Universidade do Minho
Escola de Medicina

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Microbiota Regulation of Cancer Immune Surveillance

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

Regulação da Vigilância Imunitária contra o Cancro pelo Microbiota

O cancro representa um dos principais problemas de saúde a nível mundial, prevendo-se um aumento da sua incidência nos próximos anos. Embora o desenvolvimento de novas terapias tenha reduzido a mortalidade, é crucial determinar novos alvos terapêuticos para tratar os pacientes que não respondem aos tratamentos existentes. Assim, torna-se indispensável definir os principais intervenientes envolvidos na carcinogénese de modo a revelar novas vias de intervenção e biomarcadores. O microbiota emergiu recentemente como um regulador chave quer da carcinogénese quer da vigilância imunitária. Nesta Tese de Doutoramento exploramos o impacto de alterações dietéticas, nomeadamente a suplementação dietética com zinco, na progressão tumoral. Os nossos dados mostraram que ratinhos que receberam dieta suplementada com zinco apresentaram maior crescimento tumoral quando comparados com o grupo controlo, sujeito a uma dieta *standard*. Adicionalmente, ratinhos submetidos a um tratamento com antibióticos, previamente à exposição à dieta suplementada com zinco, não apresentaram alterações no crescimento tumoral em comparação com o grupo controlo, revelando o papel crucial do microbiota neste modelo. Mais ainda, verificamos alterações na resposta imunitária nos animais que receberam a dieta suplementada com zinco que se caracterizou pelo aumento da acumulação de linfócitos T FoxP3$^+$ nos nódulos linfáticos mesentéricos, bem como acumulação intra-tumoral de células Gr1$^+$. Importa ressalvar que as células Gr1$^+$ têm um papel crítico na promoção do crescimento dos tumores, uma vez que a sua depleção o reverteu. Mostramos ainda que o aumento dos níveis de interleucina (IL)-10 reduziu o crescimento excessivo dos tumores nos ratinhos que receberam a dieta suplementada com zinco. Uma vez que os níveis aumentados de IL-10 foram acompanhados pelo aumento da produção de imunoglobulina (Ig) A no intestino destes ratinhos, hipotetizamos que a IL-10 neutraliza a atividade oncogénica do microbiota através da promoção de produção de IgA. Por fim, exploramos a avaliação dos linfócitos intra-tumorais como biomarcador de prognóstico em amostras humanas de cancro colo-retal (CCR). Embora não tenhamos encontrado nenhuma associação entre a infiltração intra-tumoral de linfócitos com os parâmetros clínico-patológicos, a presença destes linfócitos nas margens invasivas do tumor, particularmente de linfócitos FoxP3$^+$, associou-se com marcadores de bom prognóstico. No entanto, não encontramos alterações na sobrevida dos indivíduos analisados.

Em suma, os nossos resultados mostraram um papel pro-tumoral da suplementação dietética com zinco mediado pelo microbiota, bem como um papel anti-tumoral da via IL-10-IgA. Mais ainda, mostramos que a localização e o tipo de linfócitos presentes nos tumores estão associados à patogénese do CCR.

Palavras chave: Biomarcador; Cancro; Microbiota; Vigilância imunitária.

ABSTRACT

Microbiota Regulation of Cancer Immune Surveillance

Cancer represents a major health problem worldwide, and its incidence is predicted to increase in the following years. Although the development of new therapies has decreased the number of deaths, novel therapeutic targets are required to treat patients that do not respond to current treatments. It is critical to define the key players involved in carcinogenesis, as this will unveil new pathways to be intervened and will help establish new prognostic biomarkers. In this regard, the microbiota has recently emerged as a key regulator of carcinogenesis and cancer immune surveillance. Therefore, in this Doctoral Thesis we explored the impact of dietary alterations, namely dietary zinc supplementation, in tumor progression, since diet is an important modulator of microbiota. We found that tumor-bearing mice under short-term dietary zinc supplementation had increased tumor growth when compared to mice maintained with regular chow. Interestingly, we disclosed a crucial role for microbiota, as antibiotic treated mice under dietary zinc supplementation did not display tumor overgrowth. Our data further revealed alterations in the immune response, that were characterized by increased accumulations of $FoxP3^+$ T lymphocytes in mesenteric lymph nodes and intratumor $Gr1^+$ cells in mice under dietary zinc supplementation. Importantly, $Gr1^+$ cells were critical for tumor promotion, as their depletion reverted tumor overgrowth. In addition, we showed that increased levels of interleukin (IL)-10 reduced tumor overgrowth in mice under short-term dietary zinc supplementation. Given that IL-10 overexpression accompanied by increased production of immunoglobulin (Ig)A in the gut of mice under dietary zinc supplementation, we hypothesize that IL-10 counteracts the oncogenic activity of microbiota through the induction of IgA production. With these compelling evidence on the role of tumor infiltrating immune cells, we sought to explore the prognostic potential of tumor-infiltrating lymphocytes in human colorectal cancer. While we did not find any correlation between intratumor lymphocyte infiltrations with clinicopathological parameters, we found that the presence of lymphocytes in tumor invasive margins, particularly $FoxP3^+$ lymphocytes, was associated with markers of good prognosis. However, we did not find alterations in the overall survival of these patients.

Taken together, our data unravel a novel oncogenic potential for dietary zinc supplementation mediated via microbiota as well as a critical antitumorigenic role of the IL-10-IgA pathway. Additionally, our data showed that the location and type of tumor-infiltrating lymphocytes are associated with the pathogenesis of colorectal cancer.

Keywords: Biomarker; Cancer; Cancer Immune Surveillance; Microbiota.

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LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
APCs	Antigen presenting cells
B6	C57BL/6J
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CFUs	Colony-forming units
CRC	Colorectal cancer
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
DMEM	Dulbecco's modified eagle medium
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead transcription factor box 3
HIF	Hypoxia inducible factor
HPV	Human Papillomavirus
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
IPA	Indole-3-propionic acid
JAX	Jackson Laboratories
KO	Knock-out
LLC	Lewis lung carcinoma
MDSCs	Myeloid derived suppressor cells
MHC	Major histocompatibility complex
mLN	Mesenteric lymph nodes
MMPs	Matrix metalloproteases
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells
NK	Natural Killer
OTUs	Operational taxonomic units
PBS	Phosphate-buffered saline
PD-1	Programmed death 1
PD-L1	Programmed death-ligand 1

PRRs	Pattern-recognition receptors
RagKO	B6.129S7-Rag1^{tm1Mom}/J
RT	Real time
SCFA	Short-chain fatty acids
SEM	Standard error of the mean
TAC	Taconic Farms
TAMs	Tumor associated macrophages
TANs	Tumor associated neutrophils
TcraKO	B6.129S2-Tcra^{tm1Mom}/J
TGF	Transforming growth factor
Th	T helper
TIGIT	T cell immunoglobulin and ITIM domain
TME	Tumor microenvironment
TNF	Tumor necrosis factor
Tregs	Regulatory T lymphocytes
VEGF	Vascular endothelial growth factor

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Chapter I

General Introduction

Part of this chapter has been published in:

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1. Cancer

The first description of aberrant growing masses in women's breast was reported in the Edwin Smith Papyrus, an ancient Egyptian papyri of trauma surgery, with a detailed description of this phenomenon and the procedure to remove it [1]. Later, Hippocrates dedicated part of his studies describing different types of this disease and conceived the terms *carcinos*, the Greek term for *cancer*, and *carcinoma.* Despite the theories that have emerged throughout centuries, it was only in 19th century, when the genetic component of cancer was recognized by the zoologist Theodor Boveri, that this disease started to be deeply explored [2]. Boveri suggested that chromosomal mutations could generate cells with unlimited replicative potential and that these mutations may be caused by radiation, traumas, or chemical insults, as well as by pathogenic microorganisms [2]. It is then crucial for the organism to have a tight regulation of cell growth and death to prevent unnecessary cell proliferation. However, whenever this balance is corrupted, aberrant cells start to proliferate and accumulate, leading to the formation of tumor masses. Broadly, tumors are composed of agglomerates of cells with highly proliferative capacity as well as other cells, including epithelial and immune cells, and are divided into benign or malignant (cancer) tumors; for convenience the terms tumor and cancer will be used as synonyms.

Malignant tumors are distinguished from benign counterparts due to their capacity to invade both nearby and, upon entering the blood or lymphatic stream, distant tissues [3]. These distant newly-formed tumor niches are called metastases and are responsible for the majority of deaths caused by cancer [4].

Given the tremendous impact of cancer in society, this section will be dedicated to exploring the incidence of cancer, examining the main features of malignancy, and characterizing all the constituents of tumor masses that, together, create the so-called tumor microenvironment (TME).

1.1. The incidence of cancer

Cancer comprises more than 100 different types that can appear in all tissues of the body, representing a significant burden. Despite all the efforts put on cancer research and the variety of different treatments available in the clinic, cancer is one of the leading causes of death worldwide and its incidence is increasing. In fact, in 2000, 10 million new cases and 6.2 million deaths were reported, while by 2020 these numbers had almost doubled [5,6]. The most common types of malignancy include breast, lung, colorectal (CRC), prostate, skin and stomach cancers [6]. In women, the most prevalent types are breast cancer (24.5%) followed by CRC (9.4%). In men, lung cancer represents 14.3% of diagnosed cases closely followed by prostate cancer (14.1%). In Portugal, the most prevalent types of cancer in men are prostate and CRC cancer while in women the most prevalent ones are breast and CRC. Regarding its mortality,

lung cancer and CRC represent 20% of cancer-related deaths, leading the mortality ranking [6]. Specifically, in men, lung and liver cancer are the leading cause of death and, in women, breast cancer and CRC lead the mortality ranking [6].

While population growth and aging are sufficient to explain the predicted increase in cancer incidence over the next decade [6], cancer burden is expected to exceed these projections due to the adoption of risk factors by the population, such as tobacco consumption and sedentary lifestyles. As such, it is vital to implement new preventive and screening methodologies to detect cancer at an early and treatable stage. In addition, the disclosure of more mechanisms associated with carcinogenesis will improve the identification of new therapeutic targets and the development of additional therapies.

1.2. Hallmarks of Cancer

While DNA mutations are a common characteristic of uncontrolled cellular proliferation, the transformation towards malignancy is dependent on a variety of phenomena and mechanisms, that, together, represent the Hallmarks of Cancer (Figure 1).

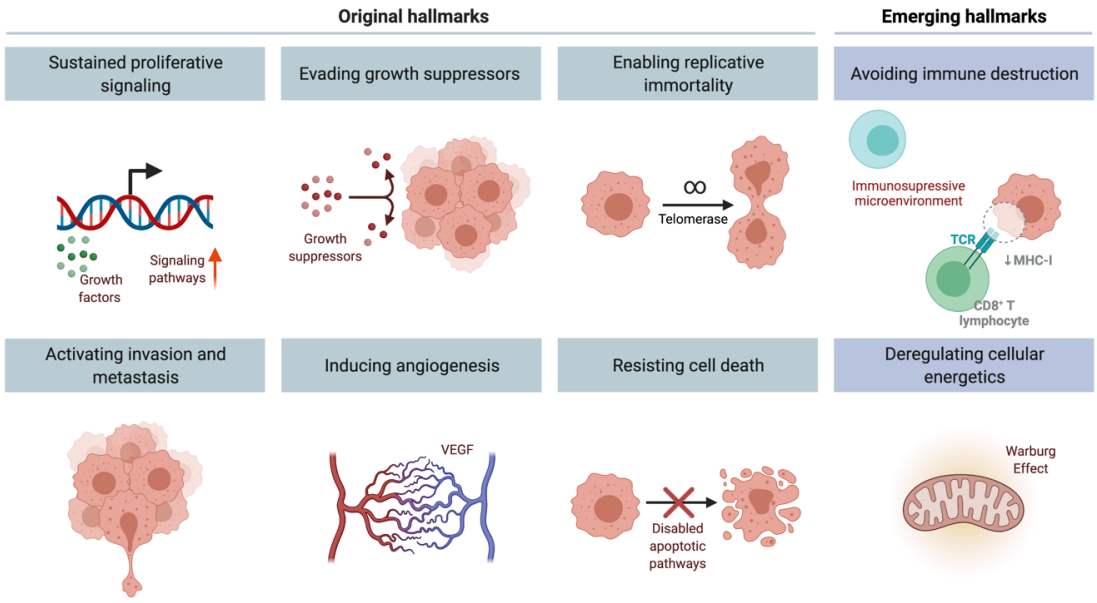

Figure 1. Hallmarks of Cancer. The combination of these eight capabilities, that are acquired as cancer initiates, are essential for its progression. Adapted from Hannah and Weinberg [8]. Created with BioRender.com

The first six hallmarks of cancer were defined in 2000 by Hanahan and Weinberg and included: sustained proliferative signaling; evasion of growth suppressors; activation of invasion and metastization; replicative immortality; induction of angiogenesis; and resistance to death (Figure 1) [7]. The revised version, published in 2011, included two new emerging hallmarks: the immune destruction avoidance;

and the dysregulation of cellular energetics [8]. Taken together, these characteristics ensure the survival and proliferation of malignant cells, even in adverse situations, as in the case of hypoxic conditions.

The sustained proliferative signaling of cells can be maintained through different mechanisms. On one hand, cancer cells can produce their own growth factors and, through autocrine regulation, activate downstream signaling cascades, such as Akt or MAPK pathways, master regulators of essential cellular processes like proliferation [9]. One of these cases is the production of epidermal growth factor receptor (EGFR) ligands, whose activation leads to the initiation of several signaling transduction cascades that culminate in cell proliferation [10]. Specifically, it has been reported that silencing the production of EGFR ligands amphiregulin or transforming growth factor (TGF)- α results in decreased tumor growth in a breast cancer model [11]. On the other hand, cancer cells can induce the production of growth factors by neighbor cells [12]. Indeed, the binding of platelet derived growth factors to stromal cells induces production of insulin-like growth factor 1, which in turn benefits the growth and survival of cancer cells [13]. In addition to this, cancer cells evolved to display constitutive activation of downstream signaling pathways to sustain the proliferative rate even in the absence of increased levels of growth factors. For instance, it has been shown that a large percentage of human melanomas has mutations that affect the structure of the B-Raf protein, which results in constitutive signaling of the MAPK pathway [14]. Cancer cells not only ensure the activation of proliferative pathways, but also display mechanisms to avoid growth suppressors, the negative regulators of cell proliferation. These signals include soluble growth inhibitors, such as TGF-β, that prevent the activation of other molecules, such as c-Myc, responsible for the cell cycle machinery [15,16]. In cancer, mutations that truncate the TGF-β receptor are commonly found in colon, gastric and esophageal patients [17]. Indeed, in a chemical-induced colon cancer model, the authors showed that the ablation of TGF-β receptor type II led to increased proliferation of tumors [18] thus indicating the suppressive activity of TGF-β signaling.

Perhaps more decisively, cancer also holds mechanisms to prevent its self-destruction and resist to cell death. Apoptosis is a form of programmed cell death mediated by a family of cysteine proteases called caspases. The activation of caspase-3 and -7, both responsible for the cleavage of cellular substrates, results in cell death [19]. Although cancer cells are under constant stress, a condition that should activate apoptosis, they are able to avoid this mechanism by disabling apoptotic pathways. Accordingly, it has been shown that the inactivation of caspases accelerates tumor formation in mice [20]. Cancer cells can also induce post-translational changes on pro- and anti-apoptotic proteins, which

impacts the induction of apoptotic pathways [21]. Together, these mechanisms favor tumor proliferation by preventing the self-destruction of malignant cells.

Another crucial characteristic of cancer cells is their increased replicative capacity, which makes them virtually immortal. It is defined that the replicative capacity of each cell is limited and is controlled by the length of telomeres, the DNA sequences that protect the end of chromosomal DNA [22] representing an apparent impediment for the sustained proliferation. Interestingly however, while normal cells have residual levels of telomerase, the enzyme responsible for the elongation of telomeric DNA, cancer cells have augmented levels of this enzyme [23]. This leads to the constant elongation of telomeres, consequently avoiding one of the triggers of cells' senescence thus providing cancer cells of an unlimited replicative capacity.

The combination of the above-mentioned characteristics helps cancer cells to survive and proliferate. As such, the activation of mechanisms to supply the energetic needs of these cells is imperative. One of these mechanisms relies on the constant sprouting of new blood vessels, a process known as angiogenesis. A key angiogenic activator is the vascular endothelial growth factor (VEGF) that contributes to tumor neovascularization. Among the VEGF family, VEGF-A, -B, -C and -E are responsible for angiogenesis, while both VEGF-C and -D are involved in lymphangiogenesis [24]. VEGF-A has the ability to stimulate all the pathways required for blood vessel proliferation and is commonly overexpressed in a variety of cancers [25–27]. The neovascularization not only ensures the distribution of nutrients to cancer cells but also facilitates the spreading of these cells to other tissues. Indeed, as previously discussed, the aptitude to invade other tissues is the key characteristic that distinguishes malignant from benign tumors. The process of invasion and metastasis requires the local invasion and then the intravasation of cancer cells into blood or lymphatic vessels [28]. Once in circulation, these cells escape into the parenchyma of distant tissues forming the metastases, upon a process known as colonization [28]. The impact of angiogenesis in cancer progression has been proven in the clinic with the success of bevacizumab, a monoclonal antibody targeting VEGF-A, in treating types of cancer [29] such as metastatic breast cancer [30], non-small-cell lung cancer [31], ovarian cancer [32], glioblastoma [33] and cervical cancer [34].

As previously discussed, the most recent version of the Hallmarks of Cancer [8] includes two emerging events: the reprogramming of energy metabolism and the evasion of immune destruction as primordial characteristics that cancer cells should also reunite to thrive in the organism [8].

Regarding metabolic alterations, it was first reported by Otto Warburg that cancer cells increase the glucose uptake with the production of lactate as final product, even in the presence of oxygen and

functional mitochondria [35]. It has been hypothesized that this occurs to meet the rapid energetic and biosynthesis demand that cancer cells require [36]. In addition, it has been also speculated that cancer cells take advantage of this pathway to modulate the TME. Specifically, it has been shown that lactate released by cancer cells participates in the differentiation of macrophages towards a pro-tumor phenotype, thus creating a tumor-permissive microenvironment [37]. Other evidence also suggest that cancer cells can consume lactate that, in turn, contributes to the generation of Krebs cycle intermediates [38], which is advantageous when the levels of glucose in the TME are low [38–41]. Although the primordial purpose of Warburg Effect is still not completely understood, it is likely that the combination of all the alterations that this metabolic preference triggers create improved conditions for cancer cells to thrive.

While the aforementioned mechanisms are primarily directed towards survival, proliferation, and subsistence of cancer cells, these cells also display mechanisms to avoid immune destruction. In the last decades, researchers have dedicated their work to disclose the immune evasion mechanisms of malignant cells, as well as the immune mechanisms involved in pro- or anti-tumor responses. Indeed, the immune system has become one of the key targets for cancer therapies that were revolutionized with the development of immunotherapies. The interaction between cancer and the immune system will be discussed in the following section (2. The immune system).

1.3. TME and its constituents

One of the major goals of cancer research is to determine all the players involved in tumor formation and progression to increase the therapeutic targets that can be explored. While at the beginning of cancer research the attention was mainly directed towards malignant cells, multiple studies have contradicted the idea that tumors were only composed of an agglomerate of homogeneous malignant cells. In fact, it has been shown that tumors are composed of different types of cells that, together, form the TME.

In addition to malignant cells, the TME is mainly composed of endothelial cells, pericytes, fibroblasts, and immune cells, and soluble factors, such as growth factors, chemokines, and cytokines, that mediate intercellular communications [42].

Endothelial cells constitute a continuous layer that lines the blood vessels having critical functions on regulating the passage of nutrients and oxygen to the periphery and in maintaining the integrity of the vessels. However, in cancer, these cells are usually found to have an irregular shape and size thus resulting in openings in the vessels, which facilitates the infiltration of malignant cells. In addition to its

aberrant architecture, endothelial cells also release angiocrine factors with the potential to facilitate metastization [43]. Along with endothelial cells, pericytes also collaborate in the stabilization of the blood vessel's structure and permeability and are recruited as a response to endothelial cells' signaling. However, during cancer, the recruitment of pericytes varies from non-recruitment to excessive recruitment. Indeed, it has been shown that increased pericyte coverage was associated to poor prognosis of CRC and renal cell carcinoma [44–46]. Contrarily, its ablation favors blood vessel invasion and, therefore, metastatic spread [47]. As such, while increased pericyte coverage stabilizes tumor vasculature and allows the access to nutrients, consequently favoring tumor progression, in their absence the vasculature is more permissive, which facilitates tumor cell invasion and metastases. Recently, Wong *et al* found that in the absence of β3-integrin, pericytes produce chemokine (C-C) motif ligand (CCL)2 that have a paracrine activity on tumor cells by stimulating the Mek1 signaling pathway and, therefore, promoting tumor cell survival and growth indicating that the activity of pericytes is not restricted to blood vessels [48].

Another markedly cellular component present in TME are fibroblasts. These cells are responsible for the production of different extracellular matrix compounds, including collagen and laminin, creating the structural foundation of TME. In cancer, these cells are associated with poor prognosis [49], therapy resistance [50], and disease relapse [51]. In fact, fibroblasts are known to enhance tumor growth by secreting growth factors, promoting angiogenesis, and supporting tumor invasion [52–54].

The recruitment of immune cells to the TME occurs in response not only to aberrant antigens but also to the stimulatory signals released by the other TME constituents. Importantly, along with the direct impact on cancer cells, the aforementioned cells (endothelial cells, pericytes and fibroblasts) also contribute to cancer progression by modulating the immune response. For instance, endothelial cells overexpress the endothelial-β receptor that suppresses T lymphocyte adhesion and extravasation to tumor sites, while the deletion of the *Rgs5* gene in pericytes enhanced the infiltration of cytotoxic T lymphocytes, resulting in improved mouse survival [55]. Additionally, endothelial cells enter a stage of endothelial anergy, characterized by a lack of response to pro-inflammatory molecules, preventing cell adhesion and migration through the endothelium [56]. Furthermore, Ersek *et al* [57] reported that melanoma-associated fibroblasts impaired the cytotoxic T lymphocytes' functions with reduced granzyme production and dysregulated nuclear factor kappa light chain enhancer of activated B lymphocytes (NF-kB) signaling, an important transcription factor for the expression of several pro-inflammatory cytokines [58].

While it has been shown that the immune system has a fundamental role in limiting tumor growth in different types of cancer, either the phenotype or the kinetics of the immune response may lead to tumor progression. The next section will explore the immune mechanisms involved in cancer.

2. The immune system

The immune system is classically divided in innate immune system and acquired immune system, that, together, comprise different cell lines such as myeloid and lymphoid lines, including macrophages, neutrophils, dendritic cells, T and B lymphocytes [59]. These cells act in collaboration to maintain homeostasis and protect the host from external aggressions, such as infections caused by bacteria, parasites or viruses, and internal aggressions, including tissue damage or the appearance of aberrant cells.

The immune response is initiated upon the recognition of danger signals, classically known as foreign immunogens. For instance, microbial components, known as pathogen-associated molecular patterns, trigger the activation of pattern recognition receptors (PRRs) expressed in different immune cells, including macrophages and dendritic cells [60,61 that lead to the activation of the immune response [62], such as the activation of microbicidal and proinflammatory responses, required to contain infectious agents [63]. Importantly, PRRs are designed to not only recognize foreign molecules but also endogenous signals, that are classified as danger-associated molecular patterns [64,65]. This proficiency allows the recognition of cancer cells and the initiation of an immune response against cancer [66]. Briefly, upon the recognition of a foreign immunogen, the antigen-presenting cells (APCs) migrate to secondary lymph nodes to present it to naïve T lymphocytes in the context of major histocompatibility complex (MHC) molecules, leading to the activation and initiation of acquired immune responses [59].

The acquired immune response is orchestrated by T and B lymphocytes. T helper (Th) lymphocytes, distinguished by the expression of cluster of differentiation (CD) 4, recognize antigens in the context of MHC-II molecules and produce cytokines that activate and control immune responses [67]. On the other hand, T cytotoxic lymphocytes, expressing CD8 molecules, recognize antigens in the context of MHC-I molecules and eliminate damaged or infected cells. Conversely, other immune components act as regulators of the immune response, mostly through immunosuppressive mechanisms [59]. This regulation is achieved through the action of different cytokines and cellular populations that are recruited to reduce the pro-inflammatory activity of inflammatory cells [59,68]. One key population are the regulatory T lymphocytes (Tregs), usually characterized by the expression of forkhead transcription factor

box 3 (FoxP3) [69,70]. Tregs are immunosuppressive in nature and are responsible for the expression of anti-inflammatory cytokines, such as interleukin (IL)-10 [71,72]. IL-10 regulates the function of innate immune cells by downregulating the expression of MHC-II and the production of pro-inflammatory cytokines, such as IL-6 and IL-12 [73]. Additionally, its effects are extended to acquired immune cells, where IL-10 acts directly on $CD4^+$ T lymphocytes inhibiting their proliferation and the production of other proinflammatory cytokines, including IL-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α [73,74]. IL-10 also decreases the antigen sensitivity of $CD8^+$ T lymphocytes by modulating cell surface glycoproteins, which reduces the downstream signal transduction of the T cell receptor thus impacting T cell activation [75]. More recently, work from Tekguc *et al* showed that the expression of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), an immune checkpoint inhibitor, by Tregs facilitates the formation of an immune synapse with APCs. In turn, this allow the depletion of CD80/CD86 costimulatory molecules on APCs, that are critical for the complete activation process of T lymphocytes [76]. As such, the immune response must be tightly regulated to allow the clearance of the aggression without compromising the integrity of the host [77,78].

As discussed above, during their lifespan, cells can suffer mutations that can ultimately lead to cancer. If this is indeed true, then almost everyone would develop cancer. However, this is clearly not the case suggesting that malignant cells are eliminated in most situations. In the early years of the XIX century, Paul Elrich hypothesized that abnormal cells were controlled by what he called “organism’s positive mechanisms” [79]. However, due to the lack of resources, he was unable to prove his theory. In 1970, the physician Sir Frank Burnet developed the theory of “tumor immune surveillance” that implied the participation of immunological components in the elimination of malignant cells [80].

Indeed, it has been clearly demonstrated that the immune system detects and eliminates malignant cells through antigen-specific responses initiated against antigens expressed by tumor cells [81–83]. Seminal studies from Kaplan and others showed that the absence of one or several immune system components increased cancer incidence, both in mice and in humans [84–86]. One crucial observation that unraveled the relevance of the immune system in the fight against cancer was in mice lacking perforin, a component of cytolytic granules of cytotoxic T lymphocytes [87]. In a set of different experimental approaches, perforin-dependent cytotoxicity was demonstrated to be a crucial mechanism of resistance to implanted tumor cell lines as well as viral and chemical-induced carcinogenesis [87]. Also in mice, the blockade of IFN-γ was associated with enhanced Meth A tumor cell growth [88]. The critical role of the immune system in limiting carcinogenic events is also highlighted by several studies associating

the presence of tumor-specific T lymphocytes in the blood, bone marrow or tumors with good prognosis [89–93].

Despite this, pre-clinical studies have also shown that pre-conditioning of mice with IFN-γ resulted in increased B16 melanoma colonization of the lungs [94]. This enhanced colonization was attributed to the effects of IFN-γ in B16 cells, which caused a decreased sensitivity of these cells to the activity of natural killer (NK) cells [94]. In line with these data is the *in vitro* observation that IFN-γ induces the expression of markers that are usually associated with more aggressive phenotypes of melanoma cells [95]. Furthermore, the presence of different types of immune cells infiltrating the tumor has been associated with tumor progression [96–99]. An example is the association between the accumulation of tumor-associated macrophages (TAMs) with lymphatic involvement, blood vessel invasion and reduced survival in a cohort of breast cancer [99]. Taken together, these studies suggest that the protective effect of the immune response may be, at least in some cases, dependent on the type of tumor and on the profile of tumor-infiltrating immune cells [81]. Therefore, it is understandable the duality of the immune responses that either can act towards cancer clearance or progression.

2.1. The immune response in tumor control

Tumor-infiltrating leukocytes are an essential component of the TME. Among these leukocytes, TAMs are one of the most represented cells [100]. TAMs differentiate locally from recruited peripheral blood monocytes in response to cytokines and growth factors produced by stromal and tumor cells in the TME [100]. In response to these factors, and because of their plasticity, macrophages can polarize towards an M1- or M2-like phenotype [101,102]. Usually, M1-like macrophages are differentiated by pro-inflammatory mediators including lipopolysaccharides and IFN-γ, while M2-like macrophages are differentiated by the immunomodulatory cytokines IL-4, IL-10 and IL-13, and TGF-β [102]. However, it is important to note that these two macrophage phenotypes are a simplistic view of the intricate mechanisms underlying macrophage differentiation and activation [103]. Indeed, macrophage polarization is not definitive, as it can be altered in response to the microenvironment [104], but these macrophage phenotypes are not mutually exclusive, and M1- and M2-like macrophages can coexist in the TME, which is important to prevent dysregulated responses [103].

In the initial steps of tumor formation, M1-like macrophages have been shown to play anti-tumor roles by producing pro-inflammatory cytokines such as IL-12, which promotes the differentiation of Th1 lymphocytes [100,105]. In turn, Th1 lymphocytes produce IFN-γ and other pro-inflammatory cytokines and chemokines that enhance antigen presentation and promote the expression of inducible nitric oxide

synthase (iNOS), an enzyme actively associated with cytostatic and growth inhibitory effects [106]. On the other hand, M2-like macrophages are characterized by their pro-tumor role [107], as discussed in Section 2.2.

In addition to macrophages, neutrophils are another critical cellular component of the TME that has received increasing attention over the past years. In this regard, recent data show that neutrophils can also have two distinct phenotypes in response to the cytokines of the TME [108]. As discussed above for TAMs, neutrophils can also have an anti-tumor (N1-like) or pro-tumor (N2-like) phenotypes [109]. N1-like neutrophils contribute to the anti-tumor immune response through the production of antimicrobial molecules with cytotoxicity against cancer cells [110–112], the inhibition of TGF-β [108], or by actively promoting the recruitment and stimulation of T cell proliferation [113] and IFN-γ secretion [114]. Indeed, recent studies using human samples of early-stage CRC cancer have associated the infiltration of neutrophils with a good prognosis and increased overall survival [115,116]. Furthermore, neutrophils have recently emerged as important targets of immunotherapy [117]. A recent study by Zhang *et al* found that the ablation of Tollip, an innate immune-cell modulator, from neutrophils enhances the anti-tumor immune response in a mouse model of CRC induced by azoxymethane–dextran sulfate sodium salt [118]. This study showed that Tollip-deficient mice exhibited a marked reduction in both microscopic and macroscopic polyps compared with wild type mice [118]. Furthermore, this phenotype was associated with an increased expression of CD80 and a downregulation of immunosuppressive molecules by Tollip-deficient neutrophils, which enhanced the activation and survival of T lymphocytes [118]. While these data suggest a crucial role for neutrophils in the anti-tumor immune response, most studies investigating the role of these cells in cancer have relied on animal models or circulating human neutrophils. As such, only limited information is available on the roles of tumor-associated neutrophils (TANs) in cancer patients. Therefore, further studies are required to better define the mechanisms and pathways whereby neutrophils modulate tumor immunity.

NK cells are another innate population of the TME playing a critical role in the elimination of abnormal cells [119,120] both in mice [121,122] and in humans [123–125]. The effector function of NK cells to eliminate tumor cells relies on the expression of molecules that can act either directly on tumor cells, such as perforins [126–128] and granzymes [127,128] or initiate the recruitment of other immune cells that contribute to tumor clearance [129–131].

Regarding adaptive immune cells, lymphocytes are also crucial in the TME. The T lymphocyte populations that infiltrate the TME include $CD4^+$ T lymphocytes, specifically the phenotypes Th1, Th17

and Tregs, and $CD8^+$ cytotoxic T lymphocytes. The anti-tumor mechanism of action of $CD8^+$ T lymphocytes is particularly important as it involves antigen-specific cytotoxicity, in addition to IFN-γ and TNF-α secretion [132]. This mechanism is supported by $CD4^+$ T lymphocytes, particularly Th1 lymphocytes, through the production of IL-2, IFN-γ, and TNF-α [132]. In some circumstances, Th2 lymphocytes have been shown to prevent tumor progression [133] mainly through the recruitment of eosinophils. In fact, the production of IL-18 by eosinophils has been shown to mediate the death of Colo-205 mouse tumor cells by the upregulation of adhesion molecules that facilitate the interaction between effector and target cells [134]. Additionally, eosinophils can also produce cytotoxins, namely granzyme-A, that promote tumor elimination [135]. In addition to conventional T lymphocytes, NK T cells also participate in tumor clearance [136]. Their effector mechanism depends on the secretion of granzymes and perforin that destroy tumor cells [137]. Moreover, NK T cells also secrete IFN-γ, which promotes the activation of $CD8^+$ T lymphocytes and the activation of M1-like macrophages [132]. The relevance of T lymphocyte populations in tumor control is highlighted by data showing that tumor-infiltrating lymphocytes are generally associated with a good prognosis [132,138]. Interestingly, tumor infiltration by $CD3^+$ and $CD8^+$ T lymphocytes in CRC patients is currently used as a prognostic tool (Immunoscore®), where patients with high tumor infiltration of these lymphocytes have been suggested to have better prognosis than patients with low infiltrations [139]. Further studies are imperative to determine the prognostic potential of immune infiltrates in other types of tumors to improve clinical approach.

Together, the above data show that the protection conferred by the immune system is critical to prevent cancer development and progression. Indeed, in recent years we have witnessed an increased development of cancer treatments targeting different components of the immune system, including dendritic cells [140] and checkpoint blockade therapies [141–143]. However, both the components and the by-products of the TME can modulate the immune response towards pro-tumor phenotypes, thus facilitating tumor progression (Figure 2).

2.2. The immune response in tumor progression

As discussed above, the immune system plays a crucial role in controlling tumor development and progression. However, tumors can develop even in the presence of a functional immune system suggesting that tumor cells and the microenvironment created by them can modulate the anti-tumor activity of the immune response. Indeed, as mentioned previously, the ability of tumor cells to avoid immune action has been considered in the last updated version of the *Hallmarks of Cancer* [8]. The cancer immunoediting theory also states that more than avoiding immune destruction, the tumor

microenvironment components can modulate the immune response towards pro-tumor phenotypes, thus facilitating the progression of cancer [81]. Briefly, cancer immunoediting is divided into three parts: the elimination phase, corresponding to the elimination of cancer cells by a plethora of immune mechanisms; the equilibrium phase wherein the immune response is sufficient to contain, but not eliminate cancer cells that survived the elimination phase; and the escape phase characterized by the growth of cancer cells with genetic and epigenetic alterations that allow them to escape the immune system. Importantly, during the escape phase, the recruitment of immunosuppressive cells and the consequent establishment of an immunosuppressive microenvironment also facilitate cancer progression [81].

The induction of immune suppression mechanisms is a complex process that requires the involvement of tumor and immune cells that act synergistically to dampen the anti-tumor immune response. As discussed in the previous section, the differentiation of TAMs in the TME can contribute to their pro-tumor characteristics [100,144]. In this regard, several meta-analyses have demonstrated that an increase in the number of TAMs is generally associated with poor prognosis [145,146]. During cancer progression, several mechanisms have been identified to overcome anti-tumor response by TAMs, including the secretion of prostaglandin E2 [147], the immunosuppressive cytokine IL-10 [148], the angiogenic factors such as IL-1ß, that through up-regulation of the hypoxia inducible factor (HIF)-1α protein increases VEGF secretion [149], VEGF [150], endothelin-2 [151] and epidermal growth factor family ligands [152]. TAMs may also play a role in tumor aggressiveness by releasing matrix metalloproteases (MMP-2 and MMP-9) that damage the extracellular matrix and the basal membrane, facilitating tumor invasion and metastases [152–154]. A mouse model of colon carcinoma also demonstrated that the expression of arginase and production of nitrogen monoxide by TAMs contribute to tumor progression by inducing apoptosis of $CD8^+$ cytotoxic T lymphocytes [155].

TANs may also adopt an immunosuppressive phenotype in the context of tumors by producing chemokines that promote the recruitment of Tregs [108,156]. In addition, N2-like neutrophils also produce cathepsin G and arginase [109], further promoting immunosuppressive status. As described above, most studies on the role of neutrophils in cancer have relied on mouse models. Therefore, it is unclear if the N1-/N2-like profile described in mouse models can be directly applied to humans. However, some of the genes associated with the phenotype and function of neutrophils in mice have also been reported in humans. For example, CCL2 and CCL17 initially found as part of the N2-like neutrophils signature in mice were also associated with increased tumor progression in humans [157,158]. Accordingly, patients with low CCL2 or CCL17 TAN counts had substantially better outcomes than patients

with higher numbers of these cells [156]. Furthermore, patients diagnosed in advance stages of cancer have been reported to present neutrophilia, which was associated with poor prognosis [159,160]. TANs produce several molecules that promote invasion and metastasis such as MMPs and VEGF [161,162]. Accordingly, it has been shown that neutrophils promoted lung metastases by breast cancer cells [163] and their depletion in murine models of melanoma and fibrosarcoma reverts tumor growth, angiogenesis and metastasis [163,164]. More studies are required to determine the impact of neutrophils, and neutrophil polarization, particularly in human cancers.

In addition to TAMs and TANs, myeloid-derived suppressor cells (MDSCs) are another population associated with the suppression of anti-tumor immune response [165]. In mice, MDSCs are characterized by the expression of Gr1 and are divided into cells expressing low levels of Ly6C, morphologically resembling polymorphonuclear granulocytes; and cells expressing high levels of Ly6C with monocytic phenotype. In humans, the identification of these cells is more difficult due to the lack of specific markers, but the overexpression of CD124 has been suggested as a potential marker for human MDSCs. In fact, it has been reported that, in melanoma patients, overexpression of CD124 was correlated with an immunosuppressive phenotype of myeloid cells [166,167]. Additionally, the suppressive capacity of these cells appears to be enhanced as they infiltrate tumors, pointing to the crucial role of the TME in modulating their function [168]. MDSCs are characterized by their ability to suppress the immune response through different mechanisms, including the inhibition of T cell functionality associated with the expression and activity of arginase 1 [169,170], or production of reactive oxygen and nitrogen species, promoting the differentiation of Tregs [171], and pro-tumor TAMs [172,173]. Recent work in humans also showed that MDSCs can promote angiogenesis via MMP-9 secretion [174].

In the context of an adaptive immune response, Tregs have been identified as central suppressors of anti-tumor immune responses [175,176]. The immunosuppressive function of Tregs is mediated through the secretion of IL-10 and TGF-β, which hamper the activity of effector T lymphocytes and their ability to eliminate tumor cells. Accordingly, increased accumulation of Tregs in the TME has been associated with poor prognosis in multiple types of cancer [138,177,178]. In addition to Tregs, Th2 lymphocytes have also been shown to favor tumor progression through different mechanisms, including the production of IL-10 [179–181] that inhibits anti-tumor immune responses, as well as the production of arginase-1 by M2-like TAM [182] differentiated in a context of a Th2 response, that inhibit cytotoxic T lymphocyte activity.

Another mechanism whereby the tumor microenvironment contributes to the suppression of anti-tumor immunity is through the induction of checkpoint proteins, such as CTLA-4, programmed-death 1 (PD-1) and programmed death-ligand 1 (PD-L1). These molecules work as a "brake" of the acquired immune response and, while checkpoint proteins play key roles in preventing autoimmunity and tissue damage during an immune response, their dysregulation during tumorigenesis can hamper the activation of immune cells, thus preventing their anti-tumor activity [183,184].

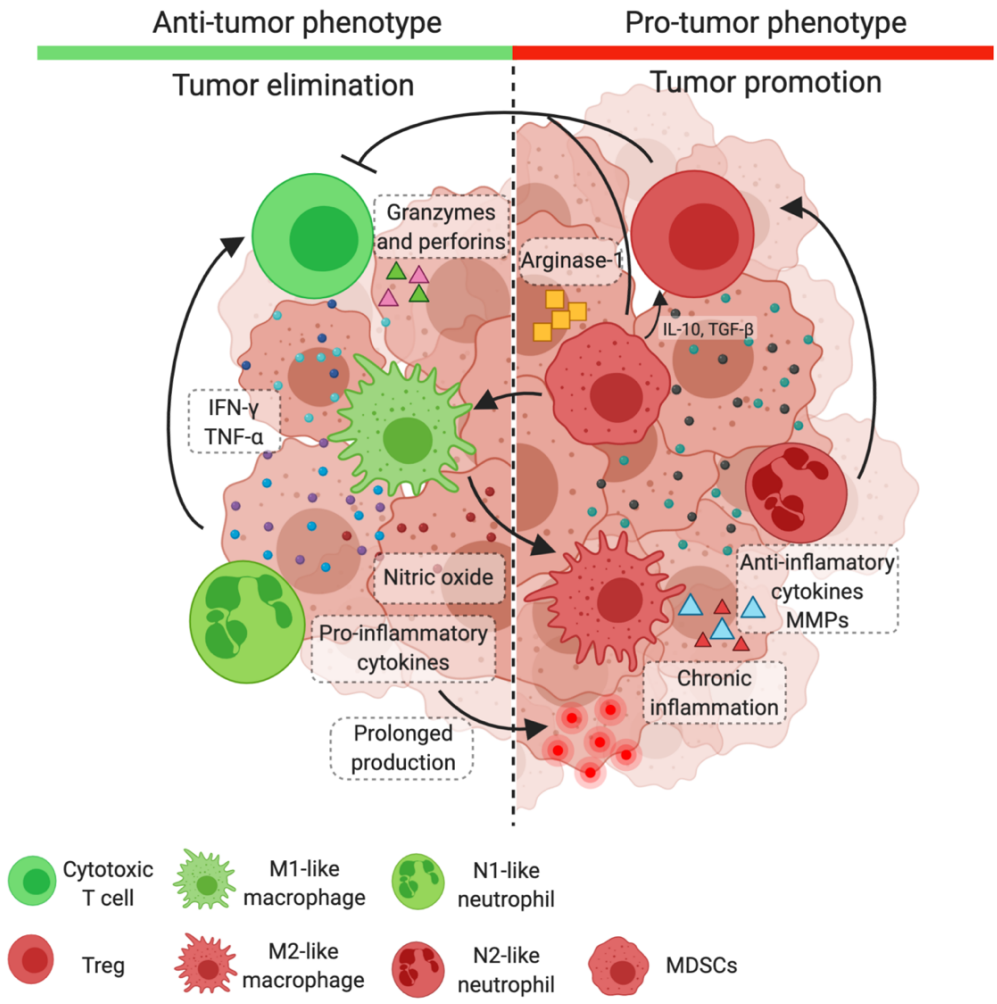

Figure 2. The anti-tumor and pro-tumor immune responses generated during cancer. Tumor infiltrating immune cells can participate in tumor elimination through different mechanisms including the production of nitric oxide and the release of granzymes and perforins that directly kill malignant cells. Moreover, through the production of pro-inflammatory cytokines, immune cells amplify the immune response in an attempt to control the tumor. However, the prolonged production of pro-inflammatory molecules can lead to chronic inflammation, which is associated to tumor promotion. On top of this, upon entering the TME, immune cells, including macrophages, are prone to be modulated towards a pro-tumor phenotype, characterized by the production of anti-inflammatory cytokines and MMPs, that facilitate tumor invasion. MDSCs and Tregs also participate in tumor promotion, as they dampen the response of anti-tumor cells, including cytotoxic T lymphocytes. Created with BioRender.com

While the data described above show multiple immune mechanisms associated with the inhibition of anti-tumor immune responses, it is important to note that this inhibition is not only accomplished through suppressive mechanisms. Indeed, an exaggerated immune response can promote dysregulated inflammation, which favors cancer development. As already mentioned, tumor-promoting inflammation has been included as an enabling hallmark. It was Virchow that suggested the first association between inflammation and cancer, over 150 years ago [185]. After this initial observation, several pieces of evidence confirmed the association between prolonged inflammation and increased risk of cancer [186,187]. As the inflammatory process promotes the further recruitment of inflammatory immune cells to damaged sites, the constant production of angiogenic factors, pro-inflammatory cytokines and reactive oxygen species creates the conditions to drive normal cells into tumorigenic cells [188,189]. In addition, the infection by pathogens instigates inflammation that promotes the development of cancer. Importantly, infections have been estimated to cause approximately 20% of all cancers [190,191].

3. The microbiota in cancer

Right after birth, microorganisms (including bacteria, fungi, and viruses) start colonizing all the human body surfaces that are exposed to external environments, such as skin, respiratory and gastrointestinal tracts. The complete collection of microorganisms inhabiting these tissues is called microbiota and the collection of their genomes is called microbiome. Despite having been neglected for decades, the development of new methodologies for nucleic acid sequencing helped unravel the diversity and vital functions of the microbiota, particularly bacterial communities [192].

The gastrointestinal tract harbors the most complex and well-studied microbiota of the human body, with more than 1000 different bacterial species already identified [193]. The upper part of the gastrointestinal tract, including stomach, duodenum, and jejunum, is enriched in aerobic gram-positive bacteria from the *Lactobacillus* and *Enterococcus* genera [193]. In the ileum, the concentration of bacteria increases, with the representation of coliforms [193]. Finally, the distal region of gastrointestinal tract (cecum and colon) harbors the most diversity with species of bacteria belonging to the genera *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* [193].

Although the microbiota composition remains relatively stable after three years of host's age [193], changes in lifestyle, such as diet [194,195] or consumption of antibiotics [196,197] can lead to microbiota imbalances, a condition known as dysbiosis, which can trigger several disorders [198]. Among all the diseases associated with microbiota, tumorigenesis is one of the most studied [199] and microbiota has been linked to local gastrointestinal cancers and other distal cancers [200].

The gastrointestinal microbiota has long been recognized to play crucial roles in the digestion of nutrients, the production of vitamins and other essential molecules, as well as in preventing the colonization and systemic infiltration by pathogenic microorganisms, deeply influencing host's homeostasis [201]. Additionally, more recent data show that the microbiota plays a critical role in the development and function of the immune system [202–204]. Therefore, in the following sections, the interaction of gut microbiota in the context of cancer as an anti- or pro-tumor influence will be discussed.

3.1. The microbiota in cancer control

The first attempt to fight cancer using bacteria was made in 1891 by the surgeon William Coley that injected *Streptococcus* into a cancer patient to stimulate the immune system [205]. While Coley, and others, directed their research towards immunotherapy, this first observation indicated the potential of bacteria in cancer clearance.

Nowadays, it is known that the microbiota plays crucial roles in controlling tumor development and progression through the production of metabolites that fuel the immune cells or promote the development of anti-tumor environments (Figure 2). Specifically, fiber-rich foods have been suggested to have health benefits through the maintenance of a healthy microbiota capable of production of short-chain fatty acids (SCFA) [206–208]. Accordingly, recent data show that butyrate and propionate, two SCFA produced by different microbiota species, display anti-tumor capacity against CRC [209] or hepatic cancer [210]. The protective capacities of these metabolites have been shown to be mediated directly in cancer cells or indirectly by modulating the immune response and the tumor microenvironment. Specifically, in cancer cells, several *in vitro* studies show that these metabolites inhibit the activity of histone deacetylases, which are critical to the gene transcription control [211–213]. In line with these data, Zagato *et al* elegantly showed that the endogenous murine microbiota member *Faecalibaculum rodentium* and its human homologue, *Holdemanella biformis*, were involved in CRC prevention [214]. This study showed that the production of SCFA, specifically butyrate, inhibits calcineurin-mediated NAFTc3 transcription factor activation through its inhibitory effect at the histone deacetylase level [214]. These data corroborated the results from Peuker *et al* that identified the contribution of calcineurin activity to tumor growth and proliferation [215]. Regarding the indirect effects of SCFA, a recent study showed that butyrate plays a key role in regulating colonic inflammation and, therefore CRC development [216]. In this study, the protective role of butyrate was mediated by promoting anti-inflammatory properties in colonic

macrophages and dendritic cells, which stimulated the differentiation of Tregs and IL-10-producing T lymphocytes [216]. Accordingly, mice deficient in the receptor for butyrate in the colon (GPR109A encoded by Niacr1) were more susceptible to the development of colon cancer [216]. Importantly, Niacin, a pharmacological Gpr109a agonist, suppressed colitis and colon cancer [216]. Furthermore, in an *in vivo* model of lymphoma, Wei *et al* reported that butyrate promoted tumor cells' apoptosis and histones acetylation, thus preventing tumor growth [217].

In addition to SCFA, microbiota also produce other molecules with anti-tumor potential. For example, the production of pyridoxine, a form of vitamin B, by a broad group of bacteria stimulates the anti-tumor immune response [218,219]. Additionally, Konishi *et al* showed that the production of ferrichrome by *Lactobacillus casei* induces the apoptosis of colon-derived tumor cells through the activation of the JNK-mediated apoptosis [220]. Lactic acid-producing bacteria have also been shown to play key roles in controlling tumor cell growth by stimulating anti-tumor immune responses [221–223]. Specifically, the supplementation of *L. casei* in a chemical-induced model of intestine injury was associated with increased production of lactate, which decreased the production of myeloperoxidases and TNF-α [224]. Another study corroborated these data and showed that *L. casei* supplementation ameliorate chronic inflammatory bowel disease by downregulating the production of pro-inflammatory cytokines, such as IL-6 and IFN-γ by intestinal lamina propria mononuclear cells [225].

Another important mechanism whereby microbiota promote regulation of inflammation, and therefore tumor control, is through the production of metabolites that are agonists of the aryl hydrocarbon receptor (AhR). This receptor is expressed at barrier sites, such as the gastrointestinal tract, acting as a sensor of environmental chemicals, including dietary compounds. Recent data show that mice treated with AhR antagonists develop severe symptoms upon induction of colitis and that patients with ulcerative colitis had reduced expression of AhR [226]. Furthermore, Alexeev *et al* showed that the levels of indole-3-propionic acid (IPA), a metabolite of the microbial tryptophan catabolism recently identified as an AhR agonist, were reduced in both ulcerative colitis patients and mice [227]. Importantly, the supplementation of mice with IPA enhanced the production of IL-10 and inhibited the production of pro-inflammatory cytokines [227]. Nonetheless, the expression of AhR can be an indicator of poor prognosis in certain types of cancer [228–231]. Indeed, Shimba *et al* reported that A549 tumor cells expressing high levels of AhR had accelerated cell growth due to shortening of late M to S phases of the cell cycle [228].

The microbiota composition also plays an important role in the efficacy of cancer therapies, such as immunotherapies. Two recent pre-clinical studies have reported the association between microbiota composition and the efficiency of CTLA-4 and PD-1 blockade. Specifically, the presence of *Bacteroidales*

and *Burkholderiales* were essential to promote the generation of anti-tumor antigen-specific Th1 responses and restored the efficiency of CTLA-4 blockade [232]. Furthermore, the efficacy of PD-1 blockade, was associated with the presence of the bacteria from the genera *Akkermansia*, *Enterococcus*, *Bifidobacteirum* and *Faecalibacteirum* [233–236]. As such, the manipulation of the microbiota in cancer patients is likely to act as an adjunct approach to therapeutic schemes. Indeed, it has been reported the supplementation of mice with *B. fragilis* and *B. cepacia* ameliorated immunotherapy-associated toxic effects [232], which thus suggest the usefulness of microbiota not only on the modulation of immunotherapy efficacy but also in its associated toxicity.

Together, these data show that there is an intimate crosstalk between microbiota and the immune system triggering important anti-tumor pathways and controlling cancer progression. However, the disruption of this symbiotic relationship can lead to a drastic remodeling of the intestinal environment, including immune responses.

3.2. The microbiota in cancer progression

As discussed above, uncontrolled chronic inflammatory contexts are characterized by increased pro-inflammatory cytokines and other molecules that promote tumor development and progression (Figure 2). The microbiota plays a crucial role in these contexts, as the outgrowth of specific commensal bacteria has been associated with chronic inflammation and cancer development [237,238]. Indeed, infectious agents are currently estimated to be responsible for ~20% of human cancers [190,191]. While a large percentage of these occurrences are due to viral infections, such as the Human Papillomavirus (HPV) that causes cervical cancer, the contribution of bacteria is usually relegated to a second plane [239,240]. However, in recent years an increasing number of studies has identified an important role of bacterial infections in carcinogenesis [241,242]. For example, bacterial vaginosis has been associated with the prevalence of HPV infection [243–246]. Although the mechanisms underlying this association are not fully understood, the disruption of the vaginal milieu by bacterial vaginosis likely prompts a decrease in protective species of the genus *Lactobacillus* and an increase in both strict and facultative anaerobic bacteria, namely *Gardnerella vaginalis* [244–246]. These bacteria in the vaginal mucosa are likely to promote the activation of downstream inflammatory pathways that influence mucosal susceptibility for HPV [247–249].

One of the first associations between cancer and bacteria was reported in the 1980s [250]. These initial studies identified *Helicobacter pylori* as a causative agent of infectious gastritis, a chronic stomach inflammation [251]. This chronic inflammatory state promotes a niche that prompts tumor development. Accordingly, the World Health Organization now classified this infectious agent as a carcinogen class I [252]. Disseminated through the planet, it has been estimated that more than 50% of the world's population harbors these bacteria [253]. Owing to its ability to neutralize gastric acidity through the expression of urease, *H. pylori* can survive and proliferate in the stomach and infect the gastric mucosa [253]. Moreover, the expression of specific cytotoxins, mostly VacA and CagA, promote the production of the two classical pro-inflammatory cytokines IL-8 and IL-6 by gastric epithelial cells [254,255] and activate the NF-kB pathway [256,257], thus enhancing and perpetuating the inflammatory response.

Another common member of the human microbiota that has been shown to enhance tumorigenesis by producing an enterotoxin is *Bacteroides fragilis* [258,259]. Haghi *et al* reported a higher prevalence of *B. fragilis* in CRC patients when compared to the control group [260]. Interestingly, the authors also showed an increased expression of the *B. fragilis* enterotoxin gene in advanced stages of the disease [260]. In line with these data, Purcell *et al* associated the presence of enterotoxigenic *B. fragilis* to the abundance of early-stage carcinogenic lesions in colorectal tissue [261]. Furthermore, *B. fragilis* enterotoxin has been shown to induce persistent colitis in mice, promoting chronic inflammatory status and cancer development [262]. This inflammation is mediated by increased IL-8 production and β-catenin signaling [258,263]. Recently, *B. fragilis* toxin was also shown to modulate the Wnt pathway and promote cell proliferation [264].

Also associated with increased CRC incidence is *Fusobacterium nucleatum* [265,266]. The pathogenesis of these bacteria has been associated with the expression of FadA and Fap2 virulence factors [267,268]. These virulence factors induce the expression of oncogenes and promote the growth of cancer cells [267]. A recent study identified a tumor-based immune evasion mechanism that is dependent on the interaction of Fap2 protein with the inhibitory receptor T cell immunoglobulin and ITIM domain (TIGIT), expressed by all human NK cells and some T lymphocyte populations, inhibiting their function [269]. Additionally, the expression of pro-inflammatory cytokines, such as IL-8, TNF and IL-6, associated with high levels of *F. nucleatum*, may also contribute to establishing a microenvironment amenable for cancer progression [265], including the recruitment of MDSCs and the differentiation of M2-like macrophages and TANs, which sustain the immunosuppressive environment [270,271].

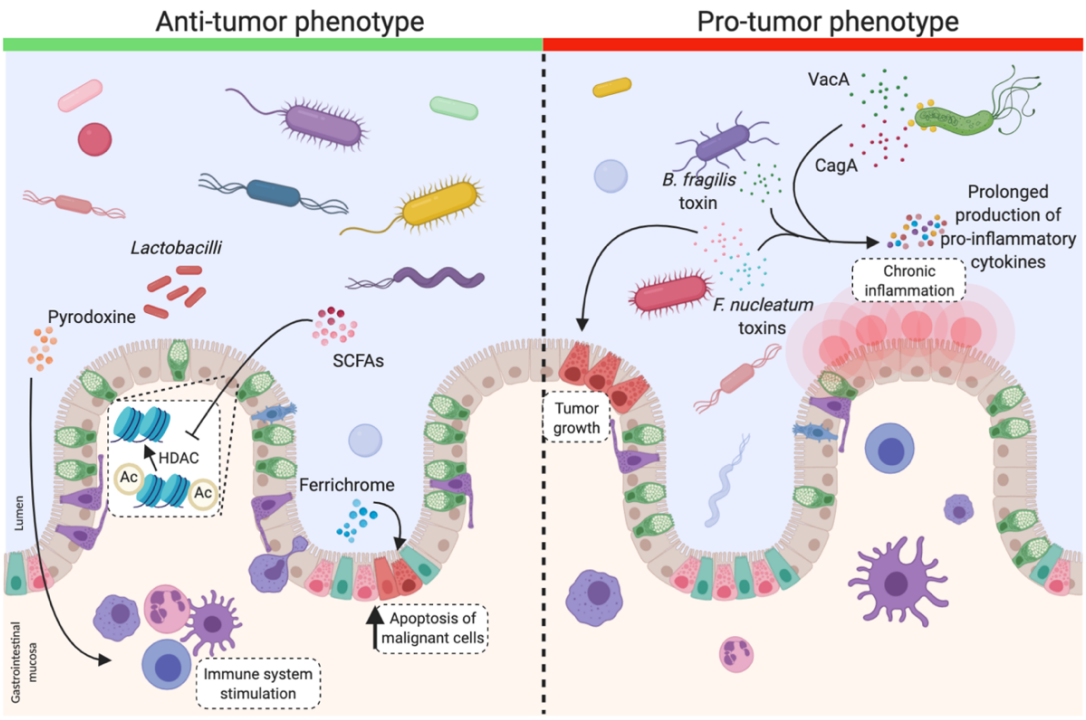

Figure 3. The anti-tumor and pro-tumor impact of microbiota in cancer. Microbiota has emerged as a key modulator of carcinogenesis. On one hand, their ability to produce several molecules, including SCFA, pyridoxine and ferrichrome, granted them mechanisms to prevent cancer progression. On the other hand, the presence of certain species, such as *H. pylori* or the production of toxins by other commensal microorganisms are associated to the promotion of constant inflammatory stimuli that facilitate cancer initiation and progression. The manipulation of microbiota is being explored as a potential pathway to tackle cancer. Created with BioRender.com

Together, these data highlight the importance of microorganisms in governing both homeostatic or pathogenic conditions. While in the last decades, we have witnessed increased attention to the impact that the host microbiota has at different dimensions, further studies are important to consolidate the knowledge on this topic and to take advantage of the usage and manipulation of microbial species for the benefit of the host.

4. Aims

Recent seminal studies in animal models and in humans have demonstrated that the infiltration of immune cells in tumors is associated with improved survival of cancer patients. However, these cells are subjected to modulation by the TME, and this modulation can promote tumor progression. Recently, the gut microbiota has emerged as an important factor in modulating the immune response to cancer. In this regard, while the composition of the gut microbiota is relatively stable throughout adulthood, several lifestyle behaviors can cause alterations in its composition and function. Among them, diet is one of the most impacting factors that can change the profile of gut microbiota leading to the modulation of cancer immune responses. As tumor infiltrating immune cells represent one of the first lines of defense against developing cancers, several studies aim to determine their prognostic potential to help clinicians stratify and treat patients. To explore further the prognostic potential of different immune subsets it is critical to consolidate their role in the TME, as their function can be modulated by the microbiota.

Taking this into consideration, this Doctoral Thesis had the following aims:

Aim 1: To investigate the impact of dietary zinc supplementation in tumor progression using a pre-clinical model. Zinc is an essential micronutrient for the homeostasis of the organism. Interestingly however, in cancer, its function is not consensual; it has been shown that it has a role in tumor control but also in tumor progression.

Aim 2: To elucidate the impact of high levels of IL-10 in tumor progression. IL-10 is an anti-inflammatory cytokine crucial to control exacerbated inflammatory responses, particularly microbiota-associated inflammation. In cancer, several studies reported the involvement of IL-10 in tumor promotion and elimination.

Aim 3: To determine the prognostic significance of the infiltration of $CD3^+$, $CD8^+$ and $FoxP3^+$ T lymphocytes in a cohort of CRC patients. In the literature, high tumor infiltrations of $CD3^+$ and $CD8^+$ T lymphocytes are associated to better prognosis of CRC patients. However, the contribution of $FoxP3^+$ T lymphocytes is yet to be disclosed.

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Chapter II

Dietary zinc supplementation modulates cancer immune surveillance through microbiota alterations

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Dietary zinc supplementation modulates cancer immune surveillance through microbiota alterations

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Abstract

An expanding body of evidence indicates that changes in the intestinal microbiota allow environmental risk factors to initiate and promote cancer. Considering the key role of diet as well as dietary supplements in the microbiota, in this work we evaluated the impact of micronutrient zinc in tumor growth. Using a pre-clinical model, we found that dietary zinc supplementation promotes the syngeneic LLC and MC38 tumor overgrowth. As microbiota depletion prevented the tumor-overgrowth effects of dietary zinc overdose, we analyzed overrepresented microbial communities in mice under dietary zinc supplementation. We found a significant increase in the genus *Bacteroides*, and the species *B. acidifaciens* and *B. dorei*. While we did not find alterations in the permeability of the intestinal barrier, we found increased regulatory T cells in the mesenteric lymph nodes and an increased accumulation of myeloid derived suppressor cells (MDSCs) in the tumors of mice under dietary zinc supplementation. Importantly, MDSCs depletion prevented tumor overgrowth.

These data show that short-term dietary zinc supplementation promotes a remodeling of the microbiota communities, which modulate the tumor immune response and promote extraintestinal tumor overgrowth.

1. Introduction

Despite the significant therapeutic advances of the past decade, cancer remains a leading cause of death worldwide. In 2020 alone, over 19 million new cases were diagnosed and almost 10 million people died of cancer [1]. The rapidly growing incidence and mortality of cancer worldwide reflects the growth and aging of the population, as well as changes in the prevalence and distribution of the main risk factors, including obesity, diet, and physical inactivity [2,3]. Recently, microbes inhabiting the human body have been recognized to influence many aspects of our health including the development of cancer [4–6]. Indeed, the most recent available data estimate that infectious agents, including viruses and bacteria, are the cause of at least 2 million new cases of cancer [7]. However, infections are not the only mechanism by which microorganisms participate in carcinogenesis [8]; indeed, recent data unraveled a crucial role for the microbiome in enhancing or inhibiting cancer initiation and progression [5,9]. As the microbiome is influenced by lifestyle factors which are important risk factors for cancer, most notably diet, it is critical to disclose the causal links between microbiota and cancer, as well as the underlying mechanisms, to provide new insight to the therapeutic field.

The gut microbiota plays pivotal roles in different processes that maintain homeostasis, including the development of the immune system and the metabolism of dietary components [10–12]. More recently, imbalances in the composition of the microbiota, a condition known as dysbiosis, have been recognized to enable the appearance and/or the outgrowth of bacterial species that have the potential to promote or enhance cancer development [13]. In this regard, the increased presence of the opportunistic pathogen *Fusobacterium nucleatum* in human colorectal cancer (CRC) tissue has been associated to poor prognosis [13]. Likewise, the enterotoxigenic commensal colonic *Bacteroides fragilis* has been reported to be most prevalent in cancerous colonic tissue when compared to healthy tissue [14]. While the mechanisms linking microbiota with carcinogenesis are not yet fully disclosed, a growing body of evidence points to their interaction with the immune system as a pathway that drives carcinogenic events. Indeed, *F. nucleatum* restrain the anti-tumor activity of the immune system through the expression of Fap2 proteins which interact with the inhibitory receptor TIGIT, expressed by T lymphocytes and Natural Killer cells [15,16]. Accordingly, mice fed with *F. nucleatum* displayed accelerated tumor development and increased accumulation of intratumor MDSCs [17], an heterogeneous population of myeloid cells associated with tumor-permissive microenvironments [17–19]. In line with these data, the presence of *Bacteroides* has been associated with poor anti-tumor responses characterized by increased systemic MDSCs and regulatory T lymphocytes (Tregs) [20], another population capable of suppressing anti-tumor

immunity [21]. In contrast with the tumor-promoting capacities of the bacteria described above, *Bifidobacterium* species can enhance the activity of dendritic cells and the priming of cluster of differentiation (CD)8$^+$ T lymphocytes with anti-tumor function [22]. Likewise, the presence *Faecalibacterium* augments anti-tumor responses associated with enhanced infiltrations of intratumor CD8$^+$ T lymphocytes [20]. Taken together, these evidences show that the microbiota composition may drive to the development of immune responses that either promote or hamper tumor progression. Therefore, exploring the factors that modulate the microbiota and its activity will be critical for the development of new therapeutic strategies, as we move forward to a personalized medicine era.

Zinc imbalance is the most common single nutrient disorder worldwide [23,24]. This essential micronutrient is a required co-factor for many enzymes, transcription factors, and replication factors, critical in many enzymatic and metabolic functions in the body [25]. Indeed, in noncritically ill patients, zinc supplementation improves markers of immune function [26]; moreover, plasma zinc concentrations are low in critically ill children, which correlate with measures of inflammation associated with the degree of organ failure [27]. Zinc-based treatments are also employed to manage infant diarrhea, suggesting an impact of this micronutrient in the gut microbiota [28]. Accordingly, recent data showed that dietary zinc is an essential mediator of microbial community structure and that both zinc deficiency and overdose can generate a dysbiosis in the gut microbiota [29]. As such, it is not surprising that dietary zinc supplementation has been suggested to impede cancer progression [30,31]. However, there is also literature pointing to a pro-tumor effect of dietary zinc [32,33]. While zinc insufficiency is an essential health problem in developing countries, the amount of zinc consumed in developed countries can exceed the nutrition requirements, particularly among supplement users [34,35]. As few studies have been conducted to understand the impact of zinc overdose in cancer progression, in this work we explored the short-term impact of dietary zinc supplementation in a pre-clinical model of cancer.

We found that dietary zinc supplementation promotes Lewis lung carcinoma (LLC) and MC38 tumor overgrowth through a mechanism dependent on the microbiota. Indeed, depletion of the microbiota through broad-spectrum antibiotics prevented tumor overgrowth. The microbial community analysis revealed a reduced bacterial diversity, but an overrepresentation of the genus *Bacteroides,* and the species *B. acidifaciens* and *B. dorei.* Importantly, these microbiota alterations did not impact the permeability intestinal barrier, suggesting that extra-intestinal tumor overgrowth was caused by modulation of the anti-tumor immune response. In support of this hypothesis, tumor-bearing T

lymphocyte-deficient mice under dietary zinc supplementation display similar tumors to those of control mice, suggesting a key role for T lymphocytes in the axis microbiota-tumor overgrowth. In line with these data, we found increased Tregs in the mesenteric lymph nodes (mLN), as well as an increased accumulation of MDSCs in the tumors of mice under dietary zinc supplementation. Crucially, MDSCs depletion reverted tumor overgrowth. These data show that short-term dietary zinc supplementation promotes a remodeling of the microbiota communities, which modulate the anti-tumor immune response and promote extraintestinal tumor overgrowth.

2. Methods

2.1. Mice

C57BL/6J (B6), B6.129S7-Rag1tm1Mom/J (RagKO) and B6.129S2-Tcratm1Mom/J (TcraKO) were bred at the ICVS animal facility from stock purchased from Charles River Laboratory (Barcelona, Spain) and The Jackson Laboratory, respectively. Mice were given drinking water supplemented with different concentrations of zinc sulphate, as indicated in the figures' legends, and 20g/L of sucrose. Control mice received drinking water containing 20g/L of sucrose. Both male and female mice between the ages of 8 to 12 weeks old were used for experimental procedures.

All procedures involving live animals were performed in accordance with the European Directive 86/609/EEC and approved by the Subcomissão de Ética para as Ciências da Vida e da Saúde (SECVS 074/2016) and the Portuguese National Authority Direcção Geral de Veterinária (DGAV 014072).

2.2. Cell line and tumor models

The murine LLC and MC38 cell lines were kindly provided by Dr. Massimiliano Mazzone (Vesalius Research Center, Belgium). Cells were cultured in T75 cm^2 tissue culture flask at 37°C in a humidified atmosphere containing 5% CO_2 in complete Dulbecco's Modified Eagle Medium (cDMEM, DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% HEPES, 1% sodium pyruvate and 1% penicillin/streptomycin (all from Gibco).

Cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS), and $1x10^6$ of LLC cells or $5x10^5$ of MC38 cells were injected subcutaneously into the right flank of recipient mice in a volume 200µL of endotoxin-free PBS. Tumor-bearing mice were monitored daily for signs of distress and the tumor volumes were monitored from day 5 onwards by measuring two bisecting diameters of each tumor with calipers and their volume was calculated using the formula: $(\pi x d^2 x D)/6x1000$; d=small diameter and D= large diameter), as previously described [36]. At day 18 post tumor implantation, mice were sacrificed by CO_2 asphyxiation and the tumor and other tissues were harvested for analysis.

2.3. Zinc quantification

The zinc levels in different tissues were determined using the zinc assay kit (Abcam), following the manufacturer's instructions. Briefly, tumors, intestine, and spleen tissues were aseptically harvested and mechanically disrupted with glass beads using a Bead Beater (Biospec). Samples were then centrifuged at 2000 rpm for 5 min and the supernatants were collected and assayed. One hundred µl of the supernatant was mixed with 100 µL 7% TCA solution provided in the kit to precipitate proteins.

Centrifugation at 16000xg for 5 min was performed to remove protein precipitates. Supernatant (50 µl) was then mixed with 200 µL salicyladoximde mixture in 96-well plates to detect zinc levels using Tecan Infinite 200 equipment (Tecan) with the wavelength at 560 nm. To minimize pipetting error, each sample was duplicated and averaged for zinc quantification. Serum samples were assayed directly.

2.4. Antibiotic treatment

Mice were given ampicillin (1 mg/mL), streptomycin (5 mg/mL) and colistin (1 mg/mL) (all from Sigma-Aldrich) in the drinking water *ad libitum* for 2 weeks. At the end of the protocol, feces from antibiotic-treated mice and non-treated mice were collected. Antibiotic activity was confirmed by cultivating the fecal pellets resuspended in sterile PBS on Columbia agar plates with 5% sheep blood (Biomerieux) for 48h at 37°C in aerobic or anaerobic conditions. The number of colony-forming units (CFUs) was counted and the total number of bacteria per gram of feces calculated.

2.5. 16S rRNA gene sequencing and bacterial quantification

Genomic DNA from cecum of mice under dietary zinc supplementation or standard diet was extracted using the Nucleo-Spin DNA Stool Kit (Machery-Nagel) according to manufacturer's instructions. After quantification of genomic DNA by spectrophotometry at 260 nm, 16S rRNA gene was amplified and sequenced using the MiSeq platform from Illumina and analyzed with Mothur software (outsourcing service provided by LGC Genomics, Germany). 16SrRNA gene sequences were aligned after filtering by quality and length, using SILVA taxonomic framework and database. Operational taxonomical units (OTUs) were identified by the average-neighbor algorithm. Sequences with distance-based similarity of 97% or higher were grouped into the same OTU. Statistical analysis was performed using Student's t-test to evaluate α-diversity and relative abundance of phyla. Log_2 fold change (FC) and false discovery rate (FDR) were obtained through edgeR algorithm. Values were considered significant for $p \leq 0.05$ and $FDR \leq 0.2$.

To validate the relative bacteria abundance from 16SrRNA data, absolute abundance of bacteria was performed by quantification of bacterial copy number in stool DNA samples using specific primers (table 1) designed for *B. acidifaciens* and *B. dorei*. Values were extrapolated from a standard curve obtained by different copy numbers of a plasmid containing a specific insert belonging to each bacterium. For the plasmid generation, a specific DNA fragment for *B. acidifaciens* or *B. dorei* was inserted in a pJET1.2 plasmid using a CloneJET PCR Cloning Kit (Thermo Scientific). After transfection in competent *E. coli*, plasmid DNA was recovered and used as template for the standard curve.

2.6. Histology

For haematoxilin and eosin staining, intestinal tissue samples were washed to remove fecal contents with PBS and fixed overnight in 4% of paraformaldehyde, dehydrated and embedded in paraffin. Samples were then sectioned and stained. Representative bright-field images were acquired on a BX61 microscope with an Olympus DP70 camera. For Ki-67 and Caspase-3 detection, intestinal samples were frozen in O.C.T. compound and sectioned in 8μm thick sections. Cryosections were allowed to dry for 1h and fixed with 4% of PFA for 15 min. Sections were blocked with blocking solution (PBS, 5% of bovine serum albumin) and probed overnight with primary antibody against Ki-67 (clone SP6, Thermo Scientific) or Caspase-6 (clone ab2301, Abcam), diluted in PBS. Sections were then washed and incubated with Alexa Fluor 568 (Thermo Fischer Scientific) diluted in PBS for 2h. All incubations were at room temperature. DAPI was used to counterstain and detect nuclei.

Representative images were captured under fluorescence using Olympus BX6 microscope with Olympus DP70 camera using the Olympus cell^P software.

2.7. FITC-dextran intestinal permeability assay

The intestinal permeability of experimental mice was assessed using fluorescein isothiocyanate (FITC)-labelled dextran (Sigma-Aldrich; 4 kDa) administration. Briefly, food and water were withdrawn for 8 hours before mice were administered 44 mg/100 g body weight of FITC-labelled by oral gavage. Four hours later, the serum was collected, diluted 1:2 in PBS, and the fluorescence was measured by spectrophotofluorimetry (excitation: 485 nm; emission: 528 nm). FITC-dextran concentrations were calculated with the help of standard concentrations prepared by using diluted FITC-dextran in serum of untreated mice.

2.8. Real-time (RT) PCR analysis

Total RNA from tumors and intestines was extracted using TRIzol (Invitrogen) following the manufacturer's instructions. cDNA was generated from 1 ug of total RNA using the GRS cDNA Synthesis Master Mix (Grisp) following the manufacturer's instructions. The resultant cDNA template was used to quantify the expression of target genes by RT-PCR (Bio-Rad CFX96 Real-Time System with C1000 Thermal Cycler) using the following protocol: one cycle of 95°C for 3 min, followed by 40 cycles of a two-stage temperature profile of 95°C for 3 seconds and 60°C for 30 seconds. Gene expression was

normalized to Ubiquitin mRNA levels using the ΔCt method. Target gene mRNA expression was quantified using SYBR green (Thermo Scientific) and specific oligonucleotides (Table 1; Invitrogen).

Table 1 – Sequence of primers used to determine gene expression by RT-PCR

Target	Forward sequence	Reverse sequence
Ubq	TGGCTATTAATTATTCGGTCTG	GCAAGRGGCTAGAGTGCAGAGTA
Arg1	ACATTGGCTTGCGAGACGTA	ATCACCTTGCCAATCCCCAG
Vegfa	AGGGTCAAAAACGAAAGCGC	CGCGAGTCTGTGTTTTTGCA
Nos2	CTATGGCCGCTTTGATGTGC	TTGGGATGCTCCATGGTCAC
Muc1	AACGATGAAGCCCTGGAGTG	TGAGAGGTCTGGTTCCCGA
Muc2	TGGAGCAACATGTGGAACTC	GTCAGCAGCCGGTTACCA
Muc3	GCTGGCTTTCATCCTCCACT	CTGTTTTCCCCGCTTGTGGT
Muc4	AGGACCATCGTGCTCTCTCT	AGCATACTTAGGTTCAGAGCCA
Muc12	CTGGCAGCTTACCATGAGCACT	GAACTACCCACGGTCCACCAA
B. acidifaciens	AATCTCTTCTAGAGACTGGGAA	AGCTGATTGACTAAAGTAGCTGGA
B. dorei	GTTGGCTCAGCCAGATGCA	AGCCTACTCATTGGGATCATCTTG

2.9. Leukocyte isolation for flow cytometry

Aseptically excised tumors were disrupted into a single cell suspension by passage through a 70-μm nylon cell strainer (BD Biosciences). To isolate lamina propria leukocytes, ceca and the proximal colon were flushed with Ca- and Mg-free PBS with 25 mM HEPES (Gibco), 50 mM sodium bicarbonate (Sigma-Aldrich) and 5% FBS (Gibco) and cut into small fragments. These fragments were incubated in Ca- and Mg-free Hank's Balanced Salt Solution (HBSS; Gibco) containing 1.3 mM EDTA (Sigma-Aldrich), 25 mM HEPES, and 2 mM L-Glutamine (Gibco), under 200 rpm agitation at 37°C for 30 min. Fragments were then transferred to new flasks and were incubated in DMEM medium (Gibco) supplemented with 0.15 mg/mL collagenase D (Roche), 10% FBS, 25 mM HEPES, and 2 mM L-Glutamine for 40 min under 200 rpm agitation at 37°C, before passing the tissues through a 70-μm-pore- size nylon cell strainer. All single cell suspensions were centrifuged and treated with red blood cell lysis buffer (10% NH_4Cl solution and 5% of PBS in distilled water). To remove cell debris, tumor cells and lamina propria leukocytes were further processed over a 40:80% of Percoll (GE Healthcare). The resulting cell suspension was washed twice and counted. A single-cell suspension was prepared from the mLN by passing the organ through a 70μm nylon cell strainer (BD Biosciences), followed by treatment with red blood cell lysis buffer. After counting, single cells suspensions were stained with fluorochrome-conjugated antibodies for 30 min on ice. After

washing, cells were processed for intracellular staining using the transcription factor buffer set (eBioscience) following the manufacturer's instructions. Antibodies specific for CD11b (clone M1/70), CD45 (clone 30-F11), CD3 (clone 145-2C11), CD4 (clone GK1.5), FoxP3 (clone FJK-16s), CD8 (clone 53-6.7), Gr1 (clone RB6-5C1) and T-bet (clone 4B10) were from BD Biosciences, Biolegend or eBioscience. Samples were acquired on a LSRII flow cytometer (BD Biosciences) and data analyzed using FlowJo software (TreeStar).

2.10. Antibody depletion experiments

The *in vivo* depletion of Gr-1- and Ly6G-expressing cells were carried out by intraperitoneal (i.p.) injections of 200 µg of anti-mouse Gr-1 (clone RB6-8C5, BioXCell) or 200 µg of anti-mouse Ly6G (clone 1A8, BioXCell) in endotoxin-free PBS. The administration of the antibodies was performed at days 5, 7, 9 and 11 of the experimental protocol.

2.11. Cytokine quantification by ELISA

Tumor infiltrating leukocytes were plated in 96 well plates at a density of $1x10^5$ cells per well coated with 0.5ug/uL of anti-CD3 antibody (Biolegend). Cell were incubated for 72h at 37°C in a humidified atmosphere containing 5% CO2 in complete DMEM containing 0.4ug/uL of soluble anti-CD28 antibody (Biolegend). Supernatants were collected and stored at -80°C before cytokine analysis. The levels of interferon (IFN)-γ were measured by ELISA (eBioscience) following to the manufacturers' instructions.

2.12. Statistical Analysis

Data are represented as mean ± standard error of the mean (SEM). One-way ANOVA, Two-way ANOVA with Bonferroni multiple comparisons post-test or the Student's t-test were used for statistical comparisons using GraphPad Prism 9. Values were considered significant for $p \leq 0.05$ and represented with * for $p \leq 0.05$; ** for $p \leq 0.01$, *** for $p \leq 0.001$ and ****$p \leq 0.0001$.

3. Results

3.1. Dietary zinc supplementation promotes tumor overgrowth

Epidemiologic studies suggest that zinc deficiency is associated with increased risk of cancer [37,38]. While zinc insufficiency is an important health problem in developing countries, the amount of zinc consumed in developed countries often exceed the upper intake limit, particularly among supplement users [34,35]. As few studies have been conducted with zinc supplementation in animals, we wanted to explore the short-term impact of dietary zinc supplementation in a pre-clinical model of cancer. To this end, we used the LLC cell line subcutaneously implanted into the right hind flank of B6 mice. Beginning the day of tumor cell implantation, we supplemented the diet of tumor-bearing mice with zinc sulphate in the drinking water. Tumor-bearing mice maintained in normal chow were used as controls.

We began by testing four different doses of zinc sulphate, ranging from 0.2 g/L to 15g/L in the drinking water. We found that increased zinc intake was associated with tumor overgrowth (Figure 1A). Indeed, while mice that received drinking water supplemented with 15g/L of zinc displayed the biggest tumors, an increase in about 60% of dietary zinc (0.2 g/L) was enough to promote a significant increase in tumor volume when compared to control mice (Figure 1A).

With these data showing that dietary zinc supplementation promotes LLC tumor overgrowth, we asked if the tumor-promoting effects of dietary zinc were restricted to the LLC cell line. To answer this, we tested the effect of dietary zinc supplementation in the *in vivo* growth of MC38, a cell line derived from CRC cancer [39]. When we supplemented the diet of mice with the lowest and the highest doses of zinc sulphate tested for LLC (Figure 1A), we also found a significant increase in MC38 tumor growth in both groups of mice under dietary zinc supplementation when compared to control mice (Figure 1B).

Taken together, these data show that short-term dietary zinc supplementation promotes tumor overgrowth. This tumor-promoting effect was dose-dependent but independent of the origin of tumor cells.

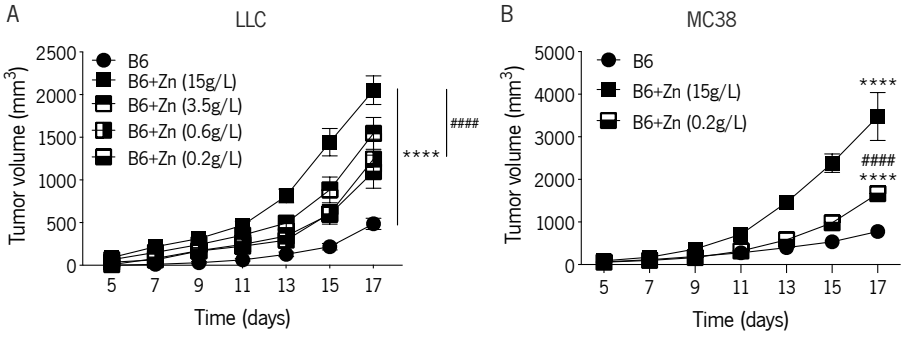

Figure 1. Dietary zinc supplementation promotes tumor overgrowth. B6 mice were subcutaneously injected in the right hind flank with either LLC or MC38 tumor cell lines and given zinc (B6+Zn) from day 0 to day 18. (A) Tumor growth of LLC or (B) MC38 cell lines was measured from day 5 and every other day until the end of the experiment. Data points represent the mean±SEM

of one representative experiment (out of 3) with 5 mice per group. Statistical analysis was performed using Two-way ANOVA with Bonferroni post-test. ****p≤0.0001 (to compare B6 – B6+Zn) #### p≤0.0001 (to compare B6+Zn (0.2g/L) – B6+Zn (15g/L)).

Dietary zinc plays crucial roles in cell-mediated immune functions, but it is also an important antioxidant and anti-inflammatory agent [40]. To explore the mechanisms whereby dietary zinc supplementation promotes tumor overgrowth, we first asked if the tumor-promoting effects of dietary zinc were mediated directly in tumor cells. To address this, we quantified the levels of zinc in tumor homogenates but found similar levels in both control mice and mice under dietary zinc supplementation (Figure 2A). These data show that excess dietary zinc does not accumulate in the tumor site, suggesting that the tumor promoting effects of zinc overdose are not directly in tumor cells.

We then analyzed the concentration of zinc in other tissues. In line with previous data showing that zinc in the whole body is maintained relatively constant while dietary zinc intakes vary [41], we did not find any alteration in the concentration of zinc in the serum (Figure 2B), and only a slight increase in the spleen of mice under dietary zinc supplementation (Figure 2C). However, when we analyzed the concentration of zinc in the gastrointestinal tract, we found a significant increase of this micronutrient in mice under dietary zinc supplementation (Figure 2D).

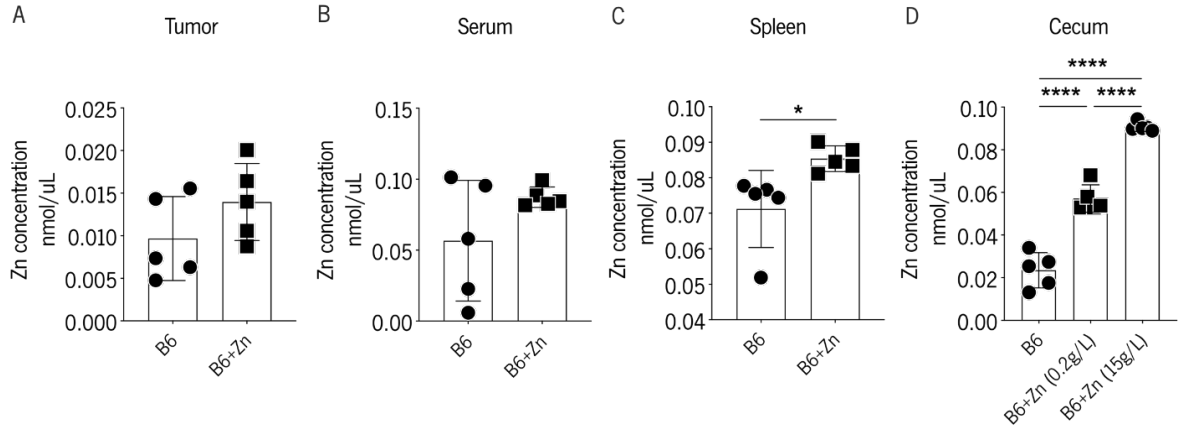

Figure 2. Dietary zinc supplementation promotes zinc accumulation in the cecum. Tissues from mice under dietary zinc supplementation (15g/L) or normal chow (control mice) were harvested and processed 18 days after tumor implantation. Zinc concentration in (A) tumor homogenates, (B) serum, (C) spleen homogenates and (D) cecum homogenates was determined. Bars represent the mean±SEM of one representative experiment (out of 2) with 5 mice per group. Statistical analysis was performed using (A-C) Student's t-test and (D) One-way ANOVA. * for p≤0.05 and ****p≤0.0001

These data suggest that dietary zinc supplementation promotes tumor overgrowth through a mechanism that is not directly dependent upon zinc accumulation in the tumor site, as the concentration of this micronutrient in the tumors of control mice is similar to that of mice under dietary zinc

supplementation. As we found a significant accumulation of zinc in the gastrointestinal tract of mice under zinc supplementation, we next questioned if the impact of dietary zinc supplementation in tumor overgrowth was mediated in the gastrointestinal tract.

3.2. Microbiota depletion prevents the tumorigenic effect of dietary zinc supplementation

Taking into consideration that (i) dietary zinc is an essential mediator of microbial community structure [29] and (ii) the microbiota has emerged as an important player in several diseases, including cancer [5,9], we hypothesized that dietary zinc supplementation promotes tumor overgrowth by acting in the gut microbiota.

To test our hypothesis, we began by depleting the microbiota of mice using the broad-spectrum antibiotics ampicillin, colistin and streptomycin, as previously described [42]. We first confirmed the depletion of the microbiota by quantifying CFUs of both aerobic and anaerobic bacteria as well as by quantitative RT-PCR for the 16S rRNA gene in the feces of mice under antibiotic treatment or non-treated mice. We found that antibiotic-treated mice displayed a reduction of 5 $\log_{10}$ in aerobic and a reduction of 7 $\log_{10}$ anaerobic cultivable bacteria when compared to control mice (Figure 3A). Furthermore, there was a reduction of 10^4 in the 16S rRNA gene quantification in antibiotic-treated mice (Figure 3B), thus confirming the microbiota depletion.

Mice were then subcutaneously implanted with either LLC or MC38 tumor cell lines and their diets were supplemented, or not, with zinc sulphate in the drinking water, as described for figure 1. We found that microbiota depleted tumor-bearing mice did not display increased tumor growth when their diet was supplemented with zinc (Figure 3C-D). Indeed, tumor growth for both LLC and MC38 was similar in microbiota-depleted mice maintained under dietary zinc supplementation or normal chow. Therefore, these data confirm our hypothesis that the tumor-promoting effects of dietary zinc are mediated through the gut microbiota.

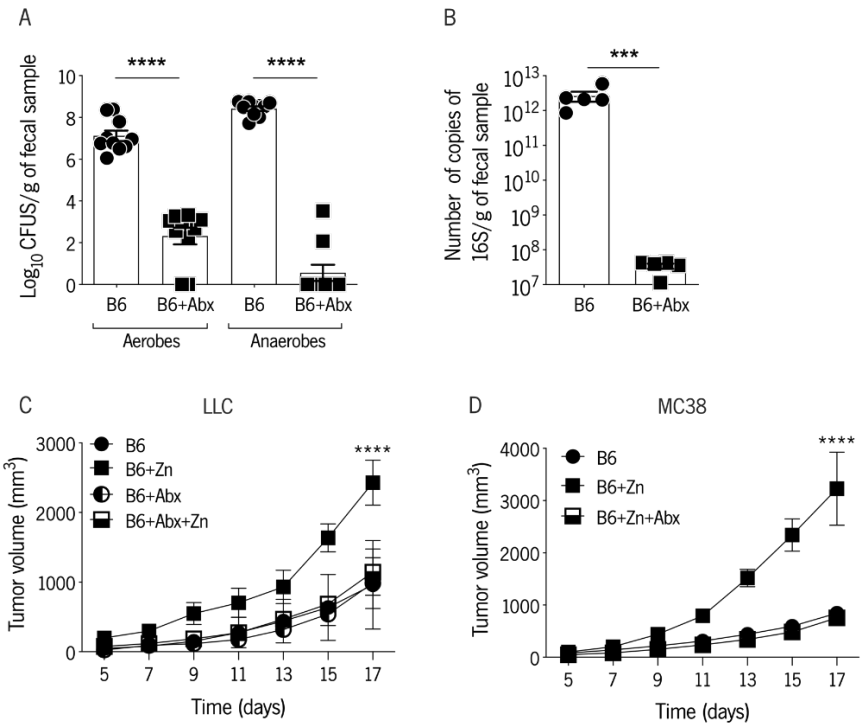

Figure 3. Microbiota depletion prevents the tumorigenic effect of dietary zinc supplementation. Mice were treated with a cocktail of antibiotics two weeks before tumor cells' implantation and dietary zinc supplementation (15g/L). (A) Number of CFUs in the fecal samples of control or antibiotic (Abx) treated mice and (B) Number of copies of 16S gene were quantified at the end of antibiotic treatment. Tumor growth of (C) LLC cell line and (D) MC38 cell line was measured from day 5 and every other day until the end of experiment. Data points and bars represent the mean±SEM of one representative experiment (out of 3) with 5 mice per group. Statistical analysis was performed using (A-B) Student's t-test and (C-D) Two-way ANOVA with Bonferroni post-test. *** for p≤0.001 and ****p≤0.0001

With our data showing the impact of dietary zinc supplementation in the microbiota-tumor axis, we next conducted a 16S rRNA sequencing to define the gut microbiota composition of tumor-bearing mice under dietary zinc supplementation or normal chow. Dietary zinc is absorbed throughout the whole small intestine [43]; importantly, during excessive intake, zinc homeostasis is maintained primarily through excretion in the feces [41,44]. Therefore, we analyzed the composition of the microbiota present in the cecum of mice, which represents one of the regions more enriched in microbial species [45] with important functions in maintaining microbial compositions in other gut compartments [46].

We began by performing a general assessment of the microbiota across the two groups of mice. We found that short-term dietary zinc supplementation promoted a significant reduction in α-diversity (Figure 4A; p=0.01), thus confirming that zinc overdose causes a shift in microbial community structure. Specifically at the phylum level, our data show that mice under dietary zinc supplementation had an increase in the relative representation of *Bacteroidetes* (9.7% vs 22.9%, p=0.03) and *Proteobacteria* (4%

vs 8.2%, p=0.01), and a decrease in *Firmicutes* (87.7% vs 65.3%, p=0.03) (Figure 4B). Minor members of the community were not significantly affected by dietary zinc supplementation, with *Actinobacteria*, *Tenericutes*, *Verrucomicrobia* and unclassified members of the community remaining constant in both groups (Figure 4B). As our data showed that microbiota depletion prevented the tumor-promoting effects of dietary zinc supplementation, we focus our microbiota analysis on the bacterial communities that were overrepresented, rather than underrepresented, in mice under dietary zinc supplementation. In this regard, analysis of the relative abundance of the dominant genera revealed that mice under dietary zinc supplementation had a consistent increase in the abundance of bacteria belonging to the genus *Bacteroides* when compared to control mice (0.1% vs 16.6%, p=0.003) (Figure 4C). Although other genera were also increased in mice under dietary zinc supplementation, the data were more variable and not consistent across mice within the same group (Figure 4C). We further addressed differences in the bacterial community at the species level. Specifically to the genus *Bacteroides*, the most overrepresented genus in mice under dietary zinc supplementation, we found 4 operational taxonomic units (OTUs), namely OTU000009 (log_2FC= 9.873; FDR=8.96x10^{-9}), OTU000015 (log_2FC=8.129; FDR=6.77x10^{-7}), OTU000020 (log_2FC= 9.902; FDR=6.42x10^{-9}) and OTU000033 (log_2FC= 7.338; FDR=1.17x10^{-7}). These increased OTUs displayed 100% of identity with two species, *B. acidifaciens* and *B. dorei.* These findings were validated by quantitative RT-PCR analysis, corroborating the increased abundance of *B. acidifaciens* (Figure 4D) and *B. dorei* (Figure 4E) in the cecum contents of mice under dietary zinc supplementation.

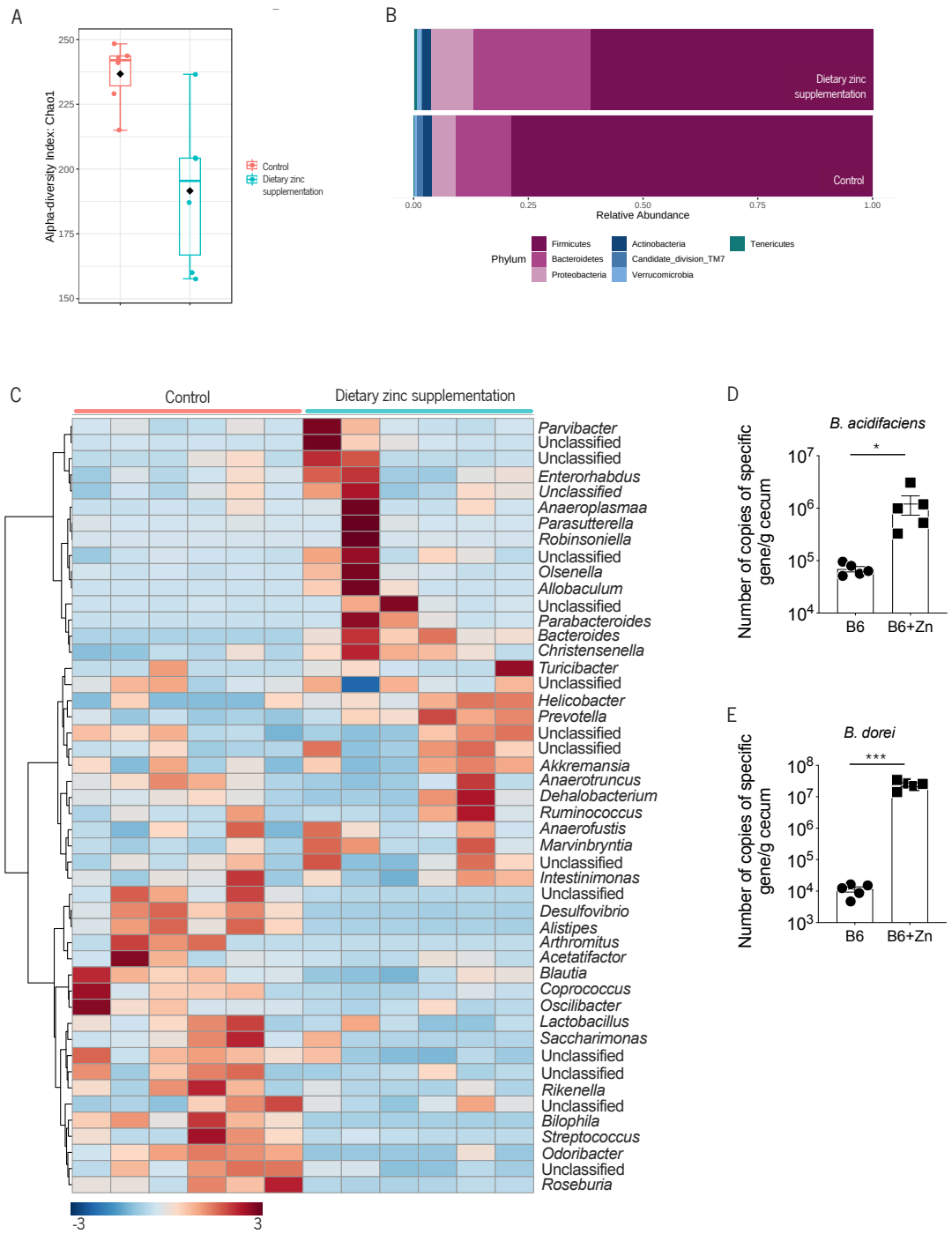

Figure 4. Dietary zinc supplementation alters the composition of gut microbiota. Cecum from mice under dietary zinc supplementation (15g/L) or control mice was harvested and processed for 16S rRNA sequencing analysis 18 days after tumor implantation. (A) α-diversity; (B) Relative abundance of the phyla; (C) Representative heat map of genera. Number of copies of specific genes from (D) *B. acidifaciens* and (E) *B. dorei* were quantified by quantitative RT-PCR. * for p≤0.05 and ***p≤0.001

Together, these data show that dietary zinc supplementation promoted a remodeling of the composition of the gut microbiota, primarily characterized by an increase in the genus *Bacteroides*, and the species *B. acidifaciens* and *B. dorei*.

3.3. Dietary zinc supplementation does not alter the architecture of the gut mucosa

With our data showing a key role of the microbiota in the tumor-promoting effects of dietary zinc supplementation, we sought to explore the mechanisms by which the zinc-modulated microbiota promoted tumor overgrowth. One of the potential mechanisms by which the microbiota can promote tumor overgrowth is by altering in the permeability of the gastrointestinal tract which facilitates the translocation of tumor-promoting bacteria to the periphery [47–49]. Therefore, we assessed the permeability of the intestinal mucosa in mice under dietary zinc supplementation.

To do this, we began by determining the level of proliferation and apoptosis of the intestinal epithelial layer. We found similar levels of Ki-67 and Caspase-3 in the epithelial layer of the intestinal mucosa, indicating similar levels of proliferation and apoptosis (Figure 5A). Additionally, we did not find alterations in the morphology of intestinal tract (Figure 5B) nor in the size of intestinal villi (Figure 5C) in mice under dietary zinc supplementation. We also evaluated the expression of mucins-encoding genes, specifically *Muc1*, *Muc2*, *Muc3, Muc4* and *Muc13,* which encode enzymes responsible for the production of mucous that prevent the direct contact between microbiota and intestinal epithelium. We found that the expression of these genes was similar in control mice and mice under dietary zinc supplementation (Figure 5D).

Taken together, these data show that the architecture of the intestine, as well as the expression of genes that play an important role in maintaining this architecture, was preserved in mice under dietary zinc supplementation.

Finally, to demonstrate that dietary zinc did not impact the permeability of the gastrointestinal tract, we fed mice FITC-dextran and assayed the presence of FITC-dextran in the serum of mice 4h later. We found a similar concentration of FITC-dextran in the serum of both groups of mice (Figure 5E), showing that dietary zinc supplementation does not alter the permeability of the gastrointestinal tract. Accordingly, we were unable to find bacterial DNA in the tumors of both control and dietary zinc supplemented mice (data not shown).

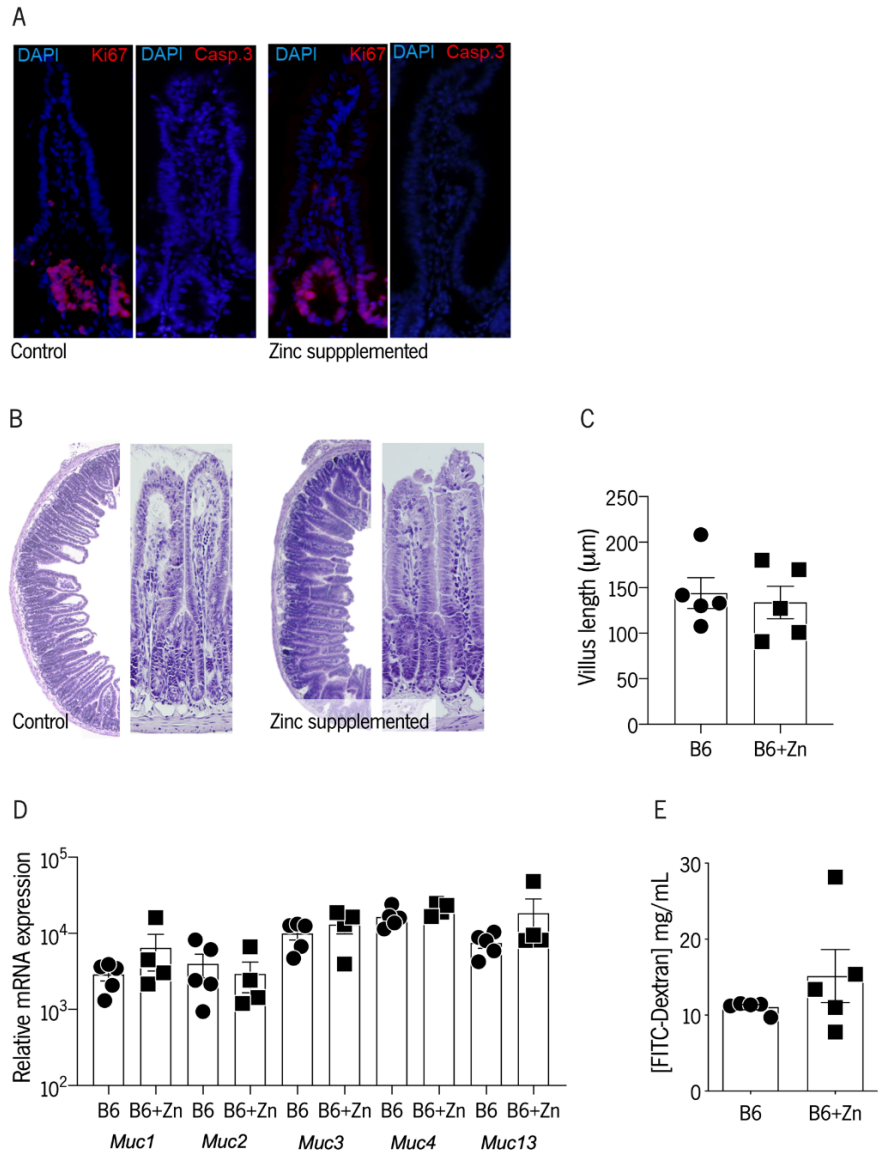

Figure 5. Dietary zinc supplementation does not impair gut architecture and permeability. Intestinal tissue from mice under dietary zinc supplementation (15g/L) or control mice was harvested 18 days after tumor implantation. (A) Immunofluorescence representative images of Ki-67 or Caspase-3 staining in frozen sections of intestinal tissue; (B) Histological representative images of gut tissue; (C) Villi length; (D) Relative mRNA expression of mucin-production associated genes; (E) Intestinal permeability evaluated by quantification of FICT-dextran administrated by oral gavage in the serum after four hours of administration. Bars represent the mean±SEM of one representative experiment (out of 2) with 5 mice per group. Statistical analysis was performed using (C-E) Student's t-test; non-significant differences were found.

3.4. Tumor overgrowth caused by dietary zinc supplementation is T lymphocyte dependent

Immune modulation is a key mechanism by which the microbiota modulates cancer development, progression, and treatment [50]. Indeed, dysbiosis has been shown to shape adaptive immunity and

trigger several immune disorders through the activity of T lymphocytes [51]. Therefore, to define the immune pathways linking microbiota alteration to tumor overgrowth we tested the impact of dietary zinc supplementation in mice deficient in key immune components. We began by testing LLC tumor growth in RagKO mice that do not produce mature B or T lymphocytes. We found that dietary zinc supplementation did not promote tumor overgrowth in RagKO mice, as the size of LLC tumors were similar in mice under a normal and zinc supplemented diets (Figure 6A). We next did the same experiment in TcraKO mice (do not produce mature T lymphocyte subsets) and found that, as for RagKO mice, dietary zinc supplementation did not promote tumor overgrowth (figure 6B). These data show that acquired-immune mechanisms, specifically T lymphocytes, play a key role in tumor overgrowth upon dietary zinc supplementation.

We then performed a broad characterization of the T lymphocyte subsets present in the large intestine and in the mLN. We found that mice under dietary zinc supplementation had no alterations in the frequency or in the number $CD4^+$ (Figure 6C-D) and $CD8^+$ T lymphocytes (Figure 6E-F). Moreover, we did not detect any alteration in $CD4^+Tbet^+$ T lymphocytes (Figure 6G-H) or $FoxP3^+$ Tregs (Figure 6I-J) subsets. Interestingly however, when we analyzed the mLN for $FoxP3^+$ Tregs, a population critical to maintain gut homeostasis [52,53] but also associated with tumor progression in mice [54] and humans [55], we found increased frequencies of $FoxP3^+$ Tregs in mice under dietary zinc supplementation, when compared to control mice (Figure 6K).

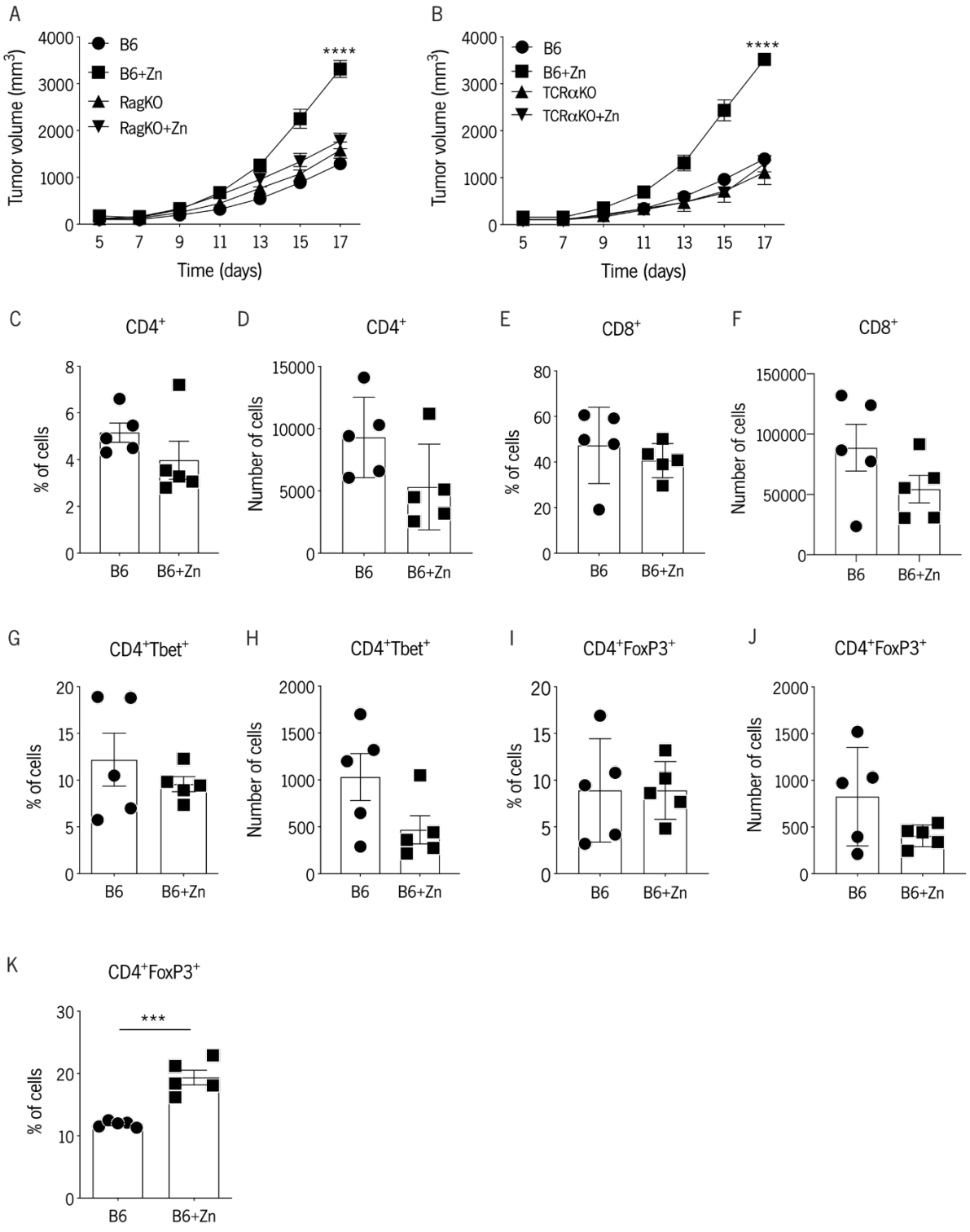

Figure 6. Dietary zinc supplementation promotes tumor overgrowth via T lymphocyte-dependent mechanisms. Tumor growth of (A) RagKO and (B) TcraKO LLC tumor-bearing mice under dietary zinc supplementation or normal chow tumor growth was measured from day 5 and every other day until the end of the experiment. LLC-tumor bearing B6 mice were sacrificed 18 days after tumor implantation and the large intestine and the mLN were collected and processed for flow cytometry. (C) Percentage and (D) Number of $CD4^+$ T lymphocytes in large intestine; (E) Percentage and (F) Number of $CD8^+$ T lymphocytes in large intestine; (G) Percentage and (H) Number of $CD4^+Tbet^+$ T lymphocytes in large intestine; (I) Percentage

and (J) Number of $CD4^+FoxP3^+$ T lymphocytes in large intestine; and (K) Percentage of $CD4^+FoxP3^+$ T lymphocytes in the mLN. Data points and bars represent the mean±SEM of one representative experiment (out of 2) with 5 mice per group. Statistical analysis was performed using (A-B) Two-way ANOVA with Bonferroni post-test and (C-F) Student's t-test. *** for p≤0.001 and ****p≤0.0001.

3.5. Increased intratumor accumulation of $Gr1^+$ cells in mice under dietary zinc supplementation promotes tumor growth

Thus far, our data disclosed a fundamental role for T lymphocytes in tumor overgrowth. We also found that dietary zinc supplementation changes the profile of T lymphocytes in the intestinal mucosa. Therefore, we then sought to analyze the intratumor profile of infiltrating T lymphocytes of LLC-tumor bearing mice. We found that, the accumulation of $CD4^+$, $CD8^+$, and $CD4^+FoxP3^+$ T lymphocytes was similar in mice under normal or dietary zinc supplementation (Figure 7A-F), suggesting that dietary zinc supplementation did not impact intratumor accumulation of T lymphocytes. Indeed, the majority of immune cells infiltrating LLC tumors were myeloid in nature (Figure 7G-H). Recent data suggest that Tregs play crucial roles in the proliferation and acquisition of immunosuppressive function of MDSCs [56]. Importantly, the crosstalk between these two immunosuppressive populations has been shown in different cancer models [57–60]. Therefore, we next analyzed the myeloid compartment paying particular attention to the presence of MDSCs, a population that can suppress immune responses against LLC tumors [61]. Our analysis revealed an increased intratumor accumulation of $Gr1^+$ cells in LLC-tumor bearing mice under dietary zinc supplementation (Figure 7I-J). To explore the role of these cells in the tumor overgrowth, we performed *in vivo* depletion assays using a monoclonal antibody against Gr1 (RB6-5C1). We found that mice dietary zinc supplementation that were treated with anti-RB6-5C1 displayed smaller tumors when compared to mice that did not receive anti-Gr1 antibody (Figure 7K). The same data was obtained with MC38-tumor bearing mice (Figure 7L). These data show that $Gr1^+$ cells play a key role in promoting tumor overgrowth in short-term dietary zinc supplementation.

To determine the granulocytic or monocytic nature of the MDSCs population [62], we treated mice under dietary zinc supplementation with anti-Ly6G antibody to deplete the granulocytic lineage specifically. Contrarily to anti-Gr1 treatment, LLC-tumor bearing mice treated with anti-Ly6G did not show alterations in tumor growth compared with dietary zinc supplemented mice (Figure 7M), suggesting that the pro-tumor $Gr1^+$ cells are monocytic in nature.

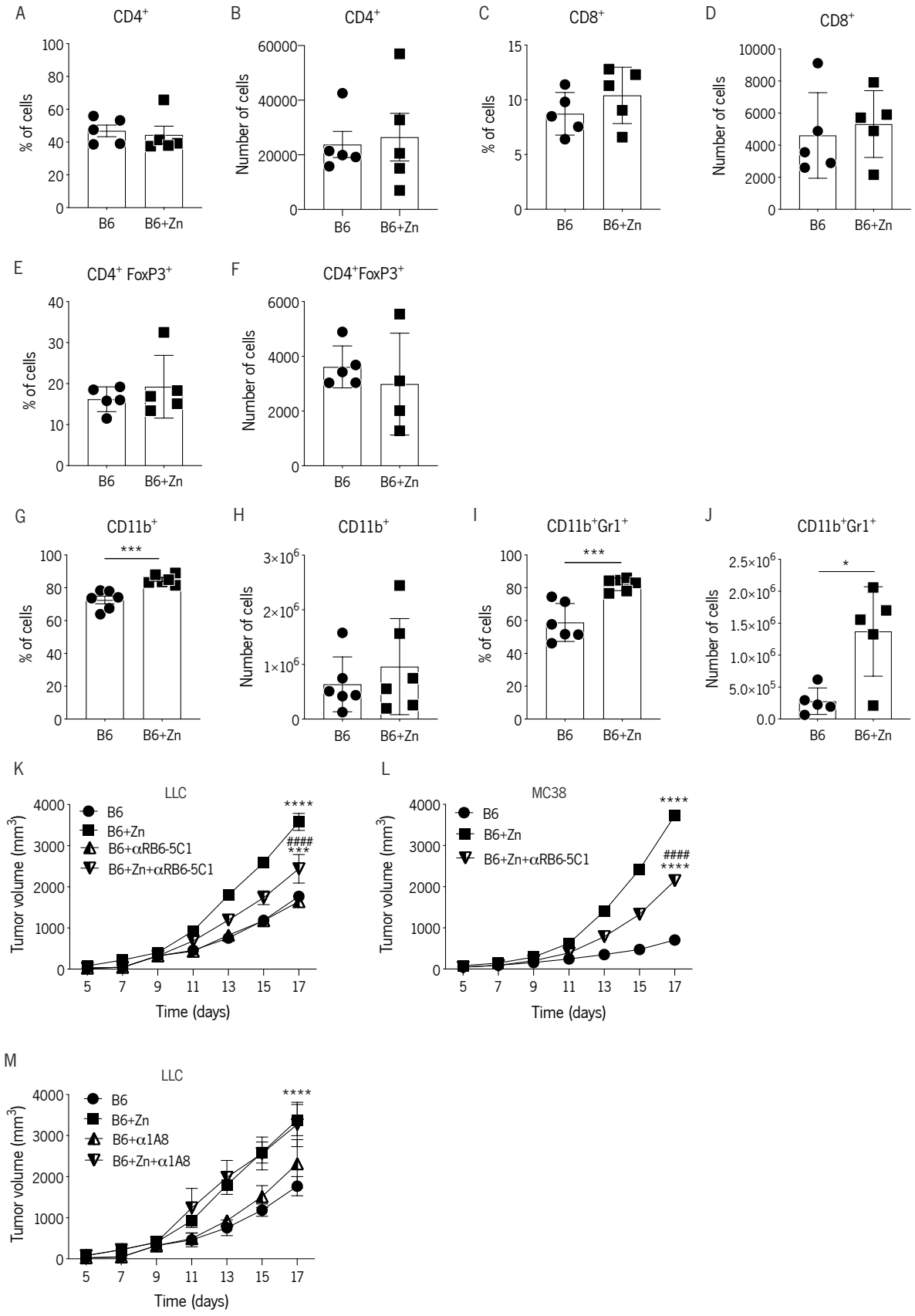

Figure 7. Increased intratumor accumulation of Gr1⁺ cells in dietary zinc supplemented mice promotes tumor overgrowth. Tumors from LLC tumor-bearing mice under under dietary zinc supplementation (15g/L) or normal chow were

harvested 18 days after tumor implantation and processed for flow cytometry analysis. (A) Percentage and (B) Number of $CD4^+$ T lymphocytes; (C) Percentage and (D) Number of $CD8^+$ T lymphocytes; (E) Percentage and (F) Number of $CD4^+FoxP3^+$ T lymphocytes; (G) Percentage and (H) Number of $CD11b^+$ cells; (I) Percentage and (J) Number of $CD11b^+Gr1^+$ cells. (K) LLC tumor-bearing miceor (L) MC38 tumor-bearing mice were treated with αRB6-5C1 antibody at days 5, 7, 9 and 11 of the experimental protocol; (M) LLC tumor-bearing mice were treated with α1A8 antibody at days 5, 7, 9 and 11 of the experimental protocol. Data points and bars represent the mean±SEM of one representative experiment (out of 2) with 5 mice per group. Statistical analysis was performed using (A-J) Student's t-test and (K-M) Two-way ANOVA with Bonferroni post-test. *** for p≤0.001 and ****p≤0.0001 (to compare B6 with B6+Zn) and #### p≤0.0001 (to compare B6+Zn with B6+Zn+αRB6-5C1)

MDSCs promote tumor growth through several mechanisms, including regulating the expression of genes encoding molecules that favor tumor growth, such as vascular endothelial growth factor (VEGF) [63], Arginase 1 [64], inducible nitric oxide synthase (iNOS) [65], or/and by suppressing anti-tumor T lymphocyte responses [66]. While we did not find altered expression of *Vegfa*, *Arg1* and *Nos2* in mice under dietary zinc supplementation (Figure 8A-C), we did find reduced production of IFN-γ by T lymphocytes isolated from tumors of dietary zinc supplemented mice (Figure 8D), thus suggesting an impairment of the T lymphocyte activity. Interestingly, mice supplemented with the highest concentration of zinc had reduced production of IFN-γ compared with mice supplemented with the lowest concentration.

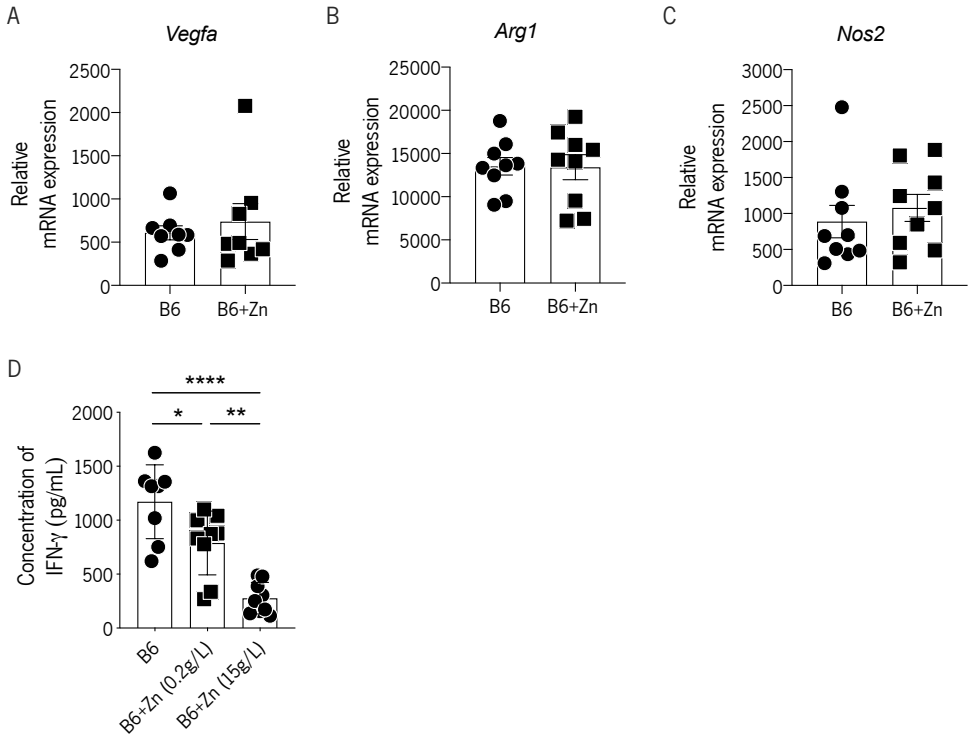

Figure 8. Mice under dietary zinc supplementation have reduced intratumor production of IFN-γ. Tumors from LLC tumor-bearing mice under supplementation (15g/L) or normal chow were harvested 18 days after tumor implantation and processed for RNA extraction or to obtain single-cell suspensions. Relative (A) *Vegfa*, (B) *Arg1* and (C) *Nos2* mRNA

expression was determined by RT-PCR (D) Concentration of IFN-γ produced by T lymphocytes after 72h of stimulation with anti-CD3/CD28. Bars represent the mean±SEM of two independent experiments with 5 mice per group. Statistical analysis was performed using (A-C) Student's t-test or (D) One-way ANOVA. * for p≤0.05, ** p≤0.1 and ****p≤0.0001

Taken together, our results indicate that dietary zinc supplemented mice display increased intratumor accumulation of Gr1-expressing myeloid cells that orchestrate tumor overgrowth likely by impairing the anti-tumor T response.

4. Discussion

Herein we show that dietary zinc supplementation promotes LLC and MC38 tumor overgrowth in a syngeneic mouse model. Our data show that the tumor-promoting effects of dietary zinc supplementation are mediated in the gut microbiota, as depletion of the microbiota through broad-spectrum antibiotics prevented tumor overgrowth. The analysis of the microbial communities revealed a reduced diversity, but an overrepresentation of the genus *Bacteroides* in the ceca of mice under dietary zinc supplementation. Importantly, these alterations did not impact the permeability intestinal barrier, suggesting that extra-intestinal tumor overgrowth was caused by modulation of the anti-tumor immune response. In support of this hypothesis, tumor-bearing RagKO or TcraKO mice under dietary zinc supplementation display similar tumors to that of control mice, suggesting a key role for T cells in the axis microbiota-tumor overgrowth. In line with these data, we found increased Tregs in the mLN, as well as an increased accumulation of MDSCs in the tumors of mice under dietary zinc supplementation. Crucially, depletion of MDSCs using anti-Gr1 antibody reverted tumor overgrowth. These data show that short-term dietary zinc supplementation promotes a remodeling of the microbiota communities, which modulate the anti-tumor immune response and promote extraintestinal tumor overgrowth.

With the disturbing perspective of an increased number of new diagnoses over the next decades, cancer is an important health problem claiming a significant number of lives worldwide [1]. While in recent years cancer research has driven the development of novel immunotherapies, a significant proportion of patients do not benefit from these therapies. Strong evidence supports the importance of the gut microbiota in the development of some malignancies, but also on responses to cancer therapies. In one hand, due to their proximity, the gut microbiota has been shown to play a crucial role in modulating gastrointestinal cancers [67]. Indeed, different species of the microbiota have been associated with the development of CRC [68,69], but also with inflammatory diseases of the gastrointestinal tract, namely Crohn's disease and ulcerative colitis, which can ultimately set the stage to cancer development [70,71]. On the other hand, the gut microbiota has also been linked to extra-intestinal cancers, including lung [72] or breast cancers [73], showing that the impact of the gut microbiota in carcinogenesis go beyond gastrointestinal cancers. While the microbiota is notably stable in adulthood [74], diet has been recognized as one of the strongest modulators of its composition [75]. In this context, over the last decades we have witnessed a significant growth of the dietary supplement market promoted by the need to overcome nutritional deficiencies but also by the desire to stimulate immunity to enhance protection against diseases, including cancer and infection [76,77].

The micronutrient zinc plays a key role in growth and development, but also in the function of the immune system [78]. The overall consensus is that zinc supplementation has beneficial effects in the prevention and treatment of cancer [40,79]. In this regard, previous published data on the effect of zinc in carcinogenesis showed that this micronutrient inhibits the production of VEGF, which was correlated with reduced tumor cell invasiveness [80]. Additionally, the combination of dietary zinc supplementation with 1,2-dimethylhydrazine, a widely used carcinogen to inducer of colon cancer in animal models, decreased the tumor incidence and tumor size in rats [30], further supporting an anti-tumor role of this micronutrient. Despite these studies suggesting an anti-cancer role for zinc, little is known about the shot-term impact of zinc in cancer progression.

Zinc is a non-toxic micronutrient to living organisms [81,82]. Indeed, dietary zinc is not stored in the body and its excessive intake results in reduced absorption and increased excretion [82]. Previous data showed that dietary zinc was not toxic to mice at dosages as high as 1000 mg/kg [81], corresponding to an intake of about 20 mg of zinc per day, a value which is similar to the highest dose that we have used in this work. Our data is also in line with previous data showing that that dietary zinc supplementation does not alter the architecture of gut mucosa [30]. Taken together, these data show that the impact of dietary zinc supplementation in tumor overgrowth is not due to zinc toxicity to mice or any direct effect of zinc to cancer cells.

Despite the well-documented anti-cancer roles of zinc, several studies have also suggested that zinc can enhance tumorigenesis. In these studies, rats maintained in zinc-restrictive diet display a markedly decreased tumor growth [32,33], suggesting that the growth of established tumors can be arrested by dietary zinc depletion. While the mechanisms whereby zinc promotes or inhibits tumor growth are not defined, the data discussed above show that the role of dietary zinc in carcinogenesis is rather complex and may depend upon several other factors. Our data now show that the impact of dietary zinc supplementation in tumor overgrowth is mediated though alteration of the microbiota, particularly the increase in species of the genus *Bacteroides,* including *B. acidifaciens* and *B. dorei.* While the effects of dietary zinc in the microbiota are well recognized [83–85] future research is important to define the role of these microbiota in tumor overgrowth. In this regard, we attempted the transplantation of pure cultures of *B. acidifaciens* and *B. dorei* (isolated or together) into mice, to determine their impact in tumor growth. However, our approach was unsuccessful as we did not find increased representation of *B. acidifaciens* or *B. dorei* after transplantation (not depicted), indicating that the reduced microbiota diversity caused by dietary zinc supplementation likely contributed to the outgrowth of *B. acidifaciens* and *B. dorei*. Additionally, we cannot exclude a role for zinc in potentiating the pathogenicity of *B. acidifaciens* or *B.*

dorei. Indeed, it has been reported that zinc impacts the toxin-associated activities of other microorganisms [85–87]. For instance, in an enteroaggregative *Escherichia coli* model of infection, zinc deficiency led to increased expression of *E.coli* virulence factors, likely contributing to increased disease severity [87]. Specifically for *Bacteroides*, it has been shown that enterotoxigenic *B. fragilis* that secrete a zinc-dependent metalloprotease toxin [88] were more prevalent in the feces of CRC patients compared to healthy controls [89]. Additionally, the presence of enterotoxin encoding genes in CRC patients diagnosed at stage III was higher than in those diagnosed at stage I and II [89]. Furthermore, it has been recently shown that the high abundance of *Bacteroides* in the microbiota of melanoma patients was associated with a poor response to anti-PD1 immunotherapy [20], further corroborating the negative impact of these microbiota in anti-tumor immune responses. Future research will be important to determine the mechanisms whereby dietary zinc alters the activity of the microbiota.

The immune system plays a pivotal role in carcinogenesis [90]. Indeed, while some cellular subsets are associated to tumor elimination, other facilitate tumor growth [91]. However, it is important to note that the role of different immune cells may depend on the type of the tumor. In this regard, increased accumulation of Tregs has been associated with poor prognosis in pancreatic, breast and lung cancers [92–94] whereas in CRC there are evidences that these cells may be beneficial [95,96]. Our data showing that dietary zinc supplementation did not promote tumor overgrowth in mice lacking T cells suggest that this population is an important link between microbiota and tumor overgrowth mediated by dietary zinc. We further suggest that the increase in Tregs may be responsible for this phenotype. Indeed, it has been previously shown that the production of the short chain fatty acid butyrate is associated to the differentiation of Tregs [97,98]. It is possible that the dysbiosis promoted by zinc supplementation results in the production of metabolites that could be involved in the differentiation of Tregs. Future studies to define the profile of metabolites of mice under dietary zinc supplementation will be important to clarify the impact of the microbiota in the differentiation Tregs.

Recently, it has been demonstrated that Tregs can orchestrate the proliferation and differentiation of MDSCs [56,99]. Furthermore, the presence MDSCs is associated with increased tumor growth [61,100], as we have seen in our model. MDSCs have been shown to modulate several pathways that facilitate tumor growth, including, enhance the production of VEGF [63], increase expression of Arginase-1 [64,101] and iNOS [65] and suppression of T lymphocyte responses [66]. While we did not find alterations in the expression of the genes encoding these molecules, we did find reduced production of IFN-γ by tumor infiltrating lymphocytes in mice under dietary zinc supplementation. Future studies will be

important to consolidate the role of $Gr1^+$ cells in our model and disclose any altered pathways associated to carcinogenesis.

Overall, our results disclose a role for dietary zinc supplementation in extra-intestinal tumor overgrowth, through microbiota and immune alterations. Importantly, future work will be directed towards clarifying the microbiota partners that drive tumor progression, as well as the immune pathways modulated. These findings may provide novel insights on the interaction between microbiota, immune system and cancer and disclose pathways that may hold potential for the development of novel therapies or personalized clinical approaches.

5. Acknowledgments

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Chapter III

IL-10 prevents tumor overgrowth in mice under dietary zinc supplementation

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IL-10 prevents tumor overgrowth in mice under dietary zinc supplementation

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Abstract

Interleukin (IL)-10 plays important roles in controlling inflammatory responses against the microbiota in the gut mucosa; however, its activity in cancer is not consensual, with reports suggesting that IL-10 acts in both tumor promotion and elimination. We have previous shown that dietary zinc supplementation promotes tumor overgrowth by modulating the gut microbiota. Therefore, in this work we used a transgenic mouse model of IL-10 overexpression, induced by dietary zinc supplementation, to test the role of this immunomodulatory cytokine in cancer progression. Our data showed that increased levels of IL-10 prevented tumor overgrowth induced by short-term dietary zinc supplementation. This protective effect was associated with the accumulation of immunoglobulin (Ig)A-producing B lymphocytes in the gut mucosa, as well as increased concentration of IgA in the intestinal lumen. Additionally, depletion of Gr1+ cells, which we previously found to be important tumor promoters in this model, prevented dietary zinc-mediated tumor overgrowth. Taken together, these data suggest that IL-10 plays a key role in controlling the oncogenic activity of the microbiota through the production of IgA in the gut mucosa.

1. Introduction

IL-10 is a nonredundant cytokine secreted by a wide variety of cells during the course of an immune response, which has been widely associated with immunosuppressive mechanisms [1]. Specifically, IL-10 suppresses the ability of antigen presenting cells, including dendritic cells and macrophages, to stimulate the proliferation and effector function of antigen-specific cluster of differentiation (CD4) T lymphocytes [2], and inhibits cytokine expression by activated macrophages [3,4].

Owing its immunosuppressive properties, one would expect IL-10 production to impair anti-tumoral immunity. Interestingly however, over the last years IL-10 has been shown to have both pro- and anti-tumor roles. In one hand, it has been reported that the blockade of IL-10 signaling enhances anti-tumor immunity [5,6] and that dendritic cells capable of producing large amounts of IL-10 induce anergy in $CD8^+$ T lymphocytes impairing their cytolytic activity towards melanoma-associated antigens [7]. On the other hand, it has been shown that tumor cells transfected with murine IL-10 grow less than control cells, and in some cases the IL-10-expressing tumor is rejected [8–11]. In humans, the role of IL-10 in tumor immunology is scarce. However, polymorphisms in the IL-10 promoter leading to a reduced IL-10 expression were associated with an increased incidence of melanoma and prostate cancer [12,13]. Furthermore, both mice and humans deficient in IL-10 activity develop inflammatory bowel disease and cancer [14,15]. In this regard, one of the critical functions of IL-10 is maintaining the homeostasis of the gut, primarily by preventing exacerbated immune responses against the gut microbiota [16,17]. Indeed, polymorphisms in IL-10 or its receptor resulting in reduced IL-10 expression have been associated with early onset of colitis in humans [14,18] and IL-10 knock-out (KO) mice develop spontaneous enterocolitis [19]. However, germ-free IL-10 KO mice does not develop spontaneous enterocolitis, suggesting that IL-10 acts to regulate pathological immune responses against the gut microbiota [19]. Taken together, these data suggest that the role of IL-10 in cancer may be context dependent and vary according to the tumor type. Considering the recently recognized role of the gut microbiota in oncogenesis, it is tempting to speculate that the role of IL-10 in tumor immunity is mediated in the gut mucosa.

Previous results from our group have disclosed a key role for dietary zinc supplementation in modulating the gut microbiome causing extra-intestinal tumor overgrowth (Chapter II). Therefore, taking into account (i) the key role of IL-10 in gut homeostasis and (ii) the controversial role of IL-10 in cancer, herein we used a novel mouse model of controlled IL-10 overexpression (pMT-10) [20] to define the role and the mechanisms whereby this cytokine modulates anti-tumor immunity.

Our data showed that pMT-10 mice are less susceptible to dietary zinc supplementation, compared to C57BL/6 (B6) mice. Importantly, the presence of increased levels of IL-10 was associated to increased production of Ig A in the gut of pMT10 mice that we suggest being the mechanism whereby these mice can better control tumor growth. While further studies are required to fully characterize the impact of IL-10 in our model, our results so far disclose an indirect anti-tumor function for IL-10 through modulation of gut immune response.

2. Methods

2.1. Mice

B6 were bred at the ICVS animal facility from stocks purchased from Charles River Laboratory (Barcelona, Spain). pMT-10 mice on a C57BL/6 background were produced by Drs. António G. Castro and Paulo Vieira, as previously described [20]. Briefly, mouse IL-10 cDNA sequence was cloned in the p169ZT vector, which carries the sheep metallothioneinc (MT) Ia promotor, a β-globin splice site and the SV40 polyadenylation signal. The resulting vector was injected in b6 eggs and transgenic founders were identified by PCR using MT specific primers. IL-10 overexpression in pMT-10 mice was induced by supplementing the drinking water with 20g/L of sucrose and 15g/L of zinc sulphate, as previously described [20]. This results in a rapid increase in the circulating levels of IL-10, which are maintained until zinc sulphate withdraw [20]. Control mice were given water containing 20g/L of sucrose. Both male and female mice between the ages of 8 to 12 weeks old were used for experimental procedures.

All procedures involving live animals were performed in accordance with the European Directive 86/609/EEC and approved by the *Subcomissão de Ética para as Ciências da Vida e da Saúde* (SECVS 074/2016) and the Portuguese National Authority *Direcção Geral de Veterinária* (DGAV 014072).

2.2. Cell line and tumor models

The murine Lewis lung carcinoma (LLC) cell line was kindly provided by Dr. Massimiliano Mazzone (Vesalius Research Center, Belgium). Cells were cultured in T75 cm^2 tissue culture flask at 37°C in a humidified atmosphere containing 5% CO_2 in complete Dulbecco's Modified Eagle Medium (cDMEM, DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% HEPES, 1% sodium pyruvate and 1% penicillin/streptomycin (all from Gibco)). Cells were harvested by trypsinization, washed in phosphate-buffered saline (PBS) and counted. $1x10^6$ cells in 200µL of endotoxin-free PBS were then injected subcutaneously into the right flank of recipient mice. Tumor volumes were monitored from day 5 onwards by measuring two bisecting diameters of each tumor with calipers and calculated using the formula: $(\pi x d^2 x D)/6x1000$; d=small diameter and D= large diameter) [21]. Tumor-bearing mice were monitored daily for signs of distress. At day 18 post tumor implantation (before tumors reach maximum volume allowed), mice were sacrificed by CO_2 and different tissues were havested.

2.3. Bacterial quantification

Genomic DNA from cecum was extracted using the Nucleo-Spin DNA Stool Kit (Machery-Nagel) according to manufacturer's instructions. Absolute abundance of bacteria was performed by quantification of bacterial copy number in stool DNA samples using specific primers for *B. acidifaciens* and *B. dorei* (table 2). Values were extrapolated from a standard curve obtained by different copy numbers of a plasmid containing a specific insert belonging to each bacterium. For the plasmid generation, a specific DNA fragment for *B. acidifaciens* or *B. dorei* was inserted in a pJET1.2 plasmid using a CloneJET PCR Cloning Kit (Thermo Scientific). After transfection in competent *E. coli*, plasmid DNA was recovered and used as template for the standard curve.

2.4. Quantitative real-time (RT)-PCR

Specific oligonucleotides for genes of interest are shown in Table 2. Quantitative RT-PCR was performed using the following protocol: one cycle of 95°C for 3 min, followed by 40 cycles of a two-stage temperature profile of 95°C for 3 seconds and 60°C for 30 seconds.

Table 2 – Sequence of primers used to determine *B. acififaciens* and *B. dorei* copy number by quantitative RT-PCR

Target	Forward sequence	Reverse sequence
B. acidifaciens	AATCTCTTCTAGAGACTGGGAA	AGCTGATTGACTAAAGTAGCTGGA
B. dorei	GTTGGCTCAGCCAGATGCA	AGCCTACTCATTGGGATCATCTTG

2.5. Leukocyte isolation for flow cytometry

Tumor single-cell suspensions and mesenteric lymph node (mLN) single-cell suspensions were prepared by passing the tissues through a 70-µm-pore- size nylon cell strainer (BD Biosciences). To isolate lamina propria leukocytes, ceca were flushed with Ca- and Mg-free PBS with 25 mM HEPES (Gibco), 50 mM sodium bicarbonate (Sigma-Aldrich) and 5% FBS (Gibco) and cut into small fragments. These fragments were incubated in Ca- and Mg-free Hank's Balanced Salt Solution (Gibco) containing 1.3 mM EDTA (Sigma-Aldrich), 25 mM HEPES, and 2 mM L-Glutamine (Gibco), under 200 rpm agitation at 37°C for 30 min. Fragments were then transferred to new flasks and were incubated in DMEM medium (Gibco) supplemented with 0.15 mg/mL collagenase D (Roche), 10% FBS, 25 mM HEPES, and 2 mM L-Glutamine for 40 min under 200 rpm agitation at 37°C, before passing the tissues through a 70-µm-pore-size nylon cell strainer. All single cell suspensions were centrifuged and treated with red blood cell lysis buffer (10% NH_4Cl solution and 5% of PBS in distilled water). Single cell suspensions from tumors and

ceca were processed over a 40:80% Percoll (GE Healthcare) gradient. The leukocyte ring in the 40:80% Percoll interface was collected, washed with DMEM. Cells were stained with fluorochrome-conjugated antibodies for 30 min on ice. After washing, the mLN cells were processed for intracellular staining using the transcription factor buffer set (eBioscience) according to the manufacturer's instructions. Antibodies specific for CD45 (clone 30-F11), CD3 (clone 145-2C11), CD4 (clone GK1.5), FoxP3 (clone FJK-16s), IgA (clone mA-6E1) and CD19 (clone 6D5) were from BD Biosciences, Biolegend or eBioscience. Samples were acquired on a LSRII flow cytometer (BD Biosciences) and data analyzed using FlowJo software (TreeStar).

2.6. IgA quantification by ELISA

Cecal contents were weighted and homogenized by mechanical disruption with glass beads in endotoxin-free PBS. The samples were then centrifuged at 4000 rpm for 5 min and the supernatants were collected and stored at -80°C. The levels of IgA were measured by ELISA (eBioscience) following the manufacturers' instructions.

2.7. Antibody treatment

The *in vivo* depletion of Gr-1$^+$ cells was carried out by intraperitoneal (i.p.) injections of 200 µg of anti-mouse Gr-1 (clone RB6-8C5, BioXCell) in endotoxin-free PBS. Antibody injections were performed at days 5, 7, 9 and 11 of the experimental procedure.

2.8. Statistical Analysis

Data are represented as mean ± standard error of the mean (SEM). One-way ANOVA, Two-way ANOVA with Bonferroni multiple comparisons post-test and the Student's t-test were used for statistical comparisons using GraphPad Prism 9. Values were considered significant for $p \leq 0.05$ and represented with * for $p \leq 0.05$; ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.001$.

3. Results and Discussion

IL-10 is a pleiotropic cytokine with anti-inflammatory and immunostimulatory functions. While in cancer IL-10 has been ascribed both pro- or anti-tumor effects [22], this cytokine plays a crucial role in down-modulating microbiota-activated mucosal inflammatory cytokines in the gut [16,17]. Taking into account our recent data showing that dietary zinc supplementation promotes extra-intestinal tumor overgrowth by modulating the gut microbiota (Chapter II), we hypothesized that the anti-tumor effects of IL-10 are, at least in part, dependent on its function in down-modulating microbiota-activated inflammatory responses. To test our hypothesis, we used a mouse model of controlled IL-10 overexpression (pMT-10 mice) which is induced by supplementing the diet of mice with 15g/L of zinc sulfate in the drinking water [20]. Therefore, B6 and pMT-10 mice were injected with LLC cells in the right hind flank and, the same day of tumor implantation, the diets of both groups of mice were supplemented with zinc sulphate in the drinking water. Tumor growth was monitored for a period of 18 days.

Our data showed that both B6 and pMT-10 mice maintained in normal chow display similar tumor g wth. As previously demonstrated (Chapter II), B6 mice under dietary zinc supplementation display increased tumor overgrowth when compared to B6 or pMT-10 mice maintained in normal chow (Figure 1). Crucially, pMT-10 mice under dietary zinc supplementation (and therefore expressing high levels of IL-10) displayed smaller tumors than B6 mice in the same diet (Figure 1). Despite this, tumor growth in this group was still significantly higher than that of B6 or pMT-10 in normal chow (Figure 1). Therefore, while the role of IL-10 in tumorigenesis is still controversial [22] with reports showing the presence of elevated levels of IL-10 in the serum of cancer patients associated with poor prognosis [23], our data show that increased levels of IL-10 systemically are associated with reduced tumor growth.

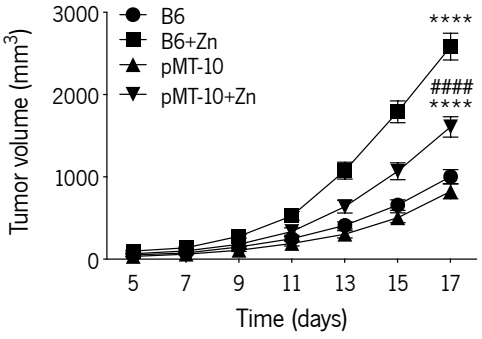

Figure 1. High levels of IL-10 prevent tumor overgrowth upon dietary zinc supplementation. B6 and pMT-10 mice were subcutaneously injected in the right hind flank with LLC cell line and given zinc from day 0 to day 18. Tumor growth was measured from day 5 and every other day until the end of the experiment. Data points represent the mean±SEM of one representative experiment

(out of 2) with 5 mice per group. Statistical analysis was performed using Two-Way ANOVA with Bonferroni post-test. ****p≤0.0001 (to compare B6/pMT-10 with B6+Zn/pMT10+Zn) and #### p≤0.0001 (to compare B6+Zn with pMT10+Zn)

Our previous data showed that dietary zinc supplementation promoted tumor overgrowth through alterations of the composition of the gut microbiota. Therefore, we tested the impact of microbiota depletion in pMT-10 mice. To do this, we depleted the microbiota of mice using broad-spectrum antibiotics ampicillin, colistin and streptomycin, as previously described ([24], Chapter II). Mice were then implanted with LLC cells in the right hind flank and tumor growth was followed over the period of 17 days. We found that tumor growth was higher in pMT-10 mice under dietary zinc supplementation than in pMT-10 mice maintained in regular chow, independently of microbiota depletion (Figure 2A). Crucially, microbiota depletion in pMT-10 mice under short-term dietary zinc supplementation prevented tumor overgrowth (Figure 2A). Another important conclusion that can be draw with this experiment is that IL-10, by itself, did not interfere with tumor growth in this model. Indeed, antibiotic-treated pMT-10 mice under dietary zinc supplementation (i.e. microbiota-depleted mice expressing high levels of IL-10) and control mice (i.e., pMT-10 mice in regular chow) display similar tumor growth (Figure 2A). In this regard, others have shown that increased intratumor levels of IL-10 is associated with reduced tumor growth [8–10]. However, in these studies the overexpression of IL-10 was accomplished by bioengineered tumor cells; as such, it is possible that the levels of IL-10 are much higher than in our model. Future studies are required to address this issue.

We next determined if dietary zinc supplementation promoted similar alterations in the microbiota composition of pMT-10 as we have reported in B6 mice (Chapter II). We found increased numbers of *B. acidifaciens* and *B. dorei* in the feces of pMT-10 mice under dietary zinc supplementation (Figure 2B), indicating that dietary zinc supplementation does induce dysbiosis in pMT10 mice; however, the increased production of IL-10 prevents, at least in part, the dysbiosis-induced tumor overgrowth.

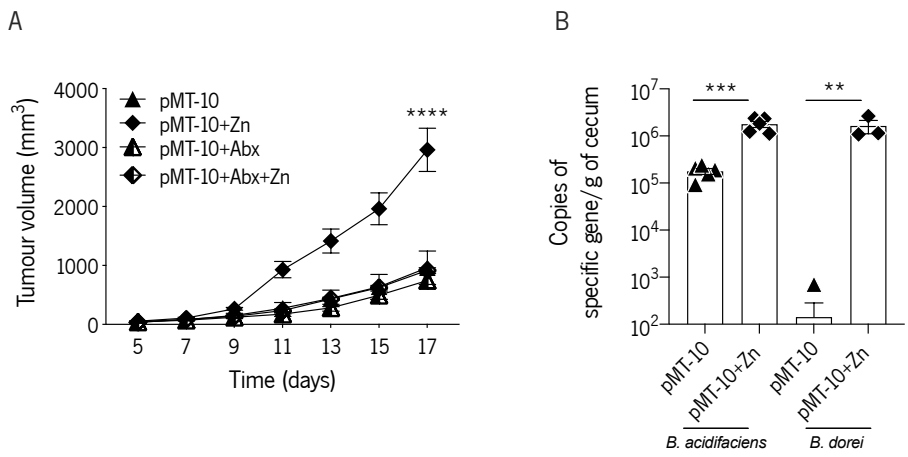

Figure 2. Dietary zinc supplementation induces microbiota alterations in pMT-10 mice. pMT10 mice were subcutaneously injected in the right hind flank with LLC cell line and given zinc from day 0 to day 18. Antibiotic-treated mice underwent a 2 week-long protocol of antibiotic treatment before tumor implantation and dietary zinc supplementation (A) Tumor growth was measured from day 5 and every other day until the end of the experiment. (B) The number of copies of specific genes from *B. acidifaciens* and *B. dorei* in the cecum of mice. Data points represent the mean±SEM of one experiment with 5 mice per group. Statistical analysis was performed using (A) Two-Way ANOVA with Bonferroni post-test and (B) Student's t test. **p≤0.01, ***p≤0.001 and ****p≤0.0001.

We have previously reported that upon short-term dietary zinc supplementation, tumor-bearing B6 mice display increased frequency of FoxP3$^+$ T lymphocytes in the mLN and increased accumulation of Gr1$^+$ cells in the tumors (Chapter II). Importantly, our data showed that the latter population are MDSCs as their depletion significantly reduced tumor overgrowth (Chapter II). Therefore, we analyzed these populations in pMT-10 mice and found that, unlike for B6 mice, dietary zinc supplementation did not display increased frequency of FoxP3$^+$ T lymphocytes in the mLN (Figure 3A). We then depleted Gr1$^+$ cells and found that tumor-bearing pMT-10 mice under dietary zinc supplementation display tumors similar to that of pMT-10 mice maintained in normal chow (Figure 3B).

Our previous data showed that depletion of Gr1$^+$ cells in B6 mice under short-term dietary zinc supplementation reduced tumor overgrowth (Chapter II). Our data now show that depletion of this population in mice overexpressing IL-10 (pMT-10 mice under dietary zinc supplementation) reverts tumor overgrowth to the same levels of control mice (Figure 3B). In addition to IL-10 overexpression, the only difference that we found in these two models is the accumulation of FoxP3$^+$ T lymphocytes in the mLN of B6 mice, which is not observed in pMT-10 mice (Figure 3A). While this population has been associated with suppression of tumor immunity, including by enhancing MDSCs proliferation and acquisition of immunosuppressive function [25], our data now suggest that the increased accumulation of FoxP3$^+$ T

lymphocytes in the mLN and Gr1$^+$ cells in the tumor are likely two independent mechanisms. Nonetheless, further studies are required to address this issue.

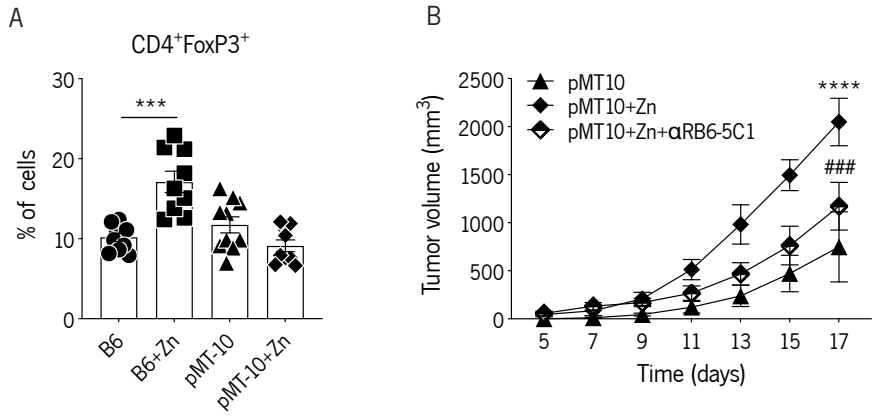

Figure 3. Depletion of Gr1$^+$cells in pMT10 mice under dietary zinc supplementation reduces tumor growth. pMT10 mice were subcutaneously injected in the right hind flank with LLC cell line and given zinc from day 0 to day 18. (A) Percentage of CD4$^+$FoxP3$^+$ T lymphocytes in the mLN; (B) Mice were treated with αRB6-5C1 antibody at days 5, 7, 9 and 11 of experimental protocol. Data points represent the mean±SEM of two experiments with 5 mice per group. Statistical analysis was performed using (A) One-way ANOVA and (B) Two-way ANOVA with Bonferroni post-test. ****p≤0.0001 (to compare pMT-10 with pMT10+Zn) and ### p≤0.001 (to compare pMT10+Zn with pMT10+Zn+αRB6-5C1)

IL-10 has been shown to play a crucial role in inducing B cell differentiation into IgA secreting cells [26,27]. In turn, IgA mediates microbial homeostasis at the intestinal mucosa by controlling bacterial growth and interaction with intraepithelial cells and immune cells, as well as neutralize their toxins [28-30]. Considering the central role of microbiota in our model, we evaluated the frequency of B lymphocytes expressing IgA in the gut mucosa and the levels of IgA in the intestinal contents of tumor-bearing B6 and pMT-10 mice.

We found that the frequency of B lymphocytes expressing IgA was similar in B6 and pMT-10 mice maintained in normal chow (Figure 4A). Importantly, dietary zinc supplementation did not alter the frequency of IgA-expressing B lymphocytes in B6 mice; however, there was a significant increase in tumor-bearing pMT-10 mice under dietary zinc supplementation (Figure 4A). These results correlated with the level of IgA in the intestinal lumen (Figure 4B). These data suggest that IL-10 overexpression enhances the differentiation of IgA-secreting B lymphocytes and the concentration of IgA in the intestinal contents of mice. While further studies are needed to determine the role of IgA, our data suggest that the elevated levels of IgA induced by IL-10 play a crucial role in neutralizing the tumorigenic effect of the microbiota from mice under dietary zinc supplementation.

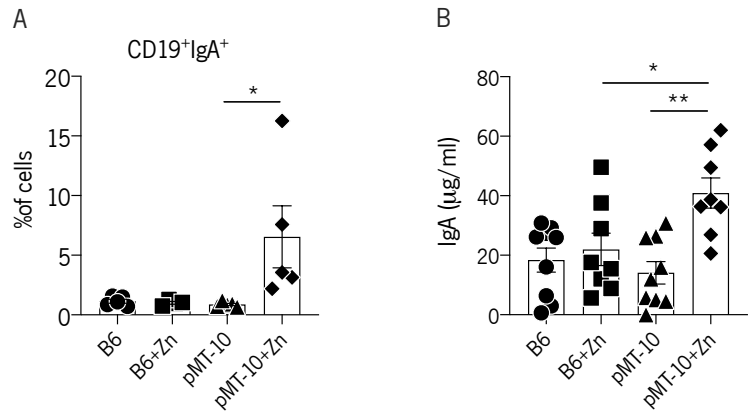

Figure 4. Increased levels of IL-10 in pMT10 were associated to increased accumulation of IgA in the intestine. pMT10 mice were subcutaneously injected in the right hind flank with LLC cell line and given zinc from day 0 to day 18. At end of the experimental protocol, large intestine was harvested and processed to recover lamina propria leukocytes; single cell suspensions were prepared for flow cytometry analysis. (A) Percentage of $CD19^{+}IgA^{+}$ B lymphocytes. Intestinal tissue was disrupted, and supernatant was collected for (B) IgA quantification. Data points represent the mean±SEM of two experiments with 5 mice per group. Statistical analysis was performed using One-way ANOVA *p≤0.05 and **p≤0.01.

In all, our data shows that increased levels of IL-10 stimulate the production of IgA in the gut mucosa that is likely preventing the tumorigenic activity of microbiota. Thus, our results unravel a potential role for IL-10 in modulating anti-tumor immune response without actively interfering in the tumor microenvironment.

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Chapter IV

Increased $CD3^+$, $CD8^+$, or $FoxP3^+$ T Lymphocyte Infiltrations Are Associated with the Pathogenesis of Colorectal Cancer but Not with the Overall Survival of Patients

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Increased CD3$^+$, CD8$^+$, or FoxP3$^+$ T Lymphocyte Infiltrations Are Associated with the Pathogenesis of Colorectal Cancer but Not with the Overall Survival of Patients

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Simple Summary: Colorectal cancer (CRC) is amongst the deadliest cancers. Surgical excision of the primary tumor is the curative intent treatment; however, recurrence occurs in approximately 20% of patients. Therefore, novel staging protocols are crucial to inform clinicians which patients will recur. In this study, we explored the prognostic potential of tumor-infiltrating lymphocytes. Our data did not reveal any association between intratumor lymphocyte infiltrations with clinical or pathological data. On the other hand, the presence of CD3$^+$, CD8$^+$, or FoxP3$^+$ lymphocyte infiltration in the tumor invasive margins were associated with markers of good prognosis. Despite this, we were not able to find any statistically significant alterations in the overall survival of patients, even though high infiltrations of FoxP3$^+$ T lymphocytes in the tumor margin resulted in an increased overall survival of 14 months. Taken together, our data show that the location and type of tumor-infiltrating lymphocytes are associated with the pathogenesis of CRC; however, only high FoxP3$^+$ T lymphocyte infiltrations are inclined to indicate favorable prognosis.

Abstract: Tumor-infiltrating lymphocytes include heterogeneous populations of T lymphocytes that play crucial roles in the tumor immune response; importantly, their presence in the tumor tissue may predict clinical outcomes. Therefore, we herein studied the prognostic significance of the presence and location of CD3$^+$, CD8$^+$, and FoxP3$^+$ T lymphocytes in colorectal cancer samples. In the intratumor analysis, our data did not reveal any association between lymphocyte infiltrations with clinical or pathological data. However, in the tumor margins, we found that the presence of high infiltrations of CD3$^+$, CD8$^+$, or FoxP3$^+$ T lymphocytes were associated with TNM stages I-II ($p = 0.021$, $p = 0.022$, and $p = 0.012$, respectively) and absence of lymph node metastases ($p = 0.010$, $p = 0.003$, and $p = 0.004$, respectively). Despite these associations with good prognostic indicators, we were not able to find any statistically significant alterations in the overall survival of the patients, even though high infiltrations of FoxP3$^+$ T lymphocytes in the tumor margins resulted in an increased overall survival of 14 months. Taken together, these data show that the presence of CD3$^+$, CD8$^+$, or FoxP3$^+$T lymphocyte infiltrates in the tumor margins are associated with the pathogenesis of CRC, but only high Foxp3$^+$ T lymphocyte infiltrations in the tumor invasive margins are inclined to indicate favorable prognosis.

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1. Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide, and its occurrence is responsible for nearly 10% of all deaths related to malignancies [1]. Historically, the incidence of CRC has been low at ages younger than 50 years; however, in recent years, there has been a rising incidence of CRC at these ages [2]. This emerging trend is prompting a rapid increase in the number of CRC cases in previously low-risk countries, a phenomenon ascribed to changes in dietary patterns and risk factors towards a more western lifestyle [3]. The cornerstone of curative intent treatment for CRC remains surgical excision of the primary tumor [4]. While this approach is curative for most patients, recurrence of CRC disease occurs in approximately 20% of patients [5]. Therefore, after tumor resection, clinicians often have follow-up appointments with patients to detect any recurrence at an early and treatable stage. These follow-ups have unraveled the need for novel predictive prognosis biomarkers and well-established staging protocols to inform clinicians which patients will recur.

The American Joint Committee on Cancer first defined the Tumor Node Metastasis (TNM) staging system to inform on patients' prognosis [6]. Currently, TNM is the most widely used staging system for CRC, and its application in the clinic has been crucial to inform patients' prognosis, also having a considerable and direct impact on the treatment that patients receive [6]. However, clinical evidence suggests that the outcome of the disease varies significantly among patients within the same TNM stage [7–9]. This is particularly noticeable at TNM stage II, wherein one-third of all patients may still die of recurrent disease [10,11]. Contrarily, patients at TNM stage III may be cured of the disease by surgery alone [12]. As such, under or overtreatment may occur between stages determined by the TNM system, as previously demonstrated [13,14]. Additionally, the lack of consensus on the application of TNM staging and the constant update of revised versions are significant hurdles in comparing different cohorts [9,15,16]. Therefore, clinical practice may benefit from the inclusion of other staging methodologies to discriminate patients who may benefit from additional therapies, such as adjuvant chemotherapies.

Over the last decade, there has been a progressive increase in our understanding of the tumor microenvironment, which prompted the identification of key players of the immune response to tumors. Particularly important and with prognostic potential in CRC are tumor-infiltrating lymphocytes (TIL), which are heterogeneous populations of T lymphocytes present in the tumor microenvironment [17–21]. In this regard, the presence of $CD8^+$ T lymphocytes has been associated with good prognosis in different types of solid tumors [22,23]. This T lymphocyte population mediates anti-tumor activity through antigen-specific cytotoxicity and by producing anti-tumor cytokines, namely IFN-γ and TNF-α [24,25]. On the other hand, increased tumor infiltration by FoxP3-expressing T lymphocytes has been associated with reduced overall survival of patients with different types of cancer, including breast [26], lung [27], and cervical cancers [28]. However, this association is not seen in all cancer types, as $FoxP3^+$ T lymphocyte infiltrates have been associated with good prognosis in other cancers, such as head and neck cancers [29]. In colorectal cancer, $FoxP3^+$ T lymphocyte infiltrates have been associated with good and bad prognosis by different studies [30–33]. Taken together, these data warrant further investigation on the prognostic potential of $FoxP3^+$ T lymphocytes in colorectal cancer.

The analysis of immune infiltrates and their correlation with patients' pathological records originated the development of staging methods based on the intratumor and invasive margin infiltration of $CD3^+$ and $CD8^+$ T lymphocytes [34]. While the predictive capacity of TNM staging is more reliable than alternative methods, such as DNA content or genetic features, the analysis of tumor immune infiltrate has been suggested to surpass

the TNM classification in multivariate analyses [35]. Indeed, after adjusting for TNM stage, recent data suggest that the density of $CD3^+$ T lymphocytes remained as an independent prognostic factor [36]. Furthermore, patients with low numbers of tumor-infiltrating $CD8^+$ T lymphocytes relapsed more independently of the T stage of the tumor [19]. These data demonstrate the high prognostic utility of TILs in staging CRC patients. However, as discussed above, the interaction between different populations of TILs in the tumor or invasive margin may influence tumor progression or control. As such, it is crucial to define the prognostic utility of the different populations of T lymphocytes.

In this work, we analyzed the presence of lymphocyte infiltrates, specifically $CD3^+$, $CD8^+$, and $FoxP3^+$ T lymphocytes, in CRC tumors including their invasive margins to evaluate their association with clinicopathological information and overall survival of patients. We did not find any associations between the presence and extent of intratumor T lymphocyte infiltrations with the clinical or pathological data of the patients. On the other hand, the infiltration of $CD3^+$, $CD8^+$, or $FoxP3^+$ lymphocytes in the tumor invasive margins were associated with the pathogenesis of CRC, but only $FoxP3^+$ T lymphocyte infiltrations were inclined to indicate favorable prognosis.

2. Materials and Methods

2.1. Patients Specimens

A total of 194 samples of colorectal cancer (CRC) at stage I to IV were used in this retrospective study. These samples were collected from patients diagnosed with CRC that underwent surgical excision of the primary tumor at the Hospital of Braga, Portugal, between January 2005 and January 2010. The CRC tissue extracted during the surgery was formalin-fixed and embedded in paraffin. Clinical and pathological data was available for 184 cases and was obtained through medical charts and pathology reports (Table 1).

Table 1. Demographic and baseline characteristics of the patients.

Parameter	N	Parameter	N	Parameter	N
Gender		Time to Diagnosis		TNM	
Male	120	<6 months	126	I-II	50
Female	64	>6 months	24	III-IV	134
Age (years)		Localization		Histological Type	
≤45	5	Colon	129	Adenocarcinoma	170
>45	179	Rectum	55	Mucinous	12
Previous tumors		CEA (ng/mL)		Signet ring and mucinous	2
Without	150	≤10	132	Differentiation	
With	34	>10	22	Well/Moderately	167
				Poorly/Undifferentiated	16
Family History		Metastasis		Lymph node metastasis	
Without	156	No	138	Without	106
With	15	Yes	46	With	77
Presentation		Tumor size (cm)			
Asymptomatic	34	≤4.5	107		
Symptomatic	150	>4.5	70		

2.2. Immunohistochemistry

Tumor-infiltrating lymphocytes were detected by immunohistochemistry using antibodies against CD3 (MCA1477, BioRad), CD8 (ab4055, Abcam), or FoxP3 (14477782, Invitrogen). Briefly, whole-tissue sections were deparaffinized and hydrated to prepare the tissue for the staining. The slides from whole-tissue sections were incubated for 30 min in citrate buffer at 96 °C followed by incubation in hydrogen peroxide for 10 min at room temperature (RT). The slides were then incubated for 1 h with blocking solution (PBS with 5% BSA and 0.05% Tween 20) before incubation with primary antibodies at 4 °C for 16 h. After washing with PBS, the slides were incubated with biotinylated-secondary antibodies for 1 h at RT. Slides were then incubated with streptavidin for 1 h followed by an incubation with chromogen (DAB; Dako) for 10 min, and then counterstained with hematoxylin. Amygdala sections were used as positive controls.

Immunostaining analysis was used to determine the presence of CD3, CD8, or FoxP3 infiltrates in the tumor, tumor invasive margins, and normal adjacent colon epithelium using an Olympus BX41 microscope. Immunostaining was considered positive whenever there was cytoplasmatic and membrane staining for CD3, membrane staining for CD8, and nuclei staining for FoxP3. Grading of the immunostaining was performed in a blind fashion by consensus of two experienced pathologists, without having prior knowledge of the pathological stage or any other clinical or follow-up data for each case. Briefly, all samples were first analyzed under the microscope at a magnification of 100× to determine the extent of infiltration for each marker. From this analysis, the grade of CD3 and CD8 infiltration was categorized as <10%, 10–50%, or >50%, and the grade of FoxP3 infiltration was categorized as <10%, 10–30%, or >30%, as the expression of this marker was less extensive than of CD3 or CD8. Ten high magnification fields (×400) from each region of the tumor were then semi-quantitatively analyzed to determine the number of lymphocytes that stained positive for each marker.

For statistical analysis, each section was then classified as either low (<50%) or high (>50%). Representative images (Figure 1) were obtained under brightfield microscopy (Olympus BX61) and were recorded with a digital camera (DP70) using the Cell∧P software.

2.3. Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social program Science (SPSS), version 24.0, SPSS Inc.®, Chicago, IL, USA.

Simple descriptive analyses were performed, determining the total number of cases and relative frequencies for each clinical-pathological factor. To assess the existence of any association of clinical or pathological data with immunohistochemistry results, Pearson's chi-square test and Fisher's exact test (scattered data) were performed. Survival analysis was performed using Kaplan-Meier curves and significant differences were determined by the log-rank test. Survival was defined between the period of analysis and death from any cause. Patients who quit the study were censored on the date of the last contact. Confidence values (p) below 0.05 were considered statistically significant.

2.4. Ethics Statement

The study was approved by the Ethics Committee for Research in Life and Health Sciences at University of Minho (CEICVS 004/2020) and by the Ethics Committee of Hospital de Braga (32/2013).

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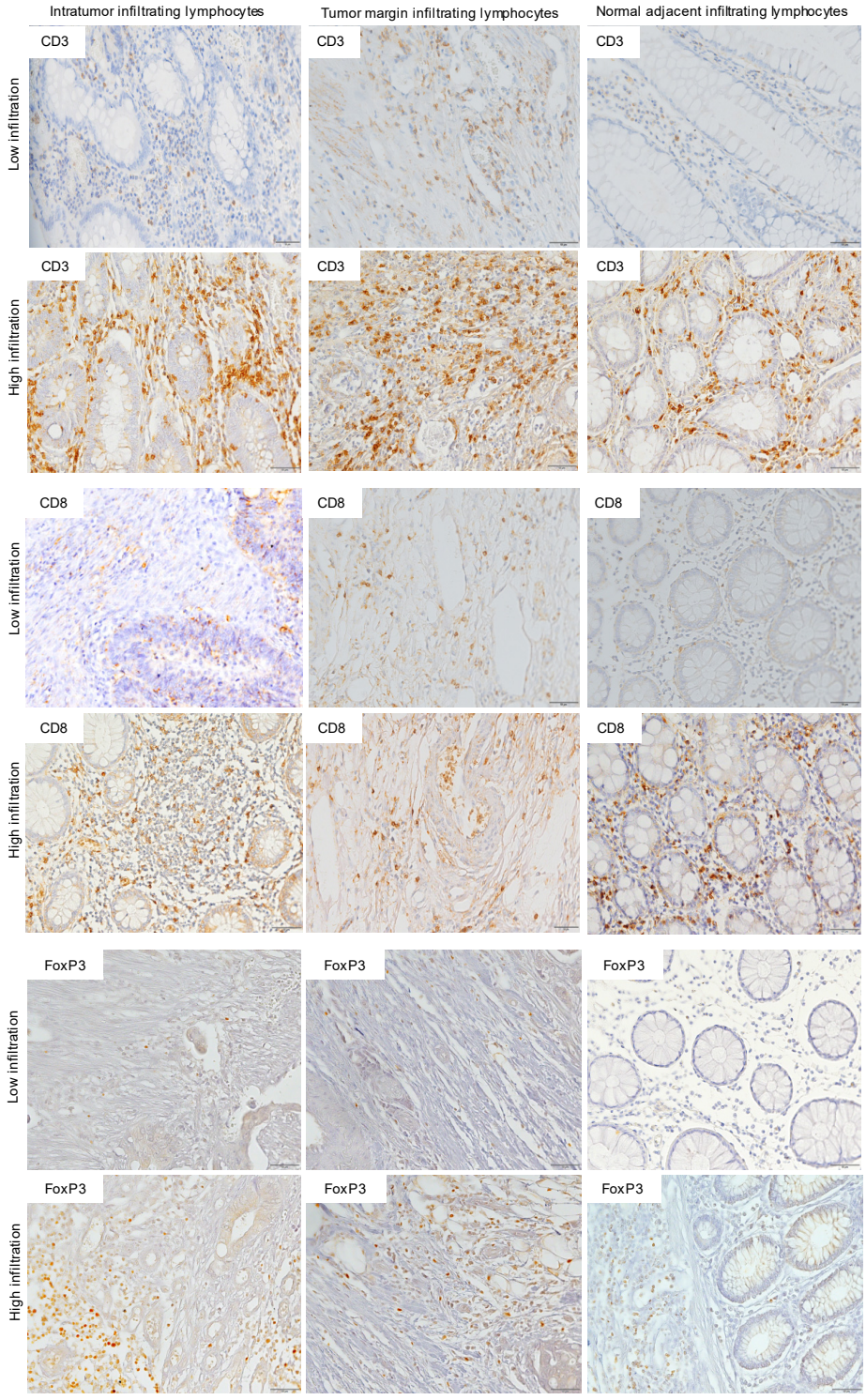

Figure 1. Immunohistochemistry (IHC) of CD3, CD8, and FoxP3 T lymphocytes in CRC. Representative IHC showing low and high $CD3^+$, $CD8^+$, and $FoxP3^+$ T lymphocytes densities. Magnification: 200×.

3. Results

3.1. CD3+, CD8+, or FoxP3+ T Lymphocyte Infiltrations Are Higher in the Tumor Tissue Than in the Normal Adjacent Tissue

To explore the association between tumor-infiltrating T lymphocyte populations with CRC prognosis, we determined the infiltration of $CD3^+$, $CD8^+$, or $FoxP3^+$ T lymphocytes within the tumor, tumor margins, and in the normal adjacent tissue by immunohistochemistry. We began by analyzing the extension of immunostaining for the different lymphocyte markers in the different regions of the samples (Table 2). This analysis revealed a high intratumor infiltration of $CD3^+$ T lymphocytes in 53% (103/194) of samples. When we compared the proportion of infiltrating lymphocytes that were $CD8^+$ or $FoxP3^+$, we found a high intratumor infiltration of $CD8^+$ T lymphocytes in 52% (97/187) and a high intratumor infiltration of $FoxP3^+$ T lymphocytes in 53% (103/196) of samples (Table 2). Regarding the infiltration of lymphocytes in the tumor margin, we also found a high infiltration of $CD3^+$ T lymphocytes in 59% (113/192), $CD8^+$ T lymphocytes in 58% (107/185), and $FoxP3^+$ T lymphocytes in 59% (114/194) of samples (Table 2). On the other hand, the extension of T lymphocyte infiltration in the normal adjacent tissue was lower than in the tumor tissue. Indeed, we found a high infiltration of $CD3^+$ T lymphocytes only in 43% (61/143), $CD8^+$ T lymphocytes in 41% (57/139), and $FoxP3^+$ T lymphocytes in 42% (62/146) of samples (Table 2).

Table 2. Distribution of cases according to the expression of CD3, FoxP3 and CD8 markers.

IHC Markers	CD3			FoxP3			CD8		
	N	Low (%)	High (%)	N	Low (%)	High (%)	N	Low (%)	High (%)
Intratumor Tissue	194	91 (47%)	103 (53%)	194	92 (47%)	102 (53%)	187	90 (48%)	97 (52%)
Tumor Margin Tissue	192	79 (41%)	113 (59%)	194	80 (41%)	114 (59%)	185	78 (42%)	107 (58%)
Normal Adjacent Tissue	143	84 (59%)	61 (43%)	146	84 (58%)	62 (42%)	139	82 (59%)	57 (41%)

We then determined the association between lymphocyte infiltration within the different tissues analyzed. As shown in Table 3, the high intratumor infiltration of $CD3^+$ T lymphocytes correlated with high infiltration of the same population in the tumor margin ($p < 0.001$). Similarly, the high infiltration $CD3^+$ T lymphocytes of the tumor margin correlated with high infiltration of the same population in the normal adjacent tissue ($p < 0.001$). However, there was no correlation between the intratumor infiltrations of $CD3^+$ T lymphocytes with the infiltration of these cells in normal adjacent tissue, indicating that these cells were actively recruited to the tumor. The same results were obtained for $CD8^+$ and $FoxP3^+$ T lymphocytes ($p < 0.001$ for all samples).

Table 3. Correlation of marker's expression according to the different regions analyzed.

Parameter	CD3 Intratumor				CD3 Tumor Margin			
	N	Low (%)	High (%)	*p*	N	Low (%)	High (%)	*p*
CD3 Tumor margin								
Low (%)	78	52 (66.7)	26 (33.3)	<0.001	-	-	-	-
High (%)	112	37 (33)	75 (67)		-	-	-	
CD3 Normal adjacent								
Low (%)	84	44 (52.4)	40 (47.6)	0.114	83	46 (55.4)	37 (44.6)	<0.001
High (%)	59	23 (39)	36 (61)		60	13 (21.7)	47 (78.3)	

Table 3. *Cont.*

Parameter	FoxP3 Intratumor				FoxP3 Tumor Margin			
	N	**Low (%)**	**High (%)**	***p***	**N**	**Low (%)**	**High (%)**	***p***
FoxP3 Tumor margin								
Low (%)	79	55 (69.6)	24 (30.4)	<0.001	-	-	-	-
High (%)	114	38 (33.3)	76 (63.7)		-	-	-	
FoxP3 Normal adjacent								
Low (%)	84	44 (52.4)	40 (47.6)	0.175	83	47 (56.6)	36 (43.4)	<0.001
High (%)	61	25 (41)	36 (59)		61	14 (23)	47 (77)	
Parameter	**CD8 Intratumor**				**CD8 Tumor Margin**			
	N	**Low (%)**	**High (%)**	***p***	**N**	**Low (%)**	**High (%)**	***p***
CD8 Tumor margin								
Low (%)	78	53 (28.8)	24 (31.2)	<0.001	-	-	-	-
High (%)	105	35 (33.3)	70 (66.7)		-	-	-	
CD8 Normal adjacent								
Low (%)	82	43 (52.4)	39 (47.6)	0.189	81	46 (56.8)	35 (43.2)	<0.001
High (%)	56	23 (41.1)	33 (58.9)		55	14 (25.5)	41 (74.5)	

Taken together, these data show that a significant proportion of samples had high T lymphocyte infiltrates and that the profile of infiltration is similar between the tumor and the margins. Furthermore, the infiltration of lymphocytes increased from the normal adjacent tissue to the tumor tissue.

3.2. High Intratumor Infiltration of FoxP3+ T Lymphocytes in Less Severe CRC Lesions

With the above data showing the presence of TIL in all tumor samples analyzed, we then sought to determine whether the degree of intratumor lymphocyte infiltration influenced CRC clinical outcome. To do this, we correlated the extension of intratumor infiltration of $CD3^+$, $CD8^+$, or $FoxP3^+$ T lymphocytes with the clinicopathological information of CRC patients.

Our analysis revealed no association between the clinical parameters with the extension of intratumor lymphocyte infiltration for all the immune populations analyzed (Table 4).

Table 4. Association of intratumor infiltration of $CD3^+$, $FoxP3^+$, and $CD8^+$ T lymphocytes with clinical data.

Parameter	CD3				FoxP3				CD8			
	N	**Low (%)**	**High (%)**	***p***	**N**	**Low (%)**	**High (%)**	***p***	**N**	**Low (%)**	**High (%)**	***p***
Gender												
Male	120	57 (47.5)	63 (52.5)	0.273	123	60 (48.8)	63 (51.2)	0.391	116	57 (49.1)	59 (50.9)	0.477
Female	64	25 (39.1)	39 (60.9)		64	27 (42.2)	37 (57.8)		62	27 (43.5)	35 (56.5)	
Age (years)												
≤45	5	3 (60)	2 (40)	0.481	5	3 (60)	2 (40)	0.540	5	3 (60)	2 (40)	0.561
>45	179	79 (44.1)	100 (55.9)		182	84 (46.2)	9 (53.8)		173	81 (46.8)	92 (53.2)	
Previous tumors												
Without	150	67 (44.7)	83 (55.3)	0.954	152	71 (46.7)	81 (53.3)	0.915	147	69 (46.9)	78 (53.1)	0.883
With	34	15 (44.1)	19 (55.9)		35	16 (45.7)	19 (54.3)		31	15 (48.4)	16 (51.6)	
Family History												
Without	156	73 (4.8)	83 (53.2)	0.135	159	77 (48.4)	82 (51.6)	0.106	152	74 (48.7)	78 (51.3)	0.214
With	15	4 (26.7)	11 (73.3)		15	4 (26.7)	11 (73.3)		13	4 (30.8)	9 (69.2)	
Presentation												
Asymptomatic	34	18 (52.9)	16 (47.1)	0.276	34	19 (55.9)	15 (44.1)	0.226	33	18 (54.5)	15 (45.5)	0.348
Symptomatic	150	64 (42.7)	86 (57.3)		153	68 (44.4)	85 (55.6)		145	66 (45.5)	79 (54.5)	

Table 4. *Cont.*

Parameter	CD3				FoxP3				CD8			
	N	Low (%)	High (%)	*p*	N	Low (%)	High (%)	*p*	N	Low (%)	High (%)	*p*
Time to Diagnosis												
<6 months	126	57 (45.2)	69 (54.8)	0.145	129	61 (47.3)	68 (52.7)	0.101	121	59 (48.8)	62 (51.5)	0.078
>6 months	24	7 (29.2)	17 (70.8)		24	7 (29.2)	17 (70.8)		24	7 (29.2)	17 (70.8)	
Localization												
Colon	129	57 (44.2)	72 (55.8)	0.874	131	61 (46.6)	70 (53.4)	0.986	124	60 (48.4)	64 (51.6)	0.628
Rectum	55	25 (45.5)	30 (54.5)		56	26 (46.4)	30 (53.6)		54	24 (44.4)	30 (55.6)	

We then analyzed the association between the pathological parameters with the extension of intratumor lymphocyte infiltrations (Table 5). As for clinical parameters, our analysis did not find any association between pathological parameters and the extension of intratumor T lymphocyte infiltration for all the immune populations analyzed (Table 5).

Table 5. Association of intratumor infiltration of $CD3^+$, $FoxP3^+$, and $CD8^+$ T lymphocytes with pathological data.

Parameter	CD3				FoxP3				CD8			
	N	Low (%)	High (%)	*p*	N	Low (%)	High (%)	*p*	N	Low (%)	High (%)	*p*
CEA (ng/mL)												
≤10	132	57 (43.2)	75 (56.8)	0.075	134	60 (44.8)	74 (55.2)	0.153	131	60 (45.8)	71 (54.2)	0.097
>10	22	14 (63.6)	8 (36.4)		23	14 (60.9)	9 (30.1)		18	12 (66.7)	6 (33.3)	
Metastasis												
No	138	57 (41.3)	81 (58.7)	0.123	141	61 (43.3)	80 (56.7)	0.117	135	60 (44.4)	76 (55.6)	0.193
Yes	46	25 (54.3)	21 (45.7)		46	26 (56.5)	20 (43.5)		43	24 (55.8)	19 (44.2)	
Tumor Size (cm)												
≤4.5	107	49 (45.8)	58 (54.2)	0.844	110	52 (47.3)	58 (52.7)	0.942	103	49 (47.6)	54 (52.4)	0.830
>4.5	70	31 (44.3)	39 (55.7)		69	33 (47.8)	36 (52.2)		67	33 (49.3)	34 (50.7)	
Histological Type												
Adenocarcinoma	170	75 (44.1)	95 (55.9)	0.913	172	79 (45.9)	93 (54.1)	0.751	163	76 (46.6)	87 (53.4)	0.773
Mucinous	12	6 (50)	6 (50)		12	6 (50)	6 (50)		12	6 (50)	6 (50)	
Signet ring and mucinous	2	1 (50)	1 (50)		3	2 (66.7)	1 (33.3)		3	2 (66.7)	1 (33.3)	
Differentiation												
Well/Moderately	167	73 (43.7)	94 (56.3)	0.473	168	77 (45.8)	91 (54.2)	0.425	169	74 (46.5)	85 (53.4)	0.458
Poorly/Undifferentiated	15	8 (53.3)	7 (46.7)		16	9 (56.3)	7 (43.8)		16	9 (56.3)	7 (43.8)	
Lymph Node Metastasis												
Without	106	44 (41.5)	62 (58.5)	0.292	106	45 (42.5)	61 (57.5)	0.126	101	44 (43.6)	57 (56.4)	0.17
With	77	38 (49.4)	39 (50.6)		78	42 (53.8)	36 (46.2)		74	40 (54.1)	34 (45.9)	
TNM												
I-II	50	21 (42)	29 (58)	0.669	51	20 (39.2)	31 (60.8)		48	19 (39.5)	29 (60.4)	0.201
III-IV	134	61 (45.5)	73 (54.5)		135	67 (49.6)	68 (50.4)		129	65 (50.4)	64 (49.6)	
Overall survival												
Months ± SD	184	106.8 ± 7.5	93.3 ± 7.2	0.294	187	103.4 ± 7.2	98.1 ± 7.3	0.598	178	103.3 ± 7.5	94.0 ± 7.5	0.358

These data show that the extent of intratumor T lymphocyte infiltrations does not impact the pathogenesis of CRC.

3.3. High $CD3^+$, $CD8^+$, or $FoxP3^+$ T Lymphocytes in the Tumor Margins Are Associated with Good Prognostic Indicators

As we found high lymphocyte infiltrates in the tumor margins of a significant number of samples, we next determined if the accumulation of $CD3^+$, $CD8^+$ or $FoxP3^+$ T lymphocytes were associated with any CRC clinical or pathological parameters.

Our analysis did not uncover any association with clinical parameters (Table 6).

Table 6. Association of tumor margin infiltration of $CD3^+$, $FoxP3^+$, and $CD8^+$ T lymphocytes with clinical data.

Parameter	CD3				FoxP3				CD8			
	N	Low (%)	High (%)	*p*	N	Low (%)	High (%)	*p*	N	Low (%)	High (%)	*p*
Gender												
Male	118	48 (40.7)	70 (59.3)	0.303	122	51 (41.8)	71 (58.2)	0.296	115	49 (42.6)	66 (57.4)	0.529
Female	62	22 (35.5)	40 (64.5)		63	23 (36.5)	40 (63.5)		61	23 (37.5)	38 (62.3)	
Age (years)												
≤45	5	2 (40)	3 (60)	0.646	5	2 (40)	3 (60)	0.684	5	2 (40)	3 (60)	0.967
>45	175	68 (38.9)	108 (61.1)		180	72 (40)	108 (60)		171	70 (40.9)	101 (59.1)	
Previous tumors												
Without	148	58 (39.2)	90 (60.8)	0.513	151	61 (40.4)	90 (59.6)	0.488	146	59 (40.4)	87 (56.9)	0.767
With	32	12 (37.5)	20 (62.5)		34	13 (38.2)	21 (61.8)		30	13 (43.3)	17 (56.7)	
Family History												
Without	152	57 (37.5)	95 (62.5)	0.407	156	60 (38.5)	96 (61.5)	0.437	149	58 (38.9)	91 (61.1)	0.418
With	16	7 (43.8)	8 (56.6)		16	7 (43.8)	9 (56.3)		14	7 (50)	7 (50)	
Presentation												
Asymptomatic	32	10 (31.3)	22 (68.8)	0.22	33	11 (33.3)	22 (66.7)	0.254	32	10 (31.3)	22 (68.8)	0.219
Symptomatic	148	60 (40.5)	88 (59.5)		152	63 (41.4)	89 (58.6)		144	62 (43.1)	82 (56.9)	
Time to Diagnosis												
<6 months	125	52 (41.6)	73 (58.4)	0.325	128	54 (42.2)	74 (57.8)	0.424	120	53 (44.2)	67 (55.8)	0.547
>6 months	23	8 (34.8)	15 (65.2)		24	9 (37.5)	15 (62.5)		24	9 (37.5)	15 (62.5)	
Localization												
Colon	126	51 (40.5)	75 (59.5)	0.310	131	55 (42)	76 (58)	0.245	124	55 (44.4)	69 (55.6)	0.151
Rectum	54	19 (35.2)	35 (64.8)		54	19 (35.2)	35 (64.8)		52	17 (32.7)	35 (67.3)	

However, when we did the same analysis for pathologic parameters, we found that high accumulation of $CD3^+$ T lymphocytes was associated with normal levels of carcinoembryonic antigen (CEA) ($p = 0.026$), an important biomarker of different types of cancer, including CRC [37] (Table 7). High infiltration of $FoxP3^+$ or $CD8^+$ T lymphocytes also showed a tendency with normal CEA levels ($p = 0.057$ and $p = 0.053$, respectively), with *p*-values very close to the statistical threshold. Additionally, the absence of lymph node metastasis was positively correlated with a high accumulation of $CD3^+$, $CD8^+$, or $FoxP3^+$ T lymphocytes ($p = 0.01$, $p = 0.004$, and $p = 0.003$, respectively). We also found a tendency associating the presence of high $CD3^+$ infiltration with the absence of distant metastases ($p = 0.054$). Finally, the high infiltration of $CD3^+$, $CD8^+$, and $FoxP3^+$ T lymphocytes was associated with TNM stages I-II ($p = 0.021$, $p = 0.022$, and $p = 0.012$, respectively). Together, these results show that high infiltrations of $CD3^+$, $CD8^+$, or $FoxP3^+$T lymphocytes in the tumor margins are associated with good prognostic indicators.

Table 7. Association of tumor margin infiltration of $CD3^+$, $FoxP3^+$, and $CD8^+$ T lymphocytes with pathological data.

Parameter	CD3				FoxP3				CD8			
	N	Low (%)	High (%)	*p*	N	Low (%)	High (%)	*p*	N	Low (%)	High (%)	*p*
CEA (ng/mL)												
≤10	128	44 (34.4)	84 (65.6)	0.026	132	48 (36.4)	84 (63.8)	0.057	129	48 (37.2)	81 (62.8)	0.053
>10	22	13 (59.1)	9 (40.9)		23	13 (56.5)	10 (43.5)		18	11 (61.1)	7 (38.9)	
Metastasis												
No	134	47 (35.1)	87 (64.9)	0.054	139	51 (36.7)	88 (63.8)	0.078	133	50 (37.6)	83 (62.4)	0.116
Yes	46	23 (50)	23 (50)		46	23 (50)	23 (50)		43	22 (51.2)	21 (48.8)	

Table 7. *Cont.*

Parameter	CD3				FoxP3				CD8			
	N	**Low (%)**	**High (%)**	***p***	**N**	**Low (%)**	**High (%)**	***p***	**N**	**Low (%)**	**High (%)**	***p***
Tumor Size (cm)												
≤4.5	103	42 (40.8)	61 (59.2)	0.185	107	46 (43.0)	61 (57)	0.116	56	44 (44)	56 (56)	0.186
>4.5	70	23 (32.9)	47 (67.1)		70	23 (32.9)	47 (67.1)		45	23 (33.8)	45 (66.2)	
Histological Type												
Adenocarcinoma	166	62 (37.3)	104 (62.7)	0.336	170	65 (38.2)	105 (61.8)	0.248	161	63 (39.1)	98 (60.9)	0.281
Mucinous	12	7 (58.3)	5 (41.7)		12	7 (58.3)	5 (41.7)		12	7 (58.3)	5 (41.7)	
Signet ring and mucinous	2	1 (50)	1 (50)		3	2 (66.7)	1 (33.3)		3	2 (66.7)	1 (33.3)	
Differentiation												
Well/Moderately	163	62 (38)	101 (62)	0.347	166	65 (39.2)	101 (60.8)	0.279	157	63 (40.1)	94 (59.9)	0.444
Poorly/undifferentiated	15	7 (46.7)	8 (53.3)		16	8 (50)	8 (50)		16	8 (50)	8 (50)	
Lymph Node Metastasis												
Without	103	32 (31.1)	71 (68.9)	0.010	105	33 (31.4)	72 (68.6)	0.004	100	32 (32)	68 (68)	0.003
With	76	38 (50)	38 (50)		78	41 (52.6)	37 (47.4)		74	40 (54.1)	34 (45.9)	
TNM												
I-II	47	12 (25.5)	35 (74.5)	0.021	48	12 (25)	36 (75)	0.012	45	12 (26.7)	33 (73.3)	0.022
III-IV	133	58 (43.6)	76 (56.4)		136	62 (45.6)	74 (54.4)		130	60 (46.2)	70 (53.8)	
Overall survival												
Months ± SD	180	93.0 ± 8.5	106.4 ± 6.7	0.117	185	92.8 ± 8.1	106.8 ± 6.7	0.092	176	93.2 ± 8.3	100.2 ± 7.0	0.224

3.4. The Presence of High CD3+, CD8+, or FoxP3+ T Lymphocyte Infiltrations Inside the Tumor or in Its Invasive Margins Does Not Impact the Overall Survival of Patients

To determine whether lymphocyte infiltrations impacted the patients' overall survival, we performed log-rank tests and constructed Kaplan–Meier curves.

Our analysis did not reveal any statistically significant association between the extension of intratumor (Figure 2A–C) or tumor margin (Figure 2D–F) lymphocyte infiltrations with the overall survival of patients. These data indicate that, in our cohort, tumor-infiltrating lymphocytes do not impact the clinical outcome of CRC patients. Interestingly however, while not statistically significant, our analysis revealed that high infiltrations of $CD3^+$, $CD8^+$, or $FoxP3^+$ T lymphocytes in the tumor margins resulted in increased overall survival of 13.4 ($p = 0.117$), 7 ($p = 0.224$), and 14 months ($p = 0.092$), respectively. On the other hand, high intratumor infiltrations of $CD3^+$, $CD8^+$, or $FoxP3^+$ resulted in a slight decrease in patients' overall survival of 13.5 ($p = 0.294$), 9.3 ($p = 0.358$), and 5.3 months ($p = 0.598$), respectively.

These data show that, while the intratumor infiltration by $FoxP3^+$ lymphocytes and infiltration of the tumor margins by $CD3^+$, $CD8^+$, or $FoxP3^+$ T lymphocytes are associated with good prognostic indicators, they do not impact the overall survival of patients.

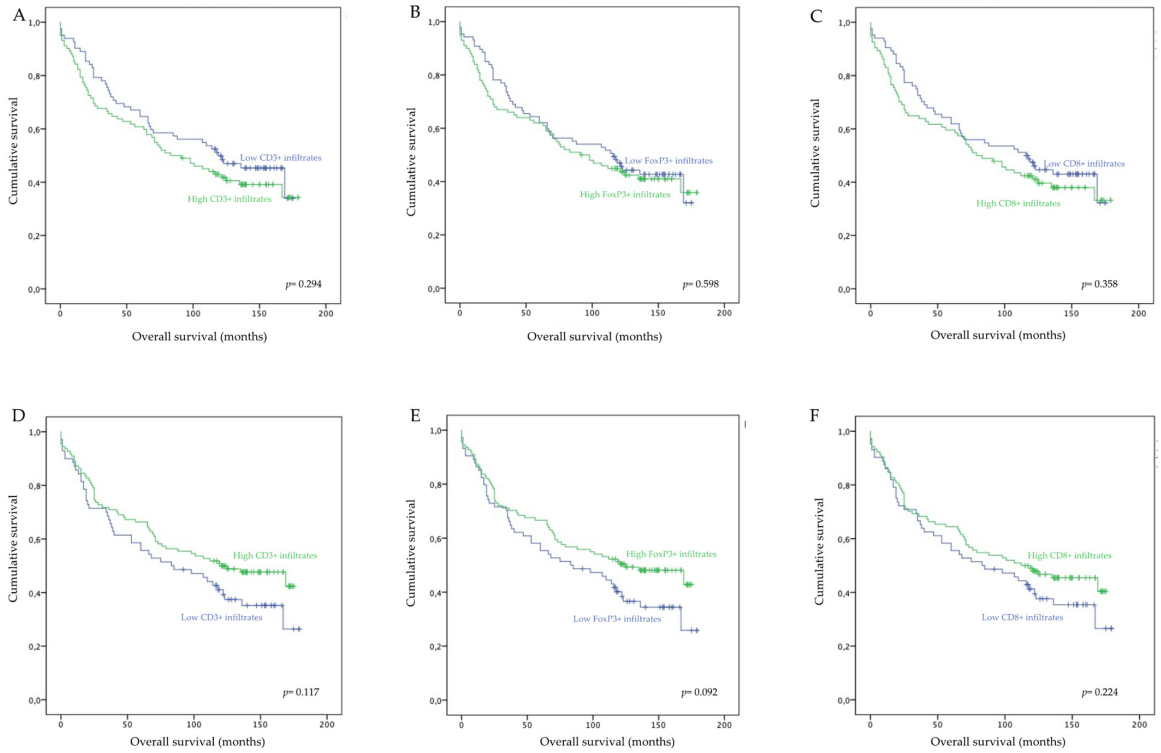

Figure 2. Overall survival of CRC patients according to low or high densities of intratumor and tumor margin infiltrating $CD3^+$, $FoxP3^+$, and $CD8^+$ T lymphocytes. (**A**) Overall survival according to intratumor $CD3^+$ T cell density; (**B**) Overall survival according to intratumor $FoxP3^+$ T cell density; (**C**) Overall survival according to intratumor $CD8^+$ T cell density; (**D**) Overall survival according to tumor margin $CD3^+$ T cell density; (**E**) Overall survival according to tumor margin $FoxP3^+$ T cell density; (**F**) Overall survival according to intratumor $CD8^+$ T cell density.

4. Discussion

The interaction between tumor cells and the immune system has prompted the quantification of immune infiltrates, particularly T lymphocytes, as prognostic markers for colorectal cancer (CRC) [35]. Herein, we analyzed the presence of lymphocyte infiltrates ($CD3^+$, $CD8^+$, or $FoxP3^+$ T lymphocytes) inside the tumor and the tumor invasive margins of CRC samples to evaluate their prognostic potential. We were not able to find any associations between the presence and extent of intratumor T lymphocyte infiltrates with the clinical or pathological data of the patients, indicating that, at least in our cohort, intratumor lymphocytes do not influence the pathogenesis of CRC. Crucially, when we perform the same analyses for the tumor margins, we found that the presence of high $CD3^+$, $CD8^+$, or $FoxP3^+$ T lymphocyte infiltrates were associated with TNM stages I-II, non-invasion of lymph nodes, and normal CEA levels. These data suggest that the presence of $CD3^+$, $CD8^+$, or $FoxP3^+$ T lymphocytes in the tumor invasive margins are associated with good prognostic indicators; however, we could not demonstrate any significant association between any of the T lymphocyte population analyzed and the overall survival of the patients.

In recent years, the analysis of the immune reaction inside the tumor and its invasive margins has been suggested to predict disease-free survival and overall survival of CRC patients, independently of the local extent of the tumor or the invasion of regional lymph nodes (TNM stages I, II, and III) [36]. The prognostic potential of the tumor immune reaction prompted the development of methodologies to quantify, in situ, the extent of immune infiltrates, particularly $CD3^+$ and $CD8^+$ T lymphocytes [35]. Several studies show

that high infiltrations of $CD3^+$ and/or $CD8^+$ T lymphocytes within CRC tumors and their invasive margins were associated with early stages of the disease (TNM stages I-II) and other good prognostic indicators, including absence of lymph node metastasis and distant metastasis [19,38]. Our study corroborated these observations but only for $CD3^+$ or $CD8^+$ T lymphocyte infiltrates in the tumor margins. Indeed, we were unable to find any association between the presence of $CD3^+$ or $CD8^+$ T lymphocyte infiltrates inside the tumor with markers of good CRC prognosis. In this regard, previous data has shown that, while $CD8^+$ T lymphocytes are directly capable of killing tumor cells and positively affect prognosis in a broad range of tumors [39–42], several other studies have shown no such correlation with prognosis [43,44]. As such, it is possible that the tumor microenvironment could modulate the effector function of $CD8^+$ T lymphocytes, and that this effect may depend on environmental variables such as the microbiome [45,46] or the tumor inflammatory status [47]. In this regard, alteration in the intestinal microbiota has been shown to increase intestinal tumorigenesis by enhancing inflammation and promoting T cell exhaustion [48]. Therefore, in addition to determining the presence of $CD8^+$ T lymphocytes, future studies should also focus on determining their effector function.

As discussed above, we also found that the high accumulation of $FoxP3^+$ T lymphocytes in the tumor margins was associated with TNM stages I-II, normal CEA levels, and, more importantly, with the non-invasion of lymph nodes. While FoxP3 can be transiently expressed by recently activated T cells in humans, the expression of this transcription factor also marks a population of regulatory T lymphocytes that can downregulate immune responses and, consequently, dampen anti-tumor immune mechanisms [49–51]. Since we did not evaluate the function of $FoxP3^+$ lymphocytes, we were not able to discriminate between the regulatory and non-regulatory populations of $FoxP3^+$ lymphocytes. This is an important limitation of our study, as it prevented us from drawing any conclusion on the prognostic potential of the regulatory population of FoxP3-expressing lymphocytes. However, we suggest that the immunosuppressive capacity of the regulatory population of FoxP3-expressing lymphocytes may be important in CRC to control continuous and aggressive inflammatory responses that may favor tumor proliferation [52,53]. Our data are in accordance with previous data showing that high frequencies of $FoxP3^+$ T lymphocytes are associated with early T stages and absence of lymph node involvement [31]. However, other studies also found associations between high $FoxP3^+$ T lymphocyte infiltrations and increased survival of CRC patients [32,54], which we did not find. Despite this, the high accumulation of these cells in the tumor margins resulted in an average gain of 14 months of the patients' life expectancy ($p = 0.092$). It is important to note that previous studies have also reported an association between high $FoxP3^+$ lymphocyte infiltrations and advanced CRC [33,55]. Additionally, the presence of $FoxP3^+$ T lymphocyte infiltrates were associated with poor prognosis in different types of cancers, including breast [26], lung [27], pancreatic [56], ovarian [57], and cervical [28] cancers. These data show that the presence of $FoxP3^+$ T lymphocytes is not always associated with a good prognosis. As this population may downregulate immune responses, we suggest that their protective effect and their prognostic potential may depend on the inflammatory status of the tumor. As such, future research is required to consolidate the prognostic significance and the context wherein $FoxP3^+$ T lymphocytes have prognostic significance.

Our data point to the location of immune cells in relation to the tumor as an important factor for prognosis. Indeed, while we did not find any association between intratumor T lymphocytes with clinical or pathological data in the tumor margins, the extent of $CD3^+$, $CD8^+$, and $FoxP3^+$ lymphocyte infiltrations were all associated with good prognostic indicators. It is tempting to speculate that the intratumor-infiltrating T lymphocytes may be modulated by the tumor microenvironment, while in the tumor margins, these populations are able to maintain their functions for longer periods. As discussed above, this may explain the lack of association between the high intratumor infiltration of $CD8^+$ T lymphocytes and markers of good prognostic or even overall survival seen in other studies [32,35,38]. Taken

together, the distribution of immune cells, as well as their functional capability, may be important in predicting patients' prognosis.

The potential limitations in analyzing tumor-infiltrating lymphocytes and perform direct comparisons with other studies include the various criteria used in different studies and the heterogeneity in the patterns of tumor-infiltrating lymphocytes [58]. In our study, we accounted for this heterogeneity by staining large samples of tumor tissue with the different lymphocyte markers, including samples with normal adjacent tissue. The assessment of the immunostainings and analysis of the extent of lymphocyte infiltration in the different areas of the tumor by an experienced pathologist ensured that the heterogeneous expression patterns of the different markers used were seen in the context of the entire tumor section. Moreover, automated counting methods, which are not available in all laboratories and have been used in multiple studies, may not yield results comparable to the usual method. In all, further studies need to take into account the heterogeneity of lymphocyte infiltrates and evaluating methods.

In conclusion, our results indicate that only the presence of high infiltrates of $CD3^+$, $CD8^+$, and $FoxP3^+$ T lymphocytes in the tumor invasive margins are associated with good prognostic indicators and potentially limit the aggressiveness and spread of CRC. The presence of lymphocyte infiltrates inside the tumor was not associated with any clinical or pathological parameter. However, while previous studies showed an association between high lymphocytic infiltrations, particularly $CD8^+$, and survival of CRC patients [35,38,59], we were unable to find any association between intratumor or tumor invasive margin lymphocyte infiltrations and overall survival. As such, while the consensus is that the tumor immune reaction may be a good prognostic indicator for CRC, and, in some cases, may even surpass the TNM staging system, our study suggests that this may not be the case for all populations.

5. Conclusions

Overall, our data show that the presence of $CD3^+$, $CD8^+$, or $FoxP3^+$T lymphocytes in the tumor invasive margin are associated with the pathogenesis of CRC. However, we did not find any association between lymphocyte infiltrations and the overall survival of CRC patients, although high $FoxP3^+$ T lymphocyte infiltrations in the tumor invasive margins resulted in an increased overall survival of 14 months.

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Chapter V

General Discussion and Future Perspectives

1. General Discussion and Future Perspectives

For a long time, cancer was thought to be simply an accumulation of fast-growing cells that resulted in tumor masses. However, the effort that the scientific community expended in cancer research, revealed that other partners are important in the formation and structuring of cancer [1]. Importantly, these advances led to the identification of new therapeutic targets and, consequently, the development of new therapies. The therapeutic field was revolutionized with the introduction of immunotherapies, which target the immune system increasing its ability to detect and kill malignant cells [2]. In fact, the ability of the immune system to eliminate malignant cells, through a process known as cancer immune surveillance, is the main reason why not everyone develops cancer [3]. Risk factors for cancer include aging, tobacco, radiation exposure, chemicals, as well as some viruses and bacterial infections [4]. Another factor closely related to cancer prevalence is dietary patterns. In this regard, it has been estimated that 30-40% of all cancers could be preventable with proper dietary habits [5,6]. The composition of the microbiota and consequent modulation of the immune response are among the proposed mechanisms by which the diet regulates carcinogenesis [7–9]. Thus, taking this into consideration, the data presented in this thesis addressed two crucial topics: i) the impact of dietary supplementation, specifically zinc supplementation, in the onset of cancer and ii) the prognostic potential of tumor infiltrating immune cells.

The role of dietary habits in microbiota is well described and its impact goes beyond modulating microbiota composition [10], since the microbiota actively participates in the differentiation and modulation of the immune response [11]. For instance, the short-chain fatty acids (SCFA), that derive from the digestion of complex carbohydrates by various microorganisms in the intestine [12], are modulators the immune response [13,14]. Indeed, the SCFA butyrate has been shown to reduce the susceptibility of mice to develop colonic inflammation as well as colon cancer though the induction of anti-inflammatory properties in colonic macrophages and dendritic cells [15]. In Chapter I, we provided insight on the different ways in which microbiota act both as a tumor protective factor, but also as a tumor inducer. Although microbiota manipulation through diet, namely through the consumption of probiotics or dietary supplements, represents an attractive strategy as an adjuvant therapy during cancer treatments [16–18], it is important to bear in mind that these manipulations, when unsupervised or unprescribed, can have unintended consequences. In this regard, although the involvement of dietary zinc supplementation in delaying tumor growth has been documented [19–21], its effects as a pro-tumor molecule are still unclear [22,23].

In Chapter II, we provide evidence that dietary zinc supplementation promotes tumor overgrowth through microbiota-mediated mechanisms. Specifically, we showed that mice under dietary zinc supplementation displayed accelerated tumor growth when compared to mice maintained on regular diet; however, when mice were pretreated with antibiotics, they did not respond to the excess dietary zinc, clearly showing that the pro-tumor action of zinc was mediated by the microbiota. In the literature there is evidence to support the pro-tumor role of microbiota [24]. For instance, the presence of *Fusobacterium nucleatum* is associated to the development of colorectal cancer (CRC) [25] and, more recently, it has also been associated with the poor prognosis of gastric cancer patients [26]. Importantly, the report from Sivan *et al* showed that the impact of alterations of the microbiota go beyond the gastrointestinal tract, by demonstrating that microbiota alterations impacted the growth of a melanoma-derived cell line in tumor-bearing mice [27]. Specifically, they showed that mice from Jackson Laboratories (JAX) had smaller tumors when compared to mice from Taconic Farms (TAC). However, when TAC mice were fed with fecal matter (enriched in *Bifidobacterium* species) from JAX mice, the tumors grew at a similar rate, indicating the protective effect of JAX mice microbiota, that they found to be mediated by increased cluster of differentiation (CD)8 T lymphocyte priming and intratumor accumulation [27]. In our study, we have found a pronounced remodeling of gut microbiota composition upon dietary zinc supplementation. Among the alterations that we found, species genus of *Bacteroides* were increased in mice under dietary zinc supplementation, namely *B. acidifaciens* and *B. dorei*. Moreover, we unraveled an important role for T lymphocytes and $Gr1^+$ cells in the tumor overgrowth. Specifically, we found that mice under dietary zinc supplementation had increased accumulation of $FoxP3^+$ T lymphocytes in the mesenteric lymph nodes (mLN). As we found that mice deficient in T lymphocytes did not display tumor overgrowth after dietary zinc supplementation, these data suggest that the presence of this subset of cells was critical for tumor overgrowth. While we cannot exclude the possibility that T lymphocyte deficient mice may have a different microbiota compared to wild type mice, as previously shown [28], we also found an increase in *B. acidifaciens* and *B.dorei* in the cecum of the mice under dietary zinc supplementation (data not shown). These data suggest that the dysbiosis pattern of these mice is similar to that of B6 mice (Chapter II, Figure 4D). Either way, future experiments will be carried out to clarify whether the lack of response to zinc supplementation of mice deficient in T lymphocytes is due to the absence of those cells or to an altered microbiota.

In addition to these data, we also showed that the intratumor infiltration of $Gr1^+$ cells are responsible for tumor overgrowth in mice under dietary zinc supplementation, as their depletion reverted tumor overgrowth. The involvement of $Gr1^+$ cells in tumor overgrowth has already been reported and, in fact,

this population is mainly characterized by immunosuppressive and pro-tumor functions [29,30]. Importantly, one of the mechanisms whereby these cells promote tumor growth is through the suppression of T lymphocyte cytotoxic responses [31,32]. Although we do not yet have the immunosuppressive assay to assess the suppressor phenotype of $Gr1^+$ cells, we have shown low production of interferon (IFN)-γ by T lymphocytes isolated from the tumors of mice under dietary zinc supplementation. In line with our finding, others have also shown that $Gr1^+$ cells inhibit IFN-γ production by T lymphocytes [32–34]. Future studies will address the immunosuppressive function of $Gr1^+$ cells. For that, we will perform co-culture assays with purified T lymphocytes and intratumor $Gr1^+$ cells isolated from tumor-bearing mice on normal diet or supplemented with zinc. In addition, dissecting the properties and functions of $Gr1^+$ cells will yield additional information about how this population stimulates tumor growth. In this sense, RNA sequencing analysis would be important to identify the molecular changes that these cells have and their functional impact on disease progression.

Together, our results from Chapter II point to an interaction between T lymphocytes and $Gr1^+$ cells since T lymphocyte deficient mice under dietary zinc supplementation i) did not show tumor overgrowth and ii) did not show increased intratumor accumulation of $Gr1^+$ cells (data not shown). It is then important to further investigate the link between T and $Gr1^+$ cells. While the capacity of $Gr1^+$ cells to modulate T lymphocytes is well documented, as exemplified by their potential to drive regulatory T lymphocyte (Tregs) differentiation [35,36], the modulation that T lymphocytes exert on $Gr1^+$ cells is not yet clear. Interestingly however, there is evidence that Tregs control the differentiation and phenotype of $Gr1^+$ cells [37]. In this study the authors showed that, in a model of murine colitis, $FoxP3^+$ T lymphocytes upregulate the expansion and immunosuppressive function of $Gr1^+$ cells through a mechanism dependent on transforming growth factor-β [37]. Because we found increased Tregs accumulation in the mLN of mice under dietary zinc supplementation, we propose to carry out co-cultures of Tregs isolated from control mice or mice under dietary zinc supplementation with monocytes to assess whether Tregs of the two groups have a distinct ability to regulate the differentiation of $Gr1^+$ cells and, even, to regulate their phenotypic function.

With our results from Chapter II critically indicating the impact of dietary zinc supplementation in the microbiota with consequences in cancer immune surveillance, we decided to determine the role of interleukin (IL)-10, a classic anti-inflammatory cytokine in this model.

The fact that IL-10 is an anti-inflammatory cytokine, its production during the tumor immune response can have diverse consequences [38]. Indeed, while it is expectable for IL-10 to promote tumor growth as it can hamper the anti-tumor T lymphocyte response [38], its ability to control the inflammatory responses can be beneficial, since exacerbated inflammation, as well as chronic inflammatory conditions, are associated to tumor promotion [38]. Thus, to address the role of IL-10, we used a genetically modified mouse model, pMT-10 mice, which overexpress IL-10 throughout dietary zinc supplementation. The report from Cardoso *et al* indicates that, in this model, high levels of IL-10 are maintained by CD45-TER119- cells in the small intestine, skin and, to a lesser extent, bone marrow [39]. Particularly in the gut, IL-10 has been shown to play a key role in maintaining intestinal homeostasis and preventing exacerbated inflammatory responses against commensal microorganisms [40].

In Chapter III, we showed that increased levels of IL-10 reduced tumor overgrowth caused by dietary zinc supplementation, thus indicating an anti-tumor function for IL-10. While the increased expression of IL-10 did not impede the dysbiosis induced by dietary zinc supplementation (Chapter III, Figure 2B), it did result in increased intestinal production of immunoglobulin (Ig)A, which is regulated by IL-10 [41]. In addition, the depletion of $Gr1^+$ cells in pMT10 mice under dietary zinc supplementation resulted in a complete abrogation of tumor overgrowth. These data, taken together with the data from Chapter II, suggest that the presence of IL-10 interferes with the capacity of $Gr1^+$ cells to promote tumor growth. Whether IL-10 reduces the intratumor accumulation of $Gr1^+$ cells or regulates their pro-tumor roles remains to be seen. Our hypothesis is that IL-10 acts by inducing IgA production that, consequently, opsonize microorganisms in the intestinal lumen, hampering their pro-tumor potential. Even though it is not completely understood how IgA regulates the interaction between gut microbiota and the host, the specificity of IgA for coating disease-associated commensal bacteria, especially those associated to intestinal inflammation, has been reported [42]. Corroborating these data, others have shown that IgA of high-affinity that selectively binds to colitogenic bacteria retarded the development of colitis in mice, thus demonstrating IgA-based treatments as a promising therapy for inflammatory bowel disease [43]. Concomitantly, our data unravels the potential of IL-10 to regulate IgA-mediated responses, which pave the way for further studies in understanding how the modulation of this cytokine can regulate carcinogenesis and other diseases, including inflammatory bowel disease.

The data that we have presented in Chapter II and III delivered key evidence on the involvement of the immune response either in promoting tumor growth, as shown by the pro-tumor effect of T

lymphocytes and Gr1$^+$ cells in Chapter II; or in preventing tumor growth, as demonstrated by the anti-tumor function of IL-10 revealed in Chapter III. These data further corroborate that the presence of specific immune cell populations can dictate the outcome of cancer. With the disturbing perspective of increased cancer incidence predicted for the next decades, it is crucial to better provide more targeted therapeutic interventions for patients. In this regard, over the last years, several lines of research have evaluated the potential of immune cells as biomarkers [44]. Specifically, these studies aim to determine whether the presence of a particular immune subset can be used as a prognostic factor to help in patient stratification and management. Among the different immune cells that infiltrate the tumors, T lymphocytes assume crucial importance in tumor control, but are also involved in tumor promotion [45–48].

To further investigate the role of this immune subset in cancer, in Chapter IV, we took advantage of a cohort of CRC patients to assess the impact of tumor infiltrating lymphocytes in CRC clinical outcome. Although we did not find found any association between intratumor T lymphocyte infiltrates and clinicopathological indicators, we showed that high infiltrations of T lymphocyte subsets in the tumor margins, namely CD3$^+$, CD8$^+$ and FoxP3$^+$ T lymphocytes, were associated with TNM stages I-II and absence of lymph node metastases. In accordance, previous studies have also reported that high infiltrations of these cells are associated with early TNM staging and the absence of lymph nodes metastases [49,50]. Despite these data indicating that the presence of these cells appears to delay disease progression, as their infiltration was associated with markers of good prognosis, we found no changes in the patients' overall survival. In contrast with our data, other reports associated CD3$^+$ and CD8$^+$ T lymphocytes tumor infiltration with good prognosis of cancer patients [51–54], including CRC patients [55–57]. Regarding FoxP3$^+$T lymphocytes, the presence of high FoxP3$^+$ T lymphocytes at the tumor margins, albeit not statistically significant, resulted in a 14 month increase in overall survival. While further studies are important to determine the phenotype of these FoxP3$^+$ T lymphocytes, since non-Tregs human cells can transiently express FoxP3 [58], our data suggest a protective role of FoxP3$^+$ T lymphocytes in CRC. Interestingly however, in other cancers such as cervical, renal, and breast cancers, FoxP3$^+$ T lymphocytes are often associated with poor prognosis [59], which indicates that the activity of these cells is likely dependent on the tumor type. Taken together, our data suggest that not only the presence of T lymphocytes, but also its distribution within the tumors, can be important for their function.

In what regards this lack of association with overall survival, we can speculate about differences across populations that can modulate the immune response, including genetic factors [60], dietary habits [61] and even microbiota composition [62,63]. When the data from Chapter II and Chapter IV are taken together, it is clear that T lymphocytes can have an important role in promoting tumor growth (Chapter

II), but, at the same time, their presence in the tumor microenvironment can be associated with markers of good prognosis (Chapter IV). It is however important to note that the pro-tumor T lymphocyte activity reported in Chapter II was dependent on dietary zinc supplementation. Indeed, if we compare tumor growth of B6 mice and mice with T lymphocyte deficiency in the absence of dietary zinc supplementation, we observe similar tumor growth, indicating that T lymphocytes only promote tumor growth when the mice were under zinc supplementation. (Chapter II, Figure 6B). As already discussed, the microbiota is an important modulator of the immune system functions. It is likely that, in our model of dietary zinc supplementation, changes in microbiota composition are modulating T lymphocyte response driving to a pro-tumor phenotype. Thus, further studies should address the involvement of microbiota composition in determining the functionality of T lymphocytes. Furthermore, our data revealed that the axis microbiota-cancer immune surveillance can be modulated through alterations in the diet. It would be therefore important to address the impact of dietary habits in this axis across the human population.

Collectively, the results presented in this thesis showed the interconnection between diet, microbiota, and cancer immune surveillance. As we move into an era of personalized medicine, these three factors may represent valuable targets, and even have potential as prognostic biomarkers, to ensure a more efficient and personalized therapeutic regimen for each patient.

2. References

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