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Role of mast cell plasticity and memory in health and disease: a 3D-based model to study the crosstalk between intestinal organoids and mast cells.

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2 ABSTRACT

Mast Cells (MCs) are long-living innate immune cells widely distributed in mucosal and connective tissues. They are located at the interface with the external environment, therefore are among the first cell type that can get in contact with pathogens. The main aim of this PhD thesis is to shed light on what underlies the heterogeneity of the MCs, to finally test the plasticity of their response to different stimulatory environments. Since microbial ligands are known to induce innate immune memory on Macrophages (Mϕ) and Natural Killer (NK) cells, to study whether it could be true also for MCs, they were stimulated for 24 hours with different inflammatory or infective stimuli, allowed to rest for 6 days and then restimulated with the same or other pathological stimuli in order to assess if an enhanced (training) or a reduced (tolerance) response was induced. In LPS primed MCs, a slight increase of TNFα and IL-6 release was induced when restimulated with living *C. Albicans*, while it was strongly reduced in LPS restimulated MCs, and this was evident also at transcriptional level. Moreover, the LPS prestimulation made MCs more prone to express MCPT-4, thus suggesting a possible role of this protease in the training or tolerance effect exerted on MCs by LPS prestimulation. Sodium Butyrate (NaB, microbial derivative) effects on MCs priming were also analysed, and it was demonstrated that it inhibited MCs degranulation (at short and long time) and reduced TNFα release after different inflammatory stimulations. Moreover, since the gut is characterized by a great cellular variability, which make it a very complex stimulating environment, MCs plasticity of response was also evaluated in the context of healthy and pathological intestinal organoid cultures. Healthy murine small intestine and colon organoids were produced as well as of inflamed (DSS derived) and tumoral (AOM/DSS derived) tissue ones, that were used for the co-culture with bone marrow derived MCs (BMMCs). Resting and IgE/Ag activated BMMCs induced different effects on healthy and pathological organoids, in terms of composition (analysed as mRNA expression of intestinal epithelial marker as Lgr5, Lyz1, Muc2, ChgA and SI) and structural architecture (analysed as mRNA expression and/or as protein expression of Cld4, Cdh1, ZO-1 and Ezrin). Moreover, BMMCs were able to perceive healthy from pathological tissue differently, responding in a specific way. Thus, protease expression profile and activation status of BMMCs indicated that tumoral organoids are capable of inducing substantial MCs activation response, in the absence of other external stimuli. In this scenario, IL-33 organoid expression and TNFα release by BMMCs seem to play a central role, creating a cytokine environment in which IL-33 stimulates BMMCs to produce TNFα that in turn induces different structural effects depending on the microenvironment. Experiments carried out with NaB (that inhibits BMMCs activation) and TNFα-/- BMMCs further supported these data. These results indicate that MCs are important mediators of tissue homeostasis, and that a different stimulatory environment can shape and direct MCs specific response towards the dampening or propagation of the inflammatory response.

3 LIST OF ABBREVIATIONS

Ag: antigen
AOM: azoxymethane
ASCs: adult stem cells
BCG: bacillus Calmette-Guerin
BM: bone marrow
BMCP: basophil, mast cell progenitor
BMMCs: bone marrow derived Mast cells
CD: Crohn's disease
Cdh-1: e-cadherin
ChgA: chromogranin A
Cldn4: claudin-4
CLRs: c-type lectins receptors
CMV: cytomegalovirus
CPA3: carboxypeptidase3
CRC: colorectal cancer
CTMC: connective tissue mast cells

Ctnn1-b: β-catenin gene
DAG: diacylglycerol
DAMPs: Damage associated molecular pattern
DSS: dextran sodium sulfate
ELISA: enzyme-linked immunosorbent assay
ET: endotoxin tolerance
GI: gastrointestinal
GMP: granulocyte/monocyte progenitor
HSC: hematopoietic stem cell
i.p.: intraperitoneal
IBD: inflammatory bowel disease
IBS: irritable bowel syndrome
IELs: intraepithelial lymphocytes
IF: immunofluorescence
Ig: immunoglobulin
IHC: immunohistochemistry
IL: interleukin
ILCs: innate lymphoid cells
INFγ: interferon γ
IP3: inositol triphosphate
iPSCs: induced pluripotent stem cells
ITAM: Immunoreceptor tyrosine-based activation motif
LAT: linker for activation of T cell
Lgr5: Leucine Rich Repeat Containing G Protein-Coupled Receptor 5
LP: lamina propria
LPS: lipopolysaccharide
Lyz1: lysozyme 1
MALT: mucose-associated lymphoid tissue
MCC: mast cells chymase
MCETs: mast cell extracellular traps
MCPT-: mast cell protease
MCs: mast cells
MCTC: mast cells tryptase and chymase
mEGF: mouse epidermal growth factor
MMC: mucosal mast cells
MOI: multiplicity of infection
Muc2: mucin 2
Mφ: macrophages
NaB: sodium butyrate
NFkB: Nuclear factor *k*-light-chain-enhancer of activated B
NK: natural killer
PAMPs: pathogen-associated molecular pattern
PIP2: Phosphatidylinositol 4,5-diphosphate
PKC: protein kinase C
PLC: phospholipase C
PRRs: pattern recognition receptors
rPCMCs: rat peritoneal cavity mast cells
SCAFs: short chain fatty acids
SCF: stem cell factor
shRNA: short hairpin RNA
SI: sucrase isomaltase
TF: transcription factor
TGFβ: transforming growth factor β
Th: t helper
TI: trained immunity
TLRs: toll-like receptors
TME: tumour microenvironment
TNFα: tumour necrosis factor α
UC: ulcerative colitis
VEGF: vascular endothelial growth factor

ZO-1: zonula occludens-1

4 INTRODUCTION

4.1 MAST CELLS UNDER THE SPOTLIGHTS

4.1.1 What are the Mast Cells?

Mast cells (MCs) are long-living, tissue-resident innate immune cells that originate from the hematopoietic precursor in the bone marrow[1] but acquire their definite phenotype once they home to the target tissues[2]. They are evolutionarily ancient cells, whose discovery dates back to 1863 when the presence of granulated cells in connective tissues of frog and tadpoles was reported by Friedrich von Recklinghausen[3]. It took about 14 years before Paul Ehrlich discovered the presence of these granulated cells also in humans. He described them as aniline-positive granulated cells located near blood vessels and nerves. Ehrlich called them "*Mastzellen*" from German "*mast*" which means "full of", which concerns the large presence of granules that were once thought to contain nutrients[4]. It was only after many years, in the 1950s, that it was found that MCs granules contained histamine and that they were the main actors in anaphylactic allergic reaction[5]. Then, in 1964 Lichtenstein and Osler first demonstrated that leukocytes of hay fever patients were able to release histamine after an *in vitro* stimulation[6], but there was a missing point represented by the responsible mechanism behind MCs activation. At the beginning of 1970 it became clear the relevance of Immunoglobulin E in this scenario, starting from K. Ishizaka and J. Ishizaka's discovery that "*the bridging of two (or more) cell-bound γE molecules by allergen may be the initial step of the reaction*"[7]. Finally, in 1989, Metzger and Kinet group's identified the structure of $Fc_{\varepsilon}RI$ and published a paper in which they stated that "*The high-affinity receptor for immunoglobulin E, $Fc_{\varepsilon}RI$, is found exclusively in mast cells and basophils*" and where they demonstrated that $Fc_{\varepsilon}RI$ binds to IgE resulting in MCs activation.[8]

Nowadays it is well known that MCs are found basically in every tissue, most abundantly near barriers where they exert a role of "Immune sentinels" during both acute and chronic inflammation[9]. Its controversial role is also reflected by the great variety of mediators that MCs contain in their cytoplasmic granules which are released through a process called "degranulation" which is triggered following the activation of the MCs. This mechanism allows the rapid and efficient release of all the pre-stored and newly synthesized mediators of the MCs[10].

MCs have been traditionally classified into two categories mostly based on their granule content and body position: the ones containing only chymase (MC_C), the ones containing only tryptase (MC_T), and the ones containing both (MC_{TC}) in human, and connective tissue MCs (CTMC) or mucosal MCs (MMC) in rodents[11]. Although this is a historically accepted classification, it's way too reductive. In light of several studies carried out in recent years, we now know that MCs are extremely heterogeneous cells, whose phenotype and function could depend on the homing

site and its microenvironment[12]. This extreme heterogeneity makes it a peculiar cell of our immune system, and the study of its different gene expression can help to better understand its role even in pathological contexts of various kinds.

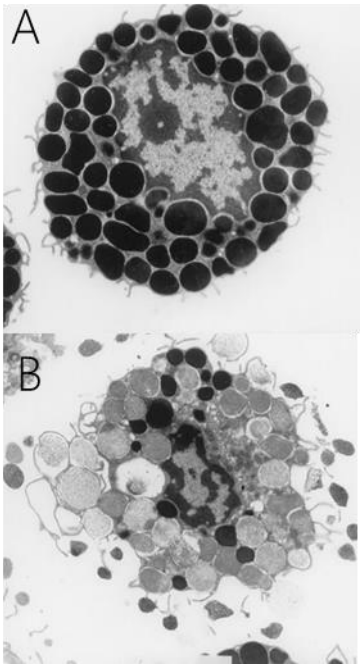


Figure 1: Transmission electron micrographs of purified peritoneal MCs. Resting (A) and degranulated (B) rat peritoneal MCs. The resting cell show a homogenous distribution of granules that contain electron-dense material. During the degranulation process, granules are fused and released. Adapted from[13].

4.1.2 MCs development

As briefly introduced, one of MCs peculiarity is the fact that they originate in the bone marrow from the hematopoietic stem cell, but then they rapidly migrate through the bloodstream to the periphery and reach the homing tissue. Here MCs terminally differentiate a tissue-specific phenotype according to the stimulatory environment in which they end up[14].

Lineage studies on MCs date back to many years and they are still quite debated, but already in the 1970s, it was clear that MCs and Basophils were cells with a similar physiological role that originate in the bone marrow but have independent cell lineages[15]. Later it was demonstrated that MC development follows the myeloid lineage: from a Kit^+ $Sca1^+$ hematopoietic stem cell (HSC) to a Kit^+ $Sca1^{lo}$ $FcγRII/III^{hi}$ Granulocyte Monocyte Progenitor (GMP) that seems to be shared by monocytes, MCs, basophils, eosinophils, and neutrophils[16]. MCs origin and development is finely regulated by transcription factors activation: GMP is characterized by the expression of C/EBPα and it is responsible for the differentiation of a progenitor with MC and basophil-forming capacity (BMCP). As it is schematized in figure 2, the expression levels of C/EPBα determine the cell lineage: while the upregulation of C/EPBα is linked to a basophil-like lineage

commitment, its downregulation together with the upregulation of mi transcription factor (MITF) are necessary for the differentiation of MCs precursors[17].

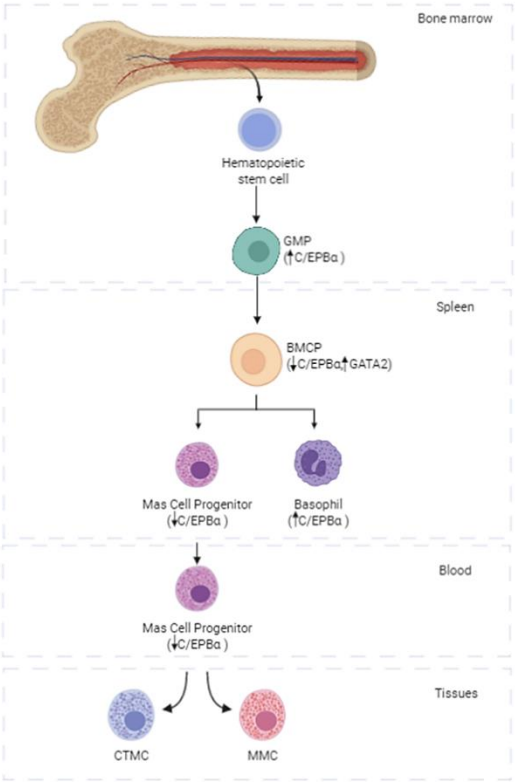


Figure 2: A short model of murine MCs lineage development. MCs progenitors originate in the bone marrow from hematopoietic stem cells (HSC) to a granulocyte monocyte progenitor (GMP) - which also gives rise to neutrophils, eosinophils, monocytes, and basophils. Moreover, a bi-potent basophil/MCs progenitor (BMCP) which can further differentiate *in vitro* to MCs or basophils, was identified in the spleen. Once released in the bloodstream, MCs quickly migrate to peripheral tissues where they complete their differentiation.

The MC precursor subsequently enters the bloodstream through which it reaches the tissue of selection where it will undergo terminal differentiation in an environmental-dependent manner. A striking discovery reversed the situation when in 2018 Gentek and colleagues, utilizing iCdh5 fate mapping, demonstrated that MCs, as Macrophages, have indeed dual hematopoietic origin: one first wave comes from the endothelium of the yolk sac, and at a later time there is a second wave of definitive MCs that gradually dilute the first one. This peculiarity of MCs origin let the authors infer that MCs probably have critical functions during the developmental stages of life, since they are not present only in the embryo but are established before the definitive haematopoiesis. Moreover, they observed that adult MCs maintenance is bone marrow (BM) independent, suggesting that the replenishment of adult MCs in the tissue comes from in situ expansion of MC progenitors or adult MCs themselves[18].

MCs can be found in many organs. The majority are in the gastrointestinal tract[19], in the peritoneum[20], in the skin and in the lungs[21], and are rarely found in other organs such as lymph nodes, spleen, brain, pancreas, kidneys, but their number can increase in case of inflammation or cancer[22]. This high tissue specificity that characterizes adult MCs makes its in vitro culture challenging, highlighting the need for specific tissue isolation protocols. Nevertheless, the bone marrow of young mice represents a rich source of hematopoietic precursors from which it is possible to obtain bone marrow-derived MCs (BMMCs), after the addition of IL-3 and SCF to drive precursors maturation and differentiation[23].

4.1.3 MCs subsets

Mouse MCs are subdivided into two main categories based on their body localization: MMCs, which predominantly localize in gut and lung mucosa, and CTMC, which are found around venules and nerves. Almost a century after Paul Ehrlich's discovery, Enerbach observed that CTMCs and MMCs differentially responded to fixative and stainings: while CTMCs could be fixed and stained with safranine and alcian blue, MMCs could be detected only with Carnoy's or, to a little extent, with alcian blue staining. This different behavior was attributed to a different granule composition, in particular to the predominant presence of heparin in CTMCs granules and its almost complete lack in those of MMCs. Even if they differ on the biochemical and functional side, CTMCs and MMCs comparably respond to IgE-mediated activation, degranulation and mediators release[14].

On the other side, human MCs are historically categorized based on their granule content. As previously anticipated, three principal subsets are recognized: MC_T, which resemble mouse MMC and is more abundant in the alveolar and gastric wall, MC_C that is less represented and doesn't have a specific localization, and

MC_{TC} that resemble CTMC and predominantly reside in intestinal submucosa and skin[24]. Related to rodents, both MMCs and CTMCs display a specific set of proteases: in particular, MMCs predominantly express transcripts for the chymases MCP-1 and -2, they contain serotonin and little or no heparin, while the chymase MCP-4, the elastase MCP-5, the two tryptases MCP-6 and -7 and carboxypeptidase A are specific of CTMCs[25]. CTMCs express both serotonin and heparin. Notwithstanding, there are some exceptions, as in the case of mouse trachea, in which it is reported that both CTMC and MMC express the same pattern of tryptases and chymases[26].

However, the protease content and expression are influenced also by the inflammatory state: for example, MMcs of *T. Spiralis* infected mice express only MCP-1[27], while CTMCs expressed both MCPT4, MCPT5, MCPT6, MCPT7, and CPA3[28]. As a result, mice lacking MCP-1 have a defective clearance in case of *T. Spiralis* infection[29].

In recent years, interesting work was published by Dwyer et al. in which different subsets of mice MCs purified from distinct anatomical sites were compared. It was demonstrated how MCs had a strong tissue-specific gene expression profile. Among the most differently expressed ones, authors reported proteases, G protein-coupled receptors, and integrins[12]. All these reported pieces of evidence highlight how MCs origin and development lead to a complex scenario, where homing and environmental stimulations lead to the terminal differentiation of an extremely heterogeneous family of cells, thus it comes difficult to make inferences on data coming from *in vitro* studies without considering their body localization.

BMMCs that are commonly used for *in vitro* studies are considered immature MCs and express intermediate characteristics between MMCs and CTMCs: they express MCPT5, MCPT6, and CPA3 (like CTMCs) but present low levels of both heparin and histamine (like MMCs). If they are cultured in presence of IL-3 and stem cell factor (SCF) on a monolayer of fibroblasts, which recreate a connective-like environment, it is reported an increase of heparin and histamine, suggesting a stronger differentiation in a CTMC way. The protease content is tuneable by adding specific cocktails of cytokines in the culture medium: for example addition of factors such as IL-9, IL-10 or IL-33 induces higher expression of MCPT1, MCPT2, and MCPT6 respectively[30,31].

4.1.4 MCs biology and activation

MCs are extremely eclectic cells, and this characteristic is also reflected by the great variety of cytoplasmic mediators and membrane receptors they are provided with[32]. Among them, MCs can interact and recognize a great variety of PAMPs through Toll-like receptors (TLRs), C-type lectin receptors (CLRs), complement receptors, and several selective cytokines and chemokine receptors[33].

MCs activation and subsequent release of specific pre-stored or newly synthesized mediators can be induced by a plethora of external stimuli like the cross-linking of IgE receptor but also the complement activation, certain neuropeptides, and toxins[34–36]. However, considering that MCs are historically associated with allergic and anaphylactic reactions, the $Fc_{\varepsilon}RI$ pathway is the most known and studied. Briefly, antigen recognition by $Fc_{\varepsilon}RI$-bound IgE induces receptor aggregation on lipid rafts, activation of SRC family kinases (SFKs), and tyrosine phosphorylation of the receptor subunits[37]. This mechanism in particular will be detailed in the following paragraph.

4.1.4.1 FcεRI pathway

The most known and deeply characterized activation pathway of the MCs is the one concerning the crosslinking of $Fc_{\varepsilon}RI$/IgE/Antigen on MCs surface. This is consistent with the fact that MCs are mostly known for their role in allergic and anaphylactic reactions, given also the fact that IgE is found in the connective tissue under epithelial layers of the skin, in the respiratory tract, and also in the gastrointestinal tract, areas heavily populated by MCs[38]. $Fc_{\varepsilon}RI$ is made up of an α-chain (binding site of IgE), a membrane-tetraspanin β-chain and a disulfide-linked homodimer of the γ-chain[39]. In the cytoplasmic side, $Fc_{\varepsilon}RI$ interacts with LYN tyrosine kinase that phosphorylates tyrosine in ITAMs on β- and γ-chain[40], and activates SYK tyrosine kinase that phosphorylates LAT1 (linker for activation of T cell), that serves as a scaffold for other protein recruitment and is fundamental for the initiation of the downstream signaling pathways[41]. In particular, LAT mediated recruitment and activation of PLCγ causes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). The production of IP3 induces a transient but sustained mobilization of calcium in the cytosol from the endoplasmic reticulum, while DAG induces the activation of PKC; these two signals will in turn activate MCs degranulation[42]. Calcium release, induce the translocation of NFkB to the nucleus where it can mediate the transcription of many cytokines, such as IL-6, TNFα and IL-13[39] (Figure 3a).

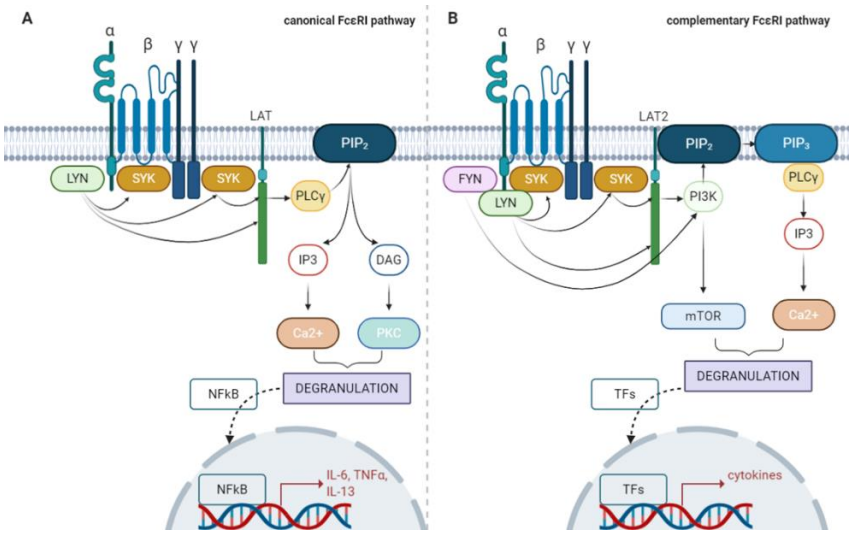


Figure 3: Canonical and complementary signaling of FcεRI in MCs: (A) The canonical FcεRI pathway starts from FcεRI aggregation (not shown), LYN interaction with ITAM domains of β- and γ-chain and consequent activation of SYK. This causes the phosphorylation of LAT that, in turn, recruits PLCγ which eventually activates PCK and induces the influx of intracellular calcium that leads to the degranulation event and cytokine production. **(B)** The complementary FcεRI pathway differs from the canonical one for the presence of FYN that activates the PI3K downstream pathway that leads to a sustained calcium influx together with the activation of the mTOR pathway that is involved in various cytokine production. Adapted from[42].

In 2004, Hernandez-Hansen and colleagues discovered that another tyrosine kinase other than LYN and SYK was necessary (and complementary) for MCs degranulation and cytokines production[43]. In this case, $Fc_{\varepsilon}RI$ activates FYN which in turn activates the PI3K pathway that eventually leads to the activation of mTOR, which underlies MCs chemotaxis and cytokines production[44] (Figure 3B). However, animal studies showed how PI3K seems to be mainly involved in the maintenance rather than in the initiation of the calcium signal that is necessary for a proper degranulation. For this reason, it has been proposed the existence of another molecule that have the same function of LAT in the assembly of the macromolecular complex necessary for FYN signaling, NTAL (also known as LAT2)[45].

4.1.4.2 Other ways of activation

Even though IgE receptors cross-linking is one of the most studied mechanisms of MC activation, it cannot be considered the most important one. MCs, indeed, are ubiquitous cells, they are found in almost any body tissue, and they in turn get in touch with several selves and external non-self molecules. As a result, MCs are

armed with a great variety of receptors for damage-associated molecular patterns (DAMPs) and for pathogen-associated molecular patterns (PAMPs), also known as pattern recognition receptors (PRRs), that can activate or prime MCs upon binding both at intracellular or at cell surface level[36].

Among the most studied PRRs, TLRs are highly represented on both human and rodent MCs. It has been shown that mice MCs express TLR from 1 to 9, of which TRL-1,-2,-4,-5,-6 are found on the cell surface, while TLR-3,-7,-8,-9 are found at endosomal level[46]. Depending on the type of receptor, tissue localization and maturation level of MCs, the binding of the receptor will induce a different profile of mediators production, that is specific for each TLR[46,47]. For example, the engagement of TLR-2, induces the production of TNFα, IL-6, IL-13, IL-4, and IL-5, while the engagement of TLR-4 is involved in the synthesis of TNFα, IL-6, IL-13, and IL-1β[48]. Interestingly, in rodents, it has been reported that a second IgE-induced activation is suppressed because of the FcεRI downregulation[49], while in humans a concomitant exposure to various TLR ligan and FcεRI stimulation induces an increased cytokine secretion without a degranulation impairment[50]. Another factor known to activate MCs are anaphylatoxins, more commonly known as the C3a and C5a fragments of the complement[36]. Their receptors, C3aR and C5aR, are found on some subpopulations of mice and human MCs, in which they have been seen to induce degranulation and hypersensitivity reactions *in vivo*[51]. Moreover, the C5a fragment is also seen to have a role in the adhesion and migration of human MCs [52]. Those above mentioned, are members of the G-protein coupled receptor (GPCRs) family, which also includes chemokine receptors that are constitutively expressed on MCs surface like for example CCR1, CCR3,4 and 5, CXCR1–4, and CX3CR1[53]. Aside from the fact that chemokine receptors are involved in chemotaxis, it has been reported that these receptors are sometimes implicated in the enhancement of FcεRI-mediated responses like reported for CCL7 in an experimental allergic conjunctivitis setting[54].

4.2 INNATE IMMUNE MEMORY

We all know that the immune response is composed of two arms: the innate immune response, which is immediate, not specific, and mediated mainly by myeloid cells, and the adaptative immune response, which is slower, specific and requires the activation of B and T lymphocytes that underlie the immunological memory. Despite this dichotomy is a cornerstone, recent studies and, above all, the discovery of PRRs have raised the possibility that the innate response is more complex than it is formally accepted, and the evidence that plants and invertebrates lacking adaptive immune system are protected from reinfection supports this theory[55,56]. An increasing body of evidence is also demonstrating that innate cells such as NK and monocytes can display adaptive characteristics[57–60]. Thus, a new term started to come out to describe this process: "TRAINED IMMUNITY"[61] (Figure 4).

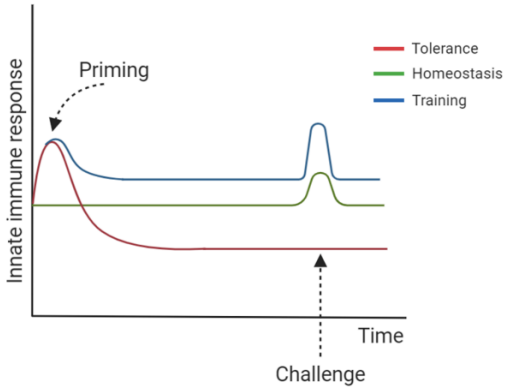


Figure 4: Proposed model of differential programming of innate immunity. Innate immune priming can cause cell reprogramming leading to a reduced (tolerance) or to an enhanced (training) activity. Adapted from[62]

Differently from the classical immune memory, trained immunity involves myeloid, natural killer (NK), and innate lymphoid cells (ILCs) which express specific receptors and cytokynes that are not commonly involved in the classical immunological memory. Another characteristic of innate immune memory is the fact that while the classical immunological memory mechanism relies on the somatic recombination of the loci encoding for immunoglobulins and T-cell receptor, different processes are involved in the innate immune memory, but it seems that the epigenetic reprogramming is most likely the mechanism involved in the enhanced or decreased capacity to respond to certain stimulations. As a result, these modifications persist for shorter periods as compared to those induced from the adaptive immunity[63].

4.2.1 Innate immune memory in vertebrates

In the last decades, a growing number of papers have pointed out that humans and mice show characteristic signs of innate immune memory in vivo. For example, an effect of β-glucan against Staphylococcus Aureus infection through training immunity was already highlighted in the 1970s[64]. Similarly, a stimulation with flagellin induces a cross-protection against Streptococcus Pneumoniae and rotavirus infections[65,66]. There are also pieces of evidence that not only microbial derivatives can trigger innate immune memory, but other molecules like cytokines can also[67]. The specific character of innate immune memory is in strong contrast with the high specificity of the adaptive immune system, suggesting a preference for non-specific innate immune mechanisms.

Other proofs of innate immune memory in vertebrates come from *in vivo* studies: mice primed with attenuated *C. Albicans* strain, PCA2, were protected against a re-infection with the virulent strain CA-6. It is interesting to note that this mechanism was independent of T and B lymphocytes recognition and activation since it was reported in a-thymic and Rag1-deficient mice (lacking B and T lineage)[68,69]. Hints of an innate immunological memory also come from viral infections: J.C. Sun and colleagues in 2009 reported that a challenge with murine CMV induced the activation of Ly49H+ NK cells which persisted in time within different organs. When a CMV re-infection occurred, these primed NK cells were able to rapidly expand, degranulate, and sustain a protective immune response[70]. Evidence arrives also from Herpesvirus, whose latency promotes the production of INFγ from activated Macrophages that in turn induces the resistance to *L. Monocytogenes* and *Y. Pestis*[71].

Another interesting example of innate immune memory comes from studies on the immunization of mice with *bacillus Calmette-Guerin* (BCG), which induces a T cell-independent protection against *C. Albicans* or *Schistosoma Mansoni* re-infection[72,73]. This phenomenon has been observed also in human: BCG was found to enhance circulating monocytes response to *M. tuberculosis, C. Albicans and S. Aureus*, and to markedly enhance macrophages response, which remain in this state for more than three months and returned only partially similar to the baseline 12 months after the immunization[74]. The beneficial effect of the BCG live vaccine is evident not only on the target disease but also on other different infections [75]. This unspecific behavior of protection against so different pathogens suggests the implications of trained immunity in the mechanism of function of BCG vaccine. Another proof for this hypothesis comes from certain malaria infections, which induce a state of hyperresponsiveness compatible with the induction of trained immunity [76,77].

All evidences suggest that priming and training of innate responses is possible, supporting the hypothesis that an innate immunological memory following reinfection exists.

4.2.2 The Endotoxin tolerance

The other side of the coin in this picture is represented by the so-called "Endotoxin tolerance" (ET). ET is a well-known mechanism that dampens the inflammation process to protect the host against a possible endotoxin shock (a pathological state caused by a strong inflammatory response to Gram-negative bacteria, that can lead to multiorgan dysfunction and eventually to death). ET has been described as the process in which cells that have already been exposed to Lipopolysaccharide (LPS) enter a refractory state to this molecule against a second encounter with it. The result is that cells became tolerant to a re-stimulation with LPS, releasing a reduced amount of pro-inflammatory cytokines, while enhancing the production of anti-inflammatory ones[78]. Quite a lot of *in vivo* studies demonstrated that the injection of a first sub-lethal dose of LPS protected mice to a second high and lethal dose of it[79,80]. Both training and ET have been well documented, but the underlying mechanism is still not completely defined. However, it is commonly accepted that the propensity towards one or the other process depends on the nature, duration, and quantity of the first stimulus that the immune cell encounters[78].
This topic will be further described in the next paragraphs.

4.2.3 Mechanisms of innate immune memory

4.2.3.1 Focusing on the molecular mechanism of trained immunity

Trained immune cells are characterized by an enhanced and qualitatively different transcriptional response when sensitized with a first pathological signal. The exact process that orchestrates this phenomenon is still not completely understood and characterized, but evidences are supporting the possibility that different regulatory network, such as chromatin remodeling, micro-RNAs (miRNA) expression and long non-coding RNAs (lncRNA) signaling pathway modulation, can contribute to it in different ways. For instance, the eventuality that epigenetic reprogramming could be at the base of monocytes and macrophages training is commonly believed[63]. Several studies reported that myeloid cells have a repressed configuration of chromatin regions encoding for inflammatory genes, limiting the accessibility to transcription factors (TFs), showing low levels of histone acetylation and no RNA polymerase II (RNA pol II) activity[81–83]. Once a first stimulation occurs, a deep modification of the chromatin structure is induced, thus increasing TFs

accessibility and RNA pol II recruitment that leads to inflammatory genes transcription. The persistence of these chromatin modifications is an open question, but a growing body of proofs supports the hypothesis that they remain persistent on specific promoters allowing a more efficient transcription in case of re-infection[83,84]. An interesting point is highlighted by histone modifications gained by latent or *de novo* enhancers[85], like in the case of human monocytes training with *β-glucan* or *C. Albicans*, where, especially at the level of the promoters of genes associated with immune signaling pathway (e.g. Myd88, Raf1, Dectin-1, Tlr4) and pro-inflammatory cytokines (e.g. TNFα, IL-6, IL-18), it is induced a stable increase in H3K4me3 levels[69]. As a result, trained monocytes showed increased p38 MAPK levels, and it's phosphorylation was quicker upon a second stimulation[69] (Figure 4). Similarly, it was demonstrated that mouse bone marrow-derived macrophages after an LPS stimulation showed a reduction of H3k9me2 levels which maintain high basal levels of some genes targets of the TF ATF7, which in this way contribute to the enhanced *S. Aureus* resistance of LPS primed mice[86]. Also, innate immune memory induced by vaccination is supposed to work in the same way. For example, BCG vaccination of healthy volunteers resulted in an increased response of monocytes to bacterial and fungal pathogens that lasted for at least 3 months, and that relied on increased H3K4me3 levels at cytokine and TLR4 promoters[74].

Despite all these experimental evidences underline the concreteness of these processes, the duration of the trained immunity remains to be clarified once and for all.

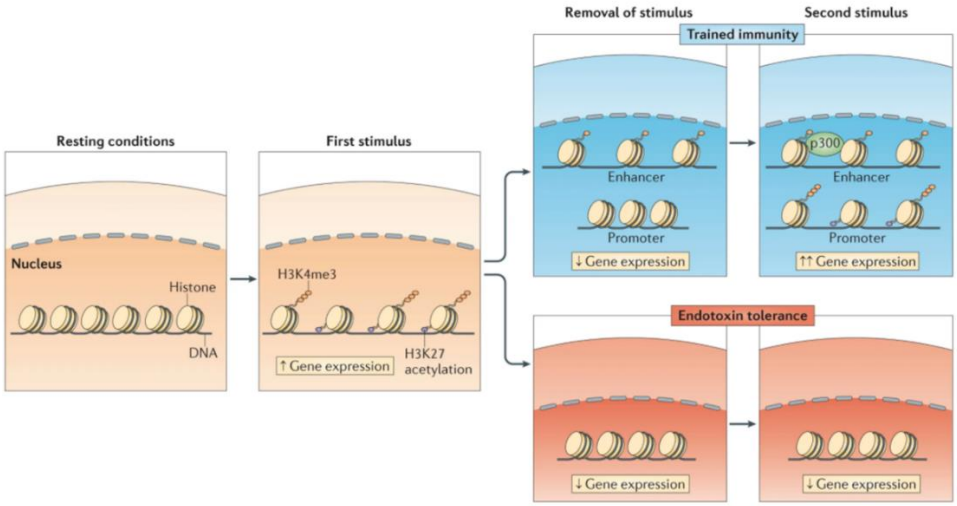


Figure 4: Epigenetic regulation of innate immune memory. A first stimulation causes the acetylation of H4 and H3K4me3 on pro-inflammatory gene promoters. In the case of trained immunity, if activation marks such as H3K4me are retained on those promoters and enhancers, it facilitates the recruitment of the transcription machinery and chromatin modifiers upon a second stimulation. In the case of ET, conversely, after the removal of the stimulus histone modifications are lost and chromatin returns to be poorly accessible. Upon restimulation, genes fail to gain histone activation marks and are not transcribed. Adapted from[87].

4.2.3.2 Focusing on the molecular mechanism of ET

Although it has been more than 60 years since researchers started talking about ET, the precise mechanism that governs it has not yet been fully revealed. As for immune training, it could depend on different processes, among which TLRs signaling rewiring, chromatin modification, gene reprogramming, and miRNA modulations[88].

In vitro studies in mouse macrophages and human monocytes identified a decreased level of TLR4-MyD88 (one of the two TLR4 intracellular adaptors) complex formation, impairment of IRAK-1 activity, and defects of MAPKs and NFkB[89], suggesting a strong implication of intracellular signaling impairment in the ET process. From that, it was demonstrated that LPS treated cells were resistant to a second LPS stimulation due to a reduced ERK1/2, JNK, and p38 phosphorylation at the moment of the second challenge. This was correlated with defective activity of AP-1 and MAPK in tolerogenic cells[90,91].

It is known that the translocation of the heterodimer p65/p50 in the nucleus is a consequence of LPS activation of the canonical NFkB pathway[92]. In this scenario, a great number of works reported that NFkB impairment in ET could be correlated

with reduced IkB-α and IkB-β (well-characterized NFkB inhibitors[93]) degradation after LPS re-stimulation, possibly due to a poor degradation of the inhibitors or to a quick replacement of the molecules[94,95]. Another way in which LPS challenge was reported to impair NFkB activity, is the induction of homodimer p50/p50 rather than heterodimer p65/p50. The homodimer lacks the transactivation domain, and it's in turn unable to bind to gene promoters and therefore to induce canonical NFkB expression genes, which results inhibited[96].
As happens during training immunity, also tolerant cells are subjected to a gene expression reprogramming (Figure 4). Foster and colleagues identified in murine tolerant macrophages two principal classes of modulated genes: one class that he called "tolerizeable" comprise inflammatory genes like IL-6, IL-1 β and Mmp13 that are strongly downregulated upon LPS re-stimulation; in the second class have been grouped genes called "non-tolerizeable" that were upregulated or remained inducible upon LPS re-stimulation, mostly represented by anti-microbial factors. In the same work, Foster demonstrated that even though a first LPS stimulation could induce H3K4 acetylation of both tolerizeable and non-tolerizeable genes promoters, it was prevented to a second stimulation with LPS and that while H3K4me3 was induced on the promoters of both classes of genes after the first challenge, it is rapidly lost on tolerizeable genes promoters, but it is maintained on non-tolerizeable ones[97].
Last but not least, a previous study demonstrated that after LPS priming, RelB, an NFkB subunit, was able to bind to promoter regions of TNFα and IL-1β orchestrating H3K9 di-methylation and silencing genes transcription, thus remodeling the chromatin[98].

4.2.3.3 Innate immune memory tracts in the mast cell

As has been extensively described above, MCs are tissue-resident, long-living, innate immune cells that localize at the interface with the external environment, where they act as "sentinels" of the body. Their intrinsic characteristics make them suitable candidates to benefit from a process such as that of the immune memory. Nevertheless, there are very few studies regarding this aspect of the MCs. In 2010, one work was published where it was shown that, as happens to macrophages, MCs stimulated with LPS entered a phase of unresponsiveness to subsequent stimulation with it, suggesting the possibility that also MCs can build an innate immune memory[99]. More recently, further studies demonstrated MCs' ability to respond to LPS stimulation releasing cytokines like TNFα and IL-6 in a p38 dependent manner. This release was strongly decreased after a re-stimulation with

LPS or with a second challenge with a TLR2 agonist, suggesting the possibility that ET in MCs could induce a cross-tolerance against different stimuli[99,100].

Interestingly, a recent work published by Poplutz and colleagues, demonstrates that MCs ET is characterized by a decreased nuclear translocation and promoter binding of p65/p50 NFkB heterodimer. This was due to the presence of H3k9me3 suppressive marker on TNFα and IL-6 promoters. Albeit H3k9me3 suppressive marker was transiently lost during LPS priming of MC, thus allowing NFkB binding and genes transcription, it remained unchanged during the second LPS stimulation. On the other hand, also IgE-mediated activation of the same NFkB pathway is suppressed in tolerant MCs, but it doesn't affect IL-6 and TNFα production. This phenomenon may be caused by IgE-mediated calcium mobilization and consequent activation of transcription factors. The authors eventually concluded that the whole process seems to be mediated by the IkB-family member BCL3, as BCL3-/- MCs failed to induce ET[101].

However, since MCs are fully equipped with an eclectic variety of receptors, and thus can be directly activated by a plethora of different stimuli, the underlying mechanism of immune memory in MCs need to be further investigated, especially in the optic of regulating their uncontrolled activation in allergic reactions and pathologic conditions like mastocytosis.

4.3 MCS IN THE GASTROINTESTINAL TRACT

The gut is the longest organ in the human body as it measures almost 16 feet,[102]. It's not just the organ where the digestion takes place, but it's more likely a complex environment, where the immune system, nervous system, and commensal microbiota converge and coexist[103]. The first line of defense in the GI is represented by the intestinal epithelium, composed of four cell lineages developed from the same stem progenitor: enterocytes (absorptive activity), Goblet cells (mucus producers), Enteroendocrine cells (Hormone producing cells), and Paneth cells (which produce antimicrobial peptides and niche factors). Then there is the lamina propria where the second line of defense of the GI is located, that is the innate immune system, mainly represented by dendritic cells, macrophages, and MCs. Finally, intermingled with gut epithelial cells, there are intraepithelial lymphocites[102].

In particular, the gastrointestinal tract (GI) comprise the largest population of MCs in the body, as they represent 2-3% of the immune cell pool in the lamina propria, but, as already well known, they are found also in the muscular and serous layers that harbor nerve and sensory fibers[104] (Figure 5). It is common knowledge that MCs exert different roles in the GI tract, from the maintenance of homeostatic conditions to the onset and propagation of different GI diseases like food allergies, parasitosis, and more debilitating diseases which are grouped under the term "Inflammatory Bowel Diseases" or IBDs. This is reflected also by the fact that MCs harbor a vast quantity of different preformed and *de novo* synthesized mediators that can exert a large variety of functions[104].

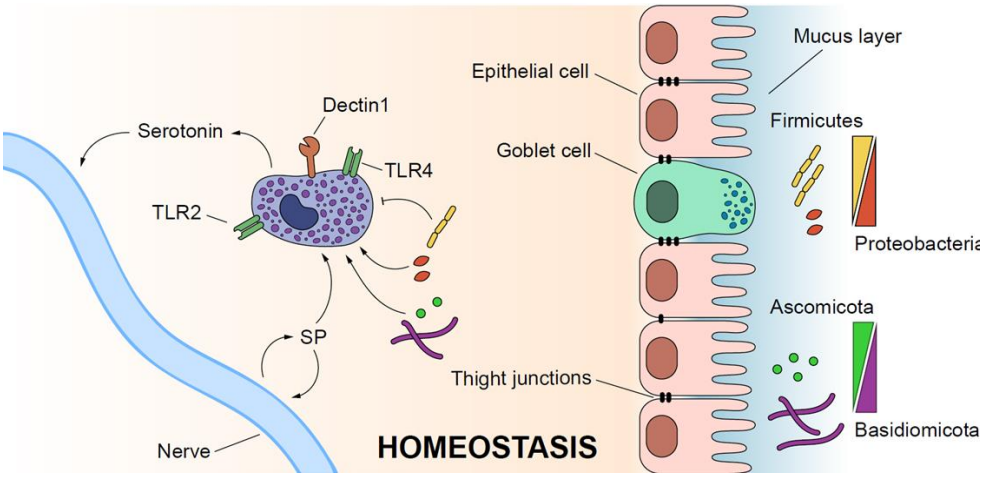


Figure 5: MC–microbiota–intestinal mucosa interconnections in homeostasis. In healthy conditions, the mutual interactions among MCs, local microbiota (bacteria and fungi), and intestinal cells guarantee the correct function of the intestinal barrier and the nervous system. Adapted from[103].

4.3.1 Heterogeneity of intestinal MCs

As discussed in 4.1.2, MCs originates in the bone marrow from a hematopoietic stem cell, which generates the MCs progenitor which in turn leave the bone marrow and enters the blood flow to reach the tissue of selection where it will terminate its maturation and will differentiate into a specific MC subtype[23]. Intestinal homing of immature MCs is in large part mediated by the expression of α4β7 integrin on MCs surface, and its interaction with ICAM-1, V-CAM, or MAdCAM-1 that are expressed on endothelial cells[19], but also by the expression of CXC chemokine receptor-2 (CXCR-2) that is specifically expressed on GI MCs[105]. Then, depending on their final locations, MCs are categorized into two main subpopulations that are connective tissue MC (CTMCs) or mucosal MCs (MMCs)[14] in rodents, and tryptase MC or chymase and tryptase MC in humans[106]. In this scenario, it becomes clear that, depending on the homing site, the same activating stimulus can induce the degranulation of different tissue-specific MCs. They will in turn release a specific set of mediators that will determine MCs' effector function depending on where they terminally differentiated[107], thus affecting also the behavior of the neighboring cells. For example, many studies reported the existence of bi-directional crosstalk between MCs and nerves within the intestinal mucosa, which is of relevance both in the maintenance of homeostatic conditions and in the perception and propagation of the pain during IBDs[108–110].

4.3.2 MCs-gut microbiota cross-talk

The gut microbiota is a set of bacteria, archaea, and eukarya estimated to be more than 10^{14} [111], colonizing the GI tract, that play a crucial role in maintaining intestinal homeostasis both at the immune and metabolic level while protecting the organism from pathogenic infections. Imbalances in the microbiota compositions are related to the onset or exacerbation of GI disorders[112]. In this scenario, a properly functioning immune system is the key to maintaining a healthy and controlled intestinal microenvironment. As MC is a versatile homeostatic cell that lives close to the commensal microbiota, it is easy to argue that also MCs-microbiota interaction can have a relevant role in maintaining intestinal homeostasis.

As can be seen from different studies carried out in recent years, one interesting point is the evidence that intestinal MCs can assist the crosstalk between the microbiota and the adaptive immune system. In 2001 one work was published by Malaviya et. al in which they demonstrated that MCs can phagocyte bacteria[113], while two more recent studies further confirmed this data and highlighted that this process can be mediated by the soluble NSF attachment receptor family protein, the synaptosomal-associated protein 29 and TLR-2[114,115]. Also in human, it has been demonstrated that an ex vivo stimulation with INFγ of human MCs isolated from cytomegalovirus infected patient, induced the expression of human leukocyte antigen complexes (HLA-DR and HLA-DM), and also of CD40 and CD80, thus allowing the uptake, processing, and presentation to CD4+ Th1 cells[116]. Microorganisms are also able to induce MCs activation, with the release of pre-stored or de novo synthesized mediators: it was demonstrated that co-culture with pathogenic microorganisms like *Francisella tularensis*[115], *S. Aureus*[117], *S. Pneumoniae*[118], and *Borrelia burdgorferi*[119] induce MCs to release pro-inflammatory cytokines (e.g. TNFα, IL-6, IL-3, MCP-1) and growth factors (e.g. vascular endothelial growth factor-VEGF-)[120]. The release of mediators can in turn have various effects on microorganisms: it was reported that the release of beta-hexosaminidase inhibits *in vitro* growth of *L. Monocytogenes*, and protects from S. Epidermidis infections *in vivo*[121,122]. Similarly, *Streptococcical* infections is reported to induce chymase and granzyme D from peritoneal MCs[123], and it was demonstrated that also the release of MCPT4 has a protective role against infections caused by a specific group of Streptococci since it was seen that it can disrupt bacterial-extracellular matrix interaction[124]. All these data support the anti-inflammatory and protective role of MCs in the field of microbial infections, as the fact that mouse and human chymases are described to disrupt and degrade several alarmins like Hsp70, IL-33, HMGB1, and biglycan that are released after *Trichinella Spiralis* infections[125]. However, this is a controversial point, since other works demonstrated how MCs were indeed dispensable for inflammatory

resolution during intraperitoneal *S. Aureus* infection or pneumococcal central nervous system infection *in vivo* since both wild type and MCs-deficient mice had no differences in bacterial clearance nor inflammatory state[117,118].

GI microbiota is rich in fungal species, as 66 genera of fungi have been identified in the so-called "microbiome", among which S. Cerevisiae, *C. Albicans*, and Cladosporidium are the most abundant ones[126]. Despite fungal species abundance in the GI tract, the literature on MCs-fungi is mostly restricted to MCs interactions with *C. Albicans*. Two studies from Trevisan et al[127] and Pinke et al[128] independently demonstrated that both rat peritoneal cavity-derived MCs (rPCMCs) and BMMCs can engulf inactivated *C. Albicans* via a mechanism that involved both TLR2 and dectin-1 (a PRR that plays an important role in antifungal innate immunity. It is a specific receptor for β-glucan mainly expressed on phagocytes)[129] which resulted in the release of reactive oxygen species (ROS)[128]. Trevisan and colleagues, in particular, pointed out the incapacity of BMMCs to kill phagocytosed yeast, while they efficiently killed the unopsonized one in the extracellular environment. The fungal challenge of BMMCs and rPCMCs also results in their degranulation and consequent activation of the NFkB pathway which in turn induces the release of TNFα, IL-1β, IL-6, IL-10, CCL3, and CCL-4[130]. This happens not only in rodents but also in humans: Lopes et al indeed demonstrated that human MC line HMC-1 underwent degranulation after *C. Albicans* stimulation. Moreover, it was shown that MCs could release MC extracellular traps, also known as "MCETs" (filiform structures of decondensed DNA in which are found active proteins from the cytoplasmic granule. They are typically found in neutrophils[131]), and kill C.Albicans in early stages of infection[132].

Microbial effects are not restricted to the activation of MCs but an increasing body of proofs started to report that they are also involved in the silencing of MCs response. One side of the story is related to pathogenic bacteria that limits MCs activation to easily evade immune response in rodents (*Y. Pestis* and *S. Typhimurium)* and in humans (*S. Typhimurium)*[133]. On the other side, commensal microbes limits MCs uncontrolled activation, resulting in an improvement of the pathology and a reduced MCs infiltration and IgE serum levels [134]. That's the case of *L. Paracaseii* and non-pathogenic *E. Coli* which have been reported to suppress murine MCs degranulation after the stimulation of IgE/Ag pathway, for example.[135,136]In this scenario, a growing interest have recently been reported on microbiota-derived metabolites like short-chain fatty acids (SCFAs) which have been proved to have different immunomodulatory effects in the GI tract[137,138]. [139] SCFAs as Sodium Butyrate, Acetate, and Propionate are considered to be beneficial to host cells, thus improving differentiation, barrier function and epithelial defense.[140] Moreover, a recent study published by Folkerts and colleagues, demonstrated that SCFAs can inhibit IgE- and non-IgE-dependent

degranulation of MCs in a concentration-dependent manner, thus highlighting a health benefit of SCFAs in allergic diseases.[141]

4.3.3 MCs in gut diseases

Dynamic interactions between epithelial cells, immune cells, and symbiotic microbiota are crucial for the maintenance of gut barrier homeostasis. Disruption of this tightly regulated balance can have major local and systemic health consequences, ranging from inflammatory bowel diseases (IBDs) to cancer[142,143]. As a matter of fact, several studies started to report that many human pathologies are related to the so-called "dysbiosis", an imbalance in the microbiota composition. A clear example of this phenomenon is provided by IBDs, in which patients tend to show a reduction in gut microbiota diversity, with an imbalance of the bacterial composition in favor of *Proteobacteria* phylum instead of the family of *Firmicutes*[143–145].
In this field, even though MCs have always been associated with allergic reactions, they are mediators of inflammation also at the intestinal level, where they not only are key effectors of allergic inflammation but are also involved in many physiological functions[23,106]. As a result, MCs in the intestine can regulate blood flow, peristalsis, mucosal secretion and mediates the crosstalk with adaptive immune cells[106,146]. It is therefore easy to deduce why MCs potentially have a role in gastrointestinal pathologies up to colorectal cancer.

4.3.3.1 MCs and IBDs

IBDs are a group of chronic inflammatory diseases of the gastrointestinal tract which include the two major and best known chronic inflammatory diseases: Crohn's disease (CD), which preferentially interests the ileum, but can affect any part of the gut, and ulcerative colitis (UC), that typically can affect all the colonic area, from the caecum to the rectum[147].
In a study published in 1996, Stephan Bischoff et al demonstrated that there wasn't a difference in MC number in histological staining of controls as compared to IBD patients, but this was since MCs underwent massive degranulation[148]. Moreover, Gelbmann et al reported that in Crohn's disease patients, MCs number was increased at fibrotic tissue sites, suggesting an MCs role in promoting fibrotic tissue generation[149]. Interestingly, not only degranulating MCs were found in ileum and colon of IBDs patients, but it was also detected an altered expression level of

pro-inflammatory mediators such as TNFα and IL-6 and of the mediators such as substance P, histamine, and tryptase in the mucosa[150].

With specific regards to Crohn's disease, Stoyanova and colleagues showed that tryptase-, substance P- and serotonin-positive MCs were located close to the basal lamina, among the epithelial cells in the gut of patients[151]. However, although all these observations may suggest a possible role of MCs in IBDs, this evidence is indirect, since triggers of MCs activation are still largely unknown. To date, there are no pieces of evidence for IgE-dependent MCs activation in IBDs onset, but there are some IgE-independent mechanisms that have been proposed to possibly be involved in the outbreak of these pathologies. One example is represented by Ig-free chains that have been demonstrated to play a role in MCs induced colitis in mice and possibly also in human IBDs[152]. Then, Kobayashi et al reported an hyperexpression of Fcγ receptor and TLR-4 on GI MCs of CD patients, highlighting that this could be involved in IBDs onset, and suggesting a possible role of bacterial products like LPS in triggering MCs activation[153]. Also, the intracellular bacterial receptor NOD2 was found to be overexpressed in MCs of active CD patients, suggesting a possible involvement of this receptor in MCs activation or other inflammatory cell recruitment[154]. All these data suggest that an altered gut function in IBDs context can be the consequence of tissue-resident MCs hyper-activation that can be caused either by a reduced GI barrier function, which allows for an increased translocation of bacterial derivatives that, in turn, can trigger MCs activation, or, ultimately, by a microbiota dysbiosis. Therefore, it is reasonable to assess that the use of MCs-stabilizing drugs can improve IBDs outcome.

4.3.3.2 MCs in Inflammatory bowel syndromes (IBS)

Despite IBS and IBD symptoms are for the most part overlapping (mainly abdominal pain and diarrhea), IBS is functional GI disorders that don't have specific diagnostic biomarkers[155]. Several studies have reported conflicting data regarding the role of the immune system, especially MCs, in the onset of the disease, nevertheless, most of the time they agree that this pathology is characterized by altered intestinal barrier marked by tight junctions disruption and increased permeability[156–158]. Since several works demonstrated that MCs tryptase might be implicated in this phenomenon and even more in the generation of GI motility dysfunctions and visceral pain in IBS, it is more likely that the grade of MCs activation is of greater importance than MCs number in the induction of clinical IBS symptoms[159,160]. In support of MCs implication in IBS, there are a lot of studies that demonstrated an improvement in IBS symptoms after administration of different MCs stabilizers like disodium cromoglycate or ketotifen.[161,162]

4.3.3.3 MC in colorectal cancer (CRC)

Colorectal cancer (CRC) incidence widely varies around the world, but it's the third most common tumor in males and the second in females according to the World Health Organization (WHO) databases. Two broad categories of CRCs can be distinguished: the hereditary CRC, characterized by adenomatous polyposis coli (APC) gene loss of function, and the sporadic CRC, mostly associated with IBD.
As it is well known, the long exposure of the GI epithelium to the pro-inflammatory microenvironment that is characteristic of the IBD pathological framework is associated with an increased risk of developing colorectal cancer (CRC). [163] In this field, the importance of lamina propria (LP) residing innate immune cells in CRC development became evident from studies conducted in $Apc^{min/+}$ mice (a mouse model that spontaneously develop intestinal adenomas) crossed with Myd88 ko mice (which have hematopoietic system defects and therefore develop immune system abnormalities). The resulting mice showed adenoma size and number significantly reduced[164]. The presence of tumor-associated inflammation is reported to be a marker of cancer progression[165], and likewise, MCs have been reported to infiltrate and accumulate in the mucosa of the inflamed intestine during tumor progression. However, the fact that MCs could have a role in CRC formation or progression is still a matter of conflict, even though there are some evidences in support of this theory[166]. For example, in 2013 Malfettone et al showed a high presence of tryptase positive MCs at the interface between rising cancer and the healthy tissue, mostly in association with blood vessels in the tumor microenvironment (TME) in human CRC tissue samples. This localization of MCs was reported to be a poor prognostic marker.[167] One possible reason for this MCs accumulation at the tumor site, could be the production of SCF from the tumor itself, which not only attracts MCs but is also able to sustain their activation and consequent release of different mediators that could eventually be responsible for different events (e.g. increased permeability, immune cells chemotaxis, extracellular matrix degradation).[168,169] Rigoni et al reported a higher MCs infiltration in most aggressive tumors developed by AOM/DSS models.[170] High MCs and MCs precursors density is also associated with CRC prognosis since these cells promote cancer progression sustaining inflammation: MCs seems to be of relevance during polyp development both in humans and in APC mutated mice models, where the autocrine production of TNFα by MCs was identified to be triggered by the tumor growth and sustain MCs proliferation.[171] Direct interactions between MCs and cancer cells have also been described: MCs was shown to release specific mediators utilizing a so-called "piecemeal degranulation" , that promoted cancer cell proliferation and invasion both *in vivo* and *in vitro*. One example is reported by a study conducted by Wang S. et al, where they showed

that during cell-to-cell interaction, MCs can induce an increased expression of MAPK, Rho-GTPase, and STAT pathways and vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGFβ) in CT26 cell line, thus influencing tumor cells proliferation and survival.[172] However, as previously mentioned, there are conflicting theories on the precise role of MCs in colon cancer. For instance, Sinnamon et al in 2008 described how MCs show a protective role in an early stage of intestinal tumorigenesis in $Apc^{min/+}$ mouse model.[173] Also Mehdawi and colleagues further investigated the protective role of MCs in human CRC and reported that a higher MC density was associated with longer overall survival in a cohort of patients.[174]

Even though the evidence of the role of MCs in CRC onset and progression is substantial, more work is required to shed light on this events. In this scenario, better and more informative models to study CRC progression could be useful to unravel the role of these particular cells.

4.4 ORGANOID TECHNOLOGY: A BREAKTHROUGH

Organoids are 3D culture systems derived from stem-cells that are able to recapitulate *in vitro* the architecture and functionality of the tissue of origin, which maintain also the same genetic background[175,176]. This is possible in the total absence of mesenchymal cells, and just by culturing them in presence of well-defined niche factors. In the past decades, great advancements have been made in this field, and organoid technology has been exploited for the study of tissue development, genetic hereditary disorders, and also cancer.[175,177–179] In this paragraph will describe the origin of organoid technology and the state of the art on its use in modeling tissue development and in the study of the pathophysiology of several GI disorders.

4.4.1 Historical background

The capacity of the mammalian stem-cells to self-organize into 3D structures was not unknown to scientists, who as early as 1946 began to use the term "organoid" to describe a 3D growing structure of cystic teratoma[180]. However, the real turning point came in 2009, when Toshiro Sato and Hans Clevers demonstrated the presence of $Lgr5^+$ (leucine-rich repeat-containing G-protein-coupled receptor 5) stem cells at the bottom of mice small intestinal crypts, from which they were able to establish a long-term culture of villus-like organoid structures that differentiated all the intestinal cell types. Sato T. also demonstrated that this was possible also in the absence of a non-epithelial cellular niche, but the right combination of growth factors was sufficient to sustain the self-organizing structures. [181] The growing conditions required an extracellular matrix, the *Matrigel*, that allowed the tri-dimensional growth of the organoids, and a complex medium supplemented with some key niche-factors, namely: WNT3a (a frizzled lipoprotein receptor-related protein-ligand), Noggin (a bone morphogenic protein inhibitor) and R-spondin (an Lgr4/5 ligand, and WNT3a agonist) which mainly allow for the stem cell expansion and maintenance, and EGF (epidermal growth factor) that promote the cell proliferation[181]. Later on, this innovative and promising system was adapted for the generation of several other 3D cultures from other murine organs like colon, stomach, liver, brain, kidney, prostate and pancreas, and ultimately from human tissues.[182–188]

Organoids can be generated from both the two main types of stem-cells: the induced pluripotent stem cells (iPSCs), which can avoid the problem coming from the poor quantity and quality of starting tissue (especially for human samples) and the adult stem cells (ASCs). both iPSCs and ASCs can establish long-term 3D organoid cultures, the difference is given by the fact that ASCs are organ-restricted and can give rise to only one organoid type (e.g. $Lgr5^+$ stem cells of the small intestinal crypt), while from the same iPSCs different organoids can be generated

just by using the right cocktail of growth factors (Figure 6). iPSCs derived organoid cultures require a strong knowledge of the factors involved in the lineage-specific differentiation, which could make it more difficult to obtain the required culture in a short time. [175,178]

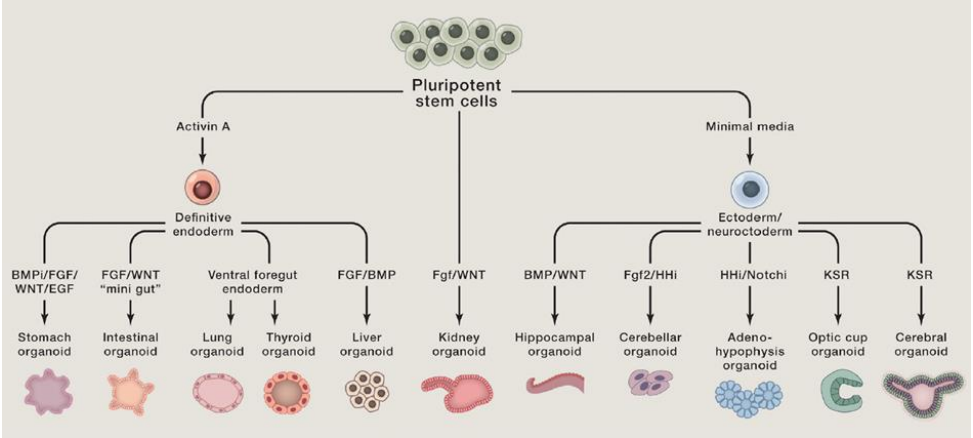


Figure 5: iPSCs derived organoids: schematic representation of different organoids generation starting from iPSCs, and developmental signals and growth factors required. Adapted from.[189]

4.4.2 Adult stem cells derived organoids

As mentioned above, the major difference between iPSCs- and ASC derived organoids is the fact that the formers exploit the developmental process for their establishment, while the latter needs the *in vitro* reproduction of the stem-cell niche to be fully generated (Figure 6).[172] In this framework, as first described in intestinal stem cells, in particular in the colon, the growth factor Wnt was found to be the most important driver and supporter of ASCs.[190] Thus, the addition of Wnt or any other Wnt pathway amplifier (like the GSK3β inhibitor, CHIR) is fundamental for successful long-term maintenance and expansion of $Lgr5^+$ stem cells derived organoid cultures. As a result, the withdrawal of Wnt results in the differentiation of the cultures.[184,191] Modifications of the medium supplemented with the growth factors that are necessary for the establishment of intestinal organoids, allowing for the generation of cultures from several other GI and non-GI tissues (Figure 6). One interesting example is represented by adult liver stem cells: the stem cell pool is reactivated after an injury of the tissue, and once fragmented and seeded in matrigel, liver bile duct fragments in presence of EGF, R-spondin, HGF (hepatocyte growth factor), FGF10 (fibroblast growth factor 10) and nicotinamide, were able to generate the so-called "cystic" (due to their swollen and hollow structure) completely stem organoid culture. After the inhibition of TGFβ and Notch signaling pathway, the culture is committed towards the hepatocyte lineage

differentiation.[184] Another example comes from adult pancreatic duct fragmented tissue, which was observed to generate cystic organoids when cultured in presence of EGF, R-spondin, FGF10, and nicotinamide. These organoids, once engrafted under the mouse kidney capsule, resulted in the final differentiation of functional pancreatic tissue, highlighting that stem cells reside in the adult pancreatic ductal compartment.[192] All these studies carried out on rodents, finally found their counterpart also in humans: indeed, in 2011 Toshiro Sato and co-workers were able to successfully develop long-term expanding organoids from both healthy and diseased tissue (from the small intestine, colon, adenoma, adenocarcinoma and Barrett's epithelium).[182] Since then, many others succeeded in the establishment of their human organoid cultures.[193–195] This opened the way to the formation of biobanks in which to store organoid cultures derived from healthy and pathological patient tissue, which represent a sort of avatar that can be preserved for a long time to evaluate their genomic stability, the similarity with the tissue of origin and above all the susceptibility to different pharmacological treatments.[196–198]

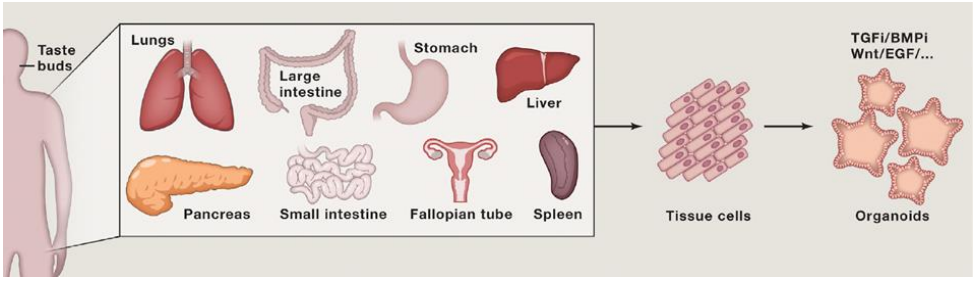


Figure 6: ASC derived organoids: schematic rappresentation of different organs from which it's possible to establish ASCs derived organoids. Adapted from.[175]

4.4.2.1 ASC-derived organoids: focusing on small intestine and colon

The GI tract is characterized by a fast epithelial turnover (3-5 days)[199], driven by the proliferation of $Lgr5^+$ stem cells residing at the crypt base[200], where they divide and move along the villus structure. Once reached the top of the villus, the epithelial cell dies and is finally released in the intestinal lumen. In intestinal organoid cultures all the differentiated cell types that are found in the tissue of origin are represented, thus absorptive enterocytes and secretory cell types like Paneth cells, goblet cells, enteroendocrine cells, and tuft cells are included[201].
As previously anticipated, once isolated and seeded in matrigel, intestinal stem cells alone (or whole crypts) needs the presence of four principal growth factors to be able to proliferate and survive for a long period. Those are Wnt, the key and most abundant mediator, Notch, that maintains the undifferentiated state of stem cells and drives the differentiation of secretory lineage once inhibited EGF, and Noggin[201]. The combination of factors that are required for the establishment and maintenance of colon and small intestinal organoids are almost the same, except

for Wnt that is not strongly required for the small intestinal crypt culture production, but it is fundamental for the colon[202]. Once seeded and cultured in presence of the required mediators, intestinal crypts can proliferate and close up to eventually form a polarized epithelium around a central lumen. In this framework, all the cell types that compose the intestinal epithelium *in vivo* are present, functional, and represented in normal ratios[181,203]. Finally, once established, intestinal organoid cultures can be passaged once a week, freeze, thaw, and remain stable both genetically and phenotypically [204].

4.4.3 Applications of the organoid technology

The major advantage of the organoid system is the possibility to obtain a stable and tending to an infinite source of patient's tissue, even when the amount of starting material is limited like often happens while working with biopsies. Organoids technology is perfectly placed between the classic 2D cell cultures *in vitro* and the studies conducted *in vivo*[202]. A great variety of essays can be carried out, from the most basic to the more complex ones, thus constituting an important window on a series of transversal studies that could allow deepening in more detail some pathological aspects that are currently difficult to understand due to the lack of sufficiently realistic *in vitro* models. Furthermore, the intrinsic characteristics of the organoid make it a much more physiologically relevant model than traditional ones, since organoids are made up of several physiologically functional cell types at the same time, and can be further engineered, modified, or implemented (immune system, inflammatory cells, microbiota, nervous system) at any time.[205] The added benefit of this system is the possibility of reconstituting the immune and inflammatory microenvironment to recreate a more physiological scenario. In this section, some outstanding organoids applications will be described.

4.4.3.1 Organoids in immunological research

As compared to other systems, organoids technology offers various advantages in the study of tissues microenvironment, which is characterized by direct and indirect interactions between epithelial cells, and non-epithelial inflammatory, immune, nervous cells and also the microbiota[205]. The cellular heterogeneity that characterizes the organoids makes this system much more reliable than that offered by the classic 2D monolayer culture. Indeed, ASCs derived organoids express all the epithelial cell types that are present in the tissue of origin in a way that is both phenotypically and genetically stable[202]. These organoids feature to allow for various experimental essays, and among them enables also the study of the crosstalk between epithelial cells and immune cells. In this scenario, organoids derived from tumoral tissue or inflammatory bowel disease samples can help to unravel the role of the immune system with a simple and reductionist approach.

Four main co-culture strategies are usually exploited to study organoid-other cell type interaction (figure 7), depending on what type of cell and interaction we want to analyze. To study the interaction of Intraepithelial lymphocytes (IELs) and intestinal organoids, for example, organoids are typically mildly desegregated and mixed with the immune population of interest; this helps the interaction between the two elements that will be then re-suspended in matrigel and plated in presence of all the required growth factors[206–208] (Figure 7a). This strategy is not completely useful to study all immune cell populations like for example circulating lymphocytes or other tissue-resident cells that don't reside at the intraepithelial level (like for example macrophages, NK cells, and also MCs). In this case, the population of interest is re-suspended in the organoid medium and placed over the organoids growing inside the matrigel, rather than mixed with them[209,210] (Figure 7b). In this way, immune cells can exert a direct effect by reaching organoids and directly interact with them, and indirectly by releasing pre-stored mediators. For instance, Dijkstra et al in 2018 demonstrated with this co-culture strategy the tumor-specific killing capacity of circulating peripheral blood lymphocytes against CRC[208]. The third generally known, and more complex, co-culture strategy makes use of the microinjector and is typically used to study the interaction of the microbiota (if present) with epithelial cells[176,211,212] (Figure 7c). This method has been used by several groups to study for example the response of the epithelium to pathogenic infections. Holokai and colleagues for example microinjected H. Pylori (Gram-negative bacterium) inside the lumen of iPSC-derived gastric organoids, where it was able to associate with epithelium and exerts all the effects that usually drives *in vivo*[212]. The fourth and last most commonly used way of co-culture organoids enables the simultaneous culture of up to three cell types in one dish. This is made possible by the use of trans-wells: organoids can be finely desegregated and seeded over the trans-well membrane where they will form a 2D polarized monolayer. In this way, the upper chamber (the apical side) can be reconstituted with elements of microbiota, and the lower chamber (basolateral side) can host the immune population of interest[213]. This co-culture system seems to be a promising substitute of the classical 2D monolayers derived from cell lines, and allows the study of an even more complex and physiologically realistic aspect of the intestinal microenvironment (Figure 7d).

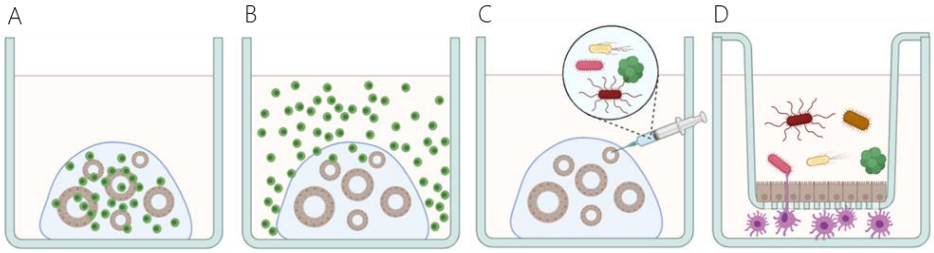


Figure 7: Schematic view of commonly used co-culture strategies: A. organoids and other cell types are cultured together inside the matrigel; **B**. organoids are cultured alone inside the matrigel while other cell types ar resuspended in the correct medium to be placed over the matrigel dome; **C.** microorganisms are microinjected inside the organoids' lumen; **D.** monolayers grown on trasnwell starting from organoids' epithelial cells, organize themselves into a luminal side and a basolateral side; this can allow the simultaneous co-culture of more than two different cell types that usually reside in different intestinal compartments (e.g. immune cells and microorganisms)

4.4.3.2 Organoids in the study of homeostatic development

As outlined in the first section of this thesis, the interaction of immune cells with epithelial cells is often needed for immune cell development, but it is also sometimes involved in the homeostatic development of border tissues like the skin and the intestine[214]. A demonstration of that comes from several studies showing that immune cell-derived cytokines and microbiota compounds can influence intestinal epithelium development. M cells, for example, that are found in the epithelium associated with the mucosa-associated lymphoid tissues (MALT) have an important role in intestinal immune responses, since it is reported that mice lacking M cells are prone to develop dysbiosis with a concomitant reduced capacity of IgA secretion[215,216]. M cell differentiation occurs only upon stimulation with the receptor activator of nuclear factor-kB ligand (RANKL) that is expressed on adjacent stromal cells like fibroblasts but also immune cells[217]. Also, immune cell-derived cytokines are involved in driving epithelial differentiation: for example, the IL-13 produced by group 2 innate lymphoid cells (ILC2) is reported to induce goblet cells (mucus-producing cells) differentiation. This is in turn due to the epithelium production of IL-33 that stimulates the ILC2 to produce IL-13[218]. IL-13 together with IL-4 was also reported to induce Tuft cells differentiation, chemosensory cells that in the intestine have been shown to act as immune sentinels for parasite infections[219–221]. In general, the culture of organoids in presence of IL-4 or IL-13, or their co-culture with activated T helper 2 cells (Th2), is associated with a decreased intestinal stem cell numbers, and the same happens in presence of pro-inflammatory cytokines like INF-γ and IL-17, or presence of inflammatory Th1 cells [217]. The opposite effect is obtained by the co-culture of organoids with anti-inflammatory cells or cytokines like IL-10 or IL-22, which eventually support epithelial regeneration upon intestinal damage[217,222].

4.4.3.3 Organoids in the study of IBDs

As now it is well known and documented, inflammation does not only contribute to the maintenance of the tumor microenvironment but in some cases, it could also be the triggering cause[223,224]. Compared to the other hallmarks of cancer, inflammation as a triggering cause of the tumor is generally little studied, probably because it is difficult to cultivate cells that are not completely mutated, in the perspective of classical 2D culture. With the advent of organoid technology, it has become much easier to study these events (and many others too), thanks to the fact that organoids can maintain *in vitro* the pathological signature of the tissue of origin and thus several types of research regarding different organs like pancreas, kidney, colorectal, and brain are already in progress[202,225,226]. In the past years, several studies have been performed in mice ASC-derived organoids to elucidate epithelial biology in an IBD background. One example comes from a work of Günther and colleagues who demonstrated that Caspase-8 could be involved in IBDs barrier dysfunction since mice deficient in Caspase-8 had a reduced number of Paneth cells and developed spontaneous ileitis. Establishing organoid cultures from both controls and Caspase-8 deficient mice they were able to demonstrate that after TNFα treatment, the organoids of Caspase-8-deficient mice underwent necrosis thus highlighting a protective role of Caspase-8 in IBD context[227]. Barrier dysfunctions were found also in organoids lacking NOD2 (nucleotide-binding oligomerization domain-2) a gene that is a major susceptibility trait for IBD[228]. Further studies have been conducted also in human-derived organoids: Dotti et al, and Pan Xu et al both demonstrated that epithelial organoids cultures (EpOCs) generated from patients with UC are possible and they agree that IBD patients derived organoids are a valuable tool for the study of pathophysiological mechanisms and barrier function defects of IBDs[229,230]. Moreover, Dotti and colleagues demonstrated that IBD patients derived organoids showed a high rate of concordance with the tissue of origin in the expression profile of different genes (among which those implicated in the antimicrobial defense like LYZ, and with secretory or absorptive functions like CLCA1 and MUC12 respectively) thus highlighting that the pathologic signature of IBD is stable[229]. All these studies underline how the organoid system can be used to unravel the pathophysiology of complex diseases.

4.4.3.4 Organoids in cancer research

After the publication of the protocols provided by Clever's group for the generation of organoids from healthy tissue, it was quickly understood that a few modifications to the original protocol were enough to produce organoids from tumoral tissue. Since then, various patient-derived organoids have been established from colon[231], brain[232], gastric tract[233], pancreas[234], prostate[187], liver[235], breast[197], oesophagus[236], kidney[237] and lungs[238]cancer tissues, which all perfectly

resembled both phenotypically and genetically the tissue of origin. Normally, organoid cultures are produced from both tumor tissue and the adjacent healthy tissue. Subsequently, they are placed in the selective medium for tumor organoids so that if in the cultures derived from healthy tissue there are any mutated cells, they are identified and avoided: for example, organoid cultures derived from colon cancers, that are often characterized by mutations in Wnt pathway, are perfectly able to grow in the absence of Wnt (and sometimes R-spondin). Wnt is also removed from the healthy tissue-derived organoid medium to see if there is any unexpected tumor cell growth. Once the cultures are confirmed as tumoral or healthy and stabilized, they can be freeze and stored in biobanks. To date, a large collection of organoids derived from healthy and tumor tissues has been generated which has begun to be used to test the efficacy, and possible adverse effects, of various anticancer therapies in development, both on tumor organoids and on the respective healthy counterpart[197,239–243].

Tumoral organoids can also be developed through the genetic-engineering of wild-type stem cells. Using for example shRNAs (short hairpins RNAs) strategies it was possible to develop tumoral-like organoids that once transplanted in mice showed to be invasive and metastatic-prone[244]. Similarly, using the shRNAs system It has been reproduced the transformation of healthy tissue to an invasive adenocarcinoma introducing the progressive silencing of APC, p53, K-ras, and SMAD4 genes[245]. A similar result has been reached also using CRISPR/CAS9 technology: two independent studies reported the possibility of reproducing the adenoma to carcinoma sequence by introducing sequential mutations in healthy human colon stem cells by means of this technique[195,246].

Taken together, these studies underline how organoid technology has marked a great step forward in the study of the pathophysiology of various diseases, and how it can be of great help in the study of personalized medicine.

5 AIM OF THE THESIS

MCs are long-living and heterogeneous innate immune cells widely distributed in almost all mucosal and connective tissues, in close contact with blood vessels and the external environment. Interestingly, each specific tissue has a distinct set of MCs, whose heterogeneity could depend on different factors such as quality, quantity, and combination of activating stimuli. They serve as the first line of defence against antigens entering the body: activation of MCs significantly modulates many aspects of physiological and pathological conditions in various settings. In particular, several works reported that MCs accumulate in the intestine of patients suffering from IBD and CRC where they can be activated and affect the integrity of the tissue, thus aggravating the disease. However, evidence of their direct effect is still missing.
In this framework my Ph.D. thesis is divided into two main parts and aims to dissect two main objectives:

i) to understand the long term effect of different stimulations on MCs development and response to a second encounter with different microbial compounds.
ii) to study the bidirectional cross-talk between terminally differentiated MCs and intestinal microenvironment, both in healthy and pathological settings.

The first aim is based on the evidence that increasing body of proof reports that MCs show classical traits of immunological memory. This aspect is of great importance since MCs are long-living, innate immune cells whose phenotype and homing are strongly influenced by the priming they receive. To this end, different inflammatory and infective stimuli were used to unravel MCs memory.

For the second purpose, organoids from healthy colon and small intestine, as well as organoids derived from different pathological tissues, have been exploited: co-cultures with resting and activated MCs have been set up. The effect of MCs on organoid architecture as well as the effect of different organoids on MCs phenotype and reactivity will be addressed.

Finally, a connection between the first and second part of the thesis will be considered by investigating whether microbial components could modulate the effect that MCs exert on organoids architecture. Therefore, the overall purpose of this Ph.D. thesis is to study what underlies the marked heterogeneity of these cells, to finally assess the plasticity of MCs response to different stimulatory environments.

6 RESULTS

6.1 Part 1

6.1.1 Mast cell priming affects their response to a second stimulation

As MCs are located in sites where their encounter with different stimuli is particularly probable and given the evidence that their terminal differentiation is strongly influenced by tissue-specific stimuli[14,104], we decided to investigate the role of microbial ligands in the modulation of MCs differentiation and response to inflammatory stimuli. Different microbial ligands are known to be involved in innate immune memory: in particular, β-glucan is able to induce trained immunity, while LPS is known to be involved in the endotoxin tolerance[58,91]. To date, these experiments have only been performed in the short-time experiment, where cells were immediately restimulated after the first stimulation leaving doubts about the duration of the effect seen.[247–249] To assess whether long-lasting effects of trained immunity or tolerance could be induced in MCs, BMMCs were differentiated for 6 weeks in IL-3 enriched media and primed for 24 hours with LPS, Curdlan (a dectin-1 agonist consisting of *b*-(1,3)-linked glucose residues), IgE/Ag, NaB (Sodium Butyrate, short-chain fatty acid, SCAFs) or left untreated. After the priming, MCs were washed and put back in culture in fresh IL-3 enriched media for one week. Each primed subset was then restimulated with a 10-fold lower dose of LPS, live *C. Albicans* yeast, Curdlan or IgE/Ag(Figure 8 A).

6.1.1.1 MCs priming affects their response to secondary inflammatory stimuli

As demonstrated by figure 8 B, LPS, Curdlan, or IgE/Ag MC priming didn't affect their capability of degranulation in response to both IgE/Ag stimulation and to the Ca^{++} ionophore ionomycin. On the contrary, in line with the literature, NaB prestimulation strongly impaired MCs degranulation capabilities. The suppressive effect of NaB on MCs degranulation is rapid since it is evident also in MCs that have been treated with or without NaB and immediately induced to degranulate (Figure 8 B and C).

In agreement with the literature, an LPS priming of MCs impaired their response to a secondary challenge with a low dose of LPS. It is demonstrated by the reduced release of both pro-inflammatory cytokines TNFα and IL-6 after 24 hours of restimulation (Figure 8 D). On the contrary, priming with IgE /Ag or Curdlan didn't affect MCs' responsiveness to a second LPS challenge. Interestingly, Sodium Butyrate pre-stimulation strongly impaired TNFα release in LPS re-stimulated MCs (Figure 8 D).

To assess if LPS priming could also be involved in a cross-tolerance against stimulations other than TLR4 mediated, BMMCs were restimulated with live *C. Albicans* yeast, Curdlan, and IgE/Ag. Strikingly, LPS priming induced an increased response to *C. Albicans*, significantly enhancing TNFα but not IL-6 release after 24 hours of stimulation (Figure 8 E). On the other hand, priming with IgE /Ag, Curdlan

or NaB didn't affect MCs' responsiveness to a live C. Albicans restimulation (Figure 8 E). In our system, Curdlan stimulation didn't exert any relevant training or tolerance effect, both as a priming stimulus and as a second stimulation (Figure 8 F). The response to IgE/Ag restimulation was also comparable between all the stimulatory groups in terms of both the release of TNFα and IL-6, except for Sodium Butyrate pre-stimulation that impaired TNFα, and to a lesser extent also IL-6, release after IgE/Ag challenge (Figure 8G).

Collectively, these data suggest that LPS pre-stimulation induces a long-term memory also in MCs. In particular, the dual role of LPS in the induction of both tolerance and training mostly in the context of TNFα release, suggest that this mechanism could depend on a multi-layered process of regulation that is dependent on the secondary stimulus, rather than relying exclusively on epigenetic reprogramming (as described for Macrophages). Moreover, data deriving from NaB treatment, suggest that MCs activation after different inflammatory challenges is rapidly reduced after a short time stimulation with this SCFA, but that it is maintained for longer periods.

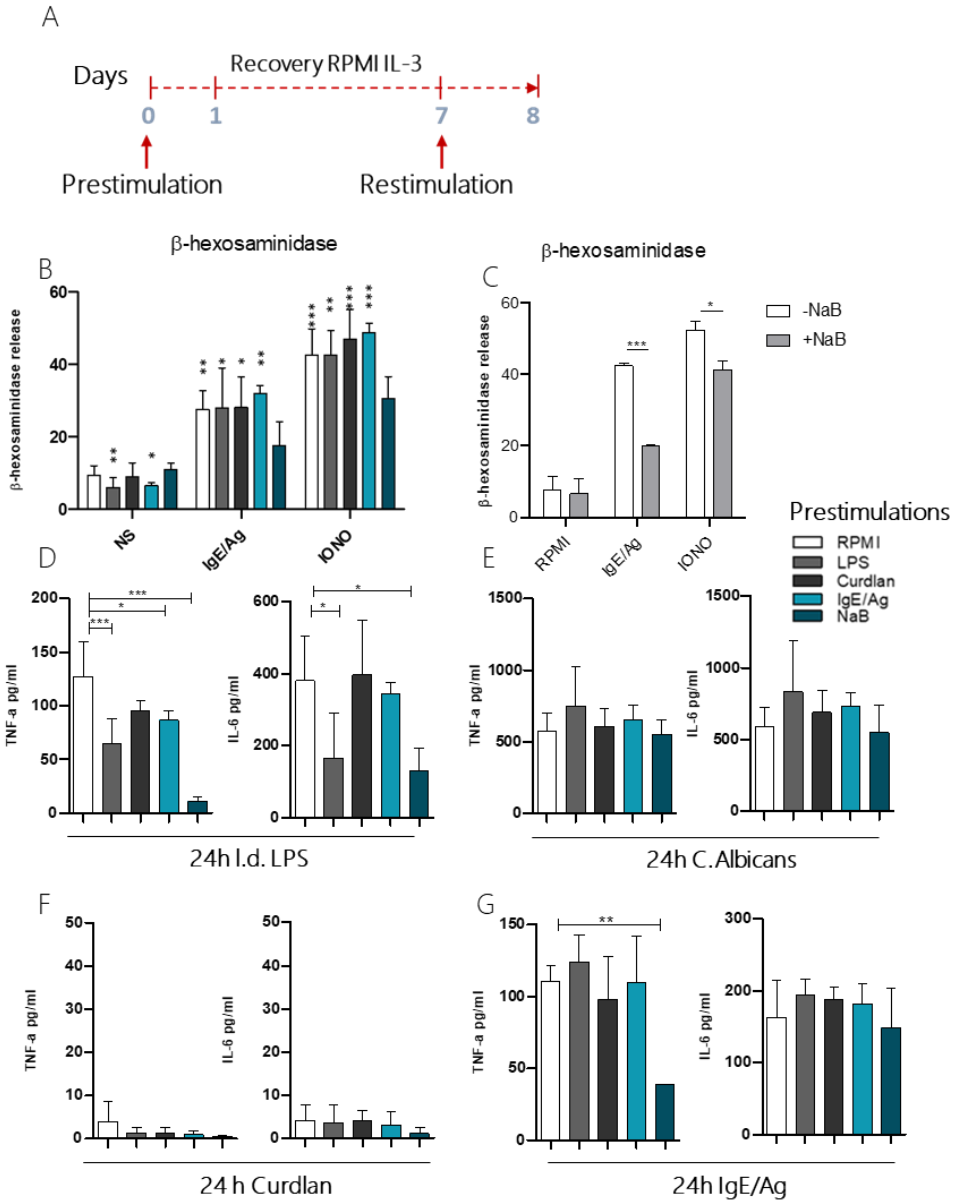


Figure 8: BMMCs priming modulate the response to secondary challenges. A. Schematic representation of the TI experimental protocol; **B.** Degranulation of BMMCs primed with LPS, Curdlan, IgE/Ag, NaB and RPMI was determined as *β*-hexoseaminidase release after IgE/Ag or ionomycin stimulation; multiple comparison test was assessed on NaB prestimulation, statistical analyses was performed with unmatched one-way Anova with Bonferroni correction on each prestimulation group; **C.** BMMCs stimulated for 30 minutes with or without 1 mM Nab and then induced to degranulate with IgE/Ag or ionomycin stimulation; Statistical analyses were performed with two-way Anova with Bonferroni correction. **D-G.** Differently primed BMMCs were restimulated with 100 ng/ml LPS (l.d. LPS, D), live C. Albicans MOI=1 (E), 10 µg/ml Curdlan (F) or IgE/Ag (G). TNFα and IL-6 levels were detected in culture supernatants after 24 hours. n>3; statistical analyses were performed with one-way Anova with Dunnet correction (*=p<0,05, **=p<0,01, ***=p<0,001).

6.1.1.2 *LPS priming modulates TNFα transcription*

The transcription of TNFα is a finely regulated process in which a lot of transcription factors are involved, and the regulation of its secretion can be independent of its transcription[250]. This statement is particularly relevant in MCs biology; indeed, the exocytosis of MCs granules and the *de novo* synthesis of mediators are tightly-regulated processes.[251,252]

To assess whether altered TNFα release is correlated with a differential transcription of the gene, qPCR analyses were performed 3 hours post restimulation. Surprisingly, unstimulated BMMCs primed with LPS showed a remarkable increase in basal TNFα transcription as compared to control (Figure 9, grey bar). Concordantly with the protein release analyzed at 24 hours, a second challenge with low dose LPS resulted in an impaired expression of TNFα transcript in LPS primed MCs (Figure 9, yellow bars). Interestingly, stimulation with live *C. Albicans* induced a huge increase in TNFα transcription levels in LPS primed MCs, greater than that observed at the level of released protein (Figure 9, blue bar). Taken together, these data demonstrate that LPS-priming induces a general higher basal transcription of TNFα in non-primed MCs. Moreover, LPS induced tolerance and training to a second challenge with a 10-fold lower dose of LPS or *C. Albicans* respectively, and it is already visible at transcription level 3 hours after the stimulation.

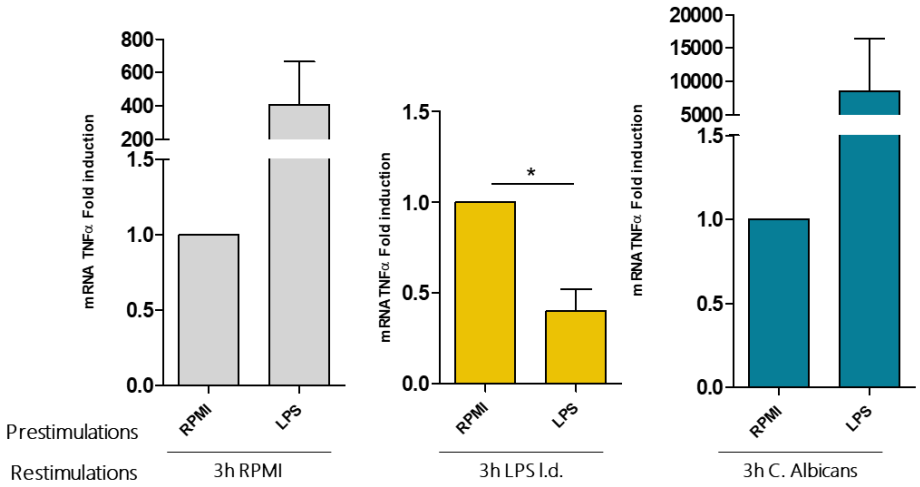


Figure 9: TNFα expression level is impaired in LPS-primed BMMCs. TNFα expression levels were measured by qPCR on LPS-primed and control MCs (RPMI) 3 hours after the restimulation with l.d. LPS or live *C. Albicans*. Data expressed as mean (SD), n=2. Statistical analyses were performed with paired Student t-test (*=p<0,05, **=p<0,01, ***=p<0,001).

6.1.1.3 *Effect of MCs priming on MC proteases expression*

As abundantly discussed above, murine mature MCs are generally subdivided into two main categories, mostly based on their localization and granule content: CTMCs and MMCs which selectively express MCPT-4, -5, -6 and -7, and MCPT-1 and -2 proteases respectively.[25] MCs are characterized by remarkable plasticity and their terminal differentiation into MMCs rather than CTMCs is strongly influenced by the stimulatory microenvironment of their homing site. To assess whether MCs phenotype and protease expression profile was influenced by LPS priming, qPCR analyses of MCPT-4, -2 and-6 transcripts were evaluated 3 hours after low dose LPS or live *C. Albicans* restimulation. As indicated in Figure 10, LPS priming induced an increased expression of MCPT-4, independently from the second stimulation. The expression of both MCPT-2 and -6 seems not to be significantly impaired by LPS priming. Taken together, these data demonstrate that LPS priming remarkably increases MCPT-4 but not MCPT-2 and -6 expression in MCs, suggesting a possible anti-inflammatory role of this protease in the impaired TNFα release to a second low dose LPS challenge, induced by LPS priming.

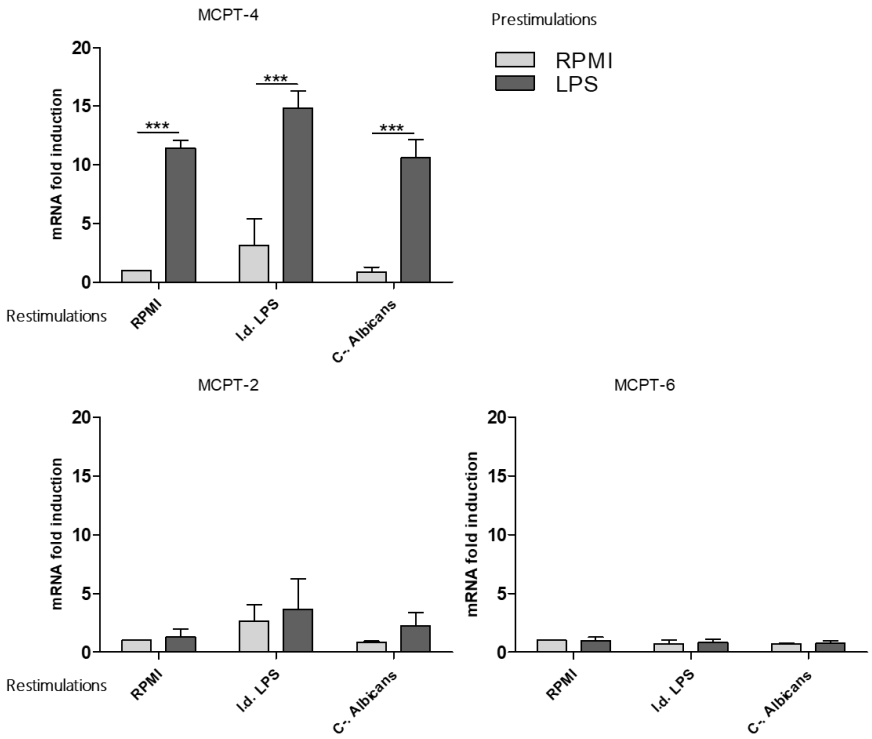


Figure 10: Mouse MC protease expression level is differently alerted in LPS-primed BMMCs. MCPTs expression levels were measured by qPCR on LPS-primed and unstimulated MCs (RPMI) in un-restimulated cells and after the restimulation with l.d. LPS or live *C. Albicans*. Data expressed as mean (SD), n≥3. Statistical analyses were performed with a two-way ANOVA with Bonferroni correction (*=p<0,05, **=p<0,01, ***=p<0,001).

6.2 Part 2

6.2.1 Cross talk between MC and intestinal cells: the MC-organoids co-culture model

The intestinal mucosa is a complex system in which several elements, namely the nervous system, the microbiota, and the immune system, coexist. All these elements are closely connected, so the variation of any one of them can lead to strong alterations of the whole system[103]. MCs, which represent about 2-3% of the resident immune cells at these sites, reach the intestine by the interaction of α4β7 integrin with several molecules expressed from the endothelial cells.[19]

Although several works [166,167,169,170,253,254]reported that there is an increase in MC numbers in the colon of patients suffering from IBDs and CRC, where they can be activated and release their pre-stored mediators which can, in turn, affect the integrity of the barriers, evidence of their direct effect is still missing. Moreover, to this increase in MCs numbers, other gastrointestinal findings can be noted, including architectural changes, such as the broadening and flattening of villi in the small bowel, and changes in crypt size, shape, and space in the colon. Most of these shreds of evidence are correlated with the increase in MCs, but the role of these cells in villous development is unknown.[255]

In the second part of this thesis, it will be illustrated the method to isolate, culture, and maintain mouse intestinal stem cells as crypt-villus forming organoids from healthy and pathological tissues, and their reconstruction with BMMCs.

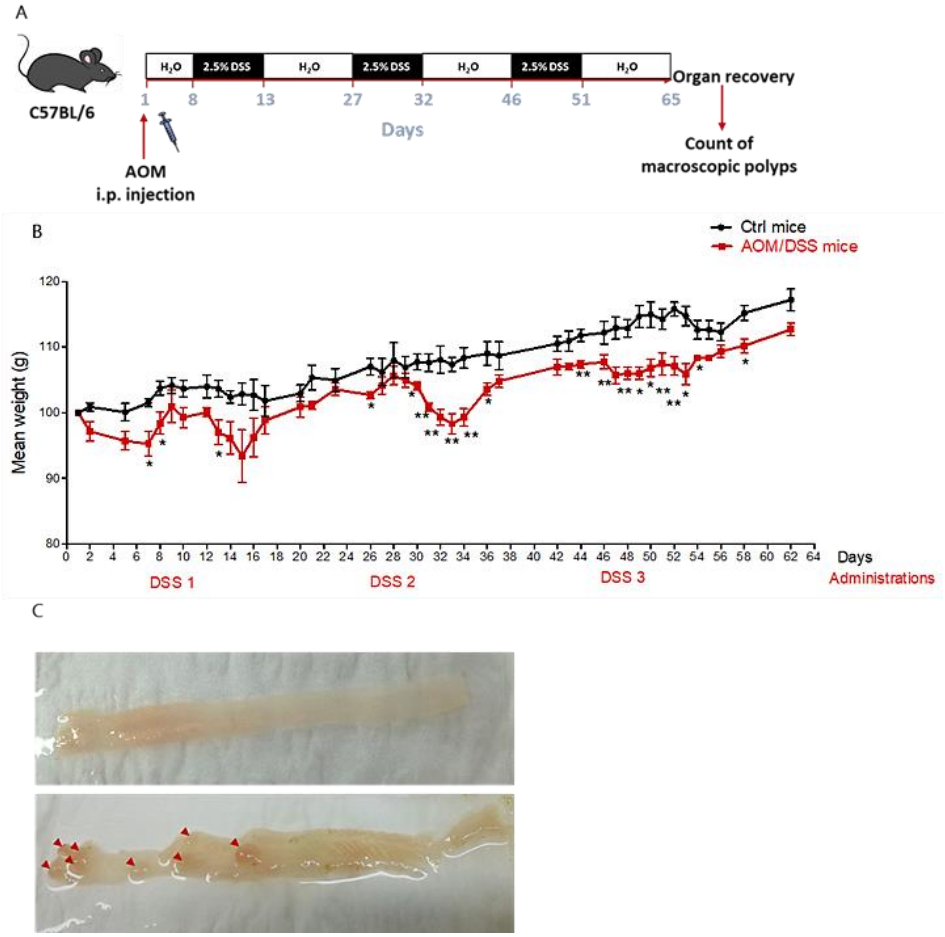


Figure 11: AOM/DSS model of colitis associated colon cancer in mice: A. protocol for the induction of AOM/DSS model of colitis associated colon cancer. **B.** Body weight loss observed in AOM/DSS-treated mice (n=4, red) compared to the control group (n=4, black) was significant. Statistical analyses were performed with unpaired Student t-test (*=p<0,05, **=p<0,01, ***=p<0,001). **C**. Evaluation of adenomas presence in the colon of AOM/DSS treated mice (lower panel, red arrows) as compared to controls (upper panel).

6.2.1.1 Set up of an animal model of gastrointestinal disease (CRC and IBD)

To obtain an organoid culture that was derived from tumor tissue, we took advantage of the AOM/DSS mouse model of colitis-associated colon cancer. In this model, colitis-associated colon cancer was induced by a single intraperitoneal injection of the mutagen azoxymethane, followed by 3 cycles of 2.5% dextran sulfate sodium salt (DSS) in drinking water, each interspersed with 14 days of recovery with normal drinking water (Figure 11 A). Animals were monitored for weight loss daily during DSS treatment and once every 2-4 days during the recovery to assess disease progression (Figure 12 B). At the end of the treatment, both controls and AOM/DSS treated mice were euthanized, colons were collected and analysed for the presence of adenomas, which were carefully isolated and used for the production of the organoid cultures (Figure 11 C).

Instead, as a model of GI inflammation, we decided to exploit the acute colitis model induced by the administration of 2.5-3% DSS dissolved in drinking water for 5 consecutive days, followed by a recovery period of 3 days with normal drinking water (Figure 12 A). Mice were monitored for stool consistency and weight loss (data not shown). At the end of the treatment controls and DSS treated mice were euthanized and the small intestines were collected; part of the tissues was used for the production of the organoid culture, and part was used for the histochemical (IHC) analysis. Haematoxylin & Eosin staining confirmed that the small intestine of DSS treated mice developed an inflammatory pathological condition characterized by shorter, wider, and thinned villi with lymphoplasmacellular infiltrate and lower epithelium (Figure 12 B, right panel), as compared to the controls which showed longer and denser villi with a thinner axis, and present but less intense infiltrate and higher epithelium (Figure 12 B, left panel).

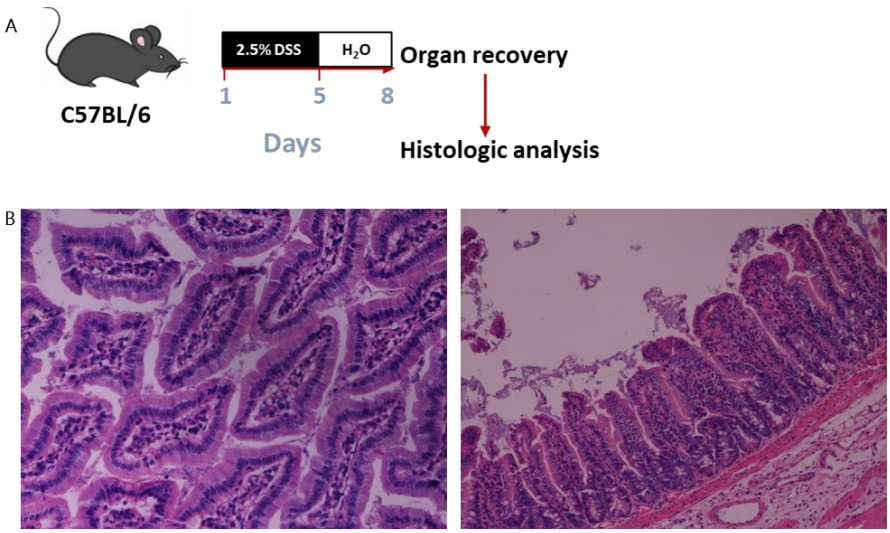


Figure 12: DSS model of acute colitis in mice: A. protocol for the induction of DSS model of acute colitis. **B**. IHC analysis of control small intestinal tissue (left panel) and DSS treated small intestinal tissue (right panel).

6.2.1.2 Set up and characterization of intestinal organoids

Mouse intestinal organoids were generated from stem cell-bearing crypts (Figure 13 A) of the colon and small intestine of healthy and AOM/DSS and DSS treated mice as described in Sato T. work published in 2009[181] with little modifications. In particular, healthy colon organoids were produced starting from the whole tissue of origin, while small intestinal cultures of both control and DSS treated mice were generated only from the proximal portion of the mouse small intestine. For the AOM/DSS derived organoids, only adenomatous tissues were processed. Crypts

were embedded in an extracellular matrix that allows the three-dimensional organization and growth of the organoid, the Matrigel. Matrigel is a finely tuned mixture of matrix proteins secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells that resembles the extracellular environment of many tissues, and

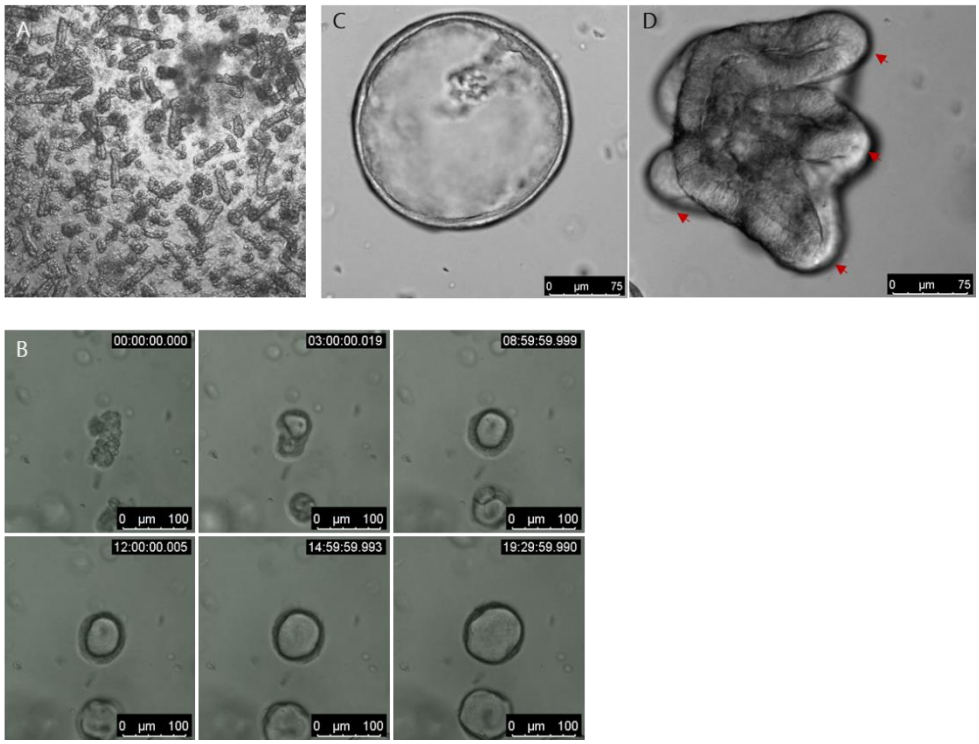
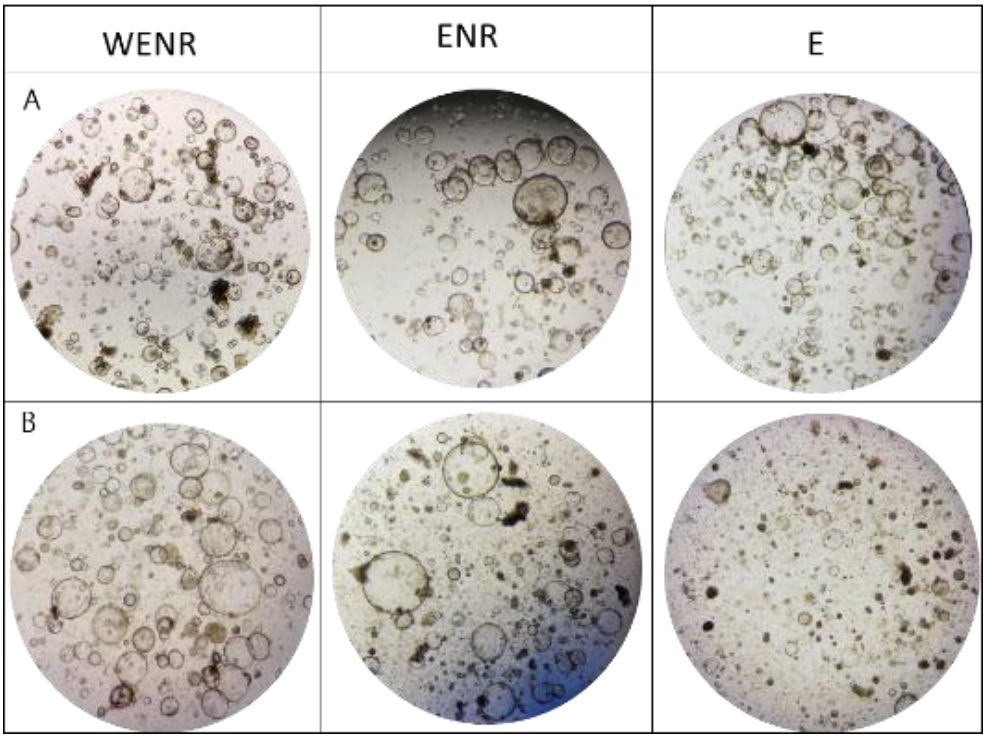


Figure 13: Establishment of a small intestinal organoid culture: from the crypt to the organoid. A. Representative image of mouse freshly extracted small intestinal crypts seeded in matrigel. **B.** Time lapse frames of a freshly isolated small intestinal crypt seeded in matrigel showing the first phases of the organoid formation. The crypt is already closed 3 hours after seeded. **C.** Representative image of a stem unpolarised small intestinal organoid; the organoid is completely formed by stem cells. **D.** Representative image of a polarised differentiated small intestinal organoid; the organoid is composed of different cell types. In the crypts (red arrows) are found the intestinal stem cells.

thus it is a suitable substrate for culturing cells and, in this case, organoids.

Intestinal crypts were cultured in a complete expanding medium enriched with Wnt, Noggin, and R-spondin, which are the three fundamental growth factors for the maintenance of the stem niche, and mouse EGF (WENR medium).[181] For the long-term expansion of normal colon and small intestinal organoids, cultures were maintained in WENR medium while for experimental conditions they were cultured in Wnt-free medium, also called ENR or differentiation medium. Once extracted and seeded in matrigel in WENR medium, intestinal crypts (Figure 13 A) close up in few hours (Figure 13 B) and finally give rise to a long-term expanding culture of stem intestinal organoids (Figure 13 C). When switched to differentiation medium, organoids start to polarize, nuclei are arranged basolaterally, while the apical side of the cells faces the lumen, in which debris, dead cells but also cellular secretions

will be released. Hence, two main compartments are distinguished: an "intestinal crypt" side (Figure 13 D, red arrows), that keeps the self-renew capacity, and a "body of the organoid" side in which all the cell types found in the tissue of origin (e.g. Goblet cells, Enteroendocrine cells, generic enterocytes) are represented (Figure 13 D).



C

AOM/DSS line	Codon	Exon	Mutated base	Genotype	Amino acid replacement	Mutation
1	34	3	GGA→ AGA	Heterozygous	Gly→ Arg	G34R
2	34	3	GGA→ GAA	Heterozygous	Gly→ Glu	G34Q
3	34	3	GGA→ GAA	Homozygous	Gly→ Glu	G34Q
4	33	3	TCT→ TTT	Heterozygous	Ser→ Phe	S33F
	37	3	TCT→ TTT	Heterozygous	Ser→ Phe	S37F

Figure 14: Set up and characterization of AOM/DSS derived organoids. A. AOM/DSS derived organoids cultured in media containing gradually fewer niche factors in order to select fully tumoral organoids growth. **B.** Healthy organoids cultured in media containing gradually fewer niche factors are unable to finish their growth and eventually die in E medium. **C.** Mutational profile of Ctnn1-b gene of the so far sequenced AOM/DSS organoids lines. All the AOM/DSS cultures analysed present hetero- or homozygous mutation in the exon 3 of Ctnnb-1 gene.

Isolated crypts from colon adenomatous tissue were cultured in a medium without R-spondin, Noggin, and Wnt, which are essential for normal organoids growth.[182,256] To further ensure cancer organoid formation in AOM/DSS samples, we cultured both healthy colon and AOM/DSS organoids in WENR medium, in medium containing R-spondin, Noggin, and mEGF (ENR), and in medium containing only mEGF (E). As demonstrated in Figure 14, AOM/DSS organoid

cultures were entirely constituted by tumoral organoids that were able to grow in the absence of the 3 fundamental growth factors Wnt, R-spondin, and Noggin (Figure 14 A), while healthy colon organoids started to differentiate in ENR medium and failed to grow in E medium (Figure 14 B).
Moreover, AOM/DSS cultures were subjected to Sanger sequencing to characterize their mutational profile. All AOM/DSS cultures we sequenced so far have a homo- or heterozygous mutation in the β-catenin gene (Ctnn1-b) (Figure 14 C).
Collectively, these data demonstrate that the AOM/DSS model of colitis-associated colon cancer successfully resulted in tumor growth and that from tumoral tissue it is possible to produce a tumoral organoid culture that resembles the tissue of origin, as evidenced by the presence of Ctnn1-b mutation which is generally induced by the AOM/DSS protocol.[257]

6.2.1.3 Colonic and small intestinal markers expression levels in controls vs pathological mouse models

The expression levels of the most studied intestinal markers were evaluated in organoid cultures. In particular, the relative expression of Lgr5, which was first discovered in 2007 by Barker et al[200], and that were subsequently exploited by Toshiro Sato in 2009 to generate "crypt-villus structures"[181], was used as a stem cell marker. The intestine is composed of a multiple varieties of cells exerting different roles, from the absorptive lineage to the secretory one. Thus, a subset of markers for each category was analyzed.
In particular, Lyz1 (Lysozyme 1) was used as a Paneth cell marker. Its expression is reported and of strong relevance in the small intestine where it exerts different roles, from the protection from parasites[258] to the production of niche factors[259]. On the contrary, Paneth cells are usually absent in the colon.
Muc2 (Mucine 2) and ChgA (Chromogranin A) were analyzed as markers of Goblet and Enteroendocrine cells respectively. These two cell types play important roles in intestinal homeostasis. Indeed, it is known that alteration of the mucus layer, which is the first line of defense of the GI tract, is linked to pathological conditions[260] and that Chromogranin A differential expression could be used as a prognostic marker in both IBDs and CRC.[261–263]
Sucrase Isomaltase (SI) is a well-known marker of the absorptive lineage[264], that is strongly expressed in the small intestine, but not in the colon, where the absorption is minimal. (Figure 15 C).
Finally, Cdh-1 (E-cadherin) was analyzed as a structural marker.
The expression of these markers was analyzed using Real time qPCR in the healthy colon versus AOM/DSS (Figure 15 A), and in healthy small intestine versus DSS organoids (Figure 15 B). As demonstrated by Figure 15 A there is a strong difference in Lgr5 expression between AOM/DSS and healthy colon organoids,

which is also reflected by upregulation of Lyz1 expression in tumoral organoids. Indeed, as mentioned above, Paneth cells are fundamental in the crypts but are normally absent in the lower gut. Nevertheless, they tend to be represented again in CRC.[265] These evidences coincide with the strong stem phenotype shown by AOM/DSS-derived cultures, that are able to grow in the absence of niche factors supplementation as demonstrated in Figure 14 A.

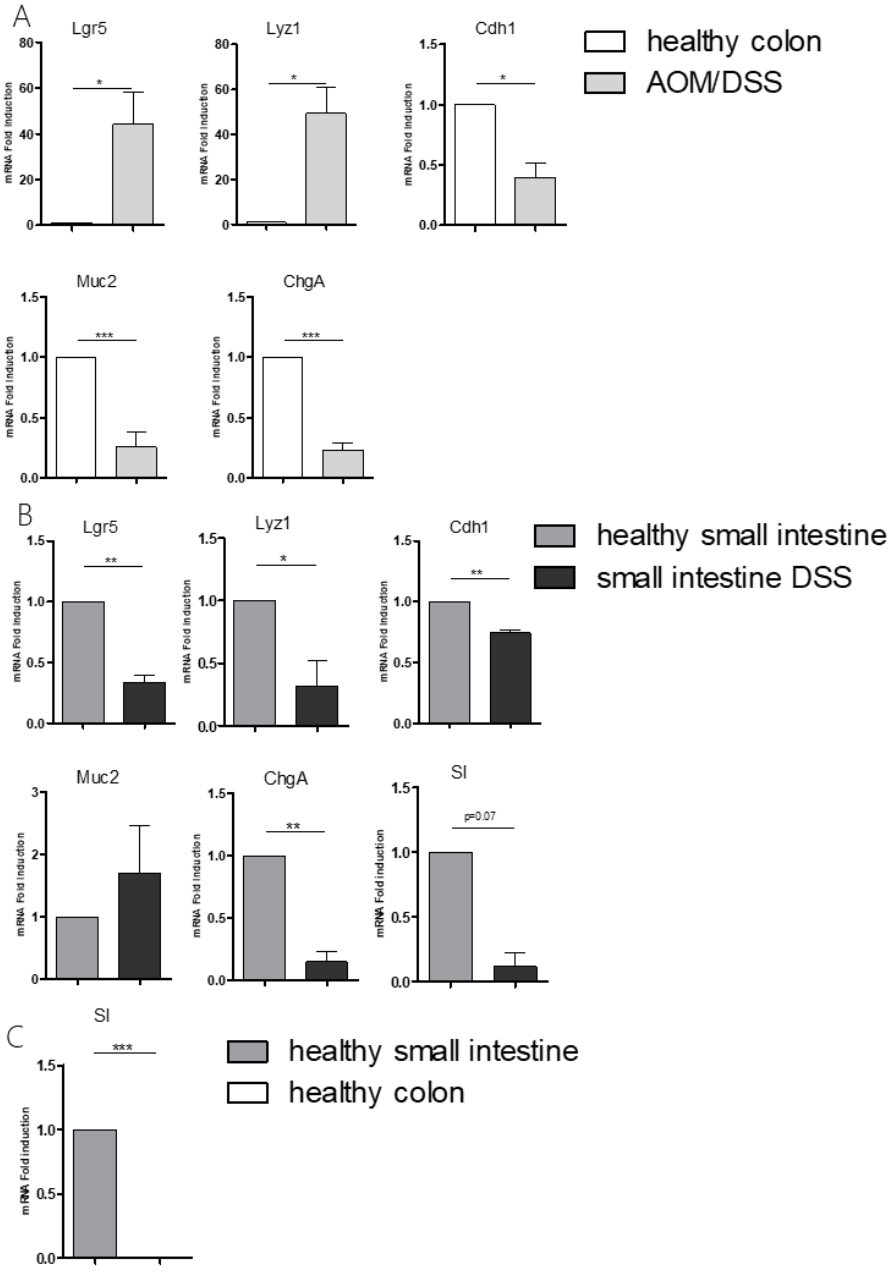


Figure 15: Differential intestinal epithelial marker expression in healthy vs pathological organoid cultures. A. Intestinal marker expression levels in healthy vs AOM/DSS organoid cultures (n≥6). **B.** Intestinal marker expression levels in healthy small intestine vs DSS organoid cultures (n≥3). **C.** SI expression levels in healthy small intestine vs colon; colon expresses negligible levels of this marker (n=4). Statistical analyses were performed with a Student paired t-test (*=p<0,05, **=p<0,01, ***=p<0,001).

As a result, other differentiation markers are less expressed in AOM/DSS as compared to healthy colon organoids (Figure 15 A).
A strong difference between the healthy small intestine and DSS derived organoids was also found (Figure 15 B). Indeed, the expression levels of the overall analyzed markers in DSS organoids are attributable to an inflammatory background.[264,266,267] Taken together, these data demonstrate that organoid cultures are a reliable system for the study of different pathological settings as well as different healthy compartments.

6.2.1.4 Set up of mouse intestinal organoids-BMMCs co-culture system

6.2.1.4.1 Type 1 co-culture

To investigate the interaction between intestinal epithelial cells and BMMCs, we first performed a co-culture system in which organoids and BMMCs were mixed and seeded together in matrigel (Figure 16 A). To this end, terminally differentiated BMMCs were stained with a cell tracker (CFSE) and mixed with desegregated colon organoids. The co-culture was performed in ENR differentiation medium. It was maintained for up to one week and images were taken at different time points. As demonstrated by Figure 16 B, mature BMMCs could reach the organoids and interact with them at the basolateral side, where the lamina propria (LP) is normally located. The same experiment was performed also with AOM/DSS derived organoids, and again, MCs were found to localize at the basolateral level (Figure 16 C). Collectively, these data demonstrate that BMMCs can be successfully co-cultured with intestinal organoids, both deriving from healthy and tumoral tissues. The co-culture can be maintained for more than one week and BMMCs can interact with organoids at the basolateral side. This is an important point since LP is the physiological localization of MCs in the gut.[104,268]

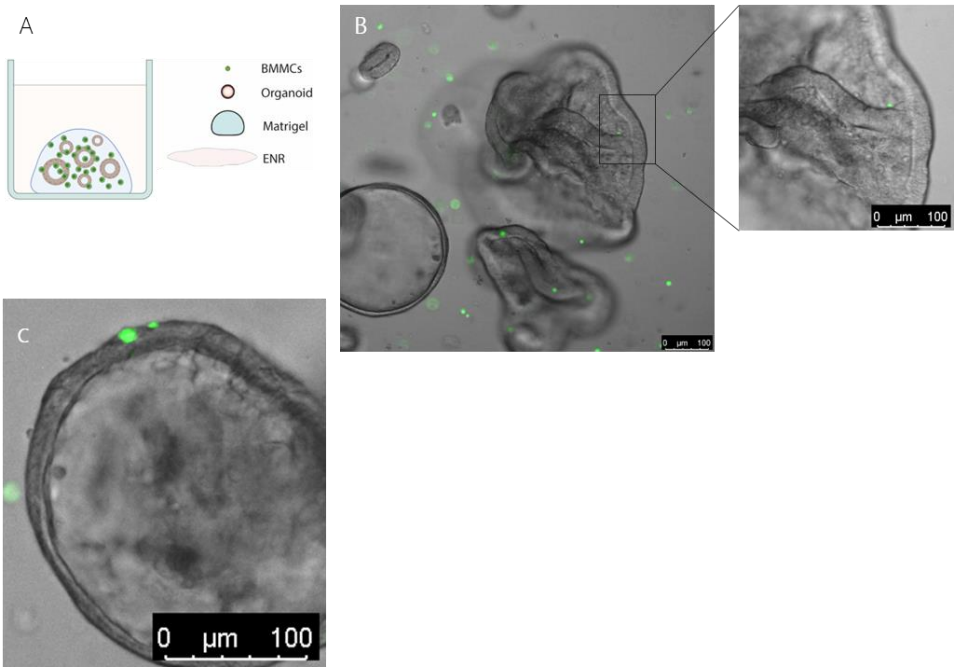


Figure 16: Intestinal organoids-BMMCs co-culture system: Type 1. A. schematic representation of type 1 co-culture system: organoids were desegregated and mixed with mature CFSE-labelled BMMCs (green); the culture was performed in ENR medium. **B.** Representative image of healthy colon organoids-BMMCs co-culture. **C.** Representative image of AOM/DSS derived organoids-BMMCs co-culture. Images were taken at day 3.

6.2.1.4.2 Type 2 co-culture

To further determine the migration capabilities of BMMCs towards intestinal organoids the second type of co-culture was set up in which only the organoids were seeded in matrigel, while BMMCs were left outside, in the medium surrounding the matrigel domes (Figure 17 A). To this end, mature BMMCs were stained with CFSE cell tracker, resuspended in ENR differentiation medium, and placed over the intestinal organoids. Also in this case, the co-culture was maintained for up to one week and images were taken at different time points. The time-lapse frames shown in Figure 17 B demonstrate that mature BMMCs can penetrate the matrigel dome and can move freely in it. Moreover, BMMCs can reach the organoids with which they interact at the basolateral side. Of relevance is the fact that not all BMMCs could migrate and interact with the organoids, but only a small percentage of them, thus mimicking in an even more truthful way the condition that is found physiologically *in vivo* where LP MCs represent the 2-3% of the intestinal mucosa.[104] Indeed, different amount of MCs have been used ($0.1x10^5$-$3x10^5$) in our co-culture system, and similar results were observed (personal observation). BMMCs cultured with matrigel in the absence of organoids didn't show any migration (Figure 17 C). Collectively, these data demonstrate that

co-cultures can be maintained for more than a week and that BMMCs are able to migrate selectively to the organoids they interact with at the basolateral level.

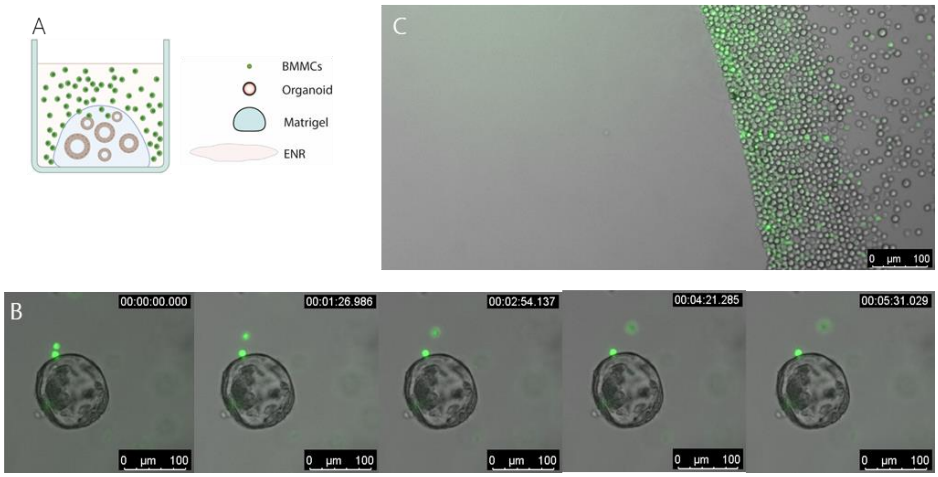


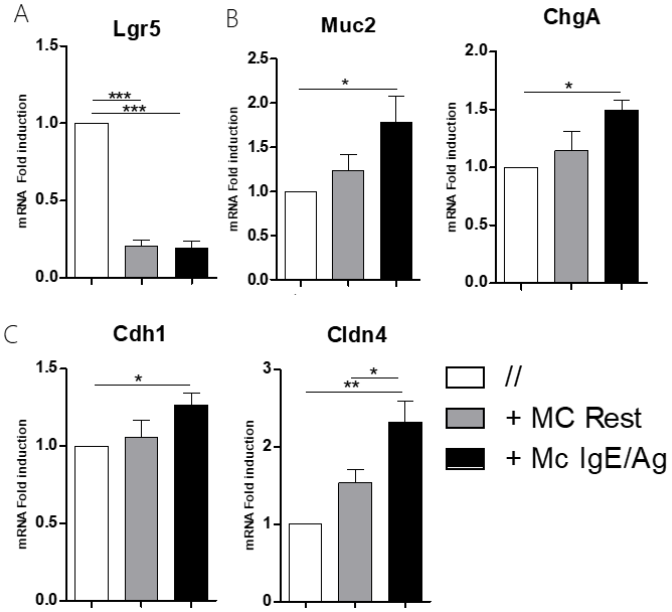
Figure 17: Intestinal organoids-BMMCs co-culture system: Type 2. A. schematic representation of type 2 co-culture system: organoids were seeded in matrigel while mature CFSE-labelled BMMCs (green) were resuspended in ENR medium that was then placed over the matrigel domes. **B.** Time-lapse frames of mature CFSE-labelled BMMCs (green) interacting with a healthy colon organoid at day 3. Images were taken every 10 seconds for a total of 5'39''. **C.** CFSE-labelled matures BMMCs at the interface with an empty matrigel dome. Image was taken at day 3.

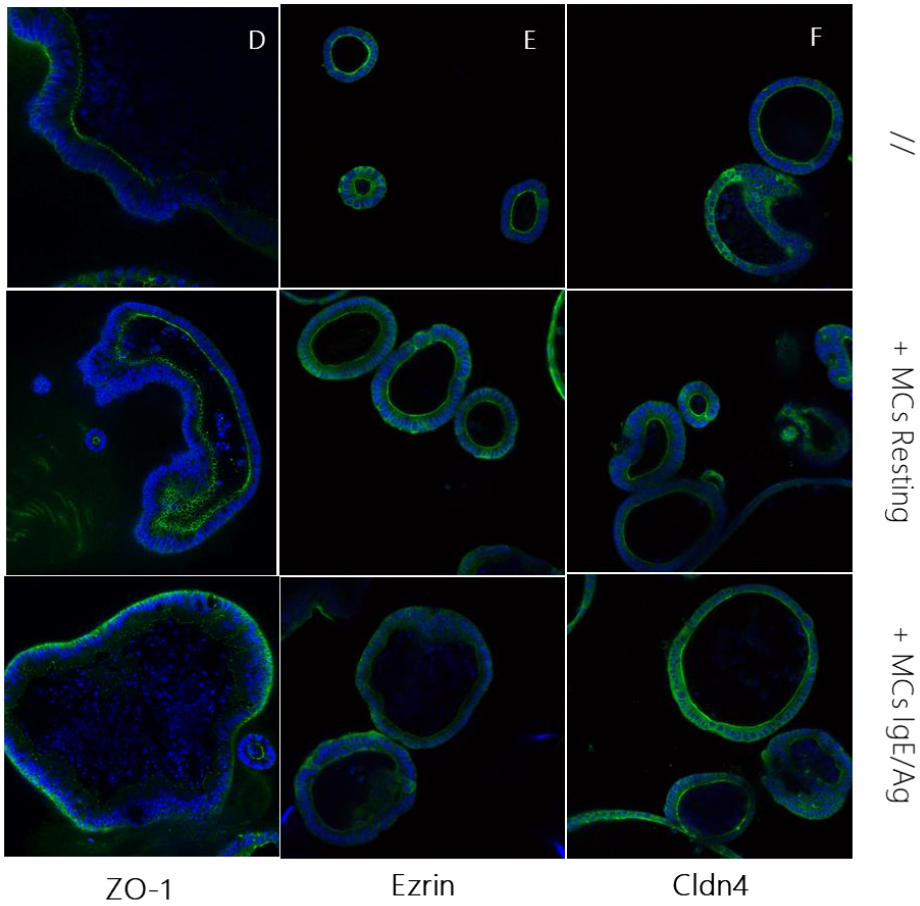
6.2.1.5 The presence of mouse BMMCs affects the organization of mouse intestinal organoids.

6.2.1.5.1 Effects on healthy colon organoids

To investigate if the presence of MCs could influence colon organoids development and constitution, qPCR analysis of organoids was performed after a type 2 co-culture with MCs. Briefly, organoids were co-cultured for 48 hours with resting or IgE/DNP (the specific antigen for the IgE) activated MCs in ENR medium. The simple presence of MCs, regardless of their activation state, induced a remarkable downregulation of the Lgr5 gene, whose expression drops by about 80%, (Figure 18 A) suggesting that MCs may be involved in driving the differentiation of major intestinal cell types along the crypt axis. In parallel, as demonstrated in Figure 18 B, in presence of resting and, above all, of IgE/DNP activated MCs the expression of the two markers of the secretory lineage Muc2 and ChgA increases respectively by 1 and 0.5 times. Moreover, the presence of activated MCs seems to affect also the architectural structure of the organoids, as demonstrated by the altered expression of Cdh-1 and Claudin 4 (Cldn4), a tight junction expressed throughout all the GI tract, but largely found in the colon[269]. In particular, in presence of activated MCs, there is an upregulation of Cdh-1 and, in

a major extent, of Cldn4, which is 1,5 fold higher in presence of activated MCs (Figure 18 C). To get more insight on this point, immunofluorescence stainings (IF) of the co-cultures





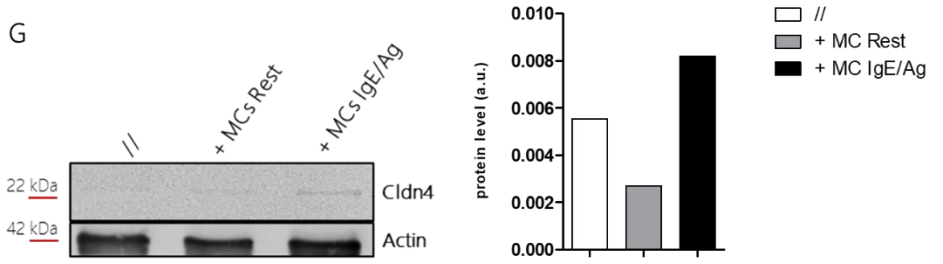


Figure 18: MCs have a role in healthy organoids composition and structural organization. A. qPCR analysis of stem cell marker Lgr5 expression in healthy colon organoids co-cultured with resting and activated MCs (n=8). **B.** qPCR analysis of secretory lineage markers Muc2 and ChgA expression in healthy colon organoids co-cultured with resting and activated MCs (n≥5). **C.** qPCR analysis of structural markers Cdh-1 and Cldn4 expression in healthy colon organoids co-cultured with resting and activated MCs (n≥3). **D.** IF staining of ZO-1 (green) in healthy colon organoids co-cultured with resting and activated MCs. Nuclei (blue). **E.** IF staining of Ezrin (green) in healthy colon organoids co-cultured with resting and activated MCs. Nuclei (blue). **F.** IF staining of Cldn4 (green) in healthy colon organoids co-cultured with resting and activated MCs. Nuclei (blue). **G**. Western Blot analysis of Cldn4 expression in healthy colon organoids cultured alone, and co-cultured with resting MCs or with IgE/Ag activated MCs. Right panel shows densitometry analysis calculated over Actin expression (n=1). Data expressed as mean (SD), Statistical analyses were performed with a one-way Anova (*=p<0,05, **=p<0,01, ***=p<0,001).

have been performed for the analysis of structural markers like ZO-1 (Figure 18 D), that is a peripheral membrane protein that contributes to the intestinal barrier integrity interacting with the other tight junctions[270], Ezrin (Figure 18 E), a member of the apical complex that links to the actin cytoskeleton to drive the intestinal epithelium organization[271], and Cldn4 (Figure 18 F).
The presence of activated MCs causes an altered localization of Ezrin and ZO-1, which are no longer found at the apical level but are delocalized at the basolateral side. Moreover, the IF of Clnd4 confirms the qPCR data, highlighting a Cldn4 upregulation in presence of activated MCs. The upregulated expression of Cldn4 in presence of activated MCs is further demonstrated by western blot analysis (Figure 18 G).
Taken together, these data suggest that MCs determine the organization and constitution of colon organoids and that their activation induces major structural alterations in healthy colon organoids, thus suggesting possible altered organoids functionality induced by activated MCs.

6.2.1.5.2 Effects on AOM/DSS derived organoids

The same co-cultures experiments were performed also with AOM/DSS derived organoids to study if the presence of resting or IgE/DNP activated MCs could induce a comparable effect also in a pathological setting. As demonstrated by Figure 19 A, the only presence of MCs in the culture caused a significant

downregulation of Lgr5 also in AOM/DSS organoids, which is reduced by 50%; a comparable effect of the activated MCs was also found on the expression of Cdh-1 and Muc2, which however does not reach statistical significance (Figure 19 A). Surprisingly, a strong difference was found in ChgA expression in AOM/DSS co-cultured with MCs as compared to what happens with healthy organoids. Indeed, the simple presence of MCs in the microenvironment leads to a strong downregulation of ChgA (Figure 19 B), which drops by 50%, inducing an opposite effect as compared to that observed in healthy organoids (Figure 18 B). Moreover, since Paneth cells are usually not expressed in the colon but it have been found that they tend to be re-expressed in a tumoral setting[265], the variation of Lyz1 expression was also evaluated. As demonstrated by Figure 19 B also in this case the presence of MCs, regardless of their activation state, seems to induce a significant reduction of Lyz1 expression in AOM/DSS derived organoid.
Together, these results suggest that MCs induce different effects in tumoral organoids than in healthy ones, and therefore may play a different role depending on the microenvironment they come into contact with.

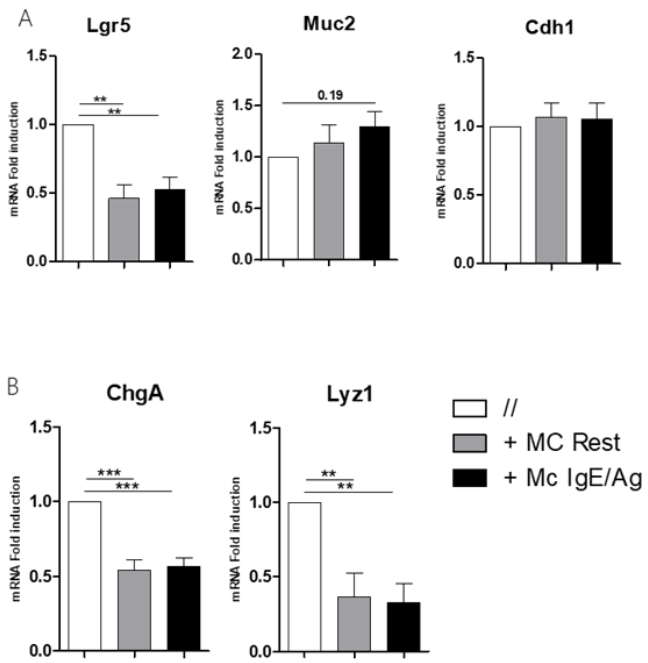


Figure 19:MCs differently affect AOM/DSS organoids composition. **A.** qPCR analysis of Lgr5, Muc2 and Cdh-1 expression in AOM/DSS derived organoids co-cultured with resting and activated MCs (n≥4). **B.** qPCR analysis of ChgA and Lyz1 in AOM/DSS derived organoids co-cultured with resting and activated MCs (n=5). Data expressed as mean (SD), Statistical analyses were performed with a one-way ANOVA (*=p<0,05, **=p<0,01, ***=p<0,001).

6.2.1.5.3 Effects on healthy small intestine organoids

Variations in intestinal epithelial markers were analysed also in healthy and DSS derived small intestinal organoids co-cultured with resting and IgE/DNP activated MCs. As demonstrated in Figure 20, the presence of MCs influenced also the small intestinal organoid cultures, inducing a differential expression of the analyzed intestinal epithelial markers. In particular, MCs caused a reduction of Lgr5 also in small intestinal organoids, similarly to the colon and both in healthy and DSS

derived organoids, where Lgr5 expression is reduced by 50% and 80% respectively. This is further demonstrated by the IF staining for CD44 (a well-known marker for the stem compartment of the intestinal crypt)[272], in healthy small intestinal organoids co-cultured with resting and IgE/DNP activated MCs: it is possible to see that in presence of both resting and activated MCs, there is an evident reduction of CD44 expression, that is not completely abolished, but it is restricted to the crypt base. Unlike in the colon, Paneth cells are highly represented in the small intestine where they exert different roles among which also the production of the niche factors.[259] The presence of MCs induced a reduction of Lyz1 in healthy small intestinal organoids that could be mirrored by reduced Paneth cell numbers, which in turn can be reflected in a reduced number of Lgr5 expressing cells. However, Lyz1 expression data coming from the co-cultures performed with DSS-derived organoids show no or negligible difference in Lyz1 expression although there is still an evident reduction of Lgr5 expressing cells. A reason for that could be that in an inflammatory setting, Paneth cells could play other roles, different from the canonical ones,[273]that, in turn, could be triggered by the presence of MCs. The small intestine harbors not only cells of the secretory lineage, but also cells of the absorptive lineage, of which Sucrase Isomaltase (SI) is a well-known marker.[264] Both resting and activated MCs induced a strong upregulation of SI in both healthy and DSS derived organoids. Concerning the other markers analyzed, there not seems to be strong differences with the healthy colon and between healthy and DSS-derived organoids.

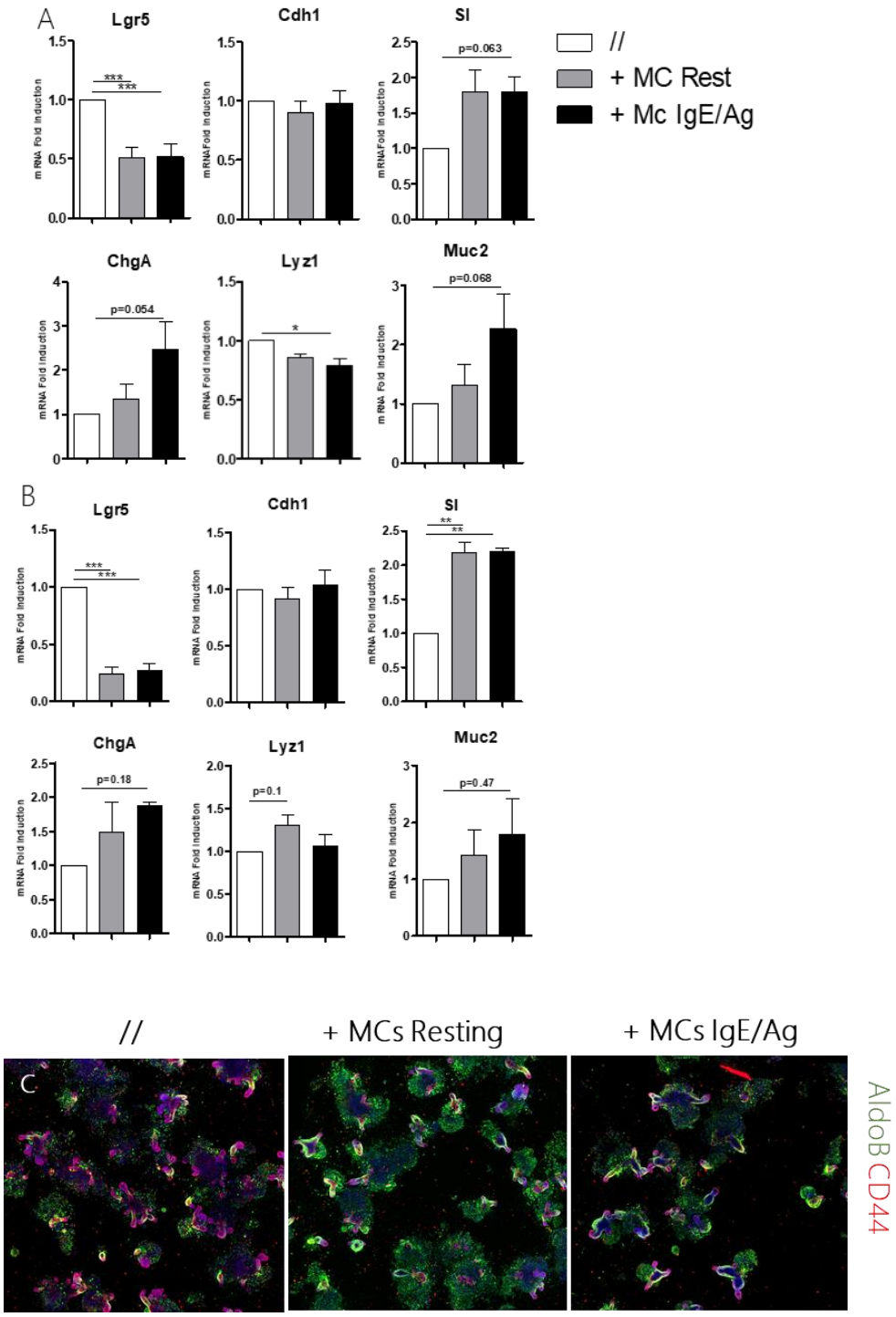


Figure 20: MCs have a role in healthy and DSS derived small intestinal organoids composition and structural organization: A. qPCR analysis of Lgr5, Cdh-1, SI, ChgA, Lyz1 and Muc2 expression in healthy small intestinal organoids co-cultured with resting and activated MCs (n≥5). **B.** qPCR analysis of Lgr5, Cdh-1, SI, ChgA, Lyz1 and Muc2 expression in DSS derived small intestinal organoids co-cultured with resting and activated MCs (n≥4). **C.** IF staining of CD44 for the stem compartment (red) and AldoB for generic enterocytes (green) in healthy colon organoids co-cultured with resting and activated MCs. Nuclei (blue). Data expressed as mean (SD), Statistical analyses were performed with a one-way Anova (*=p<0,05, **=p<0,01, ***=p<0,001).

Altogether, these data suggest that MCs could have a role also in the small intestinal organoid development. However, MCs' behavior could be influenced by the micro-environmental background.

6.2.1.6 Healthy vs pathological organoids differently affect MC biology.

6.2.1.6.1 Mature BMMCs express different proteases when co-cultured with different organoids

As already highlighted, MCs are extremely eclectic and heterogeneous cells, whose phenotype is strongly influenced by their homing site. BMMCs that are commonly used for *in vitro* studies are considered immature MCs and express intermediate characteristics between MMCs and CTMCs.[274] To study if the microenvironment recreated *in vitro* by the organoid cultures could influence MCs phenotype toward a more connective tissue or a more mucosal type, qPCR analysis of MCs co-cultured for 48 hours with healthy colon and small intestine, and AOM/DSS and DSS derived organoids were performed. MCPT-4 was used as connective tissue MCs marker, while MCPT-2 as mucosal MCs marker. As demonstrated in Figure 21 A, BMMCs cultured with healthy colon organoids downregulate MCPT-4 expression (left panel), while upregulates of almost 3 times the expression of MCPT-2 (right panel), thus leaning towards a mucosal rather than a connective tissue phenotype. On the other hand, co-culture of BMMC with AOM/DSS-derived organoids induced a huge increase of both MCPT-4 and MCPT-2 expression (Figure 21 B), suggesting that BMMCs grown with AOM/DSS-derived organoids perceive a pathological condition that could eventually lead to BMMCs activation and consequently release newly synthesized proteases.
From the analysis of MCPTs expression in BMMCs co-cultured with healthy and DSS derived small intestinal organoids (Figure 21 C and D), it emerged that MCs didn't show a differential expression of MCPTs, both in healthy and pathological settings. Indeed, they showed comparable levels of both MCPT-2 and MCPT-4 when co-cultured with healthy and DSS derived intestinal organoids.
Altogether, these data demonstrate that MCs co-cultured with healthy colon organoids tends to polarize towards a mucosal phenotype, while in presence of tumoral organoids they indifferently express high levels of both MCPT-4 and MCPT-2.

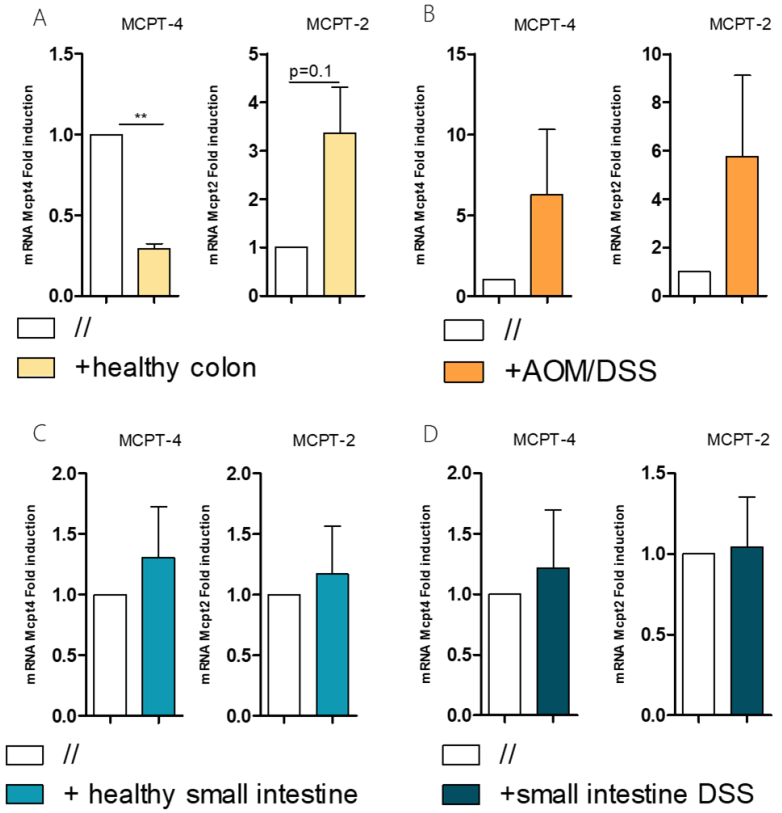


Figure 21: MCs protease expression changes in co-culture with different intestinal organoids: qPCR analysis of the connective tissue MC marker MCPT-4 and mucosal MC marker MCPT-2. MCPT-4 and -2 expression levels in MCs co-cultured with **A.** healthy colon organoids, **B.** AOM/DSS derived organoids, **C**. healthy small intestinal organoids and **D.** DSS derived small intestinal organoids. n≥3, statistical analyses were performed with a Student paired t-test (*=p<0,05, **=p<0,01, ***=p<0,001).

6.2.1.6.2 Mature BMMCs proliferate and are activated in presence of AOM/DSS derived organoids

As already well known, the presence of tumor-associated inflammation is a marker of bad prognosis, and also MCs have been reported to accumulate in the mucosa during tumor progression, where they can proliferate and undergo activation.[166–169] To get more insight into this strongly different behavior of BMMCs when co-cultured with healthy colon organoids or with AOM/DSS derived organoids, proliferation rate and the state of activation of BMMCs co-cultured with both healthy and AOM/DSS derived colon organoids were assessed. To this end, mature BMMCs were stained with CFSE cell tracker, resuspended in ENR differentiation medium, and placed over the colon organoids for 24 hours. Since the CFSE label is inherited stably by daughter cells during cell division, the proliferation rate is assessed as the percentage of CFSE low cells (Figure 22 A). As demonstrated in figure 22 A, mature BMMCs co-cultured for 24 h with AOM/DSS

derived organoids undergo a modest but statistically significant proliferation, highlighting that MCs sense a pathological setting that leads them to proliferate. Moreover, after 24h the expression of LAMP-1 (CD107a), a well-known marker of MCs degranulation[275] was further analyzed, through flow cytometry. As shown in Figure 22 B, after 24h of co-culture, MCs proliferation is accompanied by a slight activation that is demonstrated by an almost 1-fold upregulation of LAMP-1 expression in MCs co-cultured with AOM/DSS organoids. Taken together, these data demonstrate that mature BMMCs behave differently when co-cultured with healthy or AOM/DSS derived colon organoids. In particular, the co-culture with tumoral organoids leads to BMMCs proliferation and activation, thus mimicking the *in vivo* situation in a pathological setting.

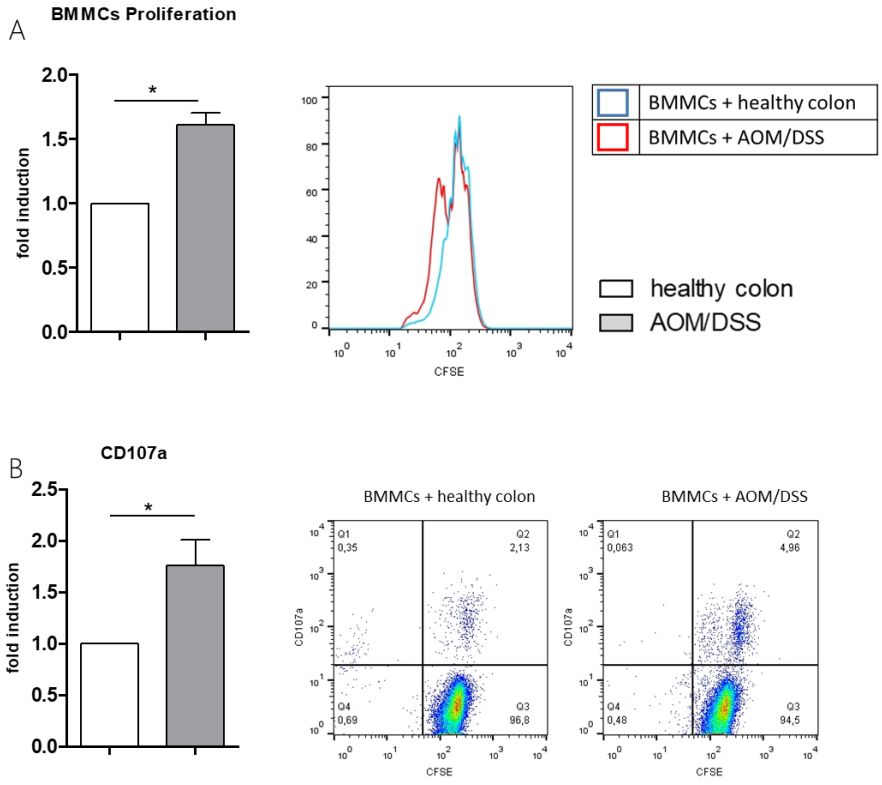


Figure 22: Mature MCs co-cultured with AOM/DSS derived organoids are induced to proliferate and degranulate. A. Fold induction analysis of proliferating MCs co-cultured for 24 h with healthy (white bar) and AOM/DSS derived (grey bar) colon organoids (left panel) n=4; Representative histogram for each experimental group. **B.** Fold induction analysis of CD107a expressing MCs co-cultured for 24 h with healthy (white bar) and AOM/DSS derived (grey bar) colon organoids (left panel) n=7; Representative flow cytometry graphs for each experimental group. Statistical analyses were performed with a Student paired t-test (*=p<0,05, **=p<0,01, ***=p<0,001).

6.2.1.7 *Effects of microbial-derived products on the organization and integrity of colon and small intestinal organoids.*

6.2.1.7.1 Preliminary data on healthy colon and small intestinal organoids

As already mentioned, within the GI tract, the host-microbiome interaction is of major importance for the maintenance of homeostasis and for a correct function. Indeed, IBD patients show a reduced bacterial diversity, that contributes to the disease outcome.[103,112] One of the roles played by the intestinal microbiota, is to

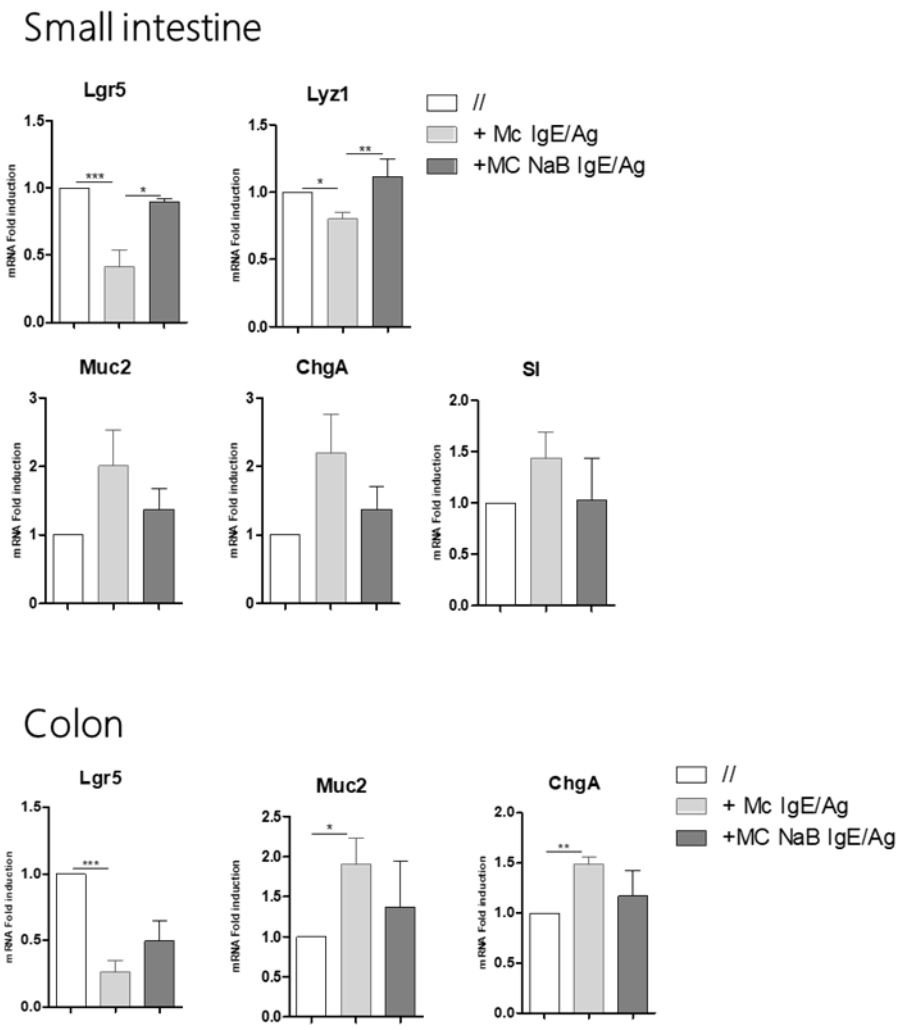


Figure 23: NaB pre-stimulation of MCs inhibits their activation effects on intestinal organoids. qPCR analysis of Lgr5, Lyz1, Muc2, ChgA and SI expression in healthy colon and small intestinal organoids co-cultured with activated MCs, and with activated MCs prestimulated with 1 mM NaB. Data expressed as mean (SD), n≥3. Statistical analyses were performed with a one-way Anova with Bonferroni correction (*=p<0,05, **=p<0,01, ***=p<0,001).

metabolize all the nutrients that cannot be directly digested by the host, thus producing the so-called "Short Chain Fatty Acids" (SCFAs).[139] SCFAs as NaB are

considered to be beneficial to host cells, thus improving differentiation, barrier function and epithelial defense.[140]
As previously demonstrated in this thesis (paragraph 6.1.1.1), MCs activation can be reduced by NaB stimulation, and this effect is maintained overtime for up to one week. (Figure 8 A and B).
To assess whether NaB prestimulation could alter the effect exerted on intestinal organoids by activated MCs, thus reverting to the normal situation, mature MCs were prestimulated with 1mM NaB 5 days before used for the co-culture with healthy colon and small intestinal organoids. The expression levels of some intestinal epithelial markers were evaluated by means of qPCR analysis. As demonstrated in Figure 23, the effect exerted by activated MCs both in the colon and small intestinal organoids is abolished when they are prestimulated with NaB. Taken together, these data demonstrate that activated MCs are responsible for the major alterations induced in intestinal organoids composition and organization and that these effects are reverted when MCs are prestimulated with microbial-derived products, such as NaB. This further highlights the beneficial role of NaB within the intestinal mucosa, and suggests that organoids are suitable for deeper study SCFAs roles in the GI tract.

6.2.1.8 TNFα could be involved in the effect exerted by the presence of MCs on the intestinal organoids organization

6.2.1.8.1 Preliminary data on healthy colon and small intestinal organoids

TNFα is known to exert an important role in the regulation of the balance between homeostasis and pathogenesis in the gut, since, depending on the microenvironment, it could mediate cell survival by triggering the NFkB pathway, as well as induce cell death.[276] As previously demonstrated in this thesis, MCs are a major source of TNFα, since it is both present as a pre-stored mediator in MCs cytoplasm but could also be *de novo* synthetized upon stimulation. In order to study if TNFα could be involved in the effects shown to be exerted by the presence of MCs on the intestinal organoids composition, qPCR of Lgr5 was performed in the healthy colon and small intestinal organoids co-cultured with resting or IgE/Ag activated wild type (wt) and TNFα-/- BMMCs. As demonstrated by the preliminary data reported in figure 24, also in presence of TNFα-/- BMMCs healthy small intestinal and colon organoids show a reduced expression of Lgr5, but it is significantly higher as compared to that exerted by wt BMMCs. Together, these data suggest that TNFα may be one of the mediators produced by MCs underlying the observed effect on the organization and architecture of intestinal organoids.

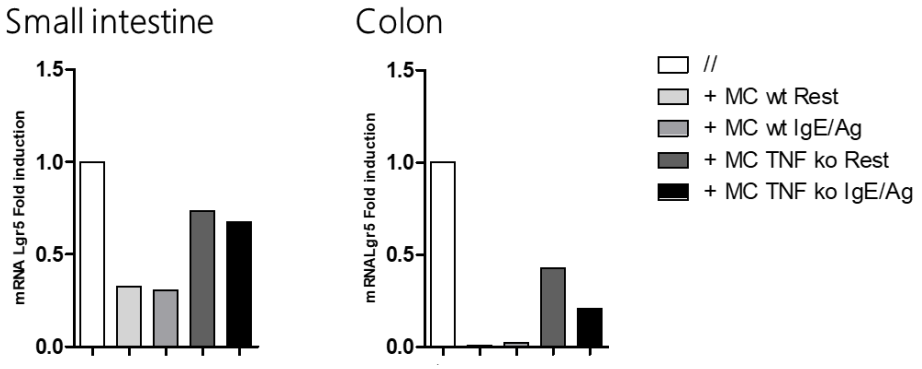


Figure 24: TNFα-/- BMMCs have a reduced ability to induce Lgr5 downregulation. qPCR analysis of Lgr5 expression in healthy colon and small intestinal organoids co-cultured with resting and activated MCs wt and TNFα-/-. Preliminary data, n=1.

6.2.1.9 Intestinal organoids express IL-33

IL-33 is a cytokine that has been associated with intestinal inflammation and CRC, and its receptor ST2 is shown to be increased in IBD.[277] It's a member of the IL-1 cytokines and it is famous for its dual role in maintaining homeostasis as well as in promoting inflammation. IL-33 is particularly relevant in the intestine where it is rapidly released from epithelial cells and fibroblasts in response to injury and tissue damage acting as an alarmin that initiates the immune response.[278]
Indeed, IL-33 and its receptor ST2 are known to be involved in the modulation of both innate and adaptive immune systems.[279] Upon barrier disruption, IL-33 is known to recruit innate immune cells which in turn are activated towards a type 2 inflammatory response with the primary goal of tissue regeneration,[280,281] but it is also known to activate intraepithelial lymphocytes, basophils eosinophils, and MCs.[282,283] Therefore, we asked whether our intestinal organoids expressed IL-33, and if so, to which extent relative to their specific healthy or pathological setting. Figure 25 demonstrates that the intestinal organoids used for the experiments of this thesis, even if in different amounts, express IL-33. In particular, Figure 25 A shows ΔCT of IL-33 mRNA and G3PDH in the healthy colon, healthy small intestine, DSS derived and AOM/DSS derived organoids. The present data demonstrate that IL-33 mRNA is expressed in all organoid cultures (note that lower values of ΔCT indicate higher expression of IL-33 mRNA). As a result, DSS and mostly AOM/DSS derived organoids respectively express IL-33 transcript, 6 and almost 50 times more than the healthy controls (Figure 25 B).
Collectively, these data demonstrate that our intestinal organoids express basal levels of IL-33 that is mostly enhanced in AOM/DSS derived organoids.

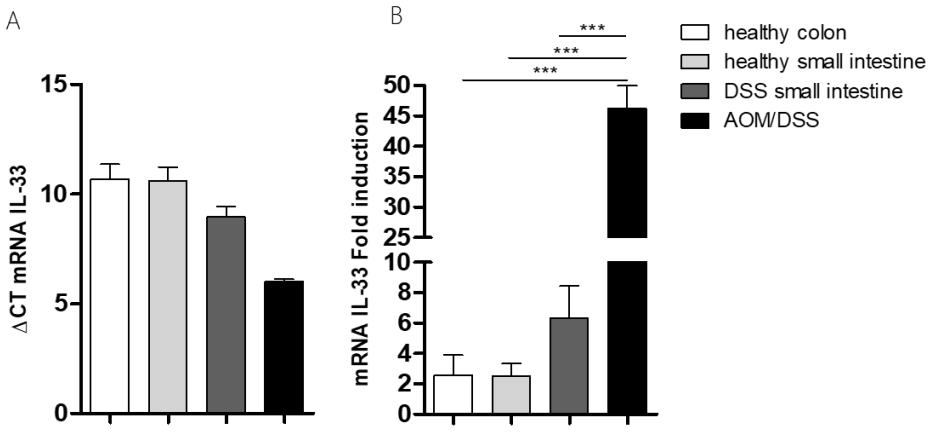


Figure 25: Intestinal organoids express IL-33. A. Comparison of ΔCT values of IL-33 mRNA in healthy colon, healthy small intestine, DSS, and AOM/DSS derived organoids. Note that lower values of ΔCT indicate higher mRNA expression. **B.** qPCR analysis of IL-33 in the healthy colon, healthy small intestine, DSS, and AOM/DSS derived organoids. n≥3, statistical analysis was performed with one-way ANOVA with Bonferroni correction (*=p<0,05, **=p<0,01, ***=p<0,001).

6.2.1.10 The presence of MCs affects the organization of human intestinal organoids

6.2.1.10.1 Preliminary data from human colon organoids-HMC-1 co-cultures

To replicate murine data in a human setting, co-cultures of human MCs and intestinal organoids have been set up. Human intestinal organoids were generated from stem cell-bearing crypts (Figure 26 A) of colon healthy tissue as described in Sato T. work published in 2011[231] with little modifications. Briefly, the healthy epithelium was isolated from other layers of fat and muscle, and after 1-hour incubation with the chelating agent EDTA, the mechanical release of crypts was induced. Stem-cell bearing crypts were counted and seeded in matrigel in presence of a complete WRENAS medium (see table 3, Materials and Methods). Since healthy tissue was isolated from the non-tumoral periphery of a CRC, some crypts were cultured in the total absence of Wnt in RENAS medium, to avoid any uncontrolled tumoral organoid formation. As a result, healthy organoids failed to grow in the RENAS medium (Figure 26 B), while formed long-term expanding stem colon organoid cultures in the WRENAS medium (Figure 26 C).

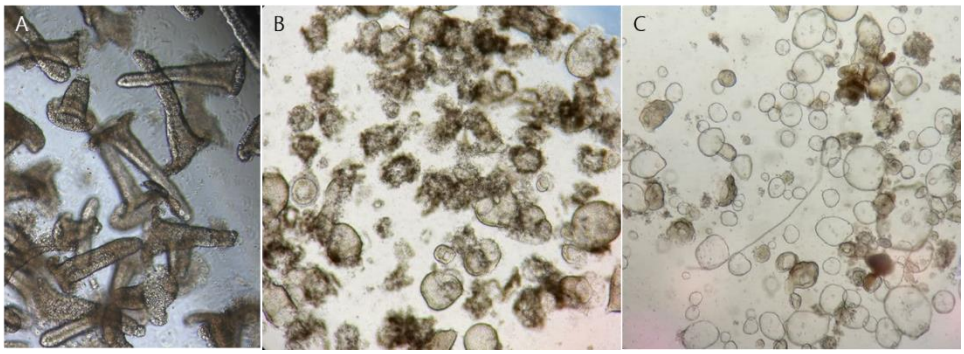


Figure 26: Establishment of a human colon organoid culture. A. Representative image of freshly isolated human colon crypts seeded in matrigel. **B.** human healthy colon organoids cultured in RENAS medium fail to grow, while **C.** healthy colon organoids cultured in WRENAS medium form long-term colon organoid cultures.

The HCM-1 cell line was used as a model of human MCs and co-cultures of MC and colon organoids were exploited in a type-2 co-culture system (Figure 17 A) with healthy colon organoids. Also, in this case, HMC-1 cells were resuspended in ENR medium and placed over the organoids for 48 hours. Then, non-interacting HMC-1 cells were thoroughly washed away, and the medium was replaced with fresh ENR medium for additional 24 hours. Finally, to see if comparable observations could be made also in human colon organoids-MCs co-cultures, the co-cultures were analyzed for the expression of the stem cell marker Olfm4 using IF staining (Figure 27). As shown in figure 27 A, when cultured alone, human colon organoids highly express Olfm4 throughout all their structure, without a specific localization of the stem crypts; when co-cultured with HMC-1 cells, human colon organoids polarize their structure, distinguishing the "crypt site" from the "body of the organoid" side (Figure 27 B). Indeed, Olfm4 expressing stem cells are restricted to the crypts when organoids are co-cultured with HMC-1 cells, as demonstrated by the high localization of this marker only in restricted sites of the organoids.
Taken together, these data confirm evidences derived from mouse experiments (Figure 20 C), and demonstrate that MCs are involved in the structural development of the healthy intestinal epithelium also in a human setting. Further studies will be needed to deepen analyze this relationship and to study MCs role also in human-derived CRC and IBD organoids.

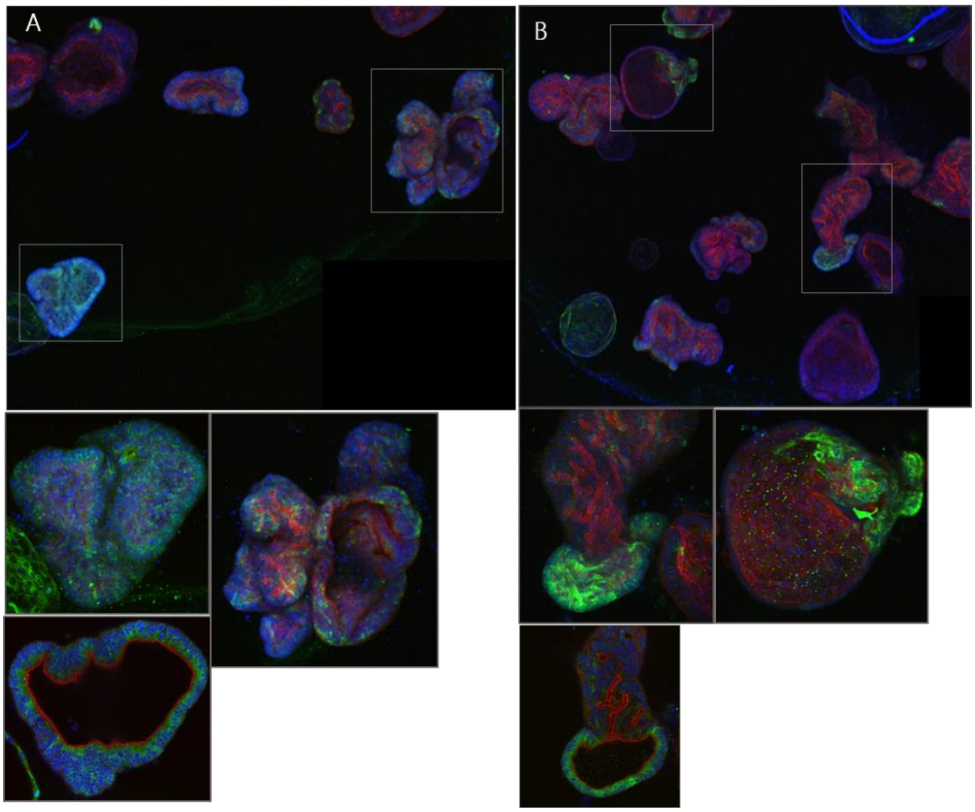


Figure 27: MCs have a role in human healthy colon organoids structural organization. A. IF staining of healthy human colon organoids cultured alone and **B.** co-cultured with HMC-1 cells in ENR medium. Olfm4 (green), actin (red), nuclei (blue).

7 DISCUSSION

MCs are innate immune cells characterized by striking plasticity. Starting from their biology, MCs are cells intrinsically sensitive to external stimuli, as their final maturation towards one phenotype rather than another is strongly influenced by the homing site. Moreover, MCs are fully equipped with an eclectic variety of receptors, and thus can be directly activated by a plethora of different stimuli. This plasticity and the strategic location in which they are normally found makes it easy to understand why MCs are involved in different roles, from the defense against pathogens to the maintenance of the tolerance and the crosstalk between the host and the microbiota. These characteristics make MCs capable of showing classic traits of trained immunity.

As a result, in the first part of this thesis, it has been demonstrated that differently primed MCs are differently susceptible to different infective and/or inflammatory stimulations. As others have demonstrated to occur in monocytes and Mϕ,[70,87,88,90] LPS primed MCs, showed a tolerant response to a second challenge with a low dose LPS. In particular, they significantly reduced both TNFα and IL-6 release after a second stimulation with LPS (Figure 8 D). On the other hand, a second stimulation with live *C. Albicans* was supposed to induce a training response in LPS primed MCs, with an enhanced release of both cytokines. *C. Albicans* restimulation of LPS primed MCs induced an enhanced response, thus suggesting a trend towards a training phenotype.

Recently, an increasing interest in the immunomodulatory effects of bacterial derivatives as the post-biotic product NaB on MCs activation has been reported.[141] Therefore NaB prestimulation effects on MCs were tested. Folkerts and colleagues[141] demonstrated that NaB strongly impairs both IgE- and non-IgE-dependent MCs degranulation after more than 12 hours of NaB stimulation, thus concluding that this mechanism preferentially relies on genetic remodeling rather than receptors rewiring. Conversely, reported data demonstrate that NaB stimulation inhibited BMMCs IgE- and non-IgE- dependent degranulation already after 30 minutes of stimulation, and that this inhibition is maintained for up to a week (Figure 8C). Moreover, NaB prestimulation was able to strongly impair the release of TNFα from MCs restimulated with l.d. LPS or IgE/Ag, but not of IL-6. These data confirm the known inhibitory effect on NaB on MCs activation, but also demonstrate that this effect is maintained in MCs for longer periods.

The dual role of LPS in inducing tolerance or training in MCs after a second stimulation with low dose LPS or live *C. Albicans* respectively is also evident at the transcriptional level. Indeed, after a short time stimulation, it is demonstrated that TNFα transcription is reduced after a second stimulation with LPS, while it is

strongly enhanced after a second stimulation with live *C. Albicans*, thus confirming the trend reported by the release of the cytokines (Figure 9).

Since specific MCs protease expression is also influenced by the stimulatory environment, another aspect that was further investigated, was if an LPS prestimulation could also be involved in a different MC proteases expression. As a result, it has been demonstrated that MCPT-4 transcription was strongly enhanced in LPS primed MCs (Figure 10). MCPT-4 is a MC protease normally found in the cytoplasm of connective tissue MCs, but it is also well-known for the anti-inflammatory role exerted. Indeed, this protease was found to be involved in the degradation of MC derived TNFα in a sepsis mouse model; thus, MCPT-4-deficient mice exhibited increased levels of intraperitoneal TNFα and higher numbers of peritoneal neutrophils. [284] Moreover, unlike mucosal MCs, connective tissue MCs are known to express high levels of TGFβ during fungal infections, thus contributing to mucosal immune tolerance,[285] but it is also studied that MCPT-4 is required for the cleavage and activation of TGFβ,[286] which in turn can activate a Th17 response that is known to be involved in fungal immunity.[287,288] Therefore, MCPT-4 could be in part contribute on one hand to the tolerogenic response of LPS prestimulated MC that show a reduced TNFα release after a second LPS stimulation, and on the other hand, it could be involved also in the enhanced response to *C. Albicans* infections. However, the exact mechanism orchestrating a tolerogenic rather than trained response in primed MCs remains unclear, as it appears to rely on a multi-layered process that most likely involves receptor signals rewiring, chromatin modification, and gene reprogramming.

This characteristic plasticity of the MC is of major importance in the gut where its encounter with the external environment and pathogenic stimuli is more likely to happen. Tissue-resident MCs in the gut are involved in multiple processes, like the regulation of the vascular and epithelial permeability, ion secretion, angiogenesis, peristalsis, fibrosis, tissue repair, defense, chemotaxis of immune cells (e.g. neutrophils), and nociception,[289] therefore, it's not surprising that MCs can be both, directly and indirectly, involved in various intestinal inflammatory pathologies. Notwithstanding, the exact role exerted from MCs in various intestinal pathologies from IBS to CRC, is still a matter of debate.

Therefore, the second part of this thesis aimed to shed light on the role of MCs in both healthy and pathologic intestinal settings, using intestinal organoids cultures. Since Toshiro Sato in 2009 for the first time described the possibility of growing self-organizing mini-organs resembling almost all the features showed by the tissue of origin starting only from a single $Lgr5^+$ intestinal stem cell, great strides have been made. Organoid technology, in particular, represents an innovative tool in cancer research as it allows the production of cultures from patient tumor samples, which constitute an avatar of the patient's tumor that can be then used for drug screening to find the right personalized therapy. [198,239,243,290] Another

aspect that lately is being studied more and more thanks to the support of organoids is the role of the immune system in the tumor microenvironment. Several works have been published on the role of adaptive immunity and of the innate immunity (mostly related to macrophages and NK cells) on healthy and tumoral derived organoids growth and development,[291–294] but, to date, nobody ever investigated the role of MCs in healthy and pathological organoids development. Hence, in the second part of this thesis, a method for the co-culture of healthy and pathologic murine intestinal organoids and BMMCs have been described. Healthy colon and small intestinal organoid cultures were obtained that correctly resembled the features of the tissue of origin, like the high expression of absorptive cells markers in the small intestine that is almost absent in the colon (Figure 15 C). More relevantly, for the pathological counterpart, organoids from pathological tissues were successfully generated. In particular, tumoral organoids were produced starting from adenomatous tissue of AOM/DSS treated mice that were mostly composed of stem cells, as demonstrated by the high expression of Lgr5, and which expressed low levels of Muc2 and ChgA, all well-known tumoral markers[257,261] (Figura 15 A). Furthermore, organoid cultures derived from DSS-treated mice were created, showing an intestinal epithelial markers expression profile typical of an inflamed epithelium[264,266,267] (Figure 15 B). Thus, organoid cultures resembling four different intestinal settings in mice have been generated and exploited: healthy colon, healthy small intestine, inflamed small intestine (DSS), and CRC (AOM/DSS).

To assess the interaction between MCs and the intestinal epithelium, mature BMMCs have been exploited for co-cultures with intestinal organoids. It has been demonstrated that BMMCs could interact with the basolateral side of intestinal organoids, namely the LP side, where MCs are normally found *in vivo* (Figure 16). Moreover, it was further demonstrated that MCs are attracted from the organoids, and can directly reach the epithelium by actively moving inside the matrigel (Figure 17). To date, no differences in the number of MCs actively interacting with healthy rather than tumoral organoids have been found. Nevertheless, it has been demonstrated that MCs generate a different response to healthy and pathological settings. BMMCs normally used for *in vitro* experiments express an intermediate phenotype between mucosal and connective tissue MCs, but we demonstrated that mature BMMCs co-cultured with healthy colon organoids preferentially polarize towards a mucosal phenotype as demonstrated by MCPT-2 upregulation. A comparable effect with healthy or DSS derived small intestinal organoids was not observed. A possible reason could be the large component of mucous-producing goblet cells which is highly represented in the colon but not in the small intestine and that might be eventually involved in a mucosal MC phenotype polarization. Strikingly, when co-cultured with AOM/DSS derived tumoral organoids BMMCs significantly upregulated the expression of both MCPT-2 and -

4, suggesting that BMMCs received a strongly activating stimulus from the tumor context, and consequently responded increasing the transcription of both proteases (Figure 21). The evidence that MCs accumulates and are activated in the tumor microenvironment is well-established knowledge.[166–169] Indeed, as demonstrated by the upregulation of the degranulation marker CD107a (LAMP-1), the co-culture with AOM/DSS organoids resulted in BMMCs activation and also proliferation (Figure 22). In this scenario, AOM/DSS derived IL-33 could be one conceivable responsible for BMMCs activation. As the literature reports, adenoma/CRC cells express high levels of IL-33 mRNA[295], and as demonstrated by figure 25 B, AOM/DSS derived organoids express 50 fold higher levels of IL-33 mRNA as compared to the healthy colon, even though restrained levels of IL-33 are expressed also by the healthy colon itself. Moreover, the enhanced expression of both MCPT-4 and -2 proteases by MCs co-cultured with AOM/DSS derived organoids, can be further exploited by the tumor itself to cleave and produce the mature form of IL-33[278]. Unfortunately, there are limitations in IL-33 released cytokine detection because, to date, there are no reliable ELISA kits capable to detect IL-33 release *in vitro*.[296]

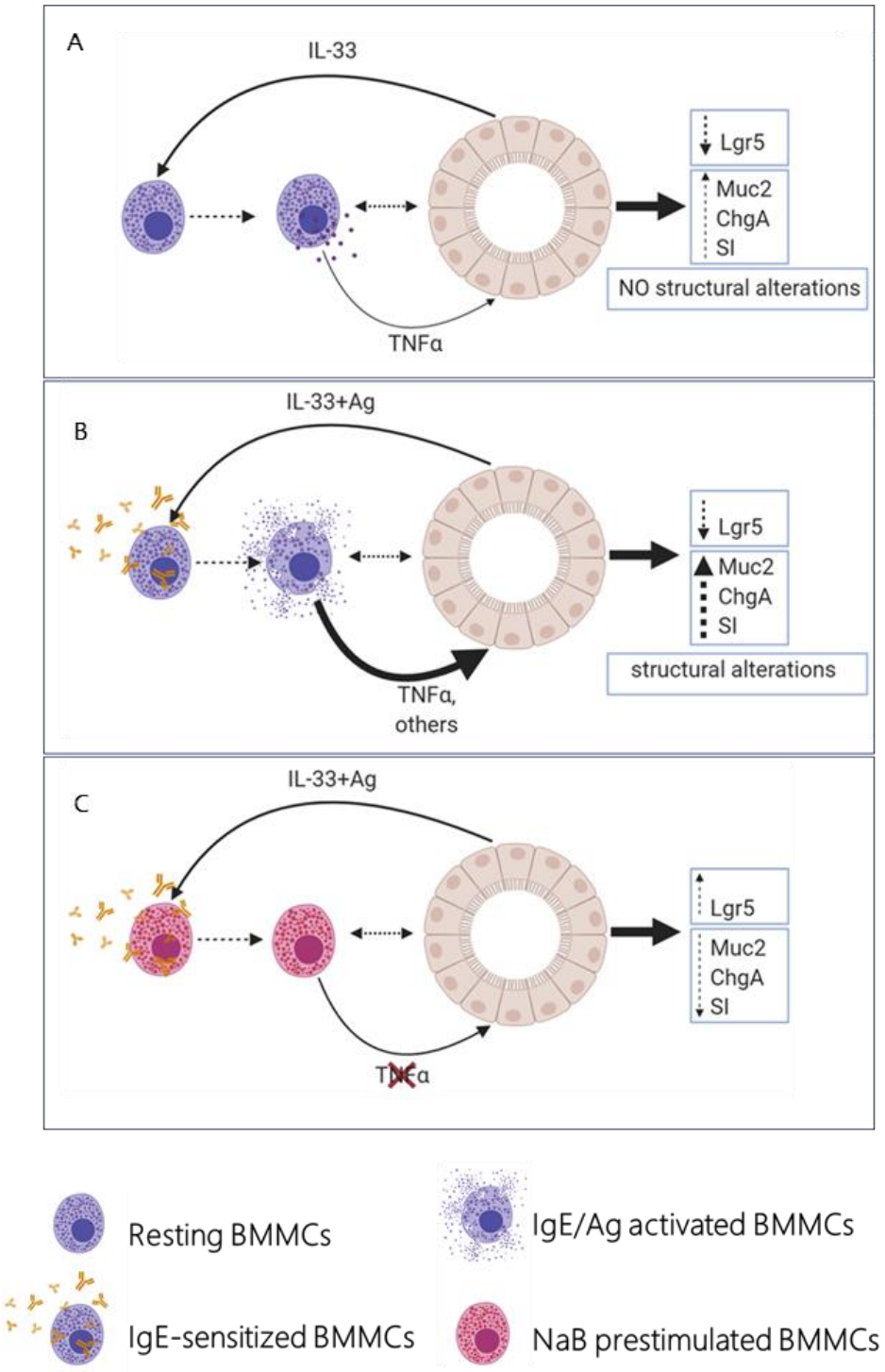


Figure 28: The proposed mechanism of BMMCs-intestinal organoids crosstalk involves IL-33 production and TNFα secretion. A. IL-33 expressing intestinal organoids induce BMMCs to release TNFα that is involved in the reduction of intestinal stem cell pool (Lgr5) and the increase of other intestinal epithelial markers (Muc2, ChgA, SI), but no structural alterations; **B.** a further IgE/Ag BMMCs activation causes the release of several other pre-stored molecules from the BMMCs that in turn induce the reduction of intestinal stem cell pool (Lgr5), the strong increase of other intestinal epithelial markers (Muc2, ChgA, SI) and structural alterations; **C.** TNFα release is inhibited in NaB prestimulated BMMCs, that in turn fail to induce a strong alteration of intestinal stem cell pool (Lgr5)and of other intestinal epithelial markers (Muc2, ChgA, SI).

There is the possibility that in the system described IL-33 in particular plays an important role in the observed effects induced by the crosstalk between intestinal organoids and MCs (Figure 28). As many published works demonstrated, IL-33 is an immunomodulatory cytokine strongly involved in molding the intestinal microenvironment, both in healthy homeostatic conditions and in pathological ones.[278,282,283,297] Mousumi Mahapatro and colleagues demonstrated an unexpected direct role of IL-33 in the reprogramming of intestinal epithelial stem cells into cells of the secretory lineage with strong innate immune functions (Paneth, Goblet, and Enteroendocrine cells). In brief, they demonstrated that after tissue damage, a specific population of pericryptal myofibroblasts release the alarmin IL-33, which directly induces the epithelium differentiation. This physiologic response of the intestinal epithelium to IL-33 secretion has the purpose of reinforcing the intestinal barrier to prevent possible damages resulting from the lesion of the epithelium (e.g. bacterial invasion).[298]

The first and clearest evidence that emerges from the reported data regarding BMMCs-intestinal organoids co-cultures, is the significant downregulation of the expression of the intestinal stem cell marker Lgr5 in presence of both resting and activated BMMCs. This effect appears to be independent of IgE-mediated activation, but to be rather induced by the sole presence of the BMMCs, in other words, something released by the organoid itself can activate BMMCs. Concerning healthy colon, healthy small intestinal and DSS derived organoids, moderate, but still relevant levels of IL-33 expression could be implicated in this interaction. Indeed, MCs constitutively express IL-33 receptors ST2 and therefore are known to be strongly activated by this cytokine.[299] Upon IL-33 stimulation MCs release a variety of mediators among which IL-13, IL-4,[300] IL-33 itself[301], and, more importantly, TNFα as many others have demonstrated.[300,302,303] In recent work, Moshe Biton and co-workers demonstrated that intestinal stem cells expressed specific receptors for Th cytokines like Interferone-γ (INFγ), IL-13, IL-4, IL-17a, IL-10, and IL-22. Moreover, they reported that the co-culture of small intestinal organoids with Th1, Th2 or Th17 cells, as well as the treatment with IL-13 or IL-17, decreased the intestinal stem cell pool; conversely, the co-culture with Tregs or the treatment with IL-10 or IL-22 had the opposite effect, expanding the stem cells pool. [301] As it was reported that healthy colon, healthy small intestine, and DSS derived organoids are characterized by moderate levels of IL-33 expression (Figure 25), it is tempting to speculate that these low but still relevant IL-33 expression levels could induce BMMCs activation and the consequent release of several cytokines among which TNFα seems to play a relevant role (Figure 28 A). As a result, a strong downregulation of Lgr5 expression was observed and further demonstrated by the IF staining of CD44 expression, showing that the intestinal stem cell pool in small intestinal organoids co-cultured with both resting and IgE/Ag BMMCs is confined to the crypt base, thus allowing the differentiation of

other intestinal epithelial cell types, as demonstrated by Aldolase B staining (Figure 20 C). Indeed, data derived from NaB pre-stimulated BMMCs indicate a major impaired release of this cytokine upon different secondary stimulations (e.g. LPS and IgE/Ag, Figure 8 D and G). This hypothesis is further supported by preliminary data derived from resting and IgE/Ag activated TNFα -/- BMMCs, which were able to induce only a reduced downregulation of Lgr5 expression both in the healthy colon and small intestinal organoids as compared to wt BMMCs (Figure 24). The consequence of a reduced intestinal stem cell pool is the expansion of the other intestinal epithelial cells. As a result, in presence of resting BMMCs, there is a slight increase of all the other markers analyzed of the secretory and absorptive lineage, namely Muc2, ChgA, and SI (Figure 28 A). These evidences suggest that the presence of resting BMMCs in the intestinal microenvironment could create a cytokine environment that drives the correct architectural organization and organoid development. As evidently demonstrated by the IF reported in Figure 18, further activation of BMMCs by IgE/Ag stimulation, conversely, caused a strong structural alteration in intestinal organoids as demonstrated by the delocalization of ZO-1, Ezrin, and Cldn4. This pattern of expression has been linked to altered function of the tissue, and in particular with an impaired permeability.[304–307] As a result, the observed significantly enhanced expression of all the analyzed secretory and absorptive markers, are essentially the reaction of the epithelium to a tissue damage, and it is aimed at maintaining barrier integrity and tissue homeostasis (Figure 28 B). Further study will be needed to assess organoids' altered functionality induced by BMMCs activation for example measuring the trans-epithelial resistance or utilizing FITC-Dextran permeability assay.

To summarize, it is hypothesized a mechanism in which intestinal organoids express low but detectable levels of IL-33 that can activate the co-cultured BMMCs. These are induced to release different factors among which TNFα, which is strongly involved in the maintenance of tissue homeostasis and the correct organization and polarization of the organoid (Figure 28 A). A further IgE/Ag-mediated activation of BMMCs induced a massive release of several mediators which in turn caused major structural alterations on the organoid cultures, that responded up-regulating intestinal epithelial markers of both secretory and absorptive lineage (Figure 28 B); as a result, when MCs are prestimulated with NaB, are refractory to both IgE-dependent and independent activation (Figure 8 G and D), and TNFα levels are not sufficient to induce any of the effect exerted by both resting and activated BMMCs, thus contributing to the maintenance of the epithelium in a more regenerative state (Figure 28 C).

Apart from the effect exerted on Lgr5 expression that resting and activated BMMCs induced in all the intestinal settings analyzed (healthy colon and small intestine, DSS and AOM/DSS derived organoids), a completely different effect of BMMCs on tumoral cultures was observed as compared to healthy and DSS

derived ones. With all probability, this could be linked to the fact that AOM/DSS cultures themselves activate BMMCs in an IgE-independent manner; thus this other way of activation, probably depending on IL-33, could prevail and drive the release of BMMCs mediators that have the effect of reducing both the expression of Lyz1 and ChgA (Figure 19). More studies are certainly needed to shed light on these effects; given the relevant role that IL-33 seems to play in this crosstalk, experiments with ST2 -/- mouse models or with IL-33/ST2 blockers would be useful to unravel its role. To conclude, MCs are homeostatic cells, that are defined as *an antenna* of the microenvironment.[9] Thus their overall effect is strongly influenced by the immune and inflammatory microenvironment of the tissue. Therefore, this co-culture system could be upgraded and other immune cells can be added to reconstitute organoids with more than one immune cell type. This would eventually help to shed light on innate and adaptive immune cells crosstalk with the intestinal epithelium in healthy and pathologic conditions. Finally, given the role of MCs activation in causing and perpetuating tissue damage in pathological settings of IBD, and considering the protective effect exerted by NaB on the intestinal epithelium and on MCs activation, the use of a diet capable of stimulating the microbiota to produce such metabolite could be considered in patients affected by inflammatory gut disorders.

8 MATERIALS AND METHODS

8.1 Mice

C57BL/6 mice were purchased from Envigo (Netherlands) and maintained at the animal facility of the Department of Medical Area (DAME) of the University of Udine (Italy). TNFα-/- femurs and tibiae were kindly gifted by Prof. Kollias (BSRC "Alexander Fleming", Vari, Greece). All animal experiments were performed in accordance to institutional guidelines and national law.

8.2 AOM/DSS treatment for the induction of colitis-associated colon cancer

For the induction of colitis-associated colon cancer, 8 weeks old C57BL/6 mice were given a single intraperitoneal injection of the mutagen azoxymethane (AOM, Sigma-Aldrich, 10 mg/kg body weight, diluted in saline) in combination with 3 cycles of 2.5% dextran sulfate sodium salt (DSS, MP Biomedicals; MW 36.000-50.000) in drinking water, followed by 14 days of recovery with normal drinking water. After the last recovery period, mice were euthanized and colon was extracted and analyzed for the presence of adenomas that were then used for the AOM/DSS organoid cultures.

8.3 DSS treatment for the induction of colitis

For the induction of colitis, 8 weeks old C57BL/6 mice were given 2.5-3% DSS dissolved in drinking water for 5 consecutive days, followed by a recovery period of 3 days with normal drinking water. At the end of the treatment, mice were euthanized and small intestines were extracted; part of the tissue was used for the DSS organoid cultures, and part was used for histologic analysis in collaboration with the pathological anatomy of the University Hospital Santa Maria della Misericordia of Udine.

8.4 BMMCs generation

Bone marrow derived mast cells (BMMCs) were obtained from 5- to 8- weeks old mice by *in vitro* differentiation of bone marrow derived progenitors obtained from mice femur and tibiae. Precursors were cultivated in complete medium supplemented with IL-3 (RPMI 1640 medium (Euroclone), 20% FBS (Sigma Aldrich), 100 U/ml Penicillin, 100 mg/ml Streptomycin, 2 mM Glutammine, 20 mM Hepes, 1X non-essential amino acids (from 100X mix, Euroclone), 1mM Sodium Pyruvate, 50 mM β-mercaptoethanol (Sigma Aldrich), 20 ng/ml IL-3 (Peprotech)) at 37°C and 5% CO_2 atmosphere. After 5 weeks, BMMCs differentiation and maturation was confirmed by flow cytometry by staining with anti-FcεRIa-PE (Biolegend) and anti-cKit-APC conjugated antibodies (invitrogen). Data were acquired with FACSCalibur cytofluorimeter and analysed with FlowJo software. BMMCs were usually ≥96% $cKit^+$ and $FcεRIa^+$.

8.5 BMMCs activation

Before the experiments, BMMCs were starved for 1 hour in IL-3 deficient complete RPMI medium (RPMI 1640 medium (Euroclone), 10% FBS (Sigma Aldrich), 100 U/ml Penicillin, 100 mg/ml Streptomycin, 2 mM Glutammine, 20 mM Hepes, 1X non-essential amino acids (from 100X mix, Euroclone), 1mM Sodium Pyruvate, 50 mM β-mercaptoethanol (Sigma Aldrich)).

For IgE-dependent activation, BMMCs were sensitized in IL-3 deficient complete medium for 2 to 3 hours with 1µg/ml of dinitrophenol (DNP)-specific IgE, washed twice and challenged with 100 ng/ml DNP (Sigma Aldrich).

For *C. Albicans* infections, BMMCs were stimulated with living *C. Albicans* yeast (1:1 ratio) at a final concentration of $2x10^6$ cells/ml in IL-3 deficient complete RPMI medium. 10 ng/ml Amphotericin B was added after 4h of culture to each well to limit fungal growth. RNA extraction was performed before Amphotericin B addition.

For the Trained Immunity (TI) experiments, BMMCs were differentiated for 6 weeks in a complete IL-3 RPMI medium and checked by cytometry for purity. BMMCs were left untreated or stimulated for 24h with 1 µg/ml LPS (LPS from E. Coli O55:E5, Sigma Aldrich), 10 µg/ml Curdlan (Invivogen), IgE/Ag (100 ng/ml DNP) or 1 mM NaB (Sigma Aldrich) in IL-3 free complete RPMI medium. After the stimulation, supernatants were collected and cells were put back in culture in fresh complete IL-3 RPMI medium for additional 6 days. Each cell subset was then re-stimulated with 100 ng/ml of LPS, live *C. Albicans* yeast (MOI=1), 10 µg/ml Curdlan or IgE/Ag for 24h.

8.6 BMMCs degranulation assay

BMMCs degranulation was determined as the percentage of β-hexosaminidase released and used as a functional test for IgE/Ag-dependent MCs activation. $0.5x10^6$ BMMCs were sensitized in complete RPMI medium for 3 hours with 1 µg/ml of DNP-specific IgE, then washed twice and resuspended in Tyrode's buffer (10 mM HEPES buffer [pH 7.4], 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 1mM MgCl2, 5.6 mM glucose, and 0.1% BSA). The enzymatic activity of the released β-hexosaminidase was assessed by the cleavage of its synthetic substrate (p-nitrophenyl N-acetyl- glucosamide, Sigma-Aldrich) in p-nitrophenol, measuring the p-nitrophenol absorbance at 405nm (in a plate spectrophotometer). Results are expressed as the percentage of β-hexosaminidase released in the supernatant over β-hexosaminidase retained in the cytoplasm.

8.7 Candida Albicans cultures

Wild-type *C. Albicans* SC5314 strain was a kind gift of Prof. Alessandra Arzese, University of Udine (Italy). Yeast was seeded on Sabouraud agar supplemented with 50 µg/ml Chloramphenicol and incubated at 30°C for 24h.

8.8 Cell cultures

HMC1 cell line was kindly gifted from Dr. Juan Rivera Niams-NIH. Cultured in complete RPMI medium (RPMI 1640 medium (Euroclone), 10% FBS (Sigma Aldrich), 100 U/ml Penicillin, 100 mg/ml Streptomycin, 2 mM Glutammine, 20 mM Hepes, 1X non-essential amino acids (from 100X mix, Euroclone), 1mM Sodium Pyruvate, 50 mM β-mercaptoethanol (Sigma Aldrich)).

8.9 Human samples

Colonic tissues were obtained upon surgical resection from the University Hospital Santa Maria della Misericordia of Udine. This study was approved by the ethical committee of University Hospital Santa Maria della Misericordia of Udine (CEUR-2017-PR-048-UNIUD) and all samples were obtained prior to informed consent.

8.10 Flow cytometry

Cells were harvested, washed with PBS and stained for 30 minutes at 4°C in the dark with monoclonal conjugated antibodies. Cells were then washed twice with PBS and acquired with a FACSCalibur flow cytometer. The antibodies used in the experiments performed in the present study are: anti-cKit-APC (Invitrogen), anti-FcεRIa-PE (Biolegend), anti-CD107a (LAMP1)-APC (Biolegend).

8.11 ELISA assay

Supernatans for cytokine quantitation were collected 24 h after BMMCs stimulation. Supernatants were assessed for the presence of TNFα and IL-6 using specific ELISA kits (eBioscience) according to manufacturer's instructions.

8.12 RNA extraction and real-time PCR analyses

Cells or organoids adequately released from matrigel were lysed with TRIreagent (Sigma-Merk) and total RNA was extracted with the phenol-chlorophorm protocol according to manufacturer's instructions. Total RNA was quantified using a NanoDrop™ spectrophotometer (ThermoFischer) and retro-transcribed with the SensiFAST™ cDNA Synthesis kit (Bioline). Quantitative PCR (qPCR) analyses were performed with SYBR Green (BioRad) using a BioRad iQ5 real-time PCR detection system. Target genes expression were quantified with the $\Delta\Delta C_t$ method using G3PDH (glyceraldehyde 3-phosphate dehydrogenase) as a normalizer. (see Table 1 for the list of primer used)

8.13 Mouse Organoid cultures

Organoids were generated from isolated crypts of murine healthy or DSS treated small intestine, healthy colon and AOM/DSS derived adenomas as described in [181] with little modifications. In brief, the whole intestine was extracted and subdivided into small intestine and colon. While the entire colon was processed, only the proximal section of the small intestine was used for the creation of organoid cultures. The intestinal lumen was washed with PBS+P/S (100 U/ml Penicillin, 100

mg/ml Streptomycin) in order to eliminate feces and to avoid contaminations. Then the tissues were opened longitudinally and the lumen was scraped with a glass in order to eliminate the villi (in small intestinal samples) and the excessive mucus (in colon samples). Regarding AOM/DSS colons, at this point the adenomas were counted, isolated from the adjacent healthy tissue and processed as described for the normal colon and healthy and DSS treated small intestine. After the removal of villi and mucus, the tissues were sliced into small fragments of 2-5 mm. The fragments were placed in a 15 ml conical tube with PBS+P/S and vigorously shacked in order to eliminate the remaining debris. The supernatant was removed and the process was repeated until the supernatant was clear. The tissues were then incubated in PBS+P/S+0,5 mM DTT (di-thiotreithol) + 5 mM EDTA for 1 h at 4°C in gentle shacking. Supernatant was removed and the pieces were resuspended in 2 ml of PBS+P/S+0,5 mM DTT and vigorously pipetted up and down for 10-15 times with a cut p1000 pipet tip in order to induce the release of the crypts in the supernatant. The crypts enriched supernatant was collected and placed in another conical tube with 2 ml of FBS. The process was repeated until a final volume of 10-12 ml was obtained. Crypts were centrifuged at 800 rpm for 3 minutes at 4°C and resuspended in cold GF- basal medium (Advanced DMEM/F12 with P/S, Glutamine and Hepes) and centrifuged again at 800 rpm for 3 minutes at 4°C. The pellet was carefully resuspended in GF- and mixed with matrigel (Corning) with 1:2 ratios and plated into pre-warmed 24 well greiner plates. (Usually mix crypts in 10µl of GF- with 20 µl of Matrigel and plate 3 x 10 µl drops in 24-well). Healthy small intestinal and colon organoids were maintained in complete WENR medium, DSS derived organoids in WENR medium, and AOM/DSS derived organoids in E medium (for medium composition see Table 2). The medium was changed every 2 or 3 days, and organoids were passaged once a week.

8.14 Mouse Organoid-BMMCs co-cultures

For the co-culture experiments with BMMCs, two different approaches were used:
A) BMMCs were starved for 3 h in complete RPMI medium with 10% FBS in resting conditions. After 3 h, BMMCs were washed twice in PBS, resuspended in GF- basal medium and mixed with already desegregated organoids. The co-culture was resuspended in matrigel and plated in a pre-warmed 24 well greiner plate, and cultured for up to 9 days in ENR medium.
B) Organoids were passaged and placed in complete WENR medium for 24 h. The next day, BMMCs were starved in complete RPMI medium with 10% FBS in resting conditions or sensitized with DNP-specific IgE. After 3 h, BMMCs were washed twice in PBS, resuspended in ENR medium and placed over the organoids-containing matrigel domes, at a ratio of 100.000 cells/matrigel dome. After 48 h, the medium was removed and BMMCs that didn't interact with organoids were pelleted and used for RNA extraction. Wells were then carefully washed with warm PBS in order to avoid matrigel disruption and to eliminate any non-interacting BMMCs from the co-culture. New ENR medium was added, supplemented where

needed with 100 ng/ml DNP to challenge IgE pre-sensitized BMMCs. After additional 24 h, medium was eliminated and organoids were washed several times in order to further eliminate residues of matrigel. Pellets were used for RNA extraction and qPCR analysis.

8.15 Human organoid cultures

The generation of human organoids from isolated crypts of healthy tissue was performed as previously described by Sato. T in 2011.[182] tissue was placed in a conical tube with ice-cold PBS + P/S and shaken 10 times. The tissue was allowed to settle down, the supernatant was removed and the process was repeated until the supernatant was clear. The tissue was then collected in a petri dish, and using scissors the epithelial layer was dissected from muscle and fat layers. The epithelium was then sliced in pieces of 2-5 mm. Tissue fragments were placed in a 15 ml conical tube with PBS + P/S + 0,5 mM DTT and pipetted up and down 8-10 times. Once the fragments settled down, the supernatant was removed and the process was repeated for 2-3 times until the supernatant no longer contained any visible debris. The fragments were then incubated 1 h at 4°C with PBS+P/S+0,5 mM DTT + 5 mM EDTA with gentle shaking. Supernatant was removed and the pieces were resuspended in 2 ml of PBS+P/S+0,5 mM DTT and vigorously pipetted up and down for 10-15 times with a cut p1000 pipet tip in order to induce the release of the crypts in the supernatant. The crypts enriched supernatant was collected and placed in another conical tube with 2 ml of FBS. The process was repeated until a final volume of 10-12 ml was obtained. Crypts were then counted and centrifuged at 800 rpm for 3 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in fresh GF- Basal medium and centrifuged once again. The cryps were finally seeded at the concentration needed (Usually mix 500 crypts in 10µl (50 crypts/µl) with 20 µl of Matrigel and plate 3 x 10 µl drops in 24-well). Normal organoids were cultured in complete WRENAS medium (see Table 3). The medium was changed every 2 or 3 days, and organoids were passaged once a week.

8.16 Human orgnanoids-HMC1 co-cultures

Organoids were passaged and cultured on ibiTreat µ-Slide 8 wells Chambered coverslip (ibidi) in complete WRENAS medium for 24 h. The next day, HMC1 cells were washed twice in PBS, resuspended in ENR medium (Table 3) and placed over the organoids-containing matrigel domes, at a ratio of 100.000 cells/matrigel dome. After 48 h, the medium was removed and wells were then carefully washed with warm PBS in order to avoid matrigel disruption and to eliminate any non-interacting HMC1 cell from the co-culture. New ENR medium was added and the co-culture was maintained for additional 24 h. Then, medium was eliminated and co-cultures were processed for Immunofluorescence staining.

8.17 Time-lapse images and fluorescence microscopy

BMMCs-organoids interaction was analysed by time-lapse microscopy using the Leica AF6000LX system (DMI6000-B microscope equipped with a DFC350FX

camera). Before the experiment, BMMCs were labelled with FAST DiO (Invitrogen) according to manufacturer's instructions. The plate was placed at 37°C in 5% CO_2 atmopshere. Co-cultures were maintained for up to 9 days and images were taken at different time points to assess the interaction. For time-lapse experiment, phase contrast images were taken every 10 seconds for a total of 6 minutes and resulting video-recorded movies were processed with LAS AF (Leica) software.

8.18 Immunofluorescence staining

For the immunofluorescence staining, Organoids-MCs co-cultures were performed on ibiTreat µ-Slide 8 wells chambered coverslip (ibidi). At the end of the co-culture, wells were washed three times in PBS for 5 minutes and fixed with 4% PFA (paraformaldehyde) for 20 minutes at RT. Then, wells were incubated for 20 minutes with NH_4Cl at RT. The permeabilization step was performed with PBS+TritonX-100 0,5% for 10 minutes at RT. Wells were then blocked for at least 1 h in PBS+1% FBS. The primary antibody was incubated O.N. in blocking buffer at the indicated concentration. The secondary antibody was used 1:400 and was incubated for 2 h in blocking buffer. Staining for actin filaments was performed with phalloidin-647 (ThermoFischer) in PBS for 20 minutes at RT: Finally, wells were incubated for 10 minutes in PBS with DAPI for nuclei staining. Each step was followed by 3 wash cycles with PBS for 5 minutes. Wells were maintained in PBS. The following antibodies were used: anti-Ezrin 1:100 (#E-AB-31393, Elabscience), anti-Claudin4 1:200 (E-AB-30945, Elabscience), anti-ZO-1 1:100 (#33-9100, ThermoFischer), anti-OLFM4 1:200 (#14369S, Cell Signaling Technology) Fluorescent images were collected using a laser scanning confocal microscope (LEICA TCS SP8, Leica Microsystems). Brightfield images were collected using a microscope (LEICA MC170 HD, Leica Microsystems).

8.19 Cell lysis and western blot analyses

Organoids were co-cultured with BMMCs as described in paragraph 8.15. At the end of the co-culture, organoids were carefully washed with ice cold PBS and adequately released from matrigel. Pellets were then washed twice with PBS and lysed in 25 µl of lysis buffer (25 mM Tris-HCl [pH7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, 1 mM Na_3VO_4, 50 mM NaF (Sigma Aldrich), and Complete Mini protease inhibitor cocktail (Roche, according to manufacturer's instructions)) for 30 minutes on ice, Lysates were then centrifuged at 12000 rpm for 20 minutes at 4°C. The protein concentration was determined by using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA).

Before western blot analysis, lysates were diluted with 4xLaemmli buffer and denaturated at 95°C for 7 minutes. Lysates were then separated on SDS 8-12% polyacrylamide gels and blotted on nitrocellulose membrane (Amersham) at 100 V and 300 mA for 90 minutes at 4°C. Blots were developed by using the NIR Fluorescence technology (LI-COR Biosciences). Images were acquired and quantified by using an Odyssey CLx Infrared Imaging System (LI-COR Biosciences).

Antibodies (clones, vendors): actin (C4, BD biosciences), anti-Claudin4 (E-AB-30945, Elabscience).

8.20 Statistical analysis

Experimental data are shown as mean ± standard error of mean (SEM). The unpaired or paired Student's t-test and the one-way or two-way ANOVA test (Prism, GraphPad Software, La Jolla, CS, USA) were used to analyse the results for statistical significance. A confidence level of 95% was used. *=p<0,05, **=p<0,01, ***=p<0,001.

Gene	Forward	Reverse
G3PDH	TCAACAGCAACTCCCACTCTTCC	ACCCTGTTGCTGTAGCCGTATTC
MCPT4	GGGCTGGAGCTGAGGAGATTA	GTCAACACAAATTGGCGGGT
MCPT2	AAGCTCACCAAGGCCTCAAC	ACACCACCAATAATCTCCTCAG
MCPT6	CGACATTGATAATGACGAGCCTC	ACAGGCTGTTTTCCACAATGG
TNFα	AGGCACTCCCCCAAAAGATG	CCATTTGGGAACTTCTCATCCC
Lgr5	AACATCAGTCAGCTACCCGC	CTAGGCGCAGGGATTGAAGG
Lyz	GTCTACAATCGTTGTGAGTTGGC	ATAGTCGGTGCTTCGGTCTC
Muc2	CTTCTGTGCCACCCTCGT	TTCGGGATCTGGCTTCTT
ChgA	CGATCCAGAAAGATGATGGTC	CGGAAGCCTCTGTCTTTCC
Cdh1	AACCCAAGCACGTATCAGGG	GAGTGTTGGGGGCATCATCA
SI	ATCCAGGTTCGAAGGAGAAGCACT	TTCGCTTGAATGCTGTGTGTTCCG
Cldn4	ACACGTTACTCCAGCGCTAC	CTCTCAATGGCCCCTCAGTC

Table 1: murine primer for qPCR analysis

Reagent	WENR	ENR	E
BASAL MEDIUM (GF-)	1X	1X	1X
B27	1X	1X	1X
N-acetylcysteine	1.25mM	1.25mM	1.25mM
Y-27632	10uM	10uM	10uM
Primocin	100ug/ml	100ug/ml	100ug/ml
EGF	50ng/ml	50ng/ml	50ng/ml
Noggin conditioned medium	10%	10%	
R-spondin conditioned medium	20%	20%	
Wnt conditioned medium	50%		

Table 2: Murine organoid culture media

Reagent	WRENAS	RENAS
BASAL MEDIUM (GF-)	1X	1X
B27	1X	1X
N-acetylcysteine	1.25mM	1.25mM
Nicotinamide	10mM	10mM
GastrinI	10nM	10nM
PGE2	10nM	10nM
Y-27632	10uM	10uM
Primocin	100ug/ml	100ug/ml
A83-01	500nM	500nM
Noggin conditioned medium	10%	10%
SB202190	3uM	3uM
EGF	50ng/ml	50ng/ml
R-spondin conditioned medium	20%	20%
Wnt conditioned medium	50%	

Table 3: Human organoid culture media

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