process between monocytes and macrophages is halted to an immature state. Differentiated monocytes remain highly sensitive to environmental signals and intensify inflammation by producing large amount of pro-inflammatory mediators. Pericryptal macrophages stimulate stem cell proliferation during resolution, which aids in mucosal healing. Concurrently, surrounding macrophages eliminate immunological and apoptotic epithelial cells, causing a phenotypic shift from pro-inflammatory to pro-resolving. Recurrent flare-ups result in persistent inflammation and fibrosis alongside the breakdown of pro-resolving pathways.

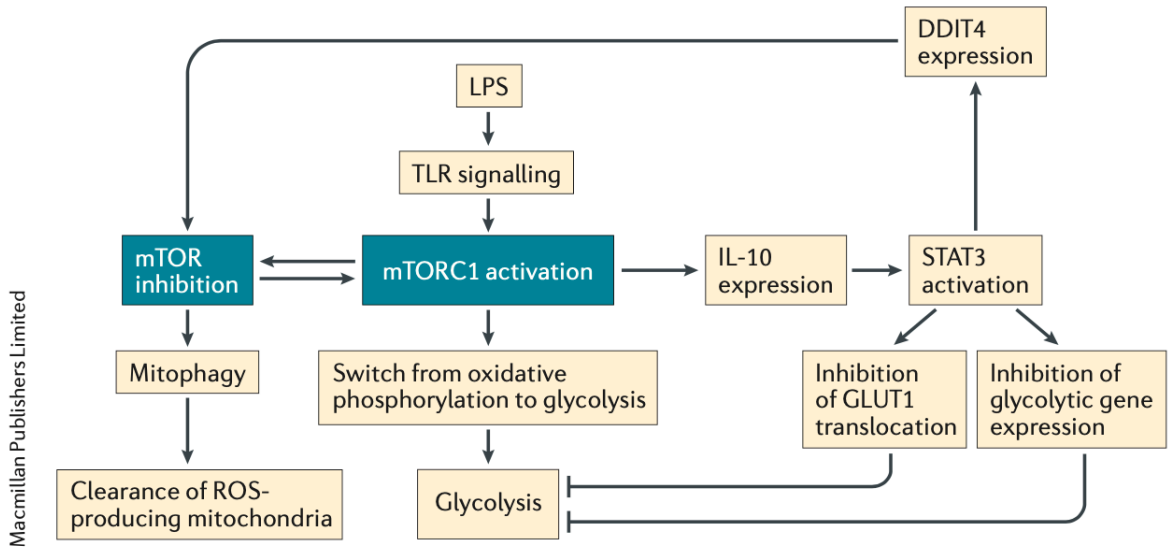


Figure 10. IL-10 targets macrophage metabolism (from Minton, 2017; original article Ip et al., 2017). IL-10 inhibits mTORC1 signalling in a STAT3–DDIT4-dependent manner, which is required for the inhibition of glycolysis. Therefore, the mTORC1-mediated effects of IL-10 on macrophage metabolism have crucial anti-inflammatory roles.

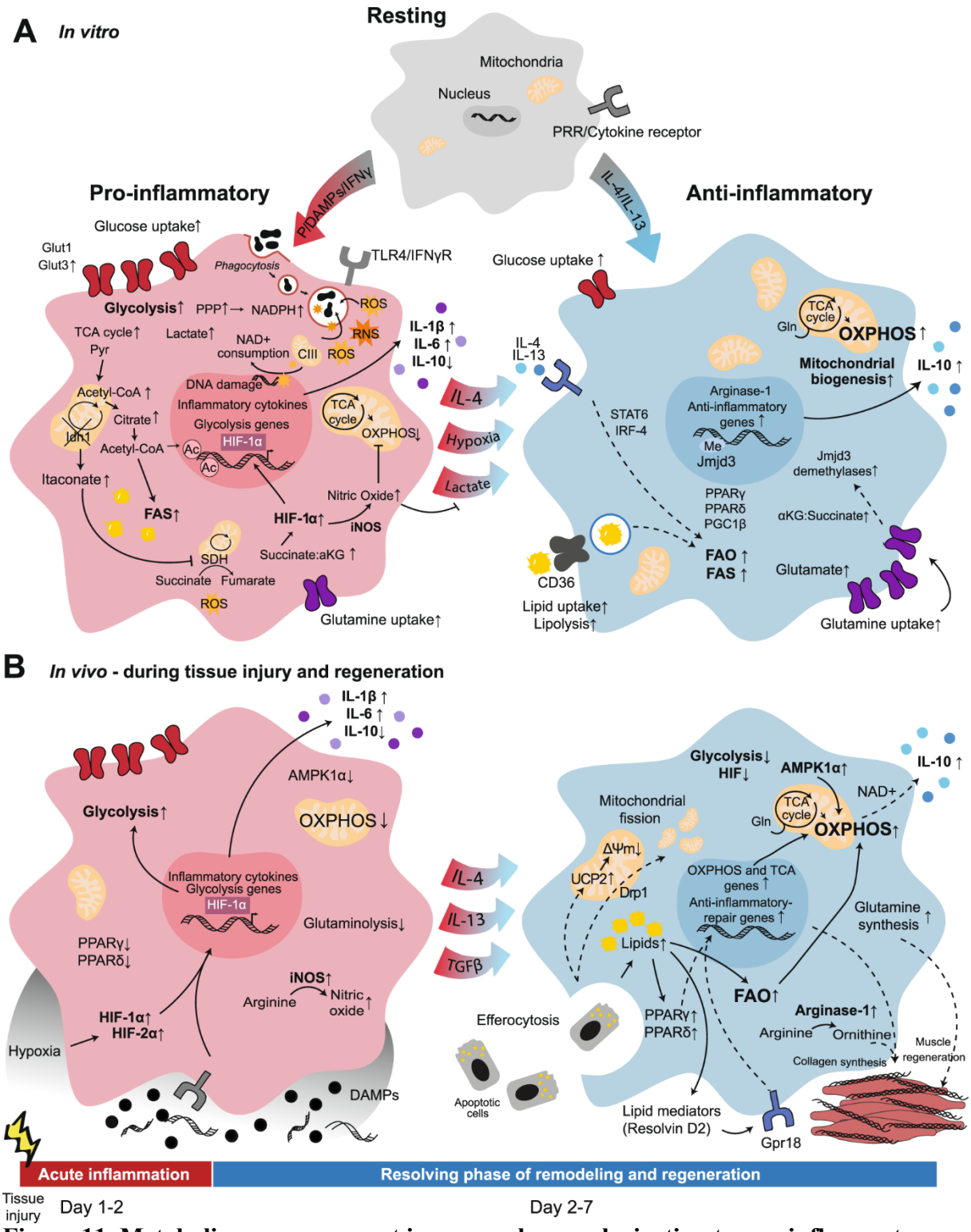


Figure 11. Metabolic rearrangement in macrophage polarization to pro-inflammatory or pro-resolving macrophages in vitro and in vivo (from Wculek et al., 2022). Both in vitro (A left) and in vivo (B left), proinflammatory polarization has been associated with enhanced glycolytic metabolism; however, the majority of the related information has been elucidated in vitro. Both in vitro (A right) and in vivo (B right), increases in OXPHOS, FAO and glutaminolysis are associated with alternatively activated macrophages.

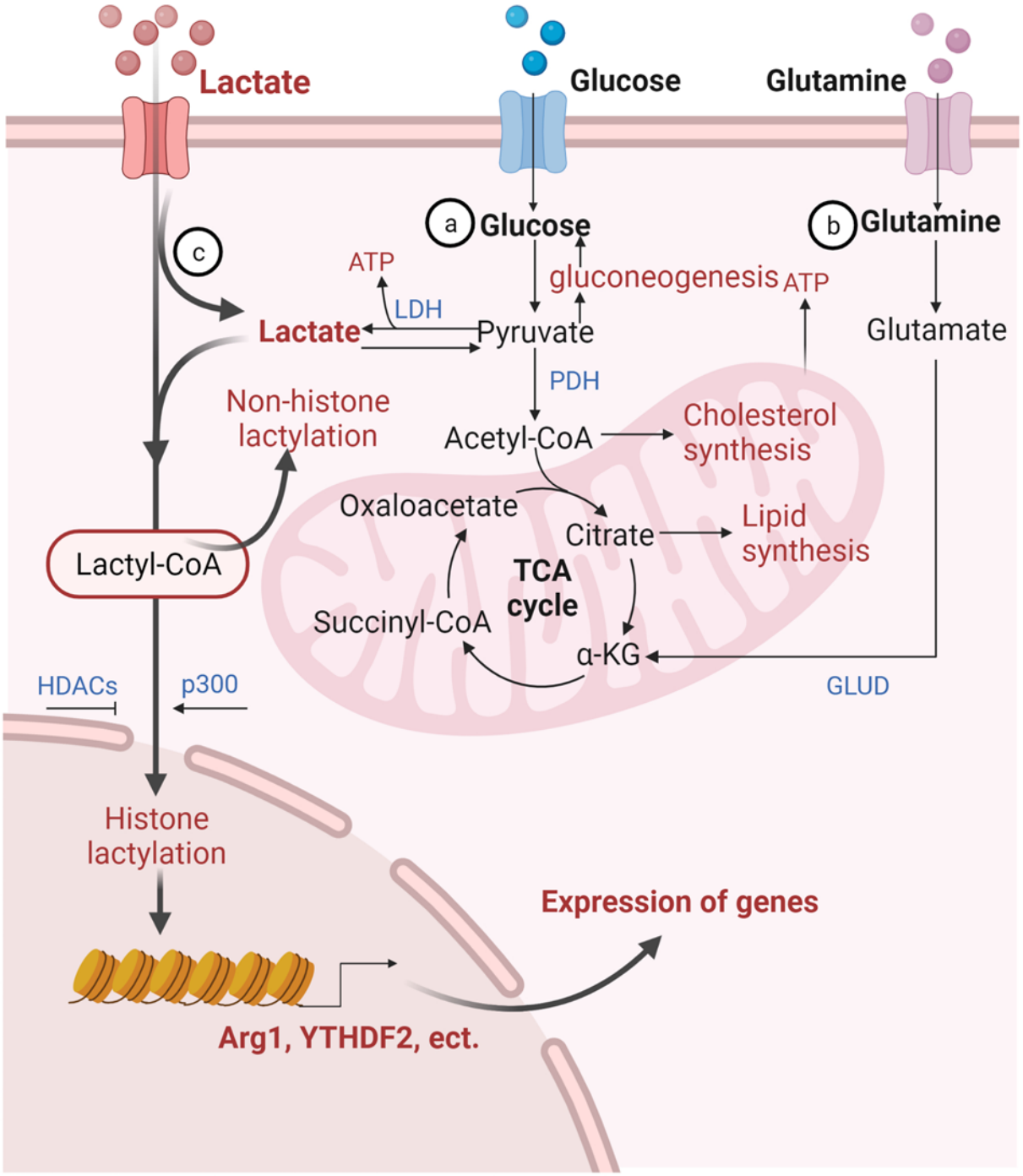


Figure 12. Lactate metabolism and lactylation in macrophages (from Li et al., 2022). In the cytoplasm, lactate is transported into cells by monocarboxylate transporter (MCTs) and is produced from glycolysis or glutamine decomposition. Lactate can be converted into lactyl-CoA and is involved in the lactylation of histones and nonhistone proteins.

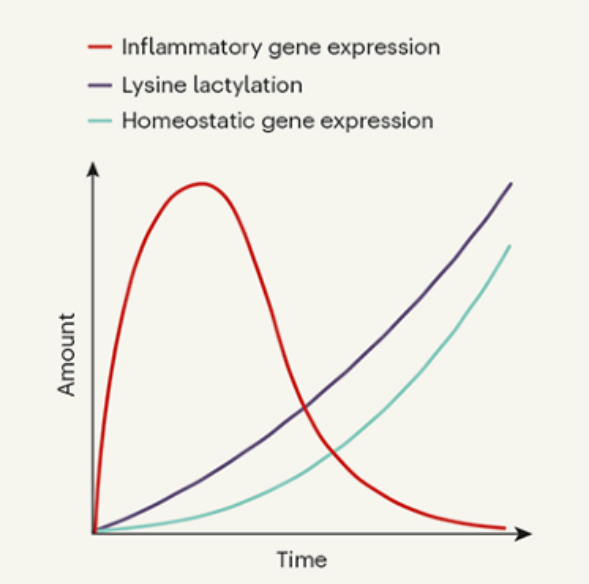


Figure 13. Lactate-clock in macrophages (adapted from Zhang et al., 2019). The transition from anaerobic to aerobic glycolysis during macrophage pro-inflammatory polarization initiates a "lactate timer" that might help repair collateral damage the host sustained during infection by inducing macrophage pro-resolving-like traits in the late phase through an epigenetic mechanism.

1.4 Colitis associated colon cancer (CAC)

Patients with IBD have an increased risk to develop colorectal cancer (CRC) (Lucafo, Curci et al. 2021). Chronic inflammation is a driver of neoplastic progression by generating oxidative stress–induced damage to DNA that may activate tumor-promoting genes and inactivate tumor-suppressing genes, resulting in dysplastic precursor lesions (Figure 14) (Itzkowitz and Yio 2004). Markers of oxidative damage and DNA double-strand breaks increase progressively in the inflammation-dysplasia-carcinoma sequence (Frick, Khare et al. 2018). Colitis-associated colon cancer shares many molecular similarities with sporadic CRC (sCRC), and preclinical investigations have demonstrated a potential role of microbiome in concert with the host immune system in the development of CAC. The same major molecular pathways that rise to sCRC, namely chromosomal instability, microsatellite instability (MSI), and CpG island methylator phenotype (CIMP), contribute to the development of CAC (Itzkowitz and Yio 2004). In CAC, the proportion of MSI-positive tumors is approximately similar to sCRC. However, CAC demonstrate a shift from the epithelial consensus molecular subtype (CMS) 2 toward the more mesenchymal CMS4 phenotype (epithelial-mesenchymal transition), with dysregulation of Wnt signaling in favor of transforming growth factor (TGF)- β activation, and an "immune-inflamed" immuosuppressive microenvironment enriched in CD4+ cells (Figure 15) (Rajamaki, Taira et al. 2021). The increased mesenchymal phenotype of CAC may be due to the remodeling of the mesenchyme surrounding the colonic crypts that is seen in patients with IBD, which likely contributes to the carcinogenesis process (Kinchen, Chen et al. 2018). Hyperactivation of Wnt pathway occurs in 80% of human CRC and is a crucial initiating step in carcinogenesis that disrupts cellular differentiation and promotes rapid proliferation. Despite reduced APC mutations, nuclear accumulation of β-catenin is prevalent in IBD-CRCs (Claessen, Schipper et al. 2010), suggesting an alternative mechanism of WNT pathway activation. CAC arises from dysbiosis among a community of commensals, involving a restriction of microbial diversity, as seen with colitis itself. *Fusobacterium nucleatum* (Fn), *Escherichia coli* containing pathogenic polyketide synthetase (pks) islands, *and Bacteroides fragilis* expressing B. fragilis toxin (BFT) have been linked to the process of human colorectal carcinogens (Boleij, Hechenbleikner et al. 2015, Tilg, Adolph et al. 2018). In particular, BFT, which binds to a specific colonic epithelial cell receptor, activates Wnt and NF-κB signaling pathways resulting in increased cell proliferation, epithelial release of pro-inflammatory mediators, and DNA damage (Boleij, Hechenbleikner et al. 2015). Interestingly, the *bft* gene sequences have been found in the mucosa of 90% of patients with sporadic colorectal neoplasia, compared with 55% of controls and in the stool of approximately 14% of patients with IBD

(Prindiville, Sheikh et al. 2000, Boleij, Hechenbleikner et al. 2015). Wnt/β-catenin pathway and inflammatory cascade share mutual co-dependence which has been confirmed through various studies. Through direct and/or indirect effects on effector T cells, T-regulatory cells, T-helper cells, dendritic cells, and other cytokine-expressing immune cells, abnormal activation of Wnt/β-catenin signaling benefits immune exclusion and hinders T-cell-mediated antitumor immune responses (Feng, Jin et al. 2019, Quandt, Arnovitz et al. 2021). For instance, persistent TCR stimulation in a Wnt-rich environment, as happens during the development of CAC, can activate Wnt–β-catenin in T cells and induce pro-inflammatory cytokine production (Quandt, Arnovitz et al. 2021). Moreover, Wnt/β-catenin signaling has been shown to be involved in crosstalk between cancer cells and tumor associated macrophages (TAMs) (Pai, Carneiro et al. 2017). The release of a Wnt-regulated soluble factor (Snail) by colorectal cancer cells stimulates the macrophage production of IL-1β which in turn increases the phosphorylation of GSK3β in colon cancer cells, increasing the availability of β-catenin (Pai, Carneiro et al. 2017).

TAMs are among the most abundant immune cells within the tumor microenvironment (TME) (Christofides, Strauss et al. 2022) (Figure 16). In CAC, macrophages not only play a role in tumor immunity, but also play a role in the inflammatory environment before tumor formation. Overactivated macrophages aggravate inflammatory damage, but also promote tumorigenesis (Onizawa, Nagaishi et al. 2009). A large number of infiltrating macrophages aggravate the mucosal damage caused by inflammation and promote the transformation from inflammation to tumor (Ji, Cao et al. 2014, Yu, Dai et al. 2022). Therefore, at early stage of tumor initiation, TAMs appear to exhibit pro-inflammatory phenotype and secrete factors like IL-6 that promote the occurrence and development of CAC through IL-6/STAT3 pathway (Sheng, Davies et al. 2022). As tumor progression, TAMs are functionally reshaped by signals derived from the tumor microenvironment (TME) and display a pro-resolving phenotype (Zhu, Zhou et al. 2018, Yuan, Gu et al. 2021). These alternatively activated macrophages, while being effective in the resolution of the inflammation reducing tissue damage and immune cell infiltration, promote angiogenesis, metastasis, and tumor immune escape according to the different stages of tumor progression (Noy and Pollard 2014, Cheruku, Rao et al. 2023). In the early stage of tumor, pro-resolving macrophages secrete a variety of growth factors and cytokines, including vascular endothelial growth factor (VEGF), TGF-β, epidermal growth factor (EGF), which promote tumor cell proliferation and neovascularization (Noy and Pollard 2014). In the middle stage of the tumor, these macrophages mainly play an immunosuppressive role, inhibiting the activity of tumor immune cells by secreting a variety of cytokines and surface molecules, including IL-

10 (Qiu, Chen et al. 2021). In the late stage of tumor, pro-resolving macrophages are mainly involved in tumor invasion and metastasis (Noy and Pollard 2014). By secreting a variety of proteases and lysosomal enzymes, TAMs can promote tumor cell infiltration and invasion of surrounding tissues (Cendrowicz, Sas et al. 2021).

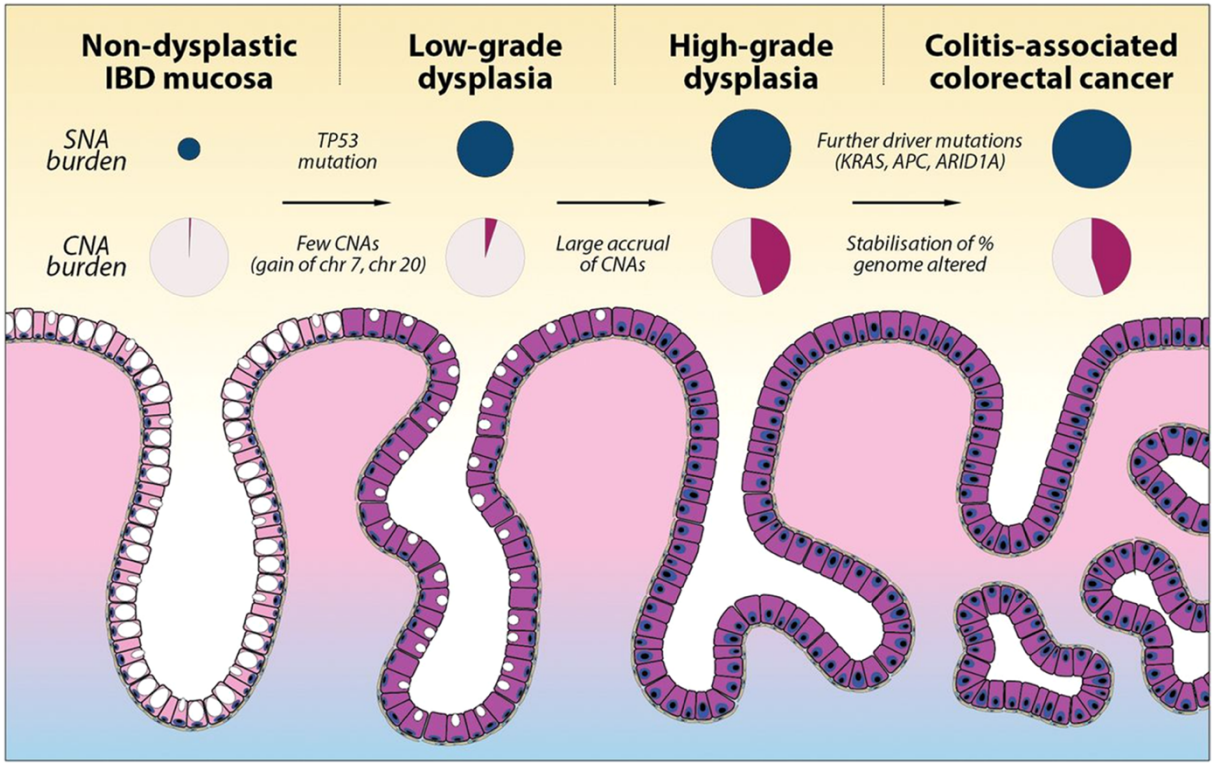


Figure 14. Mutation sequence from IBD to CAC (from Zhou et al., 2023). In contrast to the sporadic CRC (sCRC) progression, p53 loss is an early event in CAC rather than late. APC mutations on the other hand, are observed to occur after p53 loss rather than before, as classically observed in sCRC.

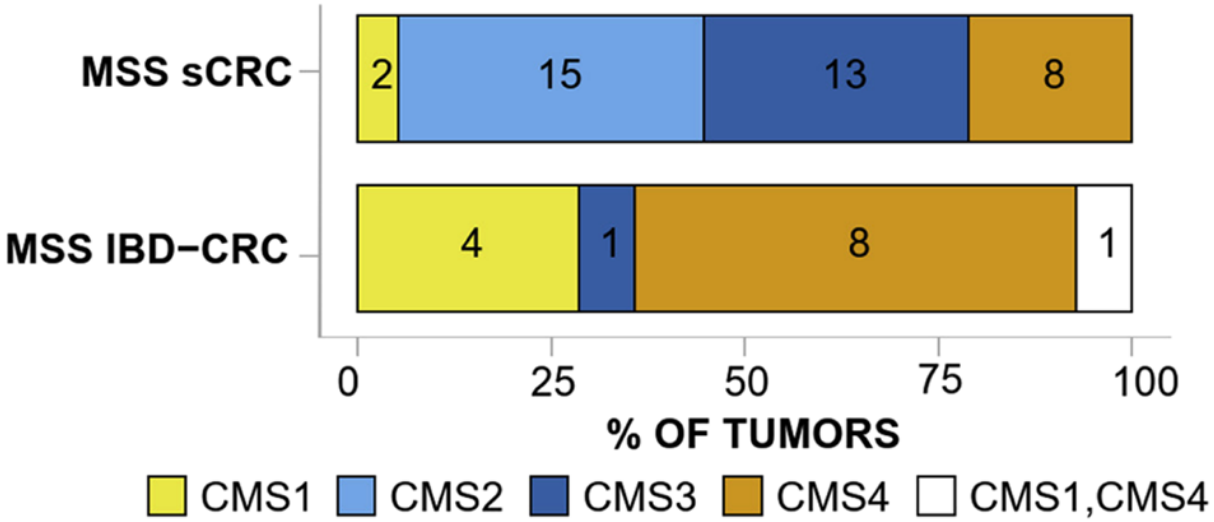


Figure 15. The consensus molecular subtypes of CAC (from Zhou et al., 2023; original article Rajamaki et al., 2021). Bulk tumor RNA-seq of independent microsatellite stable sporadic CRC (MSS sCRC) and microsatellite stable colitis-associated CRC (MSS IBD-CRC) tumors were binned into the consensus molecular subtypes of CRC. This initial study suggests CAC may most transcriptionally aligned with the CMS4 subtype, characterized by stromal, CD4 + T cell, and monocyte involvement.

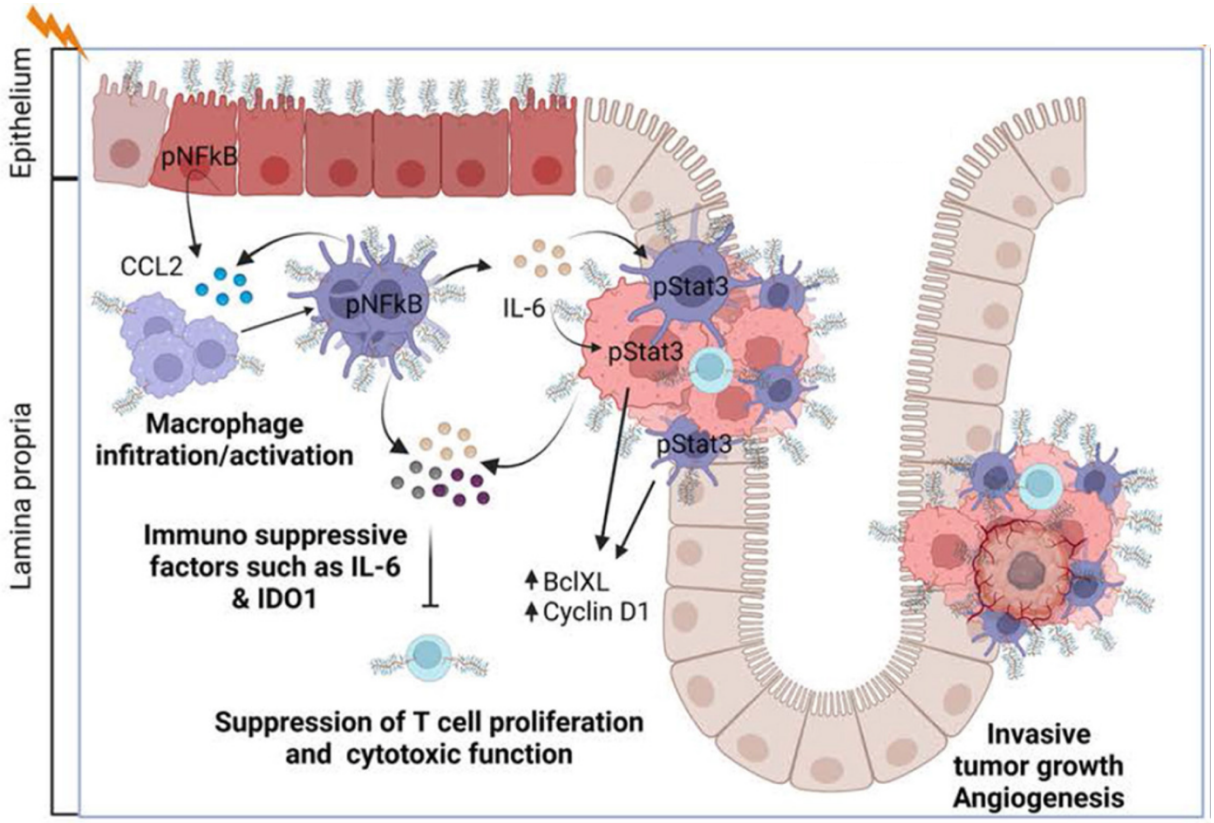


Figure 16. Macrophage activation during CAC (adapted from Sheng et al., 2022). To regulate the proliferation, survival, and progression of mutant epithelial cells, the microenvironment in the inflamed gut that leads to CAC is made up of a complex network of many cell types interacting through the autocrine and paracrine production of cytokines, chemokines, and other substances. This network is in charge of both intrinsic and extrinsic carcinogenesis and progression. Tumor necrosis factor-α and interleukin (IL)-6 are examples of intrinsic cytokines that stimulate transcription factors like nuclear factor kappa B (NFB) and signal transducer and activator of transcription 3 (STAT3) to increase the growth and survival of tumor cells. The recruitment, activation, and tumor-promoting/suppressive actions of immune and stromal cells—which encourage tumor growth through tissue remodeling and angiogenesis—are also regulated extrinsically by chemokines and cytokines.

Chapter II. The crucial role of diet in gastrointestinal disorders

2.1 Role of diet in the pathogenesis of IBD

Environmental factors (e.g., diet, smoking, medications) can alter gut homeostasis, changing microbial composition, mucosal barrier function, and immune tolerance (Khalili, Chan et al. 2018). Diet may play a causative role in IBDs, since patient nutritional status and pattern of dietary intake can influence positively or negatively the function of immune system as well as microbiome composition (Figure 17) (Ramos and Papadakis 2019). Despite diet has a key role in defining the composition of the human gut microbiota and microbial metabolites, its precise pathophysiological mechanisms remain elusive(Albenberg and Wu 2014). In addition, "western diet" has been correlated to increased mucosal inflammation and increased stool calprotectin in human subjects (Colditz, Manson et al. 1997, Serrano-Moreno, Brox-Torrecilla et al. 2022). Lastly, studies in animal models of IBD consistently with epidemiologic observations, confirm that specific factors in the diet may have direct effects on mucosal integrity and immune function, promoting immune tolerance or triggering the inflammatory response (Lee, Zhao et al. 2010, Hwang, Kim et al. 2016).

Overall, it is clear that unhealthy diet impacts metabolic responses and causes perturbations in the host–microbiota interactions, and such imbalance has harmful consequences on the intestinal barrier function and immune tolerance (Venter, Eyerich et al. 2020).

The composition of the diet is likely to significantly impact the immune system, in particular the macrophage biology, either directly or by influencing the microbiota characteristics (Figure 18). The same concept of immunometabolism demonstrates that energy, carbohydrate and lipid metabolism control innate immune responses. Moreover, direct immunomodulatory effects of micro and macronutrients have been demonstrated. For instance, a deficiency in vitamin A results in a reduction of macrophages expressing IL-10 (Erkelens, Goverse et al. 2020), and diets rich in salt can diminish the production of butyrate by microbiota (Miranda, De Palma et al. 2018). Moreover, feeding mice with a high-fat diet has demonstrated that dietary fat induces alterations in the behavior and composition of colonic macrophages, mirroring changes seen in intestinal macrophages among individuals with obesity (Rohm, Fuchs et al. 2021, Rohm, Keller et al. 2022). Importantly, these changes were not observed in germ-free mice, highlighting the intricate interplay between diet and microbiota in shaping macrophage functionality (Rohm, Keller et al. 2022).

As microbes rely on dietary substrates in the intestine, the gut microbiome is often proposed as a mediator through which foods exert their pro-inflammatory and anti-inflammatory effects (Figure 19).

Research conducted in both mice and humans suggests that additives introduced during food processing contribute to heightened gut permeability and intestinal inflammation by fostering an increase in mucolytic bacteria and endotoxins. The gut microbiome produces a large amount of short-chain fatty acids (SCFAs) from the metabolism of dietary components. SCFA areable to induce a pro-tolerogenic phenotype in macrophages, reducing the release of pro-inflammatory cytokines in context of inflammation and promoting immune homeostasis (Chang, Hao et al. 2014). Similarly, SCFA depletion during antibiotic administration in mice resulted in intestinal macrophage hyper-responsiveness to bacterial stimulation, leading to T cell dysfunction (Scott, Andrusaite et al. 2018). In conjunction with SCFAs, indole derivatives sourced from diet and gut microbiome equally play a role in regulating vulnerability to intestinal inflammation by triggering the AhR pathways within macrophages. When AhR was removed in CD11c+ dendritic cells within the intestinal mucosa and in particular subsets of macrophages, a distinct rise in the count of small intestinal epithelial stem cells occurred. This, coupled with atypical differentiation of epithelial precursors *in vivo*, subsequently increased the susceptibility of these mice to colitis induced by DSS (Chng, Kundu et al. 2016). AhR ligands are also responsible of the AHR-dependent expression of Wnt signalling components which mediate macrophage–epithelium crosstalk (Chng, Kundu et al. 2016). Finally, diet and microbial changes control gastrointestinal motility through a crosstalk between muscularis macrophages and enteric neurons (Muller, Koscso et al. 2014).

In this context, the scientific interest in diet and dietary therapies has exponentially increased in the past ten years to unveil dietary components that may further enhance immune function in sub-clinical situations or prevent specific immune-related chronic diseases from improving human health.

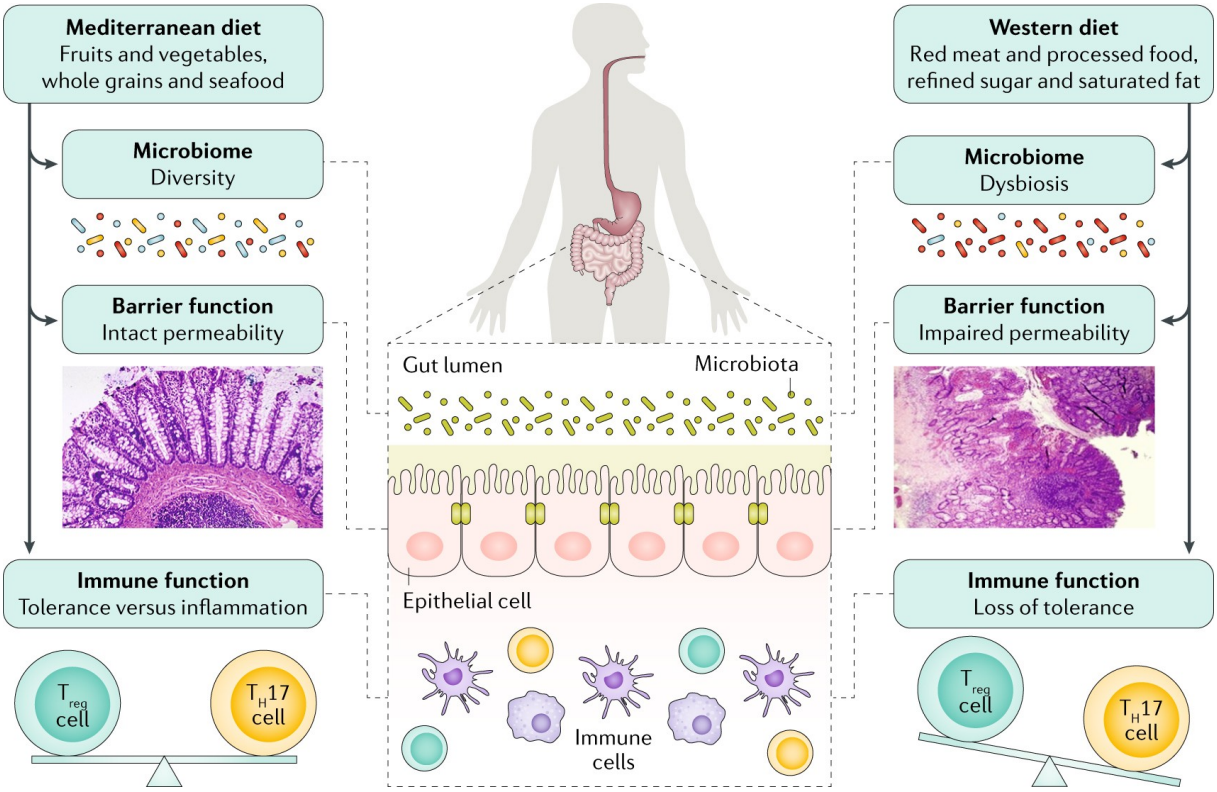


Figure 17. Possible mechanisms underlying the association between IBD and diet (from Khalili et al., 2018). Diet has been linked to changes in the gut microbiome and epithelial barrier function and seems to have a direct influence on immune function.

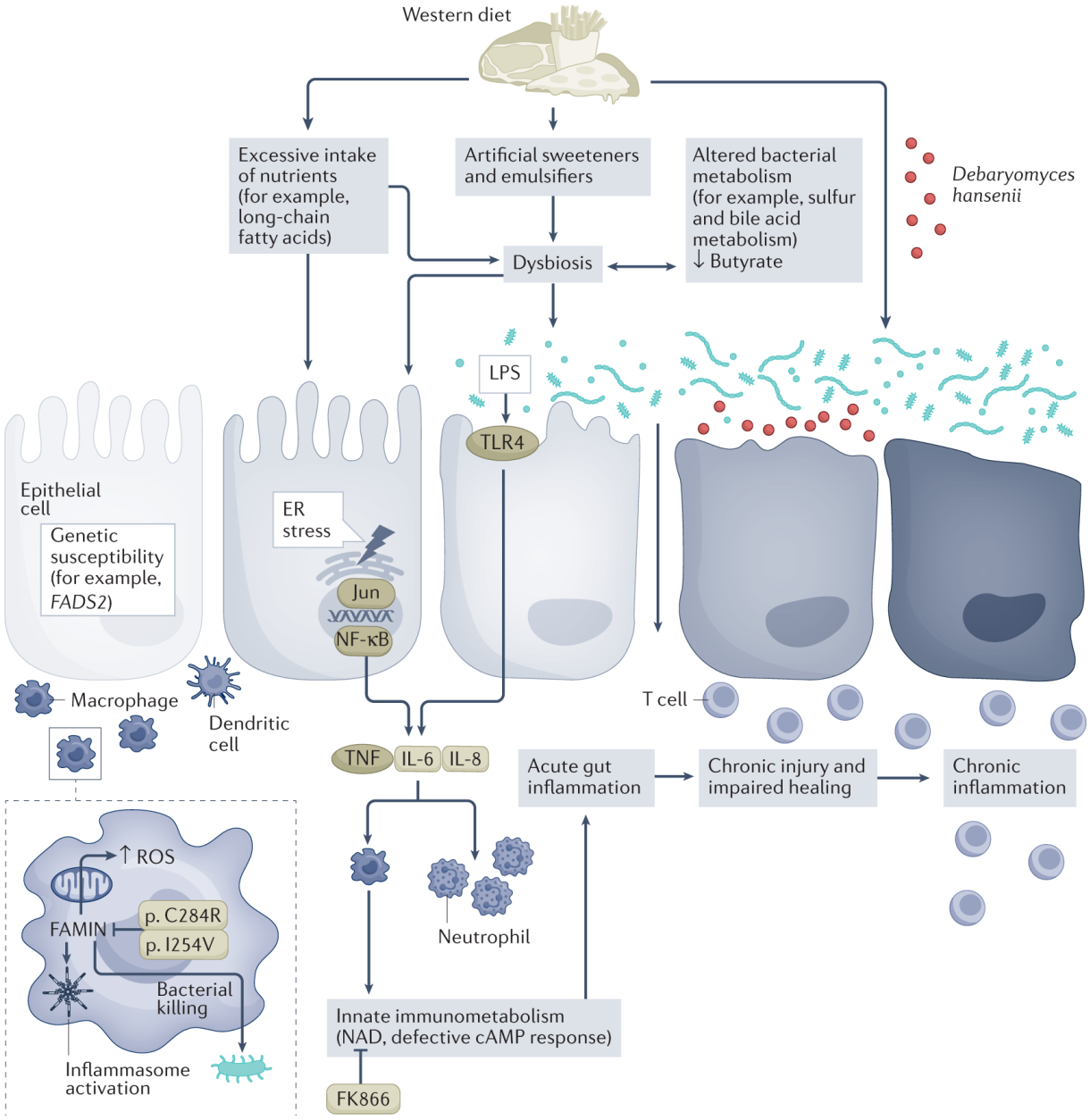


Figure 18. The metabolic nature of human IBD (from Adolph et al., 2022). Excessive intake of specific macronutrients (i.e., fat and sugar) perturbs the gut microbiota and gut barrier integrity and fuels an inflammatory response in the gut. Western diet perturbs gut microbial composition and function, and gut microorganisms or their metabolites trigger epithelial cellular stress pathways. The diet and genetic risk affect immunometabolism of gut mucosal immune cells.

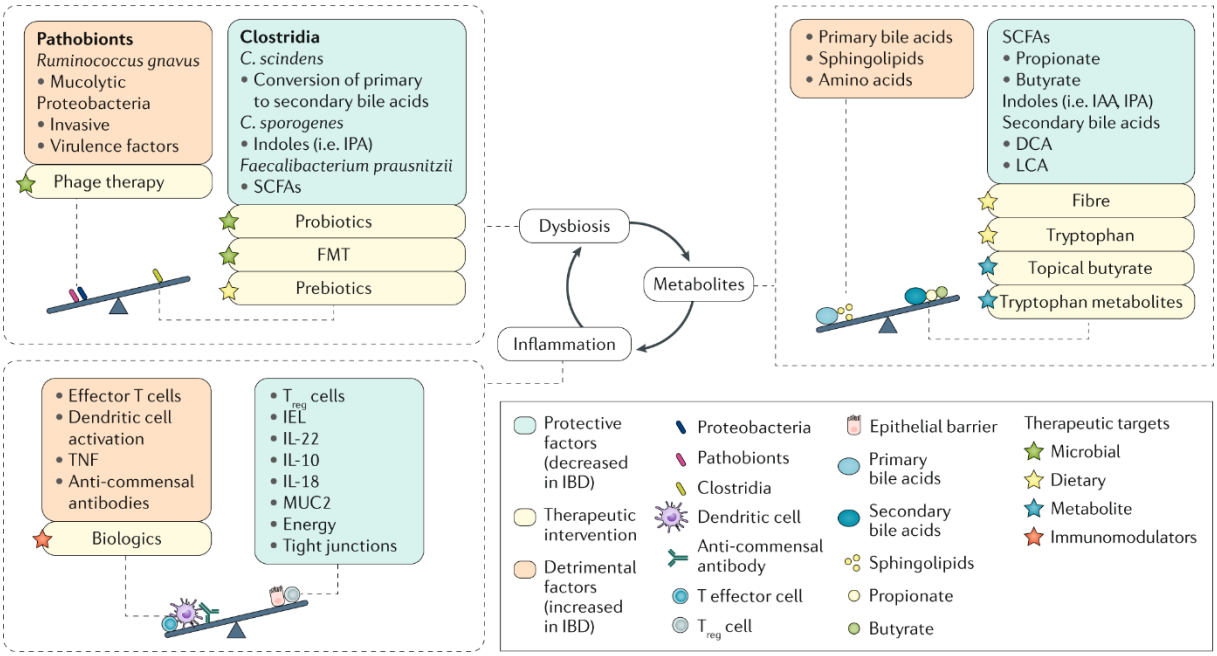


Figure 19. Gut microbiota-derived metabolites are key actors in IBD (from Lavelle and Sokol, 2020). Dysbiosis exists in a circular causality relationship with IBD. Gut microbiota-derived metabolites are a key mechanism by which the gut microbiota educates, communicates with and stimulates the host. SCFAs and tryptophan metabolites, in particular, present a relatively consistent pattern of altered metabolite profiles in IBD patients.

2.2 The role of individual dietary derived compounds

The knowledge on pro-inflammatory and anti-inflammatory capacities of single dietary factors is increasing, even if there is still limited understanding on how their synergies in dietary patterns impact the healthy and the inflamed intestine. Many dietary nutrients have been shown to help regulate mucosal immune function, such as vitamins, amino acids, and SCFAs, many of which are influenced by the gut microbiota (Venter, Eyerich et al. 2020).

It is now well established the beneficial role of dietary fiber in IBD. A fiber-rich diet modulates the composition of the gut microbiota exerting an anti-inflammatory effect. The insoluble fibers increase the colonic fermentation and promote the production of SCFAs (Tan, Macia et al. 2023). Murine experimental models of colitis have also shown that fiber might have a direct immunomodulatory effect by inhibiting the production of pro-inflammatory cytokines in peripheral blood mononuclear cells (Figure 20) (Le Leu, Young et al. 2013, Breton, Ple et al. 2015).

The essential fatty acids have been studied extensively in IBD, but their beneficial effects are still not well consolidated. Addition of PUFAs to mice standard diet had variable effects in preventing or treating colitis (Matsunaga, Hokari et al. 2008, Bosco, Brahmbhatt et al. 2013, Ananthakrishnan, Khalili et al. 2014). However, the anti-inflammatory effect of the n-3 PUFAs has been demonstrated in some clinical trials, even if their effect on relapse seems to not be confirmed. Most of amino acids (i.e., arginine, glutamine, tryptophan, threonine) have been shown to exert immunomodulatory functions and mediate responses to metabolic stress (Coeffier, Marion-Letellier et al. 2010). Both glutamine and arginine have been shown to improve colitis outcome when added to diets of mice (Xue, Sufit et al. 2011, Coburn, Gong et al. 2012, Ren, Yin et al. 2014). Animal models of inflammatory bowel disease suggest that glutamine-enriched diets can increase the bioavailability of glutamine and as a consequence the levels of several TCA intermediates, causing an alteration in the intestinal microenvironment (Kew, Wells et al. 1999). This may lead to reduce the severe intestinal damage, disease activity and weight loss, and improve nitrogen balance (Akobeng, Miller et al. 2000). Glutamine supplementation has been used in clinical practice to treat IBD patients, however a recent systematic review from European Society for Clinical Nutrition and Metabolism (ESPEN) has shown that glutamine supplementation has no effect on disease course nor on any biological parameter in patients (Severo, da Silva Barros et al. 2021). Dietary arginine supplementation in animal models of colitis also showed an effect on composition of the intestinal microbiota (Coburn, Gong et al. 2012, Singh, Gobert et al. 2019, Baier, Gansbauer et al. 2020). Dietary tryptophan (Trp) is an essential amino acid for intestinal epithelial cells. Tryptophan is a

precursor to immunoregulatory biogenic amines, such as kynurenine, xanthureic acid and 5-HT (Munn and Mellor 2013). Kynurenic acid and xanthurenic acid can activate AhR which is involved in the homeostasis of intestinal epithelial cells and gut barrier integrity (DiNatale, Murray et al. 2010, Wirthgen, Hoeflich et al. 2017, Sofia, Ciorba et al. 2018). Moreover, all the Trp metabolic pathways can modulate directly the function of various immune cells, namely T-cell differentiation and activation (Michaudel, Danne et al. 2023). It has been demonstrated that threonine supplementation impacts goblet cell number and mucus production, but this showed a variable effect on colitis development (Faure, Mettraux et al. 2006, Gaifem, Goncalves et al. 2018, Sofia, Ciorba et al. 2018). Finally, biogenic amines might also affect the immune response during intestinal inflammation. For instance, histamine derived from dietary histidine can reduce symptoms of immune-mediated colitis in mice (Andou, Hisamatsu et al. 2009).

Numerous dietary vitamins and minerals are believed to play a role in the pathogenesis of IBD, based on preclinical and clinical observational studies. Extra-skeletal vitamin D is involved in anti-inflammatory pathways, acting as an immuno-modulator, likely being beneficial in patients with IBD (Figure 21). Beneficial effects of vitamin D in intestinal inflammation are supported by pre-clinical studies, where the active form of vitamin D, 1,25-dihydroxyvitamin D (1,25-(OH)2D) has been shown to suppress the inflammatory response to luminal antigens, promoting a tolerogenic immune responses. In an observational study, patients who received supplementation of vitamin D were less likely to require surgery for IBD, than those who were vitamin D deficient (Ananthakrishnan, Cagan et al. 2013). A randomized trial of patients with CD in clinical remission confirmed that independent of other variables, lower vitamin D levels associates with a greater risk of clinical relapse (Jorgensen, Agnholt et al. 2010). Moreover, there was an associated significant reduction in inflammatory markers of colitis: both C-reactive protein (CRP) and fecal calprotectin (Nielsen, Hansen et al. 2019).

Notably, one third of IBD patients suffer from anaemia, with iron deficiency being the main cause to its development (Gasche, Lomer et al. 2004). Besides erythropoiesis, iron is also involved in immune processes, catalyzing the formation of oxygen radicals that influence macrophage and neutrophils development (Tsukamoto, Lin et al. 1999, Hentze, Muckenthaler et al. 2010, Ni, Yuan et al. 2022). Iron deficiency is associated to imbalances in the proliferation and differentiation of T and B cells (Macedo, de Sousa et al. 2004, Jabara, Boyden et al. 2016, Jiang, Li et al. 2019, Pfeifhofer-Obermair, Tymoszuk et al. 2021). On the other hand, high iron load can increase the ratio of CD8+ T cells to CD4+ T cells, cause a dysfunction of natural killer cells, impaire neutrophil cytotoxicity and reduce the responsiveness of macrophages towards IFNγ (Oexle, Kaser et al. 2003, Ban, Langonne et al. 2013, Vollger, Akong-Moore et

al. 2016, Pereira, Chen et al. 2019, Feng, Yang et al. 2021). Intracellular iron availability in macrophages is important for fighting infection, and imbalances in iron homeostasis influence cytokine production and cell mediated immune effector mechanisms. However, both monocytes and macrophages have acquired different pathways to balance iron levels, therefore they are not so much affected by iron limitation (Ganz and Nemeth 2015, Soares and Weiss 2015). Considering the direct link between iron and immunity, it is indicative that iron supplementation may be beneficial in the context of IBD. Intravenous iron supplementation in patients with active rheumatoid arthritis reduced the levels of TNF-α and peroxides in the blood and the disease activity index (Kaltwasser, Kessler et al. 2001, Weiss, Meusburger et al. 2003). However, oral supplementation with iron in animal models of IBD (Oldenburg, van Berge Henegouwen et al. 2000) as well as in patients with CD (Erichsen, Hausken et al. 2003) enhanced inflammation through a local increase of reactive oxygen species (Weiss 2011, Werner, Wagner et al. 2011).

The anti-inflammatory properties of plant-based compounds have been investigated in the context of IBD (Ung, Foshaug et al. 2010, Bruckner, Westphal et al. 2012, Liu, Li et al. 2018, Li, Han et al. 2020). In particular, polyphenols are the most studied compounds in plant-based foods, and major polyphenols in the diet include hydroxycinnamic acids (e.g. chlorogenic acids), flavonoids, stilbenes (e.g. resveratrol), and tannins. Preclinical and clinical studies in IBD patients have shown that polyphenols are effective in preventing and alleviating the symptoms of IBD (Roth, Spalinger et al. 2016, Carmona-Hernandez, Taborda-Ocampo et al. 2019, Shanmugam, Thangaraj et al. 2020). In IBD patients, polyphenols affected the composition of gut microbiota and had a direct effect on immune cell function (Figure 22) (Alrafas, Busbee et al. 2019, O'Connor, Morrissette et al. 2019). Most of the polyphenols are also metabolized by gut microbiota into beneficial microbial metabolites, whose effect during intestinal inflammation has already been established (de Ferrars, Czank et al. 2014, Braune and Blaut 2016). The effect of anti-inflammatory polyphenols on both innate and adaptative immune system is mediated by several synergic mechanisms including alteration of signaling and enzymatic processes involved in inflammation such as protein kinases, which have been known to be involved in B and T-cell activation and proliferation, inhibition of the key inflammatory mediators NFkB, iNOs, pro-inflammatory enzymes such as COX-2, modulating inflammatory mediators such as cytokines, chemokines, and arachidonic acid and scavenging of free radicals and inflammatory prooxidants (Hussain, Tan et al. 2016, Mao, Gu et al. 2017). Considering that an excessive ROS production present in inflamed mucosa is a key predisposing factor for CRC development, polyphenol-rich diet may represent a

chemopreventive treatment in CAC as well (Mileo, Nistico et al. 2019). It is still unknown whether the mechanism of action is specific to each subclass of polyphenol, and if one class is more effective than the others.

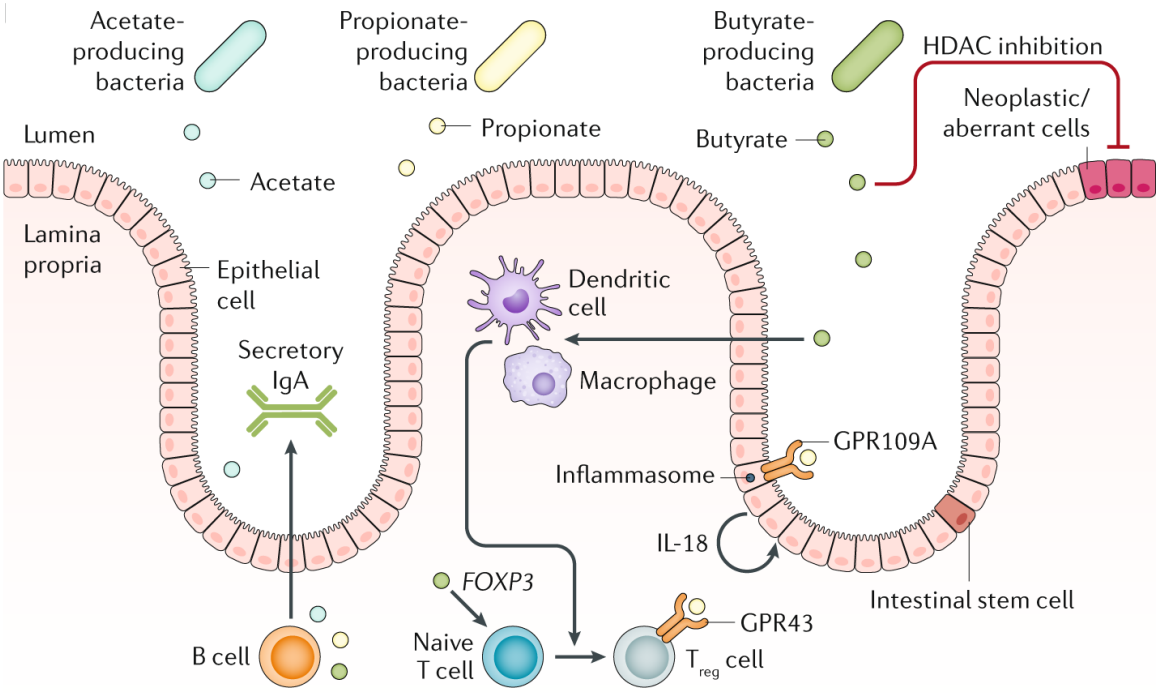


Figure 20. Effects of SCFAs on mucosal immunity (adapted from Lavelle and Sokol, 2020). SCFAs mediate diverse effects on mucosal immunity, including supporting B cell development, differentiation and expansion of regulatory T (Treg) cells, and effects on colonic dendritic cells and macrophages.

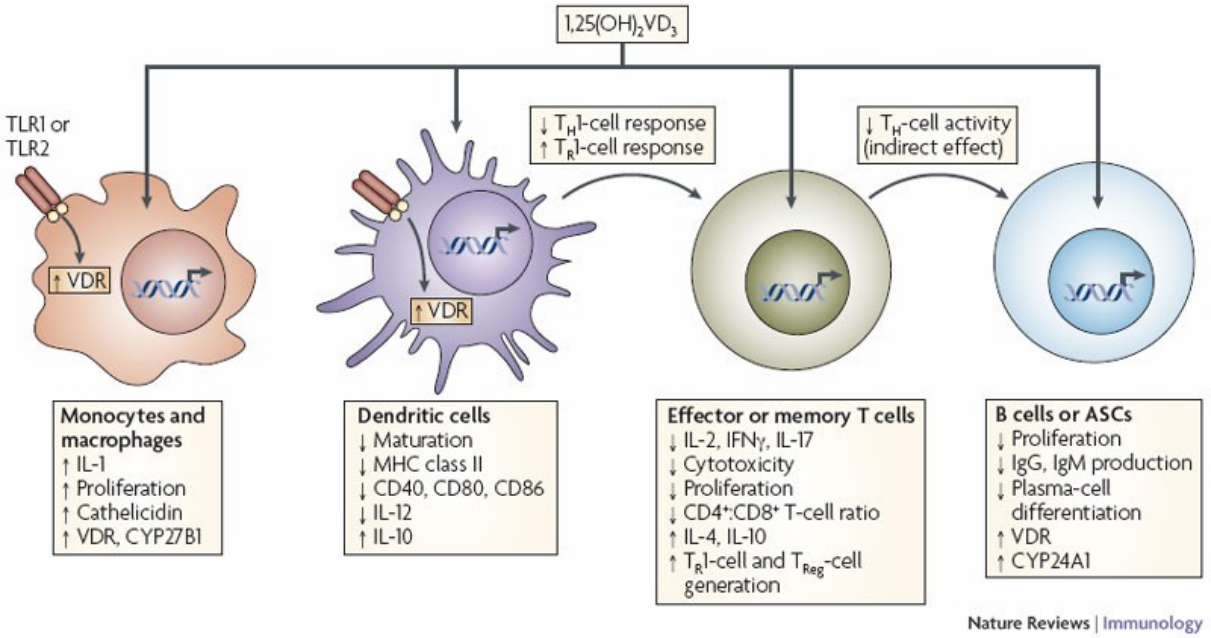


Figure 21. Mechanisms of vitamin D immunomodulation (from Mora et al., 2008). Systemic or locally produced 1,25(OH)2VD3 exerts its effects on several immune-cell types, including macrophages, dendritic cells (DCs), T and B cells.

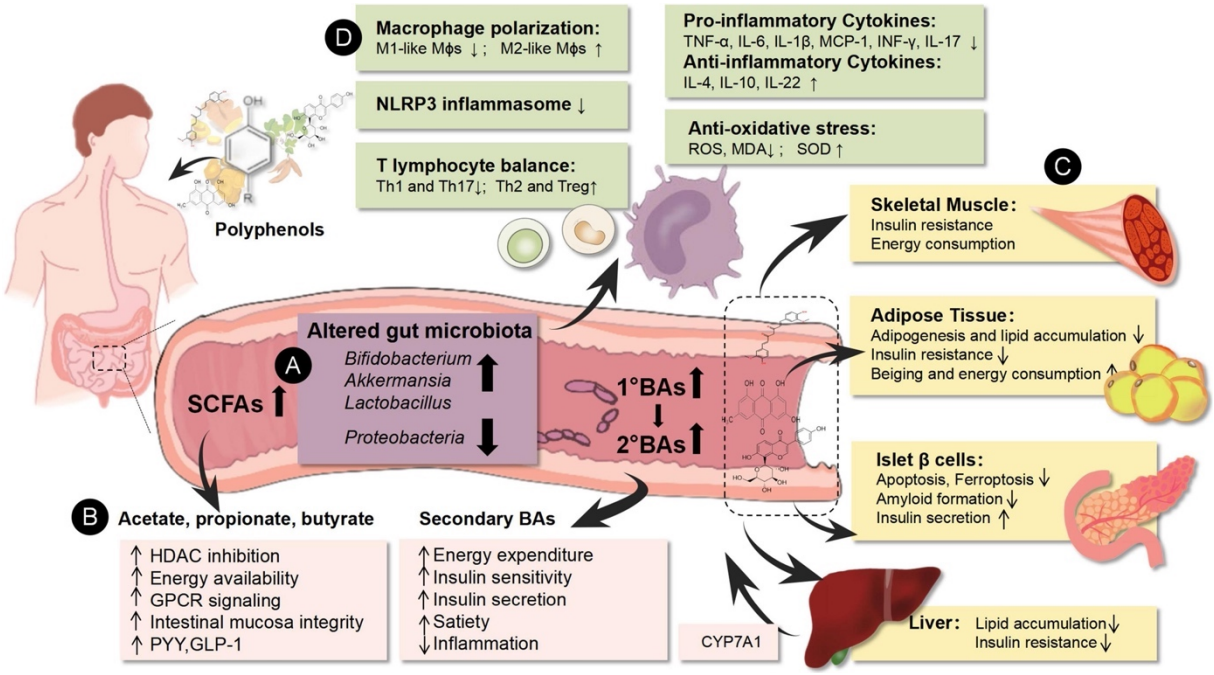


Figure 22. Multiple targets of natural polyphenols: gut microbiota, metabolism, and immunity (from Chen et al., 2022). Natural polyphenols showed multiple targets, including enrichment of beneficial gut microbiota and decrease of the abundance of harmful gut microbiota, and immunity rebalance through polarization of Mϕs and the ratio of T lymphocyte subtypes.

2.3 Dietary therapies for the management of IBD patients

Dietary intervention is becoming essential in the management of IBD patients at different stages of pathology (Figure 23). Since nutrition is expected to play a role in the pathophysiology of IBD, effectiveness of several whole food diets in IBD were studied in order to know which foods to include or avoid (Bolte, Vich Vila et al. 2021). The International Organization For the Study of Inflammatory Bowel Disease (IOIBD) and the ESPEN have developed nutrition guidelines for IBD patients (Levine, Rhodes et al. 2020, Bischoff, Bager et al. 2023). Clinical trials have suggested that a "mediterranean-like" diet, with a higher intake of fruit, vegetables and omega-3, and a reduced consumption of saturated fats, ultraprocessed foods and red meat, improves IBD symptoms (Serrano-Moreno, Brox-Torrecilla et al. 2022).

Dietary manipulation [i.e., the Crohn's disease exclusion diet (CDED), Exclusive Enteral Nutrition (EEN), and Partial Enteral Nutrition (PEN)] has been associated with higher rates of remission in both adult and pediatric patients, and a decrease in inflammatory markers such as serum C-reactive protein and fecal calprotectin (Narula, Dhillon et al. 2018, Levine, Wine et al. 2019, Svolos, Hansen et al. 2019).

EEN consists of a liquid formula diet (while avoiding all other oral intake) and has been shown to be superior to oral corticosteroids in pediatric patients diagnosed with low-mild grade CD. Considering the counter effects of steroid therapies in children, EEN is currently the treatment of choice for pediatric CD in Europe. In adults, the lower efficacy rates, standing at 45%, may be primarily attributed to suboptimal levels of adherence to therapy (Wall, Day et al. 2013, Narula, Dhillon et al. 2018). Increasing evidence supports the preoperative use of EEN, with data indicating a decrease in postoperative complications (Shariff, Moran et al. 2021). When used for longer periods of treatment, EEN has also proven effective in addressing complications associated with CD, such as strictures, abscesses, and fistulae (Hu, Ren et al. 2014, Yan, Ren et al. 2014, Yang, Gao et al. 2017). The specific EEN mechanism of action is still not well determined, but several hypotheses have been proposed, based on the results of some pre-clinical studies. EEN is likely to influence the complex interplay between host immune response and gut luminal environment (Gerasimidis, Bertz et al. 2014, Alhagamhmad, Day et al. 2017, Diederen, Li et al. 2020). Some studies have demonstrated a microbiological change in the gut following EEN, while others have proposed a direct effect on the immune cells and gut barrier (Nahidi, Corley et al. 2015, Yu, Yu et al. 2018, Sun, Zhang et al. 2019, Lunken, Tsai et al. 2021, Teng, Qi et al. 2021). Some formulas contain high levels of TGF-β (Modulen IBD® and Santactiv Digest®), which has positive effects in inducing an anti-inflammatory phenotype in immune cells (Ihara, Hirata et al. 2017).

The only real food-based dietary therapy providing compelling evidence of reducing inflammatory activity, in a way comparable to that of EEN, is the CDED with PEN (Levine, Wine et al. 2019). PEN in association with free diet is ineffective in inducing complete remission or reducing acute phase inflammatory response. The hypothesis behind the CDED is to correct the Western diet composition to reverse the disease process. Therefore, CDED avoids or reduces exposure to animal fat, dairy products, gluten, and emulsifiers and enables exposure to fiber from fruits and vegetables (Levine, Wine et al. 2019). However, the contribution of PEN even in this combination is not well determined, since a recent trial showed a similar outcome in patients receiving CDED with or without the support of PEN (Yanai, Levine et al. 2022). Nonetheless, dietary treatment combining PEN with the CDED may be a useful treatment for patients failing biological therapy and it seems to be a better tolerated alternative to EEN (Sigall Boneh, Sarbagili Shabat et al. 2017). The use of both EEN and CDED+PEN strategies for maintaining remission appears to be an unattractive option to patients due to the restrictions of this approach in social settings, therefore there is no dietary therapy that can be recommended for this phase of the disease (Yamamoto, Nakahigashi et al. 2010).

Other diets have been developed and studied in the context of IBD, i.e., the specific carbohydrate diet, based on the idea that disaccharides and polysaccharides damage the intestinal mucosa (not consistent with the scientific basis of IBD, but appeared to be effective in inducing remission in different RCTs) (Cohen, Gold et al. 2014, Obih, Wahbeh et al. 2016, Lewis, Sandler et al. 2021); the Mediterranean diet, which comprises a high intake of fruit, vegetables, legumes, wholegrains, moderate amounts of fish, dairy products and alcohol, and limited red meat; the low-emulsifiers diet, and others more (Godny, Reshef et al. 2020, Chicco, Magri et al. 2021). However, the only high-quality, evidence-based dietary therapies for reducing intestinal inflammation in patients with IBD are EEN and/or CDED (Narula, Dhillon et al. 2018).

The majority of clinical trials involving dietary therapies are susceptible to bias and methodological issues, making it impossible to determine and apply definitive guidelines (Fiorindi, Cuffaro et al. 2021). Nonetheless, almost 90% of patients changes their diet when diagnosed with IBD, and most of the times it’s self-prescribed (Larussa, Suraci et al. 2019). Therefore, a comprehensive prospective investigation into the impact of specific diets on IBD is a critical step to customize dietary therapy to diminish inflammation, alleviate symptoms, enhance nutritional status, and improve other clinical outcomes.

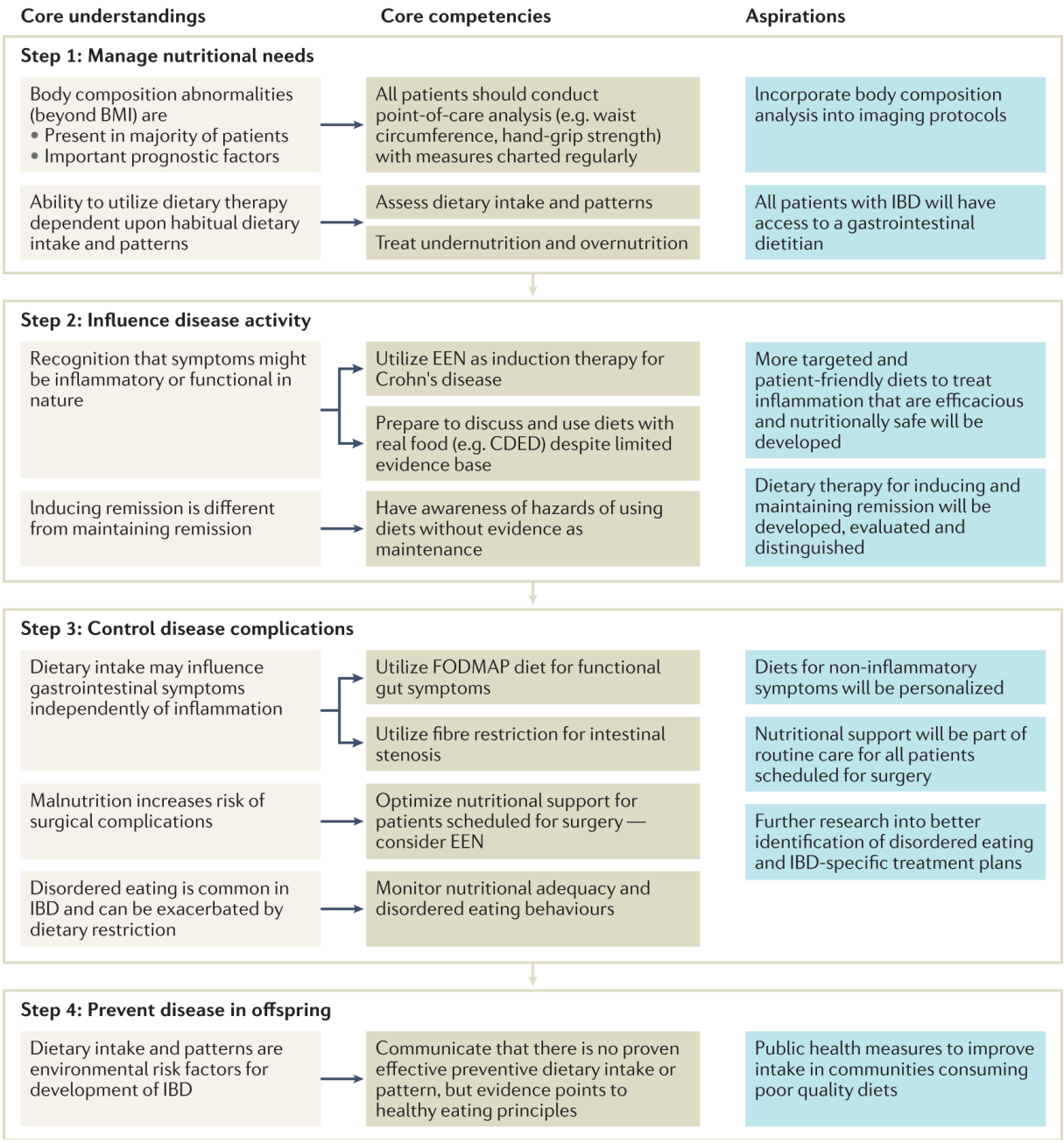


Figure 23. Roles of diet therapy in management of IBD (from Fitzpatrick et al., 2022). The four recommended stages for managing inflammatory bowel disease (IBD) are: manage nutritional needs; influence disease activity; control disease complications; and prevent disease in offspring.

Chapter III. Transient Receptor Potential (TRP) ion channels

3.1 TRP channels in gut diseases

Transient receptor potential (TRP) channels are a superfamily of ion channels found in numerous tissues and cell types, permeable to a wide range of cations such as Ca^{2+}, $Mg^{2+,}$ Na^{+}, K^{+}, and others (Figure 24). TRP channels are sensors for a variety of cellular and environmental signals and are responsible for various sensory responses including heat, cold, pain, stress, vision and taste (Clapham 2003). Mammals express a total of 28 different TRP channel proteins, which can be divided into seven subfamilies: TRPA (Ankyrin), TRPC (Canonical), TRPM (Melastatin), TRPML (Mucolipin), TRPN (NO-mechano-potential, NOMP), TRPP (Polycystin), TRPV (Vanilloid) (Clapham 2003).

TRP channels are widely distributed in various tissues, including the GI tract (Holzer 2011), and play key roles in both physiological and pathological conditions, and particularly in pain, chronic inflammatory diseases, such as IBD and its complications like visceral hypersensitivity (VHS), intestinal fibrosis, gut microbiota composition and CRC (Figure 25) (Toro, Arias et al. 2011, Balemans, Boeckxstaens et al. 2017, Inoue, Kurahara et al. 2019). The abnormal expression of TRP channels has been observed in the colon of both IBD patients and mice, as well as in patients with CRC (Stoklosa, Borgstrom et al. 2020, Du, Chen et al. 2022), identifying TRP channels as potential drug targets for the management of GI pathologies.

TRP channels are mainly expressed by extrinsic primary afferent nerves with some on epithelial, endocrine cells, and intrinsic enteric neurons in the gut (Holzer 2011, Zielinska, Jarmuz et al. 2015), but the role of extra-neuronally expressed TRP channels is emerging. In particular, in the last decades the presence of TRPs was confirmed in various cell types, including immune cells, where they are involved in the regulation of both phagocytic activity and cytokine secretion (Nassini, Pedretti et al. 2012, Romano, Borrelli et al. 2013, Khalil, Alliger et al. 2018).

Various stimuli, including heat or cold, changes in osmolarity, proton gradients, and both natural and synthetic compounds, have the potential to activate TRP channels (Cai and Chen 2023). Importantly, numerous TRP agonists and antagonists are not specific and cross-reactivity with other TRP channels has been observed. Moreover, it is widely demonstrated that TRP channels can be desensitized and therefore inactivated by their agonists, through a process based on the elevation of intracellular Ca^{2+} levels (Rohacs, Lopes et al. 2005, Mercado, Gordon-Shaag et al. 2010, Diver, Cheng et al. 2019, Liu, Yudin et al. 2020, Ningoo, Plant et al. 2021). Activation of TRP channels causes ionic changes inside and outside the cell to trigger downstream pathways (Dang, van Goor et al. 2019, Zhang, Julius et al. 2021).

Upon stimulation, TRP channels in afferents can lead to autonomic reflex responses through transmissions to the central nervous system (Veldhuis, Poole et al. 2015, Du, Liao et al. 2019). Additionally, their activation is involved in the crosstalk between the nervous and the immune system through the release of immunomodulatory neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP), evoking the so-called neurogenic inflammation (Holzer 2011, Allais, De Smet et al. 2017). Recently, it has been shown that these gut-innervating fibers can regulate the composition of the intestinal microbiota, mediating an anti-inflammatory effect during intestinal inflammation (Zhang, Lyu et al. 2022).

Besides their role in the gut nervous system, only little is known about the functional role of extra-neuronal TRP channels. In immune cells, TRPs directly contribute to immune responses, mainly affecting the mitogen-activated protein kinase (MAPK) pathway (Zitt, Halaszovich et al. 2002, Wang, Yang et al. 2019), TGF-β signaling pathway (Liu, Qi et al. 2018, Han, Tang et al. 2020, Liu, Wang et al. 2022), NF-κB pathway (Kang, Ding et al. 2017, Cao, Li et al. 2019, Yuan, Liang et al. 2021), and AMP-activated protein kinase (AMPK) pathway (Zou, Chen et al. 2019, Szrejder, Rachubik et al. 2020) (Figure 26).

TRPV1 and TRPA1 are the most expressed TRP channels in the colon, and their contribution to intestinal inflammation is long known (Boesmans, Owsianik et al. 2011). TRPV1 is predominantly coexpressed with the TRPA1 in sensory neurons innervating the gut (Story, Peier et al. 2003). Interestingly, a strong TRPA1 and TRPV1-immunopositivity within mononuclear cells infiltrating the colonic mucosa of patients with IBD has also been observed (Kun, Szitter et al. 2014). Infiltration of TRPA1+TRPV1+ T cells in the colon likely contribute to the pathophysiology of IBD, with TRPA1 exerting an anti-inflammatory effect and TRPV1 contributing to T-cell activation instead (Bertin, Aoki-Nonaka et al. 2017).

TRPV4 and TRPM2 expressing fibers show a clear pro-hypersensitivity function during colitis (Brierley, Page et al. 2008, Matsumoto, Takagi et al. 2016). Moreover, these two channels, together with TRPM7, mediate a Ca^{2+} influx in immune cells that is able to potentiate their activation, highlighting a critical pro-inflammatory role for these TRPs (Wehrhahn, Kraft et al. 2010, Schappe, Szteyn et al. 2018, Li, Fang et al. 2019).

TRPs play a crucial role also in cancer pathophysiology and studies have shown a deregulation of such ion channels in CRC patients, resulting in altered cancer hallmarks and functions. In a bioinformatic analysis of TCGA database, a TRP signature was used to make a stratification of CRC patients, and the results showed that the low-TRP score group exhibited an immunosuppressive phenotype characterized by heightened neutrophil infiltration and activation of the MAPK signaling pathway (Wang, Chen et al. 2022). Wang et al., also

demonstrated an increased sensitivity to preoperative chemoradiotherapy and was associated with a more favorable prognosis (Wang, Chen et al. 2022). Additionally, murine models have demonstrated a protective role for TRPV1 during CRC, while TRPV6 appeared as the most significantly overexpressed TRP channel in tumor cells, regulating their proliferation (Alaimo and Rubert 2019). Overall, therapeutic strategies targeting TRP channels and their signaling pathways have shown promising results for the prevention and treatment of gastrointestinal diseases.

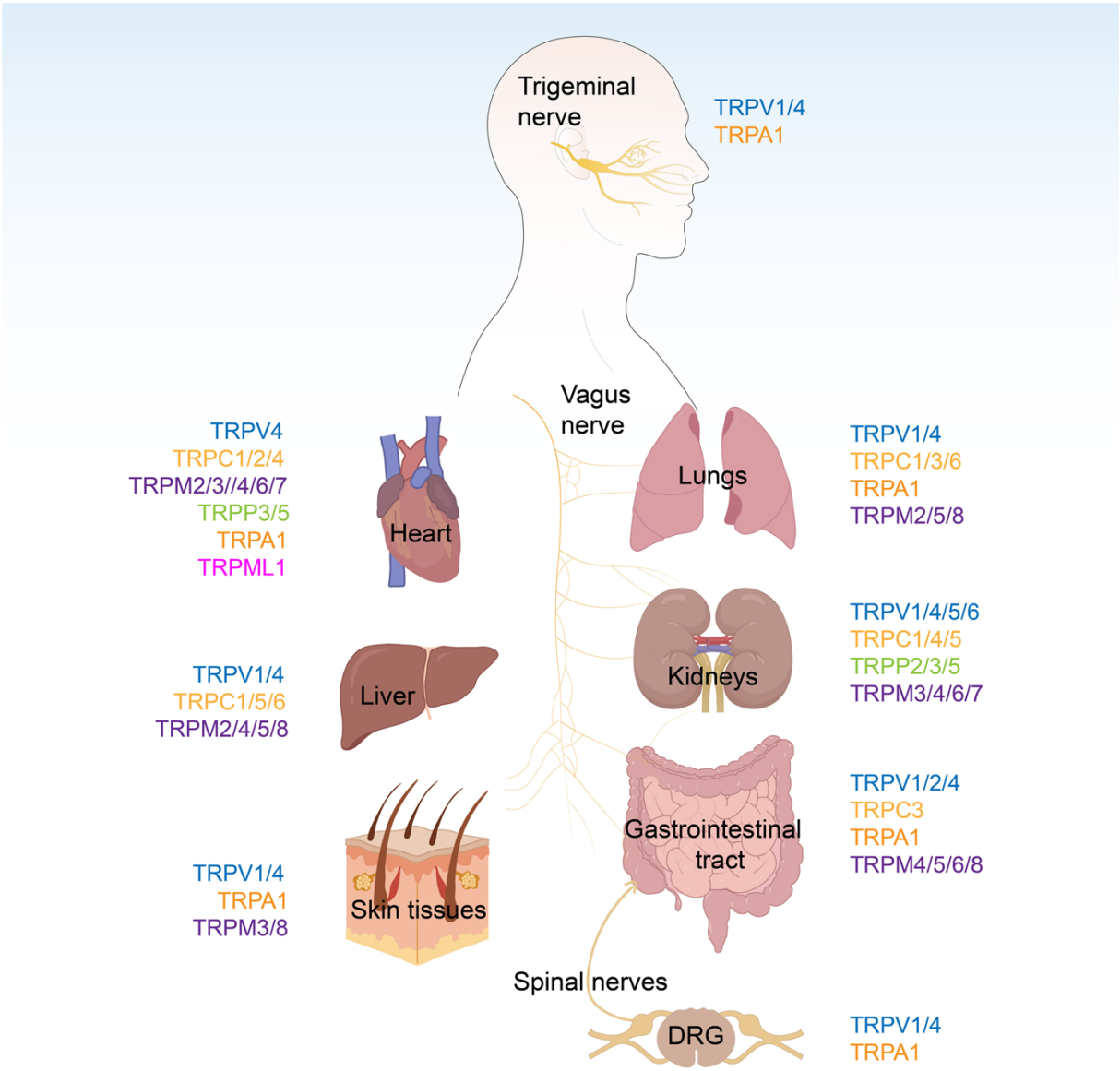


Figure 24. Distribution of TRP channels in the human body (from Zhang et al., 2023). TRP channels are widely distributed in human organs, such as lung, liver, skin, nerves, and intestine. The different colors of the words represent the different TRP families.

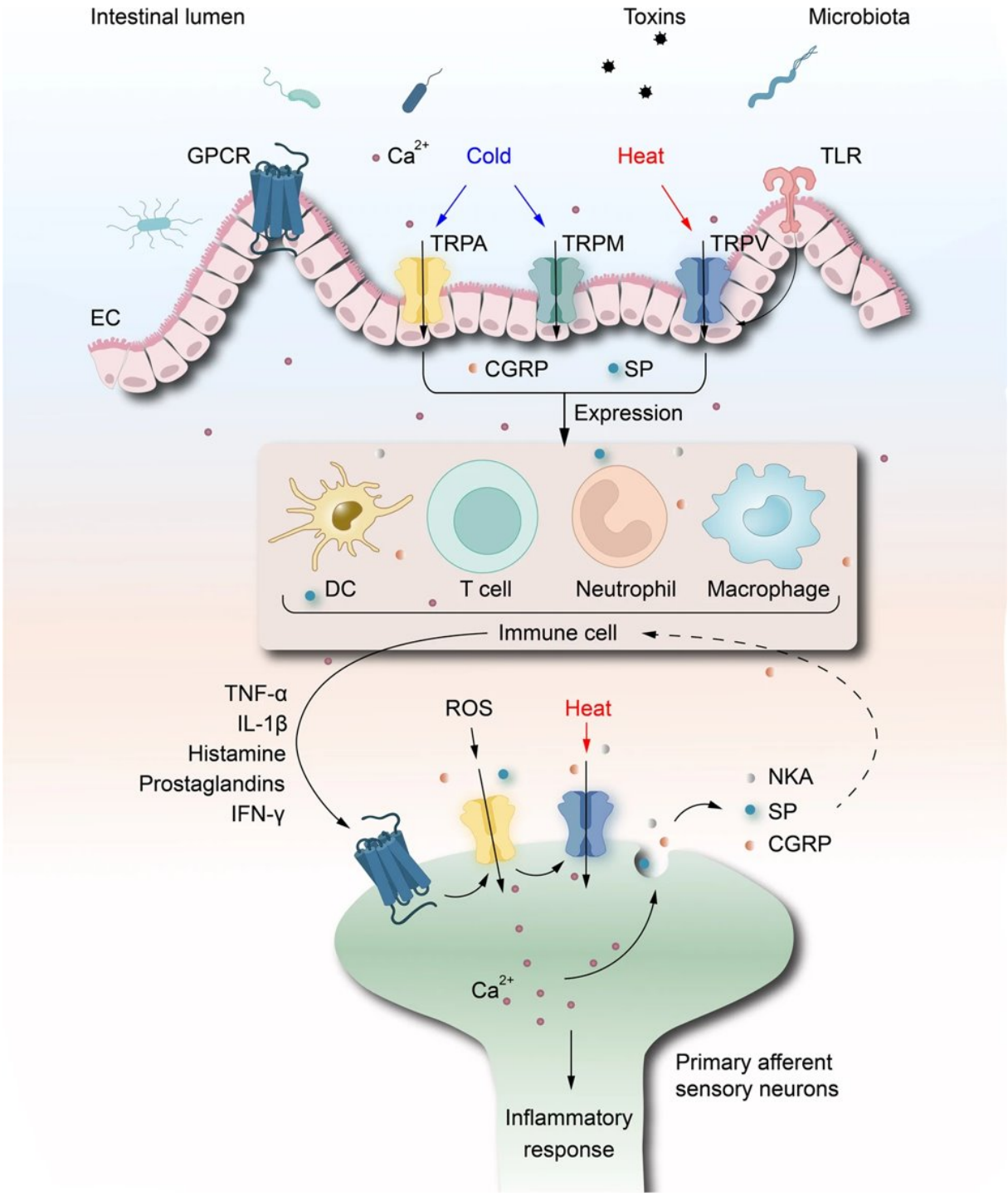


Figure 25. TRP channels in gut diseases (from Zhang et al., 2023). TRP channels can be stimulated by a variety of factors in the intestinal lumen, including temperature, chemoregulators, and inflammation, and act as auxiliary transducers for GPCR. Interestingly, TLRs play a major role in the regulation of TRPV1 signaling. Stimulation of TRP channels on EC triggers the release of the neuropeptides CGRP and SP, which activate surrounding immune cells and release more inflammatory factors such as TNF-α, IL-1β, histamine, and IFN-γ. These inflammatory factors accumulate at the GPCR of primary afferent sensory neurons. Subsequent

activation of TRP channels on neurons triggers the release of neurokinin A, CGRP, and SP, which promote pain perception and inflammatory responses.

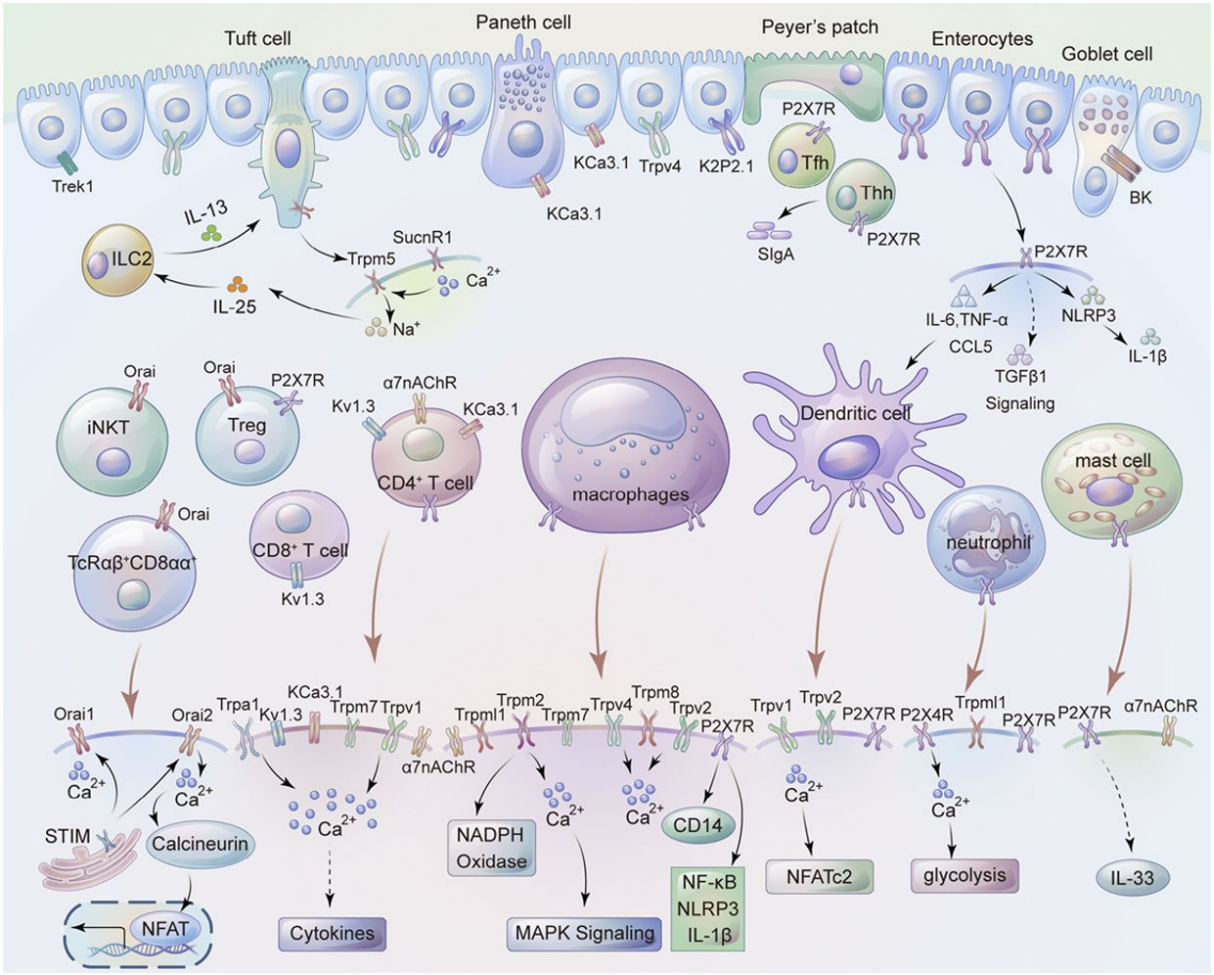


Figure 26. Summary of cell types and associated ion channels involved in gut immunity (from Feng et al., 2021). Immune cell expressing TRP ion channels regulate immune activation and cytokine/chemokine release in the intestinal inflammation through provoking Ca2+ influx and intracellular Ca2+ mobilization.

3.2 Transient Receptor Potential, subfamily Melastatin, type 8 (TRPM8)

The TRP cation channel, subfamily melastatin (M), member 8 (TRPM8), is a voltage-gated ion channel, mainly permeable to calcium, belonging to the TRP superfamily (Clapham 2003). TRPM8 channel is tetrameric protein with six transmembrane domains (S1-S6) and intracellular N-terminal and C-terminal. The S4 segment, together with the S4-S5 linker, contains the voltage sensor, and transmembrane domains S5 and S6 form the channel pore (Voets, Owsianik et al. 2007, Voets, Owsianik et al. 2007). The intracellular C-terminal has a coiled-coil domain necessary for channel tetramerization (Tsuruda, Julius et al. 2006), while a different domain is essential for channel modulation by temperature and phosphatidylinositol 4,5-biphosphate (PIP2) (Liu and Qin 2005, Rohacs, Lopes et al. 2005). TRPM8 phosphorylation in the N-terminus domain alters gating properties that negatively modulate the channel activity and modify the channel response to agonists (Phelps and Gaudet 2007, Rivera, Moreno et al. 2021). Several endogenous molecules modulate TRPM8, including testosterone and estradiol, which are able to bind TRPM8 and regulate its expression and activation, and the endocannabinoid anandamide, which acts as an endogenous antagonist of the receptor. Additionally, extracellular and intracellular pH changes can shift the threshold for TRPM8 channel activation (Andersson, Chase et al. 2004). To note, pathological conditions such as tumor and inflammation induce reported changes in pH (Ward, Meehan et al. 2020, Chen, Jaiswal et al. 2022). Besides, TRPM8 is modulated by cooling compounds, both synthetic (i.e., icilin, WS-12, AMTB), and of natural origin [i.e., menthol (from *Mentha x piperita*) and eucalyptol (from *Eucalyptus spp*.)] (Journigan and Zaveri 2013). Similarly to TRPV1, prolonged agonist exposure to TRPM8 causes desensitization and inactivation of the channel (Diver, Cheng et al. 2019). Here, increased Ca^{2+} influx subsequently leads to cleavage of membrane PIP2 which activates cellular downstream events to inactivate the channel (Figure 27) (Rohacs, Lopes et al. 2005, Sarria, Ling et al. 2011).

TRPM8 is the main receptor involved in cold sensation, being widely expressed by sensory neurons (Clapham 2003). In the gut, it is expressed in high threshold afferent fibers, where it modulates the chemosensory and mechanosensory activities of TRPA1 and TRPV1 channels (Harrington, Hughes et al. 2011). TRPM8 mRNA was also present in colonic DRG neurons (Harrington, Hughes et al. 2011). Recently, a GWAS of irritable bowel syndrome (IBS) patients showed that TRPM8 SNP were associated with slower colonic transit, constipation and IBS type C and M, probably resulting from allele specific variants of its function or expression in the GI tract (Henstrom, Hadizadeh et al. 2017). Consistently, peppermint oil-based preparations (which contain menthol as the biologically active ingredient) are already used by IBS patients

because of their analgesic, antispasmodic, and carminative effects (Henstrom, Hadizadeh et al. 2017).

Although epithelial cells commonly express low amounts of TRPM8, its expression is much higher in tumor cells (Tsavaler, Shapero et al. 2001). Upregulation of TRPM8 was observed in CRC immortalized cells (Borrelli, Pagano et al. 2014), as well as in gastric cancer patients with metastasis and in CRC patients with liver metastasis (Liu, Li et al. 2022), but also in many other cancer types (Yee, Brown et al. 2012, Kijpornyongpan, Sereemaspun et al. 2014, Hemida, Hammam et al. 2021, Xu, Kong et al. 2021). To date, either activation (by increasing cytosolic Ca^{2+} levels) or antagonism/desensitization (by decreasing cytosolic Ca^{2+} levels) of TRPM8 (Zhang and Barritt 2006) has been shown to affect *in vitro* cell growth and/or survival (Figure 28) (Okamoto, Ohkubo et al. 2012, Yee 2015, Huang, Li et al. 2020). In prostate cancer, TRPM8 androgen-dependent overexpression seems to be required for cancer cell survival (Zhang and Barritt 2004, Lunardi, Barbareschi et al. 2021), and TRPM8 has been proposed as a prognostic marker in such disease (Zhang and Barritt 2004, Zhang and Barritt 2006).

In adipocytes, TRPM8 activation has been linked to an increase in mitochondrial membrane potential, which enhances UCP1 expression, thereby promoting WAT browning and BAT thermogenesis to prevent obesity (Khare, Chauhan et al. 2019, McKie, Medak et al. 2022).

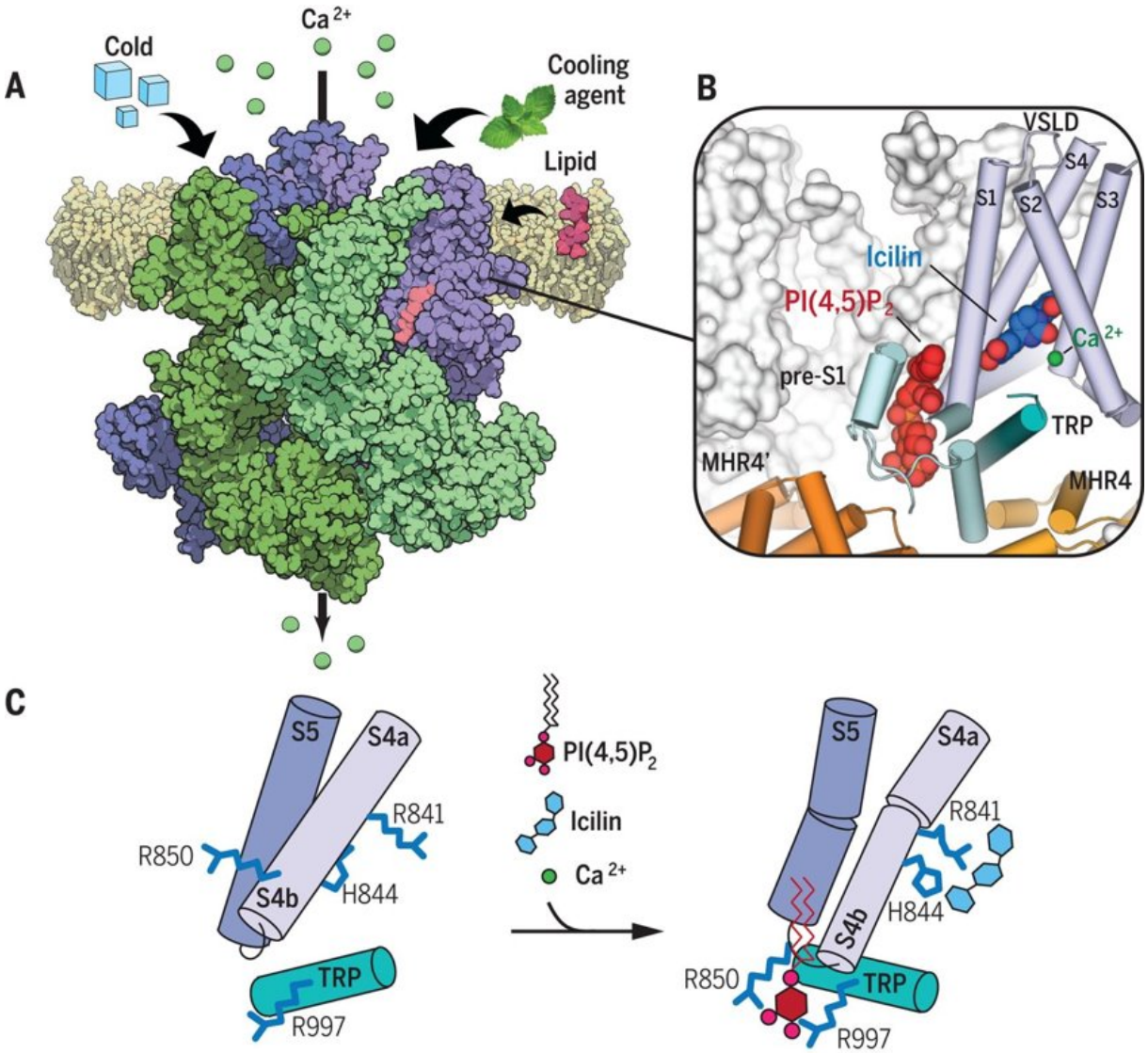


Figure 27. Structural basis of cooling agent and lipid sensing in the TRPM8 channel (from Yin et al., 2019) (A) TRPM8 is a polymodal calcium-permeable channel regulated by various physical and chemical stimuli. (B) Location of the binding site for cooling agents and membrane lipid PIP2. MHR4, melastatin homology region 4. (C) Ligand-induced structural rearrangements suggest allosteric coupling between cooling agents and PIP2.

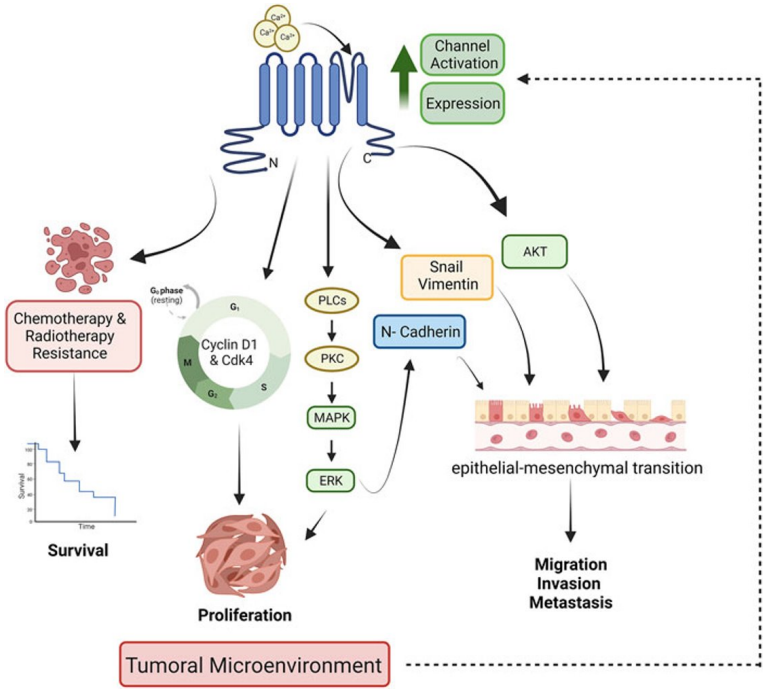


Figure 28. TRPM8-dependent mechanisms involved in tumor development (from Ochoa et al., 2023). The tumoral microenvironment releases factors that increase the expression and activation of TRPM8 channels. Channel activation leads to the activation of signaling pathways whose activation decreases patient survival and increases proliferation, migration, invasiveness, and metastasis.

3.3 TRPM8 in immune responses

Increasing evidence shows that TRPM8 might be also implicated in inflammatory disorders, and its expression in innate immune cells during intestinal inflammation has been revealed (Ramachandran, Hyun et al. 2013, de Jong, Takahashi et al. 2015, Khalil, Babes et al. 2016). TRPM8 is up-regulated in the inflamed colon of mice and CD patients (Ramachandran, Hyun et al. 2013, de Jong, Takahashi et al. 2015), as well as in activated murine peritoneal macrophages (Khalil, Babes et al. 2016). Moreover, recent findings document an involvement of TRPM8 in human monocyte differentiation and survival (Hornsby, King et al. 2022). As for others TRP channels, TRPM8 effects during inflammation could be either directly mediated in immune cells, either indirectly, through an effect on the release of immunomodulatory neuropeptides (Figure 29). Experiments conducted on peritoneal macrophages showed that LPS stimulation increased TRPM8 expression in these cells 100 folds, but its activation by menthol induced an anti-inflammatory phenotype (Khalil, Babes et al. 2016). The group of Khalil ultimately showed that *Trpm8*$^{-/-}$ mice and wild type (WT) mice transplanted with Trpm8–/– macrophages exhibited a more severe colitis compared to WT mice and Trpm8–/– mice transplanted with WT macrophages, respectively, suggesting a direct role of Trpm8 expressing macrophages during colitis (Khalil, Babes et al. 2016). On this line, TRPM8 agonists 1,8-cineol (eucalyptol) and L-menthol inhibited the production of pro-inflammatory cytokines in human monocytes and lymphocytes in vitro (Juergens, Stober et al. 1998, Juergens, Engelen et al. 2004). However, two other reports favor a dominant role for TRPM8 expression in controlling CGRP release from peptidergic sensory neurons, thus affecting the colitogenic response (Ramachandran, Hyun et al. 2013, de Jong, Takahashi et al. 2015). In particular, de Jong et al. demonstrated that *Trpm8*$^{-/-}$ mice exhibited defective release of CGRP in a DSS model of intestinal inflammation, suggesting that the role of TRPM8 was not a macrophage cell-intrinsic effect, but related to the release of CGRP from sensory neurons adjacent to immune cells in the colon (de Jong, Takahashi et al. 2015). Conversely, Ramachandran et al. showed that DSS-treated *Trpm8*$^{-/-}$ mice showed elevated colonic levels of the neuropeptide CGRP, although they exhibit no difference in terms of inflammatory parameters compared to WT mice (Ramachandran, Hyun et al. 2013). Considering this background, it seems clear that the TRPM8 mechanism of action and, importantly, pathophysiological and clinical significance in intestinal inflammation as well as in colon carcinogenesis are still largely contradictory and mechanistically undefined.

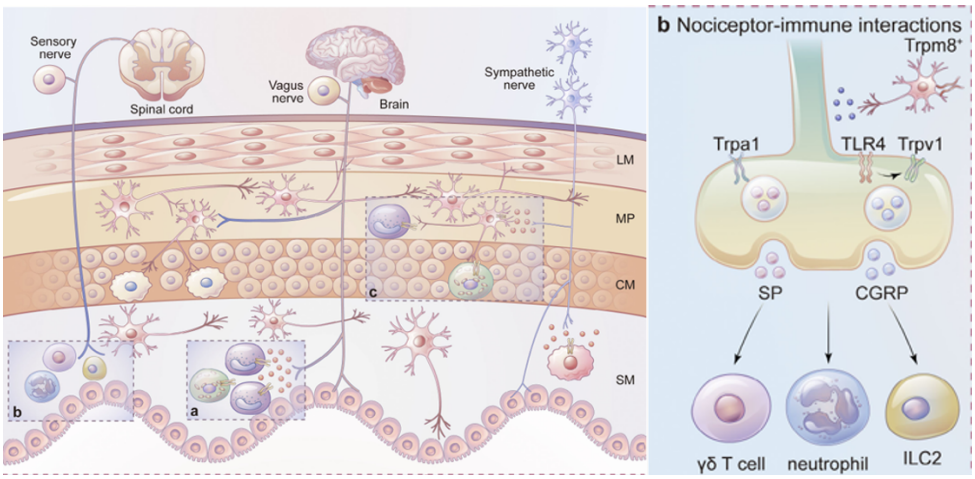


Figure 29. TRP channels in gut immunity involving crosstalk between gut-innervating neurons and resident immune cells. The GI tract is innervated by spinal cord sensory neurons, brain stem originating vagus neuron and intrinsic enteric neurons. TRP channels expressed by nociceptors control the release of neurotransmitters such as SP and CGRP, which may modulate the activities of immune cells and contribute to the nociceptor-immune interaction (b).

3.4 Dietary modulation of TRPM8

Target prediction software suggest that a significant proportion of natural products display potential pharmacophores for modulation of one or more TRP channels (Reker, Perna et al. 2014). Although experimental confirmation is required for these predictions, the data suggest that natural products present privileged scaffolds for engaging TRP ion channels. A large plethora of natural ligands and/or food-derived molecules bind and modulate TRPM8 (Rodrigues, Sieglitz et al. 2016). As seen above, menthol, extracted from several species of mint, is a prototypical TRPM8 terpene agonist (EC50= 4–80 μM), which led to the identification of the TRPM8 protein in sensory neurons decades ago (Hensel and Zotterman 1951, Bautista, Siemens et al. 2007). Menthol, upon binding to TRPM8 segment 2, shifts the voltage threshold towards negative potentials, consequently shifting the temperature-threshold towards warmer temperatures. In addition to menthol, structural analogues can modulate TRPM8. Monoterpenes are nonnutritive dietary components found in the essential oils of citrus fruits, cherry, mint and herbs. Eugenol, menthone (the precursor of menthol in its biosynthesis pathway), umbellulone and thymol also activate TRPM8, but they are less potent than menthol and also not selective (Meotti, Lemos de Andrade et al. 2014). Further, some other naturally occurring TRPM8 ligands were found in edible plants. For instance, linalool found in many spice plants, and geraniol, found in rose oil, palmarosa oil, citronella oil, geranium and lemon, have been shown to activate TRPM8 (Behrendt, Germann et al. 2004, Patel, Goncalves et al. 2014). The monoterpenes perillaldehyde and perillaketone, at a concentration of 200 μM, partially inhibited the currents induced by icilin in TRPM8-HEK-293 cells (Meotti, Lemos de Andrade et al. 2014). Sesamin (from *Sesamum indicum*), was recently identified as a TRPM8 blocker (IC50=9.79 μM), selectively over a panel of other six TRP channels, and with no cytotoxic effect up to 90 μM. Different flavones, such as chrysin, scutellarein or hispidulin, at a concentration of 10 μM, potently inhibited TRPM8 channel activation (Sui, Li et al. 2020).

Considering the widespread of TRPM8-targeting food components, dietary modulation of this receptor may be considered as a possible therapeutic strategy, keeping in mind the growing body of experimental evidence supporting the involvement of TRPM8 in many pathological as well as physiological conditions. Besides, menthol-containing foodstuffs are perhaps the most widespread and well-recognized TRP-targeting foods. In preclinical studies, dietary menthol was able to reduce inflammation following myocardial infarction in mice, through TRPM8-mediated release of CGRP (Wang, Yang et al. 2020). Moreover, dietary TRP-based tri-agonist (combination of sub-effective doses of capsaicin, cinnamaldehyde and menthol) were effective in ameliorating hyperglycemia and hyperinsulinemia in obese mice as well as in HFD-fed mice,

supporting the possibility to develop TRP-based therapeutic functional food to enhance metabolism and treat obesity (Kaur, Kumar et al. 2022, Kaur, Singh et al. 2023). Menthol preparations have also been evaluated in a clinical trial (NCT01855607) for the treatment of chemotherapy- and diabetes-induced peripheral neuropathies, respectively, in selected breast and CRC patients (Li, Zhang et al. 2022). A similar work showed that this neuropathy was relieved by menthol in patients with CRC (Li, Zhang et al. 2022).

In conclusion, taking into account the potential of menthol-based diets for clinical application, a similar approach can be taken to other TRPM8-directed bioactive compounds of dietary origin. This can be crucial for designing therapeutic strategies to pathologies in which diet plays a key role, such as IBD.

AIM

The general aim of our research is to investigate the possible involvement of Transient Receptor Potential Melastatin 8 (TRPM8) in inflammation-triggered gastrointestinal disorders, namely inflammatory bowel disease (IBD) and colitis-associated colorectal cancer (CAC).

To adequately pursue our objective, we evaluated the role of TRPM8 in both pathologies by using human biopsies, bioinformatic analysis of colorectal cancer (CRC) patient database, and *in vitro* and *in vivo* models of intestinal inflammation and colon cancer.

Moreover, considering the impact of nutrition in gastrointestinal disorders, we evaluated the possibility of targeting TRPM8 through newly identified diet-derived ligands.

More in-depth studies were performed in: i) bone marrow-derived macrophages, to evaluate the possible effect of TRPM8 on pro-inflammatory profile of these immune cells during intestinal inflammation, and ii) on CRC-patient biopsies,to evaluate TRPM8 involvement in colon carcinogenesis.

Dietary modulation and genetic ablation of TRPM8 were exploited in well-established models of IBD and CAC (i.e., DSS- induced colitis and AOM/DSS- induced tumors), respectively.

Overall, the ultimate goal of my PhD thesis is to provide potential innovative nutritional-based therapeutic strategies for the prevention and management of gastrointestinal pathologies caused by chronic inflammation.

MATERIALS & METHODS

1.1 Patient samples

Frozen tissue biopsies of 34 primary adenocarcinomas and 34 normal tissues were obtained from CRC patients following the tumor resection at the Department of Public Health (University of Naples Federico II) and were processed for mRNA and protein extraction. Healthy specimens were collected at a distance of at least 10–15 cm from the tumor lesion of the same patients. Patients with a well-established diagnosis of CRC were included (see Table 1 for tumor node metastasis classification). Exclusion criteria were age less than 35 or more than 85 years. Human studies were approved by the Ethics Committee of the University of Federico II (Naples, Italy; Protocol Number 350/19). All patients gave their written and informed consent.

1.2 Isolation of primary tumour cells from CRC biopsies and flow cytometric analysis

Fresh biopsy tissues were dissociated using the human tumour dissociation kit (RRID:SCR_020276, Cat. 130-095-929, Miltenyi Biotec, Auburn, CA, USA). Briefly, the GentleMACS dissociator (RRID:SCR_020267), which combines enzymatic disaggregation with a mechanical disruption process, was used to obtain a single-cell suspension from the bulk tumour. Thus, primary tumour cells were isolated from single-cell suspensions by using a tumour cell isolation kit (130-108-339, Miltenyi Biotec) in conformity with the manufacturer's protocols. Single-cell suspensions were analysed by flow cytometry before and after tumour cell isolation. In brief, primary cells were pelleted, washed and resuspended in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2 mM of ethylenediamine tetraacetic acid (EDTA). For surface staining, cells (5×10^5) were incubated for 10 min at 4°C with the following fluorochrome-labelled anti-human antibodies: CD326 (EpCAM)-APC (clone REA764) (RRID:AB_871664), CD45-PerCP-Vio 700 (clone REA747) (RRID:AB_2889583) and CD31-PE (clone REA1028) (RRID:AB_2660561) (Miltenyi Biotec). Cells were gated for positivity to CD326 and negativity to CD31 and CD45. The flow cytometry analysis was carried out with BriCyte E6 (Mindray, P.R. China), and data were analysed by FlowJo (Tree Star, Inc.). Cell suspension was also proceeded for the mRNA extraction by using miRNeasy Mini Kit (74104, Qiagen, Hilden, Germany), according to the manufacturer's protocol.

1.3 Data Collection and Bioinformatics Analyses

The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/tcga, RRID:SCR_003193) repository collected high-throughput and clinical data of many tumours and normal tissues from many organs. To evaluate differences in prognosis between high and low TRPM8 expressions,

survival curves were estimated by the Kaplan–Meier estimator and compared using log-rank tests. To visualize multiple genomic alteration events in a single overview together with some clinical parameters, cellular localization and immunohistochemistry (IHC), from Human Atlas tumour and normal tissues, we produced an enriched oncoprint heatmap. From UCSC Xena browser (https://xenabrowser.net/datapages/, RRID:SCR_018938) we retrieved the TCGA COAD Illumina HiSeq gene-level transcription estimates, the GISTIC2 gene-level estimates dataset and the clinical data. Using the TCGAbiolinks package, we retrieved the TCGA COAD simple nucleotide variations. In particular, after crossing RNAseq and clinical dataset, we obtained the data of 283 samples used for the survival analyses. Subsequently, the intersection of the samples among all the datasets produced a fully annotated dataset of 268 samples. Differential expression analysis performed using edgeR (Robinson, McCarthy et al. 2010) package. A gene was considered as differentially expressed (DEG) if (1) corrected (FDR) p-value < 0.05 and (2) expression change > 1.5-fold (absolute (log_2FC) > 0.58). The overrepresentation of the DEGs in MSigDB (Liberzon, Birger et al. 2015) pathways was tested applying the ClusterProfiler package (Yu, Wang et al. 2012). We considered statistically significant the gene sets resulting from the analysis of TCGA data with a FDR adjusted p-value < 0.05. The KEGG's (Kanehisa, Sato et al. 2019) WNT signalling pathways was tested and depicted applying the pathview package (Luo and Brouwer 2013). To further investigate the enrichment of the WNT signalling pathways in the expressing TRPM8 versus the practically absent TRPM8 subgroup, a GSEA was performed. To visualize multiple genomic alteration events in a single overview together with some clinical parameters, cellular localization and Immunohistochemistry (IHC) from Human Atlas (Uhlen, Zhang et al. 2017) tumor and normal tissues, we produced an enriched oncoprint heatmap (Gu, Eils et al. 2016). Statistical analysis performed using the computing environment R (R Core Team. R: A Language and Environment for Statistical Computing. 2019, R Foundation for Statistical Computing, Vienna, Austria. (http://www.R-project.org).

1.4 Drugs

Luteolin was purchased from ChemCruz (cat. sc-203119C, Santa-Cruz Biotechnology). GSK 2837808A, AMTB and Icilin were purchased from Tocris (cat. 5189, 3989, and 1531 Bio-Techne). For in vivo studies, luteolin was dissolved in 1% CMC, and for in vitro studies in 0.1% ethanol. GSK 2837808A and icilin were dissolved in 0.1% DMSO. AMTB was dissolved in mq-water. Vehicle did not affect the responses under the study.

1.5 Animal models

1.5.1 Animals

Eight weeks old female wild-type C57BL/6J (Envigo, RRID:IMSR_ENV:HSD-057), and Trpm8−/− (B6.129P2-Trpm8tm1Jul/J, Stock No. 008198; Jackson Laboratories, RRID:IMSR_JAX:008198) mice were housed, at the Department of Pharmacy, University of Naples Federico II (Italy) animal facility. Trpm8−/− mice have been previously described30 and they develop normally without any spontaneous disease emerging under the breeding conditions.51 Eight weeks old female wild-type C57BL/6J (Envigo) and IL-10fl/fl wild-type and LysMCre were kindly gifted from B. Stijlemans, Vrije Universiteit Brussel (VUB), and housed at KU Leuven animal facility (Belgium). All mice were housed under controlled conditions standard laboratory conditions (12-hr light/dark cycle, temperature of 22 ± 2°C) and fed ad libitum with commercially available chow (ssniff® R/M-H, ssniff Spezialdiäten GmbH). All animals were humanely killed by carbon dioxide and cervical dislocation. Death was further assessed by confirmation of rigor mortis. All efforts were made to minimize the number of animals used and their suffering. All experimental procedures and protocols were in compliance with National (Direttiva 2010/63/UE) laws and policies and approved by the Italian Ministry of Health (Protocol Numbers 1101/2015-PR and 481/2020-PR) and by the Animal Care and Animal Experiments Ethical Committee of KU Leuven (188/2019).

1.5.2 Azoxymethane (AOM)/dextran sulfate sodium (DSS) model of CAC

WT and Trpm8−/− mice were injected with AOM (12.5 mg·kg−1, i.p). Colitis was induced by two cycles of 2.5% DSS (molecular weight 36–50 kDa; 02160110-CF, MP Biomedicals, Canada) in drinking water for 5 days, followed by a 16-day tap water period and a final cycle of 2% DSS followed by a 5-day tap water period (Pagano, Elias et al. 2022). All animals were anaesthetized as described above at day 63 and the tumor count was determined microscopically in longitudinally cut specimens. Inflammation grade was evaluated by measuring colon weight/colon length ratio and spleen weight.

1.5.3 DSS-induced colitis

Colitis was induced in female wild-type C57BL/6J mice by DSS administration. DSS (molecular mass: 36 000–50 000 Da; MP Biomedicals, California, USA) was added to drinking water at 2.25% (w/v) for 5 days, followed by 10-day tap water period.50 Mice were monitored daily for weight loss, stool consistency and haematochezia. Disease activity index was set as the combined score of weight loss (score=0: <1%, 1: ~1%–5%, 2: ~5%–10%, 3: ~10%–20%, 4: >20%), stool blood (score=0: absence, 2: presence, 4: gross bleeding) and stool consistency (score=0: formed and hard, 1: formed but soft, 2: loose stools, 3: mild diarrhoea, 4: gross diarrhoea), as previously reported (Wirtz, Popp et al. 2017). Luteolin (3, 10, 30 mg/Kg) or

vehicle (1% CMC) was administered by oral gavage every day for the entire duration of the experiments, starting one week before DSS administration. Mice were sacrificed at day 5, 10 and 15 after colitis induction, and the colon was resected for further investigation.

1.6 Endoscopy for evaluating colon pathology

High-resolution endoscopy in live mice was assessed. Briefly, mice were anaesthetized by subcutaneous injection of alfaxolone (5 mg·kg−1) and xylazine (5 mg·kg−1) and examined with a mini-endoscope (Mainz COLOVIEW® System, Karl Storz). High-quality endoscopy video and photos were used for monitoring and grading tumours and inflammation. Scoring of colitis activity was expressed as a modified murine endoscopic index of colitis severity (MEICS) based on the observed signs of inflammation. The MEICS consisted of the following four parameters: (i) thickening of the colon, (ii) changes of the vascular pattern, (iii) fibrin visible and (iv) stool consistency (Becker, Fantini et al. 2005). Tumour development was scored by the total number as well as the size of tumours. Tumours were counted to obtain the overall number. Tumour sizes were graded as follows: Grade 1 (small but detectable), Grade 2 (tumour covering up to one eighth of the colonic circumference), Grade 3 (tumour covering up to one fourth of the colonic circumference), Grade 4 (tumour covering up to half of the colonic circumference) and Grade 5 (tumour covering more than half of the colonic circumference). Endoscopic grading was performed for each parameter (scores 0–3), leading to a cumulative score between 0 and 15 (Becker, Fantini et al. 2005).

1.7 Isolation of epithelial and lamina propria cells

After removing fat and feces, colons were opened longitudinally, washed in Hank's balanced salt solution (HBSS; cat. H9394, Sigma-Aldrich) supplemented with 1% FBS, 100 µg/mL penicillin, and 100 µg/mL streptomycin, and cut into 0.5 cm pieces. The tissue was incubated twice at 37°C with shaking for 15 min in HBSS containing 1 mM EDTA and 1% FBS. Strainer filtrates were centrifuged (5 min, 300 g) to collect epithelial cells. The remaining tissue sample was minced and incubated with prewarmed 1× Minimum Essential Medium (MEM)-α (Invitrogen) containing 2 mM L-glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin, 2-mercaptoethanol, and 10 % FBS, and supplemented with 1.25 mg/mL collagenase D (cat. 11088882001, Sigma-Aldrich), 0.85 mg/mL collagenase V (cat. C9263-1G, Sigma-Aldrich), 1 mg dispase (cat. 17105-041, Gibco) and 30 U/mL DNase (cat. 04159001, Sigma-Aldrich) for 20 min in a shaking incubator at 37°C. The resulting cell suspension was filtered through a 70 µm cell strainer and lamina propria cells were collected after centrifugation (5 min, 500 g).

1.8 Flow cytometry

Single-cell suspensions (obtained as described above) were incubated for 15 min with mouse FcR Blocking Reagent (1:100 BD Pharmingen) at 4 °C. Next, cells were labelled with anti-CCR2-BUV395 (cat. 747972, BD Biosciences, clone: M5/114.15.2, RRID:AB_2872433) and anti-CD206-AF647 (cat. 141712, BioLegend, clone: C068C2, RRID:AB_10900420) for 15 min at 37°C, then washed with FACS buffer (0.5% FBS and 2 mM EDTA in PBS). Next, cells were stained for surface markers and incubated for 20 min incubation at 4 °C, with the following antibodies: anti-CD11b-PE-Cy7 (cat. 552850, BD Biosciences, clone: M1/70, RRID:AB_394491), anti-Ly6C-BV421 (cat. 562727, BD Biosciences, clone: AL-21, RRID:AB_2737748), anti-CD45-BV510 (cat. 103137, BioLegend, clone: 30-F11, RRID: AB_2561392), anti-CD64-BV711 (cat. 139311, BioLegend , clone: X54-5/7.1, RRID:AB_2563846), anti-MHCII- APC-eFluor 780 (cat. 47-5321-82, eBioscience, clone: M5/114.15.2, RRID:AB_1548783) and anti-Ly6G-BUV563 (cat. 612921, BD Biosciences, clone: IA8, RRID:AB_2870206). Finally, cells were washed with FACS buffer (0.5% FBS and 2 mM EDTA in PBS) and resuspended in FACS buffer containing the viability marker 7-AAD (1:100 BD Pharmingen) before filtering through a 70-µm strainer. Flow cytometry analyses were performed on a BD Symphony A5 Cell Analyzer (BD Biosciences) and subsequently analysed using FlowJo v.10.8.1. (BD Biosciences). Neutrophils (CD11b+Ly6G+), monocytes (CD11b+CD64+Ly6G−Ly6C+MHCII−), immature Mφ (CD11b+CD64+Ly6G−Ly6C+ MHCII+) and mature Mφ [CD11b+ CD64+Ly6G−Ly6C−MHCII+ (CCR2+, CD206+)] cells were identified in the CD45+LD- gate (Figure 30).

1.9 Histology

For histological analyses, the entire colons were taken, and tissues were flushed with PBS, then swiss rolls were prepared as previously described (Bialkowska, Ghaleb et al. 2016). Briefly, Swiss rolls were fixed in 4% neutral buffered formalin, processed for paraffin embedding and routine hematoxylin and eosin staining. Images were acquired using a Leica DM2500M microscope and features were blindly scored for the presence of goblet cell loss, crypt density, hyperplasia, and submucosal infiltrate using the following grading system: Goblet cell loss 0 = none, 1 = <10%, 2 = 10–50%, 3 = >50%; Crypt density 0 = normal, 1 = decrease of <10%, 2 = decrease of ≥10%; Hyperplasia 0 = none, 1 = slightly increased crypt length, 2 = 2–3 times increased crypt length ,3 = >3 times increased crypt length; Submucosal infiltrate 0 = none, 1 = individual cells, 2 = infiltrate[s], 3 = large infiltrate[s] (Koelink, Wildenberg et al. 2018).

1.10 Immunofluorescence staining

Cultured WT BMDMs were fixed with methanol for 15 min at -20°C, washed with DPBS, and blocked with normal donkey serum in TBS-T for 15 min at RT. Slides of colon tissue (5 μm) were deparaffinized with xylene and rehydrated with decreasing gradient alcohol. Antigen retrieval was performed using combined Sodium Citrate Buffer at 95 °C for 20 min followed by Tris EDTA at room temperature for 20 min. Non-specific binding was blocked using 1% normal donkey serum in TBS-T for 1h. The slides were incubated in a humidified chamber overnight at 4 °C with with the following fresh primary antibodies: TRPM8 (1:250; cat. OSR00077W, Thermo Fisher, RRID:AB_2208883), IBA1 (1:100; cat. NB100-1028, NovusBio, RRID:AB_521594), diluted in 1% normal donkey serum in TBS-T. Therefore, secondary antibodies, Cy3 donkey anti-rabbit (cat. 711-165-152, Jackson ImmunoResearch, RRID:AB_2307443) and Cy5 donkey anti-goat (cat. A11055, Invitrogen, RRID:AB_2534102) diluted at 1:500 in 1% normal donkey serum in TBS-T were added and detected. 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. Slides were mounted with SlowFade Diamond Antifade Mountant (cat. S36967, Invitrogen) solution. The images were acquired and detected under 40× magnification, by using a Zeiss LSM 780 microscope.

1.11 In silico studies

Docking calculations were conducted through the Autodock Vina of PyRx 0.8 software, as previously described (Angelini, Venanzoni et al. 2020). Crystal structures of target protein were derived from the Protein Data Bank (PDB) with PDB ID as follows: 6NR3 [transient receptor potential M8 (TRPM8)]. Discovery studio 2020 visualizer was employed to investigate the protein–ligand nonbonding interactions.

1.12 Immortalized cell lines

RAW 264.7 macrophages were purchased from ATCC (cat. TIB-71, LGC Standards, RRID:CVCL_0493). Immortalized healthy human colonic epithelial cells (HCEC), derived from human colon biopsies, were kindly gifted from Fondazione Callerio Onlus (Trieste, Italy). RAW 264.7 and HCEC cells were cultured in DMEM supplemented with 10% (v/v) FBS, 1 mmol·L−1 l-glutamine, 1 mmol·L−1 sodium pyruvate, 0.1 mmol·L−1 non-essential amino acids and 100 U·ml−1 antibiotics (penicillin and streptomycin) at 37°C in 5% CO2. Human embryonic kidney 293 (HEK-293) cells were grown on 100-mm-diameter Petri dishes as monolayers in MEM (Life Technology) supplemented with non-essential amino acids, 10% FBS and 2 mM l-glutamine and were maintained under 5% CO2 at 37°C. Cell viability was evaluated by trypan blue exclusion, and cell lines were confirmed to be Mycoplasma free (by using Mycoplasma PCR Detection Kit, Cat. G238, ABM). The medium was changed every

48 h in conformity with the manufacturer's protocols. Cell viability and proliferation was assessed as described in supplementary materials. All the reagents for in vitro cell cultures and ex vivo analysis were provided by Merck Sigma (Merck Life Science), Aurogene, Thermo Fisher Scientific, Corning, and Bio-Rad.

1.13 Bone marrow derived macrophages (BMDMs)

Bone marrow was obtained from C57BL/6J wild-type (WT), Trpm8-/- mice, IL-10fl/fl WT and IL-10fl/fl LysMCre mice femurs and tibias. Cells were incubated in macrophage culture medium [Dulbecco's modified Eagle's medium (DMEM) 4,5 mg/L glucose containing 100 U/mL of penicillin-streptomycin, 1% L-Glutamine and 10% (v/v) fetal bovine serum (FBS)] supplemented with 30% L929-conditioned media for 7 days. BMDMs were then reseeded and polarized overnight toward pro-inflammatory phenotype with IFN-γ (50 ng/mL) plus LPS (20 ng/mL).

1.14 TRP calcium assays

Human embryonic kidney 293 (HEK-293) stably over-expressing recombinant rat TRPM8, rat TRPA1 and human TRPV1 were generated and subsequently cell clones were selected following published procedures (Iannotti, Hill et al. 2014). The stable overexpression of the TRPM8, TRPA1 and TRPV1 genes was periodically checked by quantitative real-time PCR following published procedures (Iannotti, Hill et al. 2014). To determine the effect of luteolin and rutin, control blank (non-transfected) and transfected HEK293 cells were loaded with the methyl ester Fluo4-AM (cat. F14201, Thermo Fisher Scientific) 4 μM in dimethyl sulphoxide (DMSO) containing 0.02% Pluronic F-127 (cat. P3000MP, Thermo Fisher Scientific) for 1 h in the dark at room temperature in MEM not containing FBS. After 1h, cells were washed twice in Tyrode's buffer (145 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl2, 1.2 mM MgCl2, 10 mM D-glucose, and 10 mM HEPES, pH 7.4), re-suspended in Tyrode's buffer, and transferred into the quartz cuvette of the spectrofluorimeter (Perkin-Elmer LS50B; PerkinElmer Life and Analytical Sciences) under continuous stirring. Change of the intracellular calcium concentration [Ca2+]i was determined in the presence of crescent concentrations of luteolin or rutin by measuring cell fluorescence at 25 °C (λEX = 488 nm, λEM = 516 nm). Curve fitting (sigmoidal concentration-response variable slope) and parameter estimation were performed with GraphPad Prism® 8 (GraphPad Software Inc.). Potency was expressed as the concentration of test substances exerting a half-maximal agonist effect (i.e., half-maximal increases in [Ca2+]i (EC50) calculated by using GraphPad®. The efficacy of the agonists was determined by normalizing its effect to the maximum Ca2+ influx effect on [Ca2+]i observed with the application of 4 μM ionomycin (cat. 11932, Cayman). Measurement of [Ca2+]i in

TRPM8 cells was performed at 22 °C with a Fluorescence Peltier System (PTP-1, Perkin-Elmer).

1.15 BMDM intracellular Calcium Assay

BMDMs were loaded with the methyl ester Fluo4-AM (cat. F14201) 1 μM, containing 0.01% Cremophor for 20 min in the dark at room temperature. After loading, the cells were rinsed with oxygenated Krebs solution and transferred to a recording chamber mounted on an inverted Zeiss Axiovert 200M microscope (Zeiss). A gravity-fed perfusion system ensured continuous and constant perfusion (1 ml/min) of the preparation with Krebs solution (at room temperature) and excess solution was removed via a peristaltic suction pump. Fluo-4 was excited at 470 nm (exposure time: 100 msec), and its fluorescence emission was collected at 525/50 nm. Images were acquired at 2 Hz using TILLVision software (TILL Photonics). Change of the intracellular calcium concentration [Ca2+]i was determined in presence of icilin (40 □M) for 60 seconds, LPS (100ng/mL) for 30 seconds, or adenosine-5'-triphosphate (ATP, 10□M) for 10 seconds, using a gravity-fed perfusion system. In order to induce pharmacological blocking of TRPM8, luteolin (10 μM) or AMTB (5 μM) were applied on the tissue surface via the gravity-fed perfusion system for 10 minute, before icilin or LPS administration. Image analysis was performed using ImageJ. Fluorescence intensity was normalized to the basal fluorescence at the onset of the recording for each region of interest (F/F0), and area under the curve of each fluorescence peak was analyzed.

1.16 Nitrite measurement by GRIESS assay

RAW 264.7 cells and BMDMs (WT and Trpm8-/-) were seeded in 24-well plates and treated with scalar non-toxic concentrations of luteolin (0,1–10 μM), stimulated or not with LPS (1 μg/mL) or IFN-γ (50 ng/mL) plus LPS (20 ng/mL), respectively. After 18h incubation, supernatant was collected and The NO_2^- concentration was measured with the Griess reagent in which naphthylethylenediamine dihydrochloride and sulphanilamide reacts with NO_2^- to form a purple azo-product. The concentration of the azo-product was measured colorimetrically at 540 nm using a microplate reader. The experiments were performed as three independent biological experiments, each with three wells per sample for incubation (24-well plate) that was split to other three wells per sample for technical replicate measurements (96-well plate).

1.17 Enzyme-linked immunosorbent assay (ELISA)

IL-6, IL-1β, TNF-α and IL-10 levels were quantified in supernatant obtained from WT, Trpm8-/-, IL-10fl/fl WT and IL-10fl/fl LysMCre BMDM cultures, using commercial ELISA kits (Invitrogen), according to the manufacturer's instructions. Results are expressed as picograms

per milliliter of protein extract. The experiments were performed as three to four independent biological experiments, each measured in a technical duplicate.

1.18 Seahorse extracellular flux assay

For extracellular flux assay, RAW 264.7 and BMDMs (WT, Trpm8-/-, IL-10fl/fl WT and IL-10fl/fl LysMCre) were treated with luteolin (10 μM) and then stimulated with LPS (1 μg/mL) or IFN-γ (50 ng/mL) plus LPS (20 ng/mL) respectively, for 18 h. ECAR, basal and compensatory glycolysis, glycolytic capacity and non-glycolytic acidification were measured using XF24 Seahorse Extracellular Flux Analyzer following the manufacturer's instruction.

1.19 NMR based metabolomics

RAW 264.7 macrophages were grown on 100-mm-diameter Petri dishes as described in supplementary materials. Upon achievement of 80% cellular confluency, the culture medium was removed and the cells were processed for the endo-metabolomic analysis. In brief, the cells were extensively washed (4 times) with ice-cold PBS 1X in order to completely remove any residue of culture medium. Then, the cell plates were immersed into liquid nitrogen upon complete freezing of the samples and then slowly thawed in an ice bath. Afterwards, 5.4 mL of PBS were added to each culture dish and cells were collected by scraping with a rubber policeman. Finally, the cells were counted, placed in Falcon tubes and the final PBS volumes were adjusted to obtain 15 x106 cells into 5.4 mL PBS (pH 7.4). Subsequently, the quenched cells were lysed by 3 short-pulse cycles of sonication of 30 s each, at maximum power, thus favoring the release of the intracellular metabolites. A dual phase extraction procedure, introduced by Bligh and Dyer56 with slight modifications, was employed to separate the polar metabolites from the non-polar compounds, as already reported elsewhere (Iaccarino, Varming et al. 2019). Briefly, 6 mL of cold methanol (-20 °C) and 6 mL of chloroform were added to the original aqueous solution (5.4 mL) containing quenched cells (volume ratio of 1:1:0.9). Then, the mixture was incubated for 20 min on ice, repeatedly vortexed to facilitate the extraction and centrifuged at 4000 g at 4 °C for 20 min, thus obtaining a two-phase extract. The skin-like layer between the two phases entrapped proteins and macromolecules. Subsequently, the upper phase, containing the water-soluble intracellular metabolites, was separated from the organic lower phase and carefully transferred into different falcon tubes. Solvents were completely removed from both fractions using a vacuum concentrator (hydrophylic phase) and under a gentle flow of N2 gas (organic phase). In this study, only the hydrophilic phase was considered, however the organic phase, rich in non-polar metabolites such as lipids, has been stored at -80 °C for future analysis. The aqueous cell extracts were dissolved in 700 μL of D2O, vortexed briefly and transferred into 5-mm NMR tubes for the NMR analysis. Detailed

description of the 1H-NMR spectra acquisition parameters, metabolites identification procedure, NMR data pre-processing and Principal Components Analysis (PCA) are provided in the supplementary materials.

1.20 Metabolite extraction and liquid chromatography-mass spectrometry analysis

Metabolites were extracted from BMDMs cell pellet by adding 800 µl 60% methanol (cat. A456-500, Fisher Scientific) containing 6.67 µg/ml glutaric acid (cat. G3407, Sigma-Aldrich) in LC/MS grade water (cat. W6500, Fisher Scientific). After addition of 500 µl chloroform (cat. C2432, Sigma-Aldrich,), samples were vortexed for 10 minutes at 4°C and then spun at 17,000 x g for 10 minutes at 4°C. The upper phase was transferred into a fresh tube and dried down in a Vacufuge plus speed-vac at 4°C. The metabolite extract was separated using an iHILIC-(P) Classic column (2.1 µm, 150 mm × 2.0 mm I.D., The Nest Group) coupled to a Thermo Scientific SII UPLC system. The autosampler and column oven were held at 4°C and 25°C, respectively. The iHILIC-(P) Classic column was used with buffer A (0.1% ammonium hydroxide, 20 mM ammonium carbonate) and buffer B (100% acetonitrile). The chromatographic gradient was run at a flow rate of 0.150 ml/minute as follows: 0–20 minutes: linear gradient from 80% to 20% B; 20–20.5 minutes: linear gradient from 20 % to 80 % B; 20.5–28 minutes: hold at 80% B. The mass spectrometer was operated in full scan, negative ion model. Mass spectrometry detection was carried out on a Q Extractive HF-X orbitrap mass spectrometer with a HESI source. For metabolite quantification TraceFinder software (ThermoFisher) was used. The lower phase after chloroform extraction was also dried down in a Vacufuge plus speed-vac at 4 °C and proteins were resuspended in 0.2M NaOH and heated at 90°C for 15 minutes. The protein concentration was determined using the Pierce BCA Protein Assay Kit Assay (cat. 23227, ThermoFisher). The metabolite levels were normalized by the total protein amount per sample in µg.

1.21 *Ex vivo* studies in colon tissues

Isolated colon specimens from WT C57BL/6J mice were maintained in a humidified incubator with 5% CO_2 at 37 ◦C for 4 h (incubation period), in RPMI buffer with added bacterial LPS (10 µg/mL), as previously described. During the incubation period, the tissues were challenged with scalar concentrations of luteolin (0.1-30 ng/mL) for 18h. Tissues were then processed for RT-qPCR analysis.

1.22 Quantitative real-time polymerase chain reaction

The RNA extraction was performed using innuPREP RNA Mini Kit 2.0 (Analytik Jena) following the manufacturer's instructions. Total RNA was retro-transcribed into complementary cDNA by qScript cDNA SuperMix (Quanta Biosciences) according to the

manufacturer's instructions. Quantitative real-time transcription PCR was performed with the LightCycler 480 SYBR Green I Master (Roche Applied Science) using the Light Cycler 480 (Roche). Results were quantified using the 2−ΔCT method. The expression levels of the genes of interest were normalized to the expression levels of the reference gene RPLP32. Polymerase chain reaction experiments were performed in 3 to 5 replicates. The primers sequences used are listed in Table 1 and refer to murine species. The primers used in Supplementary figure 6 were purchased from Life Technologies [Assays-on-Demand Gene Expression Products, Mm00478374_m1 for cyclooxygenase-2 (COX-2) gene, Mm00443258_m1 for tumor necrosis factor (TNF)-α gene, Mm00440502_m1 for inducible nitric oxide synthase (iNOS) gene, Mm00446190_m1 for interleukin (IL)-6, Mm00434228_m1 for IL-1β, Mm01288386_m1 for IL-10 gene, Mm02619580_g1 for β-actin gene].

1.23 Statistical analysis

Results are shown as mean ± standard error of the mean (SEM). Significance between two mean groups was determined by unpaired or paired two-tailed Student's t-test, while one or two-way analysis of variance (ANOVA) followed by Dunnett's or Tuckey’s multiple comparison test was performed to compare the mean of multiple groups. GraphPad Prism V.9.5.1 software (GraphPad Inc.) was used to generate graphs and to perform statistical analysis.

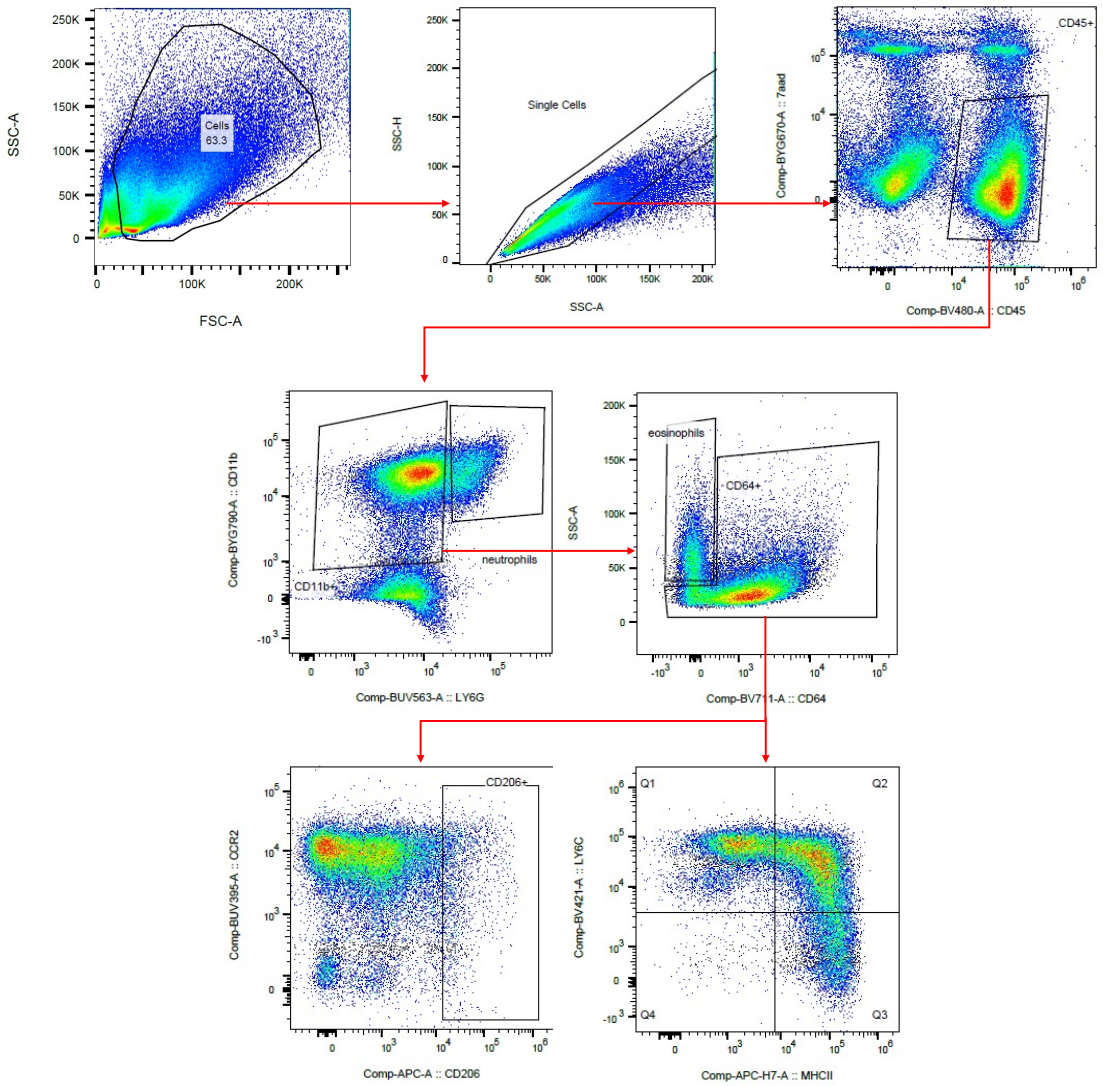


Figure 30. Flow-cytometry gating strategy for colonic lamina propria myeloid cells.

Table 1. Primer sequences

Gene	**Forward Primer**	**Reverse Primer**
il10	CCAAGCCTTATCGGAAATGA	TCACTCTTCACCTGCTCCAC
il1β	GACCTTCCAGGATGAGGACA	TCCATTGAGGTGGAGAGCTT
il6	CCATAGCTCCTGGAGTACAT	TGGAAATTGGGGTAGGAAGGA
rplp32	AAGCGAAACTGGCGGAAAC	TAACCGATGTTGGGCATCAG
tnfα	TCTTCTCATTCCTGCTTGTGG	CACTTGGTGGTTTGCTACGA

RESULTS

Chapter I. Dietary targeting of TRPM8 rewires macrophage immunometabolism reducing colitis severity

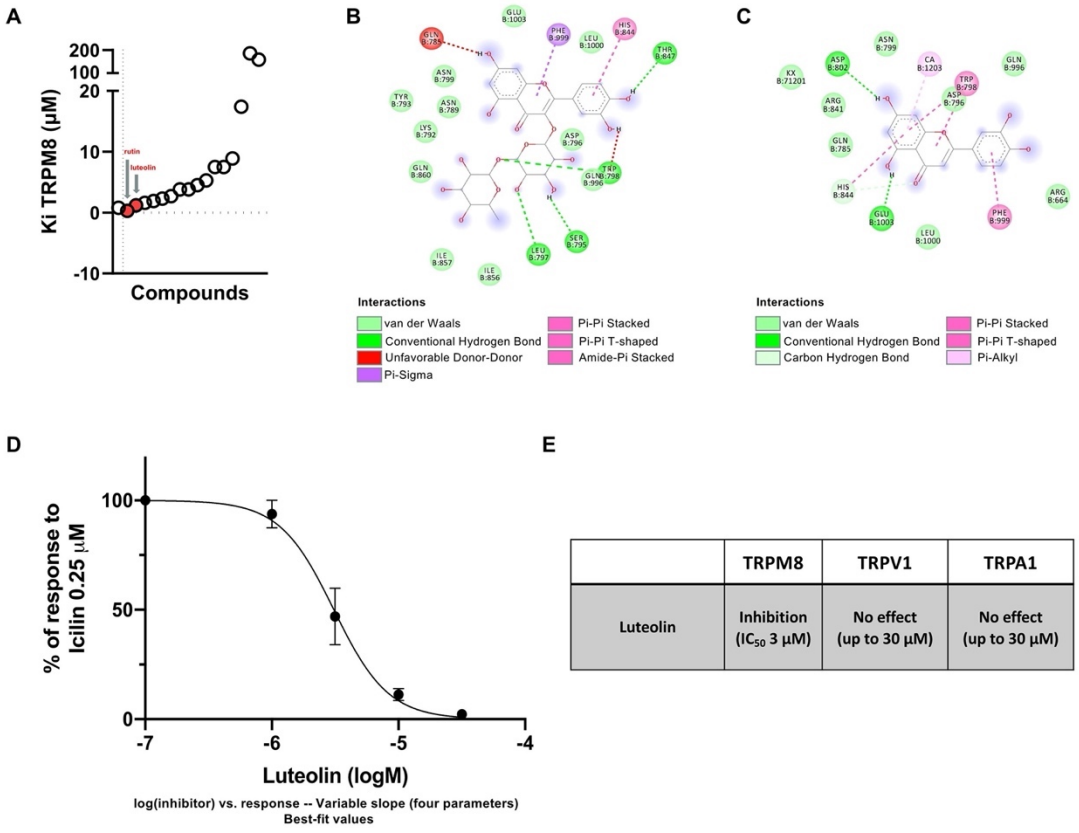
Adapted from: Cicia D. et al., under revision in Mucosal Immunology.

1.1 Luteolin is a new selective TRPM8 blocker

In order to identify novel dietary ligands for TRPM8, the affinity for the TRPM8 receptor was screened using bioinformatic tools on a subset of 17 dietary compounds (Table 2) with known anti-inflammatory properties. Menthol and sesamin are well known TRPM8 ligands and were used as references (Bautista, Siemens et al. 2007, Sui, Li et al. 2020). The affinity for the receptor is reported in terms of Ki (Figure 31A). Our analysis revealed that rutin and luteolin showed the highest affinity for TRPM8 (Figure 31B-C). To validate the in-silico results, we moved to perform *in vitro* experiments. The affinity of luteolin and rutin for TRPM8 was therefore confirmed by measuring alterations of the intracellular calcium concentration $[Ca^{2+}]_i$ in HEK-293 cells stably transfected with a plasmid encoding for the recombinant human TRPM8 protein (denoted hereafter as 'TRPM8-HEK293'). Our analysis revealed that rutin did not increase or block Ca^{2+} currents in TRPM8-HEK293 cells (data not shown). On the other hand, luteolin alone did not activate a TRPM8-mediated Ca^{2+} influx, but interestingly was able to antagonize the Ca^{2+} raise evoked by icilin (0.25μM), a potent selective agonist of TRPM8 (Voets, Owsianik et al. 2007). Specifically, icilin-mediated response was inhibited by luteolin (5-min pre-incubation) in a concentration-dependent manner, with an IC50 of 3,091±0,326 μM (Figure 31D). Non-transfected HEK293 cells were used as control. Next, to evaluate the selectivity of luteolin for TRPM8 channels, the intracellular calcium assay was also performed in HEK293 stably overexpressing recombinant TRPV1 or TRPA1. As shown in Figure 31E, luteolin did not evoke or block calcium transients in TRPV1 or TRPA1 HEK293 cells after testing it up to the concentration of 30 μM (Figure 31E). In summary, these results indicate that luteolin selectively binds and blocks TRPM8 channels.

Table 2. Subset of dietary compounds tested for TRPM8 affinity. Menthol and Sesamin were used as reference compounds.

Natural Compound	**Putative TRPM8 affinity (μM)**
Rutin	0.3
Sesamin	0.8
Luteolin	1.2
Vitexin	1.6
Kaempferol	1.9
Neochlorogenic acid	2.3
Epigallocatechin	3.8
Rosmarinic acid	4.5
Tricin	5.3
Resveratrol	7.5
Naringenin	8.9
Chlorogenic acid	17.4
Menthol	156.1
Rutamarin	3.8
Isochlorogenic acid	7.5
Vitexin compound 1	2.7
P-Cymene	184.7



E

	TRPM8	TRPV1	TRPA1
Luteolin	Inhibition (IC_{50} 3 μM)	No effect (up to 30 μM)	No effect (up to 30 μM)

Figure 31. Luteolin is a new selective TRPM8 blocker. From an evaluation of several diet-derived compounds affinity for TRPM8, luteolin emerged as a new selective TRPM8 blocker. (A) Scatter diagram of TRPM8-Ki of 17 diet-derived compounds. Each point represents a natural compound. In red are reported rutin and luteolin. (B) Rutin structure and binding site on TRPM8. (C) Luteolin structure and binding site on TRPM8. (D) Concentration-response curve of luteolin (0.1-100 μM) on intracellular Ca2+ levels in TRPM8- HEK293, measured in the presence of Icillin 0.25 μM, the TRPM8 reference agonist. Data represent the mean ± SEM of ≥5 determinations. (E) Effect of luteolin (0.1-30 μM) on intracellular Ca^{2+} levels on HEK293 cells over-expressing TRPM8, TRPV1, and TRPA1 in the presence or not of their respective agonists.

1.2 Luteolin does not affect macrophage viability and proliferation

In order to illustrate the possible effect of luteolin on macrophages *via* TRPM8, we first investigated if luteolin treatment could affect any macrophage cell function *in vitro*. Luteolin, up to the concentration of 10 μM, did not affect the viability and proliferation rate of both naïve and pro-inflammatory immortalized macrophage cell line (RAW 264.7) and WT BMDMs (Figure 32A-B, D). Of relevance, luteolin, at all concentrations tested (1–30 μM), did not affect the viability of *Trpm8*$^{-/-}$ macrophages (Figure 32C).

1.3 Pro-inflammatory macrophages express TRPM8

In accordance with previous studies (Khalil, Babes et al. 2016), TRPM8 expression was enhanced in macrophages upon stimulation with LPSand IFN-γ, compared to naïve cells (Figure 33A)**.** Consistent with these data, taking into account that TRPM8 is an ion channel mainly permeable to Ca^{2+}, the TRPM8 agonist icilin was able to induce sustained calcium transients in WT BMDMs at the concentration of 40 μM (Figure 33B-D). In *Trpm8*$^{-/-}$ BMDMs, 40μM icilin alone was ineffective in increasing the $[Ca^{2+}]_i$, confirming a TRPM8 selectivity at tested concentration (Figure 33B-D). Importantly, in WT BMDMs the effect of icilin was antagonized by luteolin (10 min pre-incubation) (Figure 33B-D), used at a non-cytotoxic concentration of 10 μM (Figure 32), further demonstrating that this flavonoid blocks TRPM8-mediated Ca^{2+} influx.

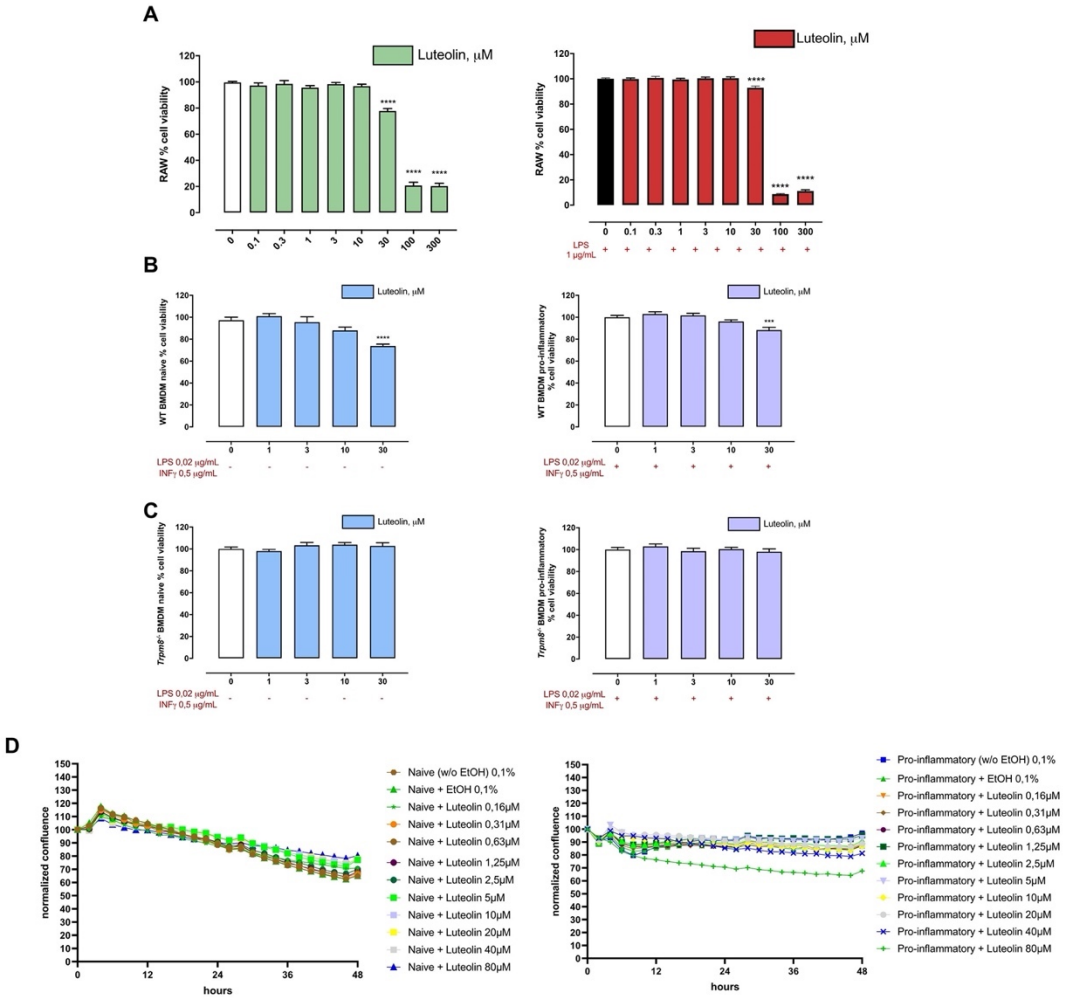


Figure 32. Luteolin does not affect macrophage viability and proliferation. (A) Cell viability rate of naïve and LPS-stimulated RAW 264.7 alone or in the presence of luteolin (0,1–300 μM) for 18h. Error bars represent ±SEM. P value was determined using one-way ANOVA followed by Tukey's multiple comparisons test. ****p<0,0001. (B-C) Cell viability rate of naïve and pro-inflammatory WT (B) or Trpm8-/- (C) BMDMs alone or in the presence of luteolin (1–30 μM) for 18h. Error bars represent ±SEM. P value was determined using one-way ANOVA followed by Tukey's multiple comparisons test. ***p<0,001; ****p<0,0001. (D) Cell proliferation rate of naïve and pro-inflammatory WT BMDMs alone or in the presence of luteolin (0,16–80 μM) up to 48h. Error bars represent ±SEM.

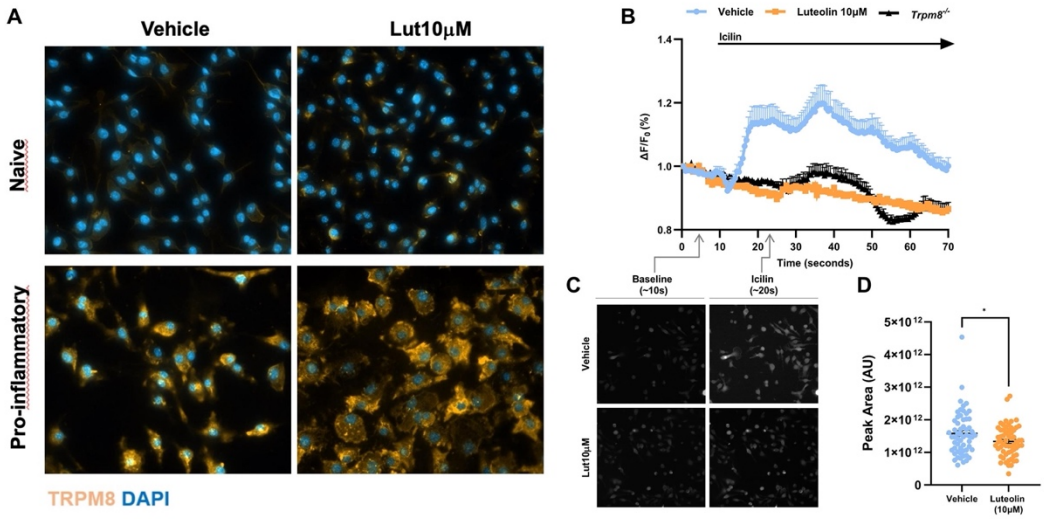


Figure 33. TRPM8 channel is overexpressed in pro-inflammatory macrophages. (A) TRPM8 (orange), and 4′,6-diamidino-2-phenylindole (DAPI) (blue) almeno dal mio pc vedo tutto nero immunofluorescence staining in naïve and pro-inflammatory WT BMDMs treated or not with luteolin (10 μM). Analysis was performed 18h after polarization with IFN-γ plus LPS. (B) Relative changes in the fluorescence of Fluo-4AM-loaded naïve WT or *Trpm8*$^{-/-}$ BMDMs, reflecting changes in intracellular Ca2+ concentration [Ca2+]i after Icilin (40μM) treatment for 1 min. Data are presented as the average of n=3 biological replicates. Error bars represent ±SEM (n = 58 for Vehicle and *Trpm8*$^{-/-}$ and n = 60 for Luteolin 10μM). (C) Representative fluorescence images from indicated time points in the experiments shown in panel B. (D) Statistical representation of total peak area of Fluo-4 fluorescence after Icilin treatment from panel B. Error bars represent ± SEM. P value was determined using Student's t-test. *p<0,05.

1.4 TRPM8 is required for LPS-induced Ca2+ influx in macrophages

Considering that TRPM8 mediates Ca^{2+} transients in macrophages, and results upregulated after LPS stimulation in our experimental conditions, we hypothesized that TRPM8 channels could directly or indirectly mediate the Ca^{2+} currents evoked by LPS. To prove this, we measured intracellular Ca^{2+} changes in BMDMs, in response to LPS (100 ng/mL). As reported, in WT macrophages we observed a robust rise in cytosolic calcium levels after LPS stimulation (Figure 34A-D). Remarkably, in *Trpm8*$^{-/-}$ BMDMs we found only a weak elevation of the $[Ca2+]_i$ following LPS stimulation, characterized also by a lower basal cell activity (Figure 34A-D). No differences were detected between WT and *Trpm8*$^{-/-}$ BMDMs in response to ATP (Figure 34E). In line with these findings, the pharmacological inhibition of TRPM8 in WT BMDMs using luteolin or AMTB (a synthetic selective TRPM8 blocker) successfully blocked LPS-induced Ca^{2+} influx (Figure 34F-K). Interestingly, both luteolin and AMTB treatment reduced basal Ca^{2+} levels in the cells before LPS stimulation (Figure 34L-M). These results suggest that most likely TRPM8 channels directly conduct Ca^{2+} to facilitate LPS-induced activation. Overall, these data support the hypothesis that TRPM8 is a key regulator of macrophage response to pro-inflammatory stimuli (i.e., LPS), by modulating intracellular Ca^{2+} uptake.

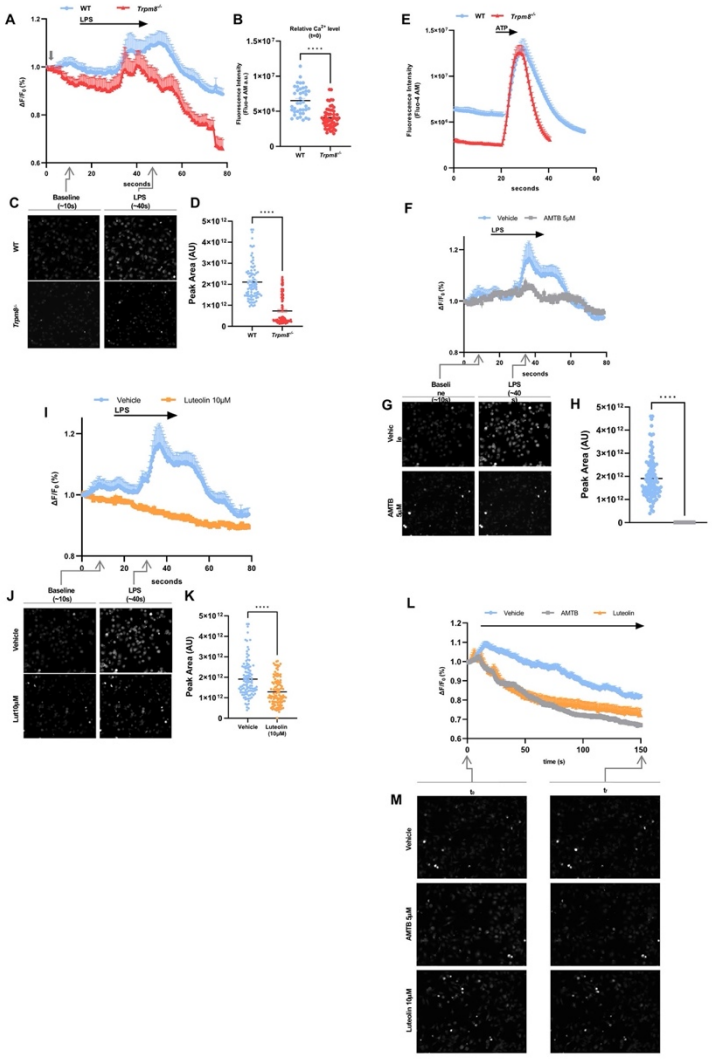


Figure 34. TRPM8 channel activity controls macrophage response to LPS. (A) Relative changes in the fluorescence of Fluo-4AM-loaded naïve WT and Trpm8-/- BMDMs, reflecting changes in intracellular Ca^{2+} concentration [Ca^{2+}]i after LPS (100 ng/mL) treatment for 30s. Data are presented as the average of n=3 biological replicates. Error bars represent ±SEM (n=86 and n=112 for WT and Trpm8-/- , respectively). (B) Quantification of basal Ca^{2+} levels in Fluo-4AM-loaded naïve WT and Trpm8-/- BMDMs. Error bars represent ± SEM. P value was determined using Student's t-test. ****p<0,0001. (C) Representative fluorescence images from indicated time points in the experiments shown in panel A. (D) Statistical representation of total peak area of Fluo-4 fluorescence after LPS treatment from panel A. Error bars represent ± SEM. P value was determined using Student's t-test. ****p<0,0001. (E) Relative changes in the fluorescence of Fluo-4AM-loaded WT and *Trpm8*$^{-/-}$ BMDMs, reflecting changes in intracellular Ca2+ concentration [Ca2+]i after ATP (10μM) treatment for 10s. Error bars represent ±SEM. (F) Relative changes in the fluorescence of Fluo-4AM-loaded BMDMs, reflecting changes in

intracellular Ca2+ concentration [Ca2+]i BMDMs after LPS (100 ng/mL) treatment for 30s. Data are presented as the average of n=3 biological replicates. Error bars represent ±SEM (n = 108 and n=144 for Vehicle and AMTB 5μM, respectively). (G) Representative fluorescence images from indicated time points in the experiments shown in panel F. (H) Statistical representation of total peak area of Fluo-4 fluorescence after LPS treatment from panel F. Error bars represent ± SEM. P value was determined using Student's t-test. ****p<0,0001. (I) Relative changes in the fluorescence of Fluo-4AM-loaded naïve BMDMs, reflecting changes in intracellular Ca2+ concentration [Ca2+]i BMDMs after LPS (100 ng/mL) treatment for 30s. Data are presented as the average of n=3 biological replicates. Error bars represent ±SEM (n = 108 and n=113 for Vehicle and Luteolin 10μM, respectively). (J) Representative fluorescence images from indicated time points in the experiments shown in panel I. (K) Statistical representation of total peak area of Fluo-4 fluorescence after LPS treatment from panel I. Error bars represent ± SEM. P value was determined using Student's t-test. ****p<0,0001. (L) Relative changes in the fluorescence of Fluo-4AM-loaded BMDMs, reflecting changes in intracellular Ca2+ concentration [Ca2+]i BMDMs after vehicle (Krebs solution), AMTB (5μM) or luteolin (10μM) treatment for ~150s. Data are presented as the average of n=3 biological replicates. Error bars represent ±SEM (n = 144 for all the experimental groups). (M) Representative fluorescence images from indicated time points in the experiments shown in panel L.

1.5 TRPM8 deletion or pharmacological targeting by luteolin reduce macrophage pro-inflammatory capacity

So far, we have shown that TRPM8 mediates, at least in part, calcium entry in macrophages in response to LPS (Figure 34). To further understand the contribution of these channel receptors to macrophage pro-inflammatory capacity, we assessed the effect of TRPM8 blockade (by using genetic ablation as well as luteolin treatment) on the production of nitrites, IL-1β, IL-6 and TNF-α in BMDMs. Whereas LPS and IFNγ treatment significantly increased the release of nitrites, IL-1β, IL-6, and TNF-α in WT BMDMs, *Trpm8*$^{-/-}$ BMDMs had a blunted response and an overall decreased expression of all the aforementioned pro-inflammatory mediators (Figure 35A). Accordingly, luteolin (10μM) significantly reduced nitrite production and pro-inflammatory cytokine release in WT BMDMs compared to vehicle-treated cells (Figure 35B). Of note, luteolin did not affect *Trpm8*$^{-/-}$ BMDMs (Figure 35B), confirming that its anti-inflammatory effect is TRPM8-mediated.

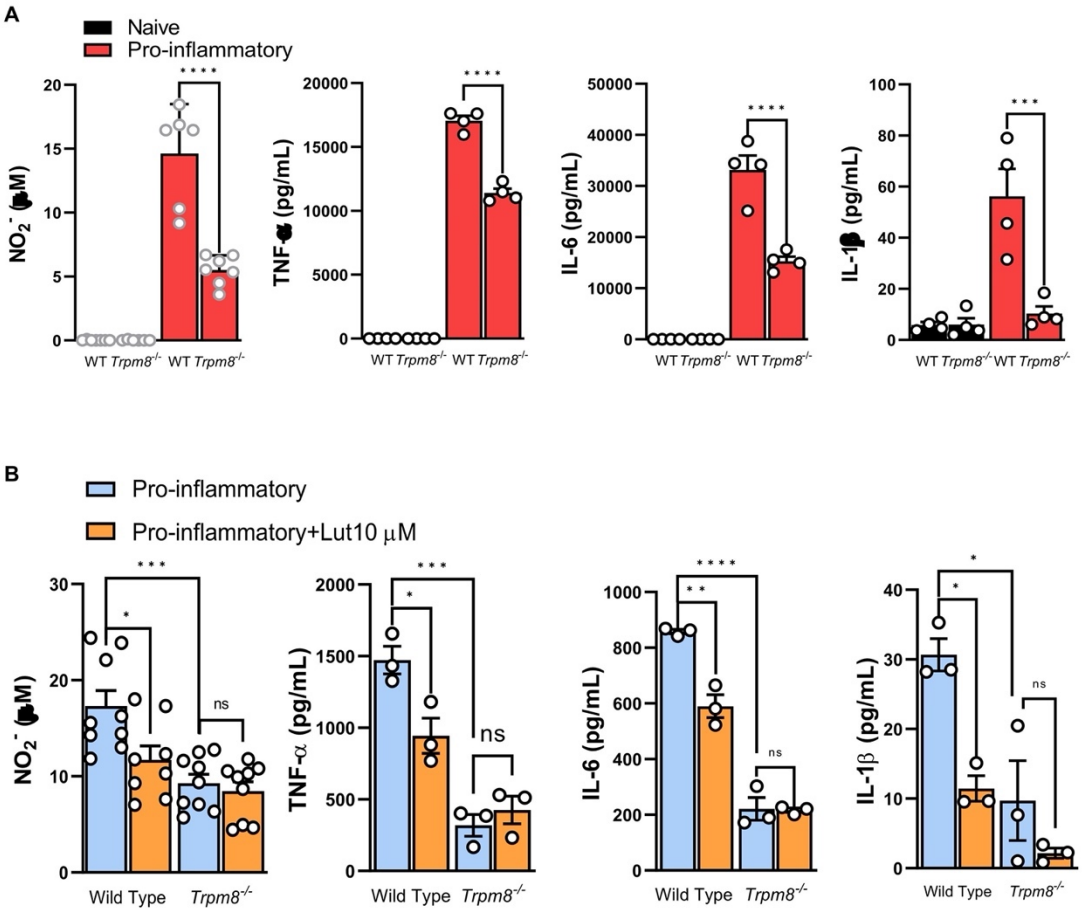


Figure 35. TRPM8 is required for pro-inflammatory polarization of macrophages. (A) Nitrite levels and TNFα, IL-6 and IL1β levels measured in the supernatant of naïve and pro-inflammatory WT or Trpm8-/- BMDMs. Error bars represent mean ± SEM. P value was determined using Two-way ANOVA followed by Šídák's multiple comparisons test. ***p<0,001; ****p<0,0001. (B) Nitrite levels and TNFα, IL-6 and IL1β levels measured in the supernatant of pro-inflammatory WT or Trpm8-/- BMDMs treated or not with luteolin (10 μM). Error bars represent ±SEM. P value was determined using two-way ANOVA followed by Šídák's multiple comparisons test. ns non significant,*p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

1.6 TRPM8 genetic or pharmacological modulation induces IL-10 pathway activation

To shed light on luteolin downstream molecular mechanism, we measured the pro-inflammatory cytokine expression in WT BMDMs at different time points (i.e., 1, 3, 6, and 12h after polarization with LPS+IFNγ). Luteolin (10 μM) was significantly more effective in reducing pro-inflammatory cytokine gene expression after 6-12h compared to untreated cells (Figure 36A). Worthy of note, blocking TRPM8 via luteolin treatment significantly increased both gene and protein levels of IL-10 in WT macrophages, at very early timepoints (i.e., 3-6h after polarization with LPS+IFNγ) (Figure 36B). In line, LPS+IFNγ– stimulated *Trpm8*$^{-/-}$ BMDMs showed higher levels of IL-10 at early timepoints (Figure 36C), supporting the hypothesis that TRPM8 inhibition can regulate pro-tolerogenic gene expression in macrophages.

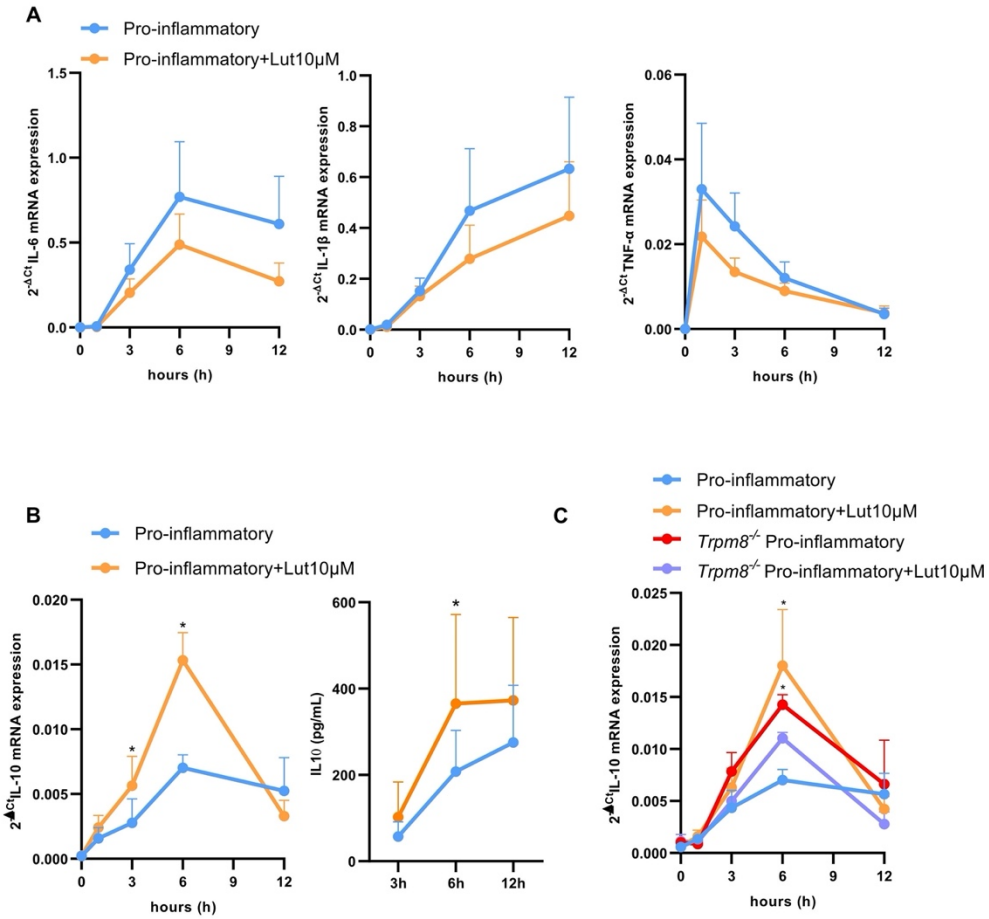


Figure 36. TRPM8 inhibition increases IL-10 levels in pro-inflammatory macrophages. (A) IL-6, IL1β and TNFα mRNA expression in pro-inflammatory WT BMDMs treated or not with luteolin (10 μM). The analysis was performed after 1, 3, 6 or 12h of polarization with IFN-γ plus LPS. Data are presented as the average of representative n=3 of n=14 biological replicates. Error bars represent ± SEM. (B) IL-10 mRNA (left) and protein levels (right) measured in pro-inflammatory WT BMDMs treated or not with luteolin (10 μM). The analysis was performed after 1, 3, 6 or 12h of polarization with IFN-γ plus LPS. Data are presented as the average of representative n=5 of n=14 biological replicates. Error bars represent ± SEM. P value was determined using Student's t-test. *p<0,05. (C) IL-10 mRNA levels measured in pro-inflammatory WT or Trpm8-/- BMDMs treated or not with luteolin (10 μM). The analysis was performed after 1, 3, 6 or 12h of polarization with IFN-γ plus LPS. Data are presented as the average of representative n=3 of n=14 biological replicates. Error bars represent ± SEM. P value was determined using Student's t-test. *p<0,05.

1.7 TRPM8 regulates macrophage glycolytic metabolism

Early IL-10 production controls the metabolic switch triggered in macrophages by pro-inflammatory stimuli (Ip, Hoshi et al. 2017). Therefore, we aimed to investigate whether luteolin could have effect on macrophage metabolic remodeling. Thus, we first determined the extracellular acidification rate (ECAR) in WT BMDMs stimulated with LPS and IFNγ, in the presence or not of luteolin (10 μM) for 18h. LPS and IFNγ stimulation increased the ECAR in WT BMDMs as previously reported (Viola, Munari et al. 2019) (Figure 37). Luteolin treatment significantly reduced both basal and compensatory glycolysis, glycolytic capacity, and non-glycolytic acidification in WT, but not in *Trpm8*$^{-/-}$ macrophages (Figure 37). Accordingly, LPS+ IFNγ- stimulated *Trpm8*$^{-/-}$ macrophages showed a significantly reduced ECAR compared to WT BMDMs (Figure 37). Overall, these results prove that TRPM8 sustains pro-inflammatory macrophage activation through a specific effect on glycolytic metabolism.

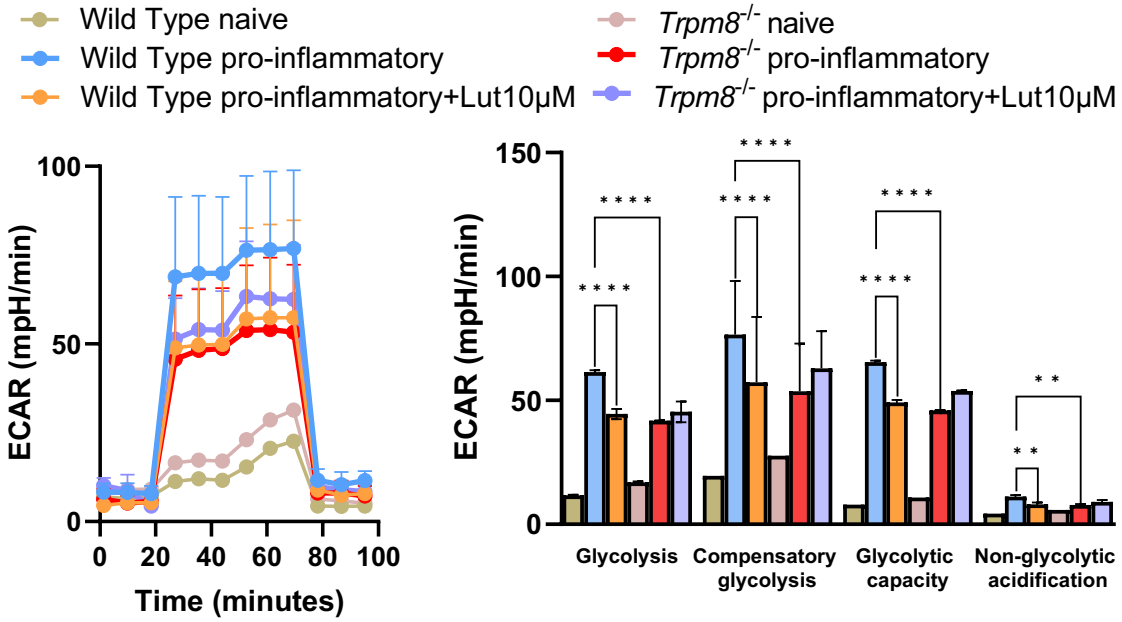


Figure 37. TRPM8 controls glycolysis in pro-inflammatory macrophages. ECAR kinetics (left) and glycolytic parameters (right) of naïve and pro-inflammatory WT and *Trpm8*$^{-/-}$ BMDMs treated or not with Luteolin (10 μM). Data are presented as the average of n=3 biological replicates. Error bars represent ±SEM. P value was determined using one-way ANOVA followed by Tukey's multiple comparisons test. **p<0,01; ****p<0,0001.

1.8 Luteolin affects the levels of the main immunomodulatory metabolites via TRPM8

To further define luteolin effect on metabolic status of macrophages, we performed a ^{1}H-NMR-based metabolomic analysis. Due to the complexity and heterogeneity of BMDMs, we initially performed an analysis on lysates of LPS-stimulated immortalized macrophages (i.e., RAW264.7) treated or not with luteolin (10 μM) for 18h. RAW264.7 cell line expresses TRPM8 (Khalil, Babes et al. 2016), and we proved that non-cytotoxic concentration of luteolin (1-10μM, Figure 32A) reduced nitrite levels, as well as ECAR, and both basal and compensatory glycolysis in LPS-stimulated cells (Figure 38A-C). Firstly, a principal component analysis (PCA) was performed to explore the differences in cell metabolome under the different experimental conditions. Interestingly, a clear separation occurs between untreated and luteolin-treated pro-inflammatory macrophages, mirroring a metabolic switch induced by luteolin (Figure 38D). These differences were driven by a differential expression of amino acids (i.e., glutamate, glutamine, glycine), but also succinate and lactate levels (Figure 38D). Specifically, LPS + IFNγ polarization induced a significant increase in succinate, glutamate, and glutamine in macrophages, whereas luteolin treatment was sufficient to prevent their increment (Figure 38E). Interestingly, glutamine can be metabolized into lactate in macrophages (Liu, Chen et al. 2023), and luteolin-treated macrophages exhibited significantly higher levels of lactate compared to untreated pro-inflammatory BMDMs (Figure 38E). Finally, luteolin treatment upregulated also the levels of glycine, the final product of the pentose phosphate pathway.

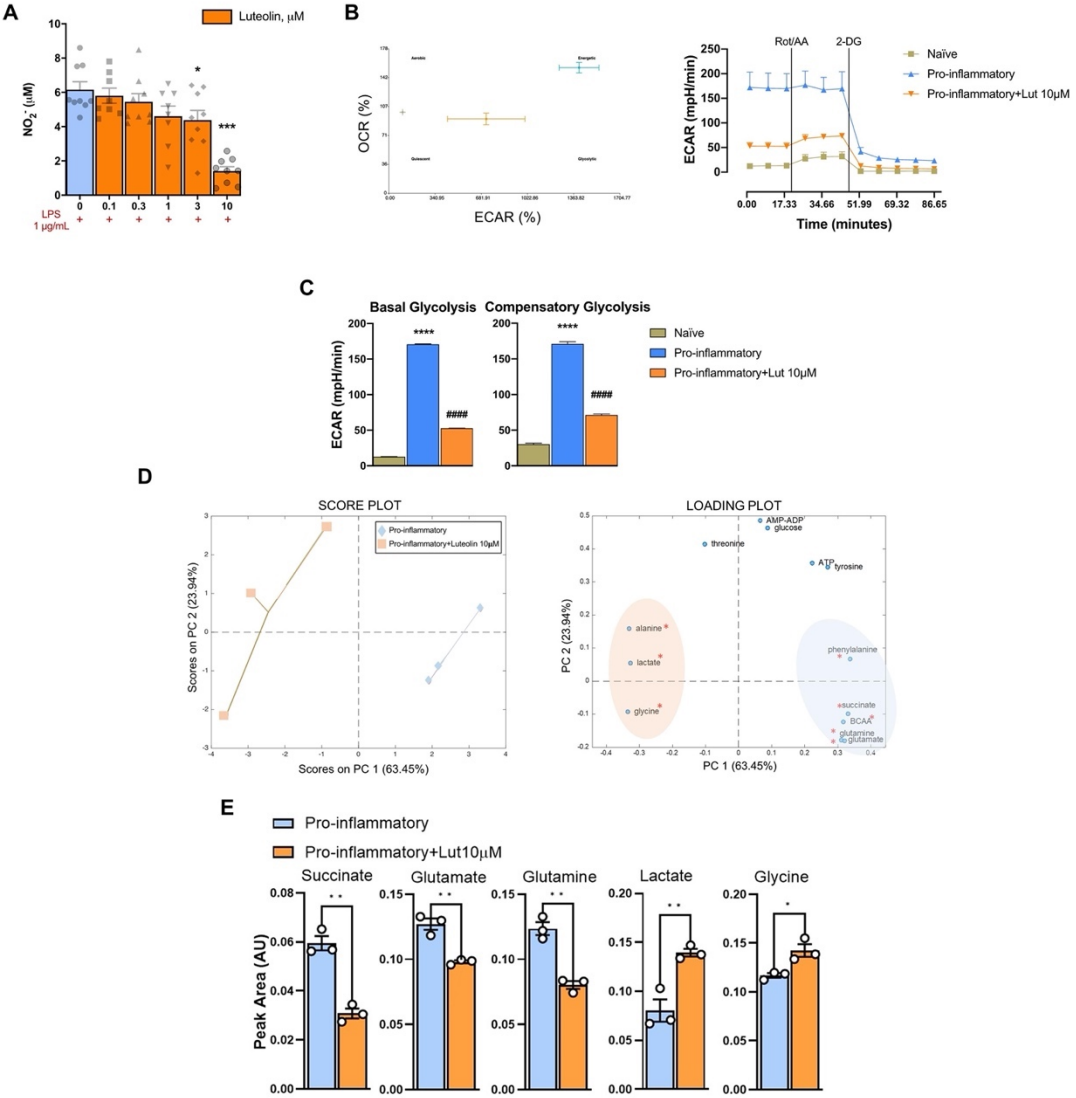


Figure 38. TRPM8 regulates immunometabolic pathways in macrophages. (A) Nitrite levels measured in the supernatant of LPS-stimulated RAW 264.7 alone or in the presence of luteolin (1–10 µM) for 18h. Error bars represent mean ± SEM. P value was determined using one-way ANOVA followed by Tukey's multiple comparisons test. *p<0,05; ***p<0,001. (B) Energetic map (left), and ECAR kinetics (right) of naïve and LPS-stimulated (pro-inflammatory) RAW 264.7 treated or not with Luteolin (10 µM). The energetic map was calculated on the basis of OCR and ECAR values measured by Seahorse analysis. Data are presented as the average of n=5 biological replicates. Error bars represent ±SEM. (C) Glycolytic parameters of naïve and LPS-stimulated (pro-inflammatory) RAW 264.7 treated or not with Luteolin (10 µM). Data are presented as the average of n=5 biological replicates. Error bars represent ±SEM. P value was determined using one-way ANOVA followed by Tukey's multiple

comparisons test. ****p<0,0001 vs naïve; #### p<0,0001 vs pro-inflammatory. (D-E) Score and loading plots derived from the PCA model (D) and succinate, lactate, glycine, glutamine, and glutamate levels (E) measured by NMR metabolomic analysis of pro- inflammatory RAW 264.7 treated or not with Luteolin (10 μM). (E) Data are presented as peak area normalized on total area. Error bars represent ±SEM. P value was determined using unpaired Student's t-test. *p<0,05; **p<0,01.

1.9 TRPM8 negative modulation induces lactate levels in pro-inflammatory macrophages

We hypothesized that luteolin mechanism of action may be partially mediated by an increase of intracellular lactate levels at early timepoints, possibly leading to the induction of tissue repair genes, such as IL-10. To verify this hypothesis, we examined the dynamics of lactate production during BMDMs polarization after the stimulation with LPS and IFNγ, in the presence or absence of luteolin (10 μM). We observed that luteolin-treated WT BMDMs exhibited a significant increase in lactate levels starting from 3h after stimulation with LPS and IFNγ (Figure 39A). In line with these results, luteolin treatment also reduced the levels of pyruvate, the primary lactate source in macrophages, at early timepoints (Figure 39B). To prove whether luteolin effect on the biosynthetic pathway of lactate is also mediated by TRPM8, we analyzed the production of such metabolites in *Trpm8*$^{-/-}$ BMDMs. Genetic deletion of TRPM8 in macrophages induced a significant increase of lactate concentration in basal conditions (t0), 3 and 6h after LPS and IFNγ (Figure 39C), confirming that the lack of TRPM8 mirrors the effect of its pharmacological inhibition.

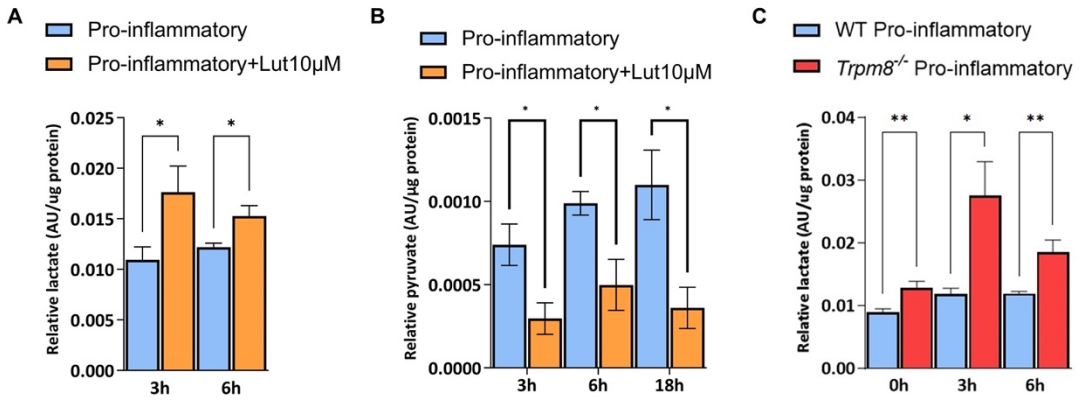


Figure 39. Luteolin, *via* TRPM8, increases lactate levels in pro-inflammatory macrophages. (A) Relative lactate levels measured by GC-MS metabolomic analysis of pro-inflammatory BMDMs treated or not with Luteolin (10 µM). The analysis was performed after 3 or 6h of polarization with IFN-γ plus LPS. Data are presented as peak area normalized on µg of protein. Error bars represent ±SEM. P value was determined using unpaired Student's t-test. *p<0,05. (B) Relative pyruvate levels measured by GC-MS metabolomic analysis of pro-inflammatory WT BMDMs treated or not with Luteolin (10 µM). The analysis was performed after 3, 6 or 18h of polarization with IFN-γ plus LPS. Data are presented as peak area normalized on µg of protein. Error bars represent ±SEM. P value was determined using unpaired Student's t-test. *p<0,05. (C) Relative lactate levels measured by GC-MS metabolomic analysis of WT and Trpm8-/- BMDMs. The analysis was performed in basal conditions (0h) and after 3 or 6h of polarization with IFN-γ plus LPS. Data are presented as peak area normalized on µg of protein. Error bars represent ±SEM. P value was determined using unpaired Student's t-test. *p<0,05; **p<0,01.

1.10 Lactate production is required for IL-10-mediated functional reprogramming in luteolin-treated macrophages

The reduction of pyruvate to lactate is catalyzed by lactate dehydrogenase (LDH), a rate-limiting enzyme for lactate production. Therefore, to corroborate our hypothesis, we pharmacologically blocked lactate dehydrogenase A (LDHa) by using the selective inhibitor GSK2837808A (Xie, Hanai et al. 2014). GSK2837808A significantly reduced the lactate production in WT BMDMs (Figure 40A), and inhibitor-treated pro-inflammatory macrophages failed to induce the expression of IL-10 upon luteolin treatment (Figure 40B). Further, lactate deprivation boosted the pro-inflammatory gene expression (i.e. IL-6, TNF-α, IL-1β) in LPS and IFNγ– stimulated macrophages, and luteolin was not effective in impairing this induction (Figure 40C). Together, these results indicate that lactate generated by LDHa activity is crucial for supporting the anti-inflammatory effect of luteolin.

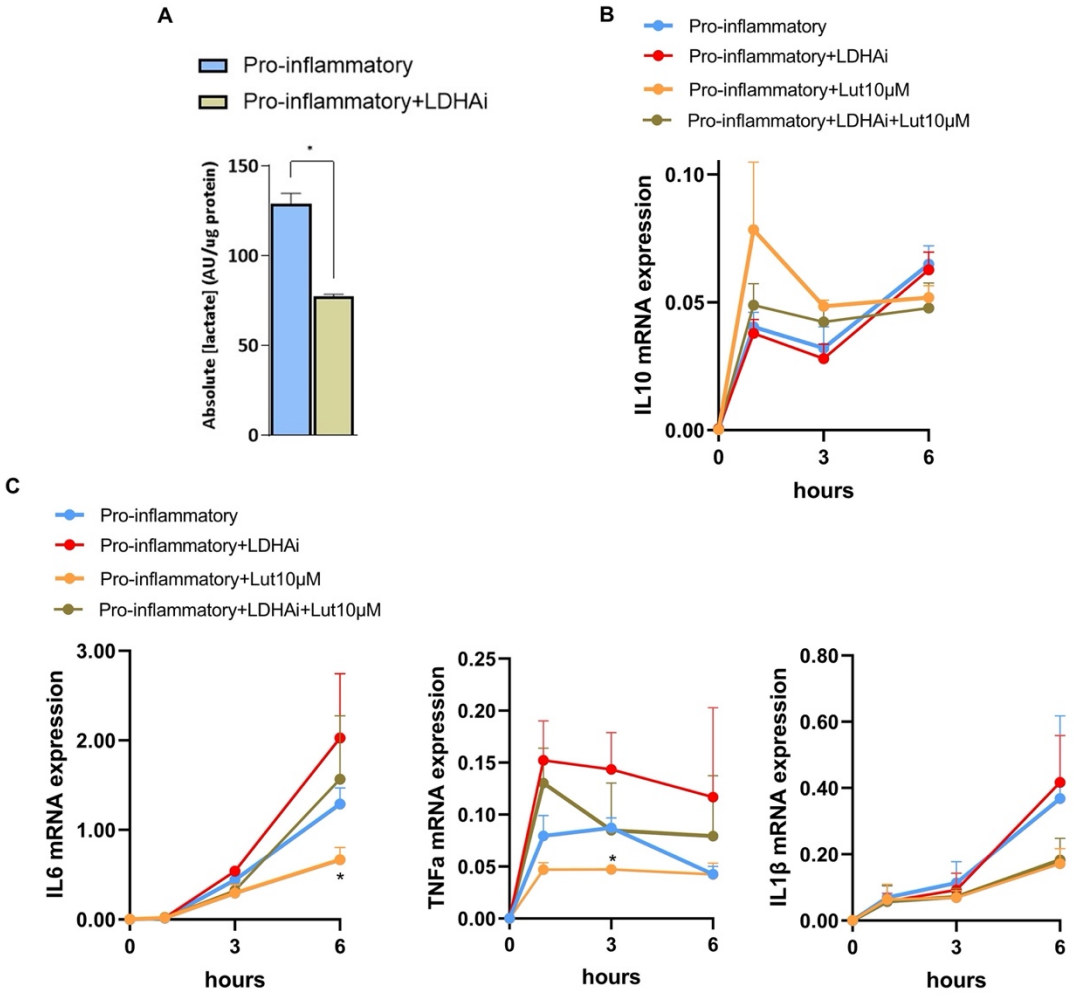


Figure 40. TRPM8-increased lactate levels are necessary for IL-10 pathway activation. (A) Absolute lactate levels measured by GC-MS metabolomic analysis of pro-inflammatory WT BMDMs treated or not with LDH-a inhibitor GSK2837808A (10 μM). The analysis was performed after 6h of polarization with IFN-γ plus LPS. Data are presented as peak area normalized on μg of protein. Error bars represent ±SEM. P value was determined using unpaired Student's t-test. *p<0,05. (B-C) IL-10 (C) and IL-6, TNF-α and IL-1β (D) mRNA expression measured in pro-inflammatory WT BMDMs treated or not with luteolin (10 μM) and LDH-a inhibitor GSK2837808A (10 μM). The analysis was performed after 1, 3 or 6h of polarization with IFN-γ plus LPS. Data are presented as the average of n=3 biological replicates. Error bars represent ± SEM. P value was determined using Student's t-test. *p<0,05 vs Pro-inflammatory.

1.11 TRPM8-mediated IL-10 increase is required for luteolin anti-inflammatory and metabolic effects in macrophages

To finally determine whether the increase in IL-10 production is responsible for luteolin anti-inflammatory and metabolic effects in macrophages, we used BMDMs derived from IL-$10^{fl/fl}$ LysMCre mice. As expected, IL10 levels were ablated in LysMcre mice (Figure 41A), and, in accordance with literature (Ip, Hoshi et al. 2017), presented higher levels of all the pro-inflammatory mediators compared to their control counterpart (IL-$10^{fl/fl}$ WT) (Figure 41B). Notably, luteolin treatment significantly reduced the pro-inflammatory cytokines release in IL-$10^{fl/fl}$ WT macrophages but was ineffective in LysMCre macrophages (Figure 41C). These data were strongly supported by the absence of luteolin effect on the metabolic rewiring induced by LPS and IFNγ stimulation in IL-10 deprived macrophages (Figure 41C). Overall, these results clearly demonstrate that lactate increase upon luteolin treatment is essential for its immunomodulatory effect, through the upregulation of the IL-10 pathway.

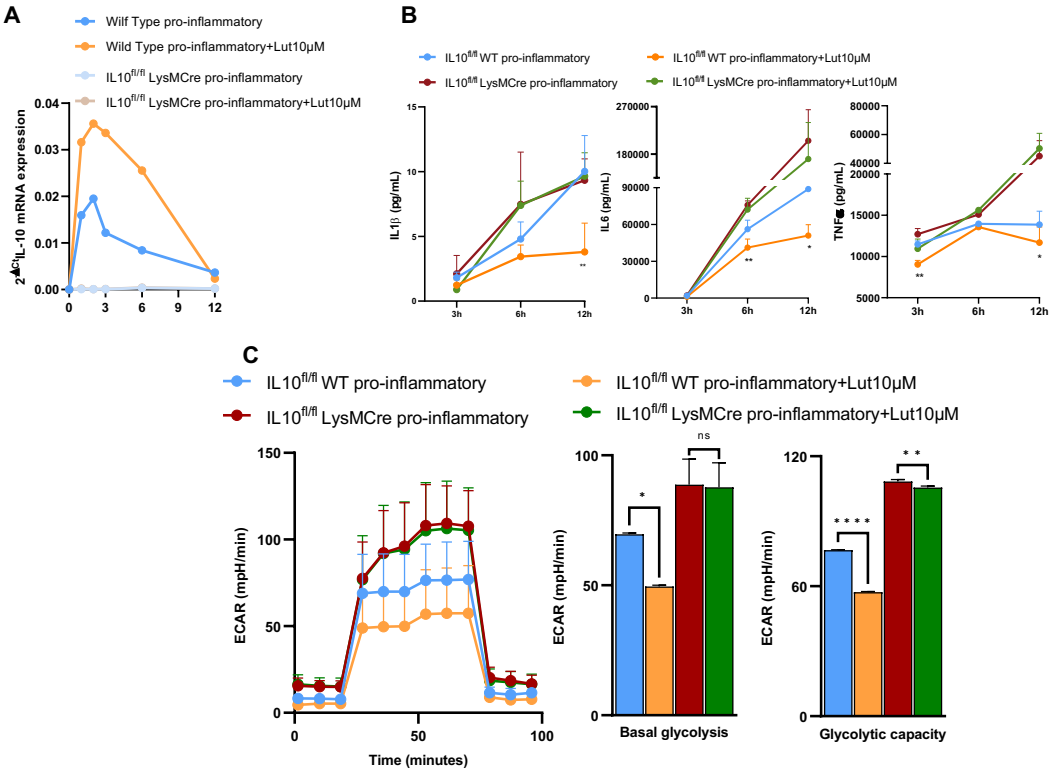


Figure 41. Luteolin anti-inflammatory effect is mediated by TRPM8 activation of IL-10 pathway. (A) IL-10 mRNA measured on IL-10fl/fl WT and IL-10fl/fl LysMCre BMDMs treated or not with luteolin (10 μM). The analysis was performed after 1, 3, 6 or 12h of polarization with IFN-γ plus LPS. Data are presented as the average of n=5 biological replicates. Error bars represent ±SEM. (B) IL-6, IL-1β and TNF-α levels measured in the supernatant of IL-10fl/fl WT and IL-10fl/fl LysMCre BMDMs treated or not with luteolin (10 μM), after 3, 6 or 12h of polarization with IFN-γ plus LPS. Data are presented as the average of n=5 biological replicates. Error bars represent ±SEM. P value was determined using Student's t-test. *p<0,05; ** p<0,01 vs IL-10fl/fl WT pro-inflammatory. (C) ECAR kinetics (left) and glycolytic parameters (right) of naïve and pro-inflammatory IL-10fl/fl WT and IL-10fl/fl LysMCre BMDMs treated or not with Luteolin (10 μM). Data are presented as the average of n=3 biological replicates. Error bars represent ±SEM. P value was determined using one-way ANOVA followed by Tukey's multiple comparisons test. n.s.=non-significant, *p<0,05; **p<0,01; ****p<0,0001.

1.12 Luteolin reduces pro-inflammatory cytokine secretion from mice colon *ex vivo*

To assess the involvement of TRPM8 in colonic inflammation, we first evaluated the modulatory effects of luteolin supplementation on the levels of inducible nitric oxide synthase (iNOS) and the main cytokines released by activated macrophages (i.e., IL-6, IL-1β, IL-10 and TNFα) in murine colon stimulated with LPS *ex vivo*. Results revealed that LPS treatment significantly increased the levels of iNOS, IL-1β, IL-6 and TNF-a. Compared to LPS alone, luteolin (10 μM) significantly reduced the expression of such cytokines, while significantly increasing IL-10 levels (Figure 42).

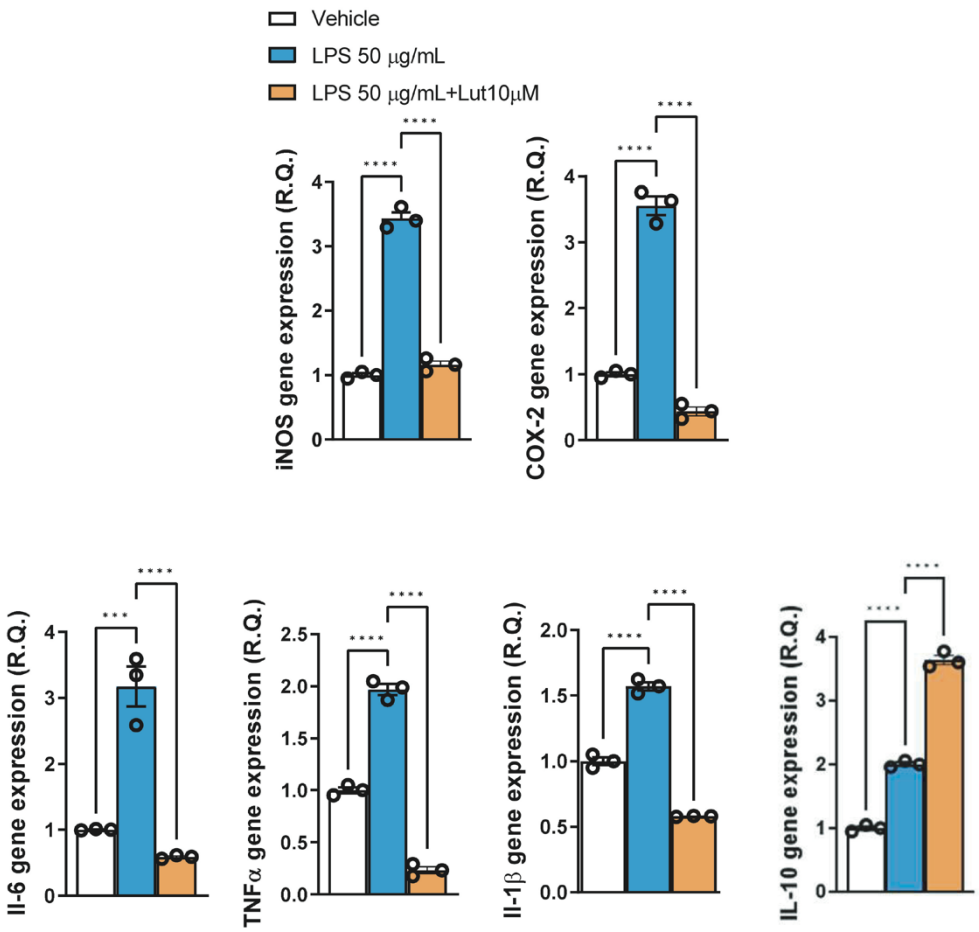


Figure 42. Luteolin reduced macrophage-secreted cytokines in murine colon *ex-vivo*. iNOS, COX-2, IL-6, TNF-α, IL-1β and IL-10 mRNA levels measured on LPS-stimulated WT mice colon treated or not with Luteolin (10 μM). Data are presented as the average of n=3 biological replicates. Error bars represent ±SEM. P value was determined using one-way ANOVA followed by Tukey's multiple comparisons test. *p<0,05; ***p<0,001; ****p<0,0001.

1.13 Luteolin oral supplementation reduces colitis severity in mice

To explore the pathophysiological significance of our findings in the context of intestinal inflammation, we aimed to evaluate the anti-inflammatory effect of luteolin in a murine DSS model of colitis. WT mice were exposed to 2.25% DSS in drinking water for 5 days (Figure 43A) and treated by oral gavage with luteolin at the doses of 3, 10, and 30 mg/kg for 21 days (Figure 43B-C). Compared to vehicle-treated mice, luteolin (30 mg/kg) counteracted the DSS-induced weight loss (Figure 43B), and reduced the disease activity index (DAI), showing earlier recovery of symptoms (Figure 43C). This reduction in colitis severity was accompanied by significant improvement in colonic histological score following luteolin (30 mg/kg) treatment (Figure 43D). Specifically, luteolin-treated mice showed a significant reduction in goblet cell loss, crypt density hyperplasia, and leucocyte infiltration compared to the vehicle group (Figure 43D). In healthy mice, luteolin treatment had no impact on gut homeostasis (Figure 43B-C). To assess the involvement of TRPM8 in colonic inflammation, we first detected its expression in the colon of DSS-treated mice by confocal immunofluorescence, which revealed no differences in terms of expression and distribution of TRPM8 in all experimental groups (Figure 43E). Considering the critical role of macrophages in IBD, we also stained the colonic sections with IBA-1, a specific marker of this immune population (Sasaki, Ohsawa et al. 2001). As shown in Figure 43E, luteolin-treated mice clearly showed a reduction in macrophage infiltration, and TRPM8 was co-localized with IBA-1 in both experimental groups (Figure 43E).

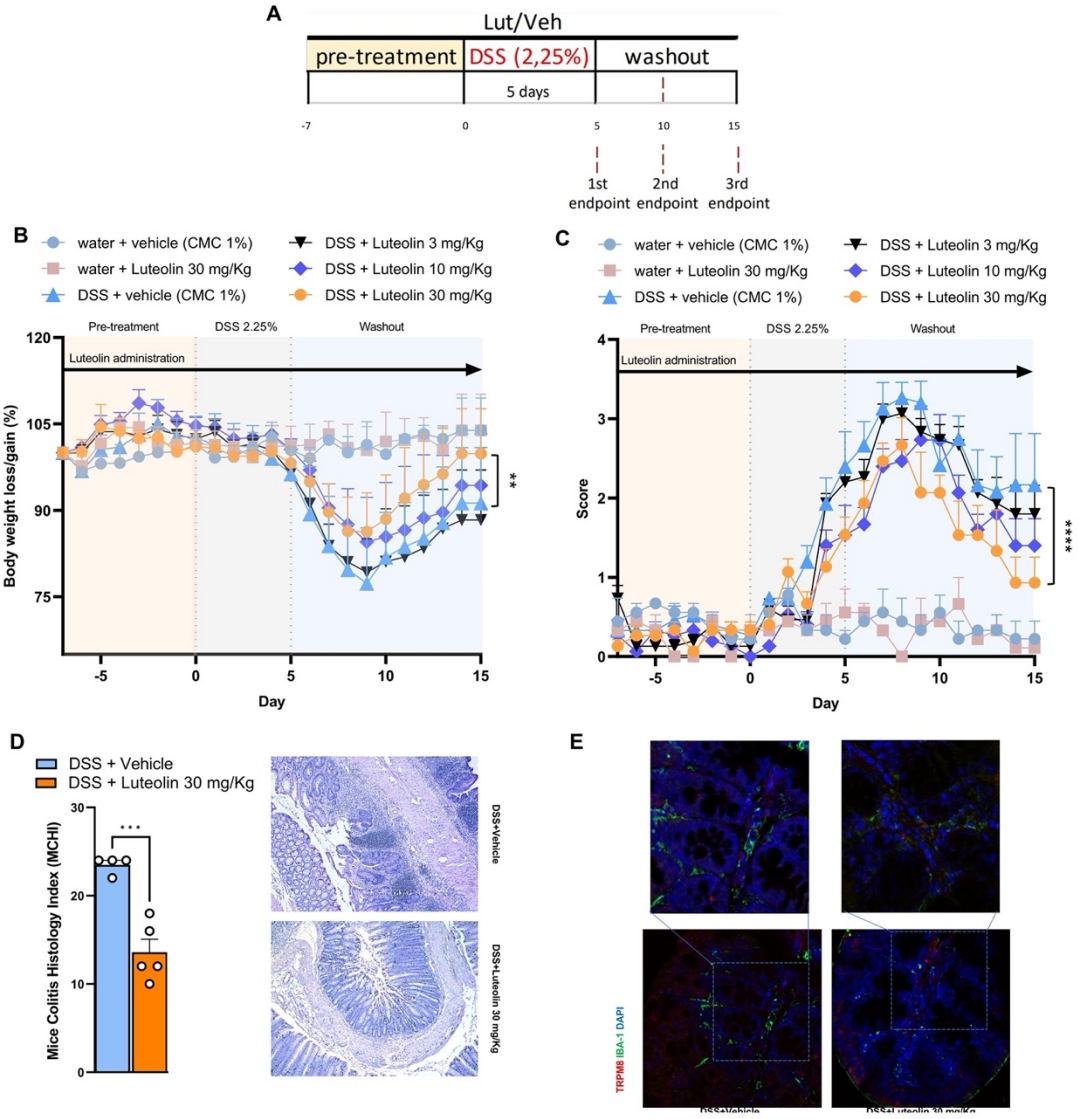


Figure 43. Luteolin reduced DSS-induced colitis in mice. (A) Schematic representation of DSS protocol and timeline. (B) Mice body weight gain/loss. Data are presented as the average of n=10 mice for each experimental group. Error bars represent ±SEM. P value was determined using Student's t-test. **p<0,01. (C) Disease activity index (DAI) of colitis severity. Data are presented as the average of n=10 mice for each experimental group. Error bars represent ±SEM. P value was determined using Student's t-test. ****p<0,0001. (D) Histological score (sx) and representative H&E images (dx) of mice colon sections. Error bars represent mean ±SEM. P value was determined using an unpaired Student's t-test. ***p<0,001. (E) Representative confocal images of TRPM8 (red), IBA-1 (green), and DAPI (blue) immunostaining in DSS+Vehicle or DSS+Luteolin treated mice, collected 15 dpt. Analyses were carried out on three mice for each experimental group.

1.14 Luteolin reduces the recruitment of inflammatory myeloid cells during intestinal inflammation in mice

We next aimed to study the effect of luteolin on the immune response in more detail. Neutrophils, eosinophils, monocytes, immature macrophages, and mature CD206+ macrophages from the colonic lamina propria of mice were studied and characterized by flow cytometry, 5-, 10- or 15-days post-treatment (dpt) (Figure 44). Flow cytometric analysis revealed that luteolin treatment did not significantly alter the immune response during the acute phase of DSS colitis (Figure 44A-B). However, a reduced amount of immune cells in the colonic lamina propria of luteolin-treated mice was observed during the recovery phase after induction of colitis compared with the vehicle group (Figure 44C). More specifically, it has been observed a significant reduction in the number of Ly6G+ neutrophils (Figure 44D) and Ly6C^{hi} monocytes and immature macrophages (Figure 44E), and a significant increase of mature Ly6C-MHCII+ monocytes and CD206+ macrophages (Figure 44E-F) in the luteolin-treated mice during DSS colitis. Taken together these data indicate that oral treatment with luteolin counteracts intestinal inflammation and mediates an impairment in the innate immune response during experimental colitis.

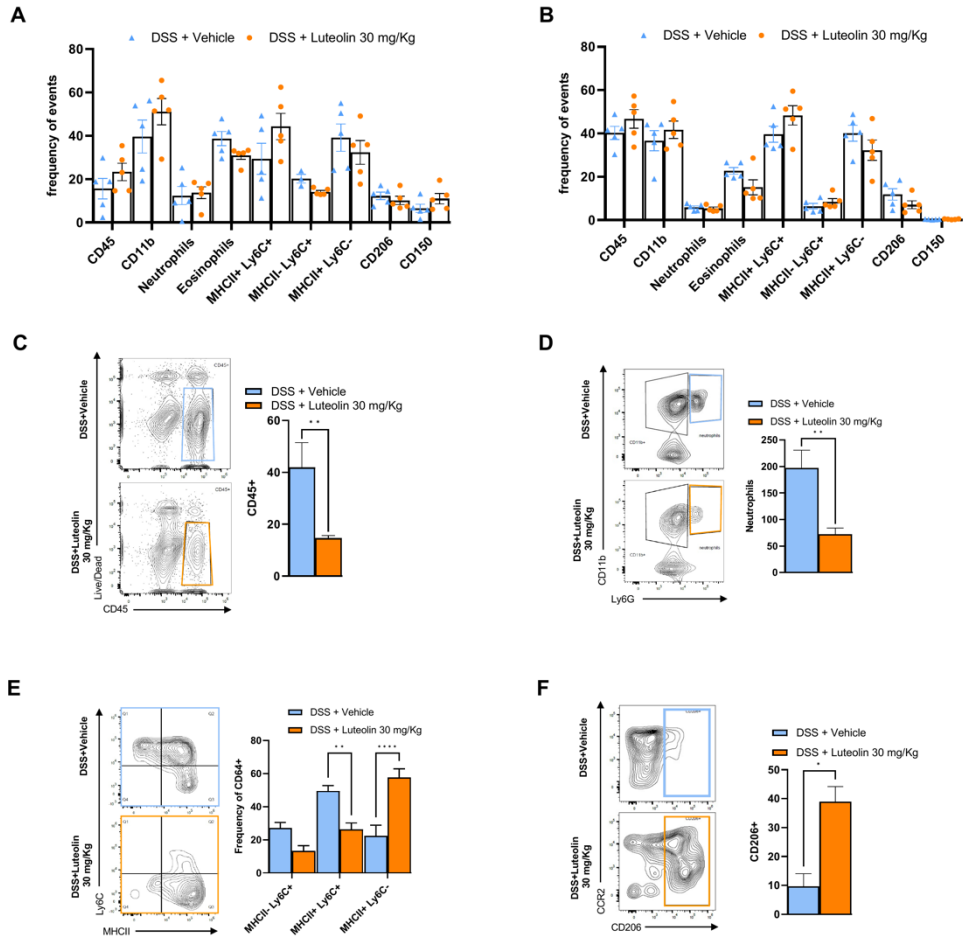


Figure 44. Luteolin reduces the recruitment of inflammatory myeloid cells during colitis. (A-B) Count of CD45, CD11b, Ly6g neutrophils, Ly6c and MHCII, CCR2 and CD206 populations in lamina propria of mice colon 5 (A) and 10 (B) dpt. Data are presented as the average of n=5 mice for each experimental group. Error bars represent ±SEM. (C-D) Representative flow plots and count of CD45 (C) and neutrophils (D) populations in lamina propria of mice colon. Data are presented as the average of n=5 mice for each experimental group. Error bars represent ±SEM. P value was determined using Student's t-test. **p<0,01 (E-F) Representative flow plots and expression of Ly6c and MHCII (E) and CCR2 and CD206 (F) by monocytes isolated from lamina propria of mice colon. Data are presented as the average of n=5 mice for each experimental group. Error bars represent ±SEM. P value was determined using Student's t-test. *p<0,05; **p<0,01; ****p<0,0001.

Chapter II. TRPM8 is crucially involved in colon tumorigenesis

Adapted from: Pagano E*, Romano B*, **Cicia D**, et al. TRPM8 indicates poor prognosis in colorectal cancer patients and its pharmacological targeting reduces tumour growth in mice by inhibiting Wnt/β-catenin signalling. Br J Pharmacol. 2023;180(2):235-251. doi:10.1111/bph.15960

2.1 TRPM8^{hi} CRC predict a significant reduction of PFI and disease-specific survival

To determine the role of TRPM8 in CRC, we looked at the expression profiles of 283 TCGA samples. TRPM8 expression is widely distributed, with Tukey's five-number summary showing the following values: 0, 0.2, 0.86, 1.65, and 8.75. However, the gene's third (upper) quartile expression (1.65) is lower than the gene's first (lower) quartile expression (3.7) of all the genes, indicating a very low TRPM8 expression. We examined the disease-specific survival and progression-free interval (PFI) of patients using the log-rank test to see if such variation in TRPM8 expression could impact the prognosis for CRC patients (Figure 45A-B). It is interesting to note that patients in the tertile with either low or no expression of TRPM8 have significantly greater disease-specific survival and PFI compared to patients with higher expression of TRPM8 (Figure 45A-B). This outcome, together with the gene modest expression, points to the possibility of a mechanism present/absent connected to the TRPM8 gene.

2.2 TRPM8 up-regulation positively correlates with higher grades of CRC

In an effort to better understand the role of TRPM8 in human CRC, we also examined the expression profile of TRPM8 in human CRC specimens and in the surrounding normal colon tissues. TRPM8 was significantly up-regulated in tumor specimens (n = 34 CRC patients) as compared to normal tissues, as emerged from RT-qPCR analysis (Figure 46A). The tumor node metastasis staging system (pT1, pT2, pT3, and pT4) was used to conduct a sub-analysis, and the results indicated that TRPM8 expression was significantly up-regulated only in stage pT4, with no significant changes observed in the pT1–pT2–pT3 stages (Figure 46B). According to immunoblot analysis, TRPM8 was considerably overexpressed in the adenocarcinoma lesions taken from CRC patients, although it was weakly expressed in the nearby normal colonic tissue (Figure 46C).

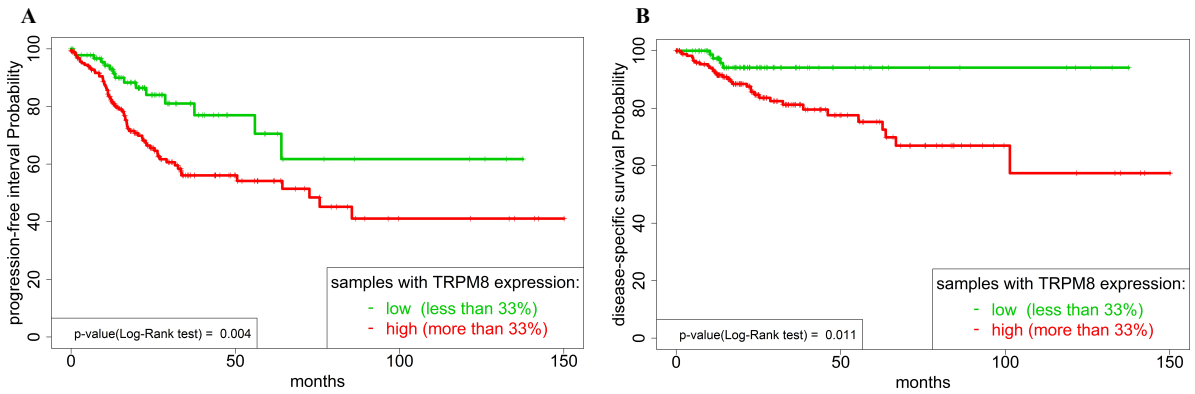


Figure 45. High expression of TRPM8 predicts low survival in patients with CRC. (A-B) Kaplan–Meier survival analysis of CRC patients with low TRPM8 expression (green) and high TRPM8 expression (red) was used to analyze the (A) progression-free survival probability and (B) disease-specific survival probability. P values were determined using the log-rank test, and the exact values are reported at the left bottom of the figures.

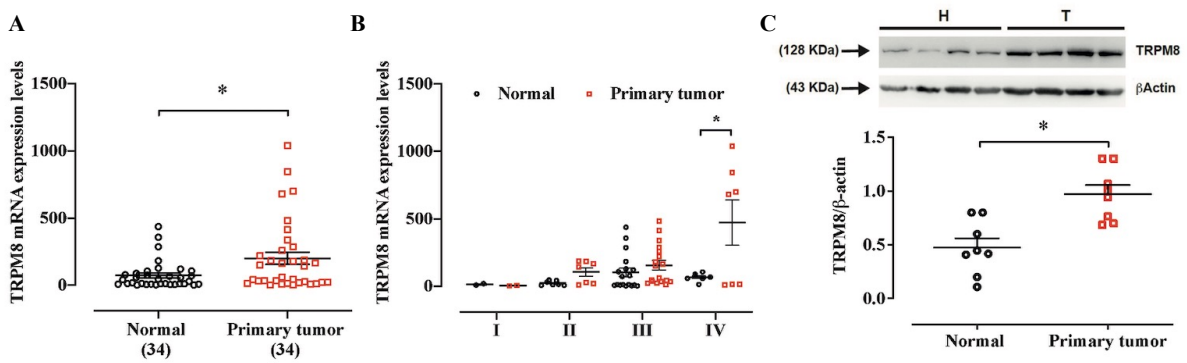


Figure 46. TRPM8 is significantly upregulated in human CRC biopsies. (A-B) TRPM8 mRNA levels in normal tissues (collected at least 10 cm from the tumor lesion) and primary tumors removed from patients with CRC diagnosis. Data are presented as the average of n = 34 different biological samples. Error bars represent ±SEM. P values were determined using paired t test. *$p < 0.05$ versus normal. (C) Representative immunoblots of TRPM8 protein levels in human CRC specimens (primary tumors) and the surrounding nontumorous tissues (normal). Lower panel shows densitometric analysis of western blot analysis. Data are presented as the average of n=8 different biological samples. Error bars represent ± SEM. P value was determined using paired t tests. *$p < 0.05$ versus normal.

2.3 TRPM8 is selectively up-regulated in tumoral cells isolated from CRC biopsies

We next dissociated human CRC biopsies to produce a single-cell solution containing roughly 10% of CD326+ tumor cells in order to determine if the overexpression of TRPM8 in the bulk tumor was associated with CRC cells or non-tumor origin cells (e.g. endothelial cells, fibroblasts and lymphocyte subpopulation) present in the tumor mass (Figure 47A). As shown in Figure 47B, using the magnetic separation of non-tumor cells, the single-cell suspension was then substantially enriched (approximately 92%) in tumor CD326+ cells (Figure 47A-B). TRPM8 was found to be up-regulated in isolated primary CRC cells, as demonstrated by a comparison between sorted CD326+ tumor cells and healthy colonic epithelial cells (HCEC) (Figure 47C). All in all, these findings demonstrate that TRPM8 is overexpressed in human CRC tissues, and that this abnormal expression is specifically linked to the tumoral epithelial compartment.

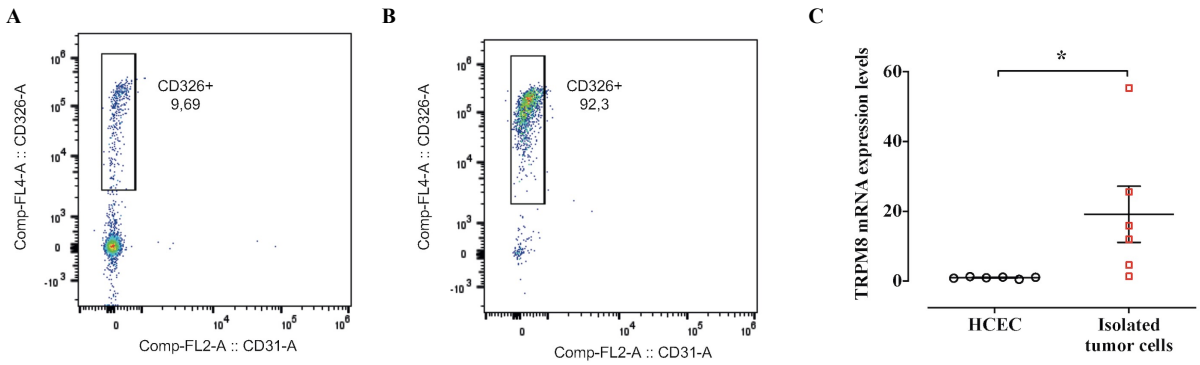


Figure 47. TRPM8 aberrant expression is selectively associated with the tumoral epithelial compartment. (A-B) Representative flow plots showing the enrichment of CD326+ human tumor cells before (A) and after (B) cells purification from primary specimens. CD31 was used to exclude the infiltration of leukocytes and endothelial cells. (C) TRPM8 mRNA levels in immortalized human colonic epithelial cells (HCEC) and primary tumor cells isolated from the bulk tumor by magnetic separation. Data are presented as the average of n = 6 independent experiments. Error bars represent ±SEM. P values were determined using unpaired t tests. *P < 0.05 versus HCEC.

2.4 TRPM8 up-regulation is linked to an overactivation of Wnt/β-catenin pathway

Given that Wnt signaling is essential for colonic regeneration and proliferation (Arnold, Tronser et al. 2020), we searched the TCGA database of 268 CRC patients for overrepresentation of the Wnt pathway. It's interesting to note that Kyoto Encyclopedia of Genes and Genomes (KEGG) Wnt signaling pathway gene set overrepresentation was shown by both gene set enrichment analysis (GSEA) and gene set analysis (GSA).

In our dataset of CRC patients, we discovered that a high expression of TRPM8 was linked with hyperactivation of the Wnt–Frizzled signaling, down-regulation of APC gene, and, surprisingly, up-regulation of NOTUM (Figure 48A-C). To summarize the Wnt pathway genetic characteristics, protein abundance, location, and drug susceptibility, we haveincluded an oncoprint heatmap of the pathway (Figure 49). By using such heatmap to find aberrations, both punctually and by sample or gene, we were able to study each gene of the KEGG pathway, even if there are no changes between the high and low TRPM8-expressing samples (Figure 49). These findings suggest that TRPM8 function in colon carcinogenesis may be directly linked to the Wnt/β-catenin pathway.

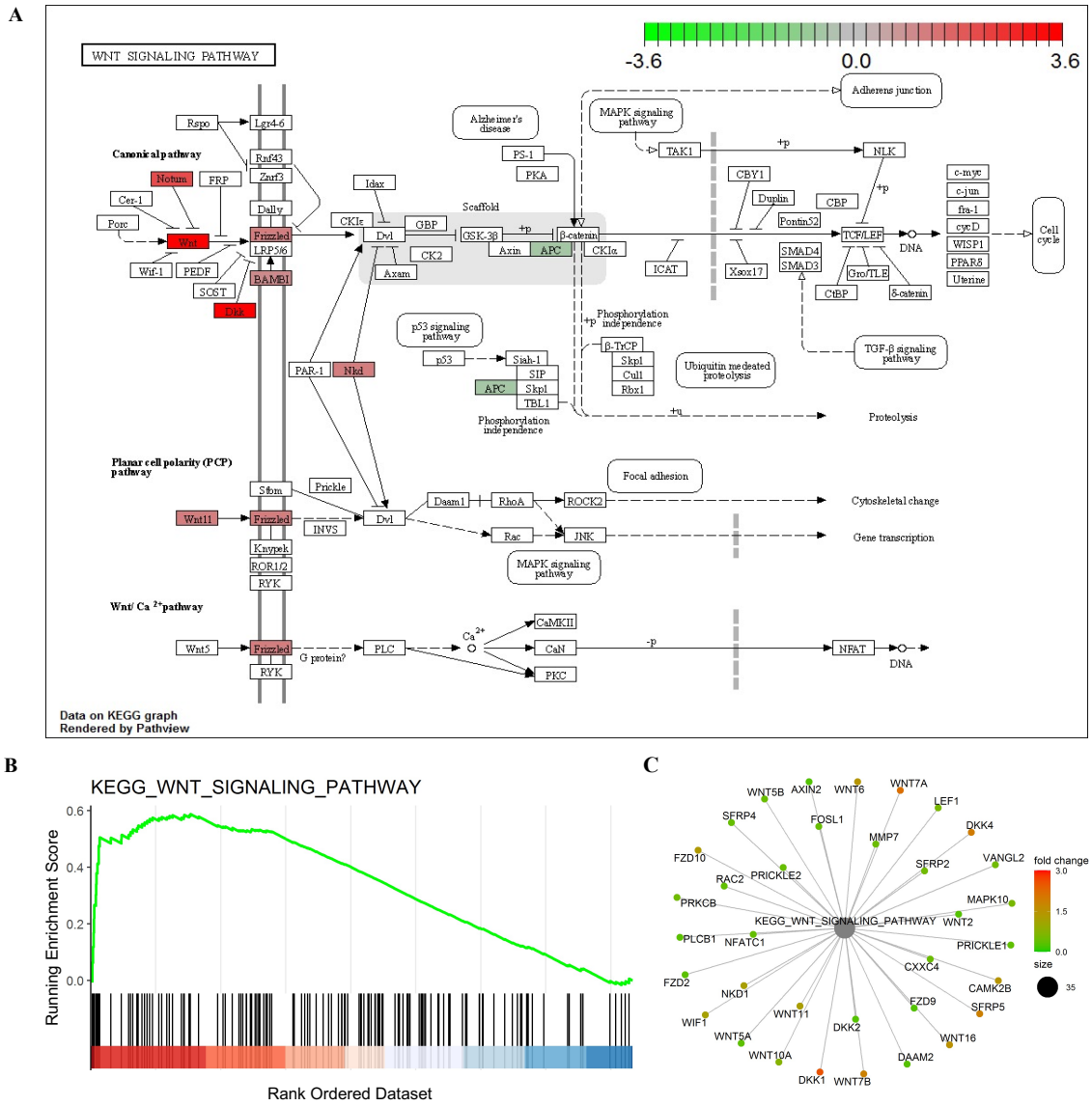


Figure 48. TRPM8 expression affects the Wnt network in colon cancer. (A) Overlay of the differentially expressed genes (DEGs) between high and low *TRPM8* expression groups on the KEGG pathway. The overexpressed and underexpressed genes are represented in red and green, respectively. (B) Gene set enrichment analysis (GSEA) enrichment plot. In green, the enrichment profile; in black on the X-axis, the hits. Colour scale on the X-axis goes from high (red) to low (blue) expression of genes in the high *Trpm8* group. (C) Cnetplot showing the linkages between genes and biological concepts. The overexpressed and underexpressed genes are represented in red and green, respectively.

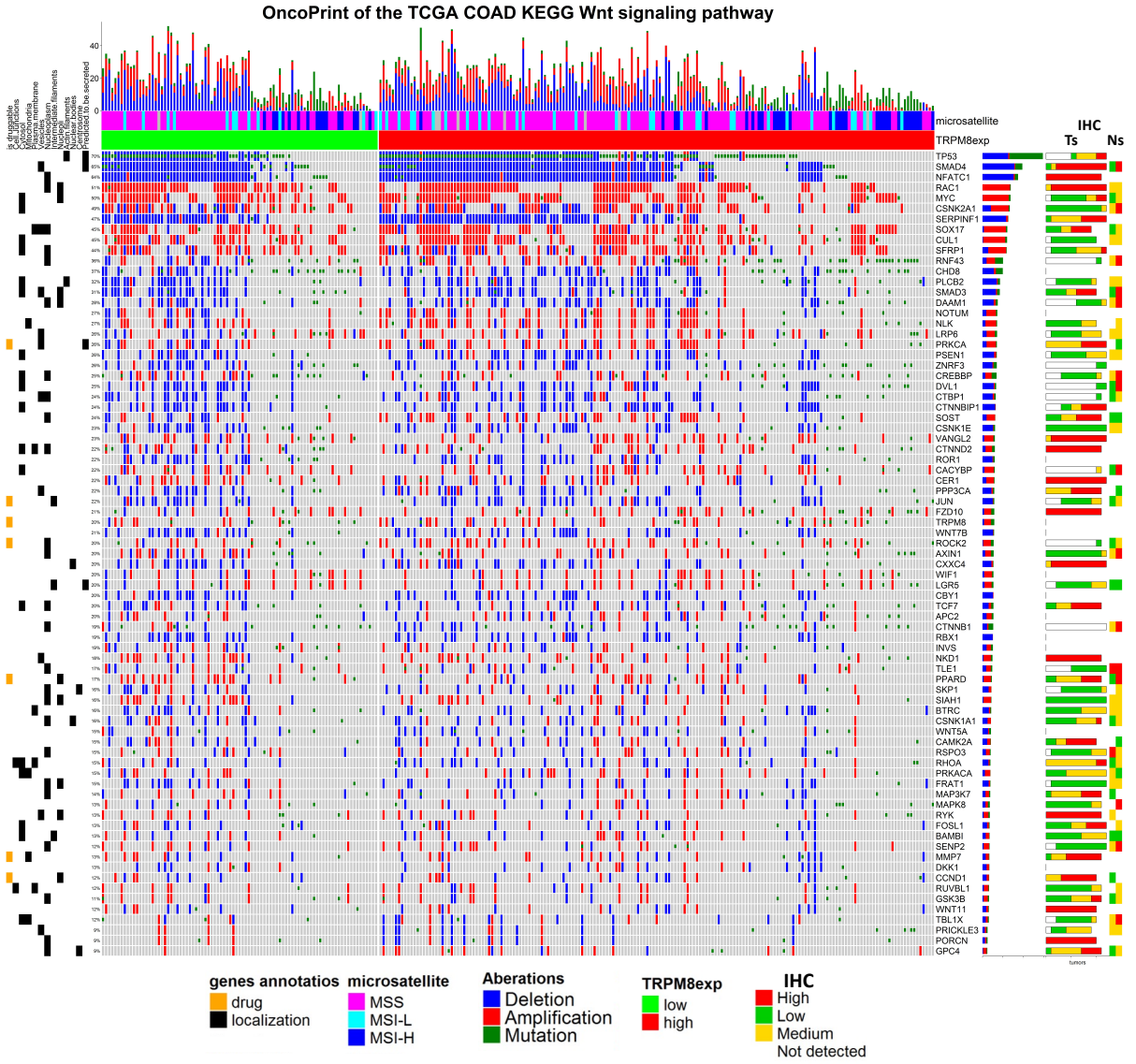


Figure 49. Oncoprint visualization of clinical and genomic features of the KEGG Wnt pathway in the 268 TCGA CRC patients. Protein abundance in both CRC and normal colorectal tissues, as well as localization and druggability are reported. In the HM, red represents amplification, blue represents deletion and green dots mutations of gene. On the left the cellular localization together with the druggability are reported for each gene. On the right, a barplot recapitulating the frequency of the aberrations for each gene and the immunohistochemistry status of the tumors and the healthy controls according to the Tumor Atlas. On top, TRPM8 subgroups microsatellite status and barplot recapitulating the frequency of the aberrations for each sample.

2.5 TRPM8 genetic deletion ameliorates CAC progression in mice

Thus, we investigated whether *Trpm8* deletion affected experimental carcinogenesis triggered by intestinal inflammation (as shown in the schematic representations in Figure 50A). Therefore, we examined WT and *Trpm8*$^{-/-}$ mice subjected to the AOM/DSS model of CAC (Figure 50A). Following the first DSS cycle, *Trpm8*$^{-/-}$ mice were subjected to a higher percentage of body weight loss in comparison to WT mice (Figure 50B). At the end of the experimental protocol, *Trpm8*$^{-/-}$ animals did not, however, exhibit statistically significant variations in any of the inflammatory markers that were examined (MEICS, colon weight/colon length ratio, and spleen weight) compared to WT mice, (Figure 50C). The first endoscopic examination was evaluated prior to the final DSS cycle (day 50) in order to investigate the evolution of CAC. *Trpm8*$^{-/-}$mice had less tumor formation, according to endoscopic examination, with a tendency toward less tumor multiplicity (Figure 50D). At 63 dpt (endpoint) AOM/DSS-treated *Trpm8*$^{-/-}$mice had considerably fewer total tumor numbers than AOM/DSS-treated WT mice (Figure 50E). Interestingly, compared to WT mice, *Trpm8*$^{-/-}$ animals showed a significantly decreased prevalence of Grade 1 tumors (Figure 50E), indicating a potential role for *Trpm8* in tumor initiation rather than progression in this experimental model. Collectively, these findings imply that *Trpm8* deletion functionally reduces the inflammation-induced colon carcinogenesis.

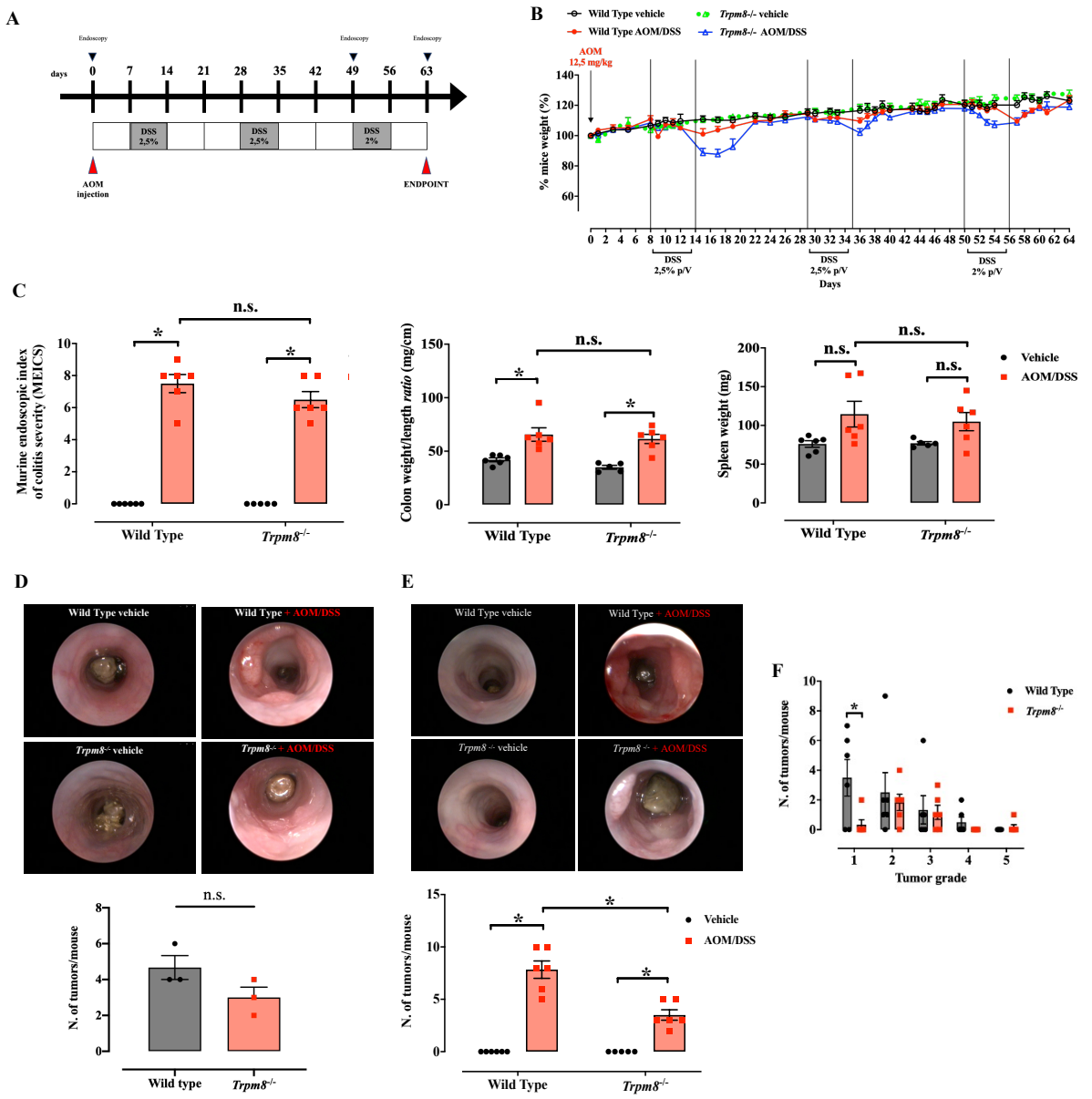


Figure 50. Loss of Trpm8 reduces azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced colitis-associated colon cancer. (A) Schematic representation of AOM/DSS protocol and timeline. Mice were injected intraperitoneally with 12.5 mg·kg−1 AOM on the first day of the experiment. DSS was administered in drinking water at the indicated doses at the indicated time. (B) Mice body weight gain/loss. Data are presented as the average of n=6 mice for each experimental group. Error bars represent ±SEM. P value was assessed by Two-Way ANOVA followed by Tukey's multiple comparisons test. *$p < 0.05$. (C) Analysis of the inflammatory parameters by the murine endoscopic index of colitis severity (MEICS), and the macroscopic inflammatory parameters colon weight/colon length ratio and spleen weight in WT and *Trpm8* $^{-/-}$ mice treated with vehicle (black) or AOM/DSS (red). Data are presented as the average of n = 6 mice for WT vehicle and WT and *Trpm8* $^{-/-}$ AOM/DSS; n = 5 mice for Trpm8 −/− vehicle. Error bars represent ±SEM. P values were determined using two-way ANOVA followed by

Sidak's multiple comparisons test. n.s. = non-significant; *P < 0.05. (D) Representative colonoscopy images and number of tumors/mouse in WT and Trpm8−/− mice carried out at day 49 of AOM/DSS protocol. Data are presented as the average of n=3 mice for each experimental group. Error bars represent ±SEM. P value was assessed by unpaired Student's t-test. Ns=non-significant. (E) Representative colonoscopy images and number of tumors/mouse counted microscopically in wild-type and Trpm8 −/− mice, treated with vehicle (black) or AOM/DSS (red), at the end of the carcinogenic protocol (Day 63). Data are presented as the average of n = 6 mice for WT vehicle and WT and *Trpm8* $^{-/-}$ AOM/DSS; n = 5 mice for Trpm8 −/− vehicle. Error bars represent ±SEM. P values were determined using two-way ANOVA followed by Sidak's multiple comparisons test. *P < 0.05. (F) Tumor grade of WT (black) and Trpm8 −/− (red) mouse tumors, evaluated by colonoscopy. Data are presented as the average of n=6 mice for each experimental group. Error bars represent ±SEM. P value was determined using two-way ANOVA followed by Sidak's multiple comparisons test. *P < 0.05.

DISCUSSION

TRPM8 is the primary receptor responsible for cold sensation, belonging to the subset of temperature-sensitive TRP channels (Clapham 2003). However, growing evidence accumulated in the past few years have shown that this channel is not only a cold sensor. While TRPM8 is widely expressed in sensory fibers, it is also found in other cell types, including murine innate immune cells and human monocytes (Khalil, Babes et al. 2016, Hornsby, King et al. 2022). Furthermore, TRPM8 has been recently implicated in chronic inflammatory conditions including inflammatory bowel disease, although its specific role is still contradictory and not mechanistically explored (Ramachandran, Hyun et al. 2013, de Jong, Takahashi et al. 2015, Khalil, Babes et al. 2016). However, TRPM8 increased expression in inflamed human or mouse colons (Ramachandran, Hyun et al. 2013) suggests that channel activation might be necessary during the inflammatory process. Notably, most TRPM8 ligands are derived from natural sources. We therefore embarked on the identification of novel dietary ligands for TRPM8 with potential anti-inflammatory properties. We firstly prove that flavonoid iuteolin is a novel potent TRPM8 blocker, antagonizing the Ca^{2+} elevation response to icilin in TRPM8-HEK293. Furthermore, TRPM8 does notactive or inhibit TRPV1- or TRPA1, the other TRP channels mainly involved in colonic inflammation (Bertin, Aoki-Nonaka et al. 2017). Luteolin is found in several vegetables such as broccoli, pepper, thyme, and celery. Like many flavonoids, luteolin exerts an anti-inflammatory effect (Hussain, Tan et al. 2016), however, its specific target and the underlying mechanism were so far unknown.

Macrophages play crucial roles in maintaining tissue homeostasis as well as in innate immunity, and affect various disease outcomes, including IBD (Delfini, Stakenborg et al. 2022). Despite the possible immunomodulatory role of TRPM8 in macrophages has been proposed (Khalil, Babes et al. 2016, Hornsby, King et al. 2022), the downstream signaling pathways resulting in its pro-inflammatory properties have not been well determined. In our research, we observe an increase in TRPM8 expression in pro-inflammatory BMDMs, sustaining the hypothesis that this channel may be a druggable target in inflammation. In line with these findings, given that TRPM8 is an ion channel mostly permeable to Ca^{2+}, we show that prolonged calcium transients occur in WT BMDMs after stimulation with icilin (i.e, TRPM8 agonist). Furthermore, luteolin can reverse this calcium influx in macrophages, further validating its role as a TRPM8 blocker. Exposure to PAMPs (such as LPS and IFNγ) brings on o to? a rapid cytosolic Ca^{2+} increase (Letari, Nicosia et al. 1991, Rada, Park et al. 2014, Vaeth, Zee et al. 2015, Schappe, Szteyn et al. 2018). Previous works showed that Orai channels, the main channels involved in store-operated Ca^{2+} entry, are not critical for LPS-induced macrophage activation (Vaeth, Zee et al.

2015). However, more recently, the LPS-mediated Ca^{2+} influx in macrophages was linked to the chanzyme TRPM7, another member of the TRPM subfamily of ion channels (Schappe, Szteyn et al. 2018), indicating that TRP channels may be involved in this mechanism instead. Here, we demonstrate that LPS-induced Ca^{2+} elevations are severely blunted in *Trpm8*$^{-/-}$ macrophages and, accordingly, TRPM8 pharmacological blockade with luteolin also reduces cytosolic calcium in response to LPS. Collectively, our findings provide strong evidence for the prominent role of this ion channel in the macrophage response to pro-inflammatory stimuli.

PAMPS-induced intracellular calcium elevation in macrophages is critical for the induction of a potent pro-inflammatory signaling, leading to an increased expression of specific genes and/or secretion of inflammatory mediators. Consistently, we reveal that innate immune cells from *Trpm8*$^{-/-}$ mice display blunted inflammatory responses upon challenging with pro-inflammatory stimuli. In accordance with these findings, TRPM8 modulation with luteolin induces a pro-tolerogenic profile in macrophages. Indeed, TRPM8 pharmacological inhibition suppresses the release of pro-inflammatory mediators IL-1β, IL-6, and TNF-α from BMDMs *in vitro* and increases the levels of early production of IL-10.

Over the past decade, it has been recognized that the profile of cellular metabolism plays a pivotal role in macrophage activation, with early IL-10 production being the primary anti-inflammatory mechanism that controls essential metabolic pathways and reduces glucose uptake (Ip, Hoshi et al. 2017). Specifically, exposure to PAMPs triggers macrophage pro-inflammatory polarization by inducing a metabolic reprogramming, characterized by elevated aerobic glycolysis and a broken tricarboxylic acid cycle, leading to accumulation of succinate and citrate (Viola, Munari et al. 2019). Our *in vitro* data show that TRPM8 pharmacological inhibition or genetic deletion reduces the metabolic switch in pro-inflammatory macrophages, restoring a naïve-like metabolism. From the analysis of metabolites, luteolin-treated pro-inflammatory macrophages produce lower levels of succinate, but higher levels of lactate from early timepoints (i.e. 3, 6h) compared to untreated cells. Lactate acts as a complex immunomodulatory molecule that controls innate and adaptive immune cell functions (Zhang, Tang et al. 2019, Irizarry-Caro, McDaniel et al. 2020, Wang, Wang et al. 2022). In pro-inflammatory macrophages, lactate-derived histone lactylation serves as an epigenetic modulation that stimulates the expression of pro-tolerogenic genes, including key immunosuppressive cytokine IL-10 (Zhang, Tang et al. 2019, Wang, Wang et al. 2022). Our data from LDHa-inhibited and IL-10 deficient BMDMs show that the suppressive effect of luteolin on LPS+IFNγ-induced pro-inflammatory cytokine production in macrophages is downstream-mediated by a lactate-dependent regulation of IL-10 pathway. In the absence of

lactate, luteolin fails to induce IL-10 expression in pro-inflammatory macrophages, and, consistently, in the absence of IL-10 luteolin is not able to induce a pro-tolerogenic profile in LPS+IFNγ stimulated macrophages.

Finally, luteolin *in vivo* ameliorates colitis severity and mucosal injury caused by DSS administration in mice. In the DSS model of colitis, the innate immune system play a more dominant role (Wirtz, Popp et al. 2017). In our data, inhibition of TRPM8 by luteolin daily oral administration mediates an impairment in the innate immune response during the recovery phase, thereby suppressing colitogenic responses. Ramachandran and Khalil's studies showed that repeated administration of TRPM8 agonists (i.e, icilin and menthol) attenuated experimental colitis (Ramachandran, Hyun et al. 2013, Khalil, Babes et al. 2016). However, the rapid desensitization (hence inactivation) of TRPM8 following repeated agonist administration has been widely demonstrated (Diver, Cheng et al. 2019). Moreover, Khalil and his group showed that global *Trpm8-/-* mice exhibited more severe acute inflammation compared to WT mice during DSS colitis (Khalil, Babes et al. 2016), inconsistently with the studies by Ramachandran et al. (Ramachandran, Hyun et al. 2013). Overall, luteolin renders mice significantly resistant to DSS-induced colitis, revealing TRPM8 as a major controller of pro-inflammatory response by myeloid cells *in vivo*.

Taking into account that the most dangerous side effect of IBD is the colitis-associated colorectal cancer (CAC), where inflammatory factors act on the intestinal mucosa to promote the growth of tumors (Rajamaki, Taira et al. 2021), the results described above prompted us to ask whether TRPM8 could have a prognostic and pathophysiological role in colon carcinogenesis. Although epithelial cells commonly express low amounts of TRPM8, its expression is much higher in tumor cells (Tsavaler, Shapero et al. 2001, Yee, Brown et al. 2012, Borrelli, Pagano et al. 2014, Kijpornyongpan, Sereemaspun et al. 2014, Hemida, Hammam et al. 2021, Liu, Li et al. 2022). Nonetheless, the TRPM8 mechanism of action and, importantly, pathophysiological, and clinical significance in colon carcinogenesis are still largely fragmented and undefined. Here, we find a substantial rise in TRPM8 expression in tumor samples from patients with stage pT4 colorectal cancer. We demonstrate a strong correlation between poor prognosis and overexpression of TRPM8 in CRC patients. More specifically, a significant reduction of PFI and disease-specific survival are predicted by high expression of TRPM8 in colon cancers (more than 33 percent of samples). Moreover, we demonstrate that the abnormal TRPM8 expression in CRC patients is selectively associated to epithelial tumoral cells isolated from primary tumors.

From a bioinformatic analysis of the biological profile of these "TRPM8$^{hi/low}$ survival" colon tumors, we observed an over-activation of Wnt pathway compared to TRPM8^{low} tumors. Wnt signaling has been widely shown to regulate the levels of the essential effector β-catenin for signal transduction (Clevers and Nusse 2012). Mutations in β-catenin have been reported in a broad range of solid tumors, including melanoma and colon cancer (Rubinfeld, Robbins et al. 1997). Consequently, we show that hyperactivation of Wnt and Frizzled and down-regulation of APC are linked to high expression of TRPM8 in CRC patients (more than 33 percent of our population). APC controls the phosphorylation and degradation of β-catenin, which in turn negatively affects Wnt signaling (Morin, Sparks et al. 1997). Nuclear accumulation of β-catenin is prevalent in IBD-CRCs (Claessen, Schipper et al. 2010). Nonetheless, in CAC molecular pathogenesis, loss of APC function is less common, and it is considered a late event while P53 mutations/loss are early, even before dysplasia (Rajamaki, Taira et al. 2021). Based on these findings, we hypothesize that the inhibition of TRPM8 could potentially decrease Wnt/β-catenin signaling. More in general, our hypothesis is that tumor cells exploit up-regulating TRPM8 by inducing an abnormal activation of β-catenin signaling pathway, which in turn promotes and maintains the growth of tumors. However, more comprehensive research is needed to illustrate the functional significance of such aberrant activation in terms of consequences on one or more cancer hallmarks (e.g., proliferation and tumor dissemination).

Ultimately, in the light of our evidence about TRPM8 role in colon cancer pathogenesis and its predictive significance in cancer diagnosis, we explored the effect of TRPM8 genetic deletion in experimental models of CAC. The loss of *Trpm8* was sufficient to significantly reduce tumor incidence and growth in inflammation-induced intestinal carcinogenesis, thus clearly featuring the critical role of TRPM8 in tumor development. Although our data in models of intestinal inflammation defined TRPM8 as an ‘anti-inflammatory’ target, in the AOM/DSS model of CAC *Trpm8* global deletion did not significantly affect the inflamed parameters related to tumor development. However, we show that TRPM8 has a prominent effect on pro-inflammatory macrophage biology during intestinal inflammation. Macrophage polarizations play different roles in inflammation and tumor, with pro-resolving macrophages playing a tumor-promoting role in the early stage of tumor formation (Zhang, Li et al. 2023). Therefore, modulation of TRPM8 may have different effects on tumor-associated macrophages during carcinogenesis.

GENERAL CONCLUSIONS

The experiments depicted in this PhD thesis highlight TRPM8 as a potential target implicated in inflammation-triggered intestinal malignancies. Moreover, by identifying novel potent dietary occurring ligands of TRPM8, we highlight the potential of targeting this ion channel through dietary interventions. Briefly, we have shown that:

- Dietary flavonoid luteolin is a new selective TRPM8 antagonist. Luteolin, *via* TRPM8, modulates the response of macrophages to PAMPs (i.e. LPS and IFNγ), diminishing their pro- inflammatory capacity and metabolic switch. This effect is downstream mediated by a lactate- dependent regulation of IL-10 activity. Accordingly, we demonstrated that genetic ablation of TRPM8 induces a pro-tolerogenic profile in macrophages. *In vivo,* luteolin oral supplementation in mice ameliorates DSS-induced colitis through an impairment in the innate immune response.
- TRPM8 overexpression correlates with poor prognosis in CRC patients and TRPM8 genetic ablation protectes mice from CAC development. Mechanistically, the carcinogenetic effect of TRPM8 seems to be correlated with an over-activation of the Wnt/β-catenin pathway.

In conclusion, this PhD thesis contributes to elucidate the physiopathological role of TRPM8 in chronic inflammatory diseases. This research is of high significance as it pinpoints specific nutrients that could aid IBD patients, paving the way for crafting specialized diets and dietary supplements tailored for managing gastrointestinal inflammatory disease.

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