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Porphyromonas gingivalis contributes to colon cancer progression by enhancing iNKT cell pro-tumorigenic functions

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“Whatever the faith may be, and whatever answers it may give, and to whosoever it gives them, such answer gives to the finite existence of man an infinite meaning, a meaning not destroyed by sufferings, deprivations, or death.”

Lev Tolstoy*, A Confession*

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List of Abbreviations

αGalCer: alpha-galactosylceramide

AOM: azoxymethane

AUC: area under the curve

APCs: antigen-presenting cells

BSA: bovine serum albumin

CD1d: cluster of differentiation 1d

CRC: colorectal cancer

CXCL: chemokine (C-X-C motif) ligand

DSS: dextran sodium sulfate

ELISA: Enzyme-linked immunosorbent assay

FasL: Fas ligand

FDR: false discovery rate

GZMB: granzyme B

HBSS: Hanks' balanced salt solution

HLA: human leukocyte antigens

IBD: inflammatory bowel disease

Ig: Immunoglobulin

IL-: Interleukin

IFN: interferon

iNKT: invariant natural killer T cell

LDH: lactate dehydrogenase

LPMCs: lamina propria mononuclear cells

MAPK: mitogen-activated protein kinase

MDSC: myeloid-derived suppressor cell

MFI: mean fluorescence intensity

MMR: mismatch repair gene

moDCs: monocyte-derived dendritic cells

MSI: microsatellite instability

NK: natural killer cell

PBMCs: peripheral blood mononuclear cells

PBS: phosphate-buffered saline solution

PD1: programmed cell death protein 1

PD-L1: programmed cell death protein ligand 1

Pg: *Porphyromonas gingivalis*

PRF: perforin

ROS: reactive oxygen species

SCFAs: short chain fatty acids

SPF: specific pathogen free

siMFI: scaled-integrated mean fluorescence intensity

TAMs: tumor-associated macrophages

TANs: tumor-associated neutrophils

TCR: T cell receptor

Tet: Tetramer

TGF-β: transforming growth factor beta

Th-: T helper

TIGIT: T cell immunoreceptor with Ig and ITIM domains

TIM3: T cell immunoglobulin and mucin domain containing-protein 3

TLRs: Toll-like receptors

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand

t-SNE: t-distributed stochastic neighbor embedding

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Abstract

Colorectal cancer (CRC) is characterized by a complex tumor microenvironment. Invariant Natural Killer T (iNKT) cells are a CD1d-restricted, lipid-specific, T cell population known to be pro-tumorigenic in CRC. On the other hand, the microbiota has gained importance for its infuence over cancer cells and, most importantly, the immune system. *Porphyromonas gingivalis* is a member of the oral microbiota linked to CRC. Although the pro-tumor functions of iNKT cells in CRC are known, how they acquire these features is not well understood. Hence, we aimed at understanding if iNKT cells become pro-tumorigenic due to signals given by CRC-associated bacteria, in particular *P. gingivalis*. For this, we used two murine models and functional assays with human iNKT cell lines. We observed that CRC cells did not induce IL-17, GM-CSF and IL-10 production, known to be secreted by pro-tumorigenic iNKT cells, but activated iNKT cell cytotoxicity via the perforin/granzyme pathway. *P. gingivalis* enhanced the expression of pro-tumor cytokines and neutrophil chemoattractant activity on tumor-infiltrating iNKT cells *in vivo*. Furthermore, we found that iNKT cells are necessary for the pro-tumorigenic effects of *P. gingivalis* in the AOM/DSS model. *In vitro* experiments revealed that *P. gingivalis*-primed cells have a neutrophil chemotactic signature and diminished cytotoxicity. Moreover, we showed that Toll-like receptor 2 and 4 signaling mediates the increase in IL-10 and GM-CSF production, whereas chitinase 3-like protein 1 is responsible for the impaired killing activity. These results identified a novel mechanism through which *P. gingivalis* contributes to CRC pathogenesis, by acting on unconventional T cell activities.

1. Introduction

1.1. Colorectal cancer

Colorectal cancer (CRC) is the second-leading cancer-related cause of death [1]. and the third most-commonly diagnosed cancer type in the world [2, 3]. Even though colon cancer (and cancer in general) is an age-related disease, with incidence substantially higher in individuals over 50 years of age, there has been a dramatic increase in cases in younger individuals [2]. Although hereditary factors, including family history and inherited germline mutations (causes of Lynch syndrome and familial adenomatous polyposis), account for ~30% of total cases of CRC, most of them arise sporadically [1]. In this regard, environmental risk factors such as alcohol intake, western diet, and lack of physical activity are among the most relevant [1, 3, 4].

Mechanistically speaking, there are three major molecular pathways in CRC (Fig. 1.1): the classic adenoma-carcinoma sequence described by Fearon and Vogelstein [5] , explains the majority of sporadic CRC cases and it is characterized by mutations in either oncogenes or tumor-suppressor genes, *i.e. APC, TP53, KRAS, BRAF* and it is associated with most chromosome instability (CIN)-high tumors [6]. On the other hand, the serrated pathway, which is associated with the activation of the MAP (mitogen-activated protein) kinase pathway, as well as the presence of the CpG methylator island phenotype (CIMP), is found in ~20-30% of sporadic tumors [7, 8]. Lastly, the inflammatory pathway, which arises from a dysplastic lesion produced by chronic inflammation, major feature of inflammatory bowel disease (IBD) patients, is responsible for 2% of all CRC cases [1, 7]. All three share a similar multi-step sequence that starts with a benign lesion (polyp, adenoma, or dysplasia), and the accumulation of genetic and epigenetic alterations leads to the transition into malignant carcinomas [1].

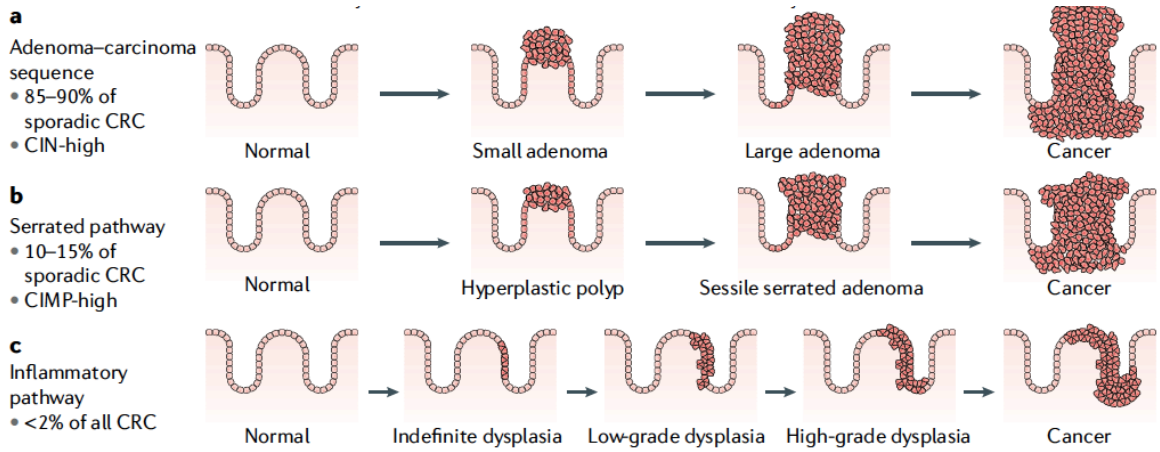

Fig. 1.1. The molecular pathways in CRC. Adapted from Keum and Giovannucci *Nat Rev Gastroenterol Hepatol* (2019) [1]

New classifications for colon cancer subtypes have been proposed in recent years. One of the most accepted is the CMS (consensus molecular subtypes) classification [9]. More importantly, one of the main features it includes is the contribution of the tumor microenvironment (TME), absent in the more traditional classification systems [9, 10]. From this, 4 CRC types arise: CMS1, which comprises 14% of the cases, are tumors characterized by strong immunogenicity (given by a high mutational burden and microsatellite instability) and immune infiltration, but patients have a negative prognosis after relapse. CMS2, also called canonical, accounts for 37% of total cases and one of its main features is the upregulation of the WNT and MYC downstream targets. The CM3 subtype, called metabolic, is characterized by a mixed microsatellite instability (MSI) status, but mostly by a general deregulation of different metabolic pathways together with several activating mutations on the *KRAS* gene. Lastly, the CMS4 subtype has a strong stromal infiltration, transforming growth factor (TGF) beta activation, and upregulation of epithelial-to-mesenchymal transition (EMT) genes, plus tending to be diagnosed at late stages and having the worst relapse-free and overall survival of all four subtypes.

This new classification system highlights the importance of the TME in CRC tumorigenesis and progression, and how it can eventually influence disease survival. It is now well known that colon cancer arises from the accumulation of genetic and epigenetic alterations that give rise to the tumor only in a tumor-promoting milieu supported by the adjacent tissue [4]. In general, the tumor microenvironment in colorectal cancer includes both cancer cell and non-epithelial cellular components, such as cancer-associated fibroblasts (CAF), the immune cell

infiltrate, endothelial cells, and the gut microbiota, as well as non-cellular elements such as cytokines, chemokines, metabolites, and the extracellular matrix [11–14].

1.1.1. Immunity in colorectal cancer

The immune system has contrasting roles in cancer formation and subsequent progression. Some populations have canonical antitumor properties, whereas other cells change their phenotype depending on the context. In this regard, whether some immune cells behave in an anti- or pro-tumorigenic manner depends on different aspects, such as nutrient availability, presence/absence of certain metabolites, oxygen gradients, microorganisms, the release of factors by cancer cells and other components of the TME, etcetera [15, 16].

On one hand, the infiltration of cytotoxic cells, especially CD8+ T cells, is widely known as a good prognostic factor in colorectal cancer. The “Immunoscore”, an index based on the density of CD8+ T and memory T cells in the core and invasive margin of CRC tumors, was created from this principle [17, 18]. In addition, natural killer cells, as well as some gamma-delta and invariant natural killer T (iNKT) cell subsets can exert killing activities against colon cancer cells [19–21]. Cytotoxic responses are promoted by interferon gamma (IFNG) and interleukin-2, cytokines produced by T helper (Th) 1 conventional CD4+ T cells, natural killer cells, as well as specific gamma-delta and iNKT cell subsets, which makes also Th1-like responses a factor for better prognosis [22, 23]. Maturation of Th1 and cytotoxic cells is also achieved by IL-12, produced by components of the myeloid lineage, such as matured dendritic cells and macrophages [24, 25]. T regulatory (Treg) cells, due to their immunosuppressive activity given by the release of IL-10, were initially presumed to promote colon tumorigenesis and progression. However, these cells might have a more ambiguous role in CRC, as some evidence shows that Treg infiltration is correlated with an increased probability of survival, as well as decreasing pro-tumorigenic inflammation in mouse models [22, 26, 27]. B cells also correlate with a positive prognosis in CRC, and they constitute a substantial fraction in the TME [22]. Herein, they are a major component of tertiary lymphoid structures (TLS), sites known for avid antigen presentation and effector and memory differentiation places for T and B cells [22]. In colon cancer, these structures are associated with a lower risk of recurrence [22].

On the other hand, like with other solid tumors, several studies have reported that IL-17, expressed by Th17 T cells, some gamma-delta and iNKT cells, as well as some cells of the myeloid lineage, is correlated with poor prognosis in CRC [28].

More specifically, IL-17 promotes tumor formation by increasing PLET (placenta expressed transcript) expression, inducing stemness, supporting pro-tumor inflammation and the recruitment of immunosuppressive granulocytes [29–32]. In concomitance with the production of IL-17, also IL-22 is thought to promote colon tumorigenesis due to the promotion of stemness [33, 34]. Some myeloid components of the TME, such as M2 macrophages and myeloid-derived suppressor cells are also associated with poor prognosis due to the promotion angiogenesis and cancer cell migration, while suppressing antitumor responses [15, 16, 27, 35–39]. Neutrophils seem to have an ambiguous role on CRC, as they are considered a good prognostic factor in some studies [40, 41], but recent evidence has shown that these cells may be also associated with an adverse outcome via various mechanisms, such as impairment of antitumor cells, promotion of epithelial-to-mesenchymal transition [42–44].

1.2. Invariant Natural Killer T cells

Invariant natural killer T (iNKT) cells are an unconventional T cell population that co-expresses some receptors typical of the natural killer (NK) lineage together with a semi-invariantly re-arranged, CD1d-restricted, lipid-specific T cell receptor (TCR) [45]. The TCR of these cells is composed by a Vα24-Jα18 (human) Vα14-Jα18 (mouse) alpha chain and a Vβ11 (human) Vβ8, -7, or -2 (mouse) beta chain [45, 46]. These cells recognize microbial and endogenous lipid antigens presented by the CD1d molecule, a non-polymorphic, MHC I-like protein present in professional antigen-presenting cells, cortical thymocytes, hepatocytes, and in the intestinal epithelium [47]. In mice, these cells are predominantly found in the liver and spleen (about 40% and 2% of the total T cells, respectively); in the intestine they can be found as intraepithelial lymphocytes (IEL) and in the lamina propria – being more abundant in the latter, where they represent ~1% of total lymphocytes [47, 48]. In humans, however, they seem to be less abundant [45, 48].

1.2.1. Activation cues

Due to the expression of some natural killer receptors and a T cell receptor, iNKT cells can be activated either in an innate or in an adaptive manner, alone or in combination [49]. Antigen-presenting cells can present both foreign and self-glycolipids when loaded to CD1d [45]. Exogenous antigens, of microbial and non-microbial origin, can enter the antigen-presenting cell through langerin (a mannose receptor), endocytosis, or along with low-density lipoprotein particles; endogenous

antigens, on the other hand, can be presented as a result of Toll-like (TLR) or nucleotide-binding oligomerization domain (NOD) receptor stimulation [49, 50]. In both cases, antigens can be processed by degradation within the lysosome, or pass through the Golgi apparatus and the endoplasmic reticulum, to be ultimately loaded onto a CD1d molecule [45]. One peculiarity of all iNKT cells, regardless of their origin, is that they recognize and are potently activated by alpha-galactosylceramide (α-GalCer), a microbial-derived lipid found in marine sponges [45].

The activation status of iNKT cells, like NK cells, is also ruled by the balance between stimulatory and inhibitory signals via natural killer and killer-cell immunoglobulin-like receptors (NKR and KIR, respectively) [49]. Through the activation of receptors such as NKG2D, iNKT cells can recognize MHC-like molecules usually expressed by stressed cells [49, 51, 52]. On the other hand, some NKRs and many KIRs recognize inhibitory HLA molecules, such as HLA-E [49, 53]. iNKT cells can also be activated by cytokines. For instance, antigen-presenting cells stimulated with TLR ligands like LPS (lipopolysaccharide), are prone to produce interleukins 12 and 18, as well as interferon-β, that can activate iNKT cells in a CD1d-independent manner [45, 46, 49, 54]. Another important cytokine is interleukin-15 – initially known to activate iNKT cells driving a Th1-like/cytotoxic response, is also involved in iNKT cell development [55, 56].

1.2.2. Functional subsets

Within minutes after activation, iNKT cells produce copious amounts of cytokines. Human and murine iNKT cells are divided into different functional subsets, which are similar to the T-helper classification [49, 50]. The NKT1 subset is similar to T helper 1 (Th1) cells, as they express the transcription factor T-bet and secrete IFN gamma and tumor necrosis factor alpha (TNFα), as well as cytotoxic molecules. Recent studies have shown that thymic NKT1 cells can be further divided into 3 groups [57, 58]: NKT1a, which seems to be a bridge state between NKT1 and NKT2 cells; NKT1b, and NKT1c, endowed with cytotoxic activities. The NKT2 program is governed by GATA3, which induces these cells to secrete IL-4 and IL-13. In the thymus, however, this subset also includes progenitors of NKT1 and 17. NKT17 cells are characterized by RORγt expression and the release of IL-17A, IL-21, and IL-22 [47, 48]. Furthermore, an NKT10 subset has been identified in adipose tissue and the intestine; these cells produce the anti-inflammatory cytokine IL-10,

but, contrary to conventional Tregs, they are governed by E4BP4 and not FOXP3 [59, 60], even if iNKT cells can express FOXP3 after stimulation with TGF-β [48, 50, 61]. Studies in mice demonstrate that whereas NKT1, 2, and 17 subsets are fully differentiated once they emigrate from the thymus, there is no precursor for NKT10 cells in this organ, suggesting that these cells acquire their properties once they reach the periphery [57].

Due to this array of cytokine programs and the co-expression of innate and adaptive receptors, iNKT cells act as a bridge between the adaptive and innate immune system. Indeed, they participate in various immune responses in lymphoid and non-lymphoid organs, both in tissue homeostasis and in diverse disease states, where they can exert either a protective or a pathogenic role depending on the tissue involved and the signals given by each microenvironment [48].

1.2.3. Antitumor activities

Invariant NKT cells can have either a direct and/or indirect antitumor effect on tumors (Fig. 1.2) [49, 62–64]. They produce granules containing granzymes and perforin, as well as the death ligands TRAIL (TNF-related apoptosis-inducing ligand) and Fas ligand (FasL) and can target tumor cells in CD1d-dependent and/or -independent manners [65–68]. Even though they can directly kill cancer cells, their main function in antitumor immunity is actually related to the regulation of innate and adaptive responses.

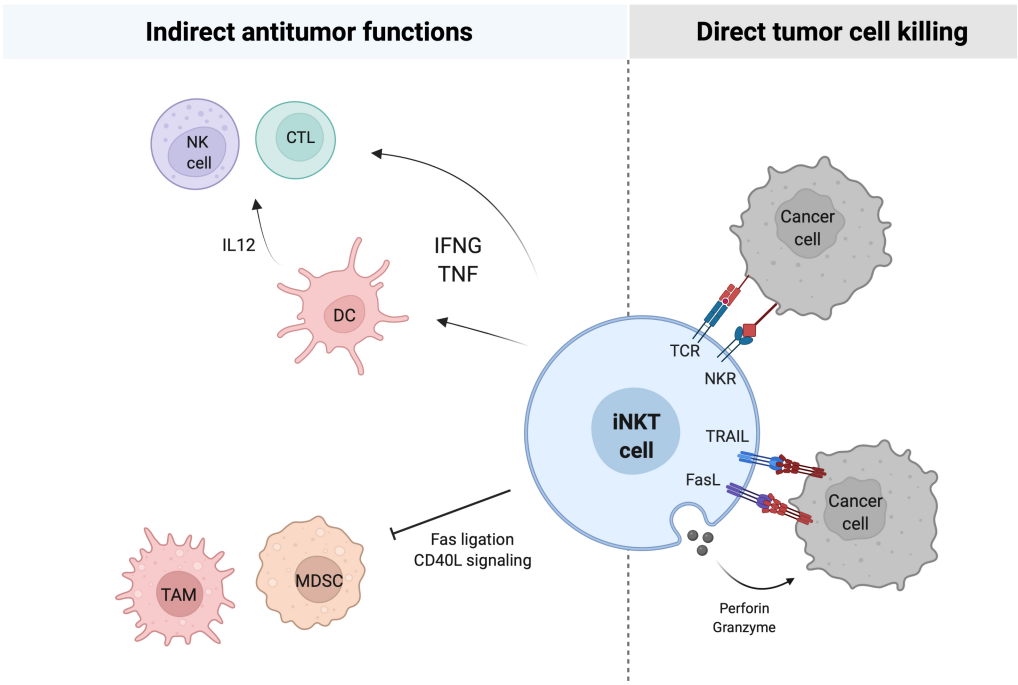

Fig. 1.2. Antitumor functions of iNKT cells. Created with Biorender.

1.2.3.1. Regulation of other immune populations

As mentioned before, iNKT cells can release several kinds of cytokines depending on the context. In cancer, it is known that they control the fates of other immune cells by the secretion of interferon gamma [49, 54, 69]. This cytokine functions by stimulating the JAK1/2-STAT1 pathway, which provokes several immunological processes, including macrophage activation, MHC-I/II pathway upregulation, co-stimulation, Treg cell inhibition, and Th1 cell differentiation and activation [70, 71]. Thanks to this, iNKT cells are involved in dendritic cell maturation, conventional T cell activation and NK cell transactivation [49, 54, 72]. Furthermore, iNKT cells can remodel the abundance and functions of myeloid populations within the tumor microenvironment [64, 73]. In particular, iNKT cells target M1-like macrophages for survival, while targeting M2 macrophages for death by the cooperative effect of CD1d, CD40 and Fas ligand [73]. Neutrophils are also influenced by activated iNKT cells in melanoma, as iNKT cells treated with aGalCer reduce the production of IL10 in neutrophils, thus downregulating their suppressive profile [74].

1.2.3.2. Direct cytotoxicity

Several lines of evidence have shown that human circulating iNKT cells, as well as murine cells from the liver, thymus, and spleen can eliminate leukemia, melanoma, lung and colorectal cancer cell lines [65–68]. Regarding the killing mechanism, some studies have indicated that the granzyme/perforin pathway is preferred in *in vitro* settings with human cells [52, 75–78]. This mechanism is based on the release of granules containing perforin and granzymes (mostly A and B) to the immunological synapse [65, 79, 80]. Then, perforin allows the entrance of granzymes, which ultimately mediate target cell death in caspase-dependent and -independent fashions [80–83]. Nonetheless, other studies have shown that Fas ligation is the preferred mechanism *in vivo* [66, 84]. The death receptor mechanism is dependent not only on the expression of death ligands (Fas ligand and TRAIL) on effector cells, but also on the presence of their co-respective death receptors (Fas and TRAIL receptors) on target cells [85]. Once the ligation occurs, death receptors provoke cell death by starting the extrinsic apoptotic pathway [85].

1.2.4. iNKT cells in the intestine

The gastrointestinal tract is one of the most complex antigenic compartments in the body, as it is constantly exposed to various stimuli from food and an enormous

ecosystem of gut microbes. To preserve tissue homeostasis, the gut harbors a highly specialized immune system, which helps prevent the translocation of gut microbes into the lamina propria, as well as inducing tolerance to food and commensal microbial antigens [86]. iNKT cells are also part of the intestinal immunity, and they seem to be crucial for gut homeostasis, prevalently in early life stages, but also in adult life. Regarding the distributions of different iNKT cell subsets in the gut, NKT1 cells are the most abundant, representing about 85% of iNKT cells in the small intestine, colon and Peyer's patches of C57BL/6 mice, whereas NKT2 and NKT17 represent ~ 10% and < 5%, respectively [87, 88].

1.2.4.1. iNKT cells and the gut microbiota

It is now known that the bi-directional crosstalk between the mucosal immune cells and the microbiota is essential for gut homeostasis and maturation of the immune system [89]. iNKT cells are not the exception, as they recognize microbial antigens and are important in host-microbiota interactions in the gut [46, 90, 91]. For instance, iNKT cell number and distribution in the intestine is regulated by the early colonization of gut commensals, as germ-free mice have higher numbers of immature and hypo-responsive cells, whose levels are then restored when animals are exposed to microbes only during the neonatal stage [90, 92]. In particular, gut microbes can regulate iNKT cell number and functions by controlling CXCL16 expression – chemokine responsible for iNKT cell recruitment, microbial antigens produced by some gut bacteria, such as *Lactobacilli* and *Bacteroides fragilis*, and indirect activation by other gut bacteria via TLR signaling and the secretion of metabolites like short chain fatty acids (SCFA, Fig. 1.3) [48, 60, 90, 91, 93–95] .

The control of the gut microbiota over iNKT cell functions becomes particularly evident in the context of intestinal inflammation. In particular, some studies have found that iNKT cells produce substantial amounts of pro-inflammatory cytokines like IFNG, TNF-α and IL17 in the presence of adherent-invasive *E. coli*, *Salmonella* Typhimurium (both associated with intestinal inflammation [96–100]), IBD patient-derived microbiota, or upon antibiotics treatment [93, 95, 96, 101]. However, we recently demonstrated that a gut microbiota rich in SCFA producers drives iNKT cell functions towards a T-reg like phenotype, with the release of IL-10 and subsequent control of pathogenic T cells, in Crohn's disease patients and in a cell transfer model of intestinal inflammation [60].

iNKT cells are also important in remodeling the gut microbiota composition. It has been observed that animals deficient for CD1d and iNKT cells are uncapable

of restricting microbial colonization in the small intestine, thus becoming more susceptible to pathogen invasion [91, 102]. Moreover, the gut microbiota from CD1d-deficient mice had pro-inflammatory properties when transplanted into wild-type mice 46. In addition, interferon gamma secretion by these cells influences the secretion of antimicrobial peptides by Paneth cells [47, 48, 102].

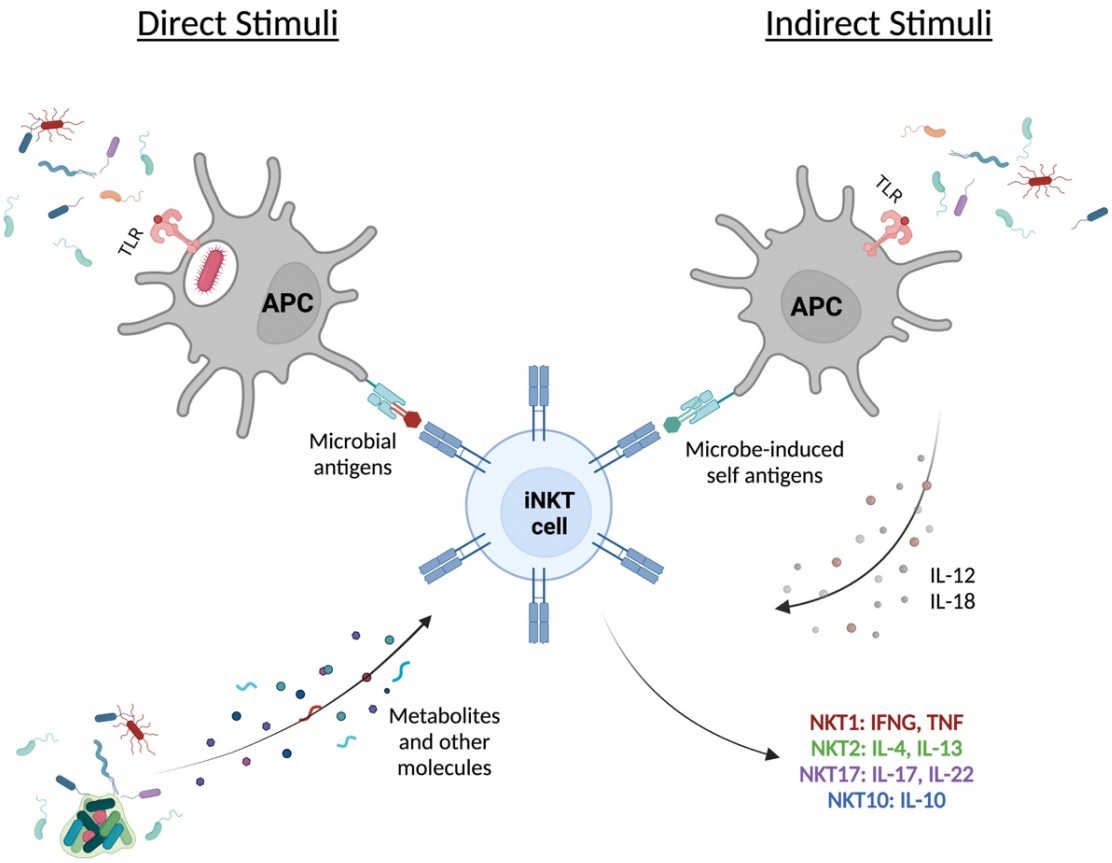

Fig. 1.3. Modulation of iNKT cell functions by the microbiota. Created with Biorender.

1.2.4.2. iNKT cells and other gut immune cells

Not only microbial-derived antigens, but also antigen-presenting cells can differentially drive tolerogenic and inflammatory responses. This is also true for iNKT cell activation, differentiation and proliferation, as lipid presentation by different $CD1d^+$ APCs can shape the outcome of their immune responses [103]. Some lines of evidence have shown that CD1d expression in $CD11c^+$ cells (which includes dendritic cells and macrophages) controls the phenotype of iNKT cells in the gut, as mice with a conditional deletion of CD1d on $CD11c^+$ cells have reduced NKT17 cell numbers in the small intestine and in mesenteric lymph nodes [87]. This, in turn, induces iNKT cells to regulate homeostasis by favoring Treg cells and controlling the composition and compartmentalization of gut microbes [87].

It is already well known that iNKT cells can also support the regulation of antibody production by B cells [104–106]. In the intestine, IgA – the most abundant antibody in the mucosae, together with IgG, cooperate to modulate intestinal immunity, as well as microbial composition and compartmentalization [107, 108]. For instance, it has been demonstrated that a subset in the Peyer's patches is crucial for the control of B cell responses [88]. Indeed, iNKT cells contribute to most of the IL-4 produced in this location and provide indirect help for class switch to IgG1. The IgA repertoire seems to be affected by iNKT cells as well, as CD1d-deficient mice have altered levels of IgA. However, it was found that such alterations are a consequence of the altered microbiota of this strain, suggesting an indirect role of iNKT cells [87].

1.2.5. iNKT cells in colon cancer

iNKT cells have gained interest in cancer research due to their antitumor potential [72, 109, 110]. However, their role in colon cancer remains highly controversial. Some studies have demonstrated a higher infiltration of iNKT cells in human CRC tumors [42, 69]. One of these studies proposed that iNKT cell infiltration was a positive prognostic factor in CRC, as iNKT cells expressed the activation marker CD69, as well as IFN gamma, perforin, granzymes, and Fas ligand in tumors [69]. However, we recently demonstrated that, both in colon cancer patients and murine models, iNKT cells are characterized by the expression IL17 and GMCSF, as well as having a strong neutrophil chemotactic signature. Indeed, iNKT cells were responsible for the recruitment of pro-tumorigenic neutrophils, thus being associated with a worse prognosis [42]. IL17 expression was also observed in the tumors of $Apc^{Min/+}$ mice, together with the expression of IL10, which in turn suppressed Th1 responses and promoted Treg cells [61]. More interestingly, iNKT cells are also thought to promote colon cancer metastasis to the liver by the release of IL22 by NKT17 cells [111].

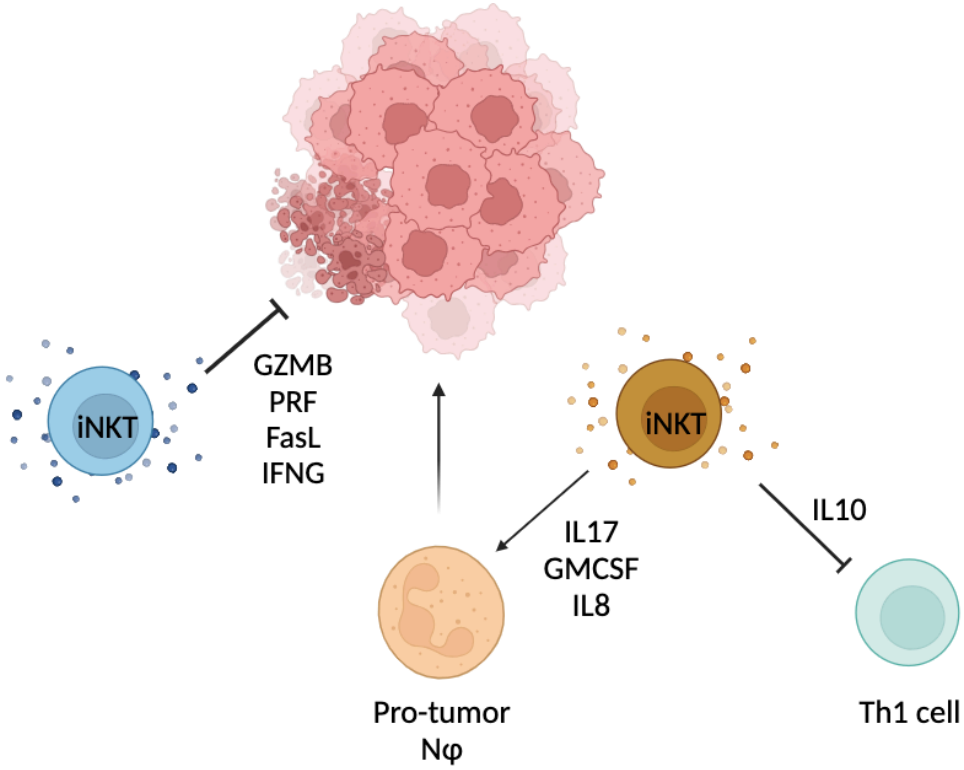

Fig. 1.4. iNKT cells in colon cancer. Created with Biorender. GZMB, granzyme B; PRF1, perforin; Nφ, neutrophil.

1.3. The gut microbiota in colon cancer

Colon cancer patients usually suffer from an altered gut microbiota composition. In general, their microbiome is characterized by a reduction of certain genera like *Clostridium*, *Bifidobacterium*, and *Bacteroides*, as well as in SCFA producers such as the *Ruminococcaceae* and *Lachnospiraceae* families, increase in, *Escherichia*, *Streptococci*, and microbes belonging to the oral microbiota such as *Fusobacterium*, *Gemella*, *Peptostreptococcus*, *Porphyromonas* and *Parvimonas*, [112–117]. Apart from changes in bacterial communities, there are also modifications in metabolic activities [118]. The role of the gut microbiota in the colon tumorigenesis and progression can be explained by various mechanisms. First, changes in the overall microbial composition (dysbiosis) can drive a non-specific inflammatory state that enhances cancer development (Fig. 1.4) [113–115, 119–121]. Second, specific bacteria, like *Fusobacterium nucleatum, pks+ E. coli*, Enterotoxigenic *Bacteroides fragilis* (ETBF), and *Enterococcus faecalis* contribute to tumorigenesis by triggering the Wnt/b-Catenin signaling, inducing Th17 responses, inhibiting cell-mediated cytotoxicity, recruitment of myeloid-derived suppressor cells, tumor-associated neutrophils and macrophages (TANs and TAMs, respectively), biofilm formation, and DNA damage [14, 114, 119, 121–124]66,75,123–126. Third, from a functional point of view, the metabolism of bile

acids, branched-chain amino acids and phenylalanine, hydrogen sulfide production, and biofilms, all known for causing inflammation and DNA damage, are increased in human CRC [116, 125, 126].

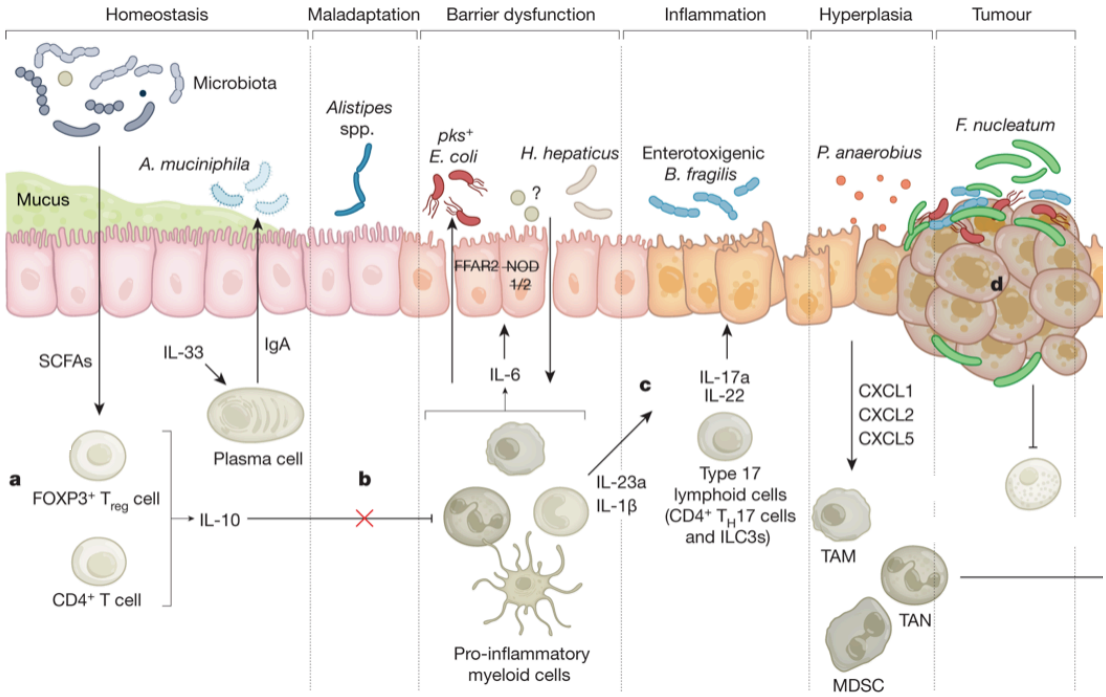

Fig. 1.5. Microbiota-mediated inflammatory mechanisms that contribute to CRC. Adapted from Janney, Powrie & Mann, *Nature* (2021) [119]

1.3.1. *Porphyromonas gingivalis*

Porphyromonas gingivalis (Pg) is a Gram-negative, non-motile, rod-shaped obligate anareobe usually found in the oral microbiota [127, 128]. It obtains metabolic energy with the fermentation of amino acids [127], and it is recognized for producing strong proteases, called gingipains, and peptidylarginine deaminases [127, 129, 130]. In fact, these enzymes represent some of the main virulence factors of *P. gingivalis* in adult periodontitis, together with its particular lipopolysaccharide and its capsule [131, 132]. Indeed, *P. gingivalis* is well-known for being one of the causative agents of this disease, being part of the Red complex, together with *Tannerella denticola* and *Tannerela forsythia* [127, 131]. Nonetheless, emerging pathogenic roles for this bacterium have also been found in Alzheimer's disease [133], rheumatoid artritis [134], ulcerative colitis [130, 135], and colorectal cancer [136–138].

1.3.2. *P. gingivalis* in the gut

Even if *P. gingivalis* is usually found in the oral cavity, it can also translocate to the intestine. Once in the gut, it mediates inflammatory responses and it is actually associated with the exacerbation of ulcerative colitis [130, 135, 139]. More specifically, it induces the release of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-17 [130, 135]. More specifically, in DSS treated mice, it was found that *P. gingivalis* regulates the Th17/Treg balance, favoring Th17 cells through TLR4 signaling [135]. However, it was also observed that the peptidylarginine deaminase seems to be responsible for the imbalance in Th17/Tregs as well [130].

1.3.3. *P. gingivalis* in colorectal cancer

As mentioned previously, in the intestine *P. gingivalis* is mostly known for its action in inflammatory bowel diseases. However, recent evidence has shown its emerging roles in colon cancer as well. Regarding its effects on cancer cells, it was observed that the total membrane and peptidoglycan fractions of *P. gingivalis* induced the up-regulation of the programmed death ligand 1 (PDL1) in CL-11 cells [140]. In addition, it can promote CRC cell proliferation by gingipain-mediated activation of the MAPK/ERK pathway [141]. Not only *P. gingivalis* influences cancer cell activities, but also other components of the tumor microenvironment. For instance, it becomes part of the tumor-associated microbiota and induces tumor-promoting senescence on fibroblasts thanks to the production of butyrate [136]. The myeloid compartment also contributes to the *P. gingivalis*-mediated promotion of colon tumorigenesis, as the activation of the NLRP3 inflammasome in this lineage given by this bacterium increases tumor burden *in vivo* [137].

2. Aims

iNKT cells are gaining importance in colon cancer research for their antitumor capabilities and for how gut microbes can modulate their functions. Even if these cells are beneficial in many types of cancer, it has been observed that they actually promote colon cancer progression by secreting pro-tumorigenic cytokines that act on other components of the tumor microenvironment [42, 61]. Nonetheless, how iNKT cells acquire this phenotype is not fully understood, even if we found that *Fusobacterium nucleatum* – an established oncobacterium, induces a pro-tumor phenotype on human iNKT cell lines [42]. Therefore, the hypothesis of this study is that iNKT cells acquire features that support colon cancer progression because of interactions with the tumor microenvironment, in particular with colon cancer-associated bacteria. To test this, I proposed the following aims:

- To assess if the activities of human iNKT cells are modulated by interactions with colon cancer cells
- To address if the iNKT cell pro-tumorigenic functions found in colon cancer is supported by *Porphyromonas gingivalis,* an emerging CRC-related bacterium
- To evaluate the mechanisms behind the effect of *Porphyromonas gingivalis* over iNKT cell functions

3. Materials and Methods

3.1. Experiments with human cells

3.1.1. Human samples

Colon samples were collected with written informed consent from all patients who underwent surgical resection at the *Ospedale Maggiore Policinico* in Milan, Italy. Collection was approved by the institutional review board (Milan, Area B) under the permission 566_2015. Clinical data are summarized in Table 3.1.

Table 3.1. Clinical data of the patients used in this study.

Parameter	
Male/female, n	11/13
Age at enrolment, mean ± SD	66 ± 12.9
Male	62 ± 14
Female	69 ± 11.6
Tumor location, n	
Left colon	9
Right colon	15
Stage	
Adenoma	2
I	2
II	6
III	14
MMR status, proficient/deficient	21/3

3.1.2. Cell lines

The cell lines used in this study, together with their growth conditions, are listed in Table 3.2. All lines were subcultured three times a week with the exception of NCM460D cells, which were split once a week. All cells were maintained in a humidified incubator with 95% air, 5% CO_2 at 37ºC. For all the experiments, cell lines were used between passages 4-10.

Table 3.2. List of the human cell lines used in this study. MSS: microsatellite-stable, MSI: microsatellite-instable. FBS: fetal bovine serum, NaP: Sodium pyruvate, Glu: Glutamine, NEAA: non-essential amino acids, P/S: penicillin/streptomycin.

Cell line	Cancer type	Growth conditions	Genetic Lesions
Caco-2	colon adenocarcinoma	Minimum Essential Medium Eagle with 20% FBS, 2 mM Glu, 1 mM NaP, 0.1 mM NEAA, P/S	CIN+, MSS, CIMP+, *TP53* (E204X), *APC* mut
DiFi	familial adenomatous polyposis	F-12 1X nutrient mix with 10% FBS, 2 mM Glu, 1 mM NaP, 0.1 mM NEAA, P/S	MSS
RKO	colon carcinoma	Minimum Essential Medium Eagle + 20% FBS, 2 mM Glu, 1 mM NaP, 0.1 mM NEAA, P/S	CIN-, MSI, CIMP+, *BRAF* (V600E), *PIK3CA* (H1047R)
HT-29	colon adenocarcinoma	Dulbecco's modified Eagle medium (DMEM) + 10% FBS, 2 mM Glu, 1 mM NaP, 0.1 mM NEAA, P/S	CIN+, MSS, CIMP+, *BRAF* (V600E), *PIK3CA* (P449T[b]), TR53 *APC* mut
Colo 205	colon adenocarcinoma, metastatic sites	RPMI-1640 with 10% FBS, FBS, 2 mM Glu, 1 mM NaP, 0.1 mM NEAA, P/S	MSS, CIMP+, *BRAF* mut, *PIK3CA* mut, *TP53* mut *APC* mut
NK-92	Non-Hodgkin's lymphoma	RPMI with 5% human serum, 2 mM Glu, 1 mM NaP, 0.1 mM NEAA, P/S, 100 IU/ml interleukin-2	-

3.1.3. Isolation of peripheral blood mononuclear cells

Buffy coats from healthy individuals were obtained thanks to the San Matteo Hospital (Pavia, Italy). Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll gradient. Briefly, blood was centrifuged for 10 min at 300 xg with slow aceleration/deaceleration to separate plasma from cellular content. Then, the cellular content was divided and diluted with phosphate buffered saline (PBS, Microgem) and layered over 13 ml of Ficoll in 50-ml Falcon tubes. Tubes were centrifuged at 300 xg for 30 min under low aceleration/deaceleration. The PBMC fraction was collected using a Pasteur pipette and washed twice with PBS to discard debris and platelets.

3.1.4. Isolation of lamina propria mononuclear cells and epithelial cells from surgical specimens

Surgical specimens from colon cancer and inflammatory bowel disease patients were obtained from the *Ospedale Maggiore Policlinico* (Milan, Italy). As soon as the specimen was obtained, the mucosa was separated using forceps and surgical scissors. Mucus was removed by carefully scrapping the mucosa with a cell

scrapper. The tissue was then pretreated with dithiothreitol (DTT, 0.1 mmol/ml, Sigma-Aldrich) for 15 minutes at room temperature under constant agitation. Later, the mucosa was washed three times (5 minutes per wash) with penicillin/streptomycin- (Gibco, Thermo Fisher) and gentamycin (Roche)-supplemented Hanks' Balanced Salt Solution (HBSS, EuroClone). To obtain the epithelial cells, the mucosa was cut in ~1 cm and incubated in ethylenediaminetetraacetic acid (EDTA, 1mmol/ml, Sigma-Aldrich) for 30 minutes at room temperature under agitation for a total of 3 incubation cycles. The tissue was then washed three times with HBSS supplemented with antibiotics. Next, it was incubated with collagenase D (400 IU/ml, Worthington Biochemical Corporation, Lakewood, New Jersey) for 5 hours at 37 ºC to digest the extracellular matrix. The resulting cell suspension was filtered in a 100 µm-cell strainer and centrifuged at 1900 rpm, room temperature for 10 minutes. Cells were separated with a 100%, 60%, 40%, 30% Percoll (Sigma-Aldrich) gradient at 1900 rpm, for 30 minutes. After the gradient was done, LPMCs were collected between the 60% and 40% layers. Cells were then washed twice with antibiotics-supplemented PBS and resuspended in complete RPMI-1640 medium.

3.1.5. Generation of monocyte-derived dendritic cells

Dendritic cells were generated from CD14+ cells isolated from PBMCs. Monocytes were isolated using CD14+ Microbeads (Miltenyi Biotec). In summary, PBMCs were counted, resuspended in MACS buffer (PBS with 0.5% w/v bovine serum albumin and 2mM EDTA) together with the microbeads, and incubated for 10 min at 4ºC. After the cells were washed to remove unbound beads, the cell suspension was passed through LS MACS columns (Miltenyi Biotec). Then, the columns were washed three times and cells were flushed out. CD14+ cells were incubated in complete RPMI medium supplemented with 1000 IU/ml granulocyte-macrophage colony stimulating factor (GMCSF, Miltenyi Biotec) and 400 IU/ml interleukin-4 (IL-4, Miltenyi Biotec) at a concentration of 1 x 10^6 cells/ml for 7 days, with cytokine refresh every 2 days.

3.1.6. Isolation of natural killer cells from PBMCs

Natural killer cells were isolated from PBMCs from healthy donors using the NK Cell Isolation Kit (Miltenyi Biotec) following the manufacturer's instructions. In brief, cells were counted and resuspended in MACS buffer in the presence of NK-cell biotin conjugated antibody cocktail and then incubated for 5 minutes at 4ºC.

Afterwards, NK cell microbead cocktail was added and the suspension was incubated for 10 min at 4ºC. The cell suspension was passed through previously rinsed LS columns (Miltenyi Biotec), collecting the flow-through, which contained the enriched population of NK cells. Columns were washed three times, and the eluent that contained the remaining NK cells was also collected. NK cells were resuspended in RPMI with 5% v/v human serum, 2 mM Glu, 1 mM NaP, 0.1 mM NEAA, 1% P/S, 100 IU/ml interleukin-2 overnight.

3.1.7. Neutrophil isolation from peripheral blood

Neutrophils were isolated from healthy donor buffy coats by dextran sedimentation plus Percoll gradient [42]. First, red blood cells were removed by resuspending plasma-free blood in 3% dextran in HBSS and allowing sedimentation on ice for 40 minutes. The remaining erythrocytes in the supernatant were lysed with ammonium-chloride-potassium (ACK) buffer for 5 minutes. For the Percoll gradient, the following layers were done in 15-ml Falcon tubes (from bottom to top): 2 ml Percoll 51%, 2 ml Percoll 42%, 2 ml cell suspension in HBSS. Then, cells were centrifuged at 400xg for 10 minutes. Neutrophils form a pellet at the bottom of the tube, which was resuspended in fresh HBSS and used for further assays.

3.1.8. Generation of primary iNKT cell lines

iNKT cell lines were generated from PBMCs of healthy donors and LPMCs from IBD patients and are listed in Table 3.3. Cells were sorted as CD45+ CD3+ hCD1d:PBS-57+ cells, as shown in Fig 3.2A. Sorted cells were resuspended in RPMI supplemented with 5% v/v human serum, 2 mM Glu, 1 mM NaP, 0.1 mM NEAA, 1% P/S, and 100 IU/ml interleukin-2, and subsequently stimulated with phytohemagglutinin (PHA, 1μg/ml, Remel) and irradiated feeders in a 1:1 iNKT:feeder ratio. Feeders were PBMCs irradiated at 12.5 Gy using a Faxitron CP160 X-ray irradiator. iNKT cells were expanded *in vitro* for 2 weeks and purity was checked *via* flow cytometry. When purity did not reach >90%, cells were re-sorted and a second stimulation/expansion cycle was performed.

Table 3.3. List of iNKT cell lines used in this study.

Name	Tissue	Type of Individual
CD1	Colon	Crohn's disease
CD2	Colon	Crohn's disease
CD3	Colon	Crohn's disease
NUN	Colon	Ulcerative colitis
PB1	Peripheral blood	Healthy donor
PB2	Peripheral blood	Healthy donor
PB3	Peripheral blood	Healthy donor
PB5	Peripheral blood	Healthy donor
PB6	Peripheral blood	Healthy donor

3.1.9. Priming of iNKT cells with bacteria

To stimulate iNKT cell lines with bacteria, moDCs were used as antigen-presenting cells in a 2:1 iNKT:DC ratio, whereas the heat-inactivated microbial content was used in a 10:1 CFU:DC ratio. Cells were stimulated for 24h at 37ºC in 48-well plates. To test the role of antigen presentation, surface Toll-like receptors and CHI3L1, priming took place in the presence of neutralizing antibodies (final concentration 10 μg/ml) and recombinant proteins listed in Table 3.4. For killing experiments with primed cells, these were first centrifuged at 200 xg for 5 minutes, supernatants were collected for ELISA assays and functional assays with neutrophils, and cells were resuspended in fresh RPMI medium supplemented with 5% v/v human serum and 100 IU/ml of IL-2.

Table 3.4. Neutralizing antibodies and proteins used during priming of iNKT cells with microbial content.

Name	Clone	Isotype	Vendor
Anti-human CD1d	CD1d42	Mouse IgG1, kappa	BD
Anti-human TLR2	B4H2	Human IgA2, kappa	InvivoGen
Anti-human TLR4	W7C11	Mouse IgG1, kappa	InvivoGen
Anti-human CHI3L1	mAY	Mouse IgG1, kappa	Merck
Recombinant human CHI3L1	-	-	R&D systems

3.1.10. Assessment of cytotoxicity

3.1.10.1. Lactate dehydrogenase release

Killing by iNKT cells was assessed using the Cytotoxicity Lactate Dehydrogenase (LDH) Assay Kit-WST (non-homogeneous assay, Dojindo EU)

following the manufacturer's instructions. All experimental conditions were performed in duplicate. Lactate dehydrogenase is a soluble and stable enzyme found in the cytosol, and its release into medium is due to cell death upon plasma membrane damage. Its measurement in culture medium is widely used in cytotoxicity assays, because the increase of LDH in supernatants is proportional to the number of dead cells. To test basal iNKT cell cytotoxicity, cancer cells (2.5 x 10^4 cells/well) were incubated at 37ºC for 4 hours with effector cells (iNKT cells, the NK-92 cell line or blood-derived NK cells) at effector:target (E:T) ratios of 8:1, 4:1, 2:1, and 1:1. Maximum and spontaneous release controls, as well as background controls, were included for calculation of the percentage of killing. Maximum release was obtained by incubating target cells with lysis buffer for the last 30 minutes of coculture. After coculture, 100 μl of supernatant was collected and plated in optically-clear, 96-well plates together with 100 μl of working solution, and were incubated for 30 min at room temperature in the dark. The working solution contains WST which, by an LDH-mediated reaction, is converted into a colored product that can be measured with a spectrophotometer. Reaction was stopped by adding 50 μl of stop solution and absorbance at 490 nm was measured using a Glomax plate reader (Promega). The percentage of cytotoxicity was calculated as follows:

$$\%\ \text{Killing} = \left(\frac{\text{Test well} - \text{mean (spontaneous release wells)}}{\text{mean (maximum release wells)} - \text{mean (spontaneous release wells)}}\right) \text{x } 100$$

For experiments with bacteria, raw values were also normalized to the values given by the presence of moDCs alone with each bacterium. For experiments in the presence of killing inhibitors and bacterial content, iNKT cells were used at 8:1 E:T ratio. The remaining supernatant was conserved at -20 ºC to perform ELISA assays, and cells were preserved for surface and intracellular staining.

3.1.10.2. Calcein release

To dissect basal iNKT cell killing, cytotoxicity was also assessed via the detection of calcein in culture supernatants. For this assay, target cells were previously incubated with Calcein AM for 1h in the dark, washed twice with PBS, and then resuspended in new medium. For these experiments, iNKT cells were used in a 8:1 E:T ratio. After the 4-hour co-incubation, 100 ul of supernatant was transferred to opaque-welled plates and calcein release was measured using a

Glomax plate reader (Promega). The percentage of dead cells was calculated as in 3.1.8.1.

3.1.10.3. Use of inhibitors and neutralizing antibodies

For experiments using inhibitors, target cells were pre-treated for 1h with anti-CD1d antibody (10 µg/ml, clone CD1d42, R&D Systems); iNKT cells were pre-treated with anti-Fas Ligand antibody (1h, 10 µg/ml, clone 100419, R&D Systems), anti-TRAIL antibody (1h, 10 µg/ml, clone 75411, R&D Systems), concanamycin A (2 hours, 10 nM, Sigma-Aldrich), or 3,4 dichloroisocoumarin (20 minutes, 40 µM, Enzo Life Sciences). Medium from pre-treated cells was replaced after the treatments. For these experiments, iNKT cells were used in an 8:1 E:T ratio.

3.2. Neutrophil migration assay

Migration assays were performed as previously described [42]. In brief, fresh neutrophils were first pre-incubated for 20 min at 37°C with RPMI-1640 + 2% FBS (basal medium) and seeded on top of a 3 mm-pore transwell (SARSTEDT). A total of 500 µl of the culture supernatant of iNKT cells (primed or not primed with *P. gingivalis*) diluted 10% v/v in basal medium, were added on the bottom of the transwell. Then, migration was allowed for 4 hours at 37°C. For these experiments, RPMI-1640 with 10% FBS was used as positive control for migration. Afterwards, migrating neutrophils found on the bottom of the plate were stained and counted using the FACSCelesta cytometer (BD Biosciences) with plate acquisition mode and defined volumes.

3.3. Growth conditions and heat inactivation of *Porphyromonas gingivalis*

Porphyromonas gingivalis ATCC 33277 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH). It was grown in Columbia broth supplemented with 5ug/ml hemin (Sigma-Aldrich) and 1ug/ml menadione (Sigma-Aldrich) for 48h, and it was used in exponential phase (OD600 0.6-0.8). For heat inactivation, bacteria were first resuspended at a concentration of 10^9 CFU/ml in sterile PBS, subsequently they were heated at 70ºC for 30 minutes and immediately stored at -20ºC.

3.4. ELISA for the detection of proteins in supernatants

High-affinity, protein-binding plates (Nunc, Thermo Fisher were coated with capture antibodies previously diluted in PBS or in Coating Buffer (8.4 g $NaHCO_3$,

3.56 g Na_2CO_3 in 1L of ddH2O, 50 µl/well) at a final concentration of 1-2 µg/ml and incubated overnight at 4ºC. The next day, plates were washed three times with PBS plus 0.05% Tween 20 (Washing Buffer). Prior to each step described hereafter, plates were washed three times. Plates were subsequently blocked with 50 µl/well of Blocking Solution/Incubation Buffer (PBS with 0.1-1% of Bovine Serum Albumin) for 1 hour at room temperature. Then, 50 µl of standards (done by serial dilutions) and samples were added to each well, and were incubated for 2 hours at room temperature. Subsequently, biotinylated antibodies were added at a concentration of 1 µg/ml in Blocking Solution/Incubation Buffer; plates were then incubated for 1 hour at room temperature. Afterwards, 50 µl/well of Streptavidin-Horseradish peroxidase (diluted 1:1000 in Blocking Solution/Incubation Buffer) were added to each well, and plates were incubated for 1 hour at room temperature. Next, 50 µl/well of Tetramethylbenzidine (TMB) Substrate Solution were added, and plates were incubated at room temperature for 20-30 minutes in the dark. Lastly, 50 µl/well of Stop Solution (H_2SO_4 1N) were added, and absorbance was measured using a Glomax plate reader (Promega) at 450 nm.

Table 3.5. List of ELISA antibodies.

Antibody	**Clone**	**Vendor**
Anti-human granzyme B, purified	GB10	Mabtech
Anti-human granzyme B, biotinylated	GB11	Mabtech
Anti-human perforin, purified	Pf-80/164	Mabtech
Anti-human perforin, biotinylated	Pf-344	Mabtech
Anti-human interferon gamma, purified	NIB42	Biolegend
Anti-human interferon gamma, biotinylated	4S.B3	Biolegend
Anti-human interleukin-10, purified	JES3-19F1	Biolegend
Anti-human interleukin-10 biotinylated	JES3-12G8	Biolegend
Anti-human interleukin-17, purified	BL23	Biolegend
Anti-human interleukin-17, biotinylated	Poly5189	Biolegend
Anti-human GM-CSF, purified	BVD2-23B6	Biolegend
Anti-human GM-CSF, biotinylated	BVD2-21C1	Biolegend
Anti-human CHI3L1, purified	384327	R&D Systems
Anti-human CHI3L1, biotinylated	Polyclonal Goat IgG, ID not indicated	R&D Systems

3.5. *In vivo* experiments

3.5.1. Mice

C57Bl/6J mice (Charles River, Italy) and B6(Cg)-Traj18tm1.1Kro/J [142] (*Traj18*-/-, gift from Paolo Dellabona, San Raffaele Institute) mice, which are deficient for iNKT cells, were housed at the European Institute of Oncology animal facility in SPF conditions. It has been observed that, even if in this model there is

lower infiltration of neutrophils in colon cancer models [42] and of eosinophils in airway inflammation [142], under steady-state conditions, this model lacks exclusively of iNKT cells [142]. All procedures were approved by the Italian Ministry of Health under the permissions Auth. 1217/2020 and 10/2021. Experiments started when mice were 7-9 weeks old and mice were always randomly assigned to each experimental group.

3.5.2. AOM/DSS Model

The scheme for this model is shown in Fig 3.1A. Eight-week old mice were weighed and injected intraperitoneally with azoxymethane (AOM) diluted in sterile saline solution at a concentration of 1 mg/ml, injecting 10 mg per 1 kg body weight. AOM stock aliquots of 10 mg/ml diluted in ddH_2O were stored at -20ºC until use. After injection, all mice were monitored for 5 days. At the start of week 2, mice received 1% w/v dextran sulfate sodium (DSS) in their drinking water for 7 days; consumption was monitored twice a week to ensure equal intake by all experimental groups. Treatment with DSS was followed by two weeks of regular water. This procedure was repeated one more time for a total of two DSS cycles.

3.5.3. Orthotopic injection model

The scheme for this model is shown in Fig. 3.1B. and performed as previously described [143]. For this model, we used the MC38 colon cancer cell line. This line was grown in DMEM medium supplemented with penicillin/streptomycin, 10% FBS, 2 mM Glutamine, 1 mM NaP, 0.1 mM NEAA., and split 3 times a week, when cells were confluent. For injection, a total of 1 x 10^5 MC38 cells were injected in the colon mucosa of 8-week old mice using a previously described colonoscopy system (Karl Storz, [143]). In brief, mice were anesthetized using 3% isoflurane during all the procedure. Injections were performed using flexible stainless-steel needles (8-inch, 30 gauge with a 45º bevel, Cadence Inc.), which were screwed to the Luer lock of the working channel of the scope. The scope was then inserted into the colon, the needle was carefully brought out of the scope and meticulously inserted in the submucosa in a flat angle, and 50 μl of cell suspension was slowly injected. To avoid liquid leakage, we waited 10 seconds before retiring the needle to ensure complete absorption. This procedure was performed by two people, one controlling the scope while the other performed the injections.

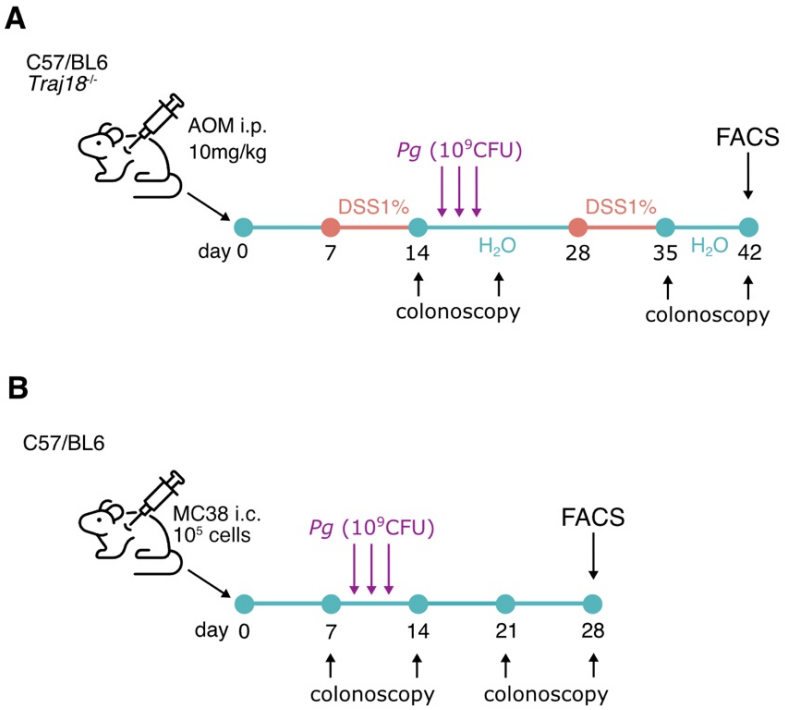

Fig. 3.1. Schemes of the murine CRC models used in this study. A. AOM/DSS model. B. MC38 cell intracolonic injection model

3.5.4. Bacteria transplantation

For each mouse model, the dosage and timing are shown in Fig. 3.1. For bacteria transplantation, they were grown one week before the procedure to guarantee an exponential growth. For these experiments, we did not summinister antibiotics prior to bacteria treatment in order to preserve the endogenous microbiota, and because antibiotics are shown to prevent tumor formation [144]. Bacteria were administered *via* oral gavage in three alternate days and diluted in sterile PBS. A total 100 μl of bacterial suspension containing 10^9 CFU were given to each mouse each day.

3.5.5. Exploratory colonoscopy and tumor endoscopic score

For exploratory colonoscopy, mice were first anesthetized using 3% isoflurane. A mouse colonoscopy system (Karl Storz) was used as previously described [143, 145]. Air was carefully insufflated into the colon in order to have clear visual of the lumen and to avoid injury due to the entrance of the scope. Afterwards, images were taken and saved for offline analyses. To address severity, we used the tumor endoscopic score documented in [145], for which we assigned values from 0 to 5 according to the percentage of lumen occupied by tumors. We performed

colonoscopy the day before sacrifice for both models, once a week for the intracolonic injection model and during the water weeks for the AOM/DSS model.

Size (avg. diameter)		Score
Just detectable		1
1/8		2
1/4		3
1/2		4
≥ 1/2		5

Fig. 3.2. Tumor endoscopic scores. Adapted from Becker *et al. Nat Protoc*. (2006) [145]

3.5.6. Isolation of murine lamina propria mononuclear cells

As soon as colons were collected, they were opened, cleaned from feces, collected in 15-ml Falcon tubes with 5 ml of PBS, and preserved on ice until processing. After being washed with ice-cold PBS, colons were cut in ~1 cm pieces and incubated in pre-warmed RPMI medium with 2% FBS, 1% penicillin/streptomycin, and 1mM EDTA for 30 min at 37ºC with agitation (to remove epithelial cells). Subsequently, medium was discarded, pieces were washed again with ice-cold PBS to be then put in GentleMACS tubes (Miltenyi Biotec) containing 2.5 ml of RPMI medium. Then, we dissociated the tissue by running 3 times the mouse intestine mixing program on a GentleMACS dissociator (Miltenyi Biotec). Colon homogenates were diluted in 20 ml of RPMI medium and serially passed through metal strainers, 100-μm, and 70-μm cell strainers to remove traces of connective tissue and other particulate material. After this, cell suspensions were centrifuged at 1500 rpm for 5 minutes, medium discarded, and pellets were resuspended in adequate quantity of medium for stimulation with PMA/Ionomycin or for respiratory burst assay.

3.6. Flow Cytometry

For experiments adressing the functional characterization of human iNKT cells and murine cells, these were stimulated with PMA (0.1 μM, Sigma-Aldrich) and Ionomycin (1μg/ml, Sigma-Aldrich) in the presence of Brefeldin A (10 μg/ml, Sigma-Aldrich) for 3h at 37ºC. For killing experiments with human iNKT cells, cells were treated with brefeldin 30 min before the end of the experiments. The antibodies used

here are listed in Tables 3.6 and 3.7. To identify and isolate iNKT cells, we used human and mouse tetramers (NIH Tetramer Facility), which consist of PBS-57, an analog of alpha-galactocylceramide (αGalCer), prototypical antigen of iNKT cells [45], coupled to fluorophore-conjugated CD1d tetramers, and specifically bind the TCR of iNKT cells. Tetramers were used as follows: mouse 0.1 μl/10^6 cells, human 0.2 μl/10^6 cells; cells were incubated for 30 min at 4ºC. Staining of loaded and unloaded tetramers are shown in Figs. 3.3C and 3.4B. Prior to staining, Fcγ receptors were blocked using anti-CD16/CD32 antibodies (clone 2.4G2, TONBO Biosciences). For surface staining, mouse antibodies were used 0.25 μl/10^6 cells, whereas human antibodies were used at 0.5 μl/10^6 cells, and it was done for 10 minutes at 4ºC. For intracellular staining, cells were first fixed with Cytofix/Cytoperm (BD) for 15 min at room temperature and permeabilized for 30 min with Perm/Wash buffer. Subsequently, antibodies diluted at a 0.5 μl/10^6 cells in Perm/Wash buffer were incubated with the cells for 30 min at room temperature.

All samples were acquired on a FACSCelesta (BD) cytometer. All data were analyzed with FlowJo software version 10, using the gating strategies shown in Figs. 3.3 and 3.4. In mice experiments, negative gates for cytokines and lineages were set based on the unstained controls and cells negative for CD45.2. For cells with human cells, negative gates were set with unstained controls.

Table 3.6. List of human antibodies for flow cytometry analysis.

Antibody	**Clone**	**Fluorochrome**	**Vendor**
human CD1d:PBS-57 Tetramer	-	PE	NIH Tetramer Facility
Anti-human 4-1BB	4B4	SB600	eBioscience
Anti-human CD1d	51.1	APC	Biolegend
Anti-human CD3	UCHT1	PE-Cy7, APC/Cy7	TONBO Biosciences
Anti-human CD15	W6D5	BV785	Biolegend
Anti-human CD45	HI30	BV510	BD Biosciences
Anti-human CD66b	G10F5	PerCP/Cy5.5	BD Biosciences
Anti-human CD69	FN50	Alexa Fluor 700	BD Biosciences
Anti-human CD95 (Fas)	DX2	BV650	Biolegend
Anti-human CD107A	H4A3	APC-Cy7	Biolegend
Anti-human Fas Ligand	NOK-1	APC	TONBO Biosciences
Anti-human CD274 (PD-L1)	29E.2A3	PE	Biolegend
Anti-human CD279 (PD1)	EH12	BV650	BD Biosciences
Anti-human EPCAM	1B7	PERCP	eBioscience
Anti-human IFNG	B27	FITC	BD Biosciences
Anti-human IL-10	JES3-9D7	BV421	Biolegend
Anti-human IL-17	eBio64DEC17	APC	eBioscience
Anti-human GM-CSF	BVD2-21C11	PerCP-Cy5.5	Biolegend
Anti-human Granzyme B	N4TL33	eF450	Invitrogen
Anti-human Perforin	dG9	BV510	Biolegend

Anti-human TIGIT	A15153G	BV605	Biolegend
Anti-human TIM-3	F38-2E2	BV785	Biolegend
Anti-human TRAIL	RIK-2	BV786	BD Biosciences
Anti-human TRAILR1	S35-934	BV605	BD Biosciences
Anti-human TRAILR2	B-K29	BV510	BD Biosciences

Table 3.7. List of mouse antibodies for flow cytometry analysis.

Antibody	**Clone**	**Fluorochrome**	**Vendor**
mouse CD1d:PBS-57 Tetramer	-	PE, BV421	NIH Tetramer Facility
Anti-mouse 4-1BB	1AH2	SB600	BD Biosciences
Anti-mouse CD1D	1B1	PERCP	Biolegend
Anti-mouse CD11B	M1-70	APC/Cy7	TONBO Biosciences
Anti-mouse CD11C	N418	BV605	Biolegend
Anti-mouse CD19	1d3	VF450	TONBO Biosciences
Anti-mouse CD3	17A2	AF700, PE/Cy7	Biolegend
Anti-mouse CD4	RM4-5	APC/Cy7	BD Biosciences
Anti-mouse CD8	53-6-7	SB600, BV650	eBioscience, Biolegend
Anti-mouse CD45.2	104	BV510	Biolegend
Anti-mouse CD69	H1.2F3	PERCP/Cy5.5	Biolegend
Anti-mouse F4/80	BM8	PE	Biolegend
Anti-mouse GM-CSF	MIP1-22E9	FITC	Biolegend
Anti-mouse IFNG	XMG1.2	BV786	BD Biosciences
Anti-mouse IL10	JES5-16E3	BV421	Biolegend
Anti-mouse IL17	TC11-18H10.1	AF700	Biolegend
Anti-mouse Ki67	16A8	Pacific Blue	Biolegend
Anti-mouse Ly6G	1A8	APC	TONBO Biosciences
Anti-mouse NK1.1	PK136	BV650	Biolegend
Anti-mouse PD1	J43	APC	BD Biosciences
Anti-mouse PD-L1	MIH5	BV786	BD Biosciences
Anti-mouse TIGIT	GIGD7	PERCP	Invitrogen

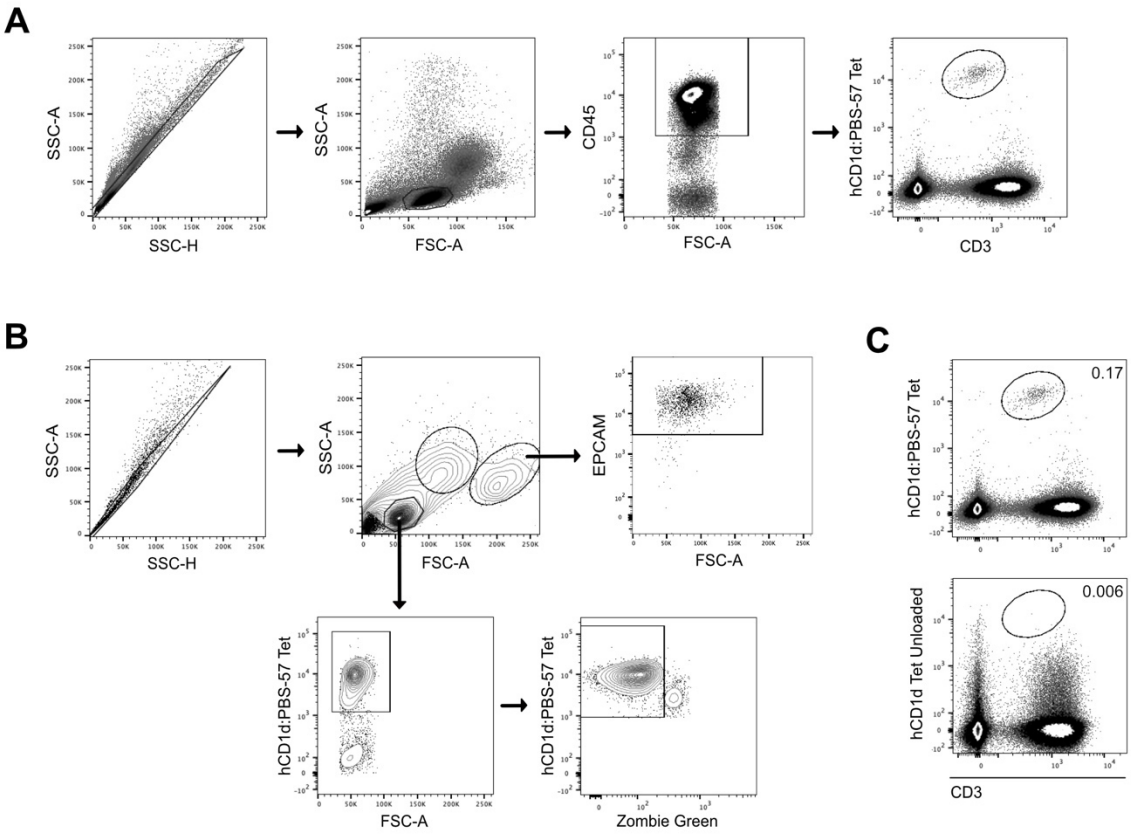

Figure 3.3. Gating strategies for the analysis human cells. A. Gating strategy to sort iNKT cells from PBMCs and LPMCs. B. Gating strategy for the analysis of iNKT and cancer cells after killing experiments. C. Representative plots of loaded and unloaded human tetramers for iNKT cell identification.

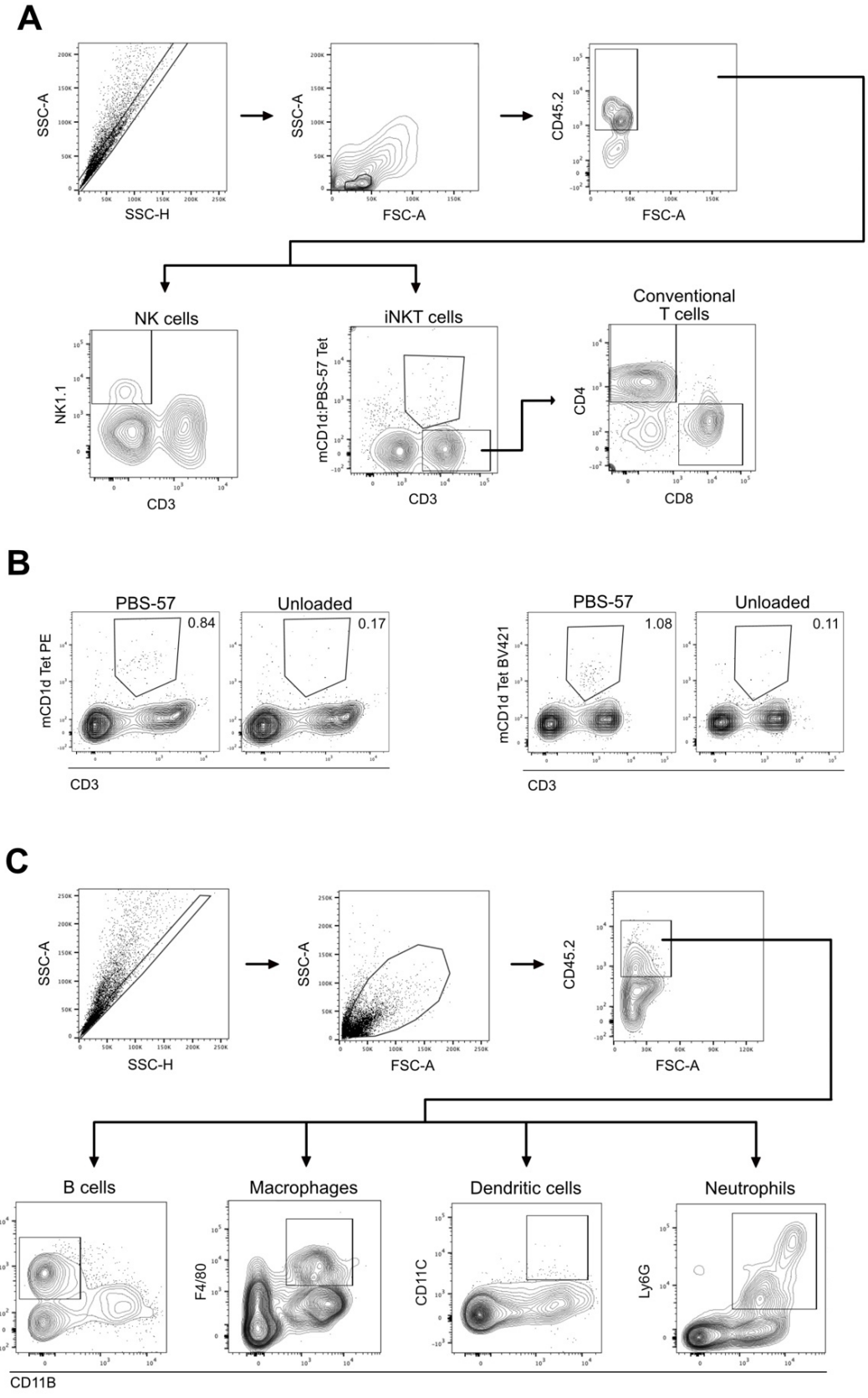

Figure 3.4. Gating strategies for murine cells. A. Strategy for lymphoid cells. B. Representative plots of loaded and unloaded mouse tetramers for iNKT cell identification. C. Strategy for APCs.

3.7. Assessment of cell viability

Cell viability was assessed using the Zombie Green Fixable Viability Kit (Biolegend) according to the manufacturer's instructions. Cells were stained with Zombie Green dye and incubated for 15 minutes at room temperature, and subsequently washed twice with 1% BSA in PBS.

3.8. Respiratory burst assay

To detect ROS production by murine cells, we used the Neutrophil/Monocyte Respiratory Burst Assay (Cayman Chemical) with some modifications. First, 2 x 10^5 cells were resuspended in 100 μl of RPMI medium with pen/strep and 2% FBS. Afterwards, 10 μl of the 10X Dihydrorhodamine (DHR) 123 stock solution were added to the cell suspensions and incubated for 15 minutes at 37ºC in the dark. Subsequently, 25 μl of 1 μM PMA working solution were added and cells were further incubated for 45 minutes at 37ºC in the dark. After this, we immediately proceeded with staining and acquisition as in 3.6. Rhodamine 123 emits at 530 nm, similar to FITC.

3.9. Bulk RNA sequencing of human iNKT cells

Total RNA (from 1 × 10^6 cells) was isolated with the RNeasy kit (Qiagen, IT) and RNA quality was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, IT). A total of 0.5-1 μg were used to prepare libraries for RNA-seq with the Illumina TruSeq RNA Library Prep Kit v2 following the manufacturer's instructions. RNA-seq libraries were then run on the Agilent 2100 Bioanalyzer (Agilent Technologies, IT) for quantification and quality control and pair-end sequenced on the Illumina NovaSeq platform.

3.10. Statistical analysis

Mann-Whitney tests were performed for comparisons between 2 groups, whereas Kruskal-Wallis tests and Dunn's tests for multiple comparisons were performed to compared among +3 groups. Normality was tested with Shapiro-Wilk test. All data were analyzed with GraphPad Prism version 9.

3.11. High-dimensional flow cytometry analysis

These analyses were performed according to [42]. In brief, FCS files were first uploaded in FlowJo software (Version 10.8) and samples were compensated manually according to the software usage. One fluorescence parameter per laser

was then queried for irregularities during acquisition by plotting it against the time parameter. Any variations in the flow stream were excluded. Samples were then cleaned for antibodies aggregates by checking each parameter in a bimodal plot. Cleaned samples were subsequently gated on singlets, viable lymphocytes and CD3+ cells. Afterwards, CD3+ populations were down-sampled to 5000 events per sample using the FlowJo DownSample plugin (Version 3.3.1).

Down-sampled populations were exported as FCS files with the compensation correction. Files were then uploaded to the RStudio environment (Version 4.1.2) using the flowCore package (Version 1.38.2). Data were first transformed using logicleTransform() function present in the flowCore package. To equalize the contribution of each marker, they were interrogated for their density distribution using the densityplot() function of the flowViz package (Version 1.36.2). Each marker was normalized using the Per-channel normalization based on landmark registration using the gaussNorm() function present in the package flowStats (Version 3.30.0). Peak.density, peak.distance and number of peaks were chosen according to the signal of each marker. Subsequently, normalized files were analyzed using the cytofkit package through the cytofkit_GUI interface. For data visualization we used the t-Distributed Stochastic Neighbor Embedding (t-SNE) method, while for clustering we used the Phenograph algorithm. t-SNE plots were visualized on the cytofkitShinyAPP with the following parameters: perplexity=50, iterations=1000, seed=42, k=50. FCS files for each cluster were generated and re-imported in FlowJo to calculate the integrated MFI (iMFI). The iMFI of all markers was scaled from 0 to 1 and used to identify Phenograph clusters.

3.12. Analysis of RNA-seq data

RNA-seq data were analyzed as in [42]. Reads were pre-processed using the FASTX-Toolkit tools. First, quality control was performed with FastQC. Pipelines for the primary analysis (filtering and alignment to reference genome) and secondary analysis (quantification of expression and differential gene expression) have been integrated and run in the HTS-flow system. Differentially expressed genes were identified using the Bioconductor DESeq2 package. p-values were corrected for false discovery rate using the Benjamini-Hochberg procedure in DESeq2. The functional enrichment analyses to determine gene ontology categories and KEGG pathways were performed using the DAVID Bioinformatics Resources (DAVID Knowledgebase v2022q2, https://- david.ncifcrf.gov).

4. Results I

4.1. Upon encounter with colon cancer cells, iNKT cells reduce their expression of interferon gamma, IL-17 and PD1

iNKT cells are characterized by the fast release of cytokines following the detection of a stimulus [146]. Therefore, we first adressed the expression of antitumor (IFNG) and pro-tumor (IL-10, IL-17 and GM-CSF) cytokines by iNKT cells after co-incubation with colon cancer cell lines (Table 3.2). For this, we isolated iNKT cells from human peripheral blood and colon specimens, expanded them and generated primary lines for functional studies (Table 3.3). As shown in Fig. 4.1, cancer cells provoked a decrease in interferon gamma and IL-17 production (A and C), while IL-10 and GM-CSF positive cells remained unaffected (Fig. 4.1B & D). We also wondered whether tumor cells could mediate changes in the expression of the activation molecule CD69 [147], as well as the inhibitory markers PD1, TIGIT and TIM-3 [148]. The percentages of activated iNKT cells (CD69+) did not vary upon coculture (Fig. 4.2A); however, there was a downregulation of PD1 (Fig. 4.2B). The frequencies of TIGIT and TIM-3 positive cells did not vary because of the presence of cancer cells. These data indicate that colon cancer cells have a negative effect on the expression of IFNG, IL-17 and PD1 by human iNKT cells.

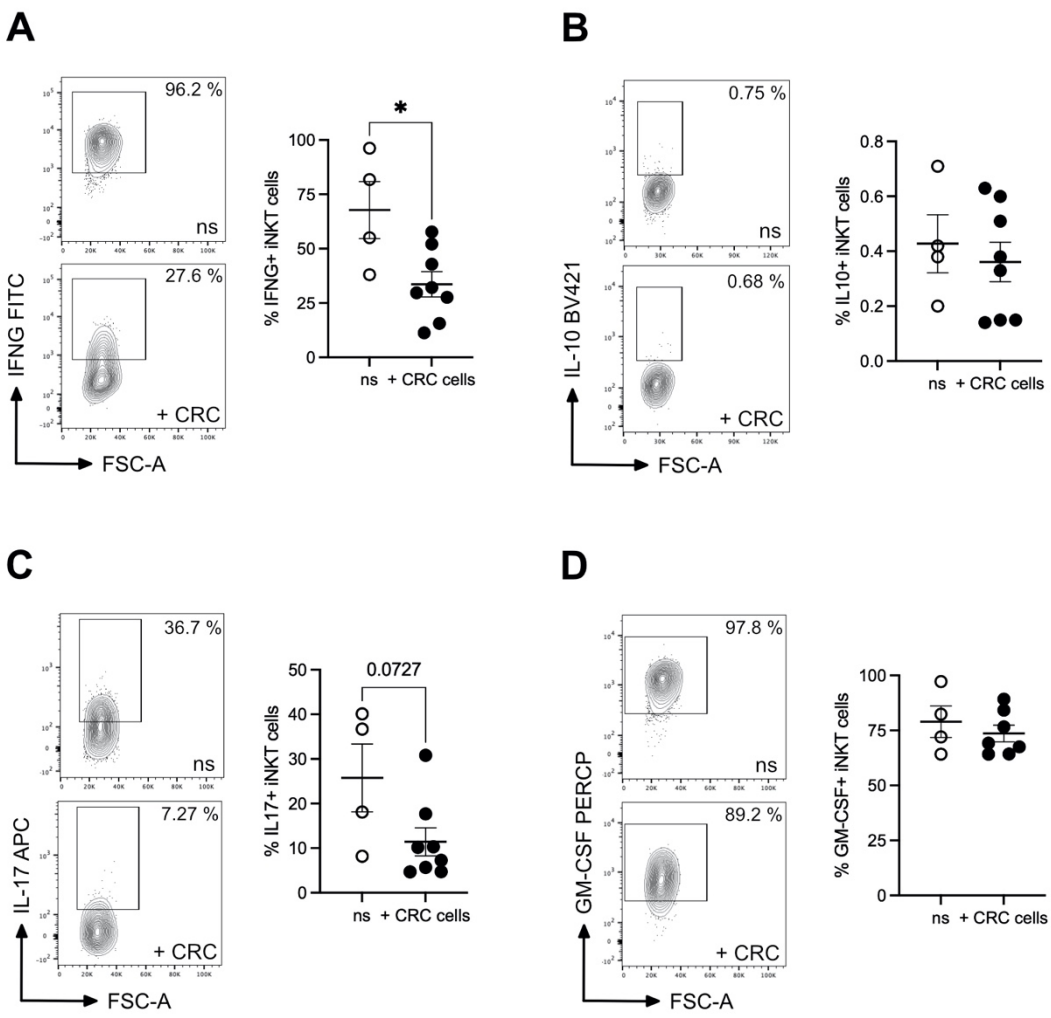

Fig. 4.1. Cytokine expression by iNKT cells at basal state and after coculture with colon cancer cells. Right, representative plots; left, frequencies of A. Interferon gamma, B. IL-10, C. IL-17, and D. GM-CSF positive cells. Mann-Whitney test. Experiments were performed on 4 iNKT cell lines and 2 CRC cell lines. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

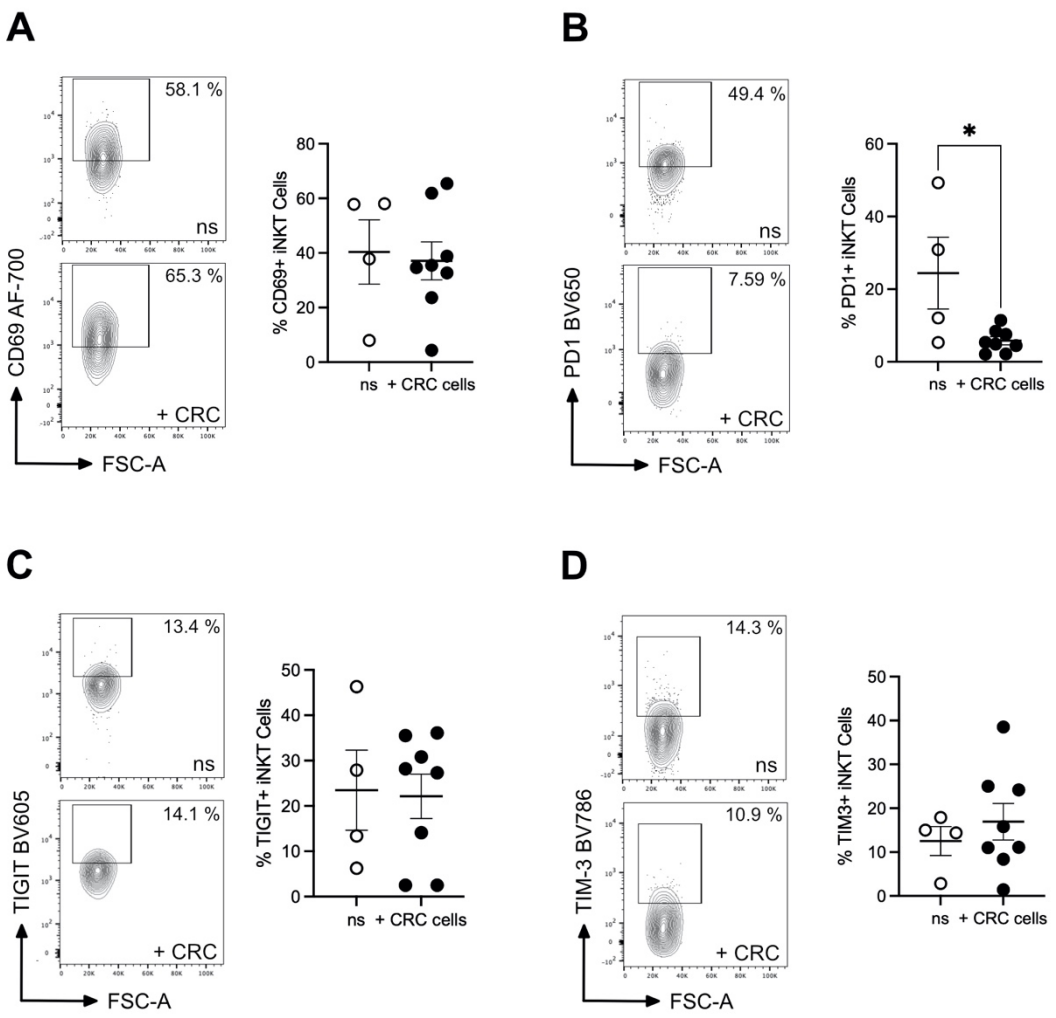

Fig. 4.2. Expression of activation and inhibitory molecules by iNKT cells at basal state and after coculture with colon cancer cells. Right, representative plots; left, frequencies of A. Interferon gamma, B. IL-10, C. IL-17, and D. GM-CSF positive cells. Mann-Whitney test. Experiments were performed on 4 iNKT cell lines and 2 CRC cell lines. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.2. Intestinal and blood-derived iNKT cells are cytotoxic against CRC cell lines

Given the negative effect of CRC cells on the expression of interferon gamma, we asked whether they would have a similar influence on iNKT cell cytotoxicity as well. For this we used the lactate dehydrogenase release assay to test the cytotoxic activity of nine iNKT cell lines (5 circulating and 4 intestinal, Table 3.3) against a panel of CRC cell lines (Table 3.2), and compared it with the killing exerted by freshly-isolated, blood-derived natural killer cells. In addition, we used the highly cytotoxic cell line NK-92 as a cell-mediated cytotoxicity control (Fig. 4.3A). Both intestinal and blood-derived iNKT cells were cytotoxic against the whole CRC line panel in a way similar to circulating NK cells, and in some cases – against Caco-2,

HT-29 and RKO cells, circulating iNKT cells were significantly more effective (Fig. 4.3A). In general, cytotoxicity of iNKT cells from both tissues did not differ, with the exception of the elimination of RKO cells, for which circulating cells were more efficient. Nonetheless, neither iNKT cells nor freshly isolated NK cells were more effective than NK-92 cells.

When iNKT cell lines were analyzed separately, we observed a substantial donor heterogeneity (Fig. 4.3B & C). In particular, iNKT CD3 and CD1 cells were the most cytotoxic among the intestinal lines, especially against Caco-2, Colo 205 and HT-29, whereas NUN cells were in general the least efficient (Fig. 4.3B). Among blood-derived lines, PB1 and PB6 cells, with PB1 cells being the most cytotoxic against Caco-2 and HT-29 cells, while PB5 and PB2 were virtually non-cytotoxic, as shown by a non-ascendent trend of their effector-to-target (E:T) ratio curves (Fig. 4.3C). Taken together, these data show that iNKT cells coming from peripheral blood and colon specimens can eliminate a variety of colon cancer cell lines with similar efficacy as circulating NK cells. However, there is a considerable donor-associated variability among iNKT cell lines.

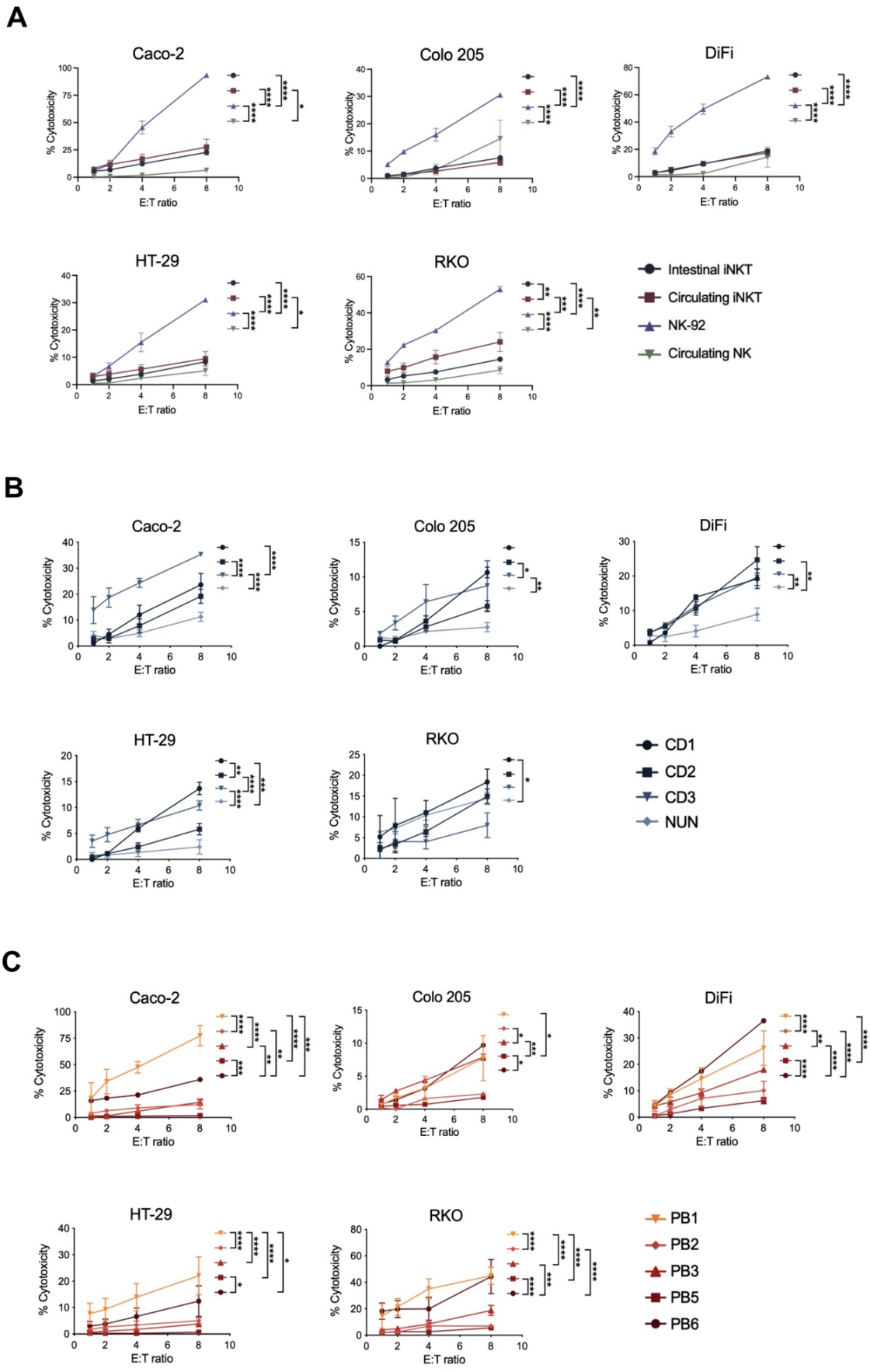

Fig. 4.3. Intestinal and blood-derived iNKT cell lines are cytotoxic against CRC cell lines. A. Effector-to-target (E:T) ratio curves of intestinal and circulating iNKT lines compared with blood-derived NK cells and NK-92 cells for each CRC cell line. B & C. E:T ratio curves of

each intestinal (B) and circulating (C) iNKT cell line for each cancer line. Two-way ANOVA. Data are means ± SEM of at least 2 experiments for each line. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.3. Colon cancer cell lines are grouped according to their sensitivity to iNKT cell-mediated killing

Next, we wondered if there are CRC lines that are more resistant to iNKT cell cytotoxicity. As shown in Fig. 4.4, there were indeed differences in the killing efficacy against each CRC line. In particular, both intestinal and circulating iNKT cells were more efficient against Caco-2, DiFi, and RKO cells than Colo 205 and HT-29 cells, shown both by E:T curves and cytotoxicity at the highest E:T ratio (Fig. 4.4A & B). To further confirm these findings, we performed killing experiments by measuring Calcein release, showing similar patterns (Fig. 4.4C). Here, we showed that some target cells are more resistant than others to iNKT cell elimination, suggesting differences in how tumor cells can activate or inhibit iNKT cell cytotoxicity.

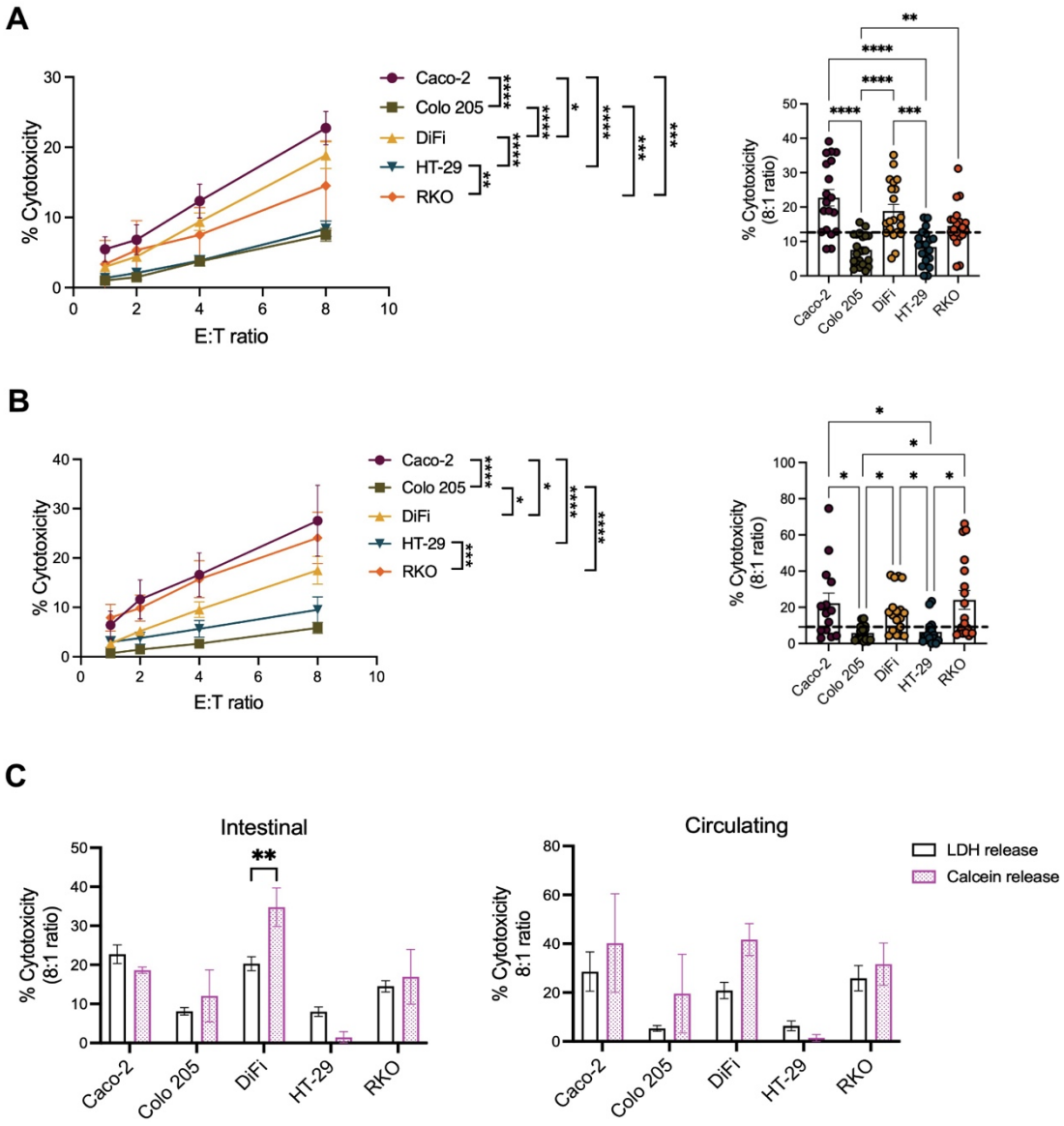

Fig. 4.4. CRC cell lines are grouped according to their sensitivity to iNKT cell-mediated killing. A-B. Left, E:T curves; right, cytotoxicity at 8:1 E:T ratio of intestinal (A) and circulating (B) iNKT cells against each CRC cell line. Dot lines indicate the median of all data. C. Comparison between LDH and calcein release for each target line at 8:1 E:T ratio. Two-way ANOVA for left A & B; Kruskal-Wallis test with Dunn's test for comparisons in right A and B; Mann-Whitney test for C. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.4. Human iNKT cells degranulate and release granzyme B and perforin when they encounter CRC cell lines

iNKT cells can eliminate transformed cells via various mechanisms [66, 149–151], being the perforin/granzyme pathway the preferred mechanism *in vitro* [65, 150, 152]. In order to assess any possible regulation of these molecules, we tested changes in surface CD107A, granzyme B and perforin release *via* flow cytometry

and in supernatants under steady-state conditions (basal) and upon coculture with CRC cell lines. We observed that intestinal iNKT cells had in general higher frequencies of degranulating cells after they encountered CRC cells (Fig. 4.5B). Conversely, blood-derived cells did not present substantial changes after the 4-hour coculture. Similar patterns were observed in the frequencies of granzyme B and perforin positive cells (Fig 4.6), for which no significant changes were observed, but trends were still evident for intestinal iNKT cells, such an increment in granzyme B numbers, but lower frequencies of perforin positive cells after coculture (Fig.4.6. A-B). Even if perforin-positive cell numbers tended to decrease, CRC cell lines promoted the release of perforin by both intestinal and circulating iNKT cells (Fig. 4.7B), and the same phenomenon occurred with granzyme B (Fig. 4.7A). In conclusion, when iNKT cells encounter colon cancer cells, they degranulate to release granzyme B and perforin.

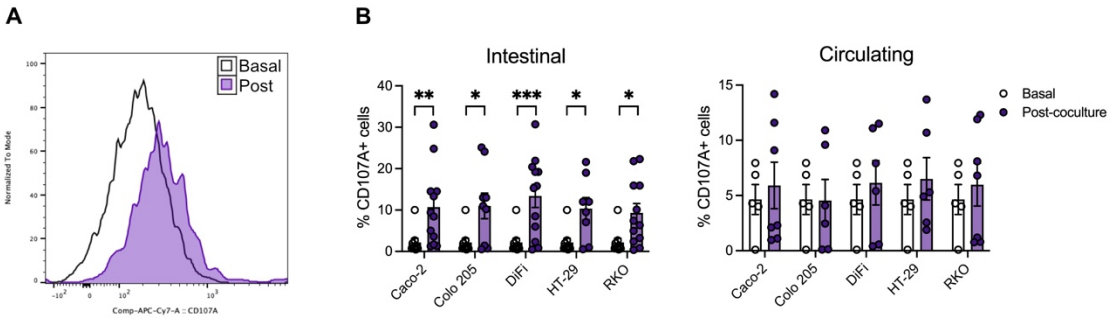

Fig. 4.5. iNKT cells degranulate when they encounter CRC cell lines. A. Representative histograms of CD107A at basal state and upon-coculture. B. Frequencies of surface CD107A positive cells at steady state and after co-incubation with CRC lines. Mann-Whitney tests. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

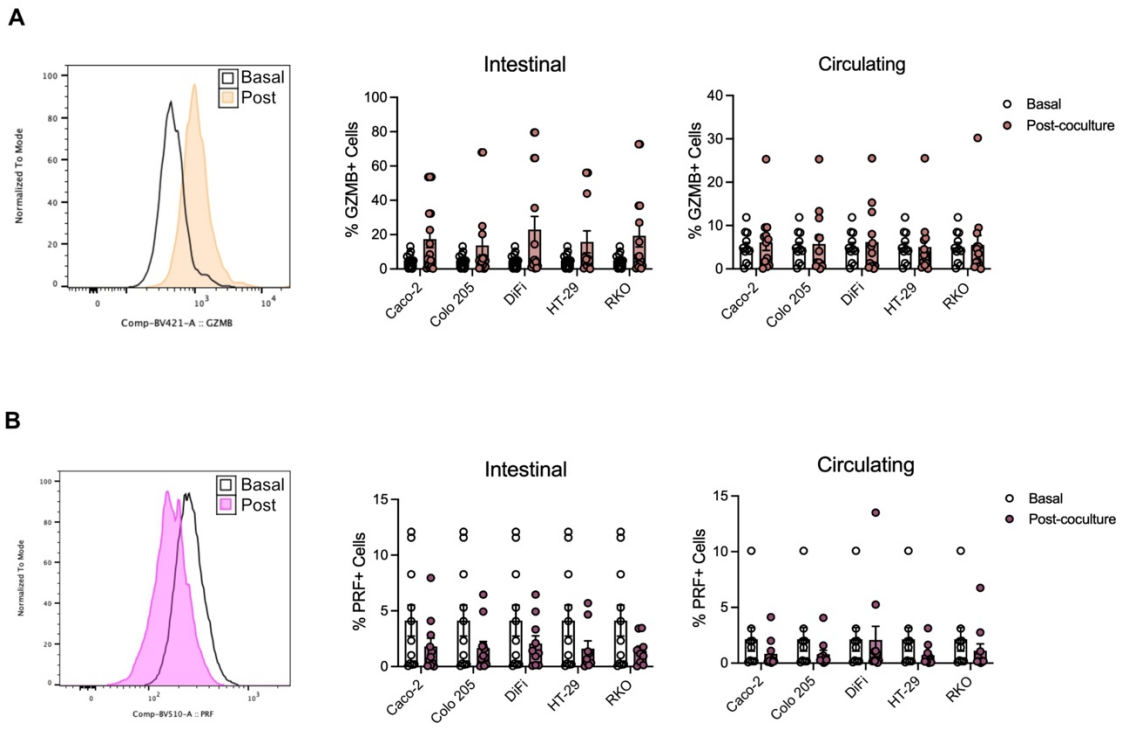

Fig. 4.6. Frequencies of iNKT cells positive for soluble cytotoxic mediators at basal state and after coculture with colon cancer cell lines at 8:1 E:T ratio. Left, representative histograms; right, frequencies of Granzyme B (A) and Perforin (B). Mann-Whitney tests. * p<0.05, ** p<0.01, *** p<0.001

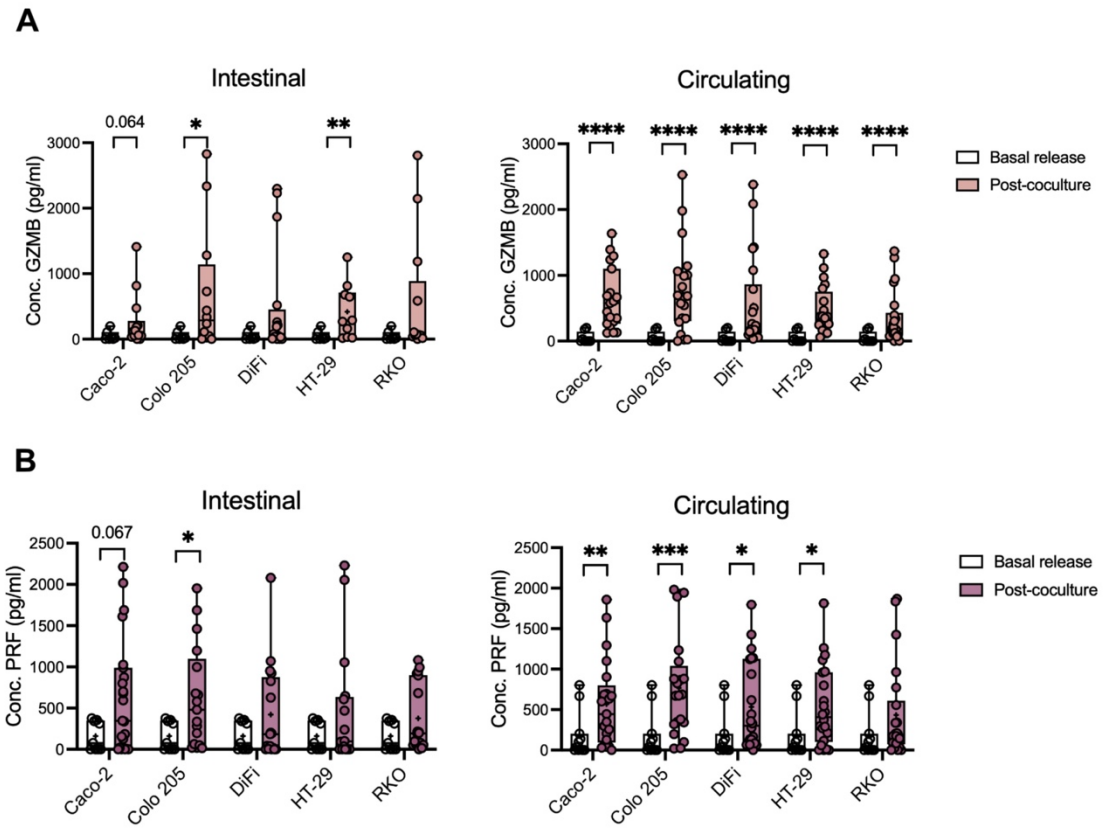

Fig. 4.7. Release of soluble cytotoxic molecules by iNKT cells at basal state and after coculture with colon cancer cell lines at 8:1 E:T ratio. A. Granzyme B and B. Perforin release. Mann-Whitney tests. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.5. The expression of death ligands by iNKT cells does not substantially change during coculture with CRC cell lines

The death ligands TRAIL and FasL are used by murine and human iNKT cells for the elimination of cancer cells *in vitro* and *in vivo* [66, 77, 84, 151]. Hence, we analyzed their expression before and after the exposure to CRC cells (Figs. 4.8A and 4.9A). While TRAIL-positive cells at steady-state conditions varied greatly among donors - ranging from 80% to less than 1% (Fig. 4.8A), positivity for Fas ligand did not surpass 5% in any of the lines studied (Fig. 4.9A). After co-incubation with CRC cells, TRAIL expression tended to increase on intestinal iNKT cells, while not having noteworthy alterations on blood-derived cells (Fig. 4.8A). A similar pattern was observed for FasL expression, for which modest modulations were observed for cells in contact with HT-29 and RKO cells (Fig. 4.9A).

On the other hand, and as mentioned previously, cell death by Fas ligand and/or TRAIL depends on the expression of death receptors on target cells. To note, the expression of all death receptors was highly variable among the target lines studied here (Fig. 4.8B & 4.9B). Regarding TRAIL receptors 1 and 2, with the exception of RKO cells, CRC lines were characterized by a low expression (Fig. 4.8B & C). Fas expression was more variable among target lines, as Caco-2 and Colo 205 cells showed low expression, DiFi and RKO cells had intermediate frequencies, whilst most HT-29 were Fas positive (Fig. 4.9B). Overall, these data indicate that human iNKT cells do not modulate the presence of TRAIL and Fas ligand upon encounter with CRC cell lines.

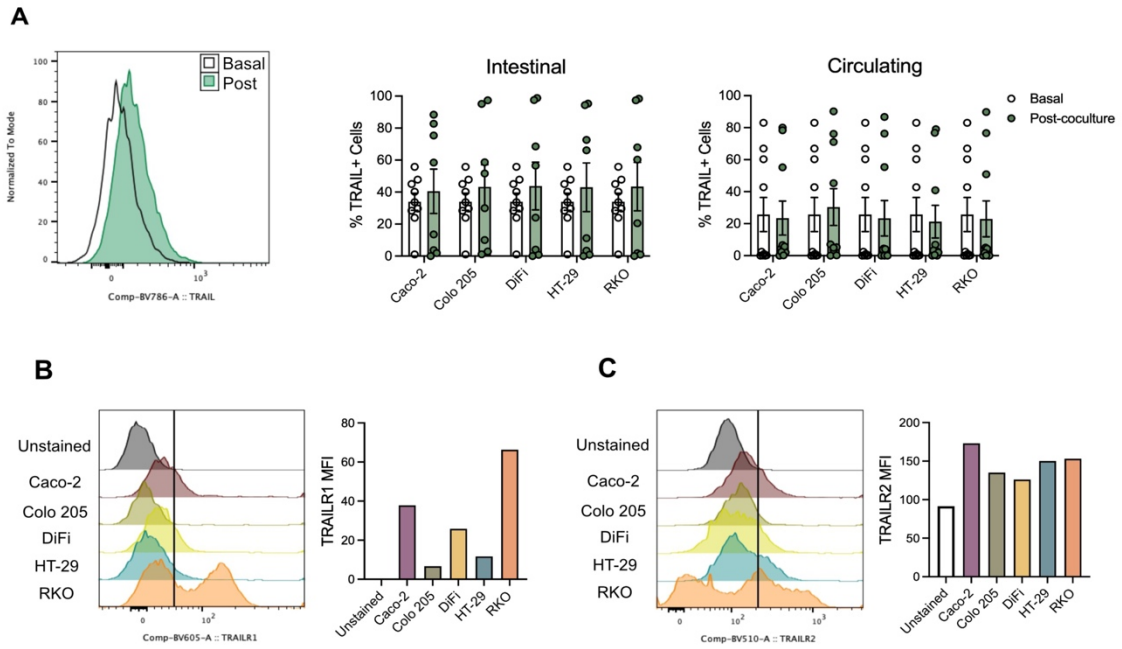

Fig. 4.8. TRAIL modulation on iNKT cells upon exposure to CRC cell lines at 8:1 E:T ratio. A. Left, representative histograms; right, frequencies of TRAIL positive iNKT cells. B-C. Representative histograms and mean fluorescence intensity (MFI) of TRAIL-R1 (B) and -R2 (C). Mann-Whitney tests in A. * p<0.05, ** p<0.01, *** p<0.001

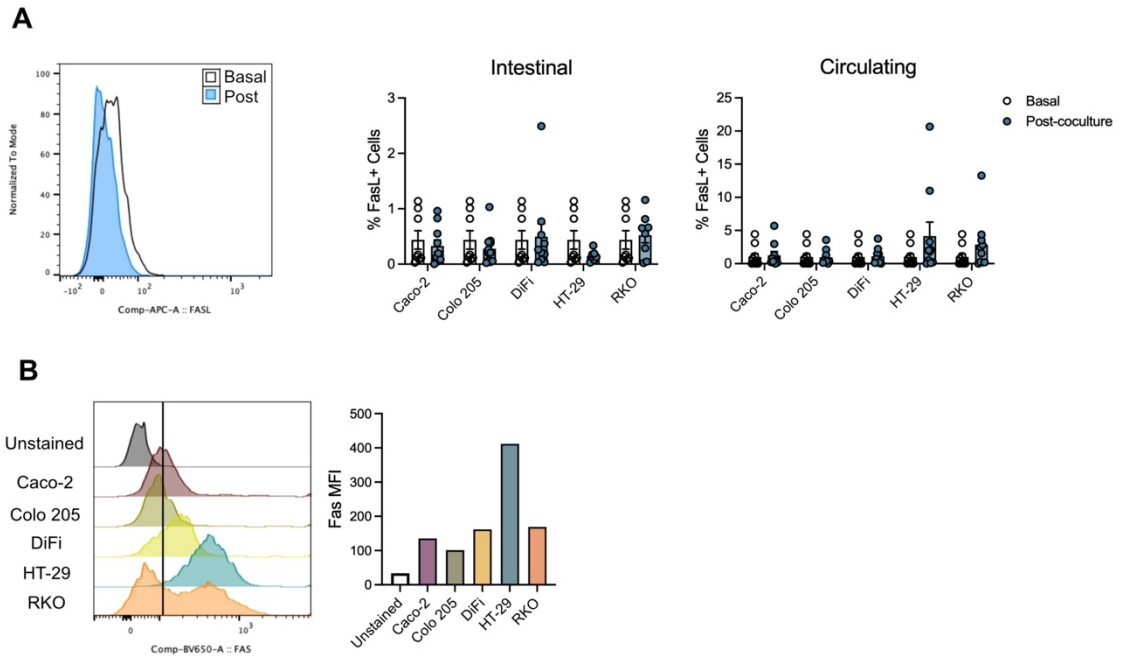

Fig. 4.9. Fas ligand modulation on iNKT cells upon exposure to CRC cell lines at 8:1 E:T ratio. A. Left, representative histograms; right, frequencies of TRAIL positive iNKT cells. B-C. Representative histograms and mean fluorescence intensity (MFI) of TRAIL-R1 (B) and -R2 (C). Mann-Whitney tests in A. * p<0.05, ** p<0.01, *** p<0.001

4.6. Cytotoxicity by human iNKT cells is dependent on perforin and granzyme release

Given the changes observed on cytotoxic molecules – mostly granzyme B and perforin, we wonder which is the actual killing mechanism used by human iNKT cells in colorectal cancer. To address this point, we pre-treated the most efficient intestinal and circulating iNKT cell lines with different inhibitors or blocking antibodies against different cytotoxic molecules (Fig. 4.10A), to then perform killing experiments in the presence of the three most sensitive CRC cell lines at the highest E:T ratio (8:1). More specifically, concanamycin A (CMA), a V-ATPase inhibitor, inhibits perforin by increasing the pH within granules and exposing it to the action of proteases [153]; 3,4 dichloroisocoumarin (DCI) is known to be a mechanism inhibitor of serine proteases, including granzymes [154]; whereas neutralizing antibodies are routinely used to block TRAIL and Fas ligand.

First, we tested for potential toxicity of each inhibitor on iNKT cells by Zombie staining. In the conditions used here, none of the pre-treatments caused toxicity on iNKT cells (Fig. 4.10B). As shown in Fig. 4.10C & D, the impairment of both perforin and granzymes drastically reduced iNKT cell cytotoxic potential. Conversely, the blockade of Fas ligand and TRAIL did not have such impact on iNKT cell killing activity (Fig. 4.10E & F). Taken together, these results indicate that the perforin/granzyme pathway is required for iNKT cell cytotoxic activity against colon cancer cells.

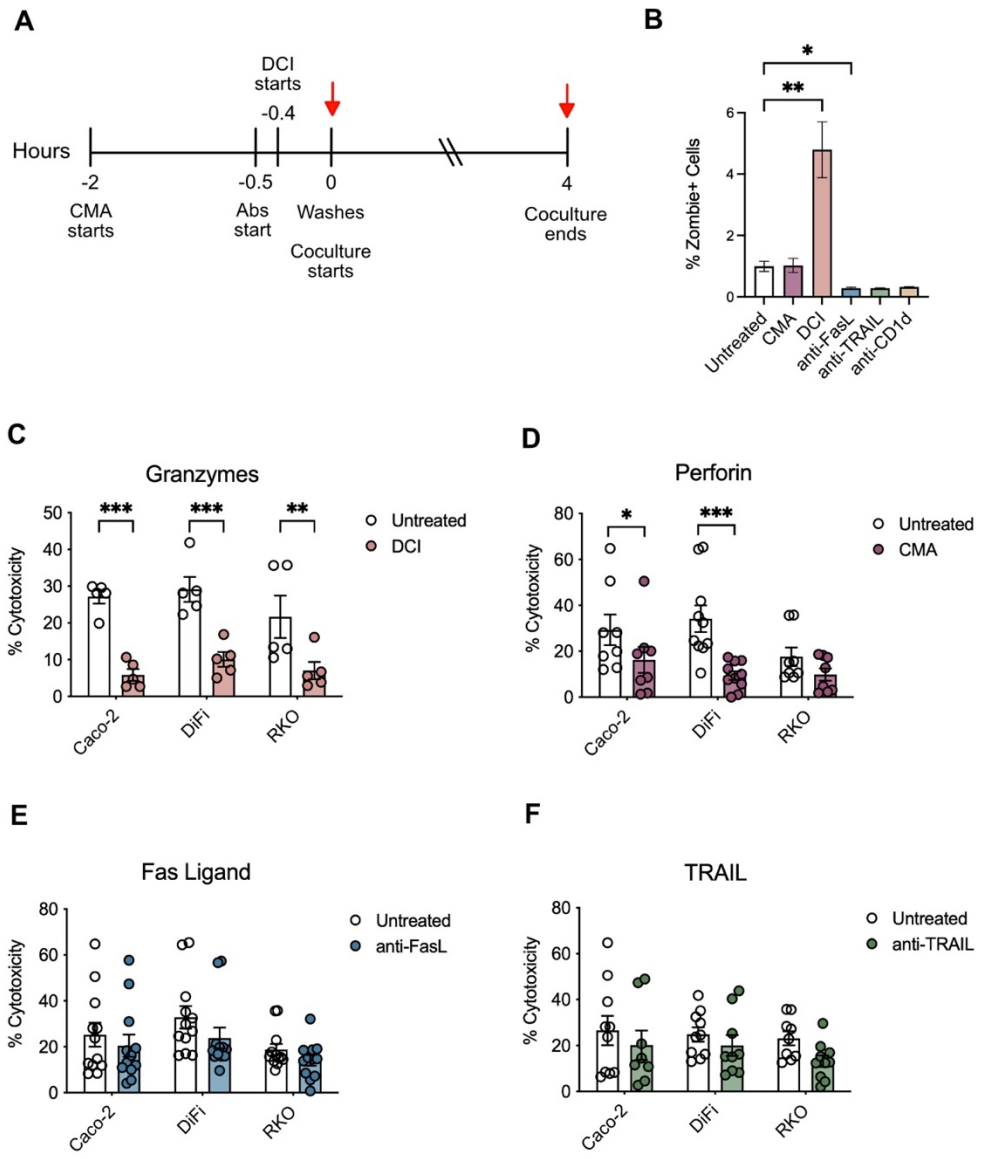

Fig. 4.10. Killing of CRC cells by iNKT cells is dependent on the perforin/granzyme pathway. A. Experimental setup. B. Frequencies of non-viable cells assessed by Zombie staining. C-F. Effect of the inhibition of granzymes (C), perforin (D), Fas ligand (E) and TRAIL (F) on iNKT cell cytotoxicity. Kruskal-Wallis with Dunn's test on A, Mann-Whitney test on C-F. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. CMA: Concanamycin A; DCI: 3, 4 dichloroisocoumarin; Abs; antibodies.

4.7. Recognition of colon cancer cell lines by iNKT cells is partially CD1d dependent

It is known that iNKT cells can target and eliminate solid tumors by either antigen-dependent or -independent mechanisms [20, 52, 66, 75, 155–158]. We thus wondered which could be the case for this context. To assess this point, we first evaluated if CRC cell lines expressed CD1d on their surfaces. All cell lines were characterized by a low presence of CD1d, with some discrete differences among

lines, being Caco-2, DiFi, and RKO cells the lines with the highest MFI for CD1d (Fig. 4.11A). Afterwards, we tested if the blockade of the presentation of self-antigens could have effects on iNKT cell cytotoxicity by pre-treating CRC cells with a CD1d neutralizing antibody. Interestingly, iNKT cell cytotoxicity was not profoundly affected by CD1d blockade, which only induced a partial decrease in the elimination of Caco-2 cells (Fig. 4.11B). Altogether, these data suggest that human iNKT cells target colon cancer cells by both CD1d-dependent and CD1d-independent mechanisms.

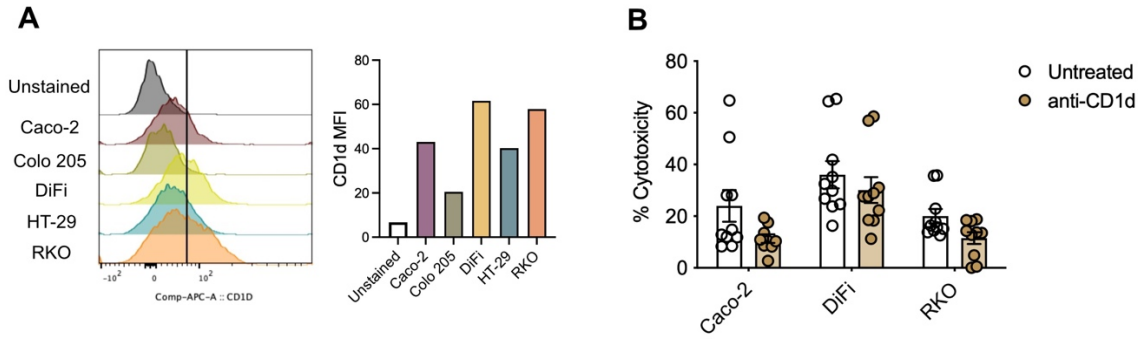

Fig. 4.11. Inhibition of antigen presentation does not profoundly affect iNKT cell cytotoxicity. A. Left, representative histograms; right, mean fluorescence intensity of CD1d on CRC cell lines. B. effect of CD1d blockade on iNKT cell killing activity. Mann-Whitney test on B. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.8. iNKT cells eliminate patient-derived colon cancer cells with the release of granzyme B and perforin

Given that all the previous experiments were done with CRC cell lines, we then aimed at dissecting if iNKT cells are also efficient at eliminating freshly isolated colon cancer cells. For this purpose, we isolated colon cancer cells from surgical specimens of four patients and incubated them in the presence of three intestinal and two blood-derived iNKT cell lines at the highest E:T ratio. As shown in Fig. 4.12A, iNKT cells from both tissues were cytotoxic against patient-derived CRC cells, being intestinal cells even more efficient. Interestingly, all iNKT cells were more efficient against these cells than against all the CRC lines used previously, reaching 100% of cytotoxicity for some patients (Fig. 4.12A & 4.4A-B). It was also observed that, in a way similar to CRC cell lines, cells from each patient were eliminated in a differential manner; cells from patients 2 and 3 were the most

sensitive to iNKT cell killing, with cells from patient 3 being completely eliminated by all iNKT cell lines (Fig. 4.12A). To note, intestinal iNKT cells had higher frequencies of cells positive for all cytotoxic molecules than blood-derived cells (Fig. 4.12B-D). In particular, the frequencies of granzyme B and perforin in intestinal cells were substantially higher compared to those found after coculture with CRC cell lines (Fig. 4.12C-D & 4.6). Such differences were also found in the levels of GZMB and PRF in coculture supernatants (Fig. 4.12C-D). Overall, these data demonstrate that human iNKT cells eliminate freshly isolated, patient-derived colon cancer cells with more efficacy than CRC cell lines by releasing greater amounts of granzyme B and perforin.

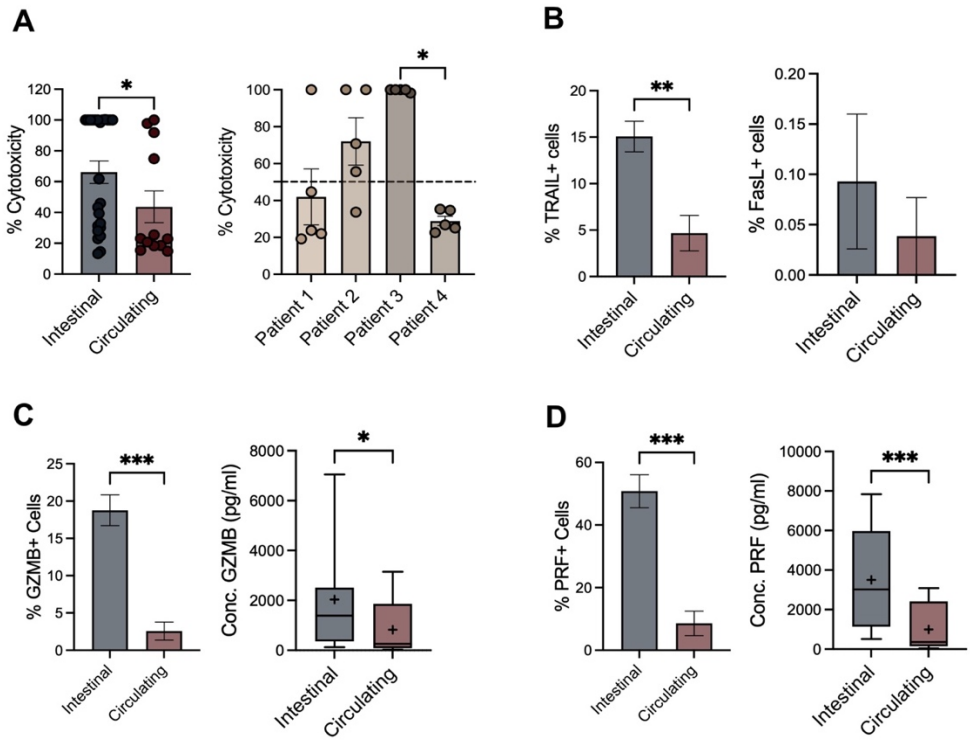

Fig. 4.12. Human iNKT cells eliminate patient-derived CRC cells. A. Left, cytotoxicity by intestinal vs circulating cells; right, percentage of dead CRC cells for each patient. B. Left, TRAIL frequencies; right, Fas ligand frequencies. C-D. Left, frequencies; right, levels in supernatants of granzyme B (C) and perforin (D). Mann-Whitney test on A left, B-D; Kruskal-Wallis and Dunn's test for A right. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

5. Discussion I

iNKT cells have gained recent interest in cancer immunotherapy [159, 160]. In particular, there are some aspects that make them suitable for cell-based therapies, such as the activation by lipid antigens, innate signals, and cytokines, and the non-polymorphic nature of their antigen-presenting molecule, CD1d [155, 157, 161, 162]. However, how these cells display their antitumor activities in human colon cancer is still largely unknown. In this study is shown that, even if colon cancer cells promoted a reduction in the levels of interferon gamma, they also provoked an increase in the release of the cytotoxic molecules granzyme B and perforin, thus influencing their elimination.

Many lines of evidence have demonstrated the importance of the fast production of IFNG by iNKT cells in activating other populations in the TME, such as CD8+ T, NK, and dendritic cells [49, 54, 72]. Therefore, one strategy used by tumors to avoid immune elimination could be the impairment in the release of this cytokine. Indeed, in models of melanoma, as well as in prostate and colon cancer patients, it has been observed that tumor-infiltrating iNKT cells produced less interferon gamma and TNF-alpha, which was reverted with αGalCer treatment [42, 163]. Here, we demonstrated that colon cancer cells are enough to induce a significant reduction in IFNG expression by iNKT cells.

Nonetheless, we also observed a decrease in IL-17 production and no changes in GM-CSF and IL-10 levels, contrary to what we recently demonstrated [42]. These findings could mean that the changes in the production of these two cytokines are not mediated by interactions with tumor cells, but by the influence of other components of the tumor microenvironment. In fact, IL-17 was primarily known to be produced by Th17 cells in response to extracellular microbes [164], and in the intestine it is induced by segmented filamentous bacteria [165] and enterotoxigenic *Bacteroides fragilis* [166]. In addition, we demonstrated that *Fusobacterium nucleatum* induced the production of IL-17 and GM-CSF by human iNKT cells [42]. Other stimuli that promote GM-CSF expression by T cells are IL-23 and IL-1β, which are produced by the myeloid compartment and epithelial cells, and are also responsible for IL-17 production [70, 167].

Surprisingly, even if CRC cells negatively influenced the production of interferon gamma by iNKT cells, they also promoted the degranulation of intestinal iNKT cells, together with the release of granzyme B and perforin, which led to CRC cell elimination. Previous evidence had demonstrated that human iNKT cells from peripheral blood iNKT cells are cytotoxic against cell lines from different types of

cancer, including colorectal cancer [20, 75, 77, 168–170]. Here, we show not only circulating, but also intestinal iNKT cells could kill both well-established CRC cell lines and patient-derived cells. In fact, this is the first study that demonstrates the cytotoxic activity of human intestinal iNKT cells.

As mentioned previously, iNKT cells are classified into subsets according to their transcriptional programs and the molecules they produce [57, 171]. In particular, the NKT1 subset has become highly relevant in cancer immunotherapy, as it is the only subset endowed of antitumor activities [58, 171, 172]. Studies in mice have shown that NKT1 cells usually migrate from the thymus to the liver, intestine, lung, and spleen [171, 173], suggesting that iNKT cells from many organs (including the colon) could eliminate cancerous cells [66, 68, 77, 84, 174]. In addition, most cytotoxicity studies using human cells have been performed with iNKT cells derived from peripheral blood [20, 65, 68, 155, 158, 168, 170], indicating that iNKT cells can eliminate tumor cells regardless of their tissue of origin.

One interesting finding here is that CRC lines were more sensitive than others to elimination by iNKT cells. Such patterns were preserved among intestinal and blood-derived iNKT cell lines, indicating similar mechanisms tumor cell recognition, even if iNKT cell lines come from different tissues. In addition, iNKT cell cytotoxicity was not significantly impaired by CD1d blockade on CRC cells. One of the hallmarks of iNKT cells is their expression of natural killer receptors, which gives them an innate nature. In fact, some studies have shown that iNKT express NKG2D, NKG2A, NKp30, NKp46, and some killer immunoglobulin (KIR) receptors [52, 149, 171, 175, 176]. The balance of the interactions of such receptors with their respective ligands establishes their activation state [177]. In this regard, it has been shown that Colo 205 cells are resistant to NK cell cytotoxicity due to their high expression of MHC class I molecules (ligands of KIR receptors, [178]), CD155 (also called PVR, ligand for CD96 and TIGIT [179]), and low expression of MICA (ligand for NKG2D, activating receptor [180]). HT-29 cells were also resistant to iNKT cell killing. In particular, even if they express MICA, they express high levels of the HLA-ABC molecules, as well as HLA-E – ligand of the inhibitory receptor NKG2A, on their surface [181–183]. On the other hand, some studies have confirmed that RKO cells are sensitive to NK killing due to the presence of the NKG2D ligands MICB, ULBP1, 2, and 3, as well as the lack of HLA-E [184–187]. Furthermore, the Caco-2 line shows low expression of the HLA molecules [183, 187], which alone could positively skew iNKT cell activation status. Therefore, the evaluation of such molecules may add important information about iNKT-CRC cell interactions.

Regarding the killing mechanism used by iNKT cells to eliminate CRC cell lines, we observed that all the iNKT cell lines used here, regardless of their tissue of origin, effectively degranulated to release granzyme B and perforin when they encountered CRC cells. In particular, we confirmed that iNKT cells depended on functional perforin and granzymes, as treatment with CMA and DCI abrogated iNKT cell killing against the colon cancer lines tested. While the perforin/granzyme pathway mostly relies on the recognition by the effector cell, death ligand pathways require the expression of death receptors on target cells and require more degranulation events, which make them slower compared to the perforin/granzyme mechanism [188, 189]. This might explain why the PRF/GZMB mechanism was favored in this coculture model. In this regard, we also showed that some iNKT lines constitutively expressed TRAIL on their membranes. Furthermore, Fas and TRAIL receptors were present in some CRC cell lines. Therefore, iNKT cells may adopt the best killing mechanism according to their activation state and switching between mechanisms over time, as it occurs with natural killer cells [190].

Although iNKT cell killing activity was not substantially affected upon the blockade of antigen presentation on tumor cells, cell elimination was still reduced for DiFi cells. These data indicate that some colon cancer cells not only express CD1d on their membranes – although at low levels, but also might present antigens that activate iNKT cell cytotoxicity, even if not as strongly as innate signals. In this regard, it is known that iNKT cells recognize and are activated by range of self-antigens [161, 191–194]. Cancer cells have altered lipid pathways which could in turn affect lipid presentation, and therefore, iNKT cell activation. For instance, endoplasmic reticulum stress – commonly found in cancer cells, influences the presentation of self-lipid antigens that activate iNKT cells [161, 194]. While CD1d is essential for iNKT cell-mediated killing in some cancer types, especially hematologic types of cancer [66, 75, 77, 170, 174], other studies, including this one, have demonstrated that in some contexts iNKT cells also make use of innate stimuli to recognize transformed cells [52, 65, 68, 157, 195].

To further confirm our findings, we assessed whether iNKT cells could eliminate patient-derived CRC cells. Interestingly, we demonstrated that freshly isolated CRC cells are efficiently killed by both intestinal and circulating iNKT cells. Furthermore, we observed that these cells were more effective against *ex-vivo* isolated epithelial cells than against CRC cell lines. Furthermore, and contrary to what observed on CRC lines, intestinal iNKT cells were significantly more

aggressive than blood-derived cells, which was confirmed by higher perforin and granzyme B frequencies and release.

These findings raise the question of how their cytotoxic activity is abrogated during colon cancer progression. As it occurred with cytokine production, the impairment of iNKT cell cytotoxicity in this context might be also mediated by the tumor microenvironment and not by cancer cells alone, even if we observed resistance also on patient-derived cells, which indicates that cancer cells still have a relevant role in the negative modulation of these activities. Some factors affecting proper elimination of cancer cells might be changes in tissue architecture that impede the formation of the immunological synapse with tumor cells, which further affects recognition and effector cell activation, the interactions with immunosuppressive components of the TME such as tumor-associated macrophages, Tregs and myeloid-derived suppressor cells, modulation by the gut microbiota, etcetera.

Altogether, these results might become of interest in the light of potential iNKT cell-based immunotherapies in CRC. They shed light about how CRC cells modulate iNKT cell antitumor functions and open the path for further research to address why these cells acquire a pro-tumorigenic phenotype in CRC. These results were published in Molecular Oncology under the doi: 10.1002/1878-0261.13104 (Appendix VI).

6. Results II

6.1. *P. gingivalis* supports cancer progression and promotes a pro-tumor phenotype on iNKT cells in AOM/DSS-treated mice

The abovementioned data demonstrated that interactions with colon cancer cells alone do not fully explain the pro-tumor cytokine profile of iNKT cells in human CRC [42]. One possible explanation for this behavior may reside in iNKT cell interactions with the microbiota within the tumor microenvironment. Therefore, we next aimed at dissecting if iNKT cell cytokine profile and chemotactic functions are mediated by bacteria associated with CRC. In this regard, we demonstrated that *Fusobacterium nucleatum* – a well-known oncobacterium, favors a IL-17+GM-CSF+, neutrophil-recruiting phenotype on human iNKT cells [42]. Thus, we wondered if other CRC-related bacteria may contribute to the promotion of pro-tumorigenic functions, further contributing to colon cancer progression. For this purpose, we focused on *Porphyromonas gingivalis* (Pg), which has been recently linked to colon cancer [136, 137, 140].

To test its role on iNKT cell functions in CRC, we first used the AOM/DSS model of colon cancer (Fig. 3.1A). For this model, we used an early time point (sacrifice at day 42, Fig. 3.1A), as it was shown that at this timepoint, murine iNKT cells best reflected the features of human tumor-infiltrating cells [42]. To follow tumor progression over time, we used exploratory colonoscopy at specific timepoints (Fig. 3.1A; for tumor endoscopic scores, see Fig. 3.2). At sacrifice, we measured tumor number, size, and we evaluated the tumor immune infiltrate. We observed that *P. gingivalis* promoted tumor progression, reflected by a higher tumor endoscopic score over time (Fig. 6.1A) and increased number of tumors of greater size at sacrifice (Fig. 6.1B).

Regarding the tumor infiltrate, we observed that tumors from Pg-treated mice had higher frequencies of iNKT cells but not of CD4+ and CD8+ T cells, as analyzed by flow cytometry (Fig. 6.1C). As shown by the gating strategy (Fig. 3.4B) and as demonstrated previously [42], murine iNKT cells are characterized by a CD3low phenotype. Next, by performing an unsupervised clustering on tumor-infiltrating iNKT cells, we observed a cluster enriched in Pg tumors characterized by the expression of GM-CSF, IL-17, and CD69 (cluster 9, Fig. 6.2A & B). These data were further confirmed by a manual gating approach (Fig. 6.2C & D). Additionally, higher frequencies of IL-10 positive cells in iNKT cell were found in Pg-treated mice (Fig. 6.2E). However, this phenotype was not observed in CD4+ T cells (Fig. 6.3). Overall, these data indicate that iNKT cell frequencies are higher in tumors in *P. gingivalis*

treated mice, and they display a pro-tumorigenic phenotype, characterized by the expression of IL-17, GM-CSF and IL-10.

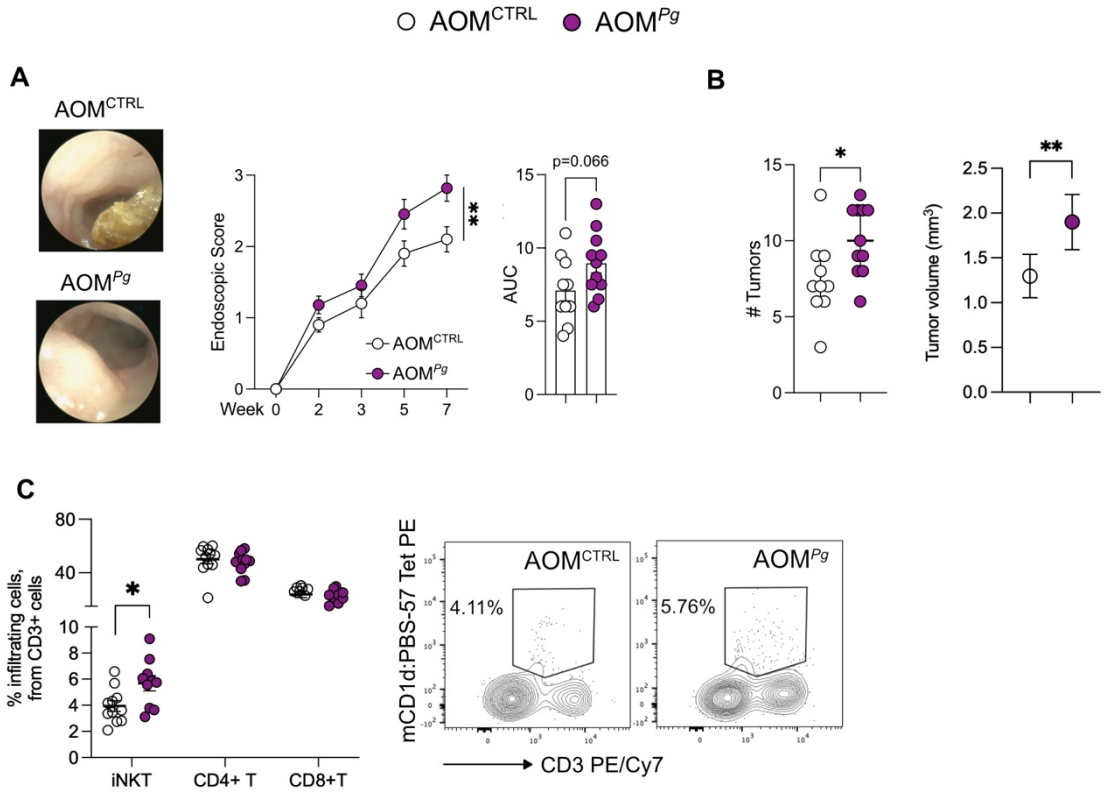

Fig. 6.1. *P. gingivalis* supports tumor progression and iNKT cell abundance in AOM/DSS-treated mice. A. Representative pictures (left), tumor endoscopic scores (center) and area under the curve of the tumor endoscopic scores (right) on AOM/DSS-treated mice. B. Number of tumors (left) and tumor size (right) at sacrifice. C. Left, frequencies of tumor-infiltrating iNKT and conventional T cells; right, representative plots of iNKT cell frequencies in tumors. Data shown are from three independent experiments. Two-way ANOVA was used in A (center), Mann-Whitney test was used in A (right), B and C. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

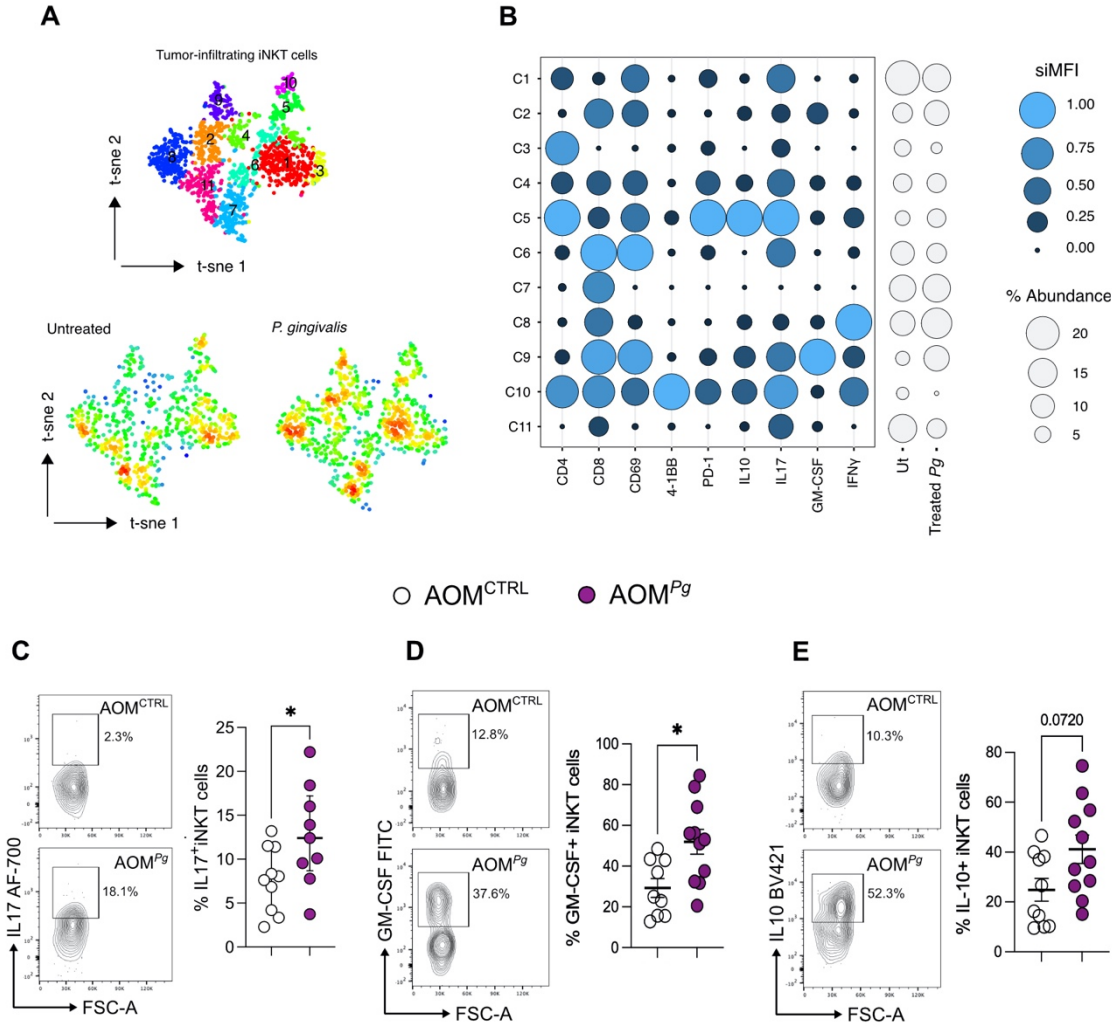

Fig. 6.2. *P. gingivalis* supports a pro-tumorigenic phenotype on tumor-infiltrating iNKT cells in the AOM/DSS model. A. t-SNE maps of iNKT cells from control and Pg-treated mice from Phenograph clustering. B. Balloon plots showing the scaled-integrated MFI (siMFi) and abundances of the clusters generated in A. C-E. Representative plots (left) and frequencies (right) of IL-17 (C), GM-CSF (D), and IL-10 (E) positive iNKT cells. Data shown are from three independent experiments. Mann-Whitney test was used in B-E. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

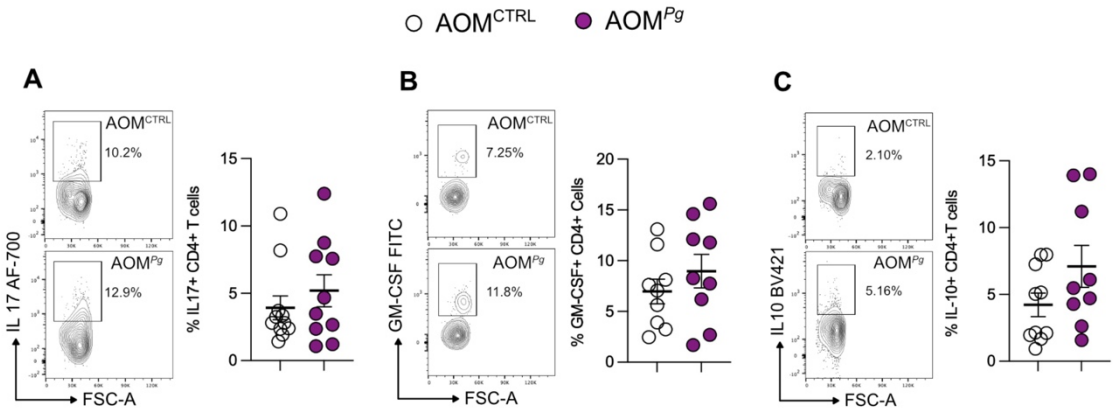

Fig. 6.3. *P. gingivalis* does not induce changes in CD4+T cell cytokine profile in AOM/DSS-treated mice. Representative plots (left) and frequencies (right) of IL-17 (A), GM-CSF (B), and IL-10 (C) positive CD4+ T cells. Data shown are from three independent experiments. Two-way ANOVA was used in A (center), Mann-Whitney test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

6.2. iNKT cells are necessary for the pro-tumorigenic effect of *P. gingivalis* in the AOM/DSS model, recruiting neutrophils with lower production of reactive oxygen species

Due to the higher infiltration of iNKT cells in tumors from Pg-treated mice and their pro-tumorigenic cytokine profile, we wondered whether iNKT cells may be required for the enhanced cancer progression given by *P. gingivalis*. For this purpose, we induced colon tumors in *Traj18-/-* mice, which are deficient for iNKT cells [142]. We observed that iNKT cells are indeed necessary for the pro-tumorigenic effect of *P. gingivalis*, as treatment with Pg in the absence of iNKT cells did not increase tumor burden (Fig. 6.4A-B). Furthermore, we found that treatment with *P. gingivalis* in knockout mice did not provoke changes in the cytokine profile of conventional CD4+ T cells, even if there was a trend towards the decrease in the frequencies of IL-17, GM-CSF, and IL-10 positive cells (Fig. 6.4C-E). This finding suggests that there is no compensatory effect by conventional CD4+ T cells in the absence of iNKT cells, and that a specific crosstalk between iNKT cells and Pg exists supporting colon cancer progression in this model of colon cancer.

Since it has been previously shown that tumor-infiltrating iNKT cells interact with myeloid cells, such as macrophages and neutrophils [42, 73, 109], we next assessed the infiltration of innate populations. In our most recent study, we reported that in concomitance with a higher iNKT cell infiltration in tumors, there was an increment in neutrophil infiltration, characterized by the presence of two populations

according to the expression of CD11b and Ly6G, upon stimulation with PMA [42]. Here we confirmed these findings, as in Pg-treated wild-type animals there was an increment in neutrophil infiltration, which was not observed in *Traj18-/-* mice (Fig. 6.5A & C). These neutrophils were characterized by a lower respiratory burst capability – measured by the oxidation of dihydrorhodamine 123 into the fluorescent rhodamine 123, suggesting an impaired cytotoxic potential (Fig. 6.5B). On the contrary, tumor-associated neutrophils (TANs) of *Traj18-/-* mice did not manifest changes in ROS production upon treatment with *P. gingivalis* (Fig. 6.5D). Taken together, these data indicate that iNKT cells are necessary for the effect of *Porphyromonas gingivalis* on tumor burden in the AOM/DSS model, and are also responsible for the Pg-mediated recruitment of neutrophils with a diminished respiratory burst capability.

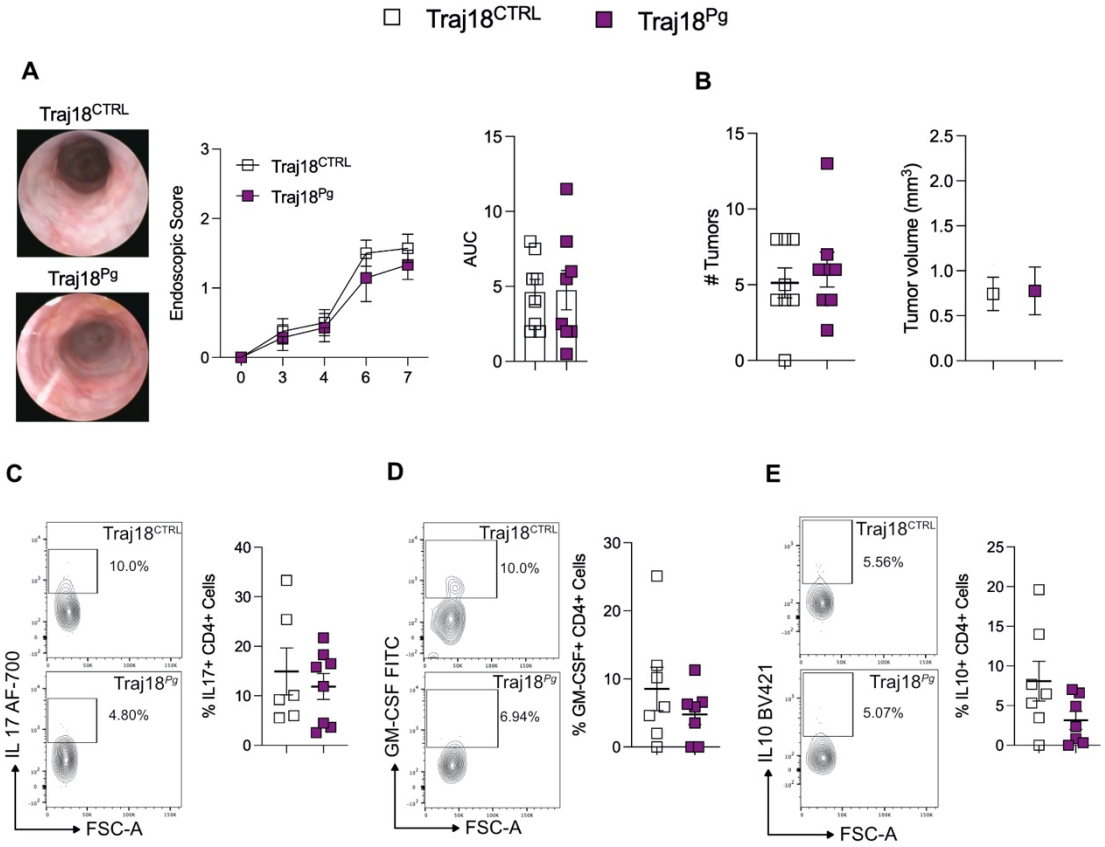

Fig. 6.4. iNKT cells are necessary for the pro-tumorigenic effect of *P. gingivalis* in AOM/DSS-treated mice. A. Representative pictures (left), tumor endoscopic scores (center) and area under the curve of the tumor endoscopic scores (right) on AOM/DSS-treated *Traj18-/-* mice. B. Number of tumors (left) and tumor size (right) at sacrifice. Representative plots (left) and frequencies (right) of IL-17 (C), GM-CSF (D), and IL-10 (E) positive CD4+ T

cells. Data shown are from two independent experiments. Two-way ANOVA was used in A (center), Mann-Whitney test was used in A (right)-E. * p<0.05, ** p<0.01, *** p<0.001

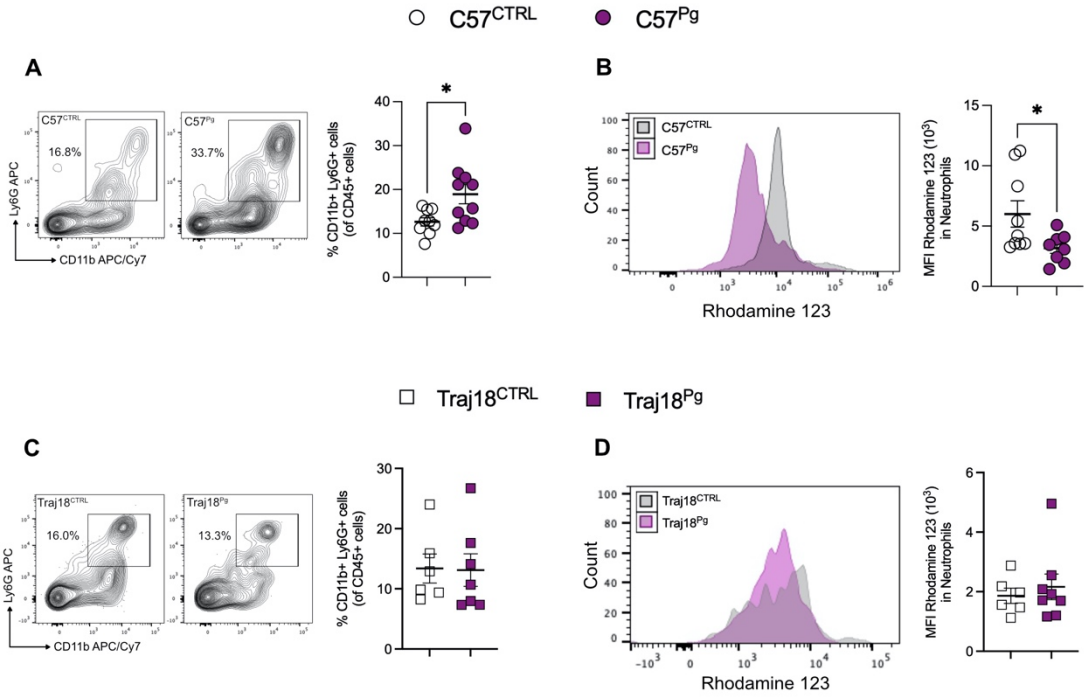

Fig. 6.5. iNKT cells are necessary for the recruitment of neutrophils with low ROS production in AOM/DSS-treated mice. A. Left, representative plots; right, frequencies of tumor-infiltrating neutrophils in wild-type mice. B. Left, representative histograms; right, mean fluorescence intensity (MFI) of rhodamine 123 on tumor-associated neutrophils from wild-type mice. C. Left, representative plots; right, frequencies of tumor-infiltrating neutrophils in iNKT cell-deficient (*Traj18-/-*) mice. D. Left, representative histograms; right, mean fluorescence intensity (MFI) of rhodamine 123 on tumor-associated neutrophils from iNKT cell-deficient mice. Data shown are from three independent experiments for wild-type animals and two experiments for *Traj18-/-* mice. Mann-Whitney test. * p<0.05, ** p<0.01, *** p<0.001

6.3. *P. gingivalis* induces a similar phenotype on iNKT cells in a sporadic model of colon cancer

The AOM/DSS model shares features with human sporadic colon cancer, such as the multi-step tumor progression, also known as the adenoma-carcinoma sequence, the presence of cancer-associated fibroblasts, different immune cell populations, as well as the activation of some pathways [196]. However, tumorigenesis is only achieved if DSS – an inflammatory agent, is used. Thus, to evaluate if the enhancement of iNKT cell pro-tumorigenic features by *Porphyromonas gingivalis* also occurs in a sporadic model of CRC, we made use of

an intracolonic injection model (Fig. 3.1B). Here, we injected MC38 cells (Fig. 6.6A left) using an endoscopy-guided system, and followed tumor progression *via* exploratory colonoscopy as in the AOM/DSS model (for tumor endoscopic scores, see Fig. 3.2). Interestingly, we observed a fast tumor progression regardless of the experimental group, arriving at the highest tumor endoscopic score (full lumen coverage) at week 3 (Fig. 6.6B), confirmed by tumor size at sacrifice (Fig. 6.6C).

Regarding the immune infiltrate, we did not observe substantial changes in T cell frequencies, both conventional and iNKT cells, between groups (Fig. 6.6D). Nonetheless, Pg treatment influenced their cytokine profile. More specifically, the frequencies of IL-17 and GM-CSF positive iNKT cells were higher in tumors from Pg-treated mice (Fig. 6.7A & B). In contrast to what observed in the AOM/DSS model, IL-10 frequencies did not undergo modifications upon *P. gingivalis* treatment (Fig. 6.7C). More interestingly, Pg treatment provoked a decrease in IL-17 and IL-10 expression by CD4+T cells (Fig. 6.8A & C), albeit not statistically significant. Similarly, neutrophil frequencies were not altered upon treatment with *P. gingivalis* (Fig. 6.9A). However, TANs from Pg-treated mice had a reduced oxidative burst capability than those from tumors of untreated animals (Fig. 6.9B). Taken together, these data indicate that *P. gingivalis* influences IL-17 and GM-CSF expression on iNKT cells and neutrophil oxidative burst also in an intracolon injection model of CRC.

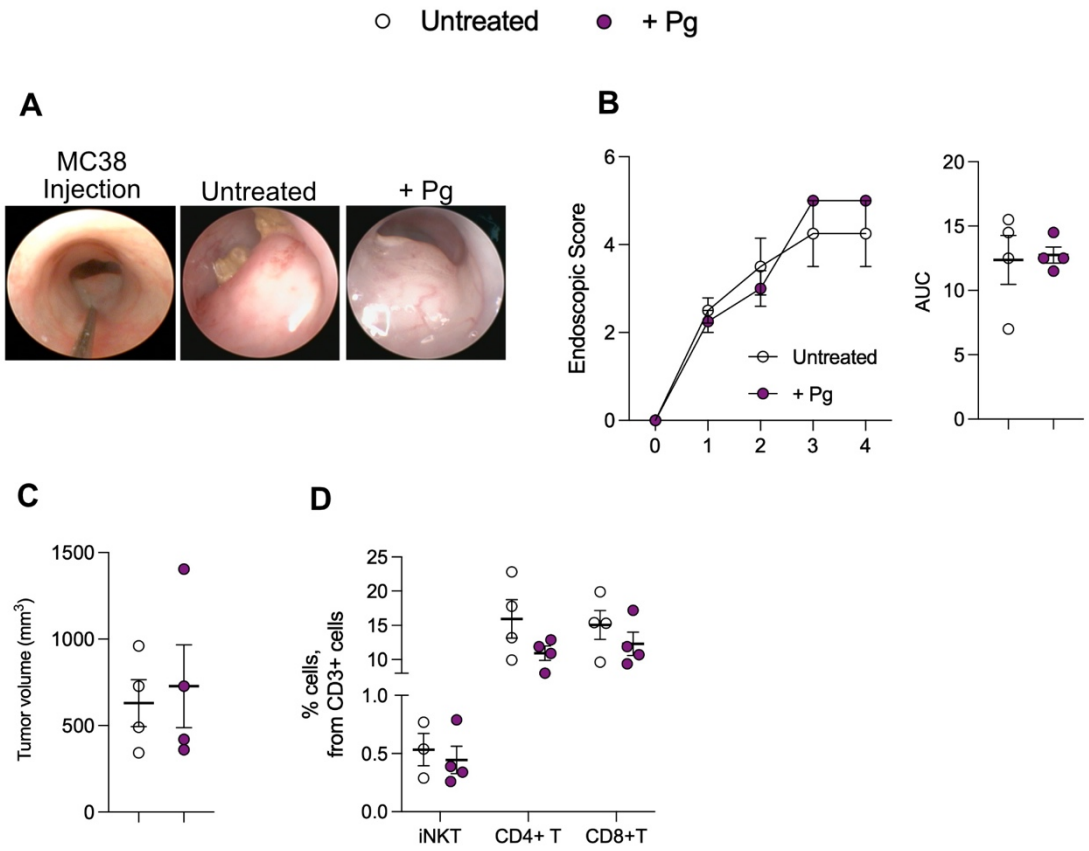

Fig. 6.6. Clinical parameters in the intracolon injection model of CRC upon *P. gingivalis* treatment. A. Representative images of the orthotopic injection (left), tumor from untreated (center) and Pg-treated mice (right) mice. B. Left, tumor endoscopic scores; right, area under the curve from the data on the left. C. Tumor volume at sacrifice. D. Frequencies of tumor-infiltrating iNKT and conventional T cells. Two-way ANOVA was used in B (left), Mann-Whitney test was used in B (right)-D. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

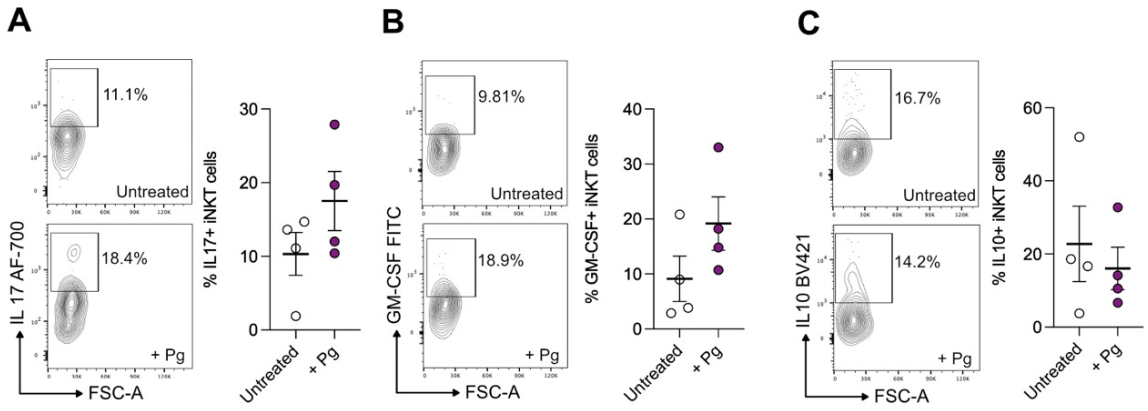

Fig. 6.7. *P. gingivalis* modifies the expression of IL-17 and GM-CSF on iNKT cells in an intracolon injection model of CRC. A-C. Representative plots (left) and frequencies (right)

of IL-17 (A), GM-CSF (B), and IL-10 (C) positive iNKT cells Mann-Whitney test. * p<0.05, ** p<0.01, *** p<0.001

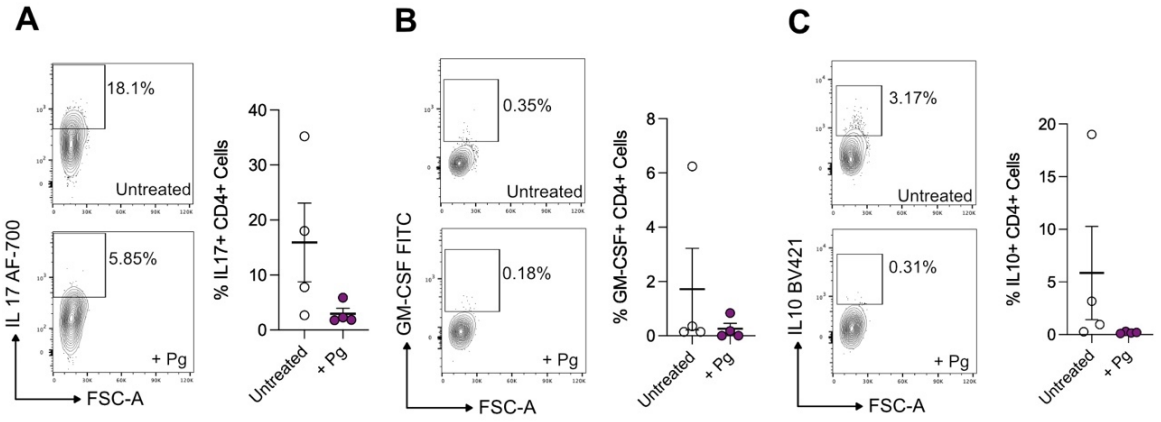

Fig. 6.8. *P. gingivalis* induces a reduction in the expression of IL-17, GM-CSF, and IL-10 on CD4+T cells in an intracolon injection model of CRC. A-C. Representative plots (left) and frequencies (right) of IL-17 (A), GM-CSF (B), and IL-10 (C) positive CD4+ T cells. Mann-Whitney test. * p<0.05, ** p<0.01, *** p<0.001

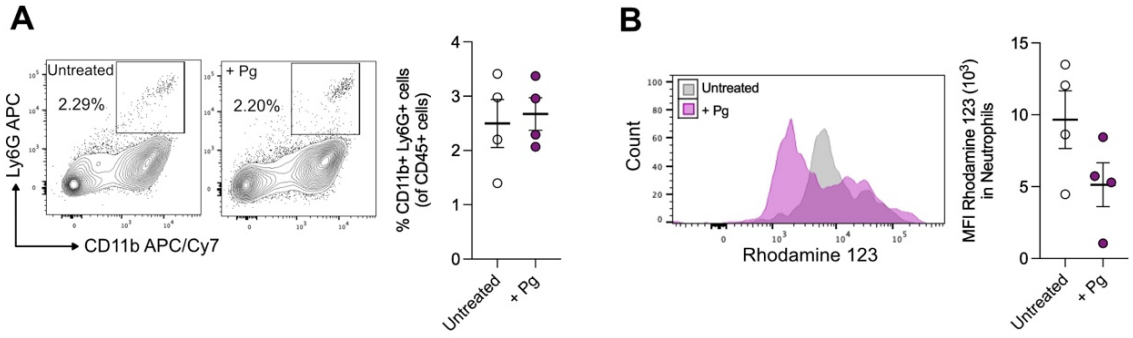

Fig. 6.9. *P. gingivalis* modifies the neutrophil oxidative burst in an intracolon injection model of CRC. A. Left, representative plots; right, frequencies of tumor-infiltrating neutrophils. B. Left, representative histograms; right, mean fluorescence intensity (MFI) of rhodamine 123 on tumor-associated neutrophils. Mann-Whitney test. * p<0.05, ** p<0.01, *** p<0.001

6.4. Human iNKT cells primed with Pg share the same characteristics with murine, tumor-infiltrating iNKT cells

Data from AOM/DSS mice showed that there is a higher co-infiltration of iNKT cells and TANs in tumors from Pg-treated animals. To address if the same phenomenon occurs in colon cancer patients, we reanalyzed data from our previous cohort [42] and observed that Pg-positive patients (assessed by 16S sequencing data) had indeed higher frequencies of iNKT cells (Fig. 6.10A) and neutrophils (Fig.

6.10B). Moreover, the *in vivo* data showed that *P. gingivalis* stimulates the production of IL-10, IL-17, and GM-CSF on tumor-infiltrating iNKT cells. In addition, we observed that iNKT cells were necessary for the Pg-mediated recruitment of neutrophils with lower ROS production. To assess whether *P. gingivalis* modulates human iNKT cell functions in a similar fashion, we primed human iNKT cell lines from the collection used previously (Table 3.2) with monocyte-derived dendritic cells (moDCs, basal) in the presence or absence of *P. gingivalis* and performed RNA-seq analysis and functional assays.

First, we observed that cells primed with Pg were characterized by a neutrophil chemotactic signature, shown by the overexpression of chemoattractant-coding genes such as *CXCL1, CXCL2, CXCL5, CXCL8* (Fig. 6.4A). To evaluate if this signature is translated into a higher neutrophil chemotaxis, we used the supernatants from primed iNKT cells to perform neutrophil migration assays. Supernatants of cells primed with moDCs alone did not modulate neutrophil migration (Fig. 6.11B). On the contrary, and in agreement with the RNA sequencing data, we observed that the supernatants from iNKT cells stimulated with *P. gingivalis* induced the migration of neutrophils at similar levels as the positive control (RPMI medium plus 10% FBS). Furthermore, these neutrophils were characterized – similarly to TANs from Pg-treated mice, by a lower production of reactive oxygen species (Fig. 6.11C), as well as an increment in the expression of PD-L1 (Fig. 6.11D).

Subsequently, we evaluated the cytokine profile of human cells exposed to *P. gingivalis*. We found that Pg-primed cells, consistent to what we had found on their murine counterpart, are more prone to produce IL-17, IL-10, and GM-CSF, detected both by flow cytometry (Fig. 6.12A-C) and ELISA on culture supernatants (Fig. 6.12D-F). Altogether, these results demonstrate that *P. gingivalis* promotes the migration of neutrophils with low respiratory burst capability *via* the induction of a neutrophil chemotactic signature on human iNKT cells, as well as inducing a pro-tumorigenic cytokine profile on iNKT cells.

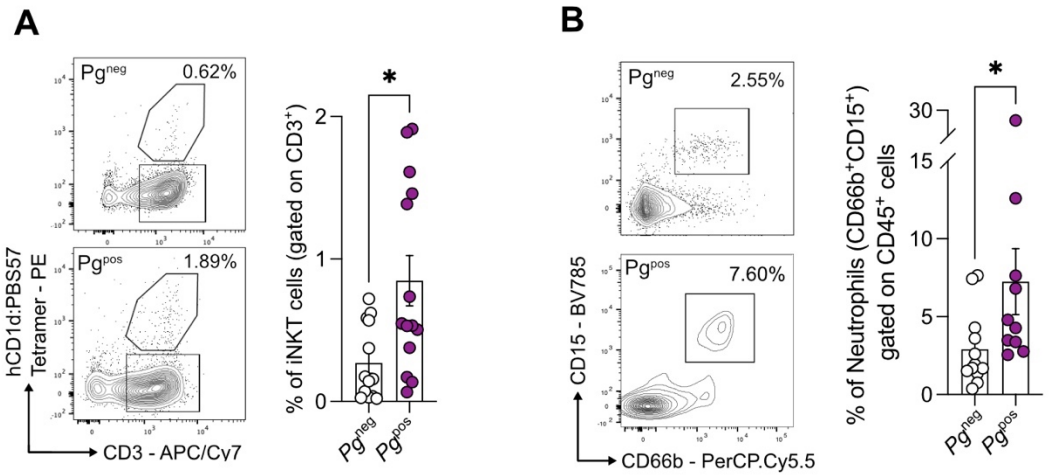

Fig. 6.10. Tumors from CRC patients positive for *Porphyromonas gingivalis* have an increased infiltration of iNKT cells and neutrophils. A. Representative plots and frequencies of iNKT cells in tumors from Pg negative and positive patients. B. Representative plots and frequencies of neutrophils in tumors from Pg negative and positive patients. Mann-Whitney test. * p<0.05, ** p<0.01, *** p<0.001

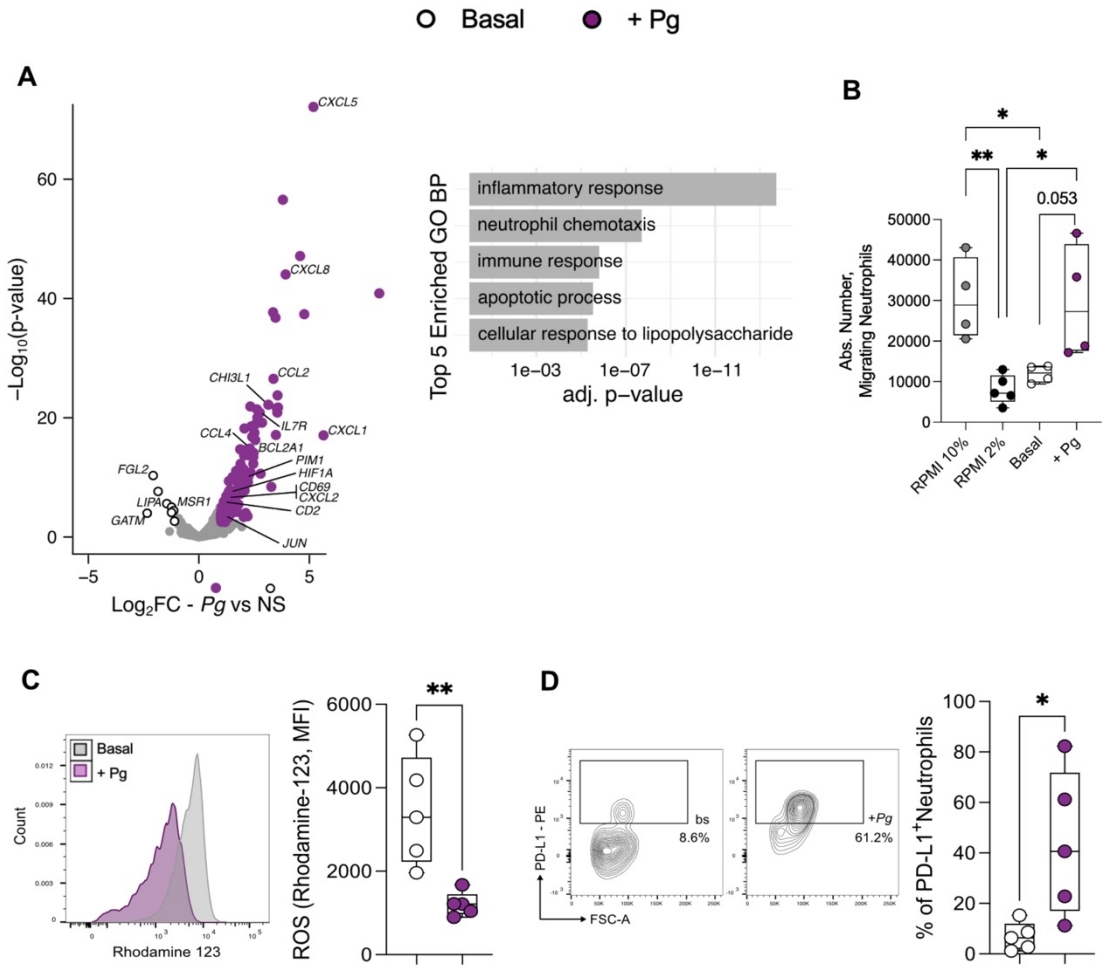

Fig. 6.11. *P. gingivalis* drives iNKT cells towards a neutrophil chemotactic gene signature, with subsequent migration of neutrophils with low respiratory burst capacity. A. Volcano plot (left) and gene ontology analysis (right) of differentially-expressed genes of human Pg vs

moDC-only-primed iNKT cells; dots (genes) in the Volcano plots show the differential expression (log_2FC) and their associated statistical significance (log_{10}p-value). B. Absolute numbers of migrating neutrophils. C. Left, representative histograms; right, MFI of rhodamine-123. D. Left; representative plots; right, frequencies of PD-L1-positive migrating neutrophils. Data presented are from 3 independent experiments. Dots in A left represent genes with an FDR-adjusted p-value of < 0.05 and log_2FC of > |1.5|; differentially-expressed genes in A right had Bonferroni-corrected p < 0.05 and log_2FC > 1. Kruskal-Wallis with Dunn's test in B, Mann-Whitney test in C and D. * p<0.05, ** p<0.01, *** p<0.001

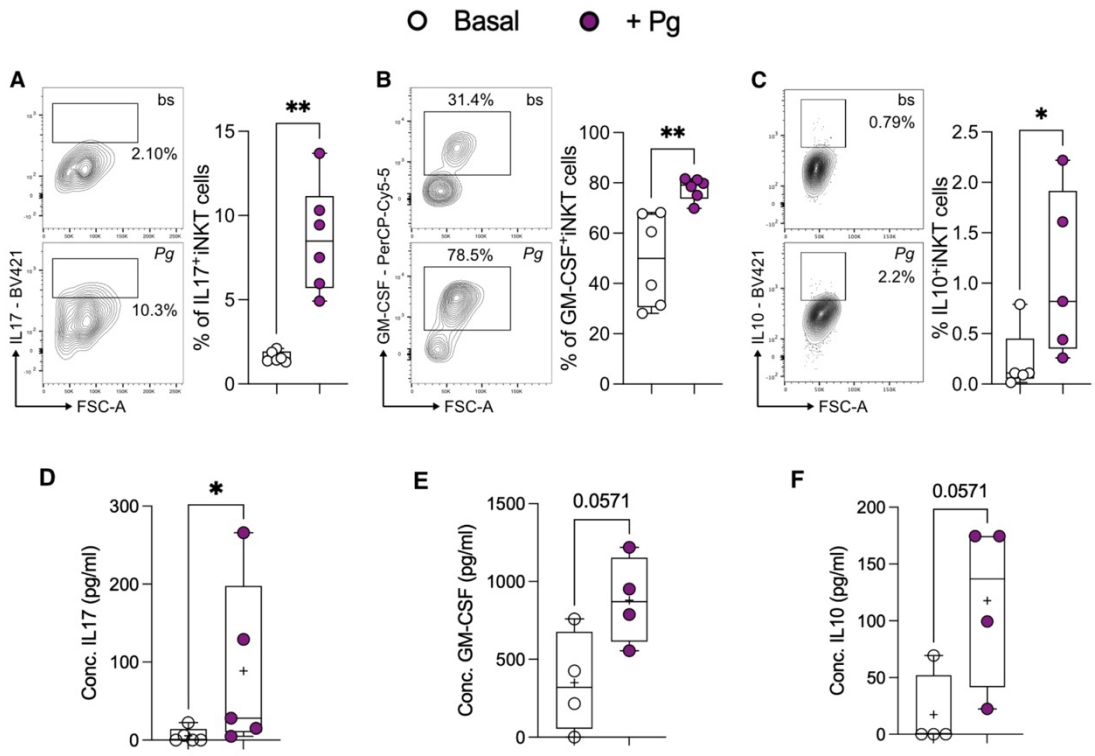

Fig. 6.12. *P. gingivalis* drives a pro-tumorigenic cytokine profile on human iNKT cells. A-C. Representative plots and frequencies from IL-17 (A), GM-CSF (B) and IL-10 (C). D-F. Release of IL-17 (D), GM-CSF (E) and IL-10 (F) by iNKT cells in culture supernatants. Data presented are from 3 independent experiments with 2 iNKT cell lines. Mann-Whitney test. * p<0.05, ** p<0.01, *** p<0.001

6.5. Blockade of TLR-2 and -4 partially abrogates the Pg-mediated IL-10 and GM-CSF production on iNKT cells, while increasing the numbers of activated cells

Some lines of evidence have shown that the upregulation of Th17 genes on CD4+ T cells by *P. gingivalis* is mediated by TLR-4 signaling [135, 197], whereas IL-10 expression seems to be mediated by TLR-2 [135, 198]. To elucidate if iNKT cell activation upon exposure to *P. gingivalis* is governed by the same mechanisms,

we primed iNKT cells with *Pg* in the presence of neutralizing antibodies for TLR-2 and -4. We observed that, whereas TLR blockade did not alter IL-17 production (Fig. 6.13A), it did have effects on GM-CSF and IL-10 secretion by iNKT cells. In particular, the inhibition of both TLRs, but especially TLR-4, provoked a reduction in the frequencies of GM-CSF and IL-10 positive cells (Fig. 6.13B-C). Moreover, TLR-2 blockade induced a significant increase in the percentages of activated, CD69+ iNKT cells, albeit TLR-4 neutralization positively influenced CD69+ iNKT cells as well (Fig. 6.13D). Taken together, these data suggest that surface Toll-like receptors 2 and 4 might mediate the effects of *P. gingivalis* on IL-10 and GM-CSF, as well as the activation status, on human iNKT cells.

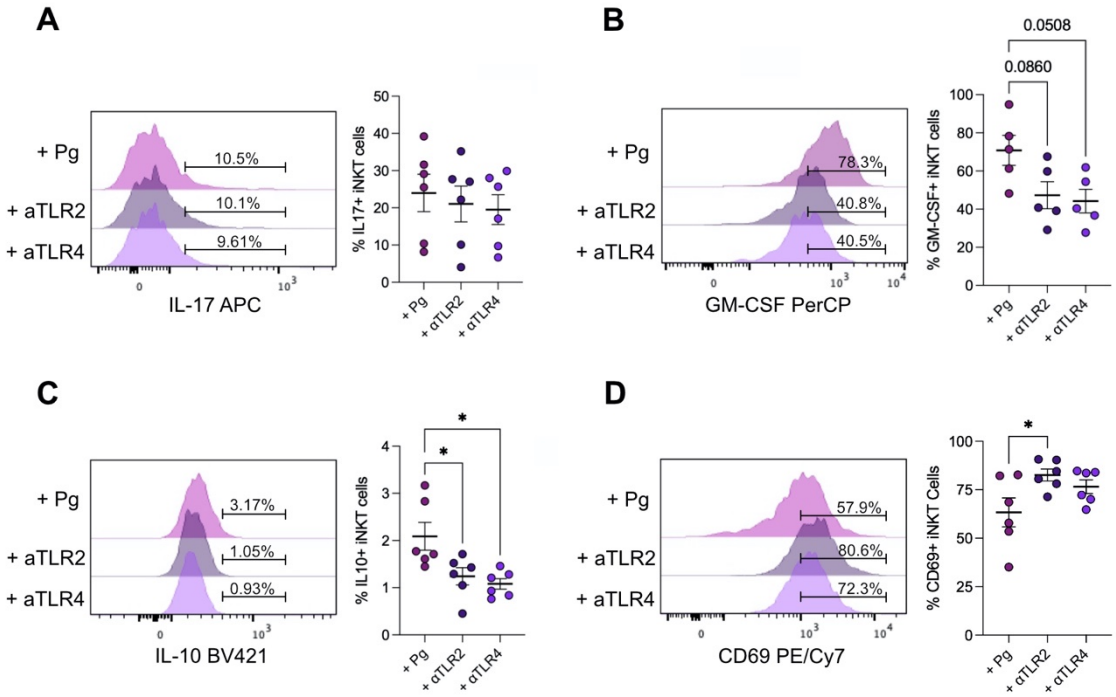

Fig. 6.13. Toll-like receptors -2 and -4 are necessary for the *P.gingivalis*-induced increase in IL-10 and GM-CSF expression by iNKT cells, as well as their activation status. A-D. Representative histograms and frequencies of IL-17 (A), GM-CSF (B), IL-10 (C) and CD69 (D) positive iNKT cells. Data from 2 independent experiments with at least 3 iNKT cell lines are presented. Kruskal-Wallis with Dunn test for multiple comparisons. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

6.6. Pg-primed iNKT cells show an impaired cytotoxic activity, given by a decrease in degranulating cells and perforin release

All previous data indicate that *P. gingivalis* can promote iNKT cell pro-tumor features both *in vivo* and *in vitro.* However, it is not clear whether it can also modulate iNKT cell antitumor functions, in particular their cytotoxic activity. To

address this point, we tested Pg-pulsed iNKT cell killing against Colo 205 and RKO cells at 8:1 E:T ratio. Interestingly, we observed that *P. gingivalis* negatively influences iNKT cell cytotoxicity (Fig. 6.14A). More specifically, *P. gingivalis* exposure is associated with a reduction in the release of perforin as well as in the percentages of degranulating (CD107A+) iNKT cells (Fig. 6.14B & C).

In addition, to assess any possible role of inhibitory antigens and/or surface TLR signaling, we stimulated iNKT cells with Pg in the presence of neutralizing antibodies for CD1d, TLR-2 and TLR-4 prior to coculture with CRC cells. We observed that the impairment of the killing activity given by Pg is both CD1d (Fig. 6.14D) and TLR-2 and -4 (Fig. 6.14E) independent. These data demonstrate that *P. gingivalis* also infuences human iNKT cell cytotoxicity independently of antigen presentation and surface TLR signaling.

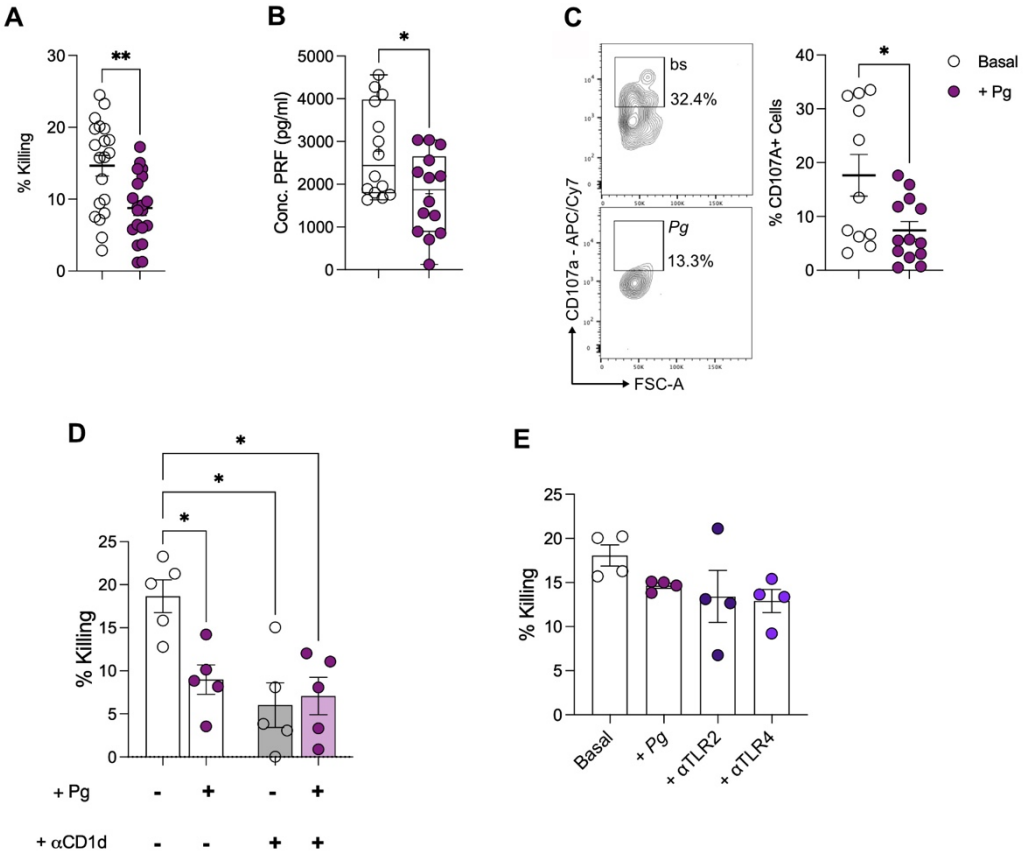

Fig. 6.14. *P. gingivalis* impairs human iNKT cell cytotoxicity in a CD1d- and surface TLR-independent manner. A. Cytotoxicity by iNKT cells upon stimulation with *P. gingivalis*. B. Release of perforin in coculture supernatants. C. Frequencies of surface CD107A+ iNKT cells. D. Effect of CD1d blockade on Pg-mediated decrease of iNKT cell cytotoxicity. E. Effect of TLR-2 and -4 blockade on Pg-mediated decrease of iNKT cell cytotoxicity. Data of at least 2 independent experiments with at least 2 iNKT cell lines are presented. Mann-

Whitney test in A-C. ANOVA with Tukey test for multiple comparisons in D-E. * p<0.05, ** p<0.01, *** p<0.001

6.7. Pg-induced chitinase 3-like protein 1 is responsible for the diminished iNKT cell cytotoxicity

Given the abovementioned data, we analyzed the RNA-seq data to gain insights about factors that may contribute to the impaired iNKT cell cytotoxicity given by exposure to *Porphyromonas gingivalis*. We found that chitinase 3-like protein 1 (CHI3L1), a known inhibitor of Th1 responses and cell-mediated cytotoxicity [199, 200] is overexpressed in Pg-primed iNKT cells (Fig. 6.11A). To fully address its role in the decrease of iNKT cell cytotoxicity, we first measured CHI3L1 protein levels in the supernatants of iNKT cells exposed to *P. gingivalis*. We observed that Pg-treated iNKT cells produced higher levels of CHI3L1 than cells at basal state (stimulated with moDCs alone, Fig. 6.15A). In addition, we found that recombinant CHI3L1 protein inhibited iNKT cell killing activity in a dose-dependent manner (Fig. 6.15B), and that its inhibition was similar to the one induced by *P. gingivalis* (Fig. 6.15C).

Next, to test if the regulation of CHI3L1 can restore iNKT cell killing potential, we inhibited its action with a neutralizing antibody. CHI3L1 blockade on Pg-primed iNKT cells not only restored their cytotoxic potential, but it actually increased it to higher levels than those given by the stimulation by moDCs alone (Fig. 6.15D). Furthermore, these levels were higher than those of the inhibition of the recombinant CHI3L1 (Fig. 6.15D). Overall, these data demonstrate that *P. gingivalis* inhibits human iNKT cell cytotoxicity via the induction of chitinase 3-like 1 production.

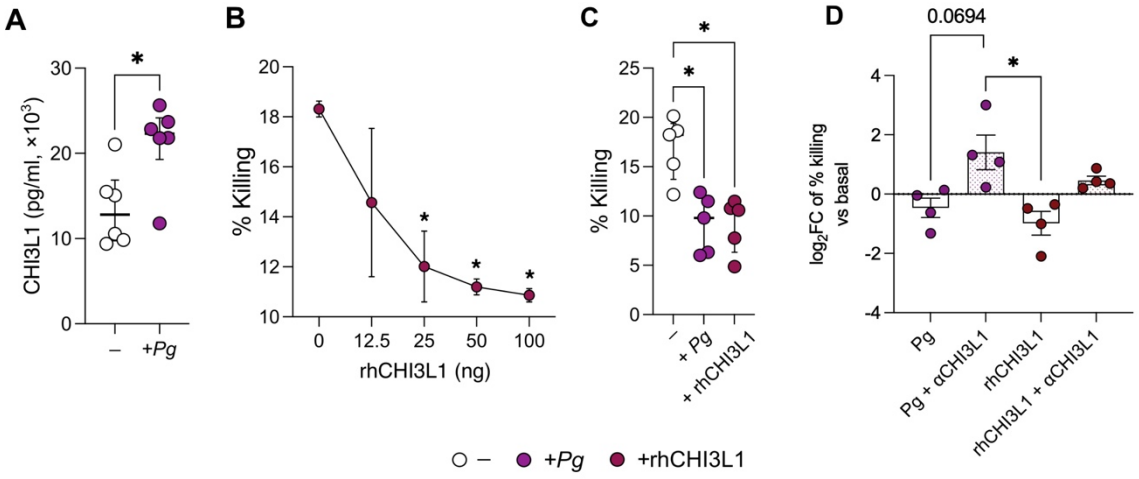

Fig. 6.15. Pg-induced chitinase 3-like protein 1 is responsible for the diminished iNKT cell cytotoxicity. A. Levels of CHI3L1 in stimulation supernatants. B. Dose-dependent effect of recombinant CHI3L1 on human iNKT cell cytotoxicity. C. Comparison of the changes in killing potential given by *P. gingivalis* versus recombinant CHI3L1 (100 ng). E. Effect of CHI3L1 blockade on iNKT cell killing activity upon Pg priming. Data of 2 independent experiments with at least 2 iNKT cell lines are presented. Mann-Whitney tests on A-B, ANOVA with Tukey tests on C-D. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

7. Discussion II

Several studies have demonstrated that members of the microbiota can modulate iNKT cell activities in inflammatory bowel diseases, where they can exert pathogenic or homeostatic functions depending on diverse microbial signals [60, 93, 201, 202]. However, how bacteria might influence iNKT cell functions in colon cancer is not well understood. Elucidating such signals has become particularly relevant, since we recently demonstrated that iNKT cell infiltration is a negative prognosis factor in human colorectal cancer [42]. More specifically, iNKT cells contribute to CRC pathogenesis due to the expression of IL-17 and GM-CSF, as well as by recruiting neutrophils with pro-tumor characteristics, such as reduced respiratory burst capability and suppressive activity, ultimately disrupting the favorable prognostic significance of neutrophils in CRC patients [42]. Here, we demonstrated that *Porphyromonas gingivalis*, an emerging CRC-associated bacterium, accelerates colon cancer progression by enhancing iNKT cell pro-tumor features. Moreover, we found that iNKT cells are necessary for the pro-tumorigenic effect of *P. gingivalis*, and that this effect is achieved *via* various mechanisms.

Porphyromonas gingivalis has gained recognition in cancer research in recent years. In particular, its role in upper digestive tract cancers is becoming more established. In these contexts, it increases cancer cell proliferation by a gingipain-mediated, non-canonical β-catenin activation [203]. It can also enhance cancer cell migration capabilities by increasing the expression of matrix metalloproteinases [204], and promote the acquisition of stem cell features [205] and resistance to apoptosis [206] by activating the NF-κB, MAP kinase pathways, and upregulating PD-L1 [207]. Additionally, some evidence has pointed at its roles in the modification of the tumor immune microenvironment by expanding myeloid-derived suppressor cells (MDSCs, [208]) and macrophage polarization towards an M2 phenotype [209].

Some of these mechanisms are also found in colon cancer cells, as *P. gingivalis* promotes MAPK activation and PD-L1 expression [140, 141]. Wang and colleagues [137] observed that *P. gingivalis* contributed to CRC by supporting an inflammatory state through NLPR3 inflammasome activation on myeloid cells, whose infiltration was also increased in tumors from Pg-treated mice [137]. In that study, authors found that the frequencies of tumor-infiltrating conventional CD4 and CD8 T cells, as well as their expression of IFNG, did not change upon *P. gingivalis* treatment. Consistent with this study, we did not find significant differences in the frequencies and in cytokine production in tumor-infiltrating CD4+ T cells. In addition, we found that iNKT cells are more abundant in tumors from Pg-treated mice in the AOM/DSS

model. More importantly, we found that tumor-infiltrating iNKT cells of *P. gingivalis*-treated mice had increased expression of IL-17, GM-CSF and IL-10, a phenotype observed also in Pg-primed human iNKT cells.

Several studies have highlighted the activation of Th17 responses by *P. gingivalis*. Mechanistically, it seems that *P. gingivalis* enhances Th17 responses by increasing IL-6, IL-23, and IL-1β expression on myeloid antigen-presenting cells while inhibiting IL-12 production, ultimately inducing RORγt expression on T cells [210–212]. Such increase, however, is mediated by various mechanims. For instance, gingipains seem to be a crucial factor for IL-17 production in aspiration pneumonia and in chronic periodontitis caused by this bacterium [211–213]. Other studies have shown that this phenomenon is instead dependent on TLR-2 and -4 signaling, indicating that fimbriae, lipopolysaccharides, and hemagglutinin B, well-known microbial virulence factors, are involved in the skewing of this response [135, 197, 214]. In the context of iNKT cells, we show that TLR-2 and TLR-4 blockade does not affect IL-17 expression, suggesting that at least these TLR ligands are not involved in the increment of this cytokine. Further studies are needed to fully decipher these interactions.

Regarding IL-10 expression on human iNKT cells, we observed that TLR-2 and TLR-4 are necessary for the increase given by *P. gingivalis*. Other studies have evidenced the importance of these receptors in the *P.gingivalis*-mediated IL-10 production on CD4+T cells [135, 198]. In particular, transforming growth factor beta (TGF-β), a known inductor for IL-10 expression [215, 216], might be responsible for the production of this cytokine upon TLR ligation, as it has been observed that fimbriae from *P. gingivalis* increase TGF-beta secretion through GARP upregulation in esophageal squamous cell carcinoma [217]. Moreover, TLR-2 and -4 ligands enhance TGF-β signaling by augmenting sensitization to TGF-beta in hepatic fibrosis [218], Langerhans cells [219], prostate hyperplasia [220], and scleroderma [221].

Less is known about the influence of *P. gingivalis* over GM-CSF secretion. Nonetheless, it has been observed that it increases GM-CSF production on oral epithelial cells [222]. Furthermore, it induced GM-CSF secretion on gingival fibroblasts upon stimulation with penta- but not tetra-acylated LPS [223], suggesting that TLR-4 can mediate this response, as it occurred on iNKT cells. Some studies have also shown that iNKT cells secrete GM-CSF upon bacterial stimuli, such as *Mycobacterium tuberculosis* and *Fusobacterium nucleatum* [42, 224].

One peculiarity of the results shown here is that the augmenting effect of *P. gingivalis* on T cell cytokine profile occurred on iNKT cells but not on CD4+ T cells. Moreover, iNKT cells seemed to be necessary for *P. gingivalis* to enhance tumor progression in AOM/DSS-treated mice. These phenomena enforce the importance of these cells as first-responders in several immune responses, including those to potentially pro-tumorigenic bacteria. In this regard, iNKT cells are characterized by a fast activation following various stimuli, similar to innate immune cells, due to the fact that they exist in a poised state [146, 225]. In addition, they are a tissue-resident and mostly non-recirculating population [48, 226], which makes them sentinels for tissue homeostasis, capable of modulating the functions of other tissue-resident populations and homing of circulating cells. However, further analyses will evaluate if these activities are specific for iNKT cells or if other innate-like, tissue-resident populations in the intestine, such as gamma-delta T and MAIT cells, are also affected by *P. gingivalis* in colon cancer.

Another important aspect found in this study is that iNKT cells not only improved tumor progression upon *P. gingivalis* treatment due to a pro-tumor cytokine profile, but also due to an enhanced recruitment of TANs. In fact, one of the most recognized interactions of iNKT cells is the one with myeloid cells. It has been observed that iNKT cells are also necessary for neutrophil recruitment in lung ischemia-reperfusion injury [227], airway neutrophilia [228], ethanol-mediated liver injury [229] and in colon cancer tumors [42]. This was achieved thanks to IL-17, IL-8, osteopontin, and MIP1-α production by iNKT cells [42, 228, 229]. We observed by flow cytometry and RNA-seq that, upon stimulation with *P. gingivalis,* iNKT cells increase the expression of IL-17, IL-8, CXCL1, -2 and -5. This bacterium is known to promote neutrophil recruitment by producing short-chain fatty acids [230], stimulating the expression of IL-8 on monocytes [204], and CXCL2 in oral squamous cell carcinoma (OSCC) [231]. In the latter, it was also found that neutrophil chemotaxis promoted OSCC progression, and *P. gingivalis*, TANs and CXCL2 predicted patient survival, being considered independent factors for adverse outcome [231]. Other studies have shown that *P. gingivalis* supports OSCC progression by promoting the infiltration of myeloid-derived suppressor cells, a phenomenon that might be mediated by CXCL2 [208, 209, 231, 232]. Hence, iNKT cells might use similar signals to mediate the *P. gingivalis*-induced recruitment of tumor-associated neutrophils in CRC.

P. gingivalis also inhibited human iNKT cell cytotoxicity, indicating that not only it promoted a pro-tumor phenotype, but also inhibited antitumor activities.

Mechanistically, we found that this phenomenon was mediated by the *P.gingivalis*-induced expression of chitinase 3-like protein 1. CHI3L1 is a pro-inflammatory protein involved in intestinal inflammation [233], Alzheimer's [234], and bacterial infections [235, 236]. In the context of cancer, it is known that its expression by T cells promotes lung metastasis by inhibiting Th1 differentiation while favoring Th2 responses [199], whereas the expression on cancer cells supports their proliferation and metastatic capacity, improves macrophage recruitment and angiogenesis in gastric cancers [237–239], and inhibits cell-mediated cytotoxicity by inhibiting JNK signaling, which results in defects in granule polarization [200]. In natural killer cells, a population phenotypically and functionally similar to iNKT cells, it was found that CHI3L1 impaired granule polarization by hindering RAGE (receptor for advanced glycation endproducts) downstream signaling [200]. This could also be the case for iNKT cells, as it has been observed that they express this receptor [227]. One study showed that gingipains from *P. gingivalis* induced the expression of the CHI3L1 analogs *chia.5* and *chia.6* in a zebrafish model of hyperglycemia [240]. In this regard, it is yet to be established which signal of *P. gingivalis* induces CHI3L1 expression on iNKT cells.

8. Conclusions and Future Perspectives

In this thesis, we first gave insights about how colon cancer cells influenced iNKT cell antitumor functions, with emphasis on their cytotoxic activities. First, we found that CRC cells impaired the production of interferon gamma, an important antitumor cytokine [22, 23]. However, these cells also stimulated iNKT cell cytotoxic activity. More specifically, we demonstrated that intestinal and circulating iNKT cells increased the release of granules containing granzymes and perforin upon exposure to CRC cell lines and patient-derived epithelial cells, which resulted in target cell death. In addition, we provide evidence about the signals required for the recognition of CRC cells by iNKT cells. This study shows that antigen presentation is not necessary for proper elimination by iNKT cells, suggesting that innate signals are used for this process. These results were published in Molecular Oncology (Appendix VI).

These results highlight the potential of iNKT cells for cell-based immunotherapies in CRC. This idea is reinforced by the fact that these cells eliminated patient-derived CRC cells more efficiently than any of the well-established cancer cell lines. Moreover, even if they represent a rare population, their infiltration is higher in CRC tumors [42, 69]. Another advantage is that iNKT cells exist in a poised state, which includes having pre-formed granules [146, 225], which makes them one of the first responders in many immune responses. Plus, the fact that they are not restricted to MHC compatibility, and that blood-derived cells were similarly efficient as tissue-derived cells, makes iNKT cells promising candidates for adoptive cell therapies. Among the current approaches using these cells in cancer there are the stimulation of autologous iNKT cells with αGalCer by administration of the antigen or pulsed dendritic cells, transfer of autologous activated iNKT cells alone or in combination with αGalCer, CAR-iNKT and recombinant TCR (rTCR)-iNKT cells [54, 72, 160]. More research will be needed to find CRC-specific antigens for next-generation CAR- and rTCR-iNKT cell therapies, even if these cells also make use of NK receptors to recognize transformed cells, as shown in this study.

One peculiarity shown here is that both cell lines and cells from different patients were not eliminated with the same efficacy by iNKT cells. We hypothesize that the differential expression of activating and/or inhibitory ligands of natural killer receptors present on CRC cells might be responsible for this phenomenon. In this regard, further studies will be done to address which signals on colon cancer cells potentiate/inhibit the antitumor functions of iNKT cells.

Nonetheless, we also observed that iNKT cells do not increase the production of IL-17, GM-CSF and IL-10 – whose expression is one of the pro-tumor functions of these cells in CRC [42, 61], upon exposure to colon cancer cells. The fact that CRC cells are not responsible for this behavior led us to hypothesize that it could be mediated by interactions with other components of the tumor microenvironment, for instance, with CRC-related bacteria. Indeed, we provided evidence about the influence of *Porphyromonas gingivalis* on the enhancement of iNKT cell pro-tumorigenic functions in two murine models of colon cancer, as well as in functional studies on human cells (Fig. 8). We observed that not only *P. gingivalis* improved the expression of IL-17, GM-CSF and IL-10 on iNKT cells, but also promoted the iNKT cell-mediated recruitment of neutrophils with low ROS production. Furthermore, we demonstrated that this bacterium could impair human iNKT cell cytotoxicity by inducing the expression of chitinase 3-like protein 1.

Using the AOM/DSS model, we observed that tumor-infiltrating iNKT cells, but not conventional CD4+T cells, were particularly affected by the exposure to *P. gingivalis.* In fact, we found that this modulation was necessary for the enhancement of tumor progression given by this bacterium, as the conditions of Pg-treated, iNKT cell-deficient animals did not worsen compared to their untreated counterparts. This is the first study that shows that the influence of *P. gingivalis* over an unconventional T cell population promotes colon cancer progression. A similar phenotype was found in an orthotopic injection model. Nonetheless, it is yet to be addressed if iNKT cells are necessary for tumor progression in this model as well. For this, a lower amount of MC38 cells will be injected in order to induce a slower tumor progression, so we can distinguish differences given by *P. gingivalis.* iNKT cell-deficient mice will be used to fully assess the role of iNKT cells on the effect given by *P. gingivalis* on this model.

By performing functional assays with human cells, we gained information about the signals of *P. gingivalis* that drive this response (Fig. 8). We showed that ligands for Toll-like receptors 2 and 4 might be responsible for the increase in IL-10 and GM-CSF expression, as well as the activation status of iNKT cells. Furthermore, we found that Pg-primed iNKT cells are prone to express several neutrophil chemokines, including IL-8 and CXCL2. However, the signals that induce IL-17 and CHI3L1 expression are yet to be dissected. In this regard, we hypothesize that gingipains might mediate these processes. To test this, we will perform experiments in the presence of gingipain-deficient *P. gingivalis* mutants. Moreover, to confirm the

role of TLR ligands, we will also perform functional assays using fimbriae-knockout mutants and Pg purified LPS.

Another important aspect to evaluate is if the *P. gingivalis* treatment used here allows its colonization in the colon and, more importantly, if it induces long-lasting changes in the gut microbiota composition and functions. The fact that we did not use a mono-colonization setting (by using antibiotics prior to treatment), and that *P. gingivalis* is found predominantly in humans [127, 128], led us to speculate that the setup used here only provoked temporary changes in the bacterial ecology. To test this, we are performing 16S sequencing on fecal microbiota of untreated and Pg-treated mice.

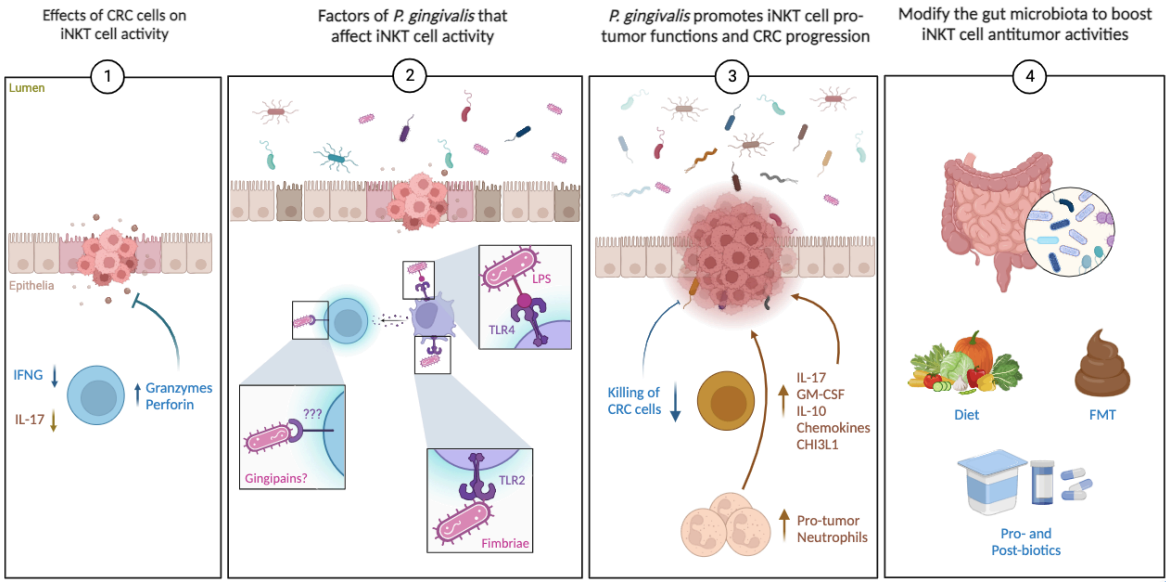

Fig. 8. Graphical abstract. Created with Biorender.

The results presented here highlight the importance of the crosstalk between colon cancer-related bacteria and iNKT cells, and how these interactions strongly influence tumor progression. They also reinforce the association between periodontal disease and colon cancer risk [241] Even if such cells are found in low numbers, we observed that this crosstalk profoundly affects tumor growth. This was achieved thanks to their influence over myeloid populations within the TME. A further understanding of these interactions could be of interest in in the clinic, as they could be exploited for immunotherapies. Such approaches have been observed for other solid tumors, in which iNKT cells have been engineered to eliminate both cancer cells and tumor-associated macrophages [73, 109].

Accordingly, understanding which bacteria and which signals drive pro-tumorigenic features on these cells is crucial for the implementation of iNKT cell-

based immunotherapies, as well as other therapeutic interventions, in colon cancer. The data shown here indicate that microbiota-directed interventions should be taken into account to avoid the pro-tumor influence of certain strains on these cells. Conversely, given the plasticity of iNKT cells upon microbial stimuli, other commensal bacteria might be beneficial to boost their antitumor activities [242-244]. More specifically, genera like *Bifidobacterium*, *Clostridium*, and butyrate-producing bacteria are now known to recruit boost antitumor immunity on NK and CD8+T cells in preclinical models of melanoma and colon cancer by increasing the expression of interferon gamma and cytotoxic molecules such as granzyme B [242-244]. In these works are highlighted the multiple forms by which the gut microbiota composition can be modified in the clinic, *i.e.*, diet, design of specific commensal consortia, probiotics, and fecal microbiota transplantation [242-244]. Such strategies are promising, but are yet to be tested to address their efficacy in boosting iNKT cell antitumor activities while inhibiting their pro-tumorigenic functions. Therefore, this opens the door for more studies about how beneficial microbiota might regulate iNKT cell features, enhancing antitumor immunity, and eventually use both as a combinatorial therapy for colorectal cancer.

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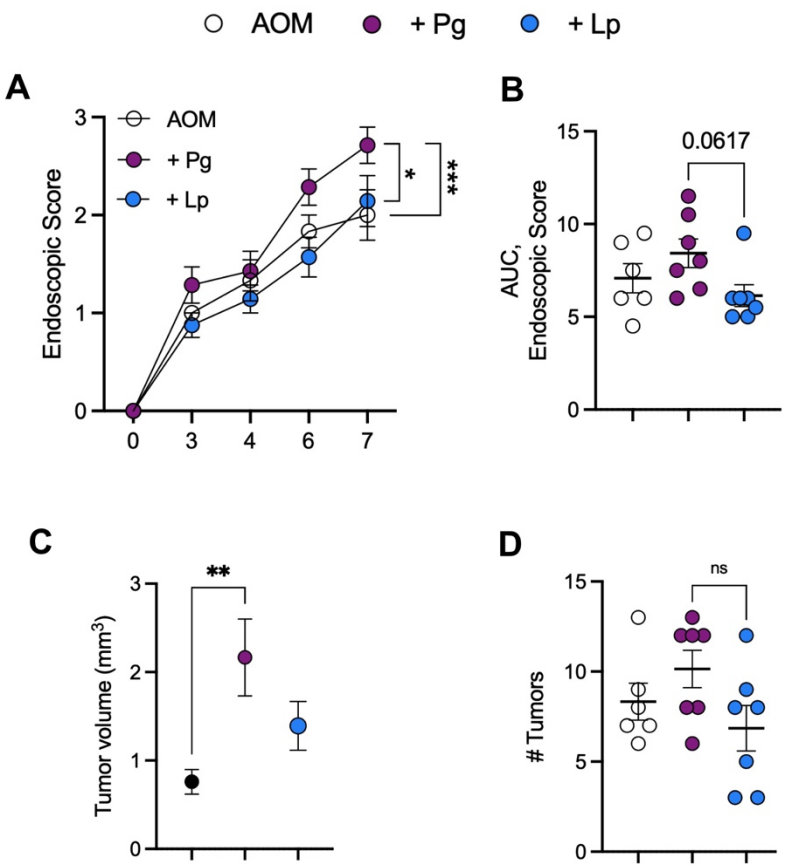
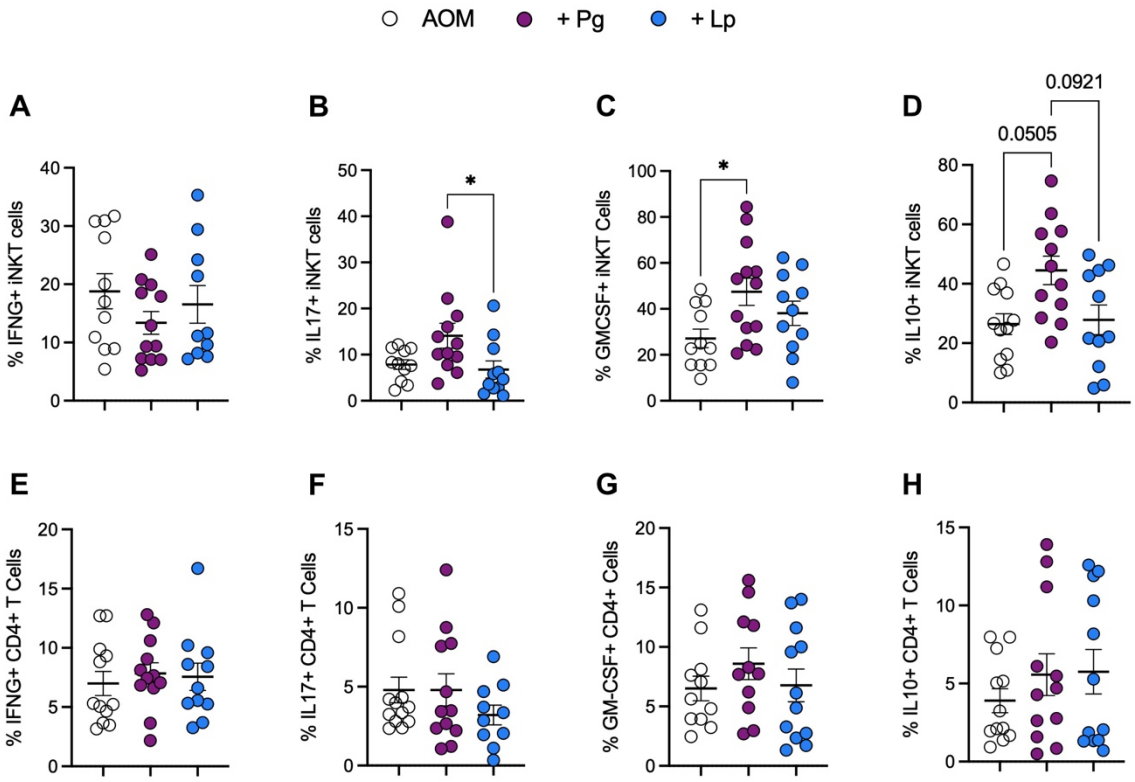
Appendix I



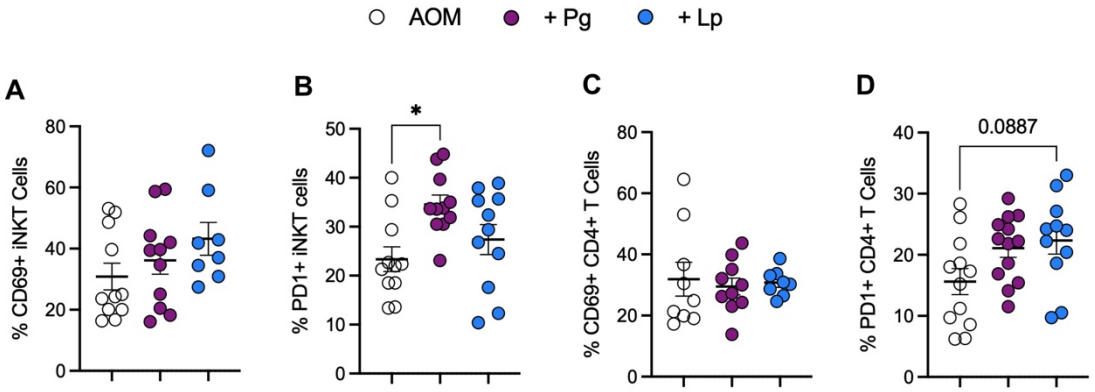
Appendix I. Clinical scores of AOM/DSS-treated mice upon treatment with *P. gingivalis* and *Lactobacillus plantarum*. A. Endoscopic scores. B. Area under the curve of the data in A. C. Tumor voulme at sacrifice. D. Number of tumors at sacrifice. ANOVA with Tukey test in A, Kruskal-Wallis with Dunn test in B-D.

Appendix II



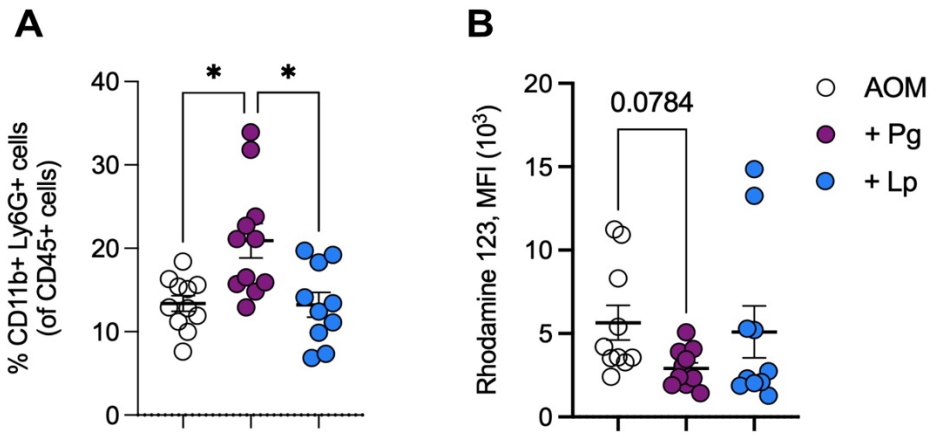
Appendix II. Cytokine profile of tumor-infiltrating iNKT and CD4+T cells upon treatment with bacteria in the AOM/DSS model. A-D. Frequencies of interferon gamma (A), interleukin-17 (B), GM-CSF (C) and interleukin-10 (D) positive iNKT cells. E-H. Frequencies of interferon gamma (E), interleukin-17 (F), GM-CSF (G) and interleukin-10 (H) positive CD4+T cells. Kruskal-Wallis with Dunn test.

Appendix III



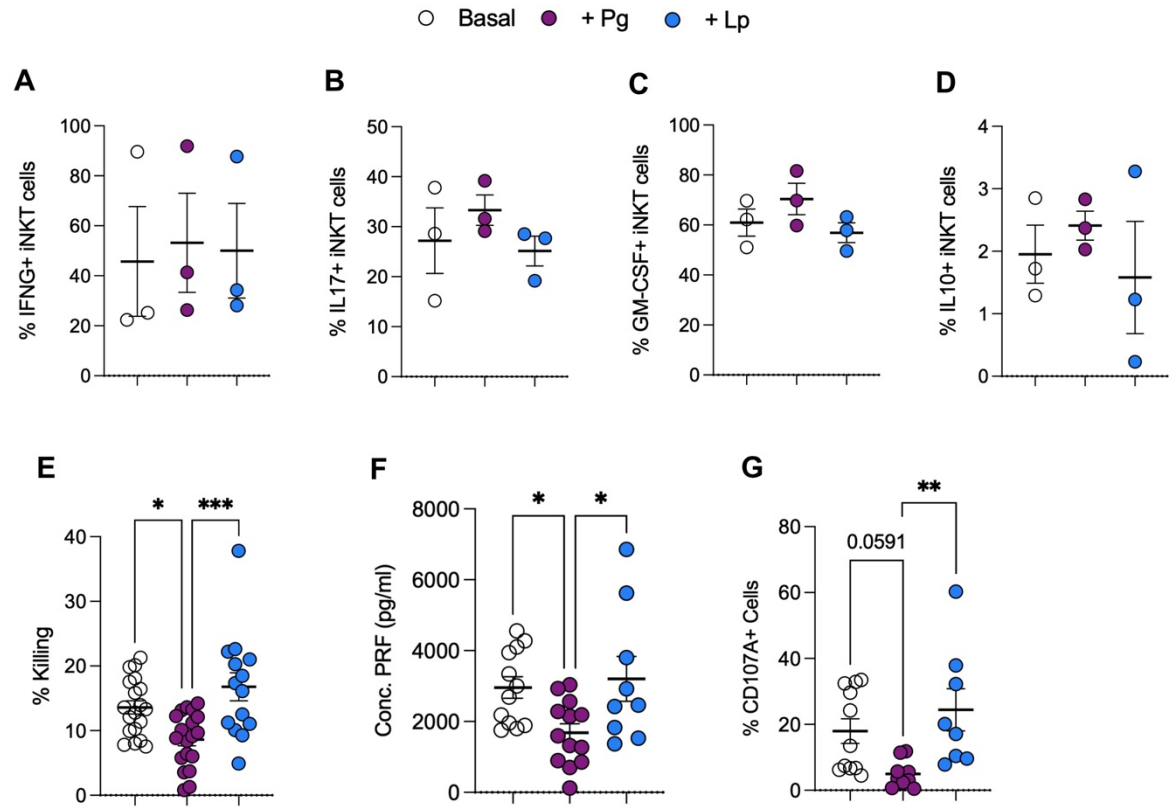
Appendix III. CD69 and PD1 expression of tumor-infiltrating iNKT and CD4+T cells upon treatment with bacteria in the AOM/DSS model. A-B. Frequencies of CD69 (A) and PD1 (B) positive iNKT cells. C-D. Frequencies of CD69 (A) and PD1 (B) positive CD4+ T cells. Kruskal-Wallis with Dunn test.

Appendix IV



Appendix IV. TANs in AOM/DSS-treated mice upon treatment with bacteria. A. Frequencies of TANs. B. MFI of rhodamine 123. Kruskal-Wallis with Dunn test.

Appendix V



Appendix V. Behavior of human iNKT cells upon treatment with *P. gingivalis* and *L. plantatum.* A-D. Frequencies of IFNG (A), IL-17 (B), GM-CSF (C) and IL-10 (D) positive iNKT cells. E. Cytotoxicity by iNKT cells upon stimulation with *P. gingivalis* and *L. plantarum*. F. Release of perforin in coculture supernatants. G. Frequencies of surface CD107A+ iNKT cells. ANOVA with Tukey test on A-D, Kruskal-Wallis with Dunn test in E-G.

Appendix VI

Molecular Oncology

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Human intestinal and circulating invariant natural killer T cells are cytotoxic against colorectal cancer cells via the perforin–granzyme pathway

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Keywords
CD1d; colorectal cancer; cytotoxicity; iNKT; perforin

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Invariant natural killer T (iNKT) cells are lipid-specific T lymphocytes endowed with cytotoxic activities and are thus considered important in antitumor immunity. While several studies have demonstrated iNKT cell cytotoxicity against different tumors, very little is known about their cell-killing activities in human colorectal cancer (CRC). Our aim was to assess whether human iNKT cells are cytotoxic against colon cancer cells and the mechanisms underlying this activity. For this purpose, we generated stable iNKT cell lines from peripheral blood and colon specimens and used NK-92 and peripheral blood natural killer cells as cell-mediated cytotoxicity controls. *In vitro* cytotoxicity was assessed using a panel of well-characterized human CRC cell lines, and the cellular requirements for iNKT cell cytotoxic functions were evaluated. We demonstrated that both intestinal and circulating iNKT cells were cytotoxic against the entire panel of CRC lines, as well as against freshly isolated patient-derived colonic epithelial cancer cells. Perforin and/or granzyme inhibition impaired iNKT cell cytotoxicity, whereas T-cell receptor (TCR) signaling was a less stringent requirement for efficient killing. This study is the first evidence of tissue-derived iNKT cell cytotoxic activity in humans, as it shows that iNKT cells depend on the perforin–granzyme pathway and both adaptive and innate signal recognition for proper elimination of colon cancer cells.

Appendix VII

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Review

License to Kill: When iNKT Cells Are Granted the Use of Lethal Cytotoxicity

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Abstract: Invariant Natural Killer T (iNKT) cells are a non-conventional, innate-like, T cell population that recognize lipid antigens presented by the cluster of differentiation (CD)1d molecule. Although iNKT cells are mostly known for mediating several immune responses due to their massive and diverse cytokine release, these cells also work as effectors in various contexts thanks to their cytotoxic potential. In this Review, we focused on iNKT cell cytotoxicity; we provide an overview of iNKT cell subsets, their activation cues, the mechanisms of iNKT cell cytotoxicity, the specific roles and outcomes of this activity in various contexts, and how iNKT killing functions are currently activated in cancer immunotherapies. Finally, we discuss the future perspectives for the better understanding and potential uses of iNKT cell killing functions in tumor immunosurveillance.

Keywords: iNKT; cytotoxicity; cancer; infections; CD1d

Appendix VIII

ARTICLE

iNKT cell-neutrophil crosstalk promotes colorectal cancer pathogenesis

Georgia Lattanzi [1,2,†], Francesco Strati [1,3,†], Angélica Díaz-Basabe [1,2], Federica Perillo [1,2], Chiara Amoroso [4], Giulia Protti [3], Maria Rita Giuffrè [1], Luca Iachini [3], Alberto Baeri [3], Ludovica Baldari [5], Elisa Cassinotti [5], Michele Ghidini [6], Barbara Galassi [6], Gianluca Lopez [7], Daniele Noviello [4,8], Laura Porretti [9], Elena Trombetta [9], Eleonora Messuti [1], Luca Mazzarella [1], Giandomenica Iezzi [10], Francesco Nicassio [11], Francesca Granucci [3], Maurizio Vecchi [4,8], Flavio Caprioli [4,8] and Federica Facciotti [1,3,✉]

iNKT cells account for a relevant fraction of effector T-cells in the intestine and are considered an attractive platform for cancer immunotherapy. Although iNKT cells are cytotoxic lymphocytes, their functional role in colorectal cancer (CRC) is still controversial, limiting their therapeutic use. Thus, we examined the immune cell composition and iNKT cell phenotype of CRC lesions in patients (n = 118) and different murine models. High-dimensional single-cell flow-cytometry, metagenomics, and RNA sequencing experiments revealed that iNKT cells are enriched in tumor lesions. The tumor-associated pathobiont *Fusobacterium nucleatum* induces IL-17 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) expression in iNKT cells without affecting their cytotoxic capability but promoting iNKT-mediated recruitment of neutrophils with polymorphonuclear myeloid-derived suppressor cells-like phenotype and functions. The lack of iNKT cells reduced the tumor burden and recruitment of immune suppressive neutrophils. iNKT cells *in-vivo* activation with α-galactosylceramide restored their anti-tumor function, suggesting that iNKT cells can be modulated to overcome CRC-associated immune evasion. Tumor co-infiltration by iNKT cells and neutrophils correlates with negative clinical outcomes, highlighting the importance of iNKT cells in the pathophysiology of CRC. Our results reveal a functional plasticity of iNKT cells in CRC, suggesting a pivotal role of iNKT cells in shaping the tumor microenvironment, with relevant implications for treatment.

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Appendix IX

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Original Article

IL10 Secretion Endows Intestinal Human iNKT Cells with Regulatory Functions Towards Pathogenic T Lymphocytes

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Abstract

Background and Aims: Invariant natural killer T [iNKT] cells perform pleiotropic functions in different tissues by secreting a vast array of pro-inflammatory and cytotoxic molecules. However, the presence and function of human intestinal iNKT cells capable of secreting immunomodulatory molecules such as IL-10 has never been reported so far. Here we describe for the first time the presence of IL10-producing iNKT cells [NKT10 cells] in the intestinal lamina propria of healthy individuals and of Crohn's disease [CD] patients.

Methods: Frequency and phenotype of NKT10 cells were analysed *ex vivo* from intestinal specimens of Crohn's disease [n = 17] and controls [n = 7]. Stable CD-derived intestinal NKT10 cell lines were used to perform *in vitro* suppression assays and co-cultures with patient-derived mucosa-associated microbiota. Experimental colitis models were performed by adoptive cell transfer of splenic naïve CD4+ T cells in the presence or absence of IL10-sufficient or -deficient iNKT cells. *In vivo* induction of NKT10 cells was performed by administration of short chain fatty acids [SCFA] by oral gavage.

Results: Patient-derived intestinal NKT10 cells demonstrated suppressive capabilities towards pathogenic CD4+ T cells. The presence of increased proportions of mucosal NKT10 cells associated with better clinical outcomes in CD patients. Moreover, an intestinal microbial community enriched in SCFA-producing bacteria sustained the production of IL10 by iNKT cells. Finally, IL10-deficient iNKT cells failed to control the pathogenic activity of adoptively transferred CD4+ T cells in an experimental colitis model.

Conclusions: These results describe an unprecedentd IL10-mediated immunoregulatory role of intestinal iNKT cells in controlling the pathogenic functions of mucosal T helper subsets and in maintaining the intestinal immune homeostasis.

Key Words: Crohn's disease; IL10; iNKT cells; SCFA; microbiota

Appendix X

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Review

Gut Microbiota Manipulation as a Tool for Colorectal Cancer Management: Recent Advances in Its Use for Therapeutic Purposes

Federica Perillo [1,†], Chiara Amoroso [1,†], Francesco Strati [1,*], Maria Rita Giuffrè [1], Angélica Díaz-Basabe [1,2], Georgia Lattanzi [1,2] and Federica Facciotti [1,*]

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Abstract: Colorectal cancer (CRC) is a multifaceted disease influenced by both environmental and genetic factors. A large body of literature has demonstrated the role of gut microbes in promoting inflammatory responses, creating a suitable microenvironment for the development of skewed interactions between the host and the gut microbiota and cancer initiation. Even if surgery is the primary therapeutic strategy, patients with advanced disease or cancer recurrence after surgery remain difficult to cure. Therefore, the gut microbiota has been proposed as a novel therapeutic target in light of recent promising data in which it seems to modulate the response to cancer immunotherapy. The use of microbe-targeted therapies, including antibiotics, prebiotics, live biotherapeutics, and fecal microbiota transplantation, is therefore considered to support current therapies in CRC management. In this review, we will discuss the importance of host–microbe interactions in CRC and how promoting homeostatic immune responses through microbe-targeted therapies may be useful in preventing/treating CRC development.

Keywords: colorectal cancer; gut microbiome; live biotherapeutic products

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“Aunque pase por el valle de sombra de muerte, no temeré mal alguno, porque Tú estarás conmigo”

-Salmo 23:4

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pero, sobre todo, superamos todos los obstáculos que se nos presentaron. Por esto y muchos más motivos, este trabajo está dedicado a ella. Quiero agradecer también a toda mi familia, en especial a mi tío Mauritxu y mi Nona por el apoyo incondicional y los valores que también he aprendido de ellos, y mi Papi porque me acercó al estudio de la música, lo que me ayudó muchísimo para tener una vida equilibrada durante este doctorado, renovar mi amor por la música clásica*, y proponerme nuevos propósitos por fuera de la ciencia.

*Shostakovich (7a), Sibelius (3a) y la hr-sinfonieorchester ayudaron también ♥