UNIVERSITÀ DEGLI STUDI DI MILANO

Scienze Biomediche Cliniche e Sperimentali

Dipartimento di Biotecnologie Mediche e Medicina Traslazionale (BIOMETRA)

CORSO DI DOTTORATO DI RICERCA IN

PATOLOGIA E NEUROPATOLOGIA SPERIMENTALI, CICLO XXVIII

MED/04

Role of intestinal CX3CR1^{+} macrophages in the development of colitis-associated cancer: focus on the relevance of the microbiome and heme-oxygenase-1

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2014/2015

Abstract

Gut represents a complex landscape in which commensal bacteria, harmless antigens and food proteins are strictly in contact with the immune system that has to be able to maintain a balance between the immune response and tolerance. In this context, mononuclear phagocytes are the most abundant population and macrophages represent a key player in this process of discrimination by allowing a peaceful coexistence. If this crucial checkpoint is lost, immune system is activated and falls in a dangerous and prolonged inflammation. Recent studies have described $CX3CR1^{+}$ cells as a population of resident macrophages able to sample the gut lumen and to produce the anti-inflammatory cytokine IL-10 in order to maintain homeostasis. These macrophages are characterized by the expression of the chemokine receptor CX3CR1; however, how this receptor is involved in the molecular mechanisms that regulate the inflammatory response is largely unknown.

The global aim of this thesis is to define the role of mucosal $CX3CR1^{+}$ macrophages during acute and chronic inflammation and in the process of intestinal carcinogenesis, and to investigate the molecular mechanisms by which these immune cells maintain gut homeostasis.

We analyzed the behavior of these macrophages in mice lacking the CX3CR1 receptor ($CX3CR1^{GFP/GFP}$) and in wild type heterozygous mice ($CX3CR1^{GFP/+}$). Firstly, we characterized them as a macrophagic population expressing CX3CR1, F4/80, CD11b and CD64 confirming the data present in literature.

As the behavior of these cells is less defined during the resolution of inflammation, we performed a model of recovery from colitis and we found that KO mice displayed higher signs of inflammation compared to WT mice, and they were not able to recover as WT mice did. From the molecular point of view, we found that the inflammatory mediators were up-regulated in KO mice as well as members of the IL-10 family cytokines. The only mediator resulting down-regulated in KO mice was heme-oxygenase-1 (hemox-1).

Hemox-1 is an anti-inflammatory enzyme over-expressed during tissue injury that is able to promote bacteria clearance. This regulation of this molecule is very complex and involves also IL-10. However, despite KO mice produced more IL-10, in an attempt to switch off inflammation, they were not able to produce an adequate amount of hemox-1.

We reproduced, *in vitro*, the model of colitis and we showed that macrophages from KO mice responded to the inflammatory stimulus (LPS) with an aberrant response, up-regulating both pro and anti-inflammatory mediators, but not hemox-1. Moreover, we observed a synergism between LPS and the chemokine Fracktalkine/CX3CL1 (FKN) in the production of hemox-1 in WT mice and also found

that stimulation with FKN alone was sufficient to produce hemox-1 in WT mice, unveiling a previously unidentified role of the chemokine receptor CX3CR1 in the regulation of hemox-1.

Using the AOM/DSS model of colitis-induced carcinogenesis (CAC), a study that has never been performed in these genetically modified mice, we found significantly higher signs of inflammation in KO mice in terms of both cytokine production and immune cell infiltration, as well as higher score of tissue damage and number of polyps. Also in the CAC model, KO mice attempt to switch off inflammation by over-producing IL-10 levels; furthermore, hemox-1 was under-expressed. We therefore decided to enhance hemox-1 production using cobalt protoporphyrin IX (coPP), a compound able to stimulate hemox-1 in the intestine. The experiments revealed that treatment with coPP was able to revert the phenotype in KO mice, resulting in marked lower inflammation and reduced tumor load. To corroborate these findings, we investigated a genetic model of intestinal tumorigenesis (APC^{min} mice) and generated APC^{min} $CX3CR1^{GFP/+}$ WT mice and APC^{min} $CX3CR1^{GFP/GFP}$ KO mice. Also in this tumor model, mice deficient for the CX3CR1 receptor demonstrated higher inflammation, lower Hemox-1 and more extensive tumorigenesis, thus overall confirming the results obtained with the sporadic CAC model.

Hemox-1 acts on macrophages by stimulating the processes of phagocytosis; accordingly, we found that bacteria phagocytosis was significantly impaired in KO mice. The sequencing of the microbiota in the stools of mice treated with the AOM-DSS model, revealed a different microbiota composition, in particular, *Akkermansia* were down represented in KO mice. *Akkermansia* is reported to be impaired during inflammatory bowel diseases, leading to the rise of other bacteria and to an overall dysregulation of the total microbiome composition. This dysbiosis might be involved in disease relapse and rise of chronic inflammation that can lead to cancer development.

Overall, we unveiled a new pathway by which intestinal $CX3CR1^{+}$ macrophages operate to maintain gut homeostasis. If the CX3CL1-CX3CR1 checkpoint is lost, the FKN-induced production of hemox-1 is missing and an aberrant inflammatory response could persist and be corroborated by a state of dysbiosis. In this landscape, chronic inflammation and the impaired resolution could, over time, promote tumor development. Finally, we confirmed that treatment with the chemical compound coPP up-regulates hemox-1 also in the absence of functional CX3CR1 receptor, and drastically ameliorates the intestinal disease. These findings open a new scenario in the use of hemox-1-inducing drugs to restore the correct intestinal homeostasis in a therapeutic context.

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Introduction

Mucosal immunology

The intestine is an exclusive tissue able to maintain balance between the immune system activation and the tolerance against the very huge amount of harmless antigens that are in contact with the intestinal lumen, including food antigens and microflora.

It is fundamental for our health that our immune system is able to respond with a robust and appropriate response to potential dangerous antigens, but the same reaction against harmless antigens, like commensal bacteria or dietary proteins, could be very dangerous and could give rise to allergies or chronic inflammation that, in the end, can lead to tissue damage or cancer development.

Many immune cells participate to the maintenance of the homeostasis in gut, with the role of understanding how an antigen is a potential danger for the organism and being silence in response to commensal bacteria and food antigens. This complex landscape includes T and B lymphocytes, natural killer cells, innate lymphoid cells, eosinophils, mononuclear phagocytes among which dendritic cells and macrophages.

Innate lymphoid cells (ILCs) are a subset of immune cells of lymphoid origin that, differently from lymphocytes, do not express antigen-specific receptors but act quickly upon cytokine stimulation. There are three different subpopulations of ILCs that mirror the T helper classification: ILC1, ILC2, and ILC3. ILC1s are present in the lamina propria and, as Th1 lymphocytes, act *via* T-bet; they are able to produce TNF-α and IFN-γ in response to intracellular pathogens. ILC2s are involved in the response against helminth and in the allergic reactions and are implicated in wound healing. They mirror the Th2 response and they activate GATA-3, upon stimulation by IL-25 and IL-33, with the consequently production of IL-4, IL-5, IL-13, IL-9 and IL-6. ILC3s are able to activate RORγt and show a heterogeneous behaviour. They produce IL-17a, IL-22 and TNF-α and are involved in the control of commensal bacteria and in the repair of lymphoid tissue. ILC3s are the most abundant ILCs in the gut. They are able to interact with epithelial cells and other mucosal immune cells in order to cooperate in

the control of gut homeostasis. Bacteria in the lumen activate epithelial cells and CX3CR1$^+$ macrophages (Mφ) that sample the lumen and play a key role in the maintenance of homeostasis. However, microbe-associated molecular patterns (MAMPs) can migrate into the Payer's patches and can activate CD103$^+$ DCs that migrate into the lymph node where they activate B and T cells. ILC3s actively participate to the formation of Payer's patches and isolated lymphoid follicle (ILFs). Here APC, T and B lymphocytes orchestrate the immune response. In this context, ILC3s are able to induce B cells in the ILF to produce IgA, with consequent mucosal protection [1]. IgA are produced by B lymphocytes that, in the intestine are specialized in the production of this type of immunoglobulin. IgAs are secreted and act as a wall to protect the intestinal epithelium from antigens. Here, IgAs can bind microbiota and food antigens, reducing their access to the bloodstream thus being able to control microbiota. Recent works have demonstrated that IL-10 and TGF-β can promote IgA secretion in the gut [2]. Moreover, IL-10 a key factor in the maintenance of the gut homeostasis, can be produced also by particular B cells called Breg. Breg production of IL-10 is an important trigger for Treg generation [3].

In the gut, the T cell mediated response plays a crucial role: when the epithelium is damaged, several molecules are released in order to activate the correct immune reaction and to protect the mucosa. Naïve lymphocytes are recruited from thymus and bone marrow and are able to enter in contact with the gastrointestinal tract *via* GALT (gut-associated lymphoid tissue). This process is mediated by the expression of complementary trafficking receptor on both endothelial and immune cells among which CCR7-CCL21, CXCR4-CXCL12, CXCR5-CXCL13: CCR9-CCL25 [4]. Once arrived in the secondary lymphoid tissues T and B naïve cells enter in contact with APC and are available for activation. The temporary expression of chemokines and chemokine receptors is fundamental for gut homeostasis: for example, the CCR6-CCL20 axis is able to recruit T lymphocytes into the lamina propria. In particular CCR6 is expressed mostly by Th17 lymphocytes. Of note, CCL20 is expressed by the intestinal epithelium only during inflammation. Instead, in healthy tissue, CCL20 expression is negligible, highlighting the fine temporary and spatial regulation of lymphocytes recruitment [5]. Another trigger example is given by IL-33. This cytokine is a member of the IL-1 family and can act as alarmin inducing the mucosa wound healing or can act as a potent inducer of inflammation. Recent studies have shown how this mechanism occurs: If IL-33 is localized in the nucleus, it is able to act as alarmin, otherwise, the loss of nuclear sequestration leads IL-33

to be released into the systemic circulation, inducing inflammation *via* activation of Th2 responses [6]. Indeed Th2 lymphocytes express the IL-33 receptor ST2 and its signalling induce the typical Th2 transcription factor GATA-3. An additional key factor in Th2 response is adenosine and its receptor A2BAR. It has been shown that the absence of this receptor impairs the Th2 cascade decreasing the recruitment of M2 Mφ and eosinophils.

Another important T subpopulation is composed by T regulatory cells (Treg) characterized by the expression of CD4, FOXP3 and CD25. It is important to note that Treg can modulate the immune response in gut not only acting in the lamina propria but also inhibiting T cell activation in lymph nodes. Treg express high level of ST2 being able to sense IL-33 that plays as alarmin. During inflammation, IL-33 is able to activate Treg cells in order to restrain intestinal inflammation. In this context it has been shown that IL-2 produced by APC is able to increase Treg response to IL-33 while IL-23 is able to limit their function highlighting the complex regulation of the inflammatory response in gut [7].

Overall, it is evident that the intestinal immune system plays a complex and crucial role in the maintenance of this balance. In this landscape, mononuclear phagocytes have a central part in the process of discrimination and allow a peaceful coexistence. It is important to note that these cells, and in particular macrophages, are able to maintain the immune system silent but, in case of need, they are able to trigger and to sustain the immune response (figure 1).

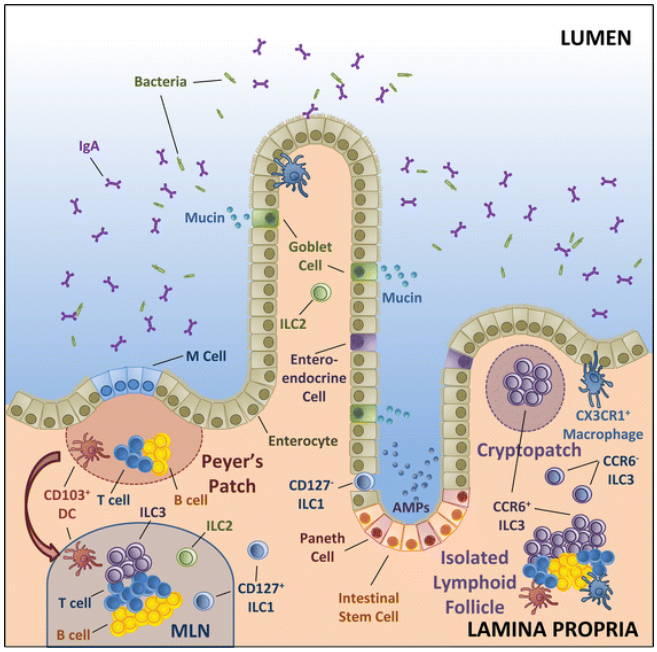


Fig.1: Many immune cells participate in the maintenance of homeostasis in the intestine [1]

Characterization of mononuclear phagocytes in gastrointestinal tract

Mononuclear phagocytes can be detected in the entire gastrointestinal tract, in particular in the *lamina propria*, close to the epithelial monolayer [8]. Two main populations of mononuclear phagocytes are present in gut: dendritic cells (DC) and macrophages (Mφ) and they represent the most abundant leukocyte population in the gut and the largest population of macrophages in the entire body. Unfortunately, it is not easy to classify these cells into a specific category. It is important to be able to distinguish between the different subsets of DC or Mφ, in order to study the ways by which they can maintain homeostasis or switch to inflammatory programs. If we can do it, we will be able to clearly identify a specific subset and, consequently, to study its mechanism of action and, in the end, to target it. For this reason, many efforts have been done in order to identify surface markers able to distinguish between DC and Mφ.

Considering mice, two different markers are used to differentiate mononuclear subsets: CX3CR1 for macrophages and CD103 for dendritic cells. CX3CR1 expression is not a feature of all tissue resident macrophages, for instance, Langherans cells, lung alveolar or epidermal macrophages do not express the receptor, on the contrary, microglia in the central nervous system and kidney macrophages do it [9]. Moreover, resident macrophages do not always express the same level of CX3CR1: it has been reported [10] that a population of $CX3CR1^{int}$ macrophages is able to expand during colitis. Bain and colleagues demonstrated that $Ly6C^{hi}$ monocytes give rise to all different types of macrophages in healthy colon and during colitis and that $CX3CR1^{int}$ cells represent a pool able to generate inflammatory or resident macrophages, depending on signals in the milieu. Similarly, in another study, Bain et al. [8] focused on the DC marker CD103, which can be used to define DC subsets in the gastrointestinal tract, expressing or not CD103. Considering this complex landscape, it is difficult to properly define the different subpopulations of mononuclear phagocytes present in the intestine. For this reason, other markers are used to characterize DC and Mφ. For instance, for resident macrophages, CD64 can be used in combination with CD11b, F4/80 and CX3CR1 [10, 11]. Fortunately, this classification seems to be valid also for human phagocytes. In particular, in association with classical markers such as CD68, CD163 and MHCII, CX3CR1 has been shown to be expressed by intestinal macrophages even in humans.

Macrophages can also be classified on the basis of their functional polarization into two major subtypes: M1-classicaly activated- or M2-alternatively activated. M1 macrophages are pro-inflammatory and sustain immune response, while M2 macrophages limit inflammation. However, this classification is difficult to apply to intestinal resident macrophages: they fail to express arginase that is considered a paradigm of M2 polarization and they produce IL-10 together with TNFα [12]. Despite this, M1 and M2 classification do not want to be a narrow organization, but describe a spectrum of *a continuum* that, sometimes, can be hard to be translated *in vivo*.

Ontogeny of resident macrophages

Macrophages in the intestinal mucosa are considered mononuclear phagocytes that originate from a common myeloid progenitor which can differentiate also in dendritic cells or granulocytes. This progenitor gives rise to a common precursor of both DCs and monocytes [13]. In mice, monocytes in the intestine were initially divided into two different subsets, depending of the expression of the combination of two markers: Ly6C^{hi}-CCR2^{hi} monocytes were considered inflammatory cells while Ly6C^{low}-CX3CR1^{hi} monocytes were considered the precursors of resident macrophages. Nowadays this classification is considered obsolete and not more valid. The majority of monocytes in blood express high levels of Ly6C and CCR2 and it has been shown that mice lacking the CX3CR1 gene do not fail to accumulate macrophages in tissues. Therefore, it has been proposed that *classical* monocytes (Ly6C^{high}) can generate both inflammatory and resident macrophages while *non-classical* monocytes expressing low levels of Ly6C and CCR2 but high level of CX3CR1 are proposed to maintain the vasculature integrity and to phagocyte in the blood stream [14-16]. In humans it has been reported the same behaviour of macrophage precursors and gene expression analyses revealed many parallels between human and mouse monocytes [17].

Monocytes in the gut have a different origin compared to precursors in other tissues. Many studies showed that lung alveolar Mφ, Langherans cells, microglia, Kupffer cells and Mφ of the peritoneal cavity derives from embryonic precursors. In yolk sac or the fetal liver and colonize tissue early in development and maintain themselves during life by *in situ*

proliferation [9, 18, 19]. Bain et al., [10] demonstrated that intestinal mouse macrophages are continuously replenished from blood stream monocytes. He showed that Ly6C^{hi} monocytes are recruited in the mucosa and here, in five days, they differentiate in mature macrophages progressively loosing Ly6C and starting to express F4/80, CD64, MHCII and CX3CR1. In the same way he demonstrated that macrophages in the adult gut have a very poor proliferative rate (figure 2).

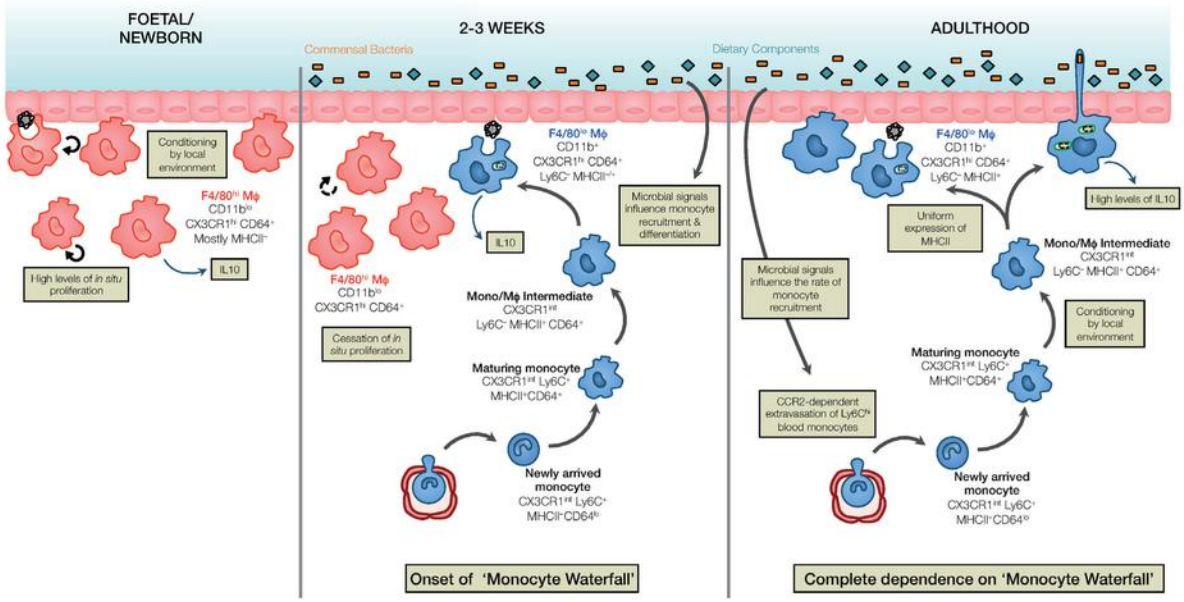


Fig.2: Ontogeny of mucosal macrophages [8]

For their recruitment monocytes need the CCR2-CCL2 axis. It has been shown that mice lacking either CCR2 or CCL2 fail to accumulate macrophages in the mucosa. However it is not completely clear how monocytes are recruited in the adult mucosa as CCR2$^{-/-}$ or CCL2$^{-/-}$ mice are not totally devoid of intestinal macrophages [20]. It has been proposed that other mediators such as TGF-β or CXC-chemokines can have an active role in this process but further studies are needed to better clarify this issue [21]. However, the meaning of this continuous replenishment is still under investigation: why does the mucosa need to incessantly replace its mononuclear phagocytes is unknown. A possibility is that this change is required to constantly monitor the persistent exposure to the commensal bacteria and food antigens; in fact, recent studies have reported that germ free mice show an impaired number of

macrophages in the colonic mucosa [22] highlighting the importance of controlling the relationship between homeostasis and microbiota.

Function of resident macrophages in the healthy mucosa

Resident macrophages are located in the *lamina propria,* in tight contact with the enterocytes that compose the crypts. As other tissue macrophages, in the gut, the main role of these cells is to execute several housekeeping functions as tissue remodelling or clearance of apoptotic cells. Moreover intestinal macrophages are able to produce several factors among which PGE2 that have a central role in the maintenance of tissue homeostasis allowing the differentiation of local epithelial progenitors to enterocytes of the crypts, regulating, in this way, the integrity of the epithelial wall [23]. Resident macrophages also play an important role in the phagocytosis of luminal bacteria and they show an efficient bactericidal capability [24]. Considering that resident macrophages express high levels of MHCII, it has been proposed that they can serve as antigen-presenting cells. Rescigno et al., demonstrated the role of CX3CR1 cells in the activation of CD4 T lymphocytes: Mφ can not migrate to the lymph node but CX3CR1 is fundamental to capture and to transmit the antigen to the dendritic cells that are able to migrate to mesenteric lymph nodes (MLN) [25]. In addition to this, they express high levels of MHCII but the exposure to antigens or bacteria does not trigger any pro-inflammatory response in these cells. This is achieved by the production of the anti-inflammatory cytokine IL-10. As a matter of fact, gut resident mecrophages in steady state produce both IL-10 and TNFα, thus maintaining the immune system in a state of "*alert anergia*", unable to trigger a pro-inflammatory response but predisposed to be activated in case of emergency. In support to this evidence, germ free mice produce less amount of IL-10 [22].

Considering that many other tissue macrophages do not express CX3CR1, it seems clear that this receptor is induced by the local microenvironment of the gut and that it plays a central role for intestinal macrophages. Which could be the process inducing CX3CR1 is still controversial: it has been proposed that TGF-β could be a candidate as well as CX3CL1. However, recent data demonstrated that $CX3CR1^{-/-}$ and WT mice have the same anatomical distribution of intestinal macrophages, excluding the CX3CR1-CX3CL1 axis in this type of

regulation [26]. On the contrary, TGF-β is very abundant in steady state but it seems that its main role is the maintenance of the hyporesponsiveness to different Toll like receptors (TLRs) in the healthy mucosa [27]. However, gut macrophages express normal levels of TLRs in both mice and humans. Many efforts are done in order to better understand the mechanisms behind the unresponsiveness of these cells. The main hypothesis is that the molecule downstream of the TLR could be the target of the regulation. In fact, if MyD88 is downregulated in intestinal resident macrophages, the molecules that inhibit the TLR signalling seem to be upregulated in these cells [28, 29].

Another function of intestinal Mφ, recently proposed, is that these cells facilitate the secondary expansion of T regulatory cells by the production of IL-10 [30]. Finally, it has been proposed that CX3CR1 is required for transepithelial dendrites (TED) formation. The mechanism proposed is that intestinal macrophages can acquire luminal bacteria by extruding TED across the epithelial barrier and maintaining homeostasis by the production of IL-10. However, recent studies were in contrast with these results and demonstrated that there were no TED in the healthy mucosa and that the uptake of lumen antigen was independent of the CX3CR1-CX3CL1 axis [31-33].

What seems to be a robust founding is that intestinal macrophages are able to maintain homeostasis in the gut by the production of IL-10. Recently, Zigmond et al., demonstrated that IL-10R is fundamental for gut Mφ to sense IL-10. If the receptor is lacking, these cells are able to produce but not to sense IL-10. It has been proposed that an IL-10-based autocrine loop acts directly on CX3CR1 intestinal macrophages. Consequently, if cells are not able to detect IL-10, the autocrine mechanism of regulation is lost and inflammation can start [34]. Importantly, also in humans, IL-10R deficiency is associated with a severe form of intestinal bowel disease (IBD) [35].

Macrophages during intestinal inflammation

The intestinal leukocyte composition changes drastically during inflammation and, in particular, the mononuclear phagocytes play a crucial role in both the early events of inflammation and in the processes of resolution. For instance in IBD, in both ulcerative colitis

and Crohn's disease, macrophages are the most abundant population in the mucosa. To understand how monocytes and macrophages change their properties during colitis is fundamental in order to target these cells for therapeutic purposes.

In many different mouse models of colitis, is reported that, during inflammation, there is a reversion in the ratio of CX3CR1^{hi} and CX3CR1^{int} macrophages. The CX3CR1^{int} Mφ have markedly a pro-inflammatory signature: they are able to produce many inflammatory cytokines as IL1β, IL6 and IL12 and they express the chemokine receptor CCR2, which allow Ly6C^{hi} precursor to enter the tissue and to differentiate into CX3CR1^{int} macrophages [28].

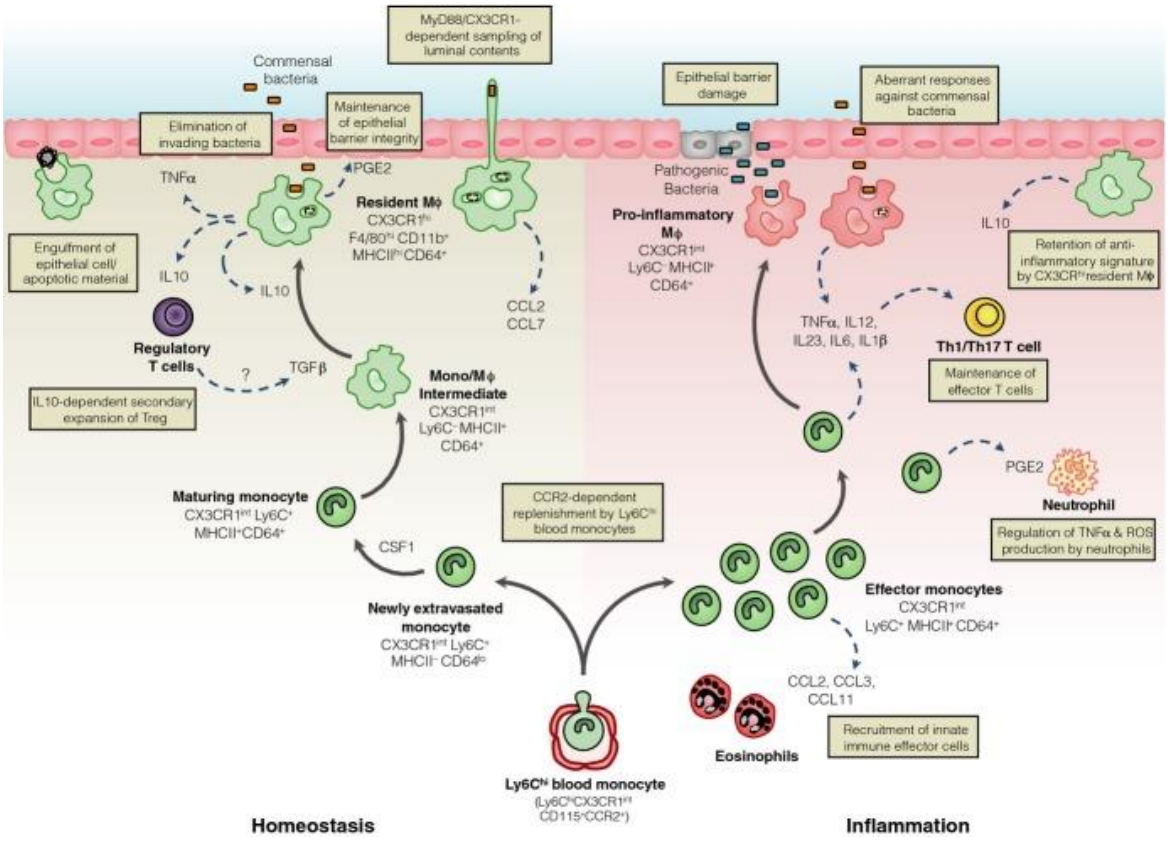


Fig.3: Changes in macrophage landscape during homeostasis and inflammation [8].

However, as discussed above, Ly6C^{hi} monocytes are the same precursor of both pro-inflammatory Mφ and resident Mφ. Bain and Mowat [8] proposed that during colitis the normal monocyte differentiation process is stopped resulting in the reversed ratio between pro

and anti-inflammatory macrophages. Interestingly, resident macrophages are not influenced by the change in the microenvironment and continue to exert their anti-inflammatory behaviour being unresponsiveness to TLRs stimuli [12]. When the inflammatory insult is finished, homeostasis must be restored in order to avoid the emergence of chronic inflammation. In this context CX3CR1^{hi} Mφ return to expand to detriment of CX3CR1^{int} Mφ that, progressively, are cleared by apoptosis [36]. How these processes take place is not fully understood. Many lines of evidence suggest that CX3CR1^{int} Mφ are recruited during inflammation from CCR2^{+} monocytes and differentiate in the mucosa. Here, the CX3CR1^{hi} Mφ maintain their anti-inflammatory role avoiding the rise of aberrant inflammation and, probably, starting the process of resolution (figure 3).

Microbiome

Bacteria, viruses, fungi, archea, parasites and phages compose the microbiome that is, more generically, the whole collection of microorganisms that resides in a host [37]. Despite many species compose the microbiome, bacteria are the most abundant and heterogeneous population. This subset of the microbiome, in healthy condition, is exclusively present in the mucosal sites and epidermis that are the surface of our body exposed to the external world. This is the case of the oral cavity and upper airways, skin, gastrointestinal and reproductive tract. To understand the importance of microbiota in human life, it is sufficient to think that only bacteria are ten times more abundant compared to host cells and that their metagenome is 100 times higher than the whole host genome [38]. Despite this important presence in our body, microbiome undergoes very strong selective phenomenon in the mucosal sites and, for instance, only nine phyla have been detected in human body, in contrast with twenty observed on plant surfaces [39, 40]. However still low is known about the microbiome: it is estimated that more than 60% of bacterial species are undetectable for the inability to be cultured and the majority of remaining species have a dynamic and complex way to grow, making the isolation and reproducibility very difficult. In case of loss of integrity of the body surfaces such as in tissue injury and healing, the composition of microbiome changes drastically and, for this reason, one of the most interesting field of investigation is how the bacteria composition influences the repair of the wound healing.

Intestinal microbiome

The microbiome of the intestine begins to form at birth. It has been proposed that bacteria enter in contact with the host *via* different ways, for example, skin and fecal microbiota of relatives and parents, bacteria from the vagina or from the milk of the mother or thanks to the contact with other children [41]. The final composition of bacteria develops during the first three years in a multifaceted way that, till now, is not completely understood [42]. What is clear is that diet or dietary changes and nutrient availability can influence the composition of the microbiome, together with antibiotics or drug administration [43]. The anatomical

distribution of bacteria is not homogeneous and it changes in the different parts of the intestine, for instance, the most abundant community of bacteria is in the large intestine. The quality of microbiome change spatially and also temporally in the gut and is different for each individual. This means that the condition of symbiosis, the relationship between the host and his microbiome, is different from person to person and, consequently, it is impossible to define a universal condition of dysbiosis that occurs when poor nutrition or disease alter the equilibrium impairing the function of microbiome [44-46]. During a wound, be it acute or chronic, the epithelium of the intestinal wall is destroyed and the quantity of mucus and lipids drastically impaired. This condition is able to alter the production of anti-microbial peptides and generates inflammation. In this way the mucosal surface is exposed to the environment creating an opportunity for non-indigenous bacteria to colonize the site and alter the equilibrium between the host and the microbial species [37]. Microbiome in wound repair has been reported to have a key role in both resolution of the injury or in the establishment of chronic inflammation, depending of the type of microbes. There is evidence that the relationship between microbiota and the epithelium wall is one of the essential point during gastrointestinal inflammation. The microbiome can influence the microenvironment producing different types of chain fatty acids, butyrate and modifying the bile. There are many mechanisms by which microbiome can influence the gastrointestinal epithelium repair. For instance, epithelial cells express high levels of TLR able to recognize bacterial PAMPS, in other way, the interaction between bacteria and epithelium can modulate β-catenin signalling or interact with the induction of NF-κB or ERK. However, it is important to remember that inflammation alters the normal epithelium layer resulting in a change in microbiome composition that can induce pathology [47-50].

Intestinal microbiome in IBD

As said above, the relationship between host epithelium and microbiome is fundamental in healthy conditions. During inflammation, things change, and there is a deficiency in mucus production that can lead bacteria to infiltrate the mucus layer and spread into tissue. Contrary to common thought, during IBD the richness in bacteria species and their diversity is reduced and also the stability in the composition of microbiota changes. This has been proposed to be one of the possible causes of the fluctuating disease in both Crohn's disease

(CD) and ulcerative colitis (UC) [51, 52]. During inflammation, as it happens in IBD, different species of bacteria colonize the gastrointestinal tract and the resulting higher risk of intestinal infection may be due to an altered intestinal environment that causes the loss of commensal bacteria and provides the colonisation of other species of microorganisms. An explicative example is given by *Akkermansia*, a mucolytic species of bacteria that is impaired during IBD. In this case, the loss of *Akkermansia* results in the rise of another mucolytic species- *Ruminococcus gnavus*- that has been observed in CD and UC and has been associated with a relapse of the disease. Moreover, being the mucus the native habitat of many species of bacteria, its alteration results also in a dysregulation of the total microbiome composition [53]. Another beautiful example is given by *Faecalibacterium prausnitzii* that is normally present in healthy gut and is an abundant butyrate-producer. In patients with UC *F. Prausnitzii* is decreased in both biopsy and fecal samples. In mice, it has been shown that *Prausnitzii* exerts an anti-inflammatory response interacting with NF-Kb and CXCL8 production, so the loss of this microorganism can eliminate an early and potentially important anti-inflammatory process [54] (figure 4).

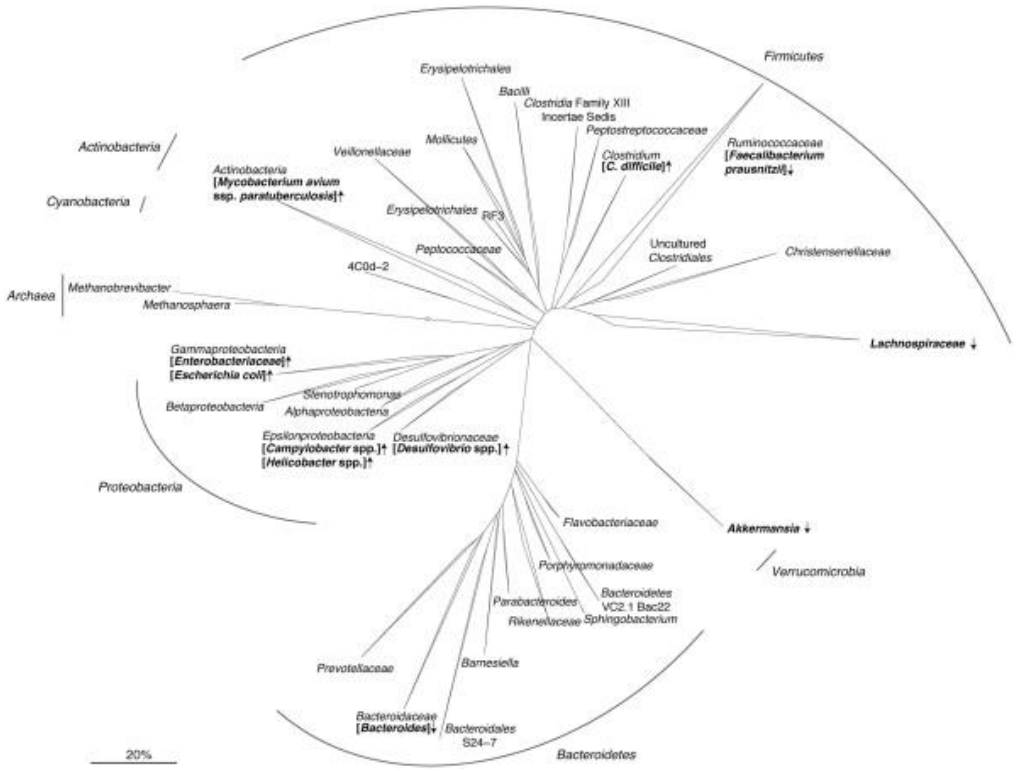


Fig.4: Changes in microbiome composition during IBD [55].

Role of macrophages in bacteria clearance

How the gut immune system is able to discriminate between pathogenic and commensal bacteria is still unknown. Commensal bacteria express many of the molecules also expressed by pathogenic bacteria, which are able to trigger the immune system. In fact, it has been shown that commensal bacteria that penetrate the epithelial barrier, are able to cause inflammation [56]. Despite the commensal role that bacteria have in healthy people, during infection or inflammation the "bad-population" of microorganisms have to be eradicated to restore the equilibrium. The key role in this complex game is mainly entrusted to macrophages and neutrophils. As discussed above, macrophages are recruited to the gastrointestinal tract largely thanks to the CCR2-CCL2 axis. Ishida et al., [57] demonstrated that CX3CR1 KO mice have impaired anti-bactericidal functions. They showed that macrophages are normally recruited in both WT and KO mice but if the CX3CR1 receptor is lacking, bacterial clearance is reduced. They supposed that CX3CL1 serves to activate macrophages and, as consequence, is able to induce the expression of NO, TNFα, IL-12, IL-1β. These mediators act in a paracrine and autocrine manner on macrophages augmenting their bactericidal functions. If cells are not able to sense CX3CL1 –as in case of KO mice-, this mechanism is lost and it results in higher levels of inflammations. Finally, Mazzini et al., demonstrated the role of CX3CR1^{+} macrophages in priming dendritic cells, highlighting, in a different way, the importance of the receptor in the maintenance of homeostasis [25].

Heme-oxygenase-1

Heme-oxygenase (HO) is a microsomal enzyme that is part of the superfamily of heat shock proteins. HO converts heme in biliverdin and carbon monoxide (CO) with the release of free ferrous iron. There are two genetically distinct isoenzymes, the inducible form (hemox-1) and the constitutive form (hemox-2). hemox-1 is expressed by different cell types, in particular epithelial cells and mononuclear phagocytes such as macrophages and neutrophils [58] while HO-2 is expressed, preferentially, by brain and testis [59]. Hemox-1 is a stress-responsive protein that can be induced quickly by different stimuli including nitric oxide, heavy metals, hemin and reactive oxygen species (ROS) [60] (figure 5).

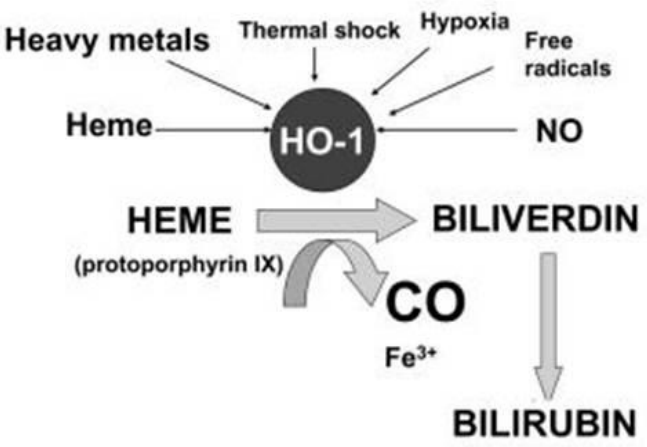


Fig.5: Induction of heme-oxygenase-1 [60]

It has been reported that the activity of hemox-1 can exert anti-inflammatory activities; in particular, biliverdin, the product of hemox-1 activity, has anti-oxidant proprieties that reduce lipid peroxidation, have anti-complement functions and reduce the mesenteric neutrophil migration and extravasation during inflammation. In parallel CO, the other product of hemox-1 activity, is able to down-regulate the expression of pro-inflammatory molecules and to modulate the leukocyte-endothelial cell interaction [61]. Moreover, murine macrophages treated with lipopolysaccharide (LPS) are able to up-regulate hemox-1 and

consequently attenuate the production of many pro-inflammatory mediators such as IL-1β, TNFα, IL-6 and cyclooxygenase-2 (COX-2) [62]. Experimental mouse model of hemox-1 deficiency demonstrated that mice are prone to develop chronic inflammation and are more susceptible to sepsis induced by endotoxin. Importantly, the same phenotype has been observed in the only event of genetic hemox-1 deficiency identified in humans [63, 64]. Despite the relationship between hemox-1 and inflammation is nowadays accepted, the mechanisms by which this enzyme exerts its immunomodulatory functions are still under investigation. Lee et al., [65] propose that the anti-inflammatory effects of hemox-1 are mediated by IL-10. In his work he demonstrated that IL-10 is able to induce hemox-1 in murine macrophages and the consequent production of CO is responsible of the anti-inflammatory effects of IL-10 such as reduction in TNFα, NO and MMP9 production after stimulation with LPS. Naito y. et al., [66] further investigated this pathway and they proposed a complicate relationship in which IL-10 and hemox-1 reciprocally stimulate each other. They demonstrated that the induction of IL-10 after LPS was significantly reduced after administration of zinc protoporphyrin IX (znPP IX) an inhibitor of hemox-1. In contrast, the administration of a hemox-1 inducer such as cobalt protoporphyrin IX (cop IX) enhanced the IL-10 production.

Heme-oxygenase-1 in the intestine

Many studies demonstrate that hemox-1 is overexpressed during experimental colitis in rats and mice and also in human specimens is possible to detect high level of the enzyme [67]. It is well known that in IBD there is a powerful expression of both ROS and iNOS and, consequently, of nitric oxide (NO). Whittle b. et al., proposed that the reactive species act inducing hemox-1 in an attempt to regulate inflammation during colitis [60]. As said above, Lee and colleagues proposed IL-10 as a greater inducer of hemox-1 [68, 69].further highlighting the importance of gut microbiota, together with IL-10, in hemox-1 production. In fact they demonstrated that microbiota is a fundamental player in this pathway: the microbiota is able to induce hemox-1 only in WT mice but not in IL-$10^{-/-}$mice. Moreover, they shown that also zebrafish maintain this kind of regulation, demonstrating that these pathways are fundamental and conserved during evolution. To summarize the current

knowledge, it seems that hemox-1, and in particular its product CO, has potent anti-inflammatory properties. The microbiome can induce hemox-1 production activating IL-10 *via* TLR signalling. The consequence of this activation is the stimulation of the production of anti-inflammatory cytokines and the inhibition of pro-inflammatory molecules such as Il-1β, IL-6 and TNFα. Finally, the increased levels of CO seems to play an important role in stimulating bacteria clearance by macrophages. Therefore, in the intestine, microbiota can act a pivotal role in the maintenance of homeostasis stimulating the production of hemox-1. This mechanism act as a loop on macrophages in order to make appropriate clearance of bacteria. If this mechanism is lost, bacteria are no longer under control and they can invade the mucosa and give rise to inflammation [70].

Heme-oxygenase-1 in macrophage polarization

In the intestine, the major producers of hemox-1 are macrophages. A lot of effort was required in the last years to establish the importance of the hemox-1 pathway in the induction of anti-inflammatory stimuli during intestinal injury (figure 6). Next to IL-10 that, as discussed above, plays a central role in macrophage life, many other effectors have to be considered. Heme is quite known to be a powerful hemox-1 inducer. Macrophages present on their surface two distinct receptors for heme that are able to interact with CD163 and to be internalised *via* endocytosis. During tissue injury, very high levels of heme can be released as a consequence of haemolysis [71]. Figueiredo et al., [72] proved also that heme can induce hemox-1 transcription binding directly TLR4 indicating that the regulation of this enzyme is dependent on heme concentration. Finally, several lines of evidence suggest that heme is able to influence macrophage polarization, enhancing M2 macrophages and suppressing M1. Another *via* to enhance hemox-1 production resulting in M2 polarization of macrophages, involves adiponectin. Recently, Mandal and colleagues [73] described a possible M2 polarization of macrophages due to adiponectin effects on hemox-1 expression. They demonstrated that adiponectin can stimulate the expression of hemox-1 improving the anti-inflammatory signature. Finally, it seems that also diet can promote hemox-1 production and in particular, some natural compounds such as curcumin, resveratrol, sulforaphane and agaro-

oligosaccharides [74]. Notably, it has been demonstrated that agaro-oligosaccharides are able to augment the expression of Mrc1, Ym1 and Arg1, typical markers of M2 activation [75]. On the contrary, Harusato and colleagues propose the transcriptional factor Bach1 (a key TF for macrophages polarization) as a potent suppressor of hemox-1 production. They demonstrated that Bach1-/- mice are protected during TNBS-induced colitis in term of both grade of colitis and pro-inflammatory cytokine production [76]. In the same way, they proved that the administration of a hemox-1 inhibitor such as tin protoporphyrin is able to potentiate the colonic damage highlighting the beneficial effect of hemox-1.

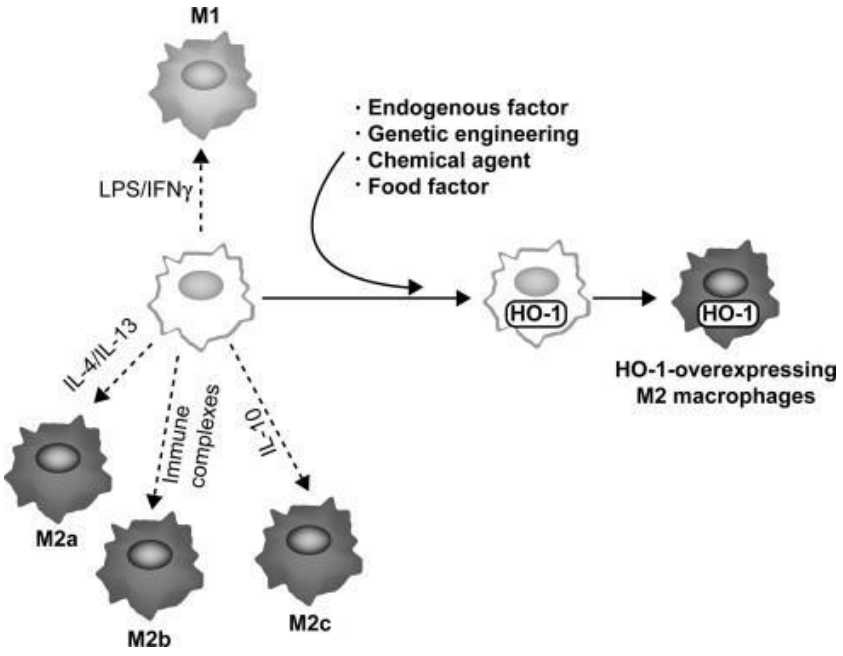


Fig.6: Hemox-1 in macrophage polarization [66]

Cancer related inflammation

Cancer is a complex multi-step process in which normal cells acquire peculiar traits that lead to malignant growth such as self-sufficiency from external signals, insensitivity to negative growth signals, resistance to apoptosis, limitless replicative potential, sustained angiogenesis, acquisition of tissue invasiveness and metastasis. Virchow, in the 19th century, firstly described the link between inflammation and cancer. His intuition was based on the observation that neoplastic tissues are infiltrated with inflammatory cells and suggested that tumors may arise at sites of chronic inflammation [77]. Nowadays it is accepted that a consistent number of malignancies worldwide are related to conditions of chronic inflammation (e.g. persistent infections, autoimmune diseases, general inflammatory conditions). Cancer-related inflammation (CRI) is currently accepted as a hallmark of cancer (figure 7).

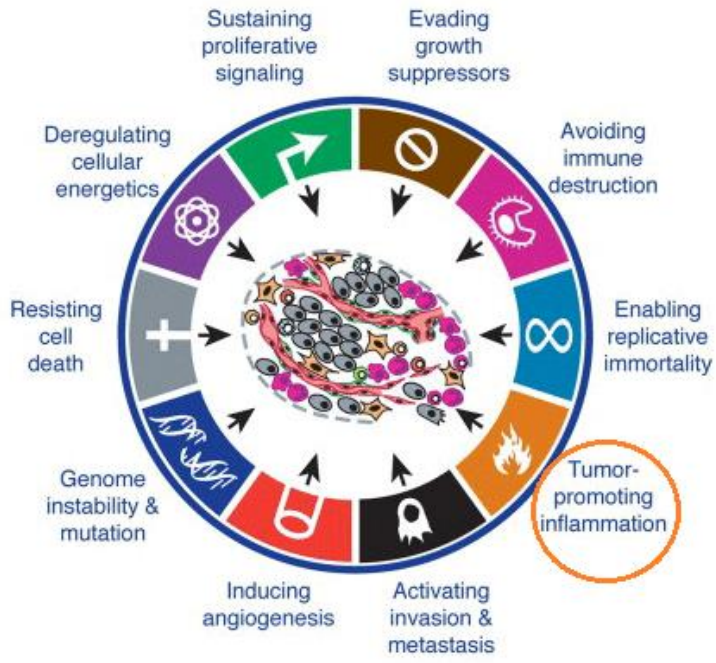


Fig.7: Recently, cancer-related inflammation has been added as a hallmark of cancer. New emerging peculiar traits of cancer are the deregulation of cellular energetics, genomic instability and the ability to evade immune destruction [77].

CRI is a smoldering, non-resolving inflammation, characterized by the infiltration of white blood cells, in particular myeloid cells, the presence of inflammatory mediators such as cytokines (e.g. TNF, IL-1, IL-6) and chemokines (e.g. CCL2 and CXCL8) and the occurrence of continuos tissue remodeling and angiogenesis [78].

Immune cells are present in the tumor microenvironment from the early stages of carcinogenesis and are deeply affected by the presence of cancer cells, which, in turn, are influenced by leukocytes in different ways. Innate and adaptive immunity leukocytes have an ambiguous link with cancer cells and can either inhibit or promote tumor growth. It is now quite clear that the immune system has a defensive role in the first acts of tumor growth and actively tries to eradicate those cancer cells that escape the immune surveillance and become antigenically "silent", overgrow, but in the end give rise to clinically evident tumors. At this stage, when tumors become detectable, cancer cells have riched a critically great mass and are able to affect the immune system. When it happens, immune cells become harmless effectors, with immunosuppressive functions and devoid of cytotoxic ability [79]. This process is known as "cancer immunoediting" and it was firstly proposed by Dunn and colleagues [80]. Cancer immunoediting can be considered as a result of three processes: elimination, equilibrium and escape (the "3E" hypothesis). During the elimination phase, the immune system recognizes transformed cells and act in order to destroy them. It can release many mediators, cytokines and chemokines able to recruit other immune effector cells at the tumor site in order to promote cancer cell death. Despite the efforts of the immune system, many neoplastic cells can survive the elimination process and enter into a dynamic equilibrium with the host immune system. During this phase, cancer cells modify their antigen expression and acquire different mutations that provide them to resist to immune attack. In the escape process, surviving tumor variants break the equilibrium evading the immune system that is no longer able to detect and to eliminate them. In this way, neoplastic cells are able to expand in an uncontrolled manner and the disease becomes clinically evident [80].

The Intrinsic and Extrinsic Pathway

Two different pathways link inflammation and cancer: the intrinsic and the extrinsic pathway (figure 8). The extrinsic pathway is driven by inflammatory or infectious conditions that increase cancer risk. On the other side, the intrinsic pathway is activated by genetic alterations that lead to a chromosome instability, reactive oxygen species (ROS) production and other alterations that can trigger inflammatory mediators and activate the inflammatory cascade [78].

In the intrinsic pathway, when genetic alterations occur (such as mutations promoting/inactivating oncogenes and tumor-suppressor genes, as well as chromosomal amplification and rearrangement), inflammatory transcriptional programs are activated in mutated cells, generating an inflammatory microenvironment. Recent studies [81, 82] showed that the activation of the protein kinase RET, due to a chromosomal rearrangement, is necessary for the pathogenesis of papillary thyroid carcinoma and directly promotes the build-up of an inflammatory microenvironment at tumor sites. Moreover, genetic alterations in tumor-suppressor genes such as VHL, TGF-β and PTEN can promote the recruitment of cells of myelomonocytic origin and represent a link to metastasis and angiogenesis [78].

In the extrinsic pathway, an inflammatory chronic response may increase cancer risk: for example, persistent infection by tumorigenic pathogens, such as *Helicobacter pylori* or hepatitis B (HBV) and C (HCV) viruses, can increase the incidence of gastric cancer, mucosa-associated lymphoid tissue (MALT) lymphoma, or hepatocellular carcinoma respectively [83].

Autoimmune diseases and inflammatory conditions as prostatitis or inflammatory bowel disease (IBD) can be also considered as triggers of chronic inflammation that increase cancer risk and progression [84].

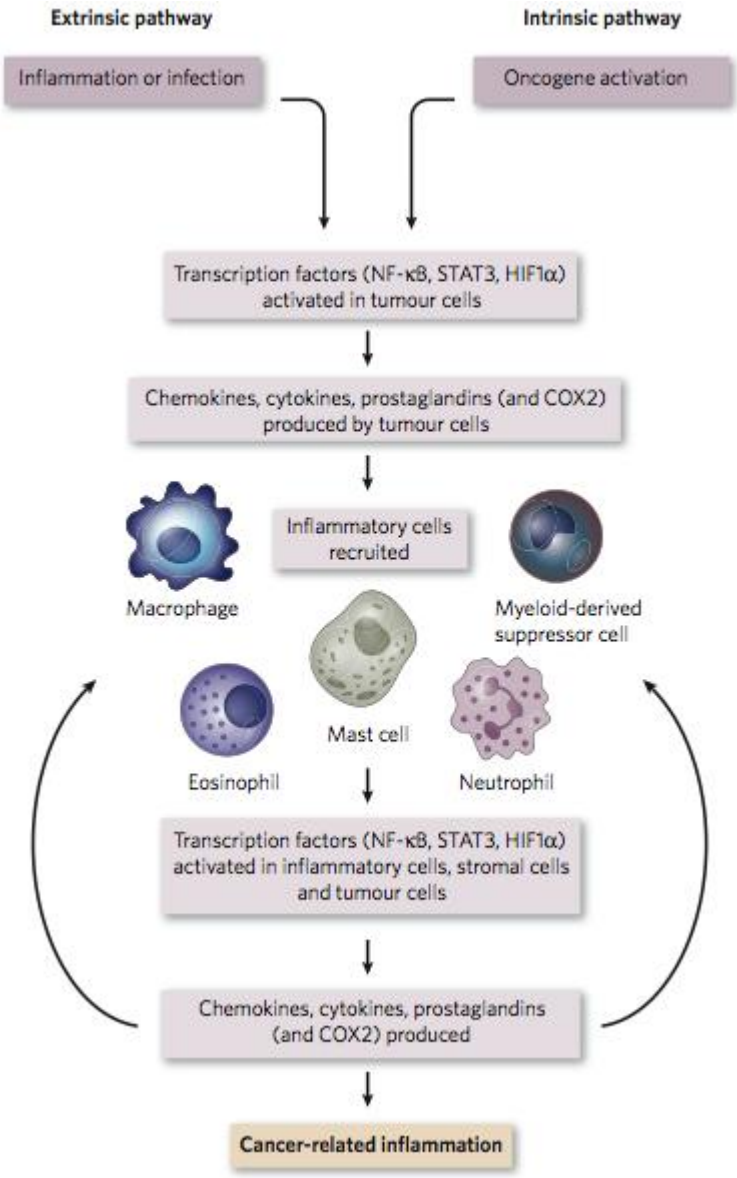


Fig.8: The link between inflammation and cancer is described by two converging pathways: the extrinsic pathway (activated by inflammatory/infectious conditions) and the intrinsic pathway (driven by oncogene activation). They both result in cancer-related inflammation and involve transcription factor activation, cytokine and chemokine release and the recruitment of infiltrating leukocytes [78].

Tumor microenvironment

The tumor microenvironment (TME) is a dynamic milieu composed by stromal cells such as fibroblasts, immune cells, soluble factors and extracellular matrix. The extensive communication between the tumor and the stroma provides a tumor niche able to sustain

tumor growth, to generate metastasis and to protect the tumor from the immune system. Moreover the niche is a controversial milieu responsible, in many cases, of therapy resistance. TME is in continuous change: it constitutes a large fraction of the whole tumor, often more than half of the tumor mass, and it is able to recruite many types of cells, inducing tissue remodeling, antigen changes and metabolic alterations [85]. Cytokines and chemokines are key components of the tumor microenvironment. They play a central role in cancer-related inflammation promoting the migration, invasion and survival of malignant cells, coordinating autocrine and paracrine interactions between tumor cells, and infiltrated leukocytes. Colotta, F. et al., recently hypothesized that inflammatory cells and their mediators, such as cytokines and chemokines, can contribute to cancer initiation and progression through different mechanisms that lead to genetic destabilization of cancer cells including DNA damage or alteration of cell cycle checkpoints and DNA repair systems [84]. The high degree of genetic heterogeneity in tumors suggests that genetic instability is an ongoing process throughout tumor development and that the inflammatory response can accelerate the somatic evolution of cancer. In the end, the tumor tissue results as a complex heterogeneous milieu in which genetic mutation are melted with genomic instability promoted by inflammatory mediators [77]. An additional critical component of the tumor microenvironment is the extracellular matrix (ECM), a highly organized three-dimensional structure whose main physiological features include: maintenance, tissue integrity, regulation cell migration, cellular differentiation and proliferation and provides a reservoir of cytokines and growth factors [86]. Five classes of macromolecules including collagen, laminins, fibronectin, proteoglycans and hyaluronans compose the ECM that can also be divided into two main groups, the basement membrane (BM) and interstitial or stromal matrix. BM is a mechanical barrier composed of dense collagen type IV and laminin; it organizes tissue and cell growth and regulates differentiation, polarity and gene expression [87]; its dissolution is one of the main features in epithelial cancer development. By contrast, the stromal matrix is composed by polysaccharide gels, proteoglycans and different fibrous proteins [86].

Tumor associated macrophages

Macrophages are plastic cells derived from monocytes that orchestrate the innate and adaptive immune responses [88]. According with Mantovani et al., macrophages can be divided into two different subpopulations depending of their functional polarization [89]. M1 classically activated macrophages and alternatively M2 macrophages (figure 9). This characterization, however, has some limitations and simplifies a complex scenario in which macrophages can show features of both populations, depending on the different stimuli they are subdued. M1 "classically activated" macrophages reflect and sustain Th1 responses. They are polarized by cytokines such as IFNγ or by microbial signals. On the other side, M2 "alternatively activated" macrophages mirror the Th2 polarization and can be generated in response to cytokines such as IL-13, IL-4, IL-10 and TGF-β [79].

M1 Mφ are pro-inflammatory cells able to produce TNFα, IL-1β, iNOS and they have bactericidal proprieties. M2 Mφ, instead, have immunomodulatory functions through the production of IL-10 and TGFβ. They are considered tissue resident and anti-inflammatory cells involved in tissue remodeling and angiogenesis [90].

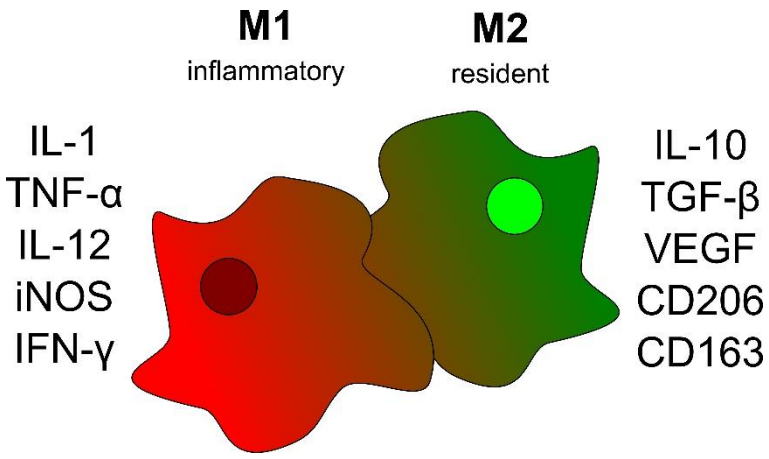


Fig.9: Macrophage polarization is driven by cytokines

In the tumoral context, Tumor Associated Macrophages (TAM) display a phenotype similar to M2 polarized Mφ. They do not exert cytotoxic activity and they actually favor tumor growth in a variety of ways, including suppressing the anti-tumoral response, enhancing tissue

remodeling and neo-vascularization (figure 10). However, recently, sophisticated analyses have revealed an abundant heterogeneity in TAM especially dependent on their localization in the tumor microenvironment [91]. TAM mainly originate from monocytes directly derived from the bone marrow. However, new evidence suggests a splenic contribution and to a local proliferation [92-94]. Incoming monocytes are preferentially recruited in necrotic or hypoxic areas within tumor stroma where they are profoundly influenced by stimuli of the tumor microenvironment and quickly differentiate in tumor-conditioned macrophages. Chemokines, for example CCL2, CCL5, CXCL12, and growth factors such as CSF-1 (M-CSF) are major determinants of monocyte infiltration in tumors. Also cytokines play a crucial role; in particular IL-10 and TGFβ can suppress the host immune response making the tumor antigenically "silent". It is believed that in the first step of tumor progression, macrophages try to fight against tumor ande exert mainly anti-tumoral functions.

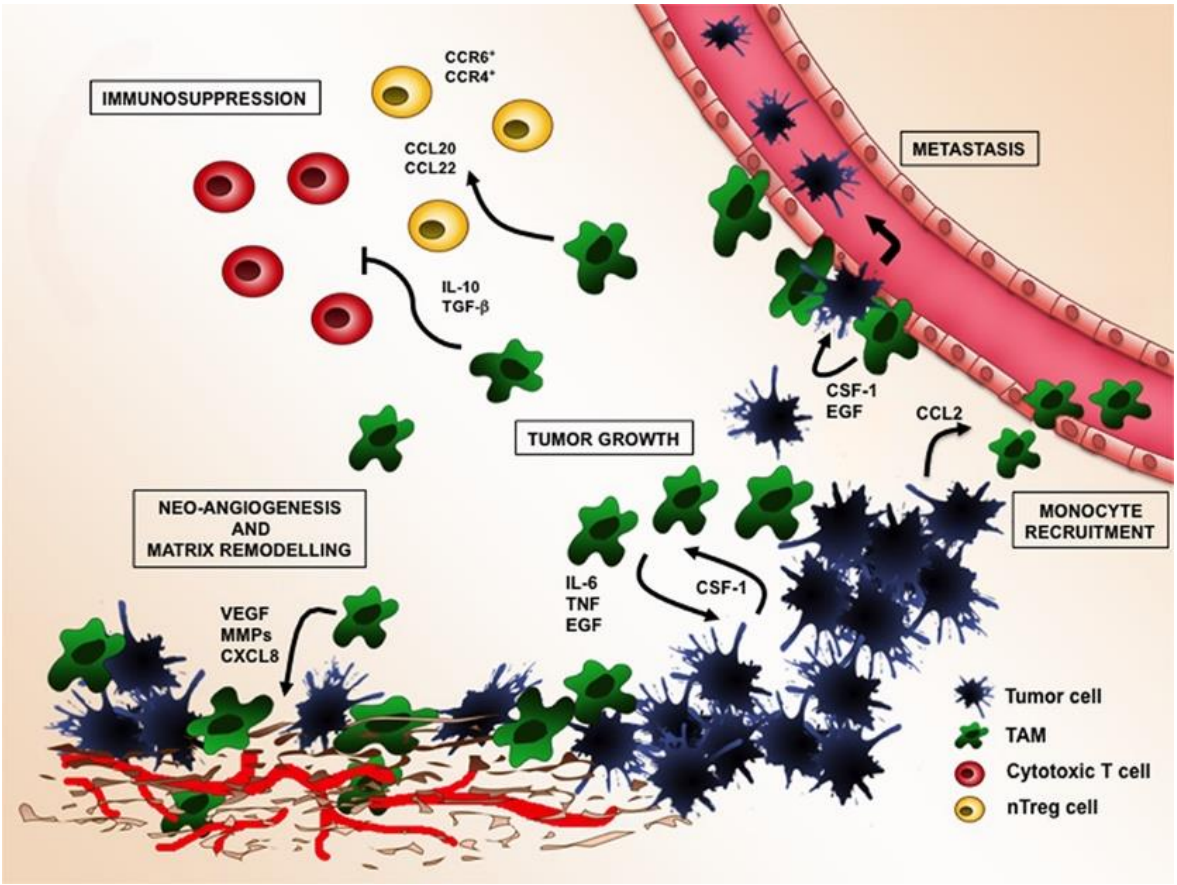


Fig.10: Pro-tumoral function of TAM [79].

However, tumor growth is able to modify the microenvironment enriching it of many suppressive molecules such as Th2-related cytokines (IL-4), IL-10 and TGF-β able to "re-educate" Mφ and allow them to acquire pro-tumoral –M2- functions [95]. In this context, TAM release trophic factors able to activate tumoral and stromal cells. TAM are able to regulate many aspects of cancer biology, such as tumor cell invasion, angiogenesis as well as immunoregulation [88] (fig). Vascularization is fundamental for tumor survival; TAM support tumor growth by triggering the acquisition of a vasculature that provides oxygenation and nutrition to tumor cells, in a process often referred to as "angiogenic switch" [77]. Indeed, TAM are able to promote neoangiogenesis and lymphoangiogenesis by releasing pro-angiogenic growth factors such as VEGF-A, VEGF-C, EGF, thymidine phosphorylase (TP) and chemokines such as CCL2 and CXCL8 [96]. Hypoxia is the best driver of angiogenesis. In tumor hypoxic areas, the up-regulation of HIF-1 and HIF-2 triggers angiogenesis inducing TAM to produce several mediators such as VEGF, FGF2, CXCL8 and proteolitic enzymes (for example matrix metalloproteinases MMP-1, MMP-7, MMP-9) that induce the ECM digestion [97].

Matrix components in the tumor microenvironment can modulate the pro-tumoral functions of TAM. TAM directly support tumor cell survivor and proliferation in a reciprocal cross talk. Indeed, tumor cells produce CSF-1 that act on TAM inducing them to secrete the epidermal growth factor (EGF), which in turn sustain tumor cell proliferation and migration. Macrophages are very important players in tissue and extracellular matrix (ECM) remodeling, being the main source of MMPs and other proteolytic enzymes. The destruction of the ECM by TAMs favor tumor cell invasion and penetration into vessels and eventually giving rise to metastatic dissemination. TAM exert their immunosuppressive role by secreting several factors such as IL-10, TGFβ and CCL22 that can suppress CD4+ and CD8+ T cell function directly or indirectly. In parallel, through the axis CCL22/CCR4 Treg cells are recruited by TAMs at tumor sites. Moreover, they can block tumor regression by inhibiting NK cell recruitment and T cell proliferation and activation *via* the PD-L1/PD1 signaling axis.

Because high TAM infiltration has been associated with poor prognosis and therapeutic failure in cancer patients, reprogramming TAM toward an antitumor M1 phenotype or inhibition of their recruitment or suppression may be beneficial in future therapeutic approaches. Indeed, in murine experimental settings different strategies have been used to

target TAM in tumor, either as therapy alone or combined with conventional or anti-angiogenetic therapies. Overall, the results obtained are very promising, confirming the promoting role of TAM in cancer progression [97]. However, even if TAM acquire M2-like features, especially in advanced metastatic tumors, is not unusual to find TAM with a M1-like phenotype in particular in early stages of tumor development or in regressing tumors. Thus, TAM are very plastic cells that can express both M1 or M2 characteristics depending on the stimuli they received from the microenvironment [98].

Tumor associated chemokines

Chemokines are chemotactic cytokines described as soluble mediators originally described to promote the directional migration of leukocytes [99, 100]. They are small proteins of 8-10 KD and, recently, it has been proposed that not only leukocytes but also epithelial and endothelial cells, fibroblasts and neoplastic cells can produce and respond to chemokines after particular stimuli, such as tissue injury or infection [101, 102].

Chemokines have been classified into four families (CXC, CC, C and CX3C) according to the position of cysteine residues in the N-terminal region; a further subdivision is based on the presence or absence of an ELR (Glu, Leu, Arg)-motif, located in front of the first conserved cysteine residue [103, 104]. All chemokines can exert their functions through binding to seven transmembrane G-protein-coupled receptors (CXCR, CCR, CR or CX3CR). The interaction of the ligand with the receptor is not unique: many chemokines can bind different receptors and viceversa. Despite redundance is a typical feature of chemokines, a small number of receptors can bind a single ligand. Moreover, chemokines can also interact with non-signaling receptors such as DARC (Duffy antigen receptor for chemokines), D6 or CCX-CKR (ChemoCentryx receptor) [105, 106].

Both receptor and ligand expression is strictly related to the differentiation and activation state of the cell and can be expressed either constitutively or after stimulation [107]. Another characteristic feature of chemokines is their ability to act in synergy; this means that different proteins can affect the same target in order to enhance the response to the stimulus. Chemokines are essential in not only pathology but also play an important role in the

maintenance of homeostasis. In steady state they regulate leukocytes trafficking and homing, mediate lymphoid tissue organogenesis and regulate hematopoiesis. When a pathological condition occurs, chemokines are produced in order to co-ordinate the correct response to injury. They are involved in all types of pathological disorders regulating both the pro and the anti-inflammatory response, the recruitment of leukocytes, tissue remodeling, angiogenesis and can influence cell survival and proliferation. Furthermore, chemokines can be activated and involved in processes such as autoimmune diseases (e.g. IBD, rheumatoid arthritis) and tumor growth and metastasis [101]. In tumors, chemokines predominantly participate in the chemoattraction of leukocytes at tumor sites and modulate tumor behaviour by several mechanisms such as activation of a tumor-specific immune response, promotion of the angiogenic switch, and stimulation of tumor cell proliferation and metastasis. Moreover, genetic mutations (such as in c-MYC gene) and the activation of transcriptional factors (e.g. NF-kB, STAT3) trigger chemokine release.

Thus, chemokines play a crucial role in tumor promotion and development, being able to affect the tumor microenvironment in terms of leukocyte recruitment, stimulation of angiogenesis, tumor proliferation and matrix remodeling. Chemokines affect tumor microenvironment; the ability of the immune system to fight against tumor is consequently influenced by chemokines. Pro or anti-tumoral response can differ depending on chemokine avaialability. Generally, pro-tumoral responses push leukocytes to produce pro-tumoral mediators and viceversa. For instance, that the interaction between ELR+ CXC chemokines (e.g. GRO, ENA-78, IL-8, GCP-2) and CXCR1 and/or CXCR2, trigger the infiltration of pro-tumoral neutrophils, while CXCR3 binding ELR- CXC chemokines (e.g. Mig, IP-10, PF-4) attracts anti-tumoral DC, T lymphocytes and NK cells. Moreover, many CC chemokines (especially CCL17 and CCL22) secreted by macrophages at tumor sites play an active role in Th2 and Treg cells recruitment promoting tumor development [101]. Finally, it is important to highlight that chemokine receptor can be expressed also by tumor cells. Autocrine and paracrine extracellular signals, genetic and epigenetic alterations, can trigger chemokine receptor expression that is correlated with an increase in cell survival and spread. Indeed, the presence of chemokine receptor such as CXCR4, CXCR1, CXCR2 or CXCR5 on tumor cells is related to an increase of invasiveness and metastasis. Muller and colleagues [108] first

described how the expression of CXCR4 in neoplastic cells mirrors the risk of metastatis development in a model of mammary carcinoma.

Notably, also in colorectal cancer the presence of the CXCR4-CXCL12 axis is related to a worst survival prognosis due to the risk of metastasis. Moreover, Popivanova et al., recently demonstrated that the expression of CCL2 correlates with CRC progression. In addition, CCL2 antagonists can inhibit COX-2 expression, attenuate neovascularization and therefore decrease the number and size of tumors [109]. Another evidence that chemokines have a central role in CRC development is given by Erreni M., and colleagues that demonstrated the up-regulation of several chemokines (CCL7, CCL20, CCL25, CXCL1, CCL26) and their receptors (CCR8, CCR6, CXCR2) in human tumor tissues of CRC [110]. Another example that chemokines and their receptors are involved in tumor metastasis is the expression of CXCR3, CXCR4 and CCR7 in primary CRC clinically correlates with tumor recurrence, patient survival and lymph node or liver metastasis [111, 112] (table 1).

Chemokines and their receptors in IBD and CRC

Name	Class/expression	Receptor	Cell attracted	IBD	CRC
CC chemokines					
CCL1	Inducible	CCR8	Monocytes, T, B		
CCL2	Inducible	CCR2	Monocytes, T, NK, immature DC	+	+
CCL3	Inducible	CCR1, 5	Monocytes, macrophages T, NK, immature DC	+	+
CCL4	Inducible	CCR5, 8	Monocytes, macrophages T, Immature DC	+	+
CCL5	Inducible	CCR1, 3 and 5	Monocytes, macrophages T, NK, immature DC	+	+
CCL7	Inducible	CCR1, 2 and 3	Monocytes, macrophages T, NK, immature DC	+	?
CCL8	Inducible	CCR2, 3 and 5	Monocytes, macrophages T, NK, immature DC	+	?
CCL9	Inducible	CCR1	DC	?	+
CCL11	Inducible	CCR3	Eosinophils, basophils Th2 T, mast cells	?	+
CCL13	Inducible	CCR1, 2 and 3	Monocytes, T, NK eosinophils, immature DC	+	?
CCL14		CCR1, 3	Monocytes, macrophages NK, T cells	?	?
CCL15		CCR1, 3	Monocytes, macrophages T, NK, eosinophils, mast cells	?	?
CCL16		CCR1	Monocytes, macrophages T, NK cells	?	?
CCL17		CCR4	Th2 T cells, macrophages T, NK cells	+	+
CCL18			T cells	?	?
CCL19		CCR7	T, B, mature DC	+	?
CCL20		CCR6	T, B, immature DC	+	+
CCL21		CCR7	T, B, mature DC	+	+
CCL22		CCR4	Th2 T cells, macrophages	+	?
CCL23		CCR1	Monocytes	?	?
CCL24	Inducible	CCR3	Eosinophils, basophils mast cells, Th2 T cells	+	?
CCL25		CCR9	Thymocytes, T cells	+	?
CCL26	Inducible	CCR3	Eosinophils, basophils mast cells, Th2 T cells	+	?
CCL27		CCR10, 2, 3	T, B, monocytes immature DC	?	?
CCL28		CCR10, 3	T cells	+	?
CXC chemokines					
CXCL1	ELR^+, inducible	CXCR2, 1	Neutrophils	+	+
CXCL2	ELR^+, inducible	CXCR2	Neutrophils	+	+
CXCL3	ELR^+, inducible	CXCR2	Neutrophils	?	?
CXCL4	ELR^-, inducible		Fibroblasts, endothelial cells	?	+
CXCL5	ELR^+, inducible	CXCR2	Neutrophils	+	+
CXCL6	ELR^+, inducible	CXCR2	Neutrophils, macrophages	+	?
CXCL7	ELR^+, inducible	CXCR2	Neutrophils	?	?
CXCL8	ELR^+, inducible	CXCR1, 2	Neutrophils	+	+
CXCL9	ELR^-, inducible	CXCR3	Th1 T cells, NK	+	+
CXCL10	ELR^-, inducible	CXCR3	Th1 T cells, NK	+	+
CXCL11	ELR^-, inducible	CXCR3	Th1 T cells, NK	+	+
CXCL12	ELR^-	CXCR4, 7	$CD34^+$ progenitor cells hematopoietic cells	+	+
CXCL13	ELR^-	CXCR5	Naïve B cells, $CD4^+$ T cells	?	+
CXCL14	ELR^-		Monocytes, immature DC	?	?
CXCL16	ELR^-	CXCR6	Naïve $CD8^+$ cells, $CD4^+$ T cells NK-T cells	+	?
C chemokines					
XCL1		XCR1	T cells, NK	?	+
XCL2		XCR1	T cells, NK	?	?
CX3C chemokine					
CX3CL1		CX3CR1	T cells, monocytes, neutrophils DC	?	+

Tab.1: Chemokines and chemokine receptors play a crucial role in IBD and CRC development, recruiting inflammatory cells [113].

Fractalkine

Fractalkine (CX3CL1) is the only member of the CX3C chemokine family. Differently from other chemokine, it can exist in two form: as a membrane-anchored molecule or in soluble form. This chemokine is firstly synthesized as membrane-anchored form and consists of a chemokine domain linked to a transmembrane domain via a mucin-rich stalk of

extracellular domain. In the presence of metalloproteases such as ADAM-10/17 or MMP2, or in the presence of the protease Cathepsin S, the chemokine domain can be cleaved and the soluble form released, exerting its chemotactic activity [114] (figure 11).

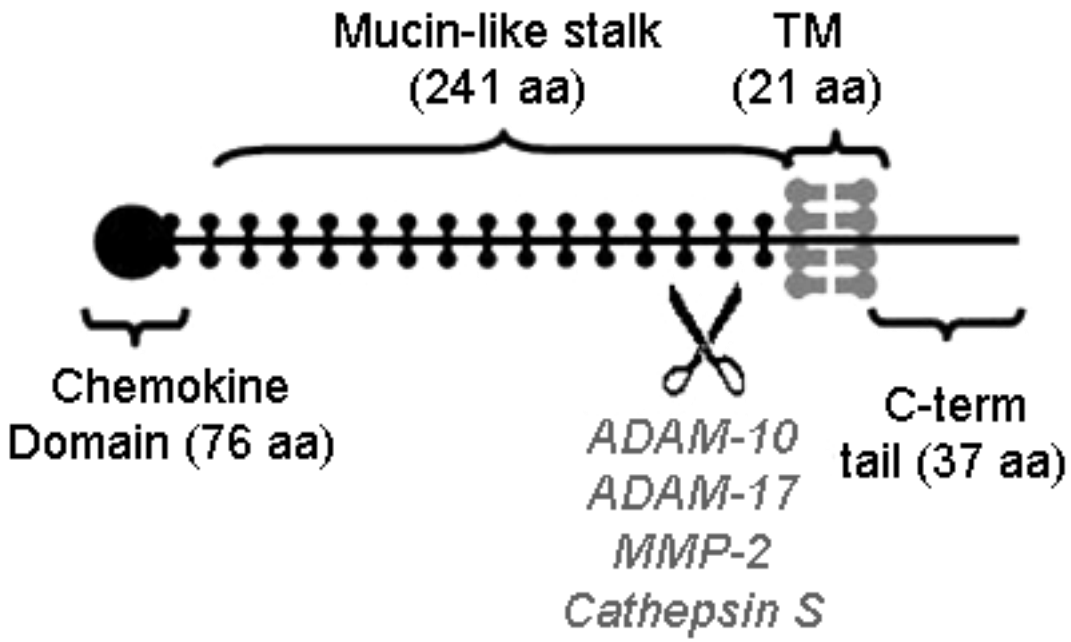


Fig.11: Secondary structure of fractalkine

http://atlasgeneticsoncology.org/Genes/CX3CL1ID46756ch16q13.html

Many soluble mediators such as cytokines (TNFα, IL-1, IFNγ) or bacterial products (LPS) can enhance fractalkine expression in different cell types such as endothelial and epithelial cells, neurons, lymphocytes, microglial cells and osteoblasts [115]. Instead, CX3CR1 is mainly expressed by macrophages but can be also present on DC, CD4 and CD8 lymphocytes, NK cells, neurons, astrocytes and tumor cells. It has been reported in literature that the CX3CL1-CX3CR1 axis play a critical role in the development of inflammatory diseases: increased levels of CX3CL1 have been detected at inflammatory sites in murine models of rheumatoid arthritis, asthma, renal disease, chronic liver disease, inflammatory brain diseases and IBD [114]. In particular for IBD, the role of this chemokine axis is quite controversial and not deeply

clarified. Kostadinova and colleagues suggested a negative role of the CX3CL1-CX3CR1 axis: in a mouse model of DSS-induced colitis they show that the absence of CX3CR1 is protective for the colonic mucosa. In particular, they reported that the presence of fractalkine and its receptor correlates with an increase in tissue damage and colitis score, promoting the recruitment of inflammatory cells, including macrophages. Therefore, they suggest that the absence of the axis could protect the epithelium from a massive macrophage infiltration thus being protective for colonic mucosa [116]. On the contrary, Medina-Contreras et al., support the idea that the CX3CR1-CX3CL1 loop has a protective role in the intestinal mucosa and that the presence of the receptor can prevent Th-17 mediated colitis. Moreover, they demonstrated that the deficiency of $CX3CR1^{+}$ macrophages contributed to enhanced colitis in $CX3CR1^{gfp/gfp}$ mice [26]. In addition, Longman et al. found that colonic $CX3CR1^{+}$ mononuclear phagocytes regulate innate lymphoid cell production of IL-22 and play a critical role in promoting mucosal healing during colitis [117]. Besides inflammatory diseases, the CX3CL1-CX3CR1 axis is also involved in cancer pathogenesis. Recently, it has been demonstrated that the presence of CX3CL1 positive cells often correlates with an increase of CX3CR1 immune cells in many neoplasia such as lung carcinoma, melanoma and gastric carcinoma. On the contrary, in some tumors, CX3CL1/CX3CR1 can play a crucial role in tumor angiogenesis, as well as in adhesion and migration of cancer cells, triggering their ability to spread and metastasize. For example, in malignant breast tissues, CX3CR1 overexpression increases the ability of tumor cells to migrate to skeleton and brain where bone stromal cells and neurons release soluble CX3CL1. In the same way, in glioblastoma patients it has been demonstrated that CX3CL1 and CX3CR1 are expressed by both cancer stem cells and progenitor cells, suggesting that this axis operates in early tumorigenesis process [118]. In colorectal cancer the role of CX3CL1/CX3CR1 axis has been poorly investigated. Zheng et al. found that CX3CR1 is expressed in tumoral cells in a histologic grade and stage-dependent manner. In addition to this they showed that CX3CR1 expression on TAM correlates with worst prognosis. They also highlighted the importance of the receptor in macrophage survival demonstrating that $CXRCR1^{+}$ Mφ are able to regulate the tumor microenvironment through the induction of survival stimuli and that the absence of the receptor inhibits macrophage infiltration in metastatic tumors through induction of macrophage apoptosis [119].

Colorectal cancer

Colorectal cancer (CRC) is a very frequent human neoplasia and represents the third most common cause of cancer death in industrialized countries [120]. The pathogenesis of CRC is a very complex process that involves both genetic and environmental factors. Considering the environmental aspects, alcohol consumption, cigarette smoke, diet, microbial or chronic infections are the most common predisposing factors to develop CRC. On the other hand, genetic alterations can occur in a sporadic or hereditary manner. Although most colorectal cancers are sporadic tumors, about 10% of CRCs are a hereditary disease. Two forms of genetic disorder are known: the hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome) and the familial adenomatous polyposis (FAP). Both HNPCC and FAP are autosomal-dominant diseases: FAP is due to a germline mutation in the adenomatous polyposis coli gene (APC) while HNPCC is caused by alterations in genes of the DNA repair system (e.g. Mismatch repair, MMR) [121].

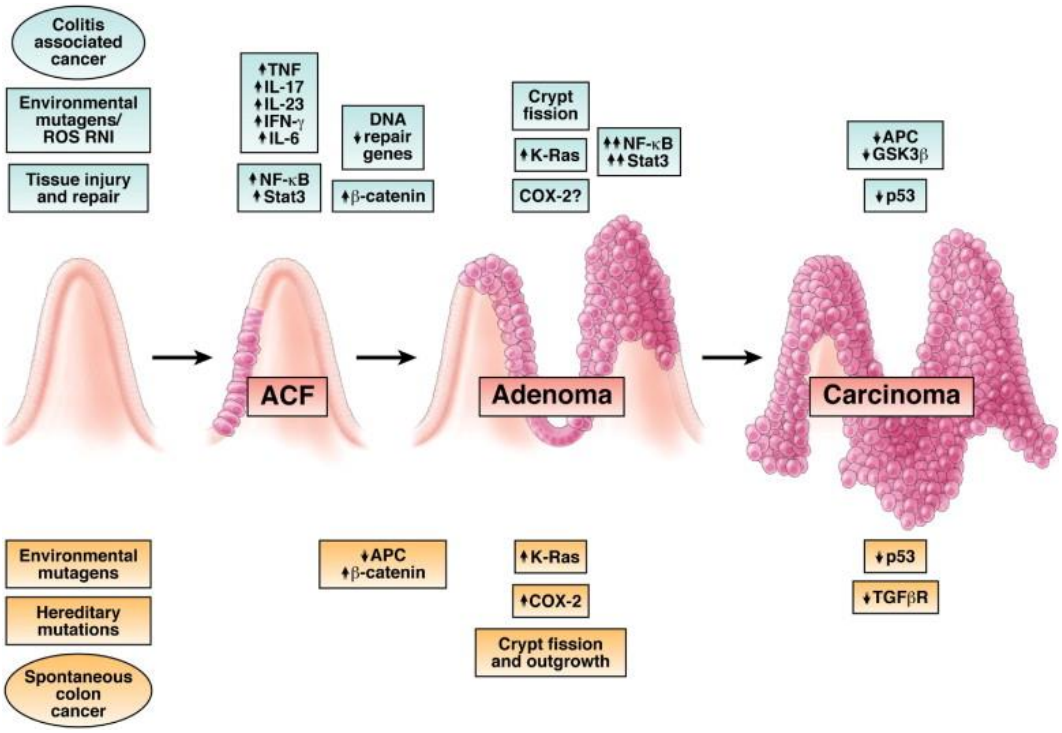


Fig.12: Colorectal carcinoma development is a multi-steps process involving the accumulation of different genetic alterations in colonic epithelial cells [122]

In most CRC cases, mutations occur as a multi-steps process that leads to the accumulation of genetic alterations in colonic epithelial cells and evolve from a benign adenoma to invasive carcinoma (figure 12).

Adenoma is an early lesion characterized by highly replicating cells forming a mass that protrudes into the lumen. The transition from benign adenoma to invasive carcinoma occurs when colonic neoplastic cells overstep the *muscolaris mucosa* and invade the submucosa, thus resulting in an invasive colorectal cancer.

CRC is characterized by many genetic alterations that can be classified based on the DNA damage that they are able to generate: genomic instability (chromosomal instability, DNA-repair defects, aberrant DNA methylation), mutational inactivation of tumor-suppressor genes (e.g. APC), activation of oncogene pathways (e.g. RAS, BRAF), and alteration of regulatory mechanisms in stem-cell and growth factor pathways [123]. The loss of genomic stability plays a crucial role in colorectal cancer development by facilitating the acquisition of multiple tumor-associated mutations: the two most common genomic instabilities in CRC are microsatellite instability (MSI) and chromosomal instability (CIN) (figure 13).

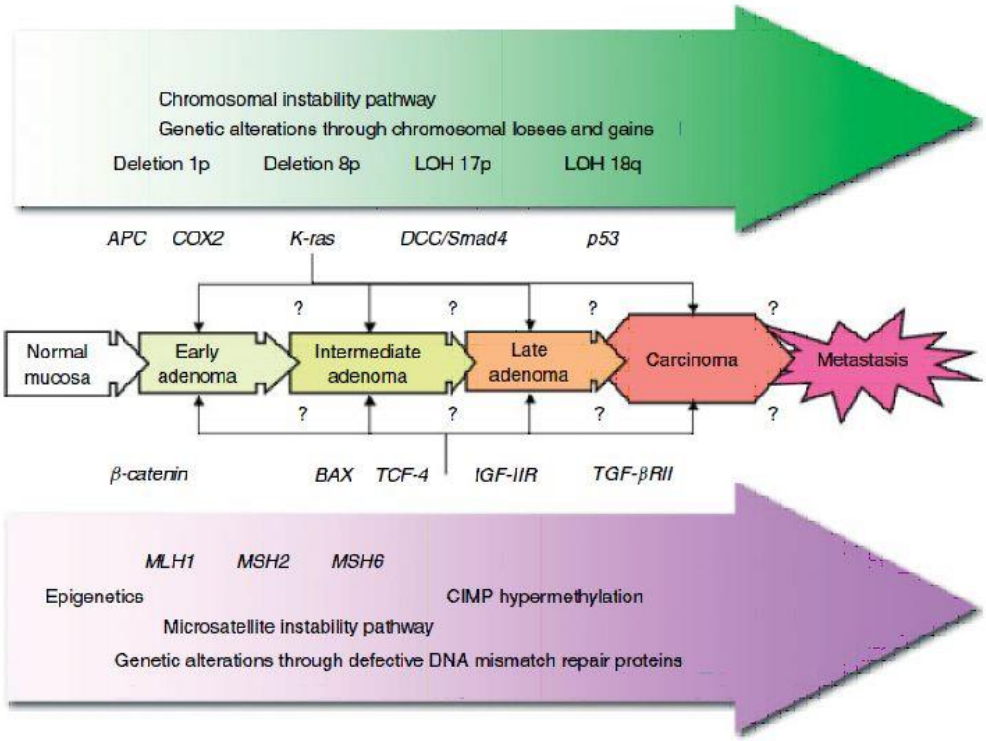


Fig.13: Schematic representation of the two major pathways (CIN and MSI) involved in CRC development and progression [124].

Microsatellite instability is a hypermutable phenotype caused by an expansion in the number of tandem repeat sequencings during DNA replication. Loss of the DNA repair system activity (Mismatch Repair Gene, MMR) is detected in about 15% of all CRCs [125]. Chromosomal instability occurs in 85% of colorectal cancer cases and is characterized by widespread imbalances in chromosome number, due to defects in chromosomal segregation. The loss of the tumor-suppressing activity caused by mutations in tumor-suppressor genes, such as APC, p53 and SMAD4, triggers neoplastic transformation and tumor cell survival, invasion and metastasis. The early mutation of APC is the most common initiating event in CRC: APC is able to bind β-catenin creating a complex that is degraded in an ubiquitin-dependent manner. In this way the wnt pathway is inhibited. In the absence of a functional APC protein, cellular activation is uncontrolled, wnt signaling is inappropriately and constitutively activated and neoplastic colon cells begin to proliferate. Beyond tumor suppressor genes, also oncogenes play a crucial role in colorectal cancer occurrence. Oncogenic mutations of RAS and BRAF activate the MAPK signaling pathway and represent 37% and 13% of colorectal cancer mutations, respectively; furthermore, one third of these tumors bear activating somatic mutations in PI3KCA [123].

Colorectal cancer and cancer related inflammation

Colorectal cancer is a paradigm of the cancer related inflammation [126] (figure 14). Epidemiological and clinical studies showed that patients affected by inflammatory bowel diseases (IBD) such as Chron's disease and ulcerative colitis (UC) have an higher risk to develop colorectal cancer: after passing through the phase of flat dysplasia and hyperplastic lesions, the inflamed mucosa evolves to carcinoma [127, 128]. In addition to genomic instability that sustains the process of tumorigenesis, the presence of a chronic inflammation, characterized by high levels of cytokines, chemokines, growth factors and reactive oxygen species, seems to be a key factor in sustaining and promoting CRC. During colorectal carcinogenesis, epithelial cells accumulate genetic mutations and release several inflammatory mediators that act on leukocytes, endothelial cells and fibroblasts, promoting the establishment of a reactive tumor microenvironment. In particular, IL-6 plays a crucial role in CRC carcinogenesis being a key component in the transition from acute to chronic inflammation [129]. IL-6 is mainly produced

by myeloid cells and once secreted it binds its receptor IL-6R leading to the dimerization of gp130 expressed by tumor cells; this event activates JAK that in turn phosphorylates STAT3, inducing the expression of crucial genes in cell cycle progression and suppression of apoptosis. Indeed, STAT3 modulates chemokine and adhesion molecule expression, supporting intestinal cell proliferation: recent studies showed that serum levels of IL-6 in colorectal cancer patients are higher than in healthy controls and correlate with a poorer survival rate [130]. On the contrary, IL-10 is a key molecule in inflammatory resolution and is downregulated during carcinogenesis development. It has been demonstrated that knock out mice for IL-10 spontaneously develop colitis while they are protected from colon cancer occurrence after exogenous administration of IL-10 [131]. Conversely, TGF-β modulates epithelial cell differentiation and inhibits cell growth. Studies have established that the TGF-β signaling pathway is mutated in CRC. In this context, TGF-β can inhibit T CD8+ lymphocytes limiting their beneficial effects on patient outcomes and promoting tumor growth and progression [132]. Finally, TNFα is another important cytokine involved in CRC pathogenesis. Several lines of evidence suggest that TNFα has a key role in IBD occurrence and promotes neoplastic cell growth, invasion and metastasis. Recent studies showed that mice lacking the type 1 TNFR-p55 are protected from the development of colorectal cancer in a model of inflammation-associated carcinogenesis [133].

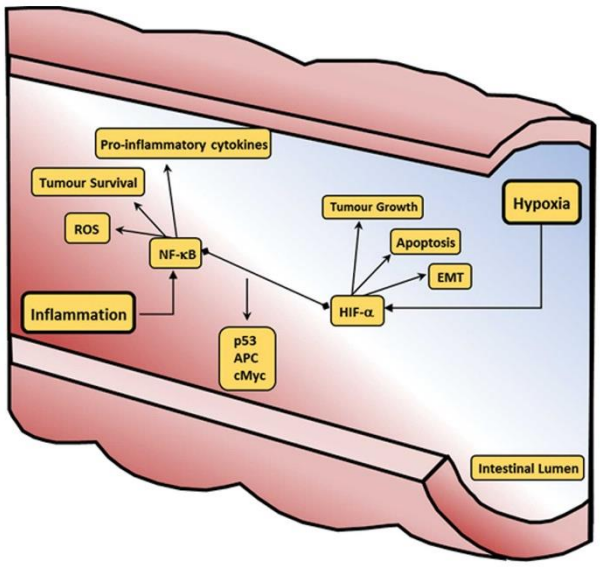


Fig.14: Inflammation can trigger cancer [134]

In CRCs, tumor-infiltrating cells predominantly include TAMs, myeloid derived suppressor cells (MDSCs), mesenchymal stem cells (MSCs), $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, regulatory T cells (Tregs), mast cells, platelets, cancer-associated fibroblast (CAFs), endothelial progenitor cells, monocytes and neutrophils. All these cells synergically interact with the extracellular matrix and matrix-associated molecules such as collagen, laminin, integtrins, MMPs, mucins and hyaluronic acid to modulate CRC progression. CAFs, are the main cellular constituents of the reactive stroma in primary and metastatic cancer and have a crucial role in CRC development: they facilitate tumor growth by secreting growth factors, are involved in the production of ECM and matrix-associated molecules and promote tumor-related angiogenesis, invasion and metastasis. Also mast cells and neutrophils play a role in CRC angiogenesis. Recent studies revealed that in CRC patients a lower number of mast cells was associated with hypovascularity and better survival rate [86, 135]; moreover, neutrophil-derived MMP-9 releases biologically active VEGF from the ECM by the cleavage of heparan sulfates [136]. Indeed, the expression levels of some MMPs is elevated in human CRC and correlates with stage of disease and/or prognosis: for example, MMP3 in CRC is associated with low levels of microsatellite instability and poor prognosis, whereas overexpression of MMP12 is related to increased survival [137]. Several studies confirmed that high levels of tumor infiltrating lymphocytes (TILs), in particular located intraepithelially, are associated with favourable prognosis in colorectal cancer. Galon and colleagues identified a dominant cluster of genes involved in Th1 immune responses that inversely correlates with tumor recurrence; moreover, they demonstrated that adaptive immunity promotes patient survival, prevents tumor recurrence and can have a beneficial effect during tumor progression [138]. In addition, Pages et al. found increased levels of mRNA of markers of Th1 effector T cells that positively correlate with prolonged survival and the absence of pathological signs of early metastatic invasion [139]. Finally, Laghi recently demonstrated the CD3+ T cell levels at the invasive margin of early tumors are associated with a lower risk of metachronous metastasis and a survival advantage [140].

Tumor associated macrophages in colorectal cancer

Considering their pro-tumoral functions, in the majority of cases, TAM infiltration correlates with tumor progression and consequently worst patient prognosis. Indeed, high density of TAM has been significantly associated with poor prognosis in the majority of tumors. In colorectal cancer, the role of TAM is still controversial. To define TAM function, their localization appears of primary importance [121]. Despite some reports describe TAM to have an anti-tumoral function, in many studies there is evidence that TAMs exert a pro-tumoral activity in CRC promoting tumor growth and metastasis by inducing angiogenesis, ECM breakdown and enhancing tumor cell migration/invasion. A massive macrophage infilitration is correlated with tumor progression, growth and disease aggressiveness. The process of tumor invasion and metastasis is supported by the release of CSF-1 by neoplastic cells. CSF-1R is expressed by macrophages and the axis is able to recruits TAM that contribute to the epithelial-to-mesenchymal transition (EMT), an initial event for cancer metastasis [86, 141]. Beside this, both TAMs and colon cancer cells secrete TGFβ that leads to EMT by activating the Smad pathway. In addition, stromal cells of the microenvironment, stimulate macrophages to secrete cathepsins, MMPs (MMP2 and MMP9) and serine proteases that cleave cell adhesion molecules, such as E-cadherin and the ECM and favor tumor spread. A further evidence of the putative pro-tumoral role of TAM in CRC was provided by Kaler and colleagues: their study reported that TAM, through IL-1β, stimulate the Wnt-β catenin signalling patwhay in colon cancer cells supporting tumor growth. Pancione et al. also reported that reduction or loss of β-catenin and peroxisome proliferator-activated receptor-γ (PPAR- γ) expression correlated with high COX-2 levels, massive TAM infilitration and tumor aggressiveness [142]. Another important pro-tumoral activity of TAM is the production of IL-23, a crucial cytokine for the polarization of Th17 lymphocytes. Recently, in some tumors, including CRC, high levels of IL-23 has been detected; this cytokine promotes tumor growth by stimulating inflammatory responses, up-regulation of MMP9, increased angiogenesis and reduced CD8+ cell infiltration. On the contrary, some studies highlight the beneficial role of TAM in CRC. For instance, Sugita and colleagues demonstrated the correlation between the number of macrophages at the tumor margin and the number of apoptotic cancer cells, suggesting a Fas ligand dependend apoptosis induced by macrophages [121]. Moreover, Funada et al. showed that high levels of

macrophages at the invasive front are linked with an increased overall survival rate; while Khorana et al. analysed the presence of VEGF-expressing TAM and found significant correlation with favorable outcome [143, 144]. Finally, Forssell et al. showed that the interaction between TAM and tumor cells is necessary to activate the anti-tumorigenic immunity and that, consequently, the macrophage infiltration at the tumor front positively influenced colon cancer prognosis [145].

Overall, these data highlight the controversial role of TAM in CRC suggesting that their pro- and anti-tumor effects may depend on their localization within cancer tissue. At the invasive margin, macrophages are located in less hypoxic areas and seem to have less exposure to tumor-derived cytokines: this condition may trigger macrophage tumoricidal differentiation instead of promoting their pro-tumoral phenotype. On the contrary, the high level of hypoxia in the stroma can trigger macrophages to be in syntony with the tumoral microenvironment and, consequently, to acquire a pro-tumoral phenotype. Reasonably, macrophages may also have different effects at different stages of tumor progression. At early stages, they cooperate with other innate immune cells and eliminate tumor cells by activating adaptive responses, while at advanced stages TAM shift toward M2-polarized cells and support tumor progression.

Mouse Models

In spite of a well-established evidence that chronic inflammatory conditions promote cancer development, the underlline molecular mechanisms are not fully elucidated. Mouse models of disease have been used and represent an useful and appropriate methodology to investigate human diseases, including colorectal cancer and it relationship with inflammation. There are many methods to induce inflammation in colon and different genetically engineered mice to investigate different aspects of this complex pathology. Indeed, using mice instead of *in vitro* methods provides the opportunity to study interrelated processes from both a pathological and a physiological point of view: to investigate the dynamic tumor microenvironment and the complex network between stromal components, inflammatory cells and neoplastic cells, the use of a whole-body system provides more valid and detailed information. Mice can spontaneously develop cancer or be susceptible to chemical induction of carcinoma. A number of genetically engineered animals, where the expression of one or more genes is selectively suppressed (*knock-out,* KO mice) are available. Many events can trigger colon cancer such as mutations in the germinal lines, sporadic mutations or toxic agents that can cause inflammation or alter the DNA integrity. In this complex scenario, KO mice are a useful model to investigate the different pathways that can lead to cancer development (table 2). For instance, IL-10 is a key regulator of the pathogenesis of IBD and the hystopathology of chronic colitis in IL-10 KO mice is very similar to that seen in IBD patients: epithelial hyperplasia, inflammatory infiltrates in the mucosa and crypts abscesses have been detected both in IL-$10^{-/-}$ mice and UC and Chron's disease patients [146]. It has to be considered, however, that IL-$10^{-/-}$ mice do not show the same involvement of genes that characterize human carcinogenesis such as APC, RAS and p53, nevertheless these animals mirror what happens in the intestine of IBD patients by spontaneously developing colitis and CAC. Another relevant model largely used is the APC^{min}. The mutation of the APC gene is an early causative event in familial and sporadic colon cancer pathogenesis, for this reason APC^{min} mice have been long used to study CRC. The APC^{min} mouse model is generated by a mutation in the tumor suppressor gene APC; APC mice, homozygous for the mutation, are not vital and show embryonic lethality, while heterozygous mice survive until 120 days [131]. The APC gene

mutation leads to mitotic defects in histologically normal crypt cells of the murine small intestine and provides changes in crypt dimensions, cell proliferation and apoptosis. Consequently, the formation of microadenomas that result from DNA hypomethilation occurs (figure 15).

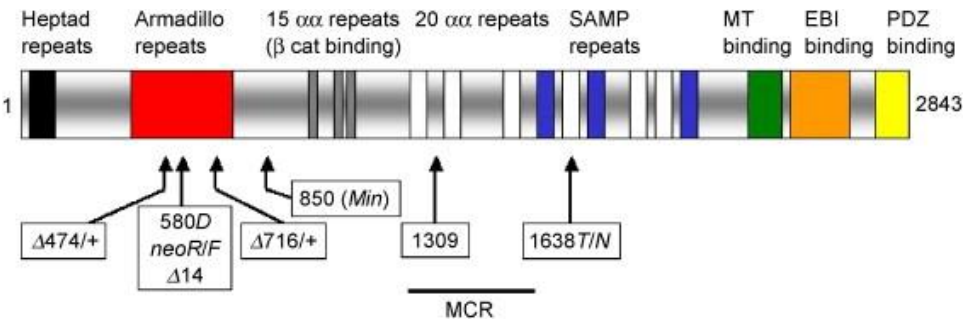


Fig.15: The APC gene is composed by 2843 aminoacids. The great number of mutations that occur in CRC are in the MCR region [147].

As for IL-10$^{-/-}$ mice, also this model shows some differences with human in the progression of the disease. For instance, APC^{min} mice spontaneously develop polyps predominantly in the small intestine, while in humans polyps and CRC develop mostly in the colon [131]. This is the reason why APC^{min} mice are often treated with chemical agents to promote polyps development also in the colon.

Model	Characteristics	Tumor histology	Tumor location	Incidence of cancer
IL-2 x β_2-M DKO	UC-like	Carcinoma	Rectum, colon	32% adenocarcinoma
IL-10 KO	CD-like duodenitis, colitis	Carcinoma	Colon, rectum	60% adenocarcinoma
RAG2 KO	Induced by infecting with *H. hepaticus*	Dysplasia, tubular adenoma, carcinoma, adenocarcinoma	Cecum, colon	100% adenocarcinoma
RAG2/Tgfβ1 DKO	Colitis	Dysplasia, adenocarcinoma	Cecum, colon	100% adenocarcinoma
TCRβ/p53 DKO	UC-like	Dysplasia, adenocarcinoma	Ileocecum, cecum	70% adenocarcinoma
Gpx1/Gpx2 DKO	Ileocolitis	Dysplasia, adenocarcinoma, signet ring cell carcinoma	Ileum, colon	28% adenocarcinoma
$G_{\alpha i2}$ KO	UC-like	Carcinoma	Colon	31% adenocarcinoma
Msh2 KO	HNPCC	Adenoma, adenocarcinoma	Jejunum	38.9% adenocarcinoma
Msh3 x Msh6 DKO	HNPCC	Adenoma, adenocarcinoma, lymphoma	GI tract	81.3% adenocarcinoma
Msh6 KO	HNPCC	Adenoma, adenocarcinoma, lymphoma	GI tract	38.5% adenocarcinoma
Mlh1 KO	HNPCC	Adenomas, adenocarcinomas	GI tract	72.0% tumor incidence 9.36% adenocarcinoma
Mlh3 KO	HNPCC	Adenomas, adenocarcinomas	GI tract	52% tumor incidence 14% adenocarcinoma
Mlh3 x Pms2 DKO	HNPCC	Adenomas, adenocarcinomas	GI tract	42% tumor incidence
$Msh2^{G674A/G674A}$	HNPCC	Adenocarcinomas	GI tract	19% adenocarcinoma
$Mlh^{G67R/G67R}$	HNPCC	Adenocarcinoma, adenoma, squamous basal cell carcinoma	GI tract	61% adenocarcinoma

Tab.2: Different mouse models used in CRC and CAC investigation [131].

Another approach to investigate the link between inflammation and CRC is to induce carcinogenesis with chemical agents (table 3). Reagents can be used in combinations or in association with genetically engineered models in order to mimic the different etiology linked to colorectal carcinoma development. The most common used model to study CAC is the

association between a carcinogen and a substance able to irritate the mucosa and, consequently, to promote inflammation. Indeed the most and frequently model used by researchers is a combination of azoxymethane (AOM), a carcinogen widely used to enhance the formation of colorectal tumors in rodents and the administration of Dextran Sodium Sulfate-induced (DSS) (tab). DSS can be used in combination with AOM -or other carcinogens- or alone. In both cases, the administration of DSS can cause a disease that quite faithfully reproduces the clinical course of UC. Because in humans the spontaneous onset of active inflammation is separated by periods of disease inactivity, DSS is usually administrate for 3-7 days in mice followed by regular water administration for 1-2 weeks to trigger colonic mucosa healing [131].

Models	Animal species	Treatment	Duration
Carrageenan	Rats	5%–10% degraded carrageenan in diet, drinking water or by stomach tube	2, 6, 9 months or up to 24 months
Dextran Sulfate Sodium (DSS)	C57Bl/6J	0.1%–0.4% DSS	60 days
	C57Bl/6J	0.7% DSS	15 cycles of: 1 week DSS followed by 10 days water
	C57Bl/6J	2.5% DSS	12 cycles of: 7 days DSS followed by 10 days water
	Mice	3% DSS	9 cycles of: 7 days DSS followed by 14 days water
	C57Bl/6J	4% DSS	4 cycles of: 4 days DSS followed by 12 days water
	Wister Rats (Male)	5% DSS	5–8 cycles of: 4 days DSS followed by 10 days water
	Swiss Webster mice	5% DSS	7 days DSS followed by 14 days water, sacrificed 120 days after the 4th cycle
DSS & AOM	Mice (ICR,Balb/c, C57Bl/6J)	AOM (10 mg/kg of body weight) & 2% DSS in drinking water	Single IP AOM injection followed by 1 week 2% DSS
DSS & DMH	Mice (BALB/c)	1 dose of DMH (20 mg/kg of body weight) & 3% DSS in drinking water	Single IP DMH injection followed by 3 cycles of 3% DSS
DSS & Iron	Mice (C57Bl/6J)	2X Fe or 2X Fe-NAC diet & 0.7%–1% DSS	12 or 15 cycles of DSS (1 DSS cycle = 7 days DSS followed by 10 days water)

Tab.3: Different combinations of chemical agents use to promote colon cancer. Protocols change based on the duration of the experiments, the way of administration and the mouse background [131].

It has been established that due to the synergic effects of AOM (a tumor-inducing agent) and DSS (a tumor-promoting agent) the AOM/DSS model mirrors colorectal carcinogenesis [148].

Importantly, the AOM-DSS-induced tumors share very similar features to human CRC even at molecular level: as in human CRC, tumors display evidence of dysregulation of the APC/β-catenin-signaling pathway due to the mutation in the β-catenin coding gene induced by AOM. This event aberrantly activates Wnt signaling pathway and leads to β-catenin cytoplasmatic and nuclear accumulation. Moreover, mutations of K-Ras and increased levels of enzymes involved in prostaglandin and nitric oxide synthesis such as COX-2 and iNOS can occur [149]. According to its particular ability in reproducing many aspects of human CRC, the AOM/DSS model is a feasible protocol that can also efficiently reproduce the multistep tumor development from microadenomas to adenocarcinomas passing through the phase of macroscopic adenomas and adenomatous polyps. For example, Tanaka and colleagues showed that already after 3-4 weeks from the beginning of the AOM/DSS protocol, luminal colon mucosa displays aberrant crypts infiltrated by a high number of inflammatory cells (including lymphocytes and plasma cells) and nascent tumors . These events have been observed also in humans were colitis-associated cancer is characterized by a dense infiltrate of immune cells (macrophages, DCs and T cells).

AIM of the thesis

The intestinal mucosa is a very peculiar environment from the immunological point of view as it is continuously exposed to foreign antigenic material and to the many commensal microbes that colonize the gut. The intestinal immune system has the difficult task of discriminating between harmless antigens and harmful pathogens, and generating tolerance or protective immunity, appropriately. Indeed, the balance between tolerance and immune response is very delicate and tightly regulated. Among the cells and soluble mediators that regulate gut homeostasis are resident intestinal CX3CR1^{+} macrophages, T regulatory cells and the anti-inflammatory cytokines IL-10 and TGFβ.

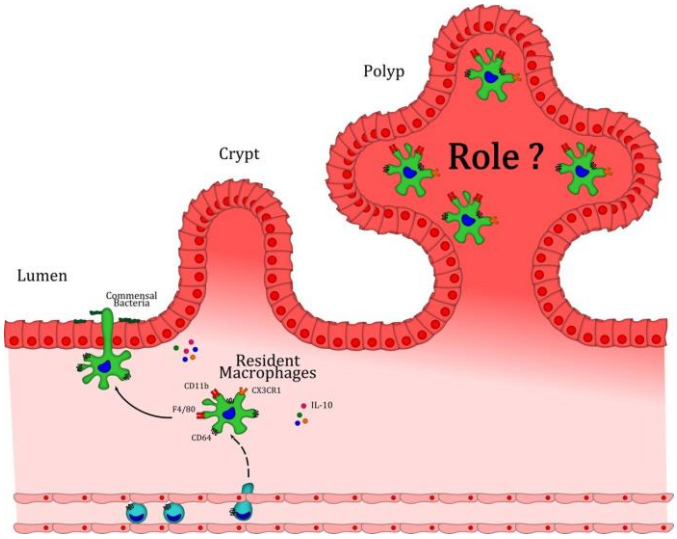
Gut resident CX3CR1^{+} macrophages play a crucial role in the maintenance of intestinal homeostasis by the production of IL-10 and the ability to sense this cytokine in an autocrine loop. These macrophages are characterized by the expression of the chemokine receptor CX3CR1; however, how this receptor is involved in the molecular mechanisms that regulate the inflammatory response, is largely unknown. Furthermore, while the function of intestinal CX3CR1^{+} macrophages in acute inflammation has been described, their role in the resolution phase and in chronic inflammation has not been investigated. Intestinal chronic inflammation is a state predisposing to colorectal carcinogenesis; indeed, it is now established that cancer-associated inflammation promotes cancer progression in all its phases: from the early events till the spread of the metastasis. It is therefore important to understand if and how these "regulators" of gut inflammation are involved in the process of colorectal carcinogenesis.

The global aim of this thesis is to define the role of mucosal CX3CR1^{+} macrophages during the process of intestinal carcinogenesis, and to dissect the molecular mechanisms by which these immune cells maintain gut homeostasis.

By using genetically modified mice for the chemokine receptor CX3CR1, we have analyzed animals deficient (CX3CR1$^{GFP/GFP}$) or proficient (CX3CR1$^{GFP/+}$) for this signaling pathway. Mouse models of chemically-induced acute and chronic inflammation, as well as of colitis-induced cancer have been performed to specifically achieve the following goals:

- Characterize the state of colonic inflammation upon acute tissue injury and during the process of recovery

- Understand the role of mucosal CX3CR1^{+} macrophages in steady state and in the inflammatory response and recovery
- Understand the role of mucosal CX3CR1^{+} macrophages in the process of chronic colitis leading to colorectal carcinogenesis
- Characterize the molecular mechanisms underlying the anti-inflammatory function of CX3CR1^{+} macrophages with a special focus on CX3CR1 signaling



Materials & Methods

Mouse models

Procedures involving animals and their care conformed to institutional guidelines in compliance with national (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 2010/63/EU, OJ L 276/33, 22.09.2010; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 2011). All efforts were made to minimize the number of animals used and their suffering.

All mice used were of the C57BL/6 background. We compared $CX3CR1^{+/GFP}$ (WT) to $CX3CR1^{GFP/GFP}$ (KO) and $CX3CR1^{+/GFP}$-APC^{min} (WT) to $CX3CR1^{GFP/GFP}$-APC^{min} (KO). Mice were maintained in a specific-pathogen free facility

DSS-induced colitis

C57BL/6 CX3CR1 WT and KO mice were used to generate a model of colitis. Six- to eight-week-old male mice with an initial weight of 18-20 g received 3% of dextran sulfate sodium (DSS-MP biomedical) dissolved in drinking water for 7 days. Mice were sacrificed at day 7. In order to assess the recovery of colitis, some mice were fed with normal water after day 7 and sacrificed at day 10 or 13. During the experiment, twice a week, weight loss was measured evaluate the level of colitis

Models of colitis-associated cancer

AOM-DSS model of colitis-associated cancer

C57BL/6 CX3CR1 WT and KO mice were used to generate a model of colitis-associated cancer (CAC).

Six- to eight-week-old male mice with an initial weight of 18-20 g received a single intraperitoneal (i.p.) injection of azoxymethane (AOM-from SIGMA-; 10 mg/kg of body weight). Water containing 3% DSS was administered to mice beginning on day 7 for 6 days,

followed by 15 days of fresh water. The treatment was repeated twice for a total of 3 rounds of DSS. Mice were euthanized on day 70 after the 10-week treatment (figure 16).

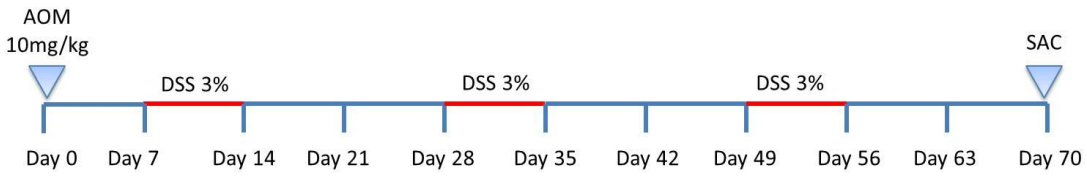


Fig 16.: AOM-DSS protocol

During the experiment, mouse weight loss and stools blood and consistency were monitored twice a week to provide an assessment of cancer-associated colitis and cancer severity during the treatment. Hemoccult test from SENSA was used to detect occult blood and to evaluate stools consistency (table 4).

Weight loss	Score	Stool blood	Score	Stool consistency	Score
< 1%	0	Negative	0	Normal	0
1-5%	1	Positive to Hemoccult	1	Soft	1
5-10%	2	Visible blood	2	Very soft	2
10-15%	3	Gross bleeding	3	Diarrhea	3
> 15%	4				

Tab 4: Disease Activity Index (D.A.I.). Every week stool blood and consistency were analyzed in respect to values reported in the table.

Induction of hemox-1 during the AOM-DSS model

Cobalt protoporphyrin IX (coPP) was prepared in dim light because of the photosensibility of the compound. It was dissolved in sodium hydroxide (NaOH) 150mM and then the pH was adjusted to pH 7 by adding equal amount of hydrochloric acid (HCl) at the final concentration of 5mg/ml. The final pH of 7,4 was achieved by further dilution with Phoshate Buffer Saline without Calcium and Magnesium (PBS-/-, BioSera). 300ug of coPP or vehicle (NaOH and HCl) in a final volume of 200ul was given to mice following the scheme reported in figure 17:

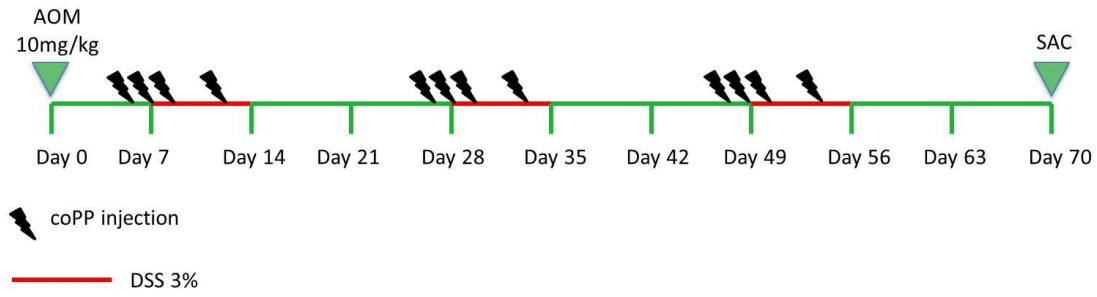


Fig. 17: The AOM-DSS protocol implemented with coPP administration

APC^{min} model of colitis-associated cancer

CX3CR1$^{+/GFP}$-APC^{min} (WT) and CX3CR1$^{GFP/GFP}$-APC^{min} (KO) mice were used to generate a genetic model of colitis-associated cancer. Four-week-old male mice with an initial weight of 17-19 g received 2,5% DSS in drinking water until day 7. Subsequently, mice were fed with fresh water for four weeks and then euthanized on day 35 after 5-week treatment.

Histological analysis

After mouse sacrifice, colons were isolated and excised for histological analysis and immunofluorescence.

Colons were harvested, flushed of luminal contents, and opened with a longitudinal cut; next, they were rolled up transversely *(swiss roll)*, with the distal region inside the *swiss roll*.

Tissues were then fixed in 4% paraformaldehyde (PFA) overnight at 4°C and dehydrated first in 30% sucrose in $PBS^{-/-}$, for 6 hours and then in 40% sucrose in $PBS^{-/-}$, overnight. Next, the colonic tissues were embedded in OCT (optimum cutting temperature compound, Diapath) and stored at -80°C.

To evaluate the histological architecture of the inflamed colon and calculate the percentage of the adenomatous area over the total tissue, 8 µm thick frozen sections were cut with cryostat, placed on a glass slide and stained with hematoxylin and eosin (H&E stain).

Sections were fixed with 4% formalin for 5 minutes, washed in tap water for 5 minute, stained with hematoxylin (3 minutes), quick washed and stained for 2 minute with eosin. The eosin excess was then removed by quick washes in water and sections dehydrated with increasing concentration of EtOH (from 70%-100% EtOH). Finally the slides were cleared with xylenes and mounted with a xylenes-based mounting medium. Slides were observed with microscope (4x) and colitis score was assessed as in the following table (5):

Score	**Description**
0	Normal mucosa
1	First signs of inflammation: few inflammatory cells, crypts well defined and equidistant.
2	Many inflammatory cells in the mucosa. Crypts are destroyed or irregular and no more equidistant. Presence of open or repaired ulcers.
3	The tissue architecture is lost. Leukocytes invade the *muscolaris mucosae.* Crypts are totally destroyed and the tissue is full of open ulcers.

Tab 5: Histological damage evaluation (colitis score)

In order to define polyps' growth, slides were scanned with VS120 Dotslide (Olympus) and analysed with OLYVIA software.

Immunofluorescence

For immunofluorescence staining, frozen tissue sections (8 μm) were firstly rehydrated with pH 7 Phosphate Buffer Saline with Calcium and Magnesium ($PBS^{+/+}$- BioSera). Sections were then fixed in 4% PFA for 15 minutes.

Subsequently, tissues were permeabilized and blocked with 0.1% Triton X-100 (Sigma Aldrich), 2% Bovine Serum Albumin (BSA), and 5% Normal Goat Serum in $PBS^{+/+}$ for 30 minutes in a dark incubation chamber. Section were washed for 5 minutes in $PBS^{+/+}$ Tween-20 0,05% (washing buffer) and subsequently incubated with the primary antibody(ies) in washing buffer (1 hr at room temperature or 4°C overnight, depending on antibody).

After three washes in washing buffer, sections were incubated with fluorophore-coniugated secondary antibody(ies) at room temperature for 1 hr in the dark (1:2000).

Finally, sections were washed four times, cell nuclei counter-stained with DAPI, washed out the excess of DAPI with water and mounted with a fluorescence preserving medium (Fluorsave, Calbiochem). Slides were then kept protected from light at room temperature. Negative controls were generated by omitting the primary antibody(ies).

Images were captured with the Olympus FluoView™ FV1000 confocal microscope. The acquired images were analysed with "(Fiji is just) ImageJ software". Multi-channel images were split to the respective components and every channel was transformed into a binary image. According to the "set measure" option, the percentage of stained tissue in each component was calculated overall the total black background of the single slide. Considering the DAPI stained tissue as the entire tissue (100%), we finally calculated the percentage of tissue stained by every single fluorophore using the mathematical proportion:

$$\%DAPI:100 = \%fluorophore: X$$

Whole mount

After mice sacrifice, animals were perfused with 2% PFA injected directly in the heart and colons excised, flushed from luminal contents and fat. Tissue were subsequently stretched and attached with fine pins to a small silicone rubber slab. Colons were fixed by immersion in 4% PFA overnight at 4°C; fixative was washed out with several changes of $PBS^{+/+}$. Samples were incubated overnight at 4°C with primary antibodies diluted in the following staining buffer: $PBS^{+/+}$, BSA 2%, Normal Goat Serum 5%, Triton X-100 0,1%, Sodium Azide 0,01% (NaN3-Merck). After incubation, tissues were washed several times in $PBS^{-/-}$ - Tween-20 0,05% for 15 minutes each time (5 hours in total) and then incubated overnight at 4°C with secondary antibodies diluited in $PBS^{+/+}$, Tween 20 0,05%, Triton X-100 0,1%, NaN3 0,01%. Finally, washing steps were repeated and tissues were mounted with Vectashield (Vector Laboratories). Slides were stored at 4°C.

Stack acquisition and morphometric analysis with Imaris software

Whole mount specimens were examined from serosa to mucosal crypts with the Olympus FluoView™ FV1000 confocal microscope. Confocal images were obtained scanning samples at a 40-150 μm depth collecting sequential XY images every 1 μm. Stacks were imported and processed using IMARIS software.

Antibodies

The following antibodies were used.

Primary antibodies: Rat anti-mouse F4/80 monoclonal antibody (eBioscience) 1:500, Armenian Hamster anti-mouse CD31 monoclonal antibody (Millipore) 1:500, Hamster anti-mouse CD103 monoclonal antibody (LsBio)

Secondary antibodies: Goat anti-rat IgG cross adsorbed Alexa Fluor® 594-conjugated (Invitrogen Molecular Probes) 1:2000, Goat anti-rabbit IgG cross adsorbed Alexa Fluor® 594-conjugated (Invitrogen Molecular Probes) 1:2000, Goat anti-rat IgG cross adsorbed Alexa

Fluor® 647-conjugated (Invitrogen Molecular Probes) 1:2000, Goat anti-armenian hamster IgG cross adsorbed Alexa Fluor® 647-conjugated (Invitrogen Molecular Probes) 1:2000.

RNA extraction and quantitative Real-Time PCR

Total RNA was extracted from colon tissue previously washed from luminal content and fragmented into small pieces (approximately 1 mm) or from cell culture.

In the CAC experiments, polyps were separated from the rest of the colon and analysed independently.

Samples were homogenized in TRIZOL (Ambion) using Tissue Lyser II (Quiagen). Homogenized tissues were then added with chloroform in order to separate RNA from genomic DNA and proteins. RNA isolation and purification required isopropanol (RNA precipitation) and 70% ethanol (RNA wash). Total RNA was resuspended in Nuclease-free water and quantified using the spectrophotometer NANODROP ND-1000. Subsequently, 1 μg of RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer's instructions. cDNA analysed through quantitative Real-Time PCR, performed on Viia-7 instrument (Thermo Fisher Scientific) using the Fast SYBR Green system. The thermal-cycling conditions for the Fast SYBR Green Master Mix are described in the table 6.

Cycling step	Temperature	Hold time	# of cycles
Initial denaturation and enzyme activation	95 °C	2:00	1
Denaturing	95 °C	0:10	40
Annealing and extension	60 °C	2:00	
Melt curve	65-95° C (in 0,5 °C increments)	0:5	1

Tab 6: The Fast SYBR Green cycling-protocol.

GAPDH gene was amplified as internal control. The others genes analysed are the follow:

GAPDH
Fw-GGCATTGCTCTCAATGACAA
Rev-ATGTAGGCCATGAGGTCCAC

IL-23
Fw-GACCCACAAGGACTCAAGGA
Rev-AGGCTCCCCTTTGAAGATGT

HEMOX-1
Fw-CAGGTGATGCTGACAGAGGA
Rev-TCTCTGCAGGGGCAGTATCT

IL-10R
Fw-CACATCCTCCACTGGAAACC
Rev-TGGATGTCATTCCAGGTTGA

IL-19
Fw-ATCCTGTCCCTGGAGAACCT
Rev-TCCTGGAACACCCTGTCTCT

IL-20
Fw-CATTGTGGGGAAGAAGCAAT
Rev-TTCTCCCAAAGCCTTTACCA

IL-22
Fw-CCGAGGAGTCAGTGCTAAGG
Rev-CATGTAGGGCTGGAACCTGT

COX-2
Fw-CCCCCACAGTCAAAGACACT
Rev-GGCACCAGACCAAAGACTTC

iNOS
Fw-GCACGATGTCATATTCCTTCCTCC
Rev-TTTGAGTAGCCTGATCC

IL-6
Fw-CCGGAGAGGAGACTTCACAG
Rev-CAGAATTGCCATTGCACAAC

IFNγ
Fw-ACTGGCAAAAGGATGGTGAC
Rev- GCTGATGGCCTGATTGTCTT

TNFα
Fw-CCACCACGCTCTTCTGTCTA
Rev-AGGGTCTGGGCCATAGAACT

IL-10
Fw-AGCCGGGAAGACAATAACTG
Rev-ATGTTGTCCAGCTGGTCCTT

TGFβ
Fw-TGGAGCAACATGTGGAACTC
Rev-AGACAGCCACTCAGGCGTAT

MMP-9
Fw-ATAGGCTTTGTCTTGGTACTG
Rev-AGGAGTCTGGATAAGTTGGG

IL-12
Fw- CTCCTGTGGGAGAAGCAGAC
Rev- CAGATAGCCCATCACCCTGT

IL-1β
Fw- GAGTGTGGATCCCAAGCAAT
Rev-TACCAGTTGGGGAACTCTGC

Quantification of the PCR signals of each sample was performed by comparing the cycle threshold values (Ct), in duplicate, of the gene of interest with the Ct values of the GAPDH housekeeping gene.

Western blot

The presence of Hemo-oxygenase-1 was tested in both inflamed colon and polyps through Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting assay. Samples were harvested after mice sacrifice and protein extracted using Urea buffer (Urea 9M, 25mM Tris-HCl pH 6,8, 1mM EDTA, 10% glycerol). 25ng of total proteins were resolved on 10% SDS-PAGE and transferred onto Trans Blot Transfer Medium, Nitrocellulosa membrane (Bio-Rad). Filters were blocked with with 5% milk in TBST (50 mM Tris, 150 mM NaCl and 0.05% Tween 20) and incubated over-night with affinity-purified rabbit anti-mouse Heme-oxygenase-1 antibody (proteintech) 1:600. After the incubation with peroxidise-conjugated donkey anti-rabbit IgG (Biosciences), the membrane were developed with chemiluminescence reagent (Bio-Rad), according to the manufacturer's instructions, and visualized with Chemidoc System (Bio-Rad).

Generation of bone marrow derived macrophages (BMDMs)

Bone marrow was harvested from femura of 8- to 12-week old mice by flushing the marrow out with IMDM supplemented with 10% Fetal Calf Serum (FCS) and ACK (100uL/bone) to lysate red cells. Flushed cells were resuspended in 20 mL IMDM, 10% FCS, 1% Penicillin/Streptavidin, 1% Glutammine and let to adhere overnight at 37°C, 5% CO_2. After incubation, cell supernatant was removed, non-adherent cells were spun down and resuspended at the concentration of $0.5x10^6$ cells/mL in complete bone marrow macrophage medium (IMDM, 10% FCS, 20 ng/mL M-CSF) in suspension culture dish (Corning) (10mL/dish). After 7 days, cells are lifted and replated for stimulations and analyses.

Cells were plated at the final concentration of $2.5x10^5$ cells/mL in 24 wells plate and stimulated with LPS (100ng/mL), recombinant Fractalkine (FKN, 300ng/mL) or LPS-FKN. To simulate acute inflammation, cells were stimulated for 18h with fractalkine and then 4h with LPS, while to simulate the recovery process, cell were stimulated with LPS for 4h and then with FKN for 18h. Subsequently, cells and supernatants were harvested for analyses.

Supernatants were centrifuged to remove cellular debris and analysed with ELISA kit for IL-6, TNF-α, and IL-10 (R&D Systems). Cells were lysate to recover RNA as already described, and used for qPCR analysis.

Isolation of mononuclear cells from lamina propria

To isolate lamina propria mononuclear cells (LPMCs), extraintestinal fat tissue was carefully removed and colons were then flushed of their luminal content with physiologic solution, opened longitudinally and cut into 1 cm pieces. Epithelial cells and mucus were removed by 40 min incubation with HBSS (without Ca2+ and Mg2+) containing 5% FBS, 2 mM EDTA at 37°C, shaking at 275 rpm. Colon pieces were then digested in HBSS-/- containing 5% FBS, 0,5 mg/ml Collagenase VIII (Sigma), for 40 min at 37°C shaking at 275 rpm. The obtained cell suspension was then pressed into a syringe needle, washed with RPMI and passed sequentially through 100 and 40 μm cell strainers. Cells were centrifuged for 6min at 1600 rpm, resuspended in RPMI and used for the subsequent analysis.

Phagocytosis Assay

In order to determine the ability of CXRCR1^{+} MO to phagocyte, peritoneal-elicited macrophages were harvested from WT and KO mice. Briefly, 1ml of thioglycolate (3% thioglycolate medium w/o dextrose, BD; Franklin Lakes, NJ) was injected intra-peritoneum (IP). After three days, mice were sacrificed and 10 ml of cold physiologic solution were injected in the peritoneum, cells were collected and kept in ice. Live cells were sorted using FACS Aria instrument based on GFP gene reporter expression. After, $2x10^5$ cells were plate on glass slides in 24 wells in DMEM overnight. In the meantime, polystyrene latex beads (Sigma-Aldrich), 3 μm in diameter were coated with LPS diluted 1:100 in bicarbonate buffer in a ratio of 1:10 cells/beads by incubating for 1h at 37°C shaking. Beads were washed one time with HBSS-/- and added to cells for 1h. The excess of beads was washed out and cells stained and fixed with DiffQuik (medion diagnostics) and mounted on glass. Beads were

counted at the microscope, analysing ten fields for each samples. The percentage of phagocytosis was calculated as follow:

n cells: n bead =100: x

Flow cytometry

Flow cytometry analyses were performed to determine the percentage of immune cells infiltrate. Briefly, cells were harvested from colon samples of both polyps, inflamed colon and healthy control as described before. $5x10^5$ cells per tube were washed with FACS buffer (PBS -/-, 2% FBS), and incubated 30 min at 4°C with antibodies. Subsequently, cells were washed twice with 1 mL of FACS buffer and suspended in 350ul of FACS buffer. Immediately, cells were read using FACS Canto II instrument and FACS Diva software version 6.1.1 (BD Biosciences). For each experiment, cells were stained with appropriate isotype control antibodies to establish background staining before calculating the percentage of positive cells.

Antibodies used for colonic lamina propria staining included: CD45 percP (BD), CD11b pacific blue (eBiolegend), F4/80 PE (Serotec), Ly6G PE-cy7 (BD), Ly6C APC (BD), TLR2 APC (BD).

Phospho-protein detection

To detect the expression of phosphorylated protein, spleen was collected from mice, smashed in DMEM and treated with ACK to lysate blood red cells. $2x10^6$ cells were suspended in DMEM at 37°C and stimuli were added (IL-10 25ng/mL, FKN 300ng/mL) to a final volume of 300ul. Cells were treated for 5', 30' or for 120'. After the treatment, 50 ul of PFA 16% were added for 10' at room temperature, to block the incubation and fix the cells. After two centrifugations, cold methanol was added for 20' to permeabilize cells. Methanol was removed with three washes in $PBS^{-/-}$ and samples were stained with pSTAT3 (py705-BD) for 30' at room temperature in the dark. After the incubation, cells were washed and suspended in 350ul of FACS buffer (PBS-/-, FBS 1%, NaN_3 0,1%) and analysed with CANTO II instrument.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software). To compare different data sets, unpaired two-tailed Student's t test was used and results were expressed as mean ± SEM or median and quartiles.

A value of $p<0.05$ (*) was considered as statistically significant (** $p<0,01$;*** $p<0,001$; $p<0,0001$****).

Results

Comparison of wild type mice and heterozygous CX3CR1$^{GFP/+}$ mice

The CX3CR1 gene was reported to be fully functional in heterozygous mice. Nevertheless, we decided to compare WT and CX3CR1$^{GFP/+}$ mice in order to establish if there were any differences between the two strains. Considering that our major interest was the understanding of the role of CX3CR1^{+} macrophages in the development of colorectal cancer, we performed our experiments using a model of colitis-associated cancer. Three physical parameters were used to establish the level of inflammation and the development of neoplasia: colon length, colitis score and the percentage of adenomatous tissue out of the total colon. As shown in figure 18, there were no differences between WT and CX3CR1$^{GFP/+}$ mice, while the variances between KO and WT or heterozygous mice were of the same extent. Based on the finding that the other allele remains functional, we adopted CX3CR1$^{GFP/+}$ mice, instead of using WT, because of the presence of the GFP gene. In this way, we were able to track CX3CR1^{+} macrophages in both strains. Therefore, in this thesis, CX3CR1$^{GFP/+}$ mice are referred as WT mice.

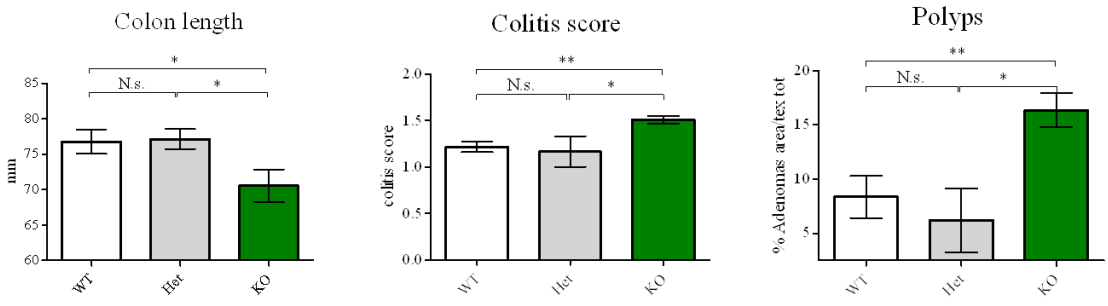


Fig.18: Comparison of WT and CX3CR1$^{GFP/+}$ mice: colon length, colitis score and the percentage of adenomatous tissue are three parameters used in the evaluation of tissue damage in mice treated with the AOM-DSS model. Here we compared WT and heterozygous mice and we found that they displayed the same behavior which is significantly different from that of KO mice.

Characterization of CX3CR1^{+} cells

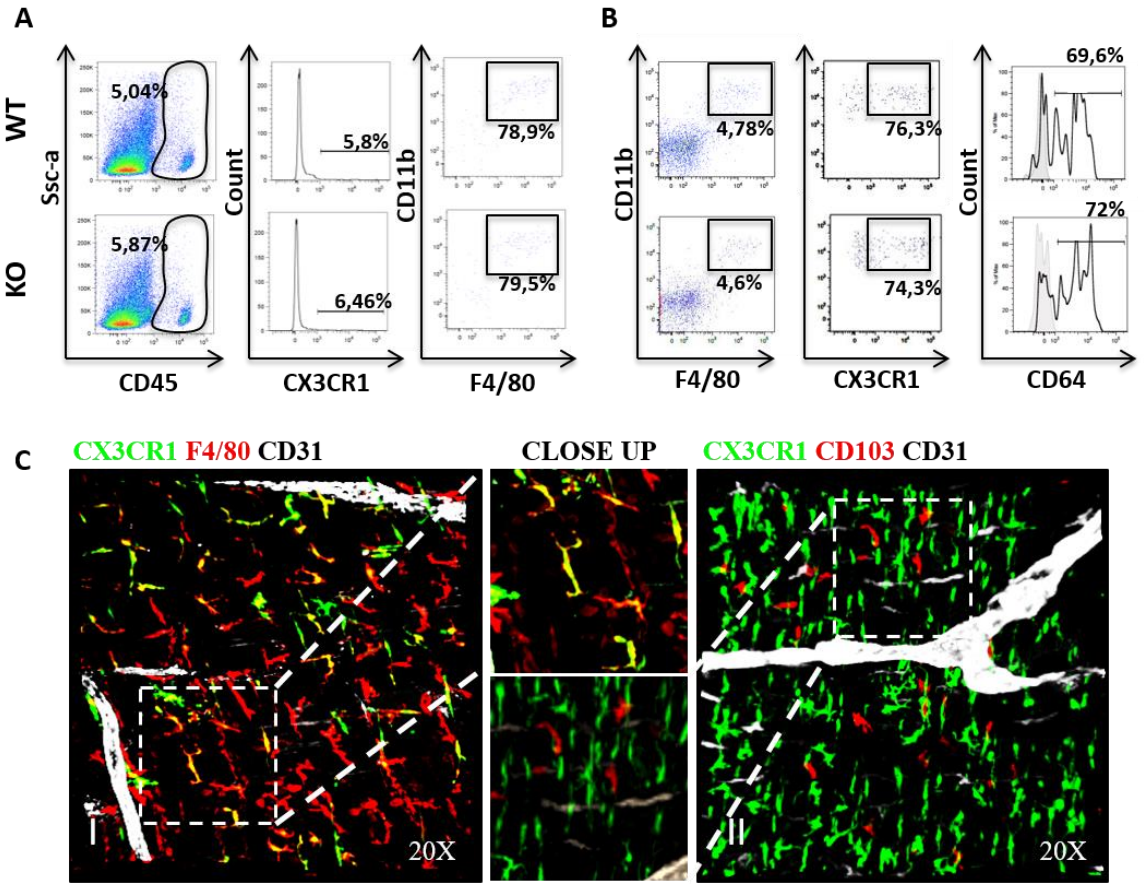


Fig. 19 Characterization of CX3CR1^{+} macrophages

Phenotype of resident macrophages in healthy colon by FACS analysis: (A) CX3CR1^{+} cells are a sub-population of macrophages in the colon expressing F4/80 and CD11b. Moreover, this subset express the marker CD64 (B). (C) Whole mount of healthy colonic tissue, magnification 20X. CX3CR1^{+} (green) cells co-localize with F4/80^{+} (red) macrophages and many spots of colocalization (yellow) are visible (I). CX3CR1^{+} (green) cells do not co-localize with dendritic cells positive for CD103 (red)(II).

Since now, the characterization of CX3CR1+ cells in the lamina propria gave controversial results. Indeed, when these cells were first characterized, they were considered dendritic cells. Nowadays, it is widely accepted that these cells are macrophages. We first wanted to confirm that CX3CR1+ cells are resident macrophages that colonize the gut mucosa. To do this, we harvested cells from healthy colon of both CXRCR1$^{GFP/+}$ and CX3CR1$^{GFP/GFP}$ mice and we performed FACS analysis. We first demonstrated that CX3CR1^{+}

cells represent 6% of all CD45$^+$ leukocytes. Among these, 80% are macrophages positive for F4/80 and CD11b. Next, we found that CX3CR1$^+$ cells are a particular subpopulation of macrophages expressing also CD64 as it was reported by Mowat and colleagues [150]. In fact, considering the percentage of positive macrophages expressing CX3CR1, we were able to demonstrate that 70% of these cells express also CD64. Interestingly, no differences between WT and KO were observed (figure 19).

In order to completely exclude that CX3CR1$^+$ cells are dendritic cells, we performed immunofluorescence analysis on whole healthy colon. We performed a double staining for F4/80, a macrophage marker, and for CD103, a dendritic cell marker. As shown in figure 19, panel C, many F4/80$^+$ cells are also CX3CR1$^+$ confirming, by mean of a different technique, that CX3CR1$^+$ cells are indeed macrophages. On the contrary, none of the CD103$^+$ cells express the receptor, definitely excluding that CX3CR1$^+$ cells are dendritic cells.

We first investigated the composition of gut resident leukocytes in WT and KO mice under homeostatic conditions. To do this, we performed FACS analysis on healthy colonic tissue and we analyzed the percentage of CD45$^+$ leukocytes, PMN and macrophages. In steady state conditions, we found no differences between WT and KO mice. Indeed, the percentage of the analyzed cells was almost the same between the two populations (figure 20). We were particularly interested in the behavior of macrophages. For this reason we evaluated different types of macrophages in order to analyze in depth this cell subset. We detected F4/80$^+$ and CD11b$^+$ macrophages and gating on these cells we analyzed the percentage of Ly6C^{high} cells to determine the rate of newly recruited macrophages. As shown in figure 20, there were no differences during the different steps of differentiation of Ly6C$^+$ monocytes in healthy colon. Finally, we looked at CX3CR1$^+$ cells and no differences were appreciated between WT and KO.

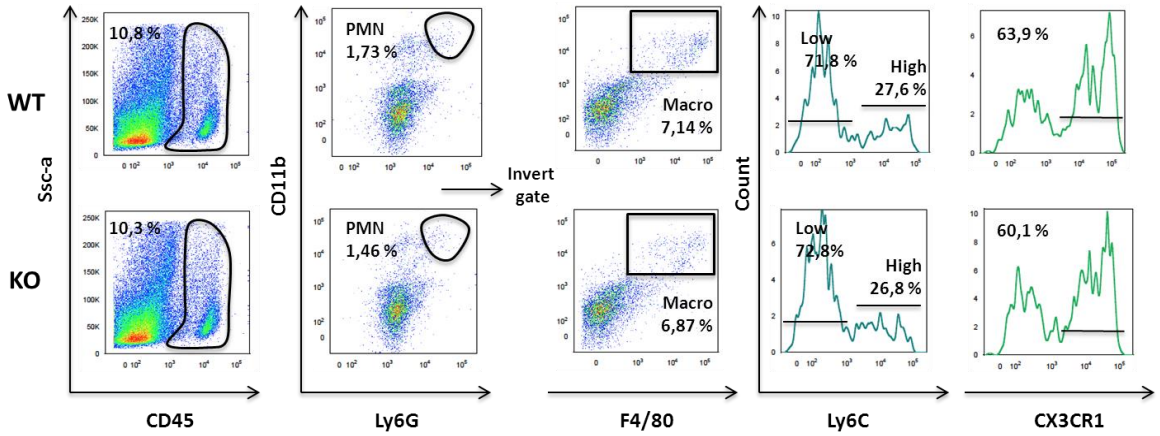


Fig. 20. Analysis of leukocytes in the healthy colon of WT and KO mice. The FACS strategy shown above was used for all the future experiments. The first gate characterized the global $CD45^+$ infiltrate. Based on $CD45^+$ cells, PMN and macrophages were detected by the expression of Ly6G or F4/80, respectively. From the latter population, macrophages expressing $Ly6C^+$ cells and $CX3CR1^+$ were studied. In steady state conditions, there were no differences in the accumulation of different immune cell types in the colon of WT and KO mice.

IL10 and CX3CL1 signalling in $CX3CR1^+$ macrophages

A very important feature of $CX3CR1^+$ macrophages is their ability to maintain homeostasis and to avoid the rise of aberrant inflammation. $CX3CR1^+$ Mφ produce a great amount of the anti-inflammatory cytokine IL-10. Recently, Zigmond et al., demonstrated that the production of IL-10 is not sufficient to maintain homeostasis: $CX3CR1^+$ Mφ need to sense the IL-10 they produce with IL-10 receptor (IL-10R) [34]. In order to confirm these results, we tested $CX3CR1^+$ Mφ for the production of IL-10 and for their ability to respond to it. We harvested bone marrow derived macrophages (BMDM), spleen and colonic macrophages from both $CX3CR1^{GFP/+}$ and $CX3CR1^{GFP/GFP}$ mice and evaluated the mRNA production of IL-10 and IL-10R. As shown in figure 21, panel A, we confirmed that macrophages produce both the cytokine and its receptor. To evaluate if the link between IL-10 and its receptor was functional and able to generate a correct signalling, we performed a phospho-flow assay to evaluate STAT3 phosphorylation after IL-10 stimulation (panel B). Macrophages from both WT and KO mice phosphorylated pSTAT3 after 5 minutes of stimulation in a super imposable manner.

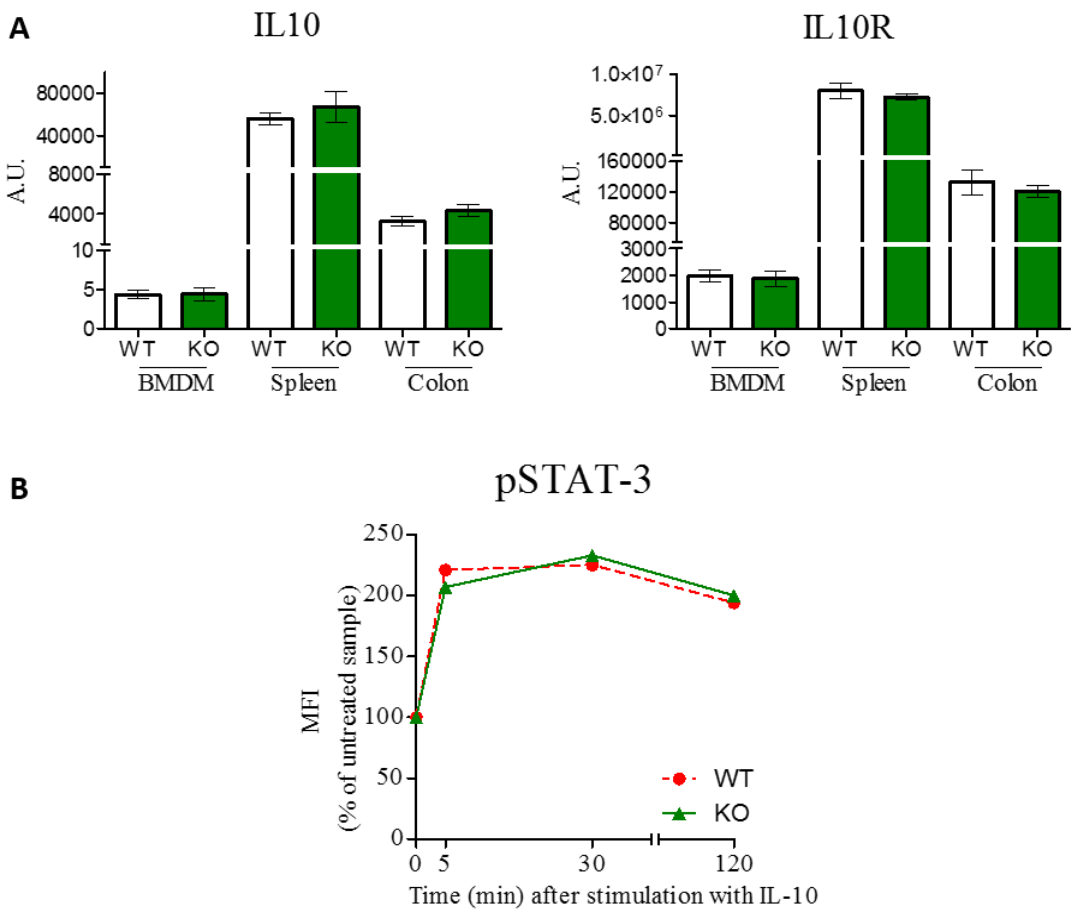


Fig 21: CX3CR1^{+} macrophages are able to produce and to sense IL10 (A) RNA quantification of IL10 and its receptor IL10R in FACS sorted CX3CR1^{+} BMDM, spleen and colonic macrophages of CX3CR1$^{GFP/+}$ and CX3CR1$^{GFP/GFP}$ mice. (B) Phospho-flow of spleen macrophages stimulated with IL10. pSTAT3 is phosphorylated after 5 minutes of stimulation and sustained thereafter, but decreases at 120 minutes.

We next demonstrated that the CX3CR1 signalling also involves STAT3. Macrophages harvested from the spleen of both WT and KO mice were stimulated with fractalkine (FKN) for 5, 30 or 120 minutes. As shown in figure 22, WT mice generated a response and pSTAT3 was phosphorylated already at 5 min. In contrast, no phosphorylation was detectable in KO mice, as expected. This finding also confirmed that CX3CR1 is the only receptor for FKN.

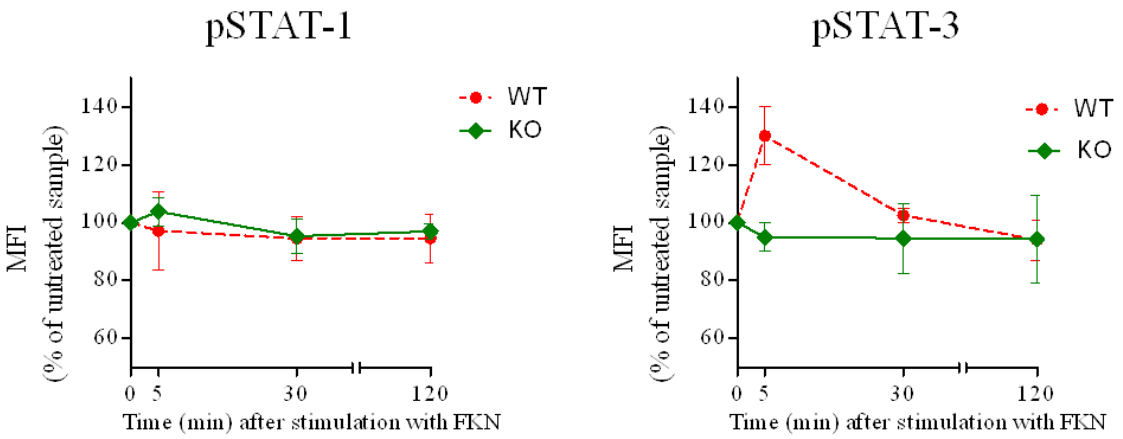


Fig.22: STAT3 phosphorylation after stimulation with CX3CL1. Spleen macrophages were used to evaluate STAT1 and STAT3 phosphorylation after stimulation with FKN. STAT1 phosphorilation is undetectable in both WT and KO mice at any time point. In contrast, WT mice, after 5 minutes of stimulation produce a response, inducing the phosphorylation of STAT3 while no response is observed in KO mice at any time point.

Acute colitis induced by DSS

Mucosal CX3CR1^{+} macrophages were investigated in a model of DSS-induced colitis. The aim was to understand if the lack of the receptor induced any difference during colitis. Both CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice were fed with DSS for seven days; along this period, loss of weight was monitored daily. At the end of the treatment mice were sacrificed and colon isolated to evaluate inflammation. KO mice showed higher signs of inflammation and lost weight rapidly, starting from day 5. Moreover, colon length was significantly reduced and evaluation of the histological score revealed how inflammation affects more severely the mucosa: colonic architecture was lost, there was a massive infiltration of leukocytes in the crypts that were, often, completely subverted (figure 23).

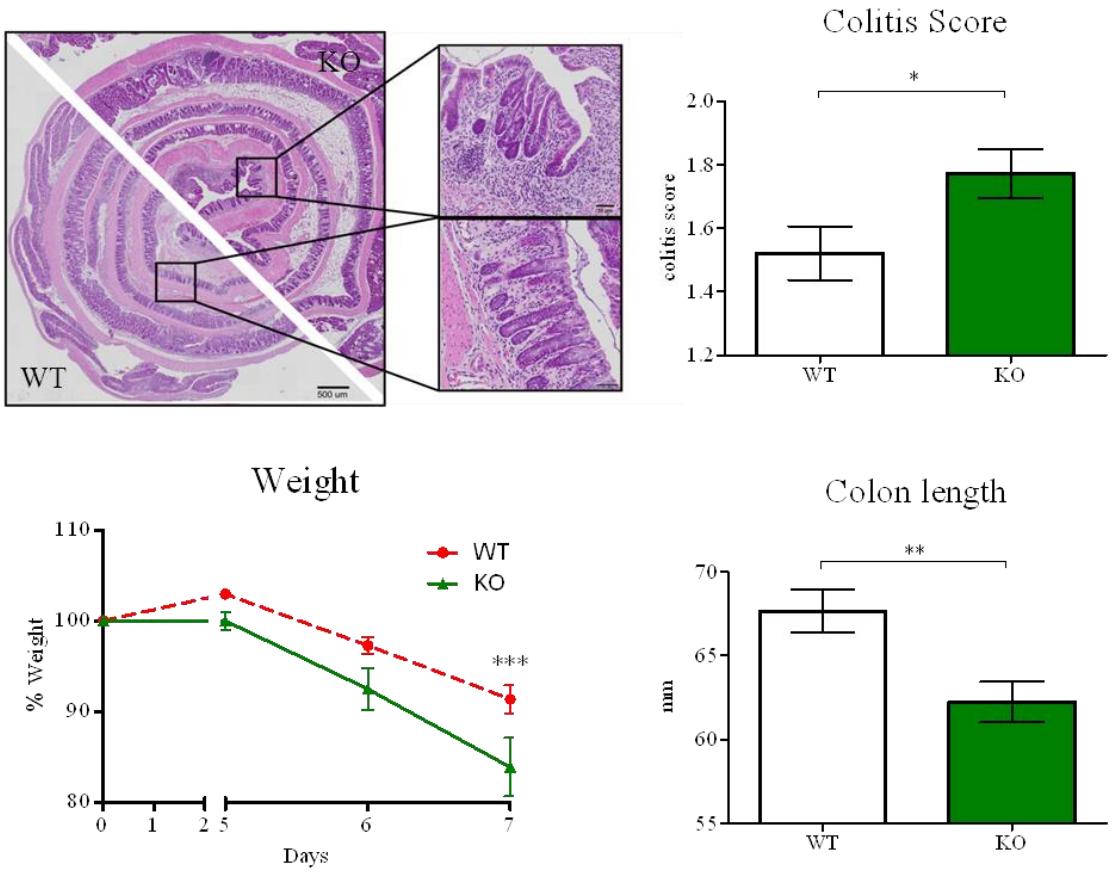


Fig. 23: Histological analysis of DSS-treated WT and KO mice. Evident signs of colitis are visible in panel A; WT mice show a lower rate of inflammation compared to KO mice in terms of colon length, colitis score and loss of weight. *p<0,05, **p<0,01

In steady state, CX3CR1^{+} macrophages are important in the maintenance of homeostasis. Considering the higher grade of inflammation in KO mice, compared to WT, we initially thought that macrophages of KO mice were not able to control inflammation because of the loss of functional CX3CR1^{+} cells in the mucosa. Surprisingly, KO mice presented a higher number of macrophages after DSS treatment. Macrophages are recruited, as reported in the literature, mainly *via* the CCR2-CCL2 axis. The absence of the CX3CR1 receptor did not prevent the recruitment of macrophages but prevented their protective functions. Therefore, more Mφ were recalled during inflammation in the attempt to obtain a response from these cells that can not be provided (figure 24).

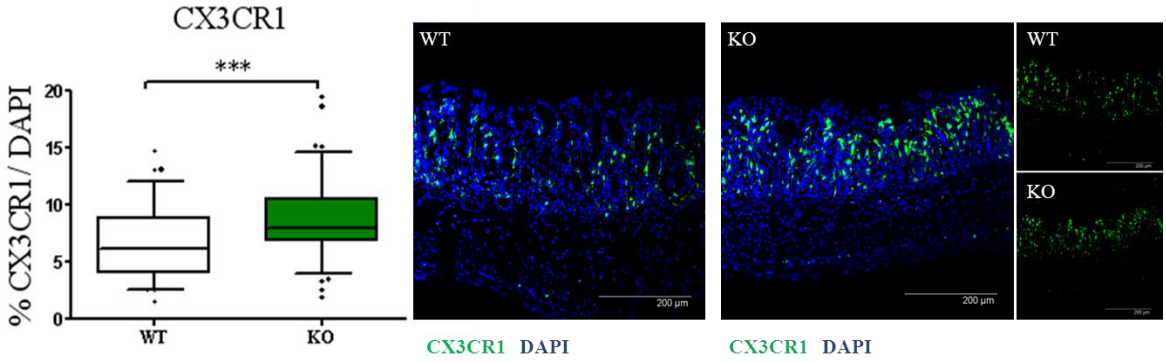


Fig. 24: Recruitment of CX3CR1+ macrophages in the colon during acute DSS-induced colitis. Immunofluorescence of CX3CR1 macrophages after treatment with DSS (day 7) shows how CX3CR1 GFP/GFP macrophages in KO mice are more abundant compared to WT. Magnification 20X, CX3CR1 green, DAPI blue; ***p<0,001

Recovery from colitis

in view of the inability of KO mice to correctly control inflammation, and considering the fact that "cancer is a wound that never heals", we analysed the behaviour of both CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice in the process of resolution of colitis. The results demonstrate that KO mice are unable to resolve inflammation. At day 10, when WT mice start to recover, KO mice still displayed high signs of inflammation in terms of both loss of weight and colon length (figure 25).

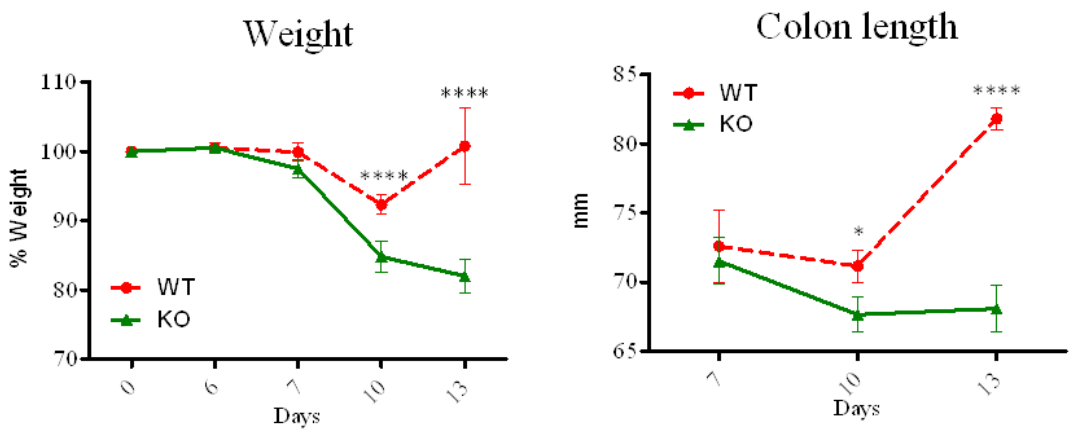


Fig. 25: Percentage of body weight and colon length of WT and KO mice at recovery, after treatment with DSS for 7 days. WT mice at day 10 began to recover from inflammation and at day 13 they had completely restored while KO mice still showed signs of inflammation. *p<0,05, **p<0,01, ***p<0,001

Moreover, we analysed the presence of different leukocyte populations in the intestine, along the experiment: at day 7, after treatment with DSS, at day 10, when WT mice begin to recover, and at day 13, when WT mice are completely healed (figure 26). We found that KO mice have an abundant macrophage infiltration: the absolute number of both macrophages and inflammatory monocytes is augmented in KO mice starting from day 10. Moreover, the percentage of macrophages and, in particular, of CX3CR1$^+$ Mφ, is higher in KO mice since day 7. Of note, the higher number of CX3CR1$^+$ and of Ly6C$^+$ monocytes underlines the current opinion that inflammatory monocytes give rise to CX3CR1$^+$ macrophages during inflammation [28].

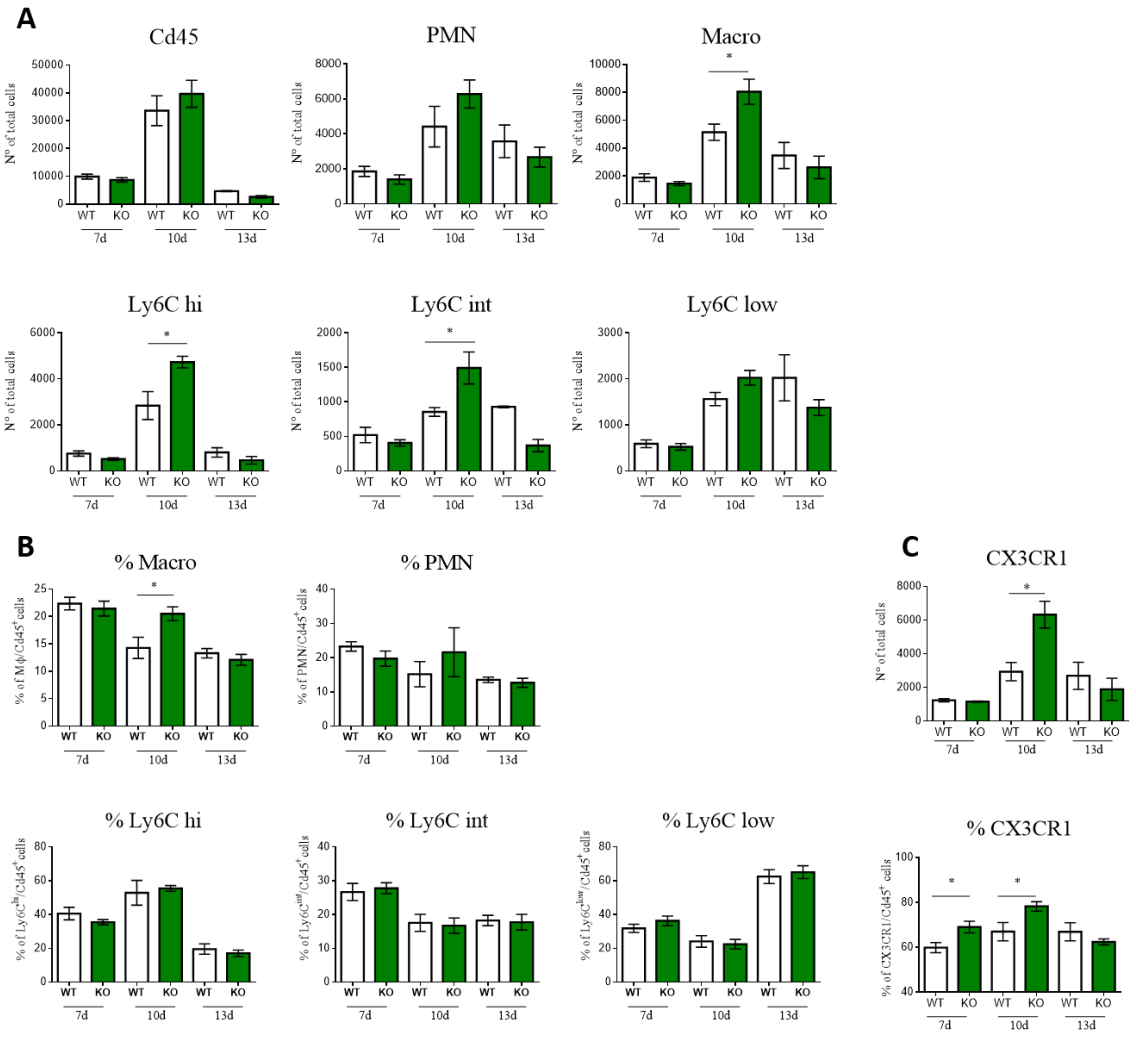


Fig. 26: Leukocyte recruitment in acute colitis and recovery. FACS analysis of colon samples from WT (white) and KO (green) mice: counts of different leukocyte populations (panel A), population percentages (panel B) and CX3CR1+ cells counts and percentages (panel C) at day 7 (at the end of DSS treatment) and in recovery at day 10 and 13. Representative results from three independent experiments are shown. *p<0,05

We next extracted RNA from colon tissues and analysed the mRNA levels of several biological mediators at day 10 and 13, comparing WT and KO mice and also comparing them with untreated mice. At day 10, in line with the histological observations, both WT and KO mice showed signs of inflammation: in particular KO mice displayed high level of the anti-inflammatory cytokines IL-10 and IL-20. The higher production of IL-10 in KO mice is likely to be an attempt to cope inflammation. However, other pathways seem to be involved in the intestine control of inflammation. Indeed, KO mice produced lower levels of heme-oxygenase-

1 (hemox-1) compared to WT. At day 13 WT mice had completely restored from colitis, while KO mice still showed high levels of inflammation: we found that the mRNA levels of TNF-α, IL-6, IL-1β, IFN-γ and COX2, together with the anti-inflammatory cytokines, were still significantly over-expressed in KO mice at day 13 (figure 27).

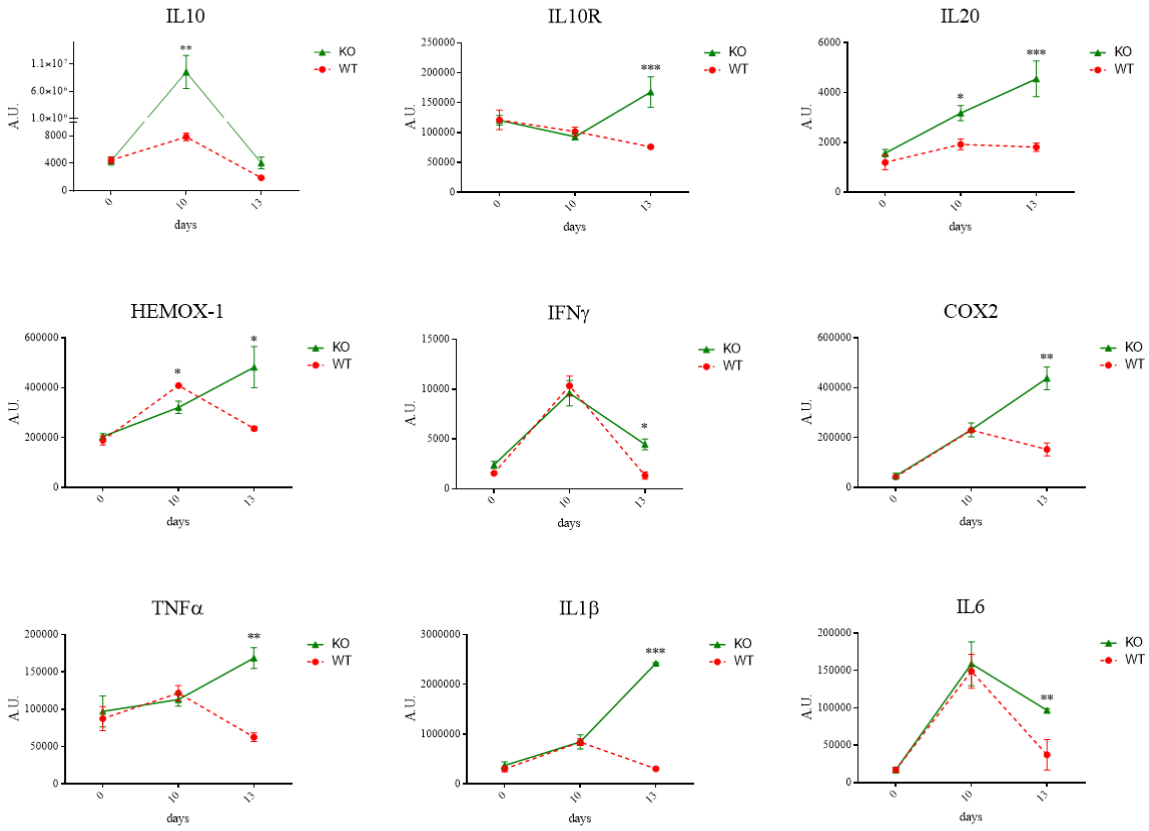


Fig. 27: mRNA expression of pro and anti-inflammatory mediators at recovery from DSS-induced acute colitis. At day 10 both WT and KO mice still show signs of inflammation. KO mice try to switch off inflammation producing higher level of anti-inflammatory cytokines. However, hemox-1 is down-regulated in KO compared to WT mice. At day 13, WT mice had completely restored while KO mice still show signs of inflammation. *p<0,05, **p<0,01, ***p<0,001

Considering the results obtained with the analysis of hemox-1, we analysed the protein levels present in the colonic mucosa at day 10 in both WT and KO mice. Figure 28 shows that protein production, as well as the mRNA expression, is dramatically down regulated in KO mice.

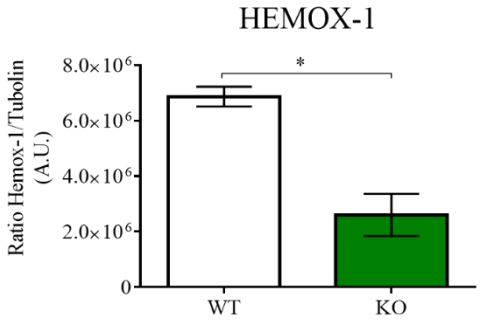


Fig. 28: Western blot of heme-oxygenase-1 at day 10 of recovery from DSS-induced acute colitis. WT mice produced higher levels of hemox-1 compared to KO mice in the attempt to control tissue damage and inflammation. Hemox-1 production is strongly impaired in KO mice.

Taken together these results suggest an inability of KO mice to control inflammation: if CX3CR1 is not functional, an important resolution checkpoint for the colonic mucosa is lost and this results in an abundant recruitment of pro-inflammatory monocytes and release of pro-inflammatory cytokines, eventually leading to an aberrant inflammatory response.

In vitro analysis of CX3CR1^{+} macrophages

To further investigate the role of the CX3CR1 receptor in the modulation of inflammation, we performed in vitro studies with bone marrow derived macrophages (BMDM) from both CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ mice. Bone marrow CX3CR1^{+} cells were FACS sorted and induced to differentiate for seven days with M-CSF. Macrophages were either left untreated, or stimulated with FKN, or with LPS or stimulated with both FKN and LPS. In the latter group, cells were pre-treated with FKN for 18 hours and then with LPS for 4 hours. After treatment, we analysed the mRNA expression of pro and anti-inflammatory mediators.

We found that both WT and KO mice were able to constitutively produce appreciable levels of TGF-β, hemox-1, IL-19 and IL-10. After treatment with FKN, hemox-1 levels were significantly increased in WT, but not in KO mice, and this increment was further augmented when cells were stimulated with the combination of FKN and LPS, suggesting a synergism of the two stimuli. As observed *in vivo*, KO mice expressed higher levels of IL-10 family members after treatment with LPS, but were unable to respond to FKN and to increase the expression of Hemox-1. In addition, TGF-β production was stimulated by FKN but not by LPS in WT mice, while KO were unable to respond to this stimulus.

Overall, these results provided the interesting information that FKN-signalling through its specific receptor induced the production of TGF-β and hemox-1, as well as the production of IL-10, highlighting the important role of this chemokine-receptor axis in limiting inflammation.

Finally, we analysed the mRNA levels of some pro-inflammatory mediators finding a higher response in KO mice for IL-6 transcripts (figure 29).

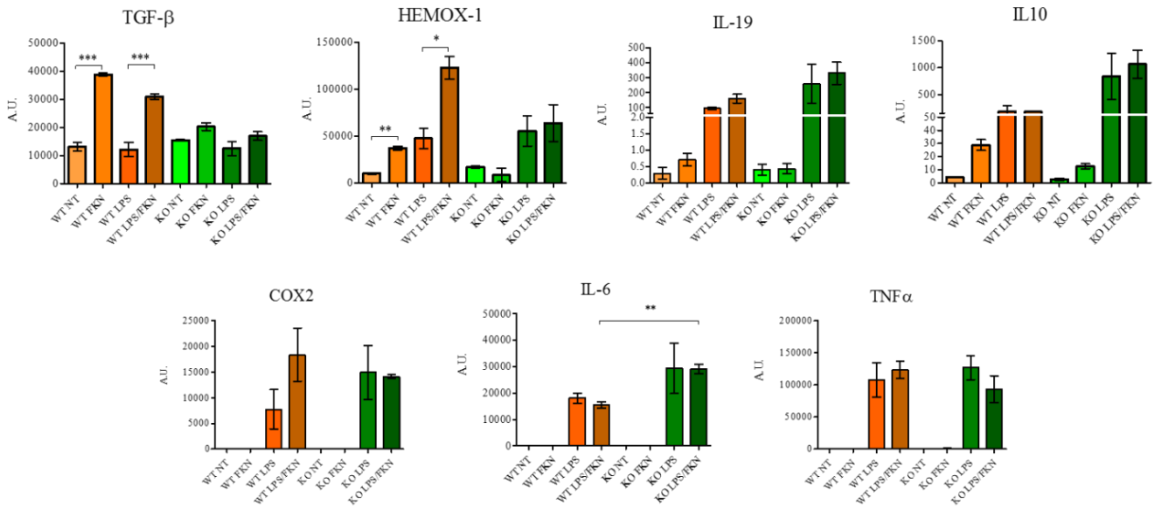


Fig.29: mRNA quantification of pro and anti-inflammatory mediators in FACS sorted BM-derived CX3CR1+ macrophages. Bone marrow derived macrophages were FACS sorted and then divided into four groups: not treated (NT), stimulated with FKN, stimulated with LPS (LPS), stimulated with both Fractalkine and LPS (LPS-FKN). WT and KO were compared. Macrophages were stimulated with FKN for 18h and then with LPS for 4h. Representative results from three independent experiments are shown. *p<0,05, **p<0,01, ***p<0,001

Furthermore, we monitored protein production of some cytokines such as TNF-α, IL-6 and IL-10 in ELISA assay and we obtained similar results as observed in RT-PCR: KO mice produced more inflammatory mediators and more IL-10 compared to WT retracing perfectly what happened *in vivo* (figure 30).

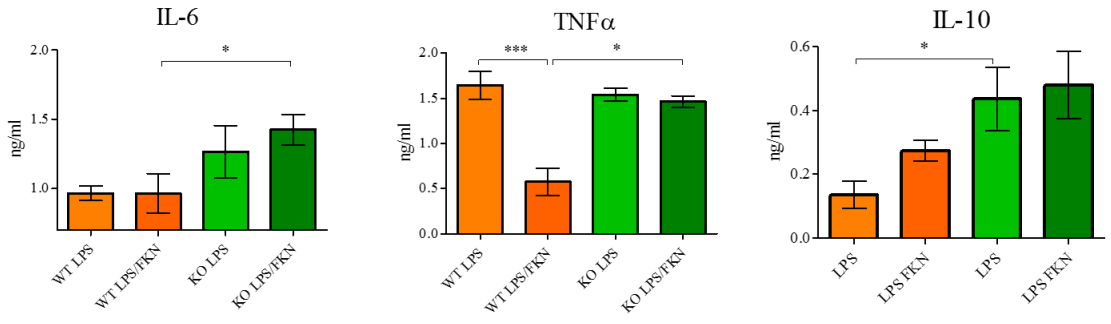


Fig.30: ELISA assay of pro and anti-inflammatory mediators in FACS sorted BM-derived CX3CR1+ macrophages. Cytokine production was detected in ELISA assay. Macrophages were stimulated with FKN for 18h and then with LPS for 4h. WT and KO cells were compared. KO macrophages displayed a higher response to the inflammatory stimulus and, in the case of TNF-α, they were not able to switch off inflammation after the administration of FKN. IL-10 is over-expressed in KO macrophages in the attempt to limit inflammation. *p<0,05, **p<0,01, ***p<0,001

To further confirm the difference in the production of inflammatory cytokines between WT and KO mice, we decided to investigate the behaviour of peritoneal elicited macrophages (PEC). We injected mice with thioglycolate, a yeast extract, and performed the same type of experiment used for BMDM. As shown in figure 31, using PEC we were able to better appreciate the differences between WT and KO. PEC from KO mice produced significantly higher levels of IL-1β, IL-6, IFN-γ, IL-12 and COX2.

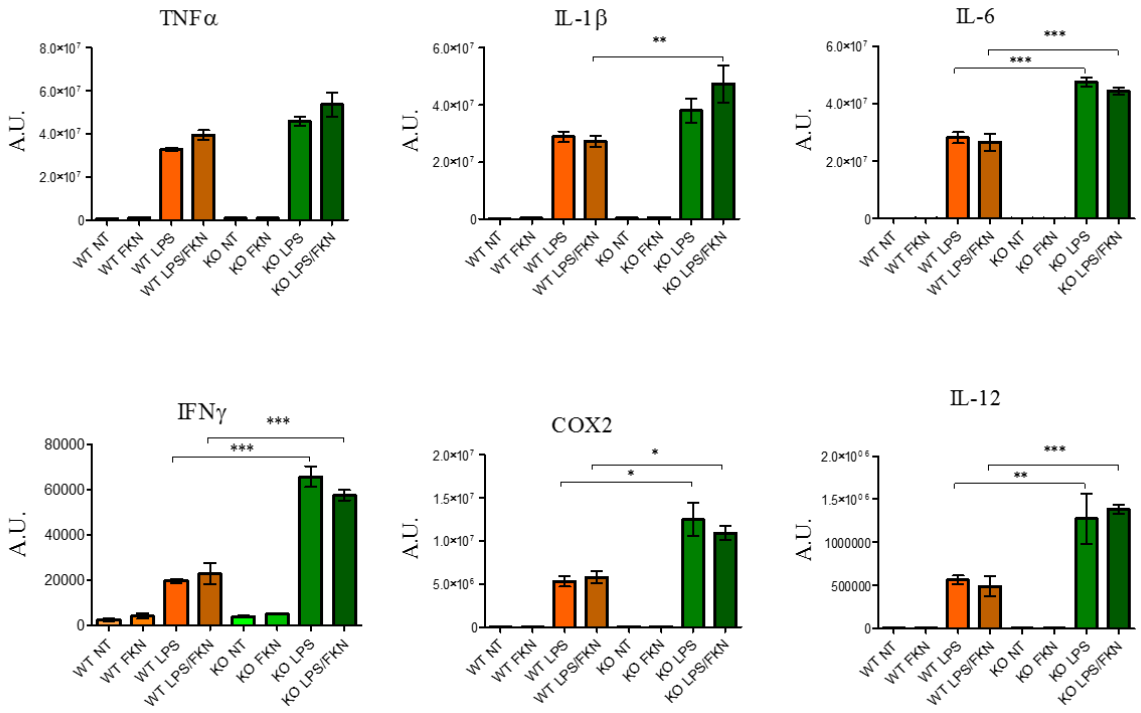


Fig.31: mRNA quantification of pro -inflammatory mediators in peritoneal macrophages. Peritoneal elicited cells (PEC) were FACS sorted and then divided into four groups: not treated (NT), stimulated with FKN, stimulated with LPS (LPS), stimulated with both Fractalkine and LPS (LPS-FKN). WT and KO PEC were compared. Macrophages were stimulated with FKN for 18h and then with LPS for 4h. Representative results from three independent experiments are shown. *p<0,05, **p<0,01, ***p<0,001

The above findings demonstrated that macrophages from KO mice have an aberrant inflammatory response. They displayed a greater response to stimuli and produced higher amount of pro-inflammatory mediators; furthermore they revealed an inability to produce adequately amount of anti-inflammatory mediators such as hemox-1, except for IL10 that was produced in a CX3CR1-independent manner.

Next, we wanted to demonstrate also *in vitro* that KO mice are not able to recover from inflammation as fast as WT mice do. Therefore, we stimulated BMDMs with LPS for 4 hours, we washed out the stimulus and we prolonged the incubation for 18 hours. As shown in figure 32 there was a delay in the recovery of KO mice. After 22 hours from LPS treatment, macrophages from KO mice still produced high levels of pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6 and iNOS. In turn, they were unable to produce TGF-β and hemox-1 at the same levels found in WT mice.

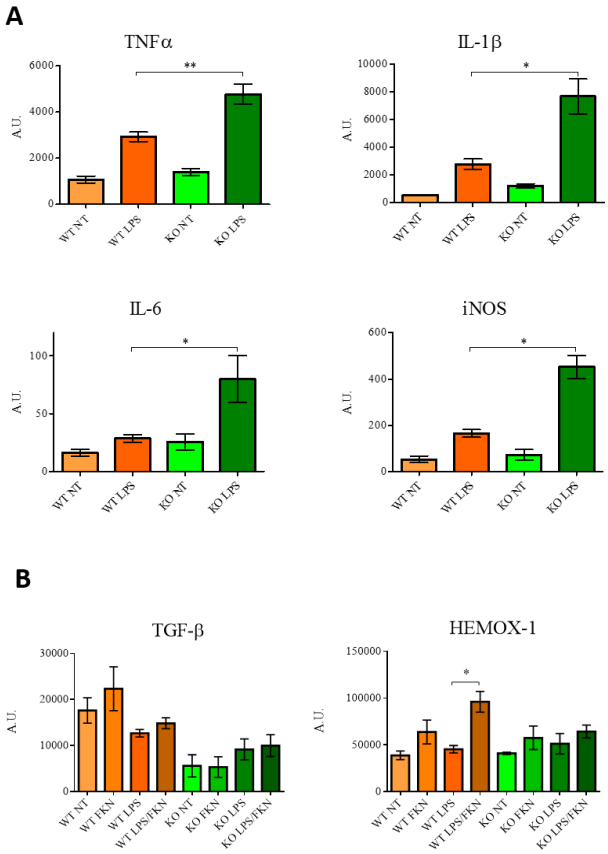
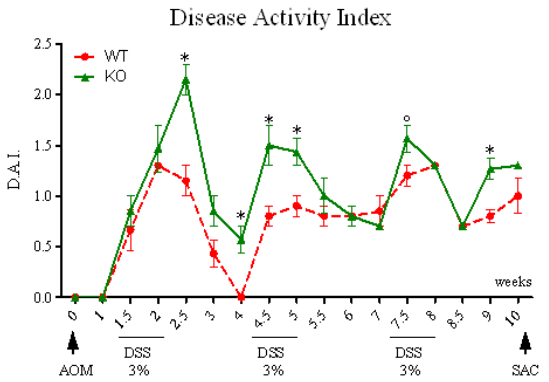


Fig.32: mRNA quantification of pro and anti-inflammatory mediators in FACS sorted BM-derived CX3CR1+ macrophages after prolonged LPS stimulation. Bone marrow derived macrophages were sorted and WT and KO cells were compared. Macrophages were stimulated with LPS for 4h then washed and cultures prolonged for 18h in order to replicate the colitis model of recovery. (A) KO macrophages show higher response compared to WT; indeed in KO macrophages the inflammatory cytokines were still over expressed compared to WT cells. (B) On the contrary, TGF-β and Hemox-1 were modulate in WT mice but not in KO. *p<0,05, **p<0,01,

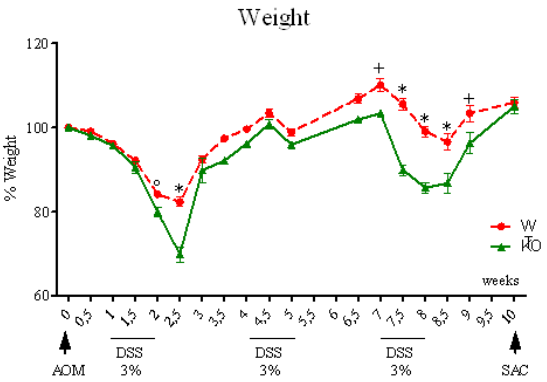
Colitis associated cancer

So far, our results demonstrated that loss of the chemokine receptor CX3CR1 leads to an altered response of mucosal macrophages in the control of inflammation during colitis and during the process of recovery. Our main interest was to evaluate whether this increased inflammation had an impact on colorectal carcinogenesis. For this reason we developed a model of colitis-associated cancer (CAC) in which $CX3CR1^{+/GFP}$ and $CX3CR1^{GFP/GFP}$ mice were intra-peritoneally injected with the carcinogen azoxymethane (AOM) and a week after, were fed with DSS followed by water for two weeks for a total of three cycles. Weekly, during the treatment, weight, stool bleeding and consistency were assessed (figure 33).

A



B



C

Weight loss	Score	Stool blood	Score	Stool consistency	Score
< 1%	0	Negative	0	Normal	0
1-5%	1	Positive to Hemoccult	1	Soft	1
5-10%	2	Visible blood	2	Very soft	2
10-15%	3	Gross bleeding	3	Diarrhea	3
> 15%	4				

Fig. 33: Disease Activity Index (DAI) in the mouse model of colitis-associated cancer: Twice a week weight and stool consistency of mice were detected. Disease activity index (D.A.I.) and weight were recorded according to parameters reported in the table. °p<0,05, $^{+}$p<0,01, *p<0,001

After the first cycle of DSS, both KO and WT mice showed loss in weight and signs of bleeding in addition to a production of soft stools. However, during the recovery with water, WT mice gained weight, stopped bleeding and stools became normal again. Instead, KO mice failed to recover: they kept losing weight and their stools resulted very soft and bleeding. During further cycles, the difference between WT and KO became even stronger, in particular for their inability to regain weight. At the end of the experiment, after 70 days, colons were harvested to be analysed. KO mice showed higher signs of inflammations: colitis score and colon length indicated that they had a stronger disease compared to WT mice and, indeed, they had a higher percentage of adenomas and a lower survival rate (figure 34).

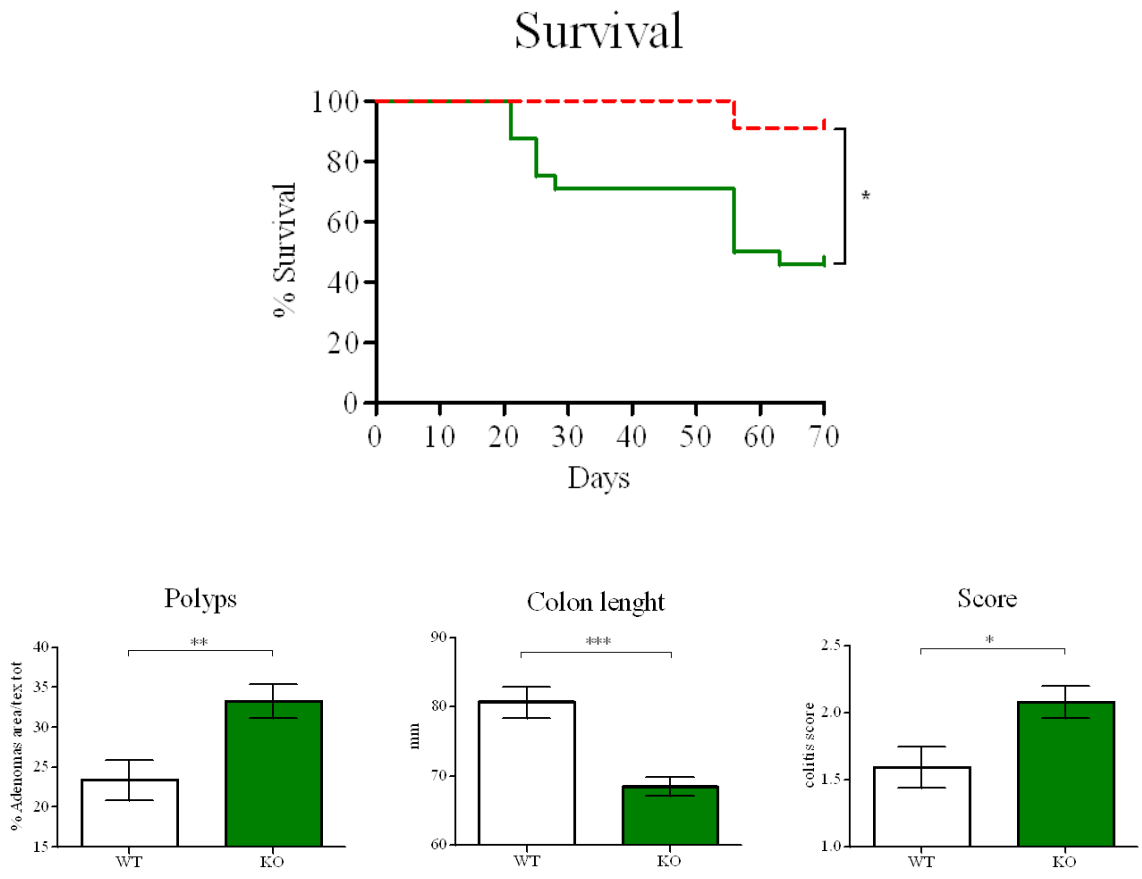


Fig.34: Histological analysis of treated mice. KO mice have a lower survival rate and developed higher numbers of polyps compared to WT mice; in contrast, WT mice show a lower rate of inflammation in terms of colon length and colitis score. *p<0,05, **p<0,01, ***p<0,001

As observed in the model of acute colitis, also in the CAC model KO mice recruited more macrophages in the adenomatous tissue. We were able to detect inside the polyp,

CX3CR1^{+} Mφ, while at adenomatous border macrophages were mostly F4/80^{+} CX3CR1^{-}, confirming that inflammatory and resident macrophages have the same precursor and that they differentiate in a context-dependent manner. In addition, we demonstrated that in polyps there was a higher infiltration of CX3CR1^{+} macrophages in KO mice (figure 35.). Moreover, FACS analysis revealed that Ly6C^{+} monocytes as well as TLR2^{+} macrophages were more abundant in the colonic mucosa of KO mice (figure 36).

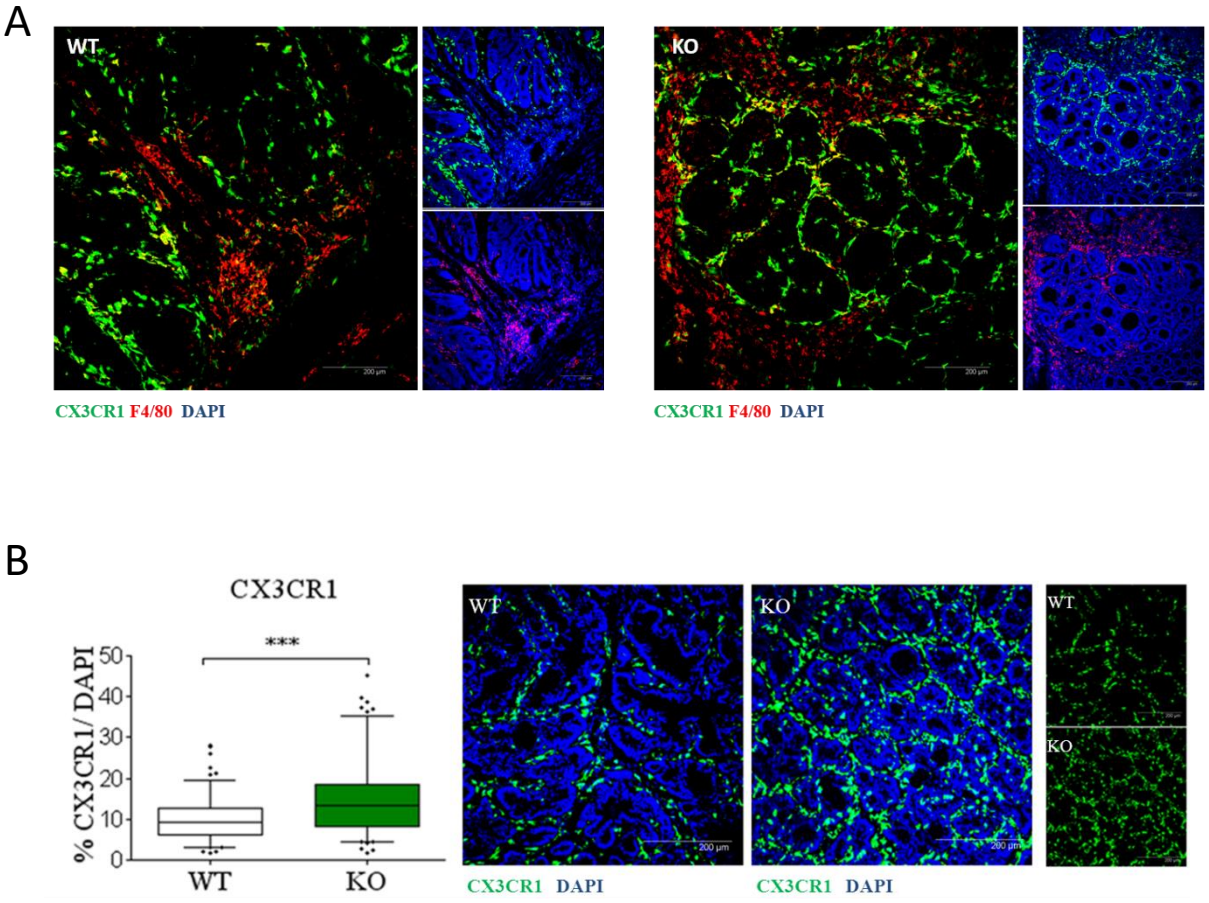


Fig.35: Recruitment of CX3CR1^{+} macrophages in polyps. Inflammatory and resident macrophages have the same precursor and they differentiate in a context-dependent fashion. (B) As observed for acute colitis, immunofluorescence of CX3CR1 macrophages after treatment with AOM/DSS shows that CX3CR1$^{GFP/GFP}$ macrophages in KO mice are more abundant compared to WT mice. Magnification 20X, CX3CR1 green, F4/80 red, DAPI blue; ***p<0,001

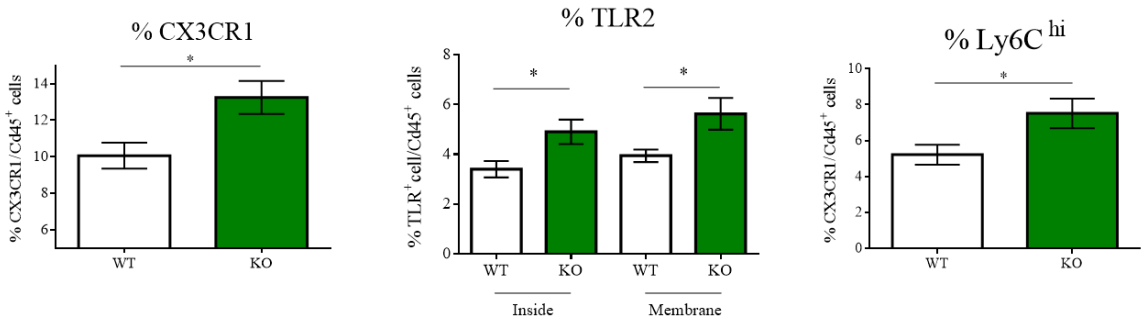
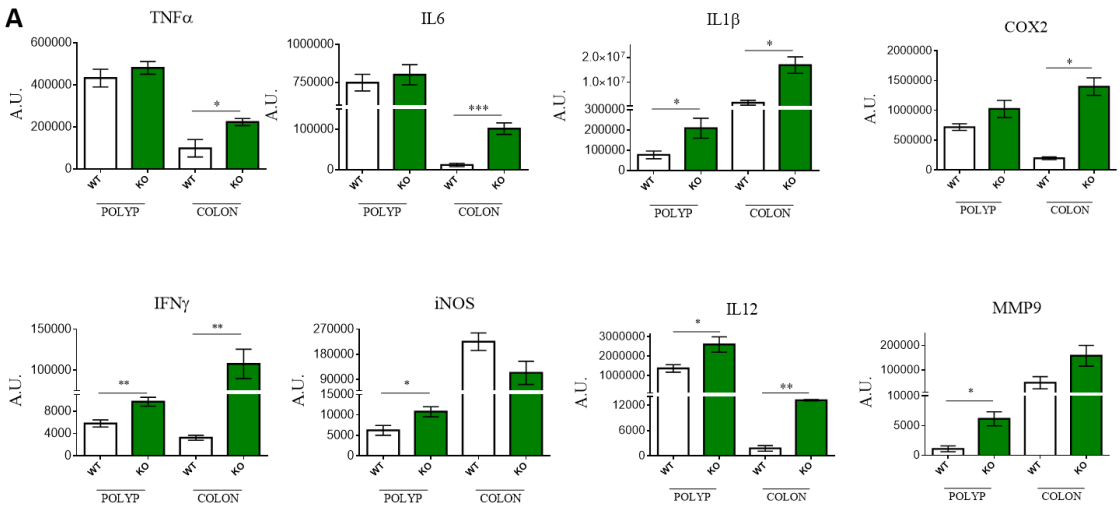


Fig.36: Macrophage recruitment in the CAC model. FACS analysis of leukocyte infiltrate in the polyps from WT (white) and KO (green) mice: the results show that KO mice had higher percentage of macrophages. Representative results from three independent experiments are shown. *p<0,05, **p<0,01, ***p<0,001

To better understand what happened in the colonic mucosa, we dissected polyps from the inflamed colon and separately analysed the mRNA levels of pro-inflammatory cytokines and anti-inflammatory mediators (figure 37).



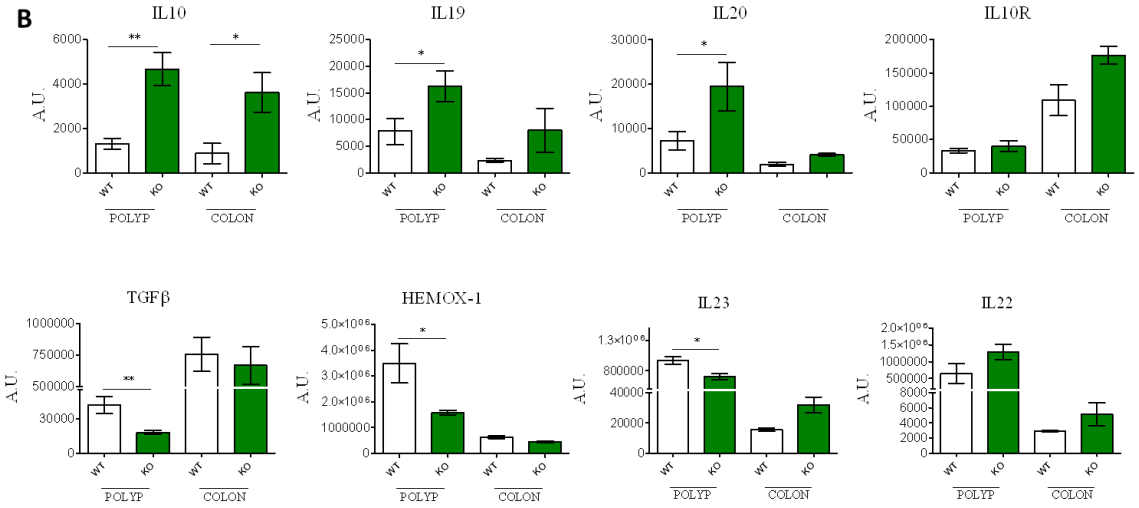


Fig. 37: mRNA expression of pro and anti-inflammatory mediators in the polyps and colon tissues of mice in the CAC model. The adenomatous polyps were separated from the rest of the colon and the two specimens were analyzed for the expression of different pro-inflammatory (A) or anti-inflammatory (B) cytokines or mediators. *p<0,05, **p<0,01, ***p<0,001

As shown in figure 37 (panel A) KO mice produced higher levels of inflammatory cytokines not only in the adenomatous tissue, but also in the inflamed mucosa. According to the extrinsic pathway of cancer-related inflammation, this higher grade of inflammation induced the development of more polyps. The higher rate of polyps in KO mice is in line with the higher levels of pro-inflammatory cytokines produced inside the transformed tissue.

In panel B it is represented the expression of anti-inflammatory cytokines and mediators. It is important to note that IL-10 family members were over-expressed in KO mice. CX3CR1 macrophages are key producers of IL-10 in the intestinal mucosa in a CX3CR1-independent manner. Indeed, KO mice are able to produce IL-10 (and to sense it, thanks to the expression of IL-10R). As KO mice have more inflammation compared to WT mice, they try to switch off inflammation by producing more IL-10, in order to limit tissue damage. Interestingly, IL-23 is down-regulated in KO mice. IL-23 is an important mediator in the colonic mucosa; it is produced by CX3CR1 macrophages and it is essential to stimulate the production of IL-22 by type 2 innate lymphoid cell (ILC2). However, there were no significant differences in the production of IL-22 between WT and KO mice, indicating that other cell types are involved in this process.

Finally, we analysed the mRNA expression of TGF-β and Hemo-oxygenase-1 and we found that these were the only two down-regulated molecules in the adenomatous tissue in KO mice.

Genetic model of colitis associated cancer

We decided to investigate a genetic model of intestinal polyposis in order to confirm the data obtained with the sporadic model of CAC. APC^{Min} mice carry a mutation in the APC gene leading to a spontaneous development of polyps in the small intestine. In inflammatory conditions, such as after DSS administration, APC^{Min} mice can develop polyps also in the colonic mucosa. We crossed $CX3CR1^{GFP/+}$ or $CX3CR1^{GFP/GFP}$ mice with APC^{Min} mice and generated mice that were either competent or not for the expression of CX3CR1 and also carried the APC^{Min} gene. Mice were administered DSS for one week and then water for four weeks. At the end of the experiment, colons were harvested in order to assess histological parameters (figure 38). As observed in the previous sporadic model of CAC, KO mice ($CX3CR1^{GFP/GFP}$-APC^{Min}) showed higher signs of inflammation and a greater number of polyps compared with WT mice ($CX3CR1^{GFP/+}$-APC^{Min}). Indeed, colons were shorter in KO mice and they showed a higher D.A.I. score. Moreover, they were not able to regain weight after DSS administration as WT mice did.

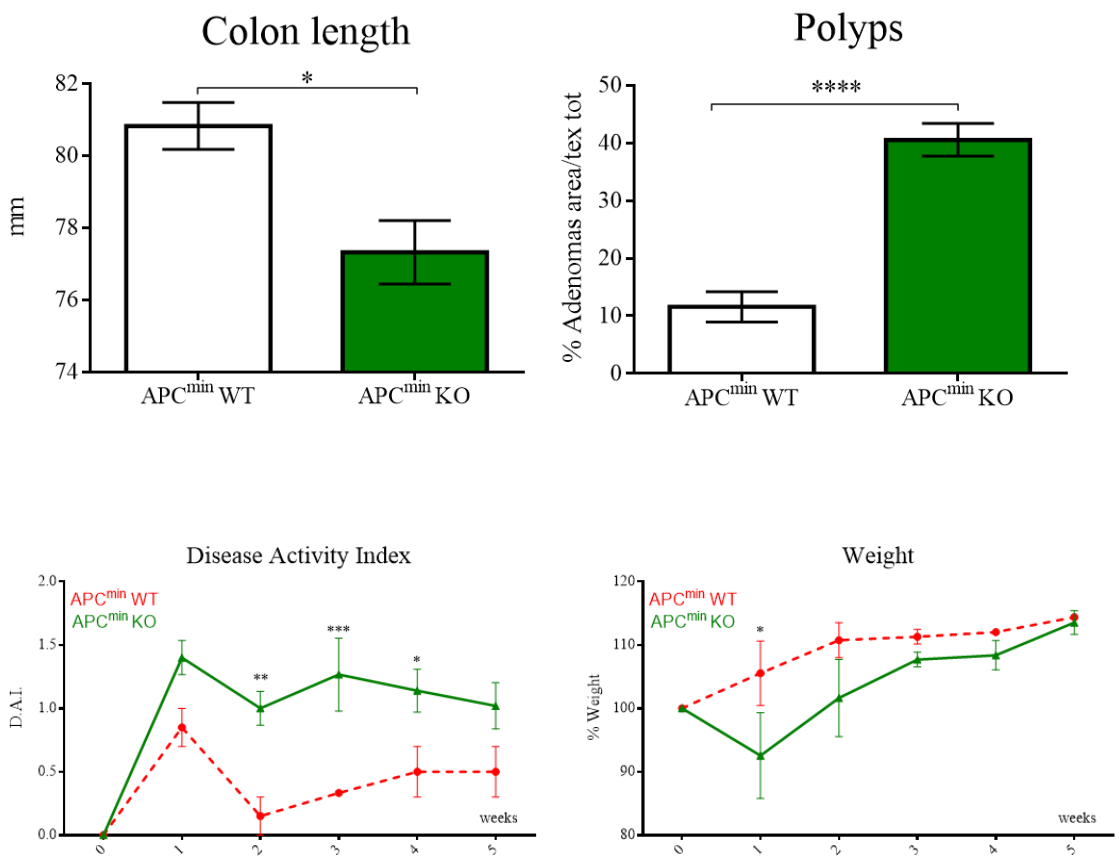


Fig.38: Histological analysis of DSS treated WT mice (CX3CR1$^{GFP/+}$-APC^{Min}) and KO mice (CX3CR1$^{GFP/GFP}$-APC^{Min}). Colons were harvested after five weeks from the beginning of the experiment, length was measured and the percentage of adenomatous tissue was calculated on the whole colon. KO mice showed higher signs of inflammation, shortness in colon length and a higher number of polyps. Disease Activity Index (DAI) and percentage of body weight of treated mice were analyzed weekly. *p<0,05, **p<0,01, ***p<0,001, , ****p<0,0001

We then investigated the composition of the tumor microenvironment in terms of both cytokine expression and leukocyte infiltration. As shown in figure 39, KO mice had higher number of CD45$^+$ cells and, in particular, more inflammatory (Ly6C$^+$) macrophages and CX3CR1$^+$ Mφ.

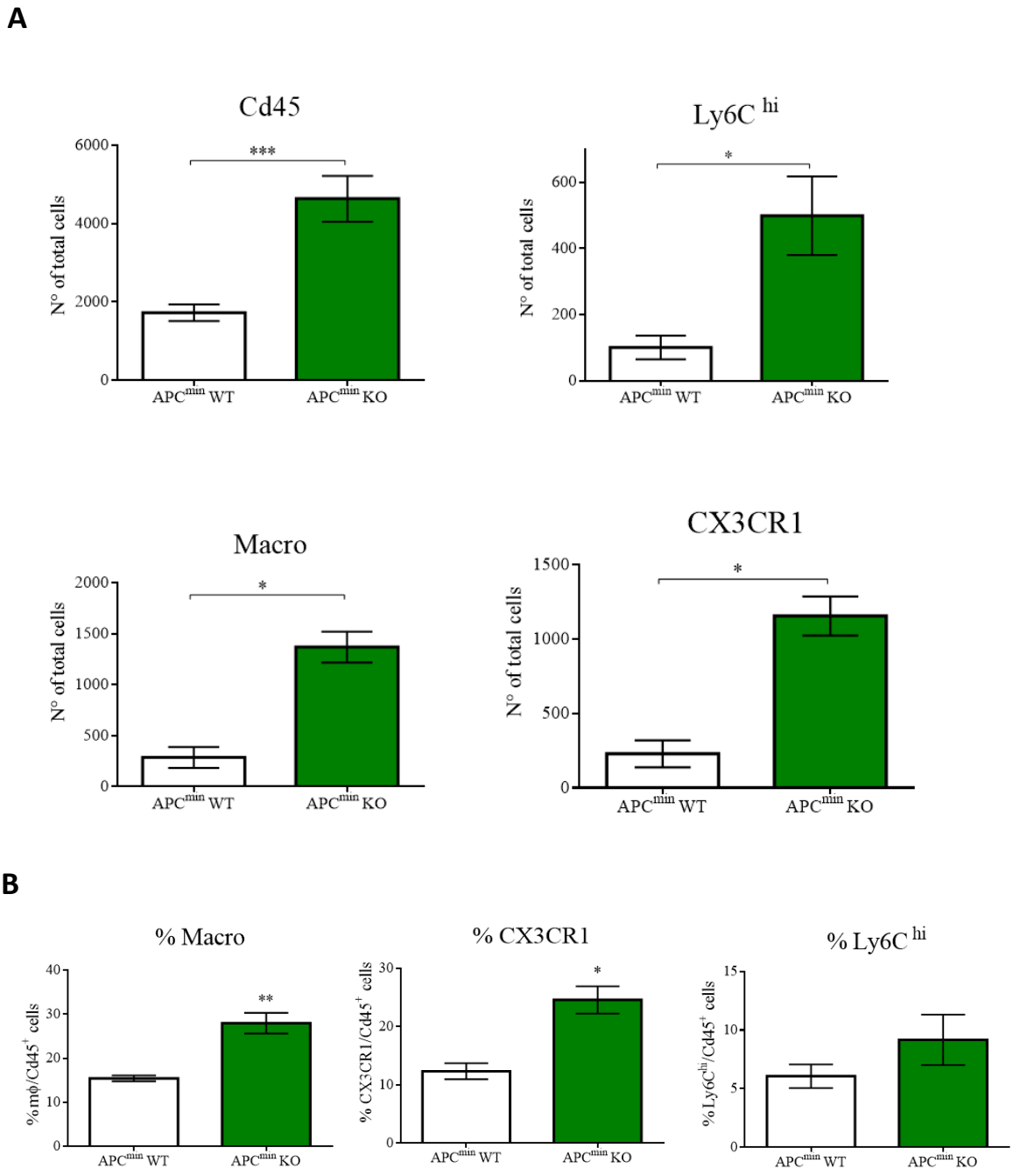


Fig.39: Macrophage infiltration in colon tissues of DSS-treated WT mice (CX3CR1$^{GFP/+}$-APC^{Min}) and KO mice (CX3CR1$^{GFP/GFP}$-APC^{Min}). FACS analysis of polyps from WT (white) and KO (green) mice. In KO mice higher number of CD45+ cells are recruited, in particular CX3CR1^{+} macrophages and Ly6C^{high} inflammatory monocytes. (A) the results are shown as absolute number of cells; (B) the results are shown as percentage of each sub-population. Representative results from two independent experiments. *p<0,05, **p<0,01, ***p<0,001

The mRNA analysis of the colonic mucosa revealed the same results obtained in the AOM-DSS model of CAC. Indeed, KO mice were not able to properly face inflammation and

this resulted in an aberrant inflammatory response. Inflammatory cytokines such as IL-6, IFNγ were upregulated in KO mice in both polyps and colonic tissue. In general, all the inflammatory cytokines appeared more expressed in the adenomatous tissue of KO mice (figure 40).

Next, we examined the expression of anti-inflammatory mediators. As it was for the previous model, also in this case KO mice attempted to switch off inflammation by producing higher amount of IL-10 that resulted upregulated in both polyps and inflamed tissues. In addition, IL-19 was higher in KO mice, although not in a significantly way, while no differences could be appreciated in the expression of IL-20 and IL-10 receptor. On the contrary a very significant difference was found in the expression of both TGFβ and hemox-1 in the transformed tissue, confirming and extending our observation in the AOM-DSS model.

In conclusion, we demonstrated that the lack of functional CX3CR1 receptor leads to the same phenotype either in the sporadic model of colitis-induced cancer and in the genetic APC^{Min} model.

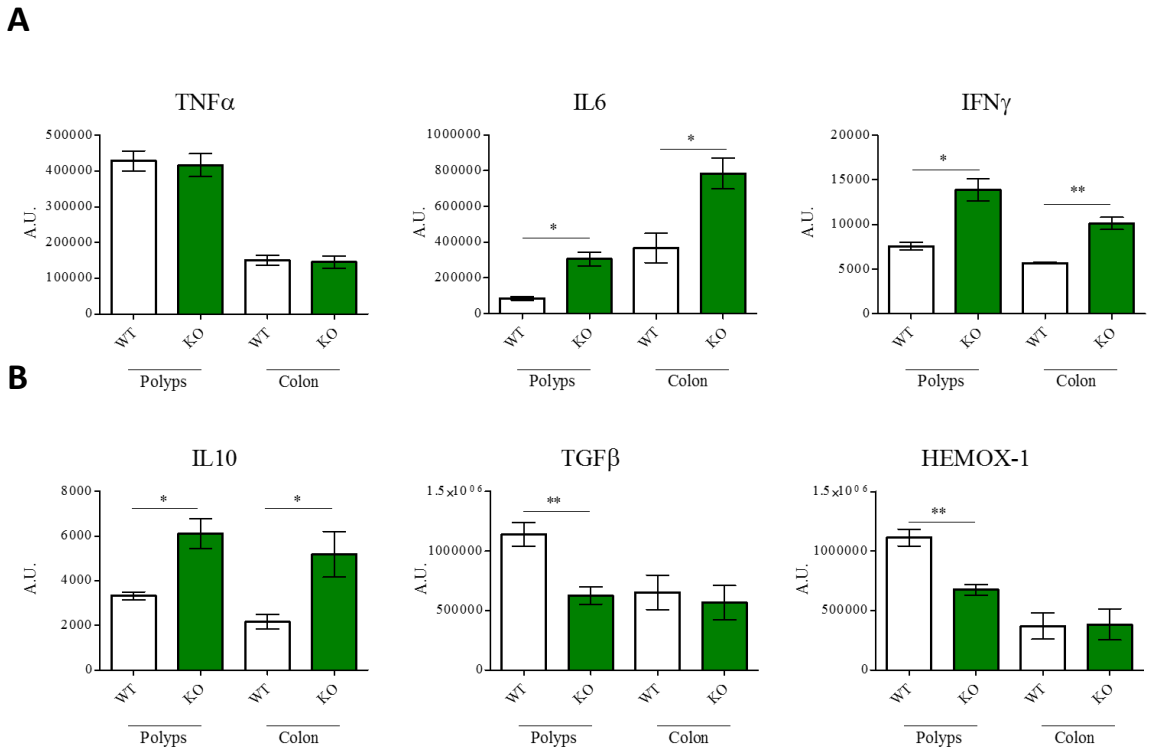


Fig.40: mRNA expression of pro and anti-inflammatory cytokines in the polyps and colon tissues of DSS-treated WT mice ($CX3CR1^{GFP/+}$-APC^{Min}) and KO mice ($CX3CR1^{GFP/GFP}$-APC^{Min}). The adenomatous tissue were separated from the rest of the colon and the two specimens were independently analyzed for the expression of different pro-inflammatory (A) or anti-inflammatory (B) cytokines or molecules. *p<0,05, **p<0,01, ***p<0,001

Induction of heme-oxygenase-1 in CAC

Having seen a decrease in the production of hemox-1 in KO mice ($CX3CR1^{GFP/GFP}$), both *in vivo* and *in vitro,* we decided to investigate in depth the mechanisms that link the CX3CR1 receptor and this molecule. We performed an AOM-DSS experiment of colitis-associated cancer in which $CXRCR1^{GFP/+}$ and $CX3CR1^{GFP/GFP}$ mice were injected with a chemical inducer of hemox-1, four times a week following the scheme in figure 17.

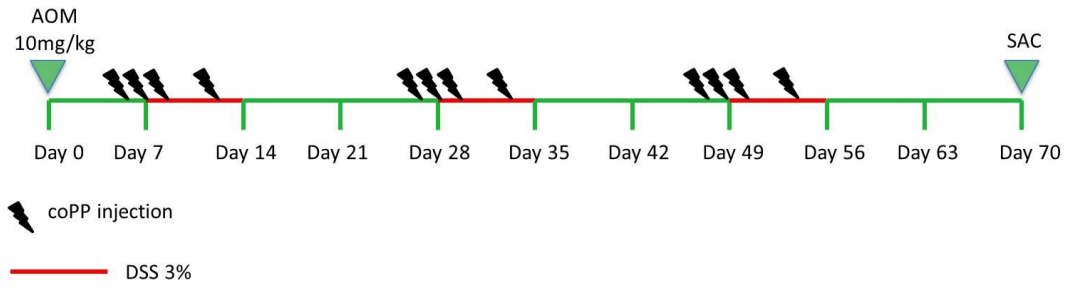


Fig. 17: Scheme of AOM-DSS experiment. Arrows indicate cobalt protoporphyrin IX (coPP) injection.

Cobalt protoporphyrin IX (coPP) is a powerful inducer of hemox-1 [67, 68]. We decided to administer the drug at day -1, 0, 3 and 6 of each cycle of DSS in order to stimulate the production of hemox-1 and its protective effects during the presence of the inflammatory stimulus. Mice were divided into four groups: WT and KO mice were treated with coPP or with vehicle. Weekly along the experiment, weight, stool consistency and bleeding were assessed in order to determine the disease activity index. Results are reported in figure 41.

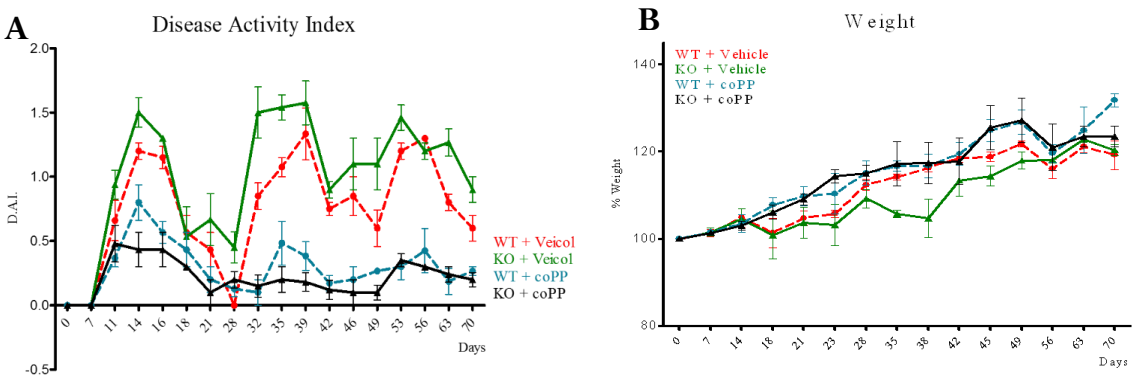


Fig. 41: **Disease score in the AOM/DSS experiment upon treatment with coPP:** (A) Disease Activity Index (D.A.I.). Every week stool bleeding and consistency were analyzed in mice treated with coPP, disease severity was milder compared to untreated mice and there was no difference between WT and KO cop-treated mice. (B) Percentage of body weight of treated mice during treatment.

As shown in figure 41, treatment with coPP was able to switch off inflammation in both WT and KO mice while in mice treated with vehicle the difference observed between WT and KO were fully conformed to the previous experiment. Indeed, in mice treated with coPP, D.A.I. score was almost always close to 0 and KO mice showed a regain in weight similar to that of WT mice. At the end of the experiment, colons were harvested for analysis. As shown in figure 42, the phenotype of coPP-treated KO mice was completely reverted: no signs of inflammation were visible and a very low number of polyps were detected. Of note, also in coPP-treated WT mice there was an amelioration of the severity of the disease. Indeed, after coPP administration KO and WT mice were indistinguishable and healthy.

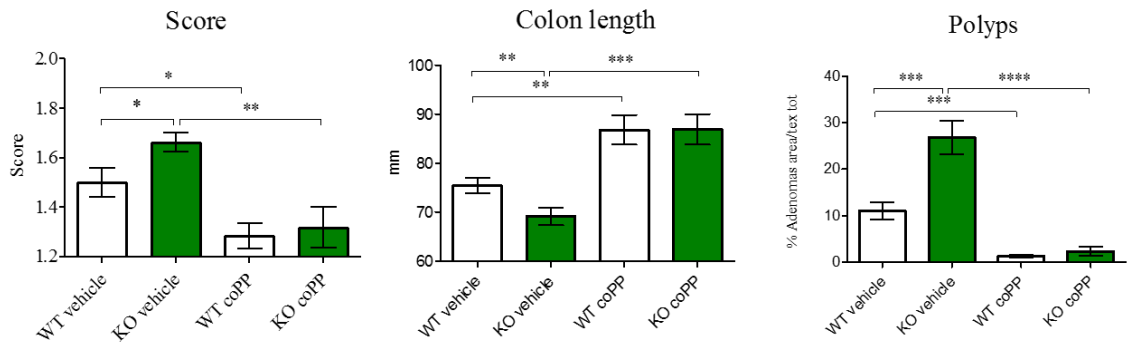


Fig.42: Histological analysis of treated mice. Mice treated with coPP revert the phenotype of the disease: for both WT and KO mice, colitis score is reduced. The number of polyps is dramatically decreased and colon length is restored. *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001

To verify that the changes observed in phenotype were due to the induction of hemox-1, we performed a western blot analysis to confirm the over-expression of hemox-1 after treatment with coPP. Shown in figure 43, panel A, hemox-1 in vehicle-treated KO mice was significantly lower than in WT mice. However, after coPP treatment, both KO and WT mice upregulated hemox-1 to a similar level. To corroborate the anti-inflammatory properties of hemox-1 we performed a gene expression of colon tissues from mice treated with either vehicle or coPP. In panel B it is reported the mRNA expression of some pro-inflammatory cytokines. While KO mice treated with vehicle showed higher levels of inflammation, treatment with coPP decreased the amount of pro-inflammatory cytokines in both WT and KO mice, confirming the anti-inflammatory effect of hemox-1.

Overall, these results demonstrate that when hemox-1 was induced via a pharmacological stimulus, the differences in the inflammatory status and disease score between the two mouse strains were abrogated. Thus, we conclude that the increased inflammation found in KO mice lacking the receptor CX3CR1 is likely due to the inability to upregulate hemox-1 *via* the FKN-CX3CR1 axis.

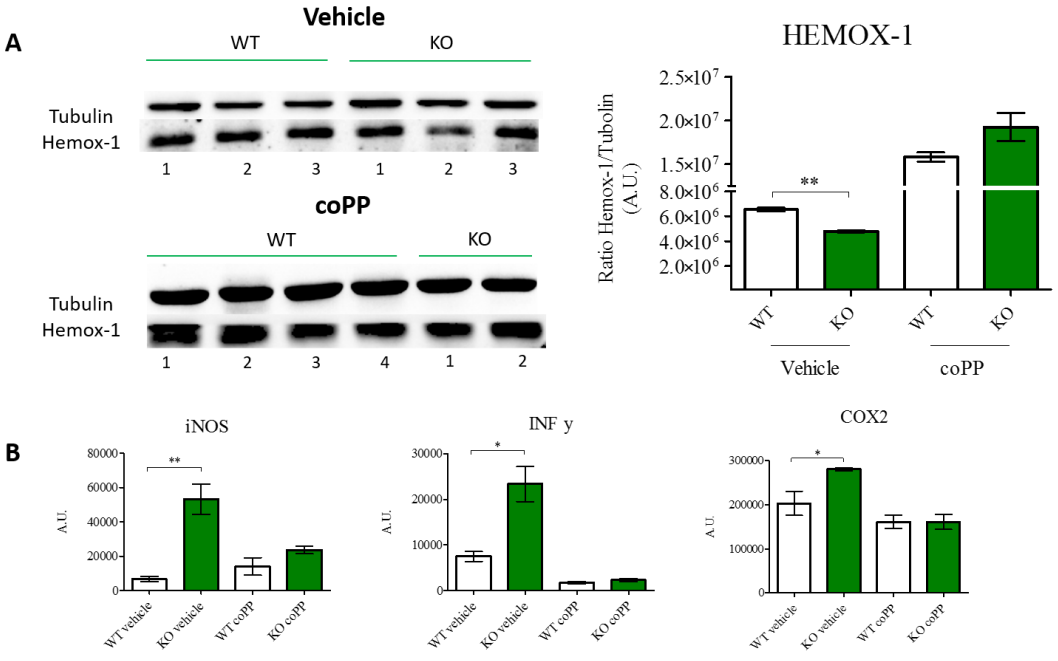


Fig.43: Molecular analysis of heme-oxygenase-1 and anti-inflammatory cytokines in coPP-treated mice. Treated colons were analyzed by western blot to detect hemox-1 (A). WT and KO mice treated with vehicle maintain the differences shown above, while the expression of hemox-1 in coPP-treated mice is increased and no differences are observed between WT and KO mice. (B) mRNA expression of pro-inflammatory mediators is increased in KO mice treated with vehicle. The treatment with coPP turns off inflammation. *p<0,05, **p<0,01, ***p<0,001

Hemox-1 is involved in the clearance of bacteria in the intestinal lumen. Considering our results obtained *in vivo,* we questioned if the ability to phagocytose was impaired in Mφ of KO mice. To do this, we harvested PEC from both WT and KO mice and we tested their aptitude to phagocytose. Mφ were co-cultured with beads coated with LPS for 1 hour and the percentage of beads phagocytosed versus the overall number of cells was evaluated as reported in figure 44. As it was expected, KO mice showed a significant reduced phagocytoses.

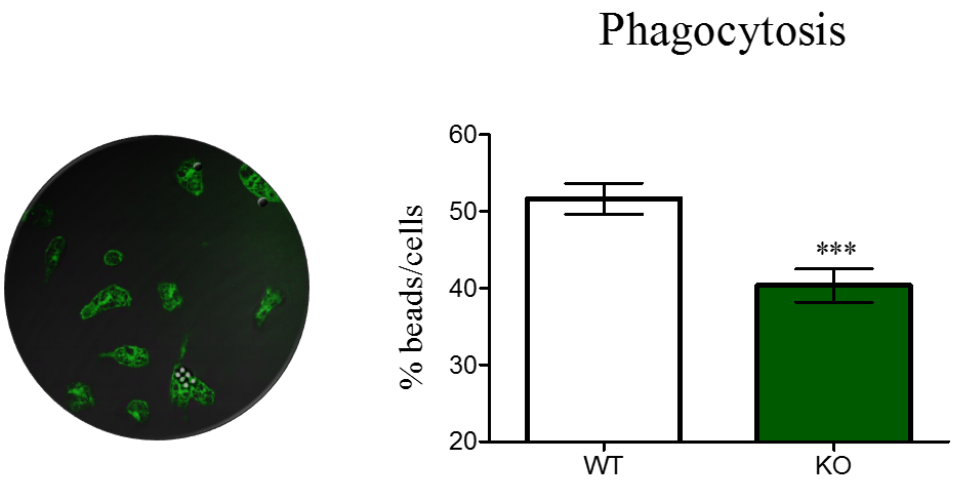


Fig.44: Phagocytosis. Hemox-1 is involved in the process of phagocytosis. Left panel shows an explicative picture of KO macrophages eating beads coated with LPS. On the right panel the graph highlights the differences in the phagocytosis ratio between WT and KO Mφ. ($p<0,05$)

Considering the differences emerged in the phagocytosis assay, we performed a deep analysis of the intestinal microbiota of WT and KO mice treated with the AOM-DSS protocol. We compared stools of mice at week 0, at week 2 (after the first cycle of DSS) and at week 10 (at the sacrifice). We sequenced 16S rRNA gene amplicons obtained from faecal metagenomic DNA and we compared WT and KO mice. We first analysed two ecological parameters: the alpha and beta diversity. Alpha diversity was studies through Chao 1 coefficient, which

estimates the intra-sample richness of operational taxonomic units (OTUs). Beta diversity, which is the inter-sample measurement of differences in OTUs diversity, was evaluated by using weighted (quantitative) and unweighted (qualitative) Unifrac algorithms. Unifranc is an algorithm that considers phylogenetic distances among OTUs; in particular, unweighted Unifrac considers the presence/absence of a specific OTU among samples, whereas weighted Unifrac considers OUT relative abundance. As shown in figure 45, at day 0, there were no differences in alpha diversity in both WT and KO mice. On the contrary, during the treatment with DSS, WT mice displayed higher rate of alpha diversity compared to KO. Both two strains tried to restore their original microbiota composition over time but, at sacrifice, WT mice still revealed higher alpha diversity. The treatment with DSS destroyed the integrity of mucosal barrier, generating inflammation. Therefore, during the administration of DSS an immune response is engaged in order to eliminate the bacteria that have invaded the tissue. KO mice displayed higher rate of inflammation respect to WT and this aberrant response cause an impairment in alpha diversity that results higher in WT.

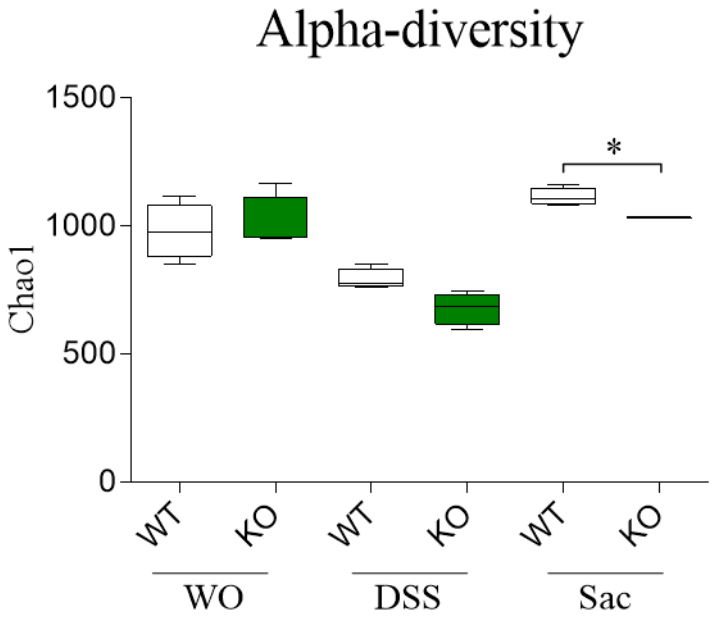


Fig.45: Alpha diversity during AOM-DSS treatment. At the beginning of the experiment, when mice were still untreated and, consequently, healthy, there were no differences in alpha diversity between WT and KO mice. This means that there were no significant differences in bacterial taxonomic richness of the healthy intestine between WT and KO mice. After the first cycle of DSS, KO mice showed a reduced alpha diversity that was maintained until sacrifice. Plausibly, the iper-stimulation of the immune response in KO mice was the reason for lower alpha diversity.

Beta diversity analyses allow the comparison of the overall taxonomic microbiota composition among sample. Figure 46 show that samples from KO mice are widely scatterd in the beta-deversity plot, indicating higher inter-sample diversity of KO than WT mice . We can speculate that the microbiota of KO mice is instable due to host immune impairment.

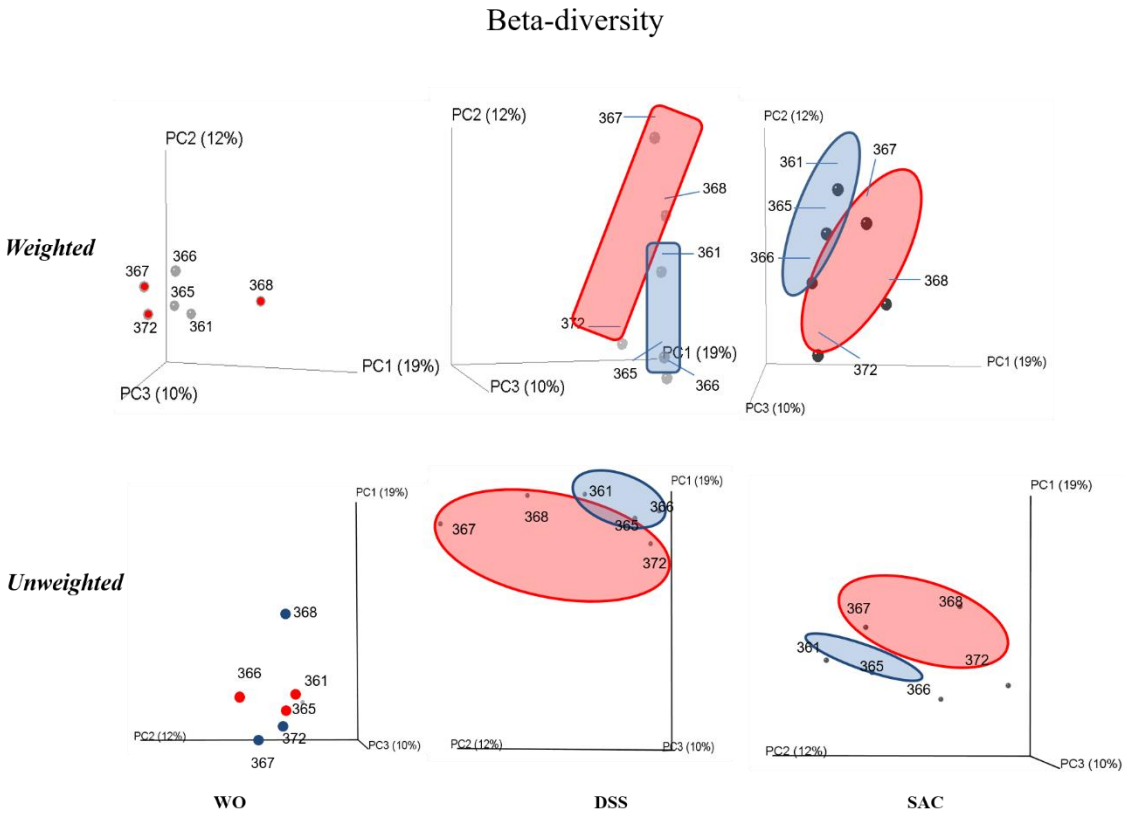


Fig. 46: Beta diversity analysis of faecal microbiota according to Weighted (top panels) and unweighted (lower panels) Unifrac. The three most significant principal coordinate have been plotted. Samples from WT and KO mice are shown in red and blue respectively.

The relative abundance of specific bacterial taxa was analysed at level of phylum, family and genus in the three time point considered. At day 0 almost no differences were detectable between WT and KO; on the contrary, one week after DSS administration, more differences became visible. In particular, at sacrifice, three genera were markedly different between KO and WT samples: *Akkermansia, Lachnospira* and *Bacteroides* (figure 47).

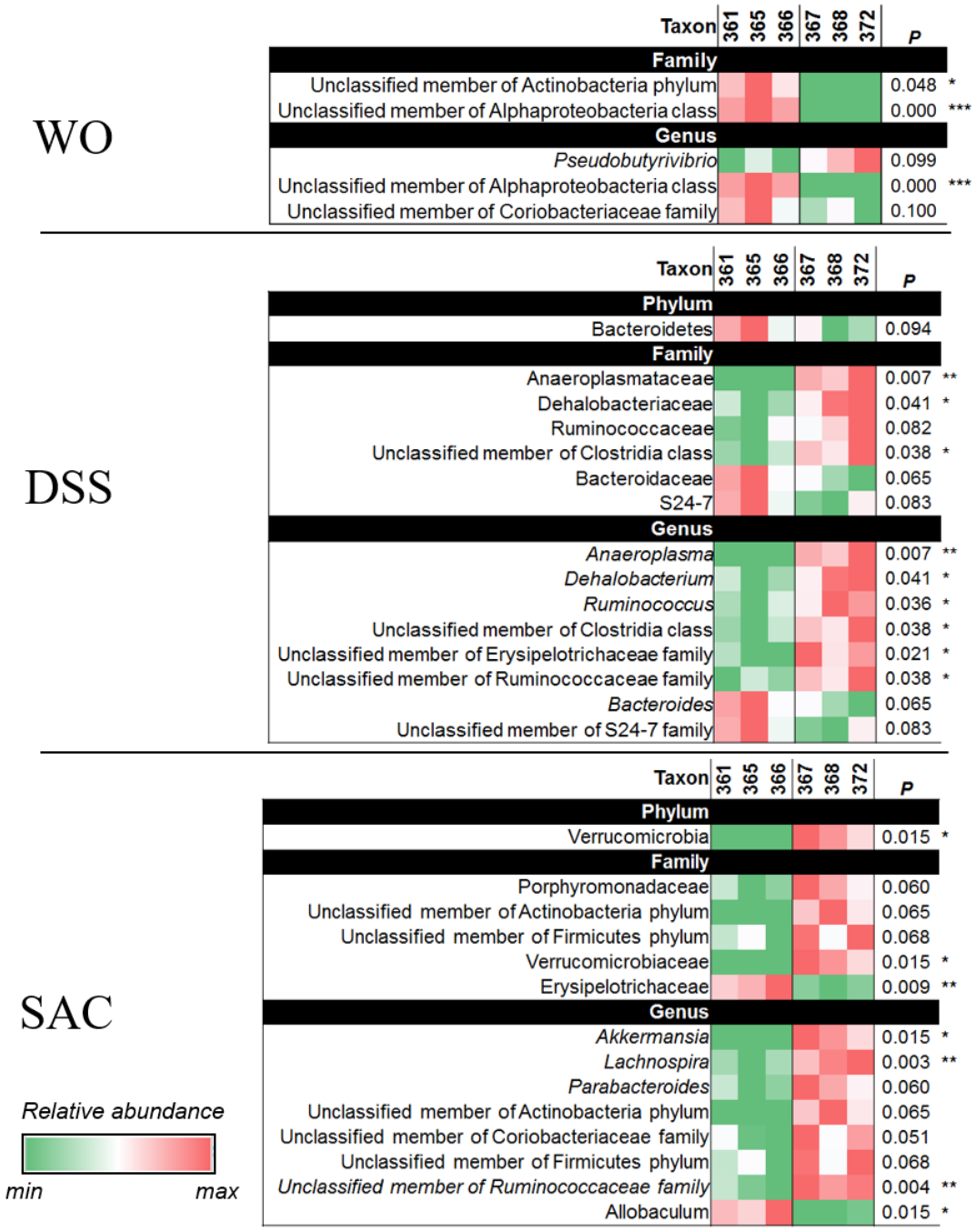


Fig. 47: 16s sequencing. Heat-map representing relative abundance of bacterial taxa of faecal samples. The taxonomic levels of phylum, family and genus were considered. At sacrifice, the main differences between the microbiota composition of KO and WT mice in genera *Akkermansia, Lachnospira and Bacteroides*.

Bacteroides is a Gram negative genus that include bacteria associated to health benefit for the host [151]. We evaluated their relative abundance in the samples at day 0 and at sacrifice but there was little difference between WT and KO, probably because the relative abundance

of this bacterium was particularly low. *Lachnospira* is a member of the Gram positive class Clostridiales; these bacteria are considered among the most active components of the intestinal microbiota and represent part of the most important intestinal producers of butyrate, a short chain fatty acid that protect mucosa and regulate immune responses in the gut. We tested the abundance of *Lachnospira* by qPCRby qPCR but, despite a tendency, no statistically significant differences between WT and KO mice were found. On the contrary, when we tested the relative presence of *Akkermansia*, we found a significant decrease in KO compared to WT mice. The presence of *Akkermansia* in untreated samples was identical between WT and KO mice but, at sacrifice, WT mice had a higher presence of this bacterium compared to KO mice (figure 48). *Akkermansia* is a Gram negative bacterium able which efficiently degrade intestinal mucins. Its role in IBD is now under investigation but it was reported that in both UC and CD the presence of *Akkermansia* is drastically diminishing [53].

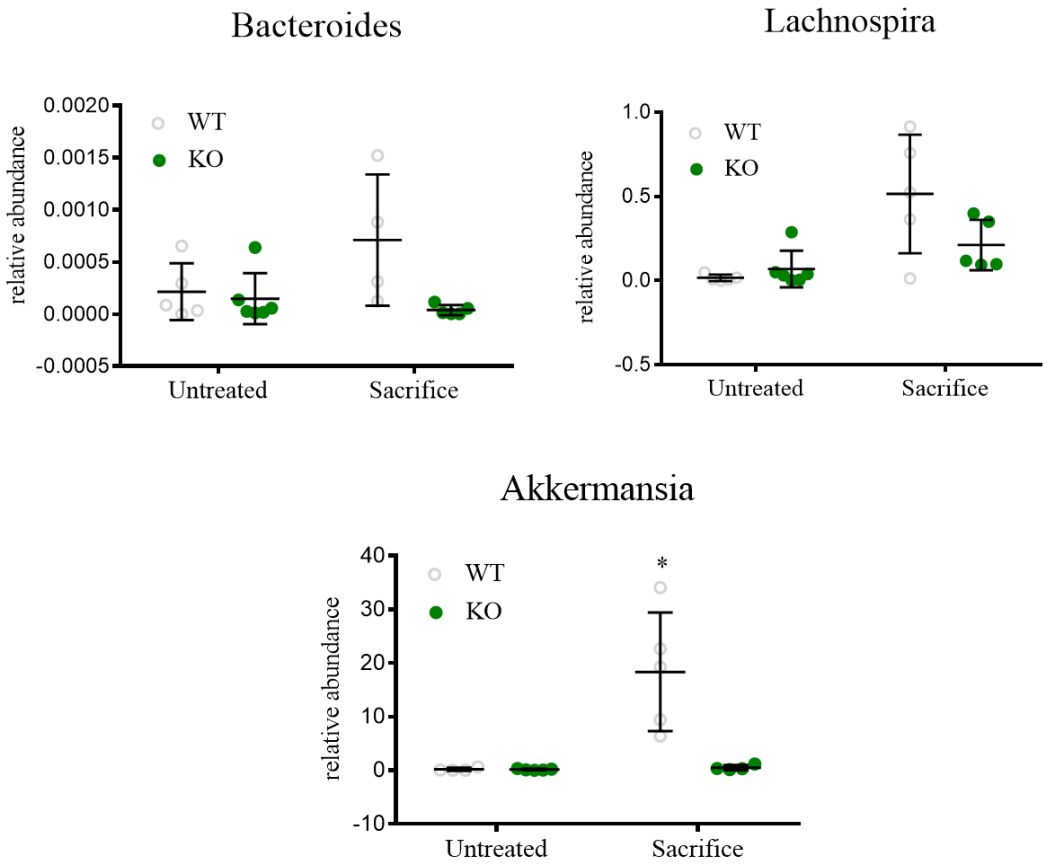


Fig.48: Relative abundance of genera *Akkermansia*, *Lachnospira* and *Bacteroides*. Data refer to the relative proportion of target bacteria relative to total bacteria calculated by qPCR using genus specific and universal (Eub) primer targeting *16S rRNA* gene. Only the relative abundance of A*kkermansia* was significantly differentbetween KO and WT mice at sacrifice.

In order to test if the intestinal microbiota can influence the microenvironment, we performed an AOM-DSS experiment co-housing WT and KO mice. Mice were put together at the time of weaning in cages with two mice WT and two mice KO. Weight and D.A.I. score were registered every week and are represented in figure 49 compared with a classical non-co-housed AOM-DSS experiment. It was clear that the co-housing altered the behaviour of mice during treatment: both weight and D.A.I. did not change between WT and KO mice indicating that gut microbiota of the two strains is able to mutually influence each other.

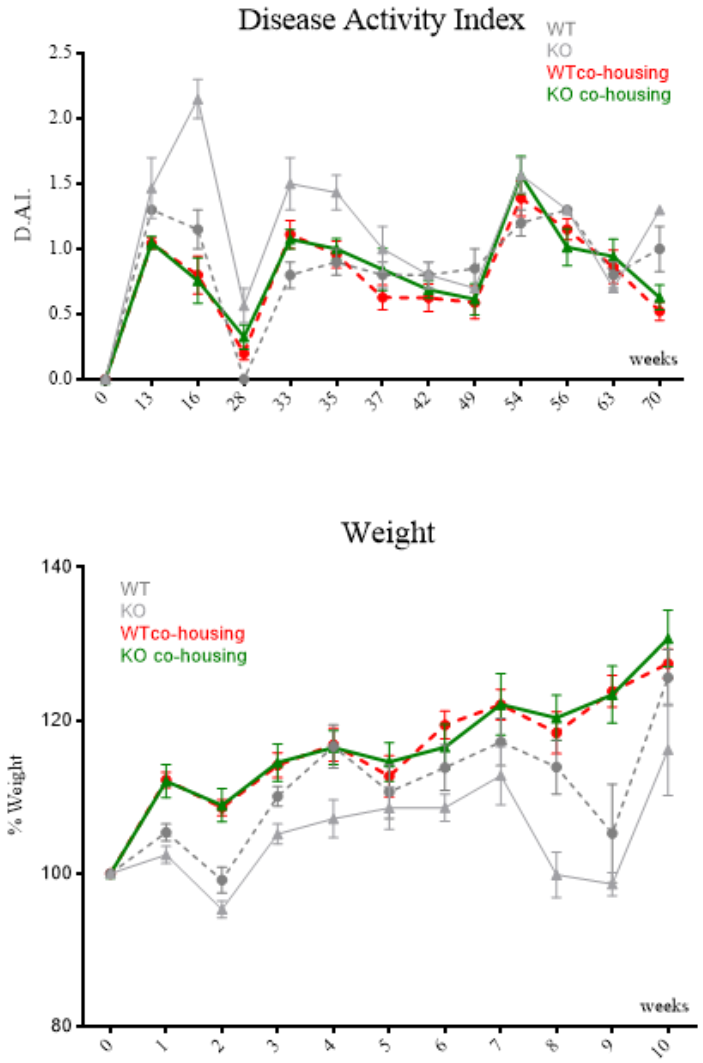


Fig. 49: Co-housing in the AOM-DSS model: Panel A reports weight of mice during treatment while in panel B it is represented the disease activity index (D.A.I.). The grey lines represent mice treated with the AOM-DSS protocol without co-housing while the red and green lines represent WT and KO mice co-housed. In both panels it is evident how co-housing altered the response to treatment.

At the end of the experiment, colons were harvested, measured and polyps and colitis score were assessed. If compared with the classical AOM-DSS experiment, both WT and KO mice displayed a lower percentage of adenomatous tissue and of colitis score together with a higher colon length. Nevertheless, some differences remained between WT and KO mice in term of polyps' formation and colon length (figure 50).

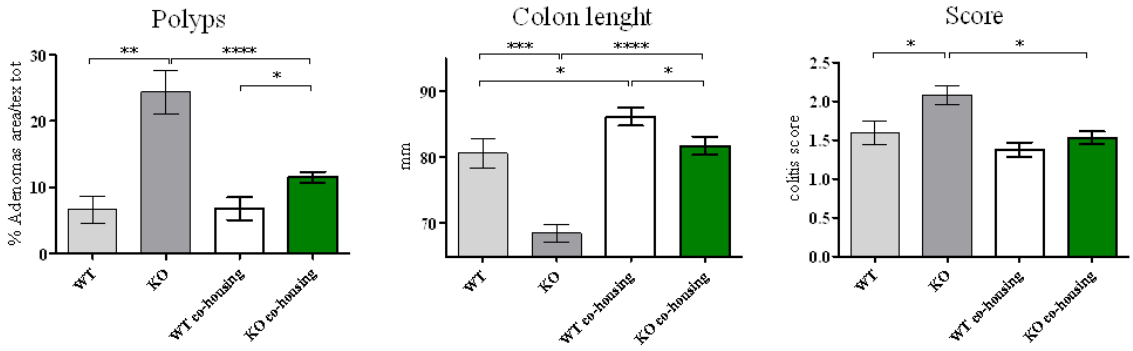


Fig. 50: Histologic analyses of co-housed mice: in green are represented co-housed WT and KO mice of the AOM-DSS experiment. Despite some differences remained between WT and KO mice, they were more similar if compared to the classical AOM-DSS experiment (*p<0,05; **p<0,01; ***p<0,001; ****p<0,0001).

In order to better understand how the co-housing could affect weight, D.A.I. and polyps formation, we extracted mRNA from polyps and we determined the levels of both pro and anti-inflammatory cytokines. As shown in figure 51, the differences observed in the classical AOM-DSS model became less evident. In the top panel the only molecule down-regulated in KO mice was hemox-1 and the only pro-inflammatory cytokine was IL-12. These preliminary results indicate that the microbiome is mutually influenced between WT and KO mice. This resulted in an attenuation of the severity of the disease reflected in a lower rate of polyps, colitis score and in a higher capability to regain weight after DSS administration. In support to this hypothesis, both pro and anti-inflammatory cytokines were modulated in both WT and KO mice and no significantly alterations could be appreciable. In this landscape only hemox-1 remained down-regulated in KO mice highlighting the need of the receptor to activate this crucial signalling. Indeed, KO mice exhibited higher rate of polyps even if co-housed. (However, a microbiota profiling of co-housed mice would be necessary).

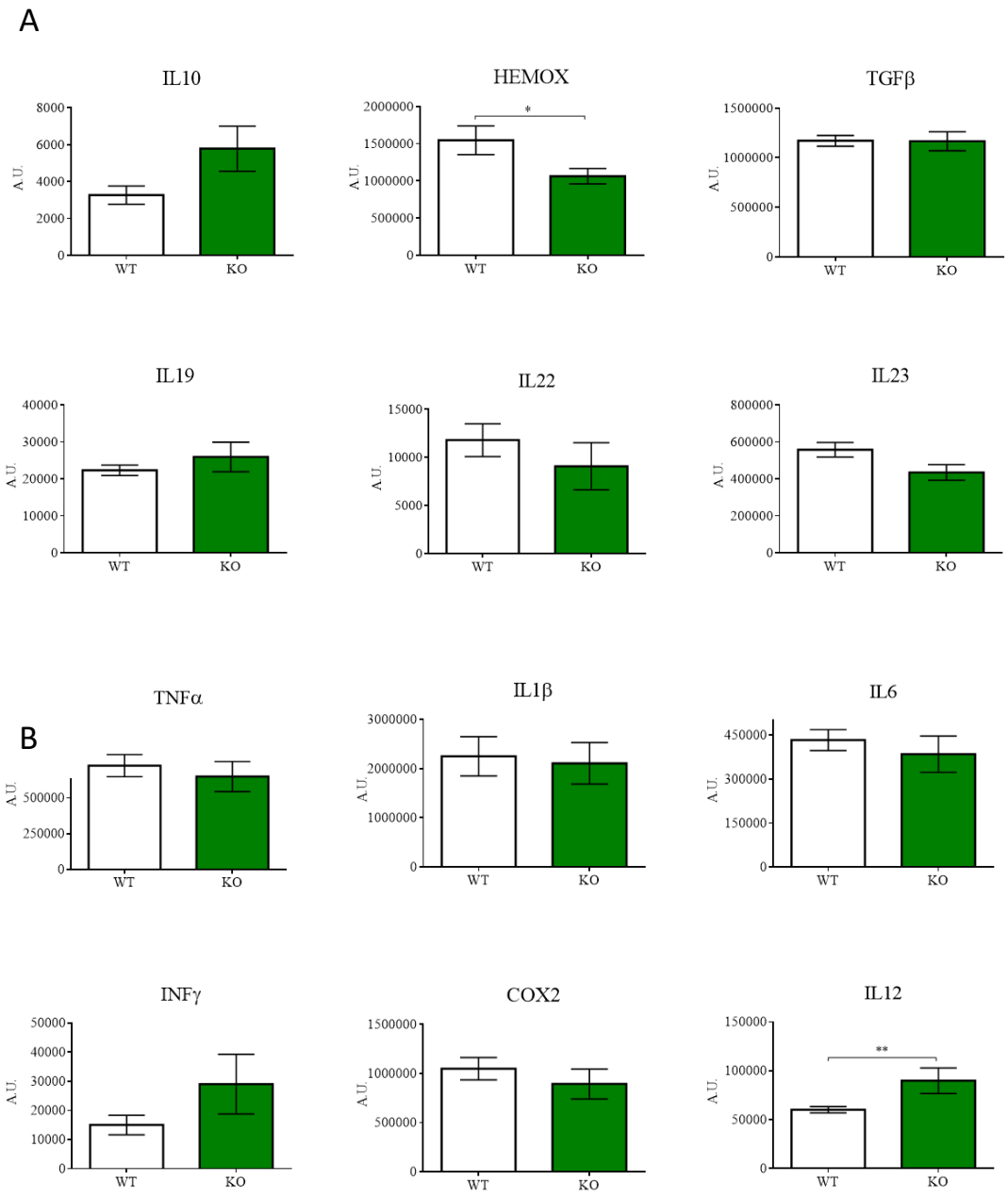


Fig. 51: Pro and anti-inflammatory cytokines in the colonic mucosa of co-housed mice during the AOM-DSS treatment. In both panels there are a few differences between WT and KO mice. It is important to note in panel A that hemox-1 is down-regulated in KO mice as it was in the classical AOM-DSS model. Therefore, even considering the importance of the microbiome, in this context this enzyme assumes a crucial role for tumour development.

Discussion

The intestine is an exclusive site able to maintain the balance between immune system activation and tolerance against the very huge amount of harmless antigens that are in contact with the intestinal lumen, including food antigens and microflora. During inflammation, the immune system has to trigger a pro-inflammatory response and to be able to restore homeostasis in order to avoid aberrant responses that can lead to chronic inflammation and tissue injury. In this complex landscape, mononuclear phagocytes, and in particular CX3CR1^{+} macrophages, represent the most abundant population and exert a key role in the maintenance of homeostasis.

Our aim was to investigate the role of CX3CR1^{+} macrophages in both steady state and inflammation with a particular focus on the resolution of inflammation and the development of intestinal tumorigenesis. Indeed an impaired resolution could favor tumor development.

We first characterized the features of CX3CR1^{+} cells in the colonic mucosa. We defined them as resident macrophages expressing high levels of the CX3CR1 receptor together with F4/80, CD11b and CD64 reproducing the data obtained by Bain and colleagues [10]. CX3CR1^{+} MO have the primary purpose to maintain homeostasis in the complex intestinal microenvironment. It is well known the key role that IL-10 plays in this context; therefore we tested mucosal macrophages, in mice deficient or competent for the CX3CR1 receptor, for their ability to produce IL-10 and, according to the recent finding of Zigmond and colleagues {Zigmond, 2014 #266, to sense IL-10 thanks to the expression of its receptor. They demonstrated that loss of the IL-10 receptor causes severe colitis. In the gut, CX3CR1^{+} Mφ produce IL-10 in order to maintain the immune system in a state of *"alert anergia"*. The IL-10 produced acts in an autocrine loop on the same macrophages that express the IL-10 receptor. Based on these findings, we analyzed the transcriptional levels of IL-10 and IL-10 receptor in bone marrow derived macrophages, spleen Mφ and in the lamina propria Mφ of both WT and KO mice in steady-state conditions. We found that no differences were present, between WT and KO mice, in any tissue and that macrophages were able to sense IL-10 because STAT-3 resulted phosphorylated after IL-10 stimulation. These first results revealed that in steady state CX3CR1^{+} Mφ are recruited in the normal mucosa, in both WT and KO mice, and here they are able to serve their main role of maintenance of homeostasis producing an equal amount of IL-10 and being able to sense it.

How the mucosal macrophages reach the colonic mucosa is still unclear. Bain et al., {Bain, 2014 #251}proposed that Ly6C^{hi} monocytes are the precursors of both resident and inflammatory macrophages. Here we demonstrated that loss of CX3CR1 receptor does not impair the accumulation of resident macrophages in the resting mucosa. The current hypothesis is that macrophages are constantly replenished by CCR2^{+} monocytes and in the mucosa they upregulate CX3CR1, acquiring their anti-inflammatory properties.

Very few and controversial studies have been reported about the role of CX3CR1^{+} macrophages in acute colitis. Two studies unveiled different results, Kostadinova et al. analyzed the behavior of CX3CR1^{+} Mφ in Balb/c mice and suggested a negative role of the CX3CL1-CX3CR1 axis in colonic protection. They argued that the ligand-receptor axis induces the production of iNOS that they considered the main trigger of inflammation [116]. In contrast, Medina-Contreras et al. showed in C57BL/6 mice that the absence of the receptor provokes a higher bacteria translocation in the mesenteric lymph nodes (mLN) resulting in higher tissue damage during experimental colitis. Their results reveal that both CX3CL1 and CX3CR1 KO mice failed to accumulate macrophages in the intestinal mucosa and this results in an increased activation of Th17 lymphocytes *via* IL-17, in addition to a higher translocation of commensal bacteria in the mLN. After the administration of DSS, KO mice displayed higher signs of CD4-mediated inflammation compared to WT [26]. Our results are consistent with the data obtained by Medina-Contreras. In our colitis experiments, we observed more inflammation in KO mice, in terms of both histological parameters and quantification of soluble mediators. In contrast to the observations made by M-C, we found higher accumulation of macrophages and, in particular, of CX3CR1 Mφ in the colonic mucosa of CX3CR1 KO mice. As mentioned above, in the *lamina propria*, CX3CR1^{+} macrophages are involved in the maintenance of homeostasis. Weber and colleagues hypothesized that even during inflammation, CX3CR1^{+} Mφ are able to maintain their anti-inflammatory behavior, avoiding the rise of chronic inflammation [12]. Our results are consistent with this hypothesis: KO mice failed in maintaining homeostasis because of CX3CR1 loss and, consequently, recruit more macrophages in the attempt to switch off inflammation.

Considering the role of CX3CR1^{+} Mφ in steady state, we next analyzed their behavior in the process of recovery from colitis. The evaluation of weight revealed that at day 10, WT

mice began to recover from colitis and at day 13, they had completely restored their original phenotype. In contrast, KO mice were unable to recover and displayed, even at day 13, high signs of inflammation with a marked reduction in colon length. At day 10, both monocytes and macrophages were more abundant in the mucosa of KO mice. The characterization of the inflammatory mediators released in the microenvironment was performed as mRNA quantification: while WT mice were able to switch off inflammation, KO mice failed in this process and over-expressed all the pro-inflammatory mediators up to day 13. We then measured IL-10, which is the key regulator of inflammation and the hallmark of CX3CR1^{+} macrophages [8, 30, 34]. We found that KO mice were able to produce even more IL-10 than WT mice. This finding indicated that IL-10 production is not dependent from the CX3CL1-CX3CR1 axis. Our hypothesis is that, due to the higher amounts of inflammatory cytokines, KO macrophages respond to this disequilibrium by producing higher levels of IL-10, in an attempt to restore homeostasis. However, this does not occur, and this result suggests that loss of the receptor and consequently its signaling, has a profound impact on the macrophage control of the mucosa.

Considering this point, we further investigated why loss of receptor leads to higher inflammation in KO mice. We found that the only molecule down-regulated in KO mice at day 10 was heme-oxygenase-1. Intestinal macrophages have high bactericidal properties [24] and hemox-1 is a key regulator of phagocytosis. Hemox-1 is reported to be an anti-inflammatory molecule, regulated by many factors, including IL-10 [65]. Many studies suggest a synergism between IL-10 and the microbiome in the induction of hemox-1 [60], [68, 69]. It seems that the microbiome stimulates hemox-1 production *via* IL-10, and the consequent CO formation is able to stimulate bacteria clearance by macrophages. Moreover, Inui et al [152] reported that CXRCR1^{+} Mφ could produce hemox-1 in a model of inflammation induced by toxin A. Our results obtained in a model of recovery of colitis unveil that at day 10, when WT mice began to restore, there is a strong decrease of hemox-1 in KO mice, both as mRNA and protein levels, electing it as a key molecule in CX3CR1 Mφ for the control of colonic inflammation. Lee demonstrated that induction of hemox-1 is dose-dependently induced by IL-10. In our study we demonstrate that IL-10 production is not sufficient: KO mice are in fact able to provide IL-10, but they failed to produce a relevant amount of hemox-1. These results

suggested the importance of a correct CX3CL1-CX3CR1 signaling in the modulation of hemox-1.

To deeper investigate the role of hemox-1 in this context and the potential involvement of the receptor CX3CR1, we moved *in vitro* and reproduced the inflammatory conditions observed *in vivo*. We FACS-sorted CX3CR1^{+} BMDM and stimulated them with LPS and/or FKN, obtaining the same results of the *in vivo* model of colitis. Indeed, after stimulation with LPS we detected more pro-inflammatory mediators in KO mice compared to WT. LPS and FKN synergized in the production of hemox-1 in WT but not in KO mice; most importantly, stimulation with FKN alone was sufficient to produce Hemox-1 in WT mice, unveiling a previously unidentified role of the chemokine receptor CX3CR1 in the regulation of Hemox-1. Even in an experiment of prolonged LPS stimulation (trying to mimic the process of recovery from colitis) we observed up-regulation of hemox-1 production in WT mice but not in KO mice.

These *in vitro* results overall confirm the data obtained *in vivo* with the DSS model of acute colitis, with higher inflammatory mediators and lower hemox-1 in KO mice. The ability of our CX3CR1^{+} macrophages to respond to LPS is in contrast with the previous observation by Bain et al. [10]. In their study, they demonstrated that CX3CR1^{+} macrophages are unresponsive to TLR stimulation and the Authors considered this a key feature for the maintenance of homeostasis in the colonic mucosa. A possible explanation for the different results might be the presence, in the inflamed colonic mucosa, of a population expressing intermediate levels of CX3CR1 (CX3CR1^{INT}), recently recruited from circulating monocytes. The cells upon inflammation are able to exert pro-inflammatory features; further on, CX3CR1^{+} macrophages expand and begin to recover the tissue [10].

Furthermore, in the vitro experiment, BMDM from KO mice showed an increment in the expression and production of IL-10, retracing the behavior observed in vivo. Overall, we demonstrated both in vivo and in vitro the same attitude of CX3CR1 macrophages. In WT mice these macrophages maintain the homeostasis, they correctly respond to inflammatory stimuli being able to induce inflammation but also to restore the steady state at the end of the inflammation. In contrast, KO mice respond to inflammatory stimuli with higher levels of pro-inflammatory mediators and are not able to recover at the end of the inflammatory insult. KO

mice respond to inflammation also by up-regulating IL-10 in order to switch off the aberrant response, but this appears a useless attempt. Moreover the impaired production of hemox-1 results in a prolonged tissue damage.

Considering the behavior of CX3CR1 macrophages in acute colitis and in the recovery from colitis, and considering that tumor is a "wound that fails to heal", we decided to investigate the role of $CX3CR1^+$ Mφ in the development of colorectal cancer, a study that has never been performed, to the best of our knowledge. We used the AOM-DSS model to induce a colitis-associated carcinogenesis. Weekly, during the experiment, we assessed D.A.I. score considering the loss of weight, the stool consistence and bleeding. Along treatment, in correspondence with DSS administration, KO mice showed a higher degree in D.A.I. score. Indeed, at the end of the experiment, they displayed a lower survival rate and the histological analyses revealed higher signs of inflammation in terms of colitis score and shortness of the colon. The most relevant result was the higher polyps formation in KO mice in comparison to WT. In line with the model of acute colitis, we detected more $CX3CR1^+$ macrophages and $Ly6C^+$ monocytes in the polyps of KO mice. Moreover, we analyzed the composition of macrophages at the tumor border and we demonstrated that the newly recruited macrophages entering the tissue are not $CX3CR1^+$, while those that have already reached the tissue express the receptor. These data confirmed what is already published in the literature about the recruitment of macrophages in the colonic mucosa: the current hypothesis is that Ly6C monocytes are recruited *via* CCL2-CCR2 in the tissue where they are able to differentiate in resident or pro-inflammatory macrophages in a context-dependent fashion [28] [10].

Considering the higher level of inflammation and the higher number of polyps detected in KO mice compared to WT, we looked at inflammatory cytokines and anti-inflammatory mediators in both polyps and inflamed tissues. In both specimens, pro-inflammatory cytokines such as IL-1β, IFN-γ and IL-12 were up-regulated in KO mice compared to WT, while other mediators, such as TNF-α, IL-6 and COX2 were higher only in the inflamed tissue, suggesting that inflammation could be the cause of higher rate of polyps in KO mice. Instead, when we looked at anti-inflammatory mediators we obtained the same data of the acute colitis model. Indeed, IL10 and IL-10 family members, such as IL-19 and IL-20 were over-expressed in KO mice. As discussed above, KO mice are able to produce IL-10 and they try to switch off the

aberrant inflammation by expressing these cytokines. In contrast, TGF-β and hemox-1 were down-regulated in KO mice, unveiling a central role of these mediators also in polyps development.

Since hemox-1 seemed to be a key regulator of mucosal resident macrophages, we decided to enhance its production in KO mice during the AOM-DSS experiment, using a drug able to induce hemox-1 in the intestine (coPP). The results we obtained indicated that hemox-1 is a crucial player in the regulation of intestinal macrophages. The D.A.I. score of both WT and KO mice was almost assessed near 0 and they failed in losing weight after DSS treatment. These data were confirmed by the histological results: colon length was restored and the colitis score was similar to that of untreated mice. Moreover, the percentage of adenomatous tissue was drastically decreased. In addition, pro-inflammatory cytokines, such as iNOS, IFN-γ and COX-2 were dramatically reduced in coPP-treated mice. Overall, these results demonstrate that, upon pharmacological induction of hemox-1, KO mice retrace the same behavior of WT mice and the differences between the two populations disappear, revealing the importance of hemox-1 production in this context.

Moreover, we decided to study a genetic model of intestinal tumorigenesis, in order to confirm the data obtained with the sporadic model. We crossed mice bearing the APC mutation with $CX3CR1^{GFP/+}$ or $CX3CR1^{GFP/GFP}$ and we fed them with DSS in order to generate an inflamed milieu. We were able to demonstrate also in this model that the absence of the receptor leads to higher polyps formation in KO mice compared to WT. Colons of KO mice were shorter and the D.A.I. score was more severe. Moreover, the colonic mucosa of KO mice was infiltrated with a higher number of monocytes and macrophages reflecting the AOM-DSS model. Finally, also the analysis of the soluble mediators mirrored the data previously described. In particular, IL-6 IFN-γ and IL-10 were upregulated in both polyps and inflamed mucosa of KO mice, while TGF-β and hemox-1 were significantly reduced in KO mice.

Hemox-1 acts on macrophages stimulating the processes of phagocytosis. Considering the key role of this molecule in this context, we investigated if KO mice are able to phagocytose as WT do. The data we obtained revealed an impaired phagocytic ability of KO macrophages. Ishida et al., [57] demonstrated that $CX3CR1^{+}$ Mφ have impaired anti-bacterial functions.

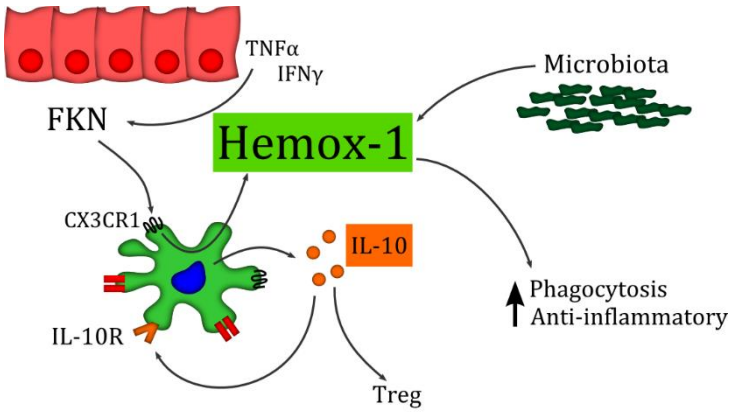
They showed that macrophages are normally recruited in both WT and KO mice but if the CX3CR1 receptor is lacking, bacterial clearance is reduced. They supposed that CX3CL1 serves to activate macrophages to induce the expression of NO, TNFα, IL-12, IL-1β. These mediators act in a paracrine and autocrine manner on macrophages augmenting their bactericidal functions. Here we demonstrated that if the CX3CR1 receptor is lacking, bacterial clearance is reduced, but we propose that this is a direct consequence of impaired hemox-1 production.

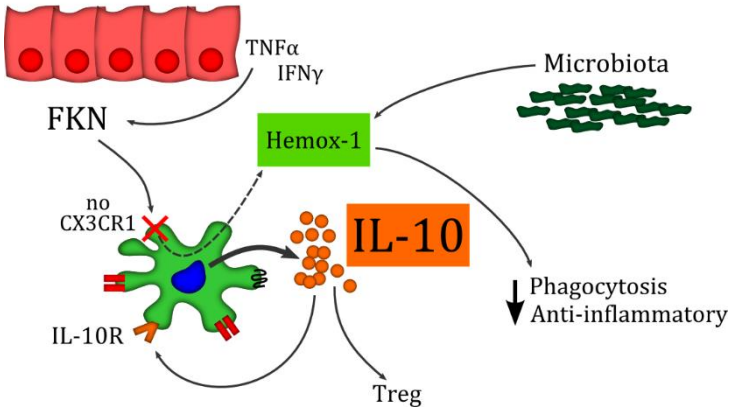
Considering the importance of microbiome in healthy and in chronic diseases such as ulcerative colitis or Chron disease, we performed a deep analysis of the intestinal microbiota of WT and KO mice treated with the AOM-DSS protocol. We compared stools of mice at week 0, at week 2 (after the first cycle of DSS) and at week 10 (at the sacrifice). We sequenced 16S rRNA gene amplicons obtained from faecal metagenomic DNA and we compared WT and KO mice. At day 0, no differences were detected between WT and KO mice. Contrary to common thought, during IBD the richness in bacteria species and their diversity is reduced and also the stability in the composition of microbiota changes [51]. In line with this evidence, we observed a higher richness in the microbiota composition of WT mice, confirming our previous results indicating that KO mice displayed a more severe disease. Our preliminary results unveiled that at sacrifice three different genera were divergent between WT and KO. *Bacteroides* is a Gram negative genus that includes bacteria associated to health benefit for the host [151]. *Lachnospira* is a member of the Gram positive class Clostridiales; these bacteria are considered among the most active components of the intestinal microbiota and represent part of the most important intestinal producers of butyrate, a short chain fatty acid that protects the mucosa and regulates immune responses in the gut. *Akkermansia* is a Gram negative bacterium able to efficiently degrade intestinal mucins. Among these three genera, only Akkermansia was significantly different between WT and KO at sacrifice. As reported by Png [53], Akkermansia is impaired during inflammatory bowel diseases. Loss of *Akkermansia* results in the rise of another mucolytic species- *Ruminococcus gnavus*- that has been associated with the relapse of the human disease. Moreover, being the mucus the native habitat of many species of bacteria, its alteration results also in a dysregulation of the total microbiome composition [53]. Therefore, the dysregulation observed in KO mice may be a consequence of their inability to produce hemox-1 and consequently to properly regulate the commensal microbiome. The resulting

down-regulation of Akkermansia could be another factor that promotes chronic inflammation and, in the absence of the regulation by CX3CR1$^+$ Mφ, ultimately lead to increased tumor development.

Finally, we analysed if the microbiome of WT mice was able to positively influence the behaviour of CX3CR1 KO mice. The results obtained with the co-housing experiment indicated that the mixed composition of WT and KO microbiome had a positive role in controlling tumor development. Indeed the differences between WT and KO mice were attenuated in this experimental model: the histological parameters were drastically ameliorated in KO mice if compared to a classical AOM-DSS experiment, the D.A.I. score was almost the same as in WT mice and the percentage of adenomatous tissue was the same between the two strains. In addition, the transcriptome analyses revealed that inflammation was also attenuated in KO mice, even if a difference in hemox-1 production remains. These results suggest that the WT microbiome exerts a positive effect on the disease of KO mice. However, a microbiota profiling of co-housed mice is necessary in order to better clarify this point.

To summarize, we described a context in which CX3CR1$^+$ macrophages play a crucial role thanks to their ability to control homeostasis and tissue inflammation. During inflammation FKN is induced by TNF-α and IFN-γ and, when the receptor is present, the CX3CL1-CX3CR1 axis is functional. This results in the induction of hemox-1 with consequent increased of phagocytosis and anti-inflammatory conditions. The microbiome contributes to hemox-1 production in macrophages. If the CX3CR1 receptor is lacking, intestinal Mφ are not able to respond to FKN and do not produce sufficient amount of hemox-1; in spite of high levels of IL-10, they are unable to switch off inflammation. In this context, the only stimulus for hemox-1 production is provided by the microbiome. However, also the microbiome is affected by the inadequate levels of hemox-1 that cause impaired phagocytosis and tissue repair. Changes of the microbiome lead to dysbiosis and favour chronic inflammation and cancer development.





Taken together these results unveil a new pathway by which intestinal macrophages act to maintain homeostasis. If the CX3CL1-CX3CR1 checkpoint is lost, the aberrant inflammation could persist and be corroborated by a state of dysbiosis. In this landscape, chronic inflammation can establish and the impaired mechanism of resolution could, with time, lead to tumor development. Finally, we confirmed that treatment with the chemical compound coPP up-regulates hemox-1 also in the absence of functional CX3CR1 receptor and drastically ameliorates the intestinal disease. These findings open a new scenario in the use of hemox-1 inducing drugs to restore a correct intestinal homeostasis in a therapeutic context. To this aim further experiments are required to validate the mechanism by which these drugs work and to better investigate the microbiome composition.

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