Immunobiology of IL-17A in human colorectal cancer

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During my PhD training I have extensively investigated the biological relevance of T helper 17 cells in human colorectal cancer.

This thesis consists of an **introduction** covering important aspects related to the development and function of T helper 17 cells in health and disease, followed by a comprehensive description of the **methods** used and the **results** obtained. Major findings and perspectives are then commented in the **discussion.**
The results of this study have been included in a manuscript recently submitted for publication.

I have also been involved in additional projects of our research group addressing the impact of innate immune cells and stromal cells in colorectal cancer.
Finally, I collaborated with the group of Prof. Schifferli, on a project investigating the immune-modulatory role of platelet-derived microvesicles. The manuscript is currently under revision.
Four publications and a submitted manuscript resulting from these additional studies are included in the **appendix**.

to L’Aquila, my hometown

Immota Manet

Summary

During the last decade tumor infiltration by immune cells has been recognized as a key factor determining clinical outcome [1]. Whereas the presence within tumor tissues of defined lymphocytic populations, including cytotoxic CD8+ T cells and IFN-γ-producing T-helper 1 cells has been univocally recognized to predict favorable prognosis [1, 2], the clinical relevance and the pathophysiological role of IL-17-producing cells remain unclear.

In some tumor types, including ovarian, prostate and lung cancer, a positive association between tumor infiltration by IL-17+ cells and prolonged patient survival has been reported [3-5]. In contrast, in colorectal cancer (CRC), IL-17 expression has been shown to predict unfavorable clinical outcome and to weaken the beneficial effect of tumor infiltration by CD8+ T cells [6, 7]. However, restricted numbers of patients were analyzed and no functional data concerning the IL-17 source and the potential mechanisms underlying its negative effect were provided by these studies.

In the enclosed work we have investigated prognostic significance, phenotype, and functional features of tumor-infiltrating IL-17-producing cells in human CRC.

Upon analysis of a tissue micro-array (TMA) including 1400 cases of primary CRC, we found that tumor infiltration by IL-17+ cells was significantly associated with lower T (tumor border) and N (lymph nodes involvement) stage, but in contrast to previous findings, did not *per se* impact on overall patients survival. Interestingly, numbers of IL-17+ cells strongly correlated with those of CD8+ and CD16+ myeloperoxidase (MPO)+ neutrophils, which were predictive of better clinical outcome in the same patient cohort [8-10]. Phenotypic analysis revealed that the majority of tumor infiltrating IL-17+ cells consisted of polyfunctional T helper 17 (Th17), producing, in addition to IL-17 a

spectrum of pro-inflammatory cytokines and chemokines such as TNF-α, IL-21, IL-22, and GM-CSF, and, IFN-γ, and IL-8. Interestingly, tumor-derived Th17 cells induced IL-8-dependent neutrophil migration and enhanced MPO release. Furthermore, tumor-derived Th17 cells favored the indirect recruitment of CD8+ T cells, by triggering chemokine release from tumor-associated endothelial cells. More surprisingly, CD8+ T cells were also directly recruited by Th17 cells in a CCL20 dependent manner. Importantly, the direct effect of Th17 proved sufficient to drive CD8+ T cells into an engineered CRC tissue-like structure.

Our data suggest that CRC infiltrating Th17 cells can favor the recruitment of clinical relevant effector cells into the tumor site, therefore contributing to a more favorable clinical outcome.

Altogether our findings unravel a positive role possibly played by tumor infiltrating polyfunctional Th17 cells in CRC and underline their pleiotropic effects beyond IL-17 production.

Table of contents

I. INTRODUCTION

1 Interleukin-17

1.1 The interleukin-17 cytokine family

Interleukin-17A (IL-17A) is the founding member of the IL-17 cytokine family, including six cytokines, termed IL-17A to IL-17F, characterized by strong inflammatory activity [11]. IL-17A, hereafter referred to as IL-17, is composed of two monomers that are linked by intramolecular disulphide bonds on cysteine residues to form a homodimer. IL-17 and IL-17F, sharing 50% of sequence identity, are by far the best-characterized cytokines of the IL-17 cytokine family.

The *il17* gene and IL-17 protein, known as cytotoxic lymphocyte-associated antigen 8 (CTLA8), were first discovered as a product of T cells in rodents[12]. One of the first studies concerning the biological activity of this cytokine demonstrated that IL-17 could induce production of IL-6, a cytokine playing a major role in inflammation and host defence, and of CXCL8 (also known as IL-8), a strong chemoattractant for neutrophils, from synoviocytes obtained from patient with rheumatoid arthritis (RA), linking its activity to inflammation [13]. Indeed, IL-17 has the ability to induce the expression of a variety of proinflammatory mediators, including IL-6, tumor necrosis factor-α (TNF-α), CXCL8, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) by stromal cells, ultimately leading to neutrophils recruitment [14].

1.2 The IL-17 receptor family

The first receptor to be identified within the IL-17 receptor (IL-17R) family, which comprise five receptor subunits, is known as IL-17RA[15]. The different IL-17R subunits are characterized by conserved structural motifs, including an extracellular fibronectin III-like domain and a cytoplasmic SEF/IL-17R (SEFIR) domain [16, 17]. Binding of IL-17 to its receptor, recruits adapter protein ACT1, which further stimulates TNF receptor–associated factor 6 to activate the NF-κB and MAPK pathways (Figure I.1) [17]. Although it is not clear how IL-17R subunits interact to form productive receptor complexes, it is now evident that IL-17RA is a common signaling subunit used by at least three members of the IL-17 family including IL-17A, -E and –F. Furthermore, it has been recently recognized that, in addition to IL-17RA, IL-17RC is required for cell signaling in response to both IL-17A and IL-17F [18-20], even though IL-17A binds IL-17RA with much higher affinity [21]. IL-17RA is expressed ubiquitously, and at particularly high levels in haematopoietic tissues [15, 22]. In contrast, IL-17RC expression appears to be restricted mostly to non-hematopoietic cells[17]. This may explain why IL-17A predominantly targets non-hemapoietic cells, while its effect in hematopoietic cells is limited.

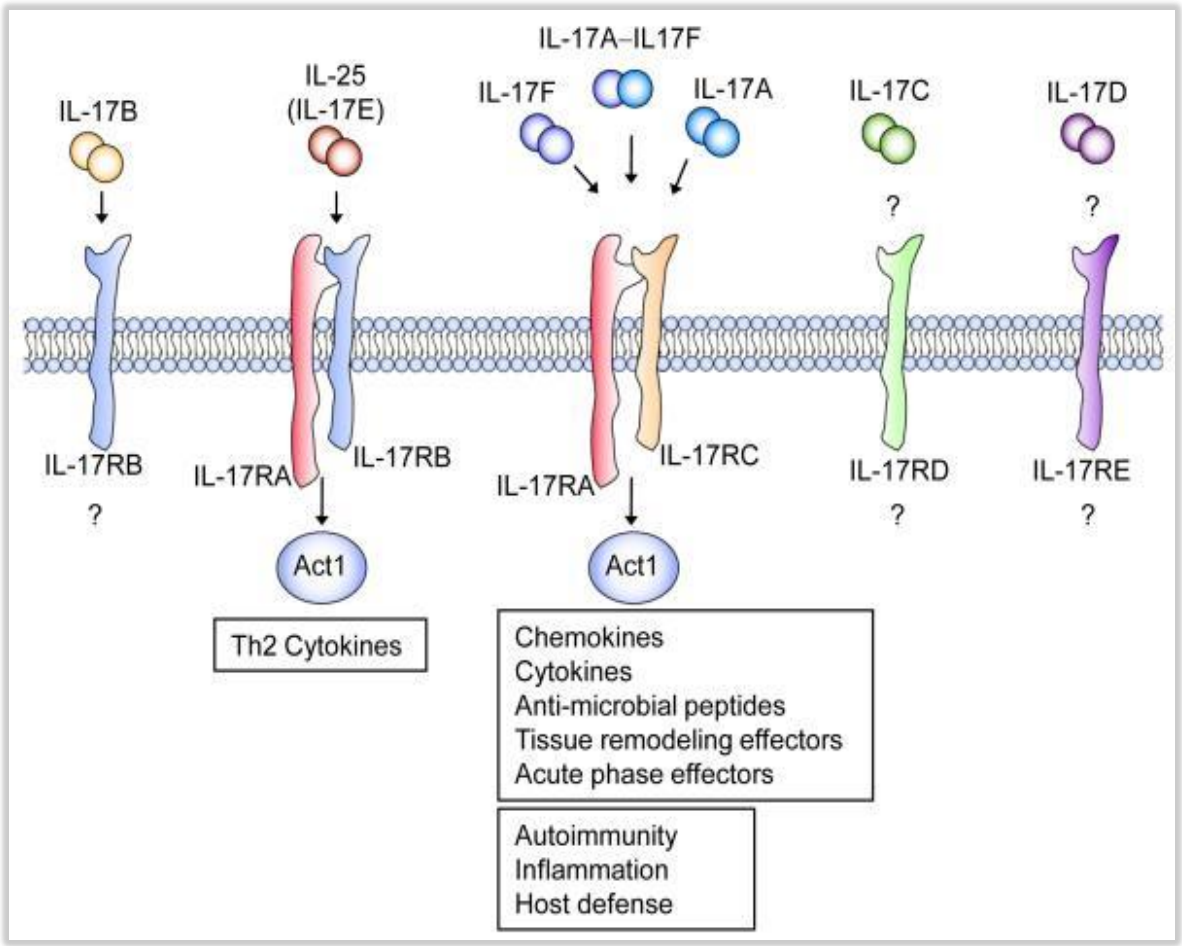


Figure I.1. IL-17 cytokine and receptor family. The IL-17 cytokine family includes IL-17A–F, which are predicted to form homo- and heterodimeric interactions that are necessary for signaling. There are also five known IL-17 receptor subunits. To date, IL-17RA, -RB and -RC are the best characterized. IL-17RA is the common receptor subunit for IL-17A, IL-17F and IL-17E (IL-25). IL-17A and IL-17F bind the receptor complex IL-17RA–IL-17RC to drive expression of inflammatory genes. IL-25 binds to the IL-17RA–IL-17RB complex and regulates Th2 homeostasis. (Gaffen S.L., Nature Review Immunology, 2009).

1.3 Key functions of IL-17

It is well established that IL-17 activity contributes to the initiation of acute inflammation. One of the earliest function attributed to this cytokine was indeed the induction of neutrophils differentiation (known as granulopoiesis) [13]. When cultured in presence of IL-17, fibroblasts, epithelial and endothelial cells were shown to release IL-6, CXCL1, CXCL2, CXCL8 (also known as IL-8), and GM-CSF, driving neutrophils differentiation and recruitment, thus contributing to the early stages of inflammation (Figure I.2) [13,23, 24]. Of note, IL-17 is not able to direct interact with neutrophils, because of their lack of IL-17RC expression [25]. In addition, this cytokine controls the expression of several molecules with antimicrobial activity, such as β-defensins, calgranulins and mucins [26-28], thereby promoting the release of natural antibiotics mostly at mucosal surfaces [29, 30]. Due to these effects IL-17 is critical for the development of mucosal immunity against extracellular bacteria and fungi [31](see below). Additional functions attributed to IL-17 concern the induction of VEGF production, and other angiogenic mediators [32], by surrounding endothelial cells and fibroblasts, and of matrix metalloproteinases production by synoviocytes, leading to irreversible cartilage damage and defective tissue repair [33, 34]. Furthermore, IL-17 also acts on osteoblasts, prompting bone destruction [35]. Importantly, it exerts an additional activity driving CCL20 (MIP3α) release by epithelial cells [27]. CCL20, acting on the cognate receptor CCR6, is a chemokine that recruits Th17 cells (see below) cells and dendritic cells (DCs) into the inflamed site [36], thus mediating a positive feedback loop for IL-17 amplification.

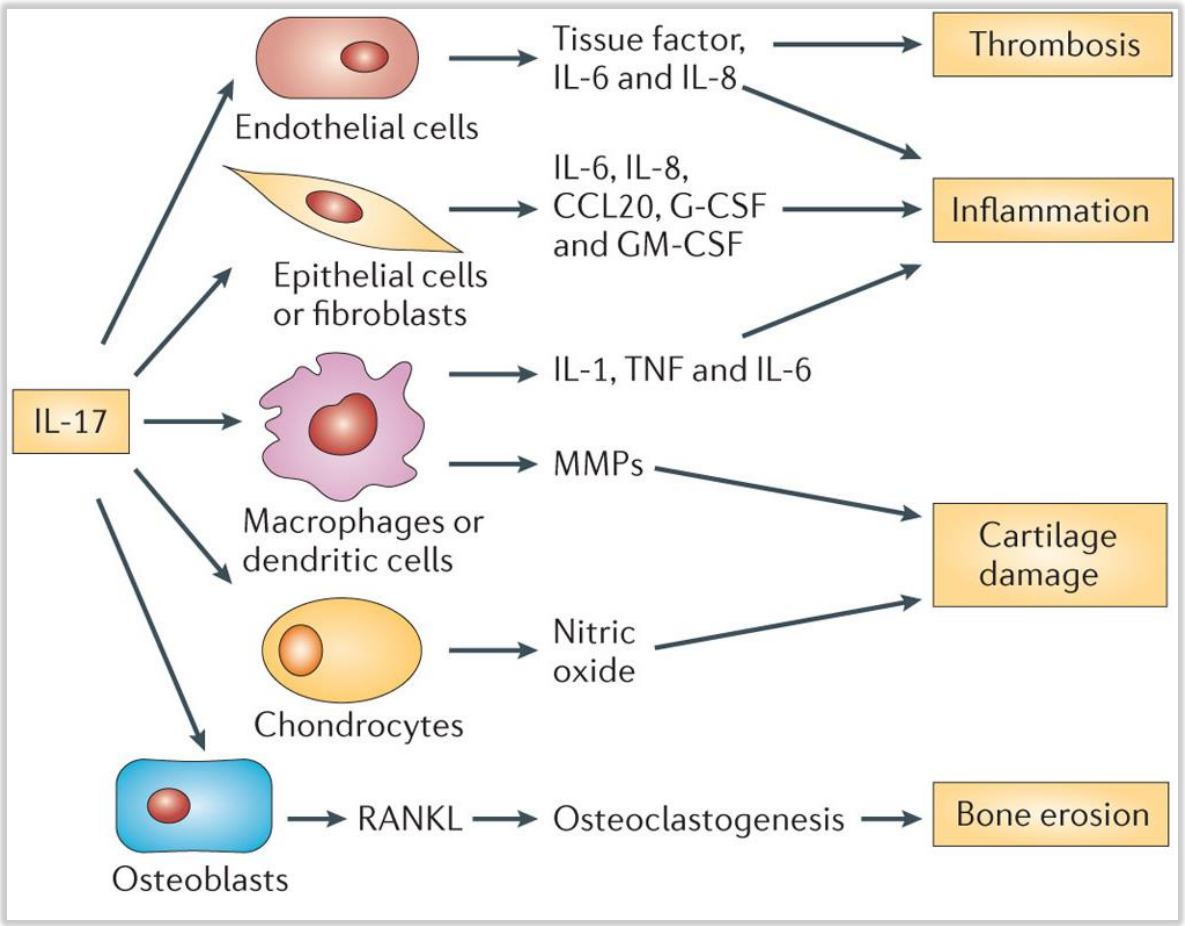


Figure I.2. IL-17 Key Functions. IL-17 induces release of several pro-inflammatory cytokines, chemokines, and metalloproteinases by different cell types including endothelial cells, epithelial cells, macrophages, chondrocytes, and osteoblasts ultimately leading to recruitment and activation of neutrophils and other immunocompetent cells. (P. Miossec & J.K. Kolls, Nature Reviews Drug Discovery, 2012).

1.4 IL-17 cellular sources

A large body of literature identifies IL-17 as a product of a specific CD4+ T helper cell subset, known as Th17 (see below). However, additional cell types within the adaptive and innate immune system have been found to release IL-17 in specific inflammatory conditions [37, 38]. In particular, a minor subset of memory cytotoxic CD8+ T cells, known as Tc17, expressing CCR6 and CCR5 molecules, and producing IL-17, has been identified in the peripheral blood of healthy donors [37]. More fascinating is the observation of the presence of a small subset of circulating and tissue resident T cells, expressing Foxp3 and IL-17 molecules [38-40], whose nature and function are not well defined (see below). Finally, innate immune cells including γδ-T, NK, invariant NK (iNK) and lymphoid-tissue inducer (LTi)-like cells, as well as neutrophils, have also been recognized to secrete IL-17 [41]. In particular, γδT cells have been shown to produce IL-17 upon exposure to IL-23, but not to IL-6 or TGF-β [42]. IL-17-producing γδT cells share specific features with Th17 cells, such as expression of CCR6, ROR-γt and the IL-23 receptor [43]. In addition, NKT cells constitutively express the IL-23 receptor and ROR-γt and rapidly produce IL-17 in an IL-6-independent manner [44]. The contribution of these innate cell populations in mucosal immunity is currently a focus of intensive research.

2 T-helper 17 cells in health and disease

2.1 T-helper cell subsets

T helper cells are essential coordinators of the entire immune system, facilitating the expansion of CD8+ T cells, the responses of B cells, and the recruiting and modulation of different component of the innate immune compartment. After recognition of foreign antigen-derived peptides presented by antigen-presenting cells, naïve CD4+ T cells undergo massive proliferation and differentiate into distinct subsets characterized by the expression of specific transcription factors, as well as of hallmark cytokines, and by specific functions (Figure I.3). Th1 and Th2 subsets were the first to be discovered. Th1 cells express the transcription factor T-bet, secrete interferon (IFN)-γ and protect the host against intracellular infection. Th2 cells express GATA-3, secrete IL-4, IL-5 and IL-13, and mediate the host defense against extracellular pathogens.

In addition to Th1 and Th2, a specialized subset of CD4+ T cells displaying high expression of CD25 and of the master regulatory transcription factor Foxp3 has been identified [45]. Transforming growth factor-beta (TGF-β) is a critical differentiation factor for this subset [46]. Since the main function of CD25+Foxp3+ T cells is to prevent auto-reactive T cells from being activated in the periphery, thereby regulating the immune homeostasis, they were named T regulatory cells (Tregs). There are two populations of CD4+CD25+ Tregs: natural Tregs, arising in the thymus under homeostatic conditions, and adaptive Tregs arising during inflammatory processes such as infections and cancers. Tregs mediated suppression through different mechanisms including cell–cell contact, local secretion of inhibitory cytokines, and local competition for growth factors[47].

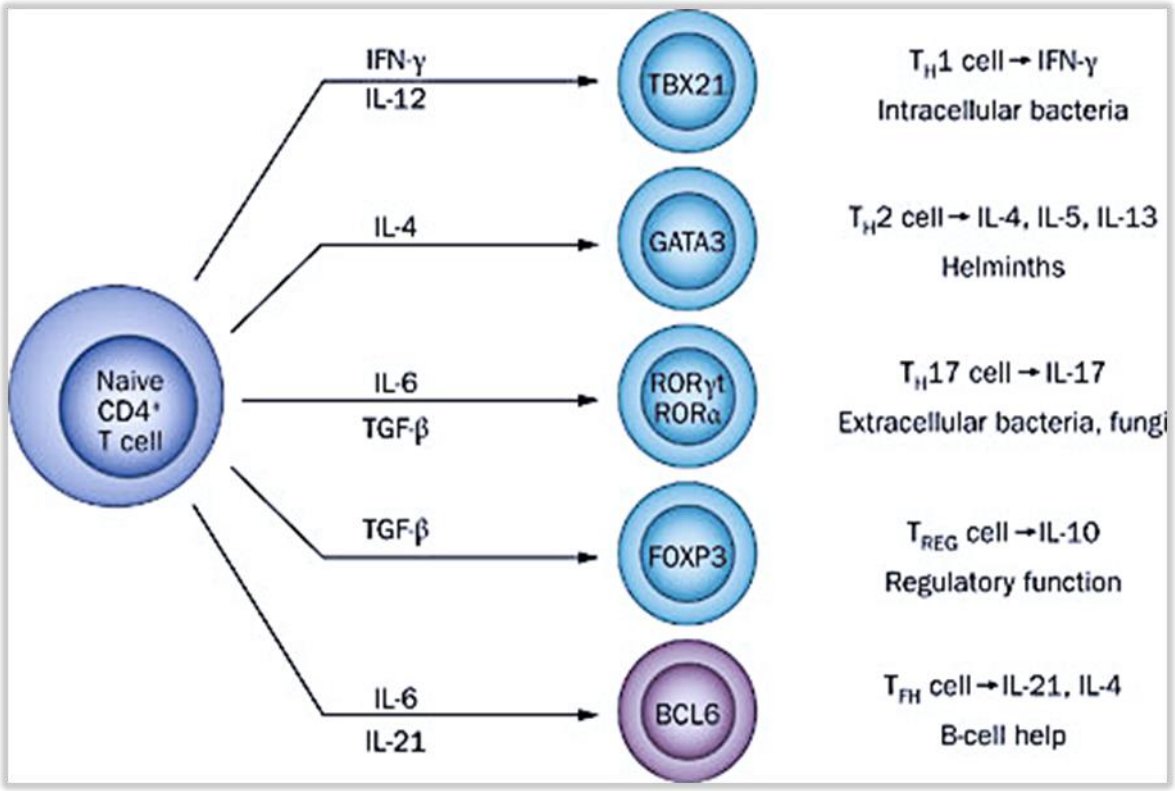


Figure I.3. T-helper subsets. Upon activation by antigen-presenting cells, naive CD4+ T cells undergo clonal expansion and differentiate into different subsets of functional effector Th cells. IL-12 triggers the differentiation of Th1 cells, which are indispensable for cellular immunity and clearing of intracellular pathogens through the production of IFN-γ. IL-4 initiates the differentiation of Th2 cells, which is characterized by production of IL-4, IL-5 and IL-13, and responsible for humoral immunity and the clearing of parasites. IL-6, IL-1 and TGF-β initiate the programming of Th17 cells, which are characterized by the production of IL-17A/F, IL-21 and IL-22. Th17 cytokines can stimulate the expansion and recruitment of neutrophils, and are critical in in host defenses against fungi and some extracellular pathogens. (Joseph E. Craft, Nature Review Rheumatology, 2012).

More recently, additional T-helper subsets have been identified. The Th17 cell population is one of the most intensively studied. Human Th17 cells are characterized by the expression of the transcription factor retinoic acid-related orphan nuclear hormone receptor C (RORC), which is a human orthologous of mouse RORγt, and are crucial for the development of mucosal immunity to extracellular pathogens including bacteria and

fungi. Although Th17 cells share several surface markers with other $CD4^+$ T cell subsets, recent results demonstrate that CCR6+CCR4+CXCR3- memory $CD4^+$ T cells are *bona fide* Th17 cells [48]. In addition, human Th17 cells are characterized by surface expression of the lectin receptor CD161[49] and IL-23 receptor [50]. These cells develop in the thymus (natural Th17 cells) and in the periphery [51, 52], and through their expression of CCR6, are largely trafficking to the mucosal surfaces.

In vitro, human and murine naïve T cells exposed to IL-1β, IL-6, and TGF-β, express RORγt, which in turn leads to the production of IL-17 [53-55]. Furthermore, IL-6 activates T cells to produce IL-21, which in synergy with IL-1β, IL-6, and TGF-β, induces the expression of IL-23 receptor on IL-17 producing cells. Subsequently, IL-23 induces IL-17 and IL-22 production by RORγt+IL-17+ cells, and suppresses the expression of IL-10 and IFN-γ. Thus, IL-23 is essential for Th17 expansion and stabilization [56].

In addition to IL-17, Th17 cells produce other effector molecules including IL-17F, involved in several pro-inflammatory responses [11], IL-22 ,essential for host defense at mucosal surface and tissue repair [57], and IL-21, playing pleiotropic action on myeloid and lymphoid populations, as well on epithelial cells [58]. Furthermore, Th17 cells display a greater degree of context-dependent plasticity, as compared to Th1, Th2 and Tregs. Indeed, depending on the environmental factors which they are exposed to, Th17 can also release additional cytokines including TNF-α, IFN-γ, GM-CSF, IL-4, IL-8, and CCL20. Interestingly, IL-17 producing cells co-expressing T-bet/RORγt, have been detected during inflammatory responses. As previously mentioned, Foxp3+/RORγt+ IL-17-producing cells have also been identified [40, 59]. Interestingly, it has been recently recognized that the development of Th17 and Tregs is reciprocally regulated. For example, at the molecular level, Foxp3 can bind physically to RORγt and RORα, thus antagonizing Th17 generation [60]. In addition, retinoic acid, by enhancing TGF-β

signaling and blocking the expression of the IL-6 receptor, preferentially induces Tregs over Th17 cells [61]. However, the precise stimuli needed for the generation of Foxp3+/RORγt+ IL-17-producing cells are not yet well defined [62, 63]. In addition, it is still unclear whether IL-17+Foxp3+ cells originate from Tregs that have gained IL-17 secretion capacity or from Th17 which have acquired Foxp3 expression.

2.2 Th17: their role in the intestine

Under physiological conditions the gut mucosa is populated by various T cell populations infiltrating epithelium and lamina propria, possibly as a consequence of the continuous exposure of the gut to a vast amount of antigens derived from food and commensal flora [64]. In the colon, intraepithelial lymphocytes are mainly comprised of "unconventional" T cells expressing either TCRαβ or TCRγδ together with the CD8αα homodimer [65]. In addition, the lamina propria contains large numbers of CD4+ T cells, including Th1, Th2, Th17 and Tregs, as well as conventional CD8 T cells, plasma cells, macrophages, dendritic cells, eosinophils and mast cells. In contrast, neutrophils are rare in the healthy intestine, but their number increases during inflammation or infection. Th17 cells preferentially accumulate in the intestinal lamina propria although at steady state their frequency is limited as compared to that of other T cell subsets [66].

Notably, Th17 cells are not present in the intestine of germ-free mice, indicating that microbiota promote their generation. In particular, in mice Th17 cells are attracted and induced by specific commensals, known as segmented filamentous bacteria (SFB) [67]. SFB reside in the intestinal lumen and do not cross the epithelial barrier. It is believed that metabolites derived from SFB can enter the lamina propria and, by inducing intestinal epithelial cells to produce serum amyloid A, can promote DC-mediated Th17 differentiation [67]. Interestingly, it has also been recently found that SFB induce the

generation of SFB- specific Th17 cells in the gut. Indeed, intestinal DCs were found to present SFB-antigens and prime Th17 cell locally [67].

The main function of the "steady-state Th17 cells" is to maintain intestinal immune homeostasis, by providing a crosstalk between the immune system and the intestinal tissue [68]. In addition, Th17 cells are also critical for the mucosal immune responses against certain pathogens, including *C. rodentium*, *Klebsiella pneumoniae, Toxoplasma gondii*, and *S. typhimurium,* mainly through neutrophil recruitment and induction of antimicrobial peptides [33]. Interestingly, a degree of functional diversity may exist between cytokine profiles of "steady state Th17" and "pathogen-specific Th17 cells".

Environmental factors present in the intestine can modulate the cytokine profile of Th17 cells. For example, in the presence of ligands of the aryl hydrocarbon receptor, abundantly present in the intestine, Th17 are activated to produce IL-22 [69], contributing to proliferation and survival of the epithelial cells [70] and to the control of bacterial replication [14, 25]. Furthermore, high levels of IL-12, IL-1β and IL-23 present in the course of colitis, may convert Th17 into highly inflammatory IFN-γ/IL−17 producing cells [71]. On the other hand, when exposed to IL-6 and TGF-β, Th17 cells produce IL-10, which is known for its strong anti-inflammatory activities [72].

Finally, several mechanisms have evolved to avoid deleterious Th17-mediated inflammatory episodes. For example, TGF-β and retinoic acid, released by intestinal epithelial cells and DCs, inhibit Th17 responses by promoting expansion of Tregs, eventually dampening Th17 activation and proliferation [73,74]. In addition, Th17 express IL-10Rα [75], thus being susceptible to IL-10 mediated suppression. When such mechanisms are perturbed, Th17 may favor development of inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis.

2.3 Th17 and diseases

Since their discovery, IL-17 and Th17 cells have been recognized to play an important pathogenic role in several human chronic diseases including IBD, psoriasis, multiple sclerosis, RA, as well as in allergy and rejection of transplanted organs.

The IBD has been initially linked to Th1-mediated immune responses. However, high expression of IL-17 in Crohn's disease and ulcerative colitis suggested involvement of Th17–mediated responses as well [76]. Indeed, it has been found that IL-17 and other inflammatory cytokines such as IL-1, IL-6 and IL-23, might synergistically act to mediate potent local inflammation and tissue damage [33]. However, it is not completely understood which of the effector cytokines released by Th17 cells are critical for their pathogenicity.

In addition, analysis of skin biopsies from patients with psoriasis revealed high levels of IL-17, IL-23, IL-6 and IFN-γ within the psoriatic lesions and high numbers of Th17 and Th1 were found to positively correlate with disease progression [33]. Th17, Th1, and IFN-γ–producing Th17 cells indeed, collaboratively contribute to the pathogenesis of psoriasis by inducing increase keratinocytes proliferation and local inflammation in the psoriatic lesions[33]. Similarly, peripheral Th17 cells are increased in patients with RA. RA is a chronic inflammatory disease characterized by bone and cartilage destruction in which Th17 cells play a pathogenic role by inducing matrix metalloproteinase and proinflammatory cytokine production from synovial fibroblasts[33].

Finally, it has been found that Th17 cells can also participate to the pathogenesis of allergic disorders by recruiting neutrophils and by inducing IgE production by B cells [33].

However, additional studies concerning the pathogenic role of IL-17 in these diseases are needed to better predict patients' responses to potential IL-17-targeted therapys.

3 IL-17 and Th17 in cancer

3.1 Cancer immunosurveillance

The potential of malignant cells to proliferate, evade apoptosis and invade tissues is largely regulated by the tumor microenvironment that is composed of extracellular matrix and of non-neoplastic host cells, including mesenchymal, vascular endothelial and immune cells. More specifically, cells from the innate and adaptive immune compartments, including macrophages, neutrophils, mast cells and lymphocytes, mediate inflammatory responses potentially leading to tumor progression or eradication [77, 77].

The contribution of the immune system to cancer growth has been a matter of debate for many years. It is now well recognized that the immune system can shape tumor immunogenicity[78]. It has been indeed observed, that tumors originated in immunocompetent mice developed at higher rate than tumors originated from immunocompromised mice, when transplanted into syngeneic immunocompetent mice [79, 80]. This finding indicates that tumors are shaped by the immune system. This process, defined as cancer immunoediting, involves three different dynamic phases: elimination, equilibrium and escape. A series of studies in mice deficient for critical components of the immune system have elucidated the fundamental role of so called cancer immunosurveillance (elimination phase) in the control of spontaneous, transplantable, virus- or carcinogen-induced tumors [79, 81, 82]. When immune cells fail to eradicate the tumor, an equilibrium phase can be observed in which the immune system only constrains tumor cell growth. Finally, tumors escape immune responses and progress.

It is now evident that the immune system can exert a tumor-suppressive function by eliminating nascent transformed cells and a tumor-promoting action by exerting a selective pressure on tumor cells and by providing a tumor-favorable microenvironment.

So far it has been shown for instance that cytotoxic T lymphocytes (CTLs), Th1 cells, DCs and type 1 macrophages mediate anti-tumor immunity [83, 84]. In contrast, Th2, type 2 macrophages, and Tregs contribute to tumor progression [85-89]. However, the role played by other immune cell subsets in tumor immunity is not completely understood. Several immune cell types can display both tumor-promoting and tumor- suppressive abilities. This is the case of IL-17 producing cells, whose functions in the context of tumor immunology remain controversial.

3.2 Role of IL-17 and Th17 cells in experimental tumor models

Studies investigating the role of IL-17 in mouse tumor models have reported contradictory results depending on the tumor type and the animal model used (Table I.1 and Figure I.4).

Table I.1. IL-17/Th17 in experimental tumor models

TUMOR TYPE	MOUSE MODEL			
	IMMUNO-DEFICIENT	IMMUNO-COMPETENT	IL-17 DEFICIENT	IL-17R DEFICIENT
Human IL-17 transfectant	PRO [90]			
Mouse immunogenic IL-17 transfectant	NONE [91] NONE [92]	ANTI [91] ANTI [92]		
Mouse non immunogenic IL-17 transfectant		PRO [32]		
Mouse immunogenic				PRO [93]
Mouse non immunogenic		ANTI* [94] ANTI* [95]	PRO [96] ANTI [97] ANTI [94]	PRO [93]
Mouse spontaneous (APC+/-)			PRO [98]	

-PRO = pro-tumoral effect ; -ANTI= anti-tumoral effect
* adoptive transfer tumor-specific Th17

Tartour et al. have shown that IL-17 transfected human cervical tumor cell lines injected in nude mice grow faster as compared to the parental tumor[90]. On the contrary, no difference in tumor growth was observed when IL-17 expressing immunogenic hematopoietic mouse tumors were transplanted in nude mice [91]. However, tumor growth was significantly inhibited when the same cell line was grafted into immunocompetent mice [91]. Furthermore, in this work, IL-17 increased the generation of specific cytolytic CD8+ T cells, thus establishing a tumor protective immunity. Interestingly, weakly immunogenic fibrosarcoma and colon adenocarcinoma cell lines expressing IL-17, promoted tumor growth also in immunocompetent recipients [32]. Thus, the impact of IL-17 appears to depend on the immunogenicity of the tumor type and on the host immune status. However, it has to be remarked that a constantly induced release of IL-17 by tumor cells does not resemble physiological conditions.

In different experimental settings, IL-17 or IL-17R deficient mice were also used; in these studies, depending on the tumor type injected into the immunocompetent animals opposite results were obtained [96,93,97] . Importantly, Martin-Orozco has shown that IL-17-deficient mice bearing a poorly-immunogenic B16-F10 melanoma, were more susceptible to developing melanoma metastasis in the lung [94]. In this model it was proved that adoptive T cell therapy with tumor-specific Th17 cells prevented tumor development, and caused a remarkable activation of tumor-specific CTLs, which were necessary for the anti-tumor effect. In addition, tumor specific Th17 cells have been shown to mediate melanoma eradication more effectively than Th1 cells, upon adoptive transfer into tumor-bearing mice [95]. Surprisingly, Th17-mediated tumor responses were found to largely depend on IFN-γ. Indeed, the effects of Th17-polarized cells were completely abrogated by the IFN-γ neutralization. Interestingly, it has been lately recognized, that Th17 cells are long-lived cells capable of maturational plasticity, thus giving rise to Th1 effector

progeny *in vivo*, a process required to effectively eliminate tumors [99].

Thus, differential host immune status and different tumor immunogenicity can only in partially explain the discrepancies observed. The latter finding suggested indeed, that the mere evaluation of IL-17 is limited and might not mirror the function of the entire Th17 subset.

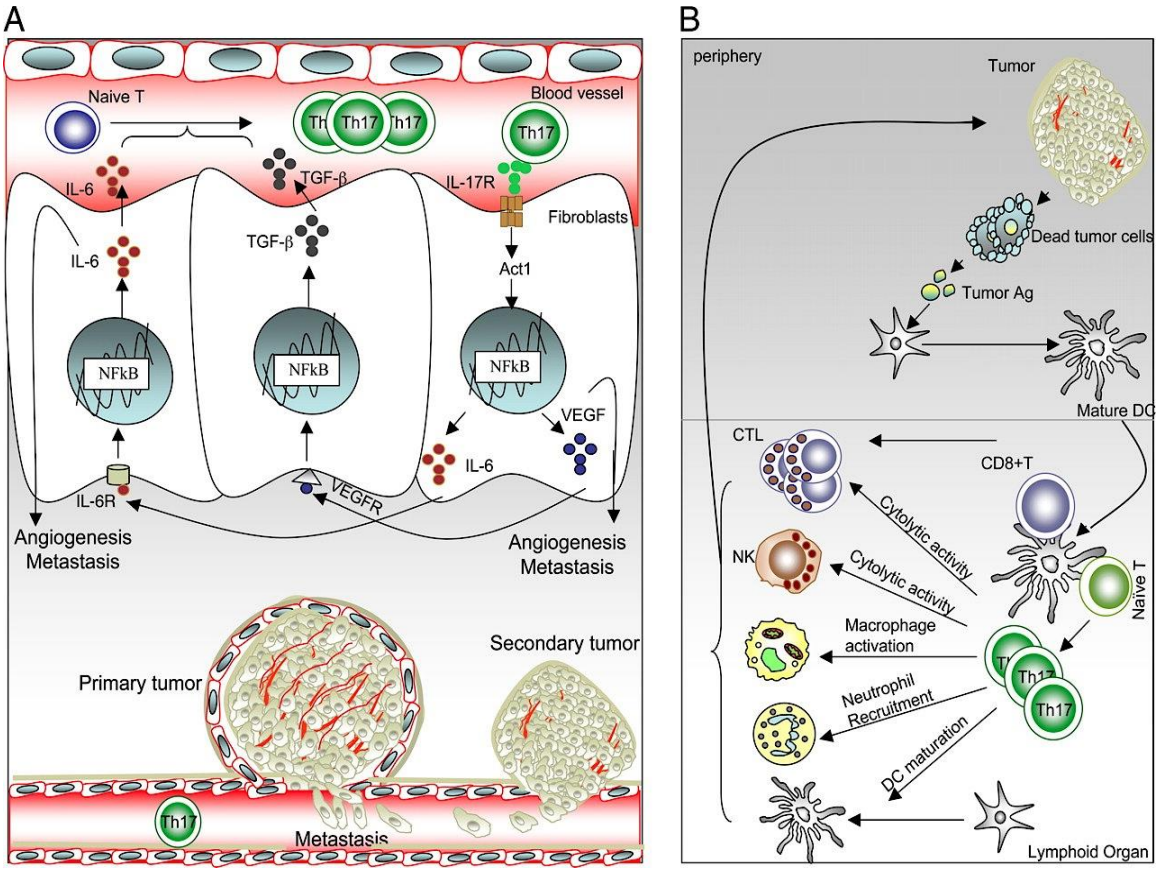


Figure I.4. Pro- tumor and anti-tumor function of IL-17. A. *Protumor function*. IL-17 signaling induces the production of both proangiogenic and protumorigenic factors. IL-6 and TGF-β further amplify Th17 differentiation and create a sustained chronic inflammatory state that may favor tumor growth and metastasis. **B**. *Antitumor function*. Differentiated Th17 cells induce recruitment and potentiate functions of both CTLs and innate cells such as NK cells and neutrophils, ultimately leading to tumor growth inhibition. (Gopal M. et al, The Journal of Immunology. 2009).

3.3 IL-17 and Th17 cells in human cancers

IL-17 producing cells have been found in many different human malignancies, including

lymphoma [100], myeloma [101], breast cancer [102], colon cancer [4, 7], gastric cancer [103, 104], hepatocellular cancer [105], melanoma [94, 102], ovarian cancer [4, 106] , pancreatic cancer [4], and prostate cancer [5]. Tumor infiltration by IL-17+ cells has been reported to correlate with either better or worse prognosis, depending on tumor the type. In particular, in ovarian, prostate and lung cancer, a positive association between tumor infiltration by IL-17+ cells and prolonged patient survival has been observed [3-5]. In contrast, in hepatocellular carcinoma and in CRC, IL-17 expression has been shown to predict unfavorable clinical outcome [6, 105,7]. Thus, the impact of IL-17 producing cells in human cancer also remains controversial.

Tumor-infiltrating IL-17 positive cells isolated from different human tumor types resemble the classical phenotype of the Th17 subset, expressing CXCR4, CCR6, CD161 and, the gut homing molecules CD49 [4, 43, 95]. In ovarian cancer, infiltrating Th17 express the memory phenotype CD45RO, but low levels of the activation markers CD25 and HLA-DR, an observation suggesting that they may not be conventional effector cells [4, 107]. In addition, human ovarian cancer-derived Th17 cells express high levels of IL-2, GM-CSF, and IFN-γ, but negligible levels of IL-10. Interestingly, IL-17 and IFN-γ synergistically induced the secretion of CXCL9 and CXCL10 chemokines by tumor cells, potentially attracting effector T cells at the tumor site [4]. Human Th17 clones, derived from melanoma, breast, and colon cancers, were also found to release other cytokines in addition to IL-17, including IL-8, TNF-α, TGF-β and, low levels of IL-10 [4, 108]. The different cytokine profiles suggested heterogeneity of tumor-infiltrating Th17 cells. A more precise phenotypical and functional characterization of these cell subsets could be useful to better depict the final impact of Th17 cells in different tumor types.

4 Human colorectal cancer

4.1 Epidemiology and genetics of colorectal cancer

CRC is a major worldwide health problem owing to its high prevalence and mortality rates. In Europe, CRC is the second cause of cancer death [109].

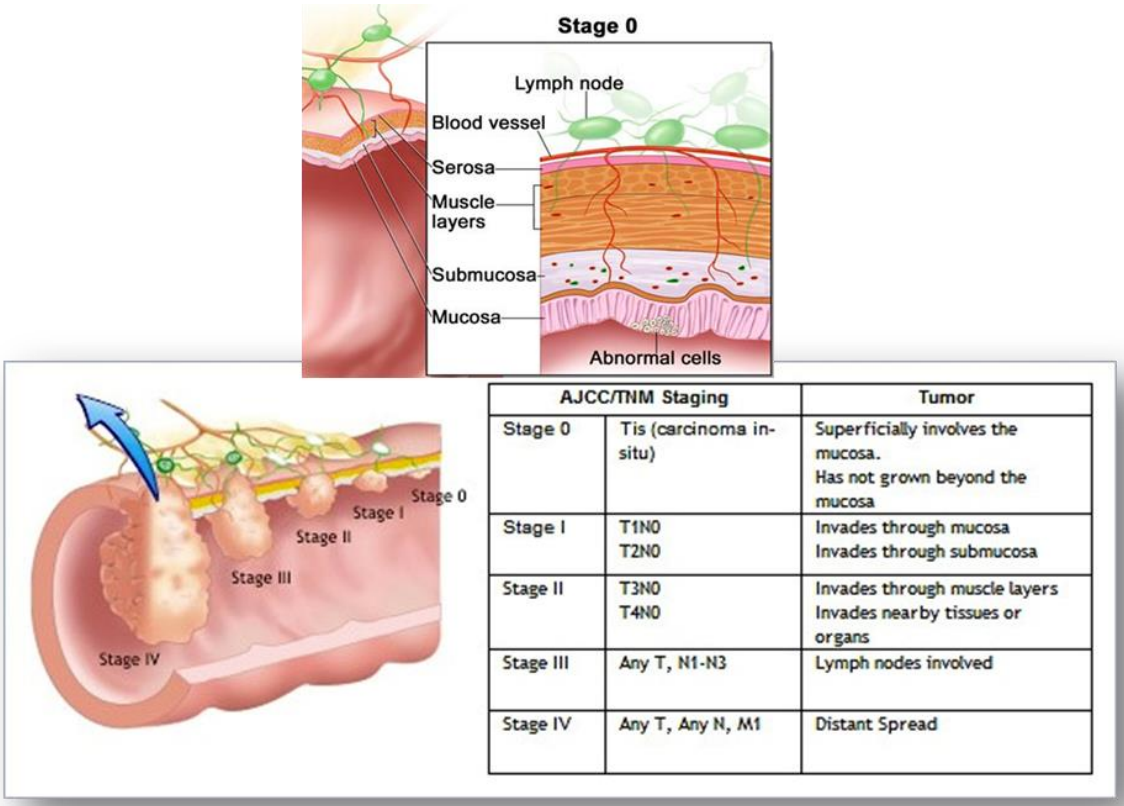
CRC arises as the result of the accumulation of acquired genetic and epigenetic changes that transform normal glandular epithelial cells into invasive adenocarcinoma. The steps leading to transformation of the normal epithelium into benign neoplasia (adenoma), followed by invasive carcinoma and eventually metastatic cancer are described in the classic tumor progression model proposed by Fearon and Vogelstein[110]. Our understanding of the molecular pathogenesis has advanced considerably since this model was proposed.

Most cases (88%-94%) of CRC are sporadic and arise through the chromosomal instability pathway characterised by aneuploidy, allelic losses, amplifications, translocations and mutation of *APC, KRAS* and *TP53,* whereas 5-10% of CRC are hereditary. Within the latter group, the two main forms are the hereditary nonpolyposis colorectal cancer (HNPCC) and the familiar adenomatous polyposis (FAP). HNPCC is an autosomal dominant disorder caused by germline mutations of mismatch repair genes (MMR). Tumors that arise in this setting have molecular characteristics called microsatellite instability, defined as frequent mutations in short repeated DNA sequences (microsatellites). FAP is also an autosomal dominant disease, with a germline mutation in the adenomatous polyposis coli (APC) gene. Mutation of the APC gene also occurs in about 85% of sporadic CRC. In addition, many other tumor suppressors and oncogenes may be involved. A 15% of sporadic CRC are characterized by mutations of MMR genes

[111]. Epigenetic mechanisms are also involved in the genesis of CRC. For instance, the so called "CpG island methylator phenotype" (CIMP) identifies a group of sporadic CRC characterized by microsatellite instability consequent to the methylation of the MMR gene hMLH1[112].

4.2 CRC prognosis

A prognostic factor is defined as a parameter evaluable at diagnosis, potentially associated with clinical outcome. The anatomic extent of the tumor burden has been the most important prognostic factor in all solid tumors including CRC[113]. The current staging system (AJCC/UICC-TNM classification) integrate data on tumor burden (T), presence of cancer cells in draining and regional lymph nodes (N), and evidence of distant organ metastases (M) (Figure I.5) [113]. TNM classification is currently used for prognostication of newly diagnosed CRC cases and to pose indications for eventual adjuvant therapy [114]. Furthermore, other tumor related features, such as venous and lymphatic invasion, tumor grade and budding, have been recognized as additional prognostic factors. Although patient survival is largely dependent on TNM stage, this system does not precisely predict clinical outcome. Indeed, patients with early stage CRCs still have approximately a 20-30% risk of recurrence [115]. Notably, these patients are not generally treated with postoperative chemotherapy, which is currently recommended only for patients in stage III [116]. On the other hand, adjuvant treatment in all patients with early disease stage would be inappropriate, due to potential toxicity and related social costs [116, 117] . The possibility to identify patients in early disease stage, which may be at risk of recurrence, represents therefore a crucial clinical need.



AJCC/TNM Staging		Tumor
Stage 0	Tis (carcinoma in-situ)	Superficially involves the mucosa. Has not grown beyond the mucosa
Stage I	T1N0 T2N0	Invades through mucosa Invades through submucosa
Stage II	T3N0 T4N0	Invades through muscle layers Invades nearby tissues or organs
Stage III	Any T, N1-N3	Lymph nodes involved
Stage IV	Any T, Any N, M1	Distant Spread

Figure I.5. TNM staging classification. The bowel wall is composed by four different layers including the mucosa, the submucosa, the muscularis propria and the serosa. In stage 0 (carcinoma in situ) abnormal cells are present in the mucosa of the colon wall. The TNM (tumor, node, metastasis) system classifies cancer with a letter and a number to describe the tumor, the node, and the metastasis. Once the values for T, N, and M have been determined, they are combined, and an overall stage is assigned.

4.3 The immune contexture

During the last decade, tumor infiltration by specific immune cell subsets has been recognized as a key factor in determining patient clinical outcome. The tumor immune infiltrate includes macrophages, DC, mast cells, NK cells, neutrophils, naive and memory lymphocytes, B cells, and effector T cells. Analysis of the *in situ* immune components and their organization has revealed a large heterogeneity between tumor types and also a

broad patient-to-patient diversity. In particular in CRC, nature, functional orientation, density, and location of adaptive immune cells (defined as the "immune contexture"), have been recognized to have a prognostic value superior to that of the TNM-classification [2]. The parameters that establish the immune contexture (Figure I.6A) comprise the density of CTLs and memory T cells (CD45RO+), their location at the tumor center and invasive margin, combined with the quality of tertiary lymphoid structures, and additional functionality factors such as Th1-related factors (IFN-γ, T-bet, IRF1, IL-12), chemokines (CX3CL1, CXCL9, CXCL10, CCL5, CCL2), adhesion molecules (MADCAM-1, ICAM-1, VCAM-1) and cytotoxic factors (granzymes, perforin, granulysin) [2, 7]. Based on the immune contexture, a simple and powerful classification, the 'Immunoscore', has been derived [118]. The Immunoscore (Figure I.6B) is based on the numeration of two lymphocyte populations (CD3/CD45RO, or CD3/CD8 or CD8/CD45RO) quantified within the tumor center and the invasive margin. A high immunoscore, corresponding to a strong lymphocytic infiltration, is associated with a better clinical outcome in human CRC [118, 119] .

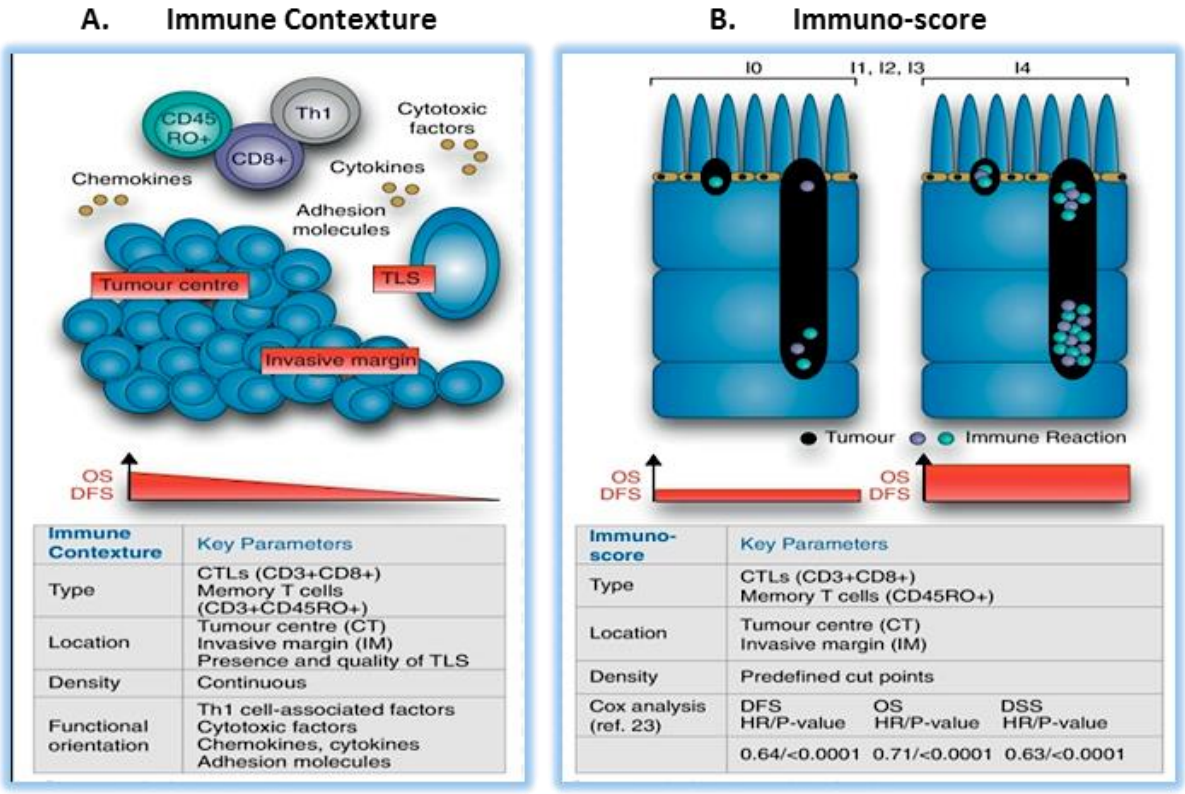


Figure I.6. Immune Contexture and Immuno-Score. A. Immune Contexture. Key parameters defining the immune contexture including type, location, density and functional orientation of the immune infiltrate. **B.** Immunoscore, illustrating the importance of the immune reaction regardless of tumor burden. Scoring system from immune-score 0 (I0), which corresponds to low densities of CTLs and memory T cells in both regions (CT, IM), to immune-score 4 (I4), corresponding to high densities of both cell populations in both regions. CT, tumor center; CTL, cytotoxic T lymphocyte; TLS, tertiary lymphoid structures; DSF, disease free survival; DSS, disease specific survival; IM, invasive margin; OS, overall survival.

4.4 Th17 and Treg cells in CRC

Whereas the infiltration by activated CD8+ memory T cells and Th1 cells within CRC strikingly correlates with favorable prognosis, the association between other subsets of T cells and clinical outcome is still under debate. In particular, the most intriguing and complex issue concerns the role played by Tregs and Th17 cells. Indeed, Tregs are known to suppress tumor-specific T cell responses, and a high frequency of intratumoral Foxp3+

cells is predictive of poor prognosis in several tumor types. Surprisingly in CRC, a positive correlation between the infiltration by Foxp3+ cells and good prognosis was detected [120, 121]. The fact that Tregs apparently play a positive role in CRC immunity has been emphasized as the "CRC paradox". This apparent contradiction could be explained by the peculiarity of the gut microenvironment. Here Tregs may play a favorable role in as much as they might damp the immune reaction induced by gut microorganisms [122]. Alternatively, in the CRC tissue Tregs may lose their suppressive capacity, and acquire pro-inflammatory function [122]. On the other hand, it has been shown that the transcription factor Foxp3 can be upregulated on activated effector T cells. Thus, the Foxp3 marker may fail to identify a population of truly suppressive cells [120]. As discussed above, also the impact of Th17 in CRC is not a black and white picture and needs additional and more precise investigation. Increased IL-17 mRNA levels and higher numbers of Th17 cells in primary CRC tissues, as compared to healthy colonic mucosa, have been described [102]. Importantly, high infiltration by IL-17+ cells has been shown to correlate with unfavorable prognosis [6, 7]. However, a comprehensive analysis of the prognostic relevance of IL-17 in CRC on a large patient cohort is still missing. Moreover, phenotypes and cytokine profiles of IL-17-producing cells remain to be fully characterized.

II. RATIONALE AND AIMS OF THE STUDY

1 Rationale

Tumor-infiltrating IL-17 positive cells have been found in several types of solid malignancies [1, 123] but their role in cancer remains debated. In mouse tumor models, transfection of the IL-17 gene into tumor cells has been shown to result in faster tumor development due to autocrine or paracrine induction of pro-angiogenic factors [32, 90, 96, 124]. Consistently, reduced tumor growth in IL-17- or IL-17R-deficient mice has been reported [93, 96, 98]. However, endogenous or ectopically expressed IL-17 has also been shown to favor the development of specific anti-tumor immunity, ultimately leading to tumor regression [91, 92, 94, 97]. Furthermore, IL-17-producing tumor-specific CD4+ or CD8+ T cells, adoptively transferred in tumor-bearing mice, prevented the development or mediated the eradication of established melanomas, more effectively than IFN-γ-producing Th1 cells [91, 94, 95, 125]. In humans, tumor infiltration by IL-17+ cells has been reported to associate to better or worse prognosis, depending on the tumor type [107, 123]. Thus, the effects played by IL-17 within the tumor microenvironment and its final impact on clinical outcome remain to be fully understood.

CRC is a leading cause of cancer-related death. Tumor infiltration by certain immunocompetent cell subsets, including cytotoxic CD8+ T cells [2, 10], CD16+ myeloid cells [9] and, unexpectedly, of Foxp3+ Tregs [120, 121], has been recognized to correlate with improved prognosis. In contrast, in a few studies based on the analysis of limited numbers of cases, high infiltration by IL-17+ cells has been reported to predict unfavorable clinical outcome [6, 7]. However, a comprehensive analysis of the prognostic relevance of IL-17 on a large cohort of CRC patients is still missing. Moreover, phenotype and cytokine profiles of IL-17-producing cells remain to be fully characterized.

2 Aims of the study

In this work we aimed to investigate:

1) The prognostic significance of tumor infiltrating IL-17-producing cells in a large cohort of CRC patients,
2) The phenotype and the cytokine profile of CRC infiltrating IL-17+ cells,
3) The role played by infiltrating IL-17+ cells in the CRC microenvironment.

III. MATERIALS and METHODS

1 Clinical specimen collection and processing

Clinical specimens were collected from consenting patients undergoing surgical treatment at Basel University Hospital, Kantonsspital Olten, Kantonspital St. Gallen, Ospedale Civico di Lugano. Tumor or control tissue fragments were snap frozen for RNA extraction or treated by enzymatic digestion in order to obtain single cell suspensions. Briefly, tissues were minced, and digested in RPMI 1640 medium supplemented with 2 mg/ml collagenase IV (Worthington Biochemical Corporation) and 0.2 mg/ml DNAse I (Sigma-Aldrich) for 1 hour at 37°C. Single cell suspensions were then filtered through cell strainers (100, 70 and 40 μm diameter, sequentially) and used for flow cytometric analysis.

2 Cell lines

The human CRC cell line HT29 was purchased from European Collection of Cell Cultures (ECACC) and maintained in McCoy's 5A medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, GIBCO), GlutaMAX-I, and kanamycin (GIBCO). The endothelial HMEC cell lines was kindly provided by Prof. T. Resink, University of Basel, Switzerland, and cultured in EBM-2 medium (Lonza).

3 Tissue microarray

The TMA used in this work was constructed by using 1420 non-consecutive primary CRC and 71 samples of normal colonic mucosa, as previously described [126]. Formalin-fixed, paraffin-embedded tissue blocks of CRC resections were obtained and one tissue cylinder with a diameter of 0.6 mm was punched from the tumor center of each donor block and brought into one recipient paraffin block (3×2.5 cm) using a home-made semi-

automated tissue array. One punched per case was used. Clinical information, listed in Table III.1, was retrieved from patient records. The use of material in this study has been approved by the local ethical committee.

Table III.1: Patient characteristics (n=1420)

Features		Frequency n (%)
Age (yrs)	Mean (range)	70 (36-96)
Tumor diameter (mm)	Mean (range)	49.1 (4-170)
Gender	Female	614 (51.9)
	Male	570 (84.1)
Tumor location	Left-sided	763 (65.2)
	Right-sided	407 (34.8)
Histologic subtype	Mucinous	97 (8.2)
	Non-mucinous	1088 (91.8)
pT stage	pT1-2	230 (19.8)
	pT3-4	932 (80.2)
pN stage	pN0	608 (53.4)
	pN1-2	531 (46.6)
Tumor grade	G1-2	1005 (86.6)
	G3	155 (13.4)
Vascular invasion	Absent	848 (73.1)
	Present	312 (26.9)
Mismatch repair status	Proficient	990 (83.5)
	Deficient	195 (16.5)
Local recurrence	Absent	262 (58.7)
	Present	184 (41.3)
Distant metastasis	Absent	370 (81.9)
	Present	82 (18.1)
Post-operative therapy	None	355 (79.4)
	Treated	92 (20.6)
Survival rate	5 year (95%CI)	57.4 (54-60)

4 Immunohistochemistry

To assess IL-17+ cells, the whole TMA (n= 1420) was stained with a goat polyclonal anti-human IL-17 (AF-317-NA, R&D Systems). Due to the entire or partial loss of tissues area within some tissue punches IL-17 expression was evaluated on a total of 1148 CRC cases (training cohort) and 39 healthy mucosa samples. A randomized subgroup of cases (validation cohort), including 661 CRC and 24 healthy mucosa cases, was also stained with a rabbit polyclonal anti-human IL-17 (H-132, Santa Cruz Biotechnology), and used as validation group. The avidin-biotin complex method was used for IL-17 immunohistochemistry. Tissue sections were deparaffinized, alcohol-rehydrated, subjected to heat-induced antigen retrieval and incubated overnight with anti-IL-17 (1:100 in PBS). Sections were rinsed in PBS and visualized using standard techniques for labeled avidin-biotin immuno-peroxidase staining (Vectostain ABC kits, Vector Laboratories) and antigens were visualized as previously described [127]. The immune reaction was revealed with 0.06 mmol/l DAB (Dako) and 2 mmol/l hydrogen peroxide. Negative controls underwent the same treatment with the primary antibody omitted.

Protein markers were scored by three independent observers by analyzing the number of positive cells per punch. No image analysis software was used. The total number and localization (intraepithelial or stromal) of immune-reactive cells within the tumor microenvironment was evaluated. Cases were classified in four groups, according to the number of positive cells per punch (0, 1-10, 11-50, >50). Protocols for staining of CD8, CD16 and MPO have been previously reported [8-10].

5 Flow cytometry and cell sorting

For phenotypic characterization of IL-17-producing cells, peripheral blood mononuclear cells (PBMC) from healthy donors or CRC patients, and single cell suspensions obtained from freshly excised clinical specimens of CRC and corresponding tumor-free colonic mucosa were incubated for a total of 5 hours with phorbol myristate acetate (PMA, 50 ng/ml) (Sigma-Aldrich) and Ionomycin (1 μg/ml) (Sigma-Aldrich) at 37°C. After 3 hours of PMA/ Ionomicin stimulation, Brefeldin A (5 μg/ml) (Sigma-Aldrich) was added for additional 2 hours. Cells were then fixed with the fixation buffer (fixation/ permeabilization kit, eBiocience) for 30 minutes at room temperature (RT) according to standard protocol, and surface stained with different fluorochrome-conjugated anti-human CD3 (clone SK7), CD4 (clone SK3), CD8 (clone SK1), CD16 (clone 3G8), CD56 (clone B159), αβTCR (clone WT31), γδTCR (clone B1), HLA-DR (clone SK10), CD45R0 (clone UCHL1), CCR6 (clone 11A9) (all from BD Biosciences), V alpha 24 J alpha 18 TCR (clone 6B11, eBioscience), CD66b (clone ASL-32, BioLegend) antibodies, for 15 minutes at 4 °C. Cells were then permeabilized with perm buffer (fixation/ permeabilization kit, eBiocience) for 10 minutes at RT, and stained intracellularly with anti-human IL-17 (clone 64DEC17, eBioscience), IFN-γ (clone 25723.11, BD Biosciences), TNF-α (clone 340511, BD Biosciences), IL-22 (clone 142928, R&D), IL-21 (clone 3A3-N2, eBioscience), IL-8 (clone AS14, BD Biosciences) antibodies or GM-CSF (clone BVD2-21C11, BioLegend) and Foxp3 (clone 206D, BioLegend) specific antibodies for 30 minutes at RT.

Chemokine receptor expression on CD8+ T cells was evaluated on PBMC and freshly excised clinical specimens from CRC patients by surface staining with anti-human CCR3 (clone 5E8) , CCR5 (clone 2D7), CCR6 (clone 11A9), CXCR3 (clone 1C6), and CXCR4 (clone 12C5) antibodies (all from BD Biosciences), for 15 minutes at 4°C. Stained cells

were analyzed by FACSCalibur flow cytometer (BD Biosciences) and data analyzed with FlowJo software (Tree Star).

In order to isolate tumor, endothelial and mesenchymal cells form CRC samples, single cell suspensions obtained from tissue digestion of freshly excised CRC samples (see material a and methods paragraph 1) were stained with specific antibodies against tumor cells (anti-EpCAM antibody, clone EBA-1), endothelial cell (anti-CD31 antibody, clone WM59) and mesenchymal cells (anti-CD90 antibody, 5E10) (all from BD Bioesciences), for 15 minutes at 4°C. The purity of the different isolated cell populations was > 97%. In some experiments mesenchymal cells were purified by plastic adherence, in alternative to cell sorting.

6 T cell expansion and cloning

T lymphocytes isolated from CRC were stimulated with 1μg/ml of PHA (Sigma-Aldrich) in the presence of autologous feeder layers, and expanded in medium supplemented with 100UI/ml IL-2 (Roche) and 5% of pooled human AB serum (provided by Blutspendenzntrum Beider basel, Basel University Hospital) for 20 days. Percentages of Th17 and Th1 cells were determined by FACS analysis after intracellular staining for IL-17 and IFN-γ, respectively. Th17 and Th1 cell clones were then generated by limiting dilution from sorted CXCR3-CCR4+CCR6+ and CXCR3+CCR4-CCR6-, respectively, as previously described[128] . Supernatants of Th1 and Th17 clones were collected after overnight T cell stimulation with plate bound anti-CD3 (10μg/ml, clone OKT3, eBioscience) and soluble anti-CD28 antibodies (1μg/ml, clone CD28.2, BD Biosciences).

7 Real-time reverse transcription PCR assay

Total RNA was extracted from stored CRC tissues or CRC-sorted cell populations using RNeasy® Mini Kit protocol (Qiagen), treated by DNAse I (Invitrogen) and reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen). Quantitative Real-Time PCR was performed in the ABI prism™ 7700 sequence detection system, using TaqMan Universal Master Mix, No AmpErase UNG (both from Applied Biosystems). Commercially available primer sequences specific for human *IL-17A* (Hs99999082_m1), *CCL3* (Hs00234142_m1), *CCL5* (Hs00982282_m1), *CCL20* (Hs00171125_m1), *CXCL9* (Hs00171065_m1), *CXCL10* (Hs99999049_m1) and *CXCL12* (Hs00171022) (all from Applied Biosystems) were used.

8 Migration assay

$CD8^+$ T cells and neutrophils were sorted from PBMC of healthy donors by magnetic microbeads (Miltenyi Biotec) and EasyStep enrichment kit (StemCell Technologies), respectively, according to manufacturer's instructions. The purity of both cell populations was > of 98%, as confirmed by flow cytometry. Chemotaxis assays were performed using 96-well transwell plates with 5-µm pore size membranes (Corning Costar) [129]. Supernatants from Th17 clones, or from HMEC cells, untreated or exposed to rIL-17 (50 ng/ml) or to Th17 clone supernatants for an overnight period, were added to the lower chamber. CD8+ T cells and neutrophils (1.5×10^4 / chamber) were placed in the upper chamber and allowed to migrate for 90 min at 37°C. Extent of cell migration into the lower chamber was quantified by flow cytometry and expressed as migration index (numbers of cells migrated towards supernatants / number of cells migrated towards control medium). In specific experiments, 10µg/ml of anti-IL-8 antibodies (R&D Systems) were added to Th17 supernatants 1h prior to chemotaxic assays. Depletion of

CCL5 and/or CCL20 from Th17 derived supernatants was obtained by specific capture antibodies (R&D Systems). Efficiency of depletion was evaluated by ELISA.

9 ELISA

Supernatants of Th1 and Th17 clones were collected after an overnight T cell stimulation as describe in paragraph 6. Supernatants of HMEC cells, untreated or exposed to rIL-17 (50 ng/ml) or to Th17 clone supernatants, were collected after overnight period. Cytokine and chemokine contents in culture supernatants were assessed by ELISA using CCL3, CCL5, CCL20, CXCL9, CXCL10, CXCL12 DuoSet ELISA (all from R&D Systems) according to standard protocols.

10 Engineered tumor-like tissue for CD8+ T cell migration

A perfusion bioreactor system, previously developed for cell seeding and culture on scaffolds [130], was used for the development of tridimensional (3D) tumor tissue. Tumor cells from the established CRC cell line HT29 were injected (1×10^6 cells /scaffold) and perfused at 400 μm/sec through a collagen scaffold (Ultrafoam Avitene Collagen Hemostat®, Davol Inc). After 24 h (cell seeding phase), the superficial velocity was reduced to 100 μm/sec. At day 7 CRC derived Th17 cells (5×10^6/scaffold) were injected and perfused overnight at 400 μm/sec. Th17 cells were then left untreated or activated by adding CytoStim (Miltenyi Biotec) to the medium. After 3h of activation the system was extensively washed and the perfusion was stopped afterwards. CD8+CD45RO+ were then injected and allowed to spontaneously migrate for an overnight period. The engineered tissue was then enzymatically digested in order to obtain single cell suspensions to be used for flow cytometric analysis. In addition, paraffin embedded sections or cryosections

were collected and used for hematoxylin and eosin staining (H&E), and immunofluorescence analysis.

11 Histological and immunofluorescence analysis

For morphological evaluation, paraffin sections (5 μm) were stained with H&E and analyzed by light microscopy. Cryosections (10 μm) were cut and fixed in methanol. Sections were incubated with rabbit polyclonal anti-human CD8 antibody (ab4055 Abcam) (1/100 in PBS + 0.3% Triton + 1% BSA) and mouse monoclonal anti-human CD4 antibody (MO716 DakoCytomation) (1/100 in PBS + 0.3% Triton + 1% BSA), for an overnight period at 4°C, followed by secondary species-specific Alexa Fluor 488- or Alexa Fluor 547 - conjugated antibodies (Invitrogen) (1/800 in PBS) for 55 minutes at RT. Nuclei were counterstained with DAPI (Invitrogen) (1/100 in PBS) for 5 minute a RT. Sections were examined under an Olympus BX61 fluorescence microscope (Olympus Switzerland) and images captured with 10x and 20x magnification using a digital camera and AnalySIS software (Soft Imaging System GmbH).

12 Statistical analysis

CRC cases were classified into four categories according to numbers (0; 1-10; 11-50; >50) of IL-17+ cells/punch. Specific cut-off values for CD8, CD16, and MPO (10, 50, and 60, respectively) were obtained by ROC curve analysis, as previously published [8-10] . Chi-Square test was used to determine the correlation between dichotomous variables. Survival analysis was depicted by the Kaplan-Meier method and compared with log rank test. Statistical analyses were performed using R (Version 2.15.2, www.r-project.org). Differences in frequencies of IL-17+ cells within PBMC of healthy donors or CRC

patients, and between tumor and control tissues were evaluated by t-test. Differences in migration rates and cytokine release were tested by one-way ANOVA. Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software).

IV. RESULTS

1 CRC-infiltrating IL-17+ cells are not predictive of clinical outcome

Infiltration by IL-17+ cells was evaluated by IHC upon staining of a well-characterized TMA [131, 132]. In particular, IL-17 stainings were performed on a training cohort (n=1148), using a polyclonal anti-IL-17 antibody purchased by R&D system, and on a validation cohort (n=649), using a polyclonal anti-IL-17 antibody purchased by Santa Cruz Biotechnology (Figure IV.1A). Results obtained from the analysis of the training and the validation cohorts were highly concordant ($p<0.0001$). Numbers of IL-17+ cells ranged from 0 to 50 per punch within normal mucosa and from 0 to 350 cells per punch within CRC samples. In addition, we evaluated the localization of immune-reactive cells infiltrating tumor and normal colonic mucosa tissues. IL-17+ cells were found within both stromal and epithelial compartments. Notably, within the latter one, their number was significantly higher in tumor as compared to normal mucosa samples (Figure IV.1B). Accordingly, parallel analysis of a group of freshly isolated clinical specimens showed increased IL-17 mRNA levels in CRC tissues than in corresponding autologous healthy mucosa (Figure IV.1C).

CRC cases were then classified in four groups, according to the number of positive cells per punch (0, 1-10, 11-50, >50), and the potential association between the infiltration by IL-17+ cells and clinico-pathological features was evaluated by univariate analysis (Table IV.1). No significant association between IL-17+ infiltrates and tumor location or tumor border configuration was found. Also, no relevant difference in the prevalence of IL-17+ cells between MMR-proficient and deficient tumors was observed. In contrast, infiltration by IL-17+ cells strongly correlated with the presence of peritumoral lymphocytic infiltration ($p<0.001$). Furthermore, a slight, although significant, increase in IL-17+ cell numbers was observed in tumors characterized by early T and N stage, low grade and absence of vascular invasion. In addition, fewer IL-17+ cells ($\leq$ 30 cells per punch) were

detected in cases positive for distant metastasis, although this difference did not achieve statistical significance, possibly due to the limited size of this patient subgroup (n=82). Thus, the presence of IL-17+ cells was linked to early stage tumors. Unexpectedly, however, no significant impact of infiltration by IL-17+ cells on overall survival was observed (Figure IV.2A,B).

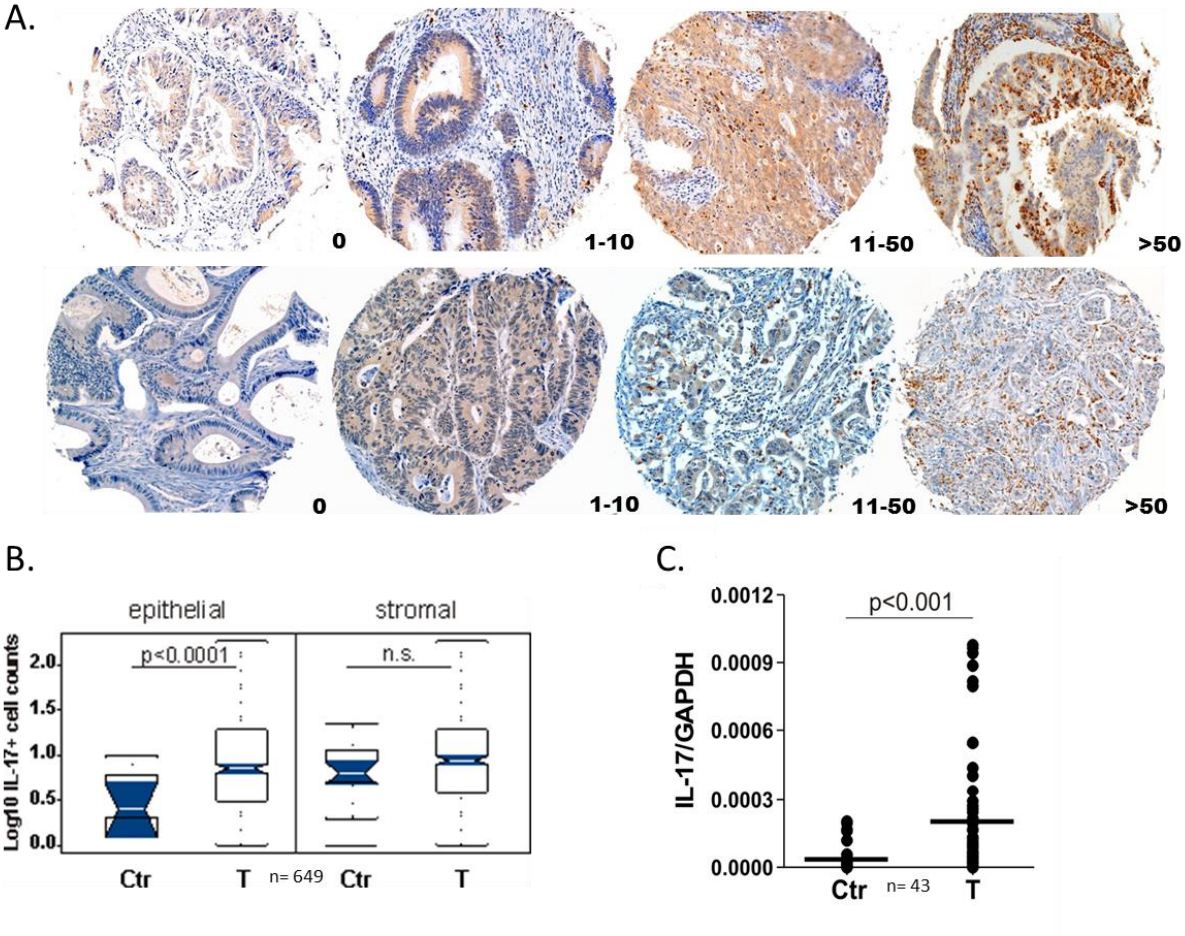


Figure IV.1: IL-17-producing cells are enriched in CRC tissues. A. IL-17 expression was evaluated by IHC. Representative pictures of IL-17 staining in the training (upper panel) and validation cohorts (lower panel) using anti-IL-17 antibody purchased by R&D Systems and Santa Cruz Biotechnology respectively. CRC cases are classified into four categories (0. 1-10, 11-50, >50) according to numbers of infiltrating IL-17+ cells. **B.** Distribution of IL-17+ cell numbers within the epithelial or stromal fraction of tumor samples (T) or healthy colonic (Ctr) tissues. Data from the validation cohort are shown. **C.** IL-17 mRNA levels assessed by quantitative PCR in tumor-free colonic mucosa (Ctr) and CRC (T) samples (n=43).

Table IV.1: Association of IL-17 lymphocyte count and clinico-pathological features in CRC

Clinico-pathological features		IL-17 lymphocyte count		p-value
		Median / Mean	Min-Max	
All		3 / 13.8	0 - 350	
Tumor location	Right-sided	3 / 12.6	0 - 200	0.94*
	Left-sided	3 / 14.3	0 - 332	0.13**
	Rectum	4 / 13.7	0 - 350	
MMR-Status	proficient	3 / 13.6	0 - 350	0.18
	deficient	4 / 14.5	0 - 167	
pT stage	pT1-2	5.5 / 15.1	0 - 150	0.03
	pT3-4	3 / 13.7	0 - 350	
pN stage	pN0	4 / 15.5	0 - 350	0.03
	pN1-2	3 / 12.3	0 - 350	
Tumor grade	G1-2	4 / 14.2	0 - 350	0.01
	G3	2 / 12.1	0 - 200	
Vascular invasion	Absent	4 / 15.1	0 - 350	0.03
	Present	3 / 10.7	0 - 250	
Tumor border	Pushing	4 / 12.3	0 - 250	0.78
	Infiltrating	3 / 14.9	0 - 350	
Peritumoral lymphocytic inflammation	Absent	3 / 13.4	0 - 350	**< 0.001**
	Present	7.5 / 15.8	0 - 250	
Local recurrence	Absent	1 / 8.5	0 - 350	0.35
	Present	1 / 6.1	0 - 200	
Distant metastasis	Absent	1 / 8.2	0 - 350	0.10
	Present	1 / 3.8	0 - 30	
Death	Censured	3 / 14.1	0 - 350	0.5
	Present	4 / 13.8	0 - 332	

Data from the training cohort (n= 1148) are shown.
p-Value calculated according to the Mann- Whitney test.
* Right-sided versus Left-sided; ** Right-sided or Left-sided versus Rectum

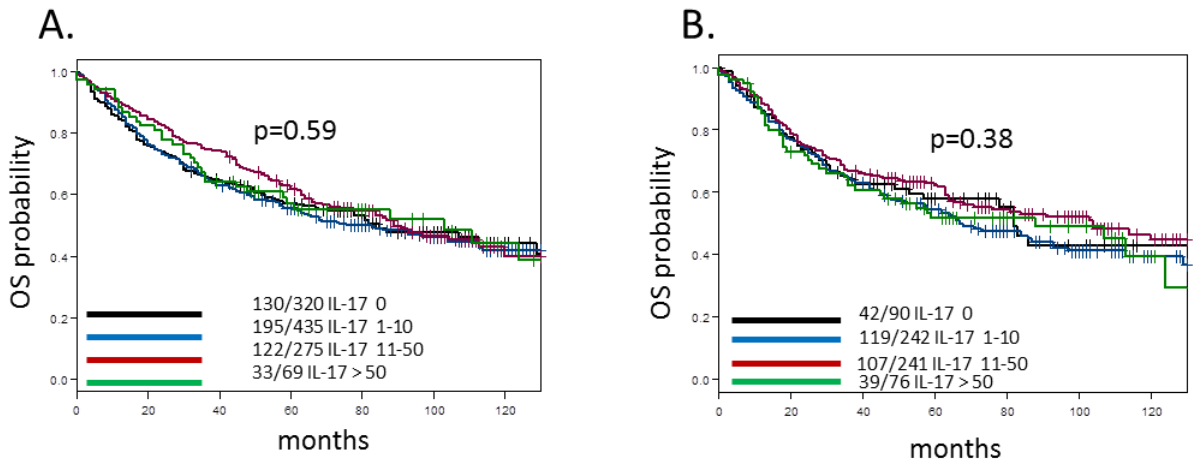


Figure IV.2 IL-17-producing cells are not predictive of survival in CRC patients. Kaplan-Meyer curves illustrating overall survival probability according to IL-17+ cell density in the training **(A)** and in the validation **(B)** cohorts. Numbers of deaths/total cases within each category are indicated.

2 Tumor infiltration by IL-17+ cells is associated to that of clinically relevant cell populations

When the correlation between IL-17 specific staining and other cell markers was evaluated, IL-17 was found to be significantly associated with CD8 and CD16 (Figure II.3). These markers were previously assessed on the same TMA, and notably were found to predict improved patients survival [8, 9, 133]. In parallel, we also investigated the clinical impact of MPO+ cells in the same TMA. We found that the infiltration of MPO+ cells predicted prolonged survival (see appendix, page 88, Droeser et al.) [8], and associated with the infiltration of IL-17+ cells (Figure II.3). MPO+ cells were found to express CD16, CD15, and CD66b, but not HLA-DR molecules, thus most likely consisting of activated neutrophils (see appendix, page 88, Droeser et al.) [8]. In conclusion, tumors with high infiltration by IL-17 producing cells are characterized by higher numbers of CD8+ T cells and neutrophils, both positively impacting on CRC outcome.

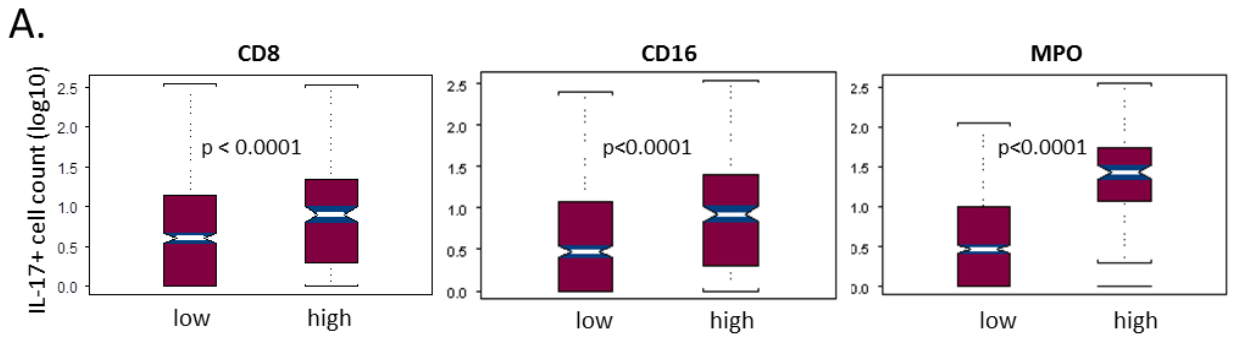


Figure IV.3. Tumor infiltration by IL-17+ cells is associated to that of clinically relevant cell populations. Numbers of IL-17+ cells within CRC cases characterized by low or high infiltration of CD8+, CD16+ and MPO+ cells, according to cut-off scores identified by ROC curve analysis.

We wondered whether the association between infiltration by IL-17+ cells and that of clinically relevant CTLs and activated neutrophils, could mask a negative prognostic effect of IL-17+ cells. However, also upon stratification of CRC cases according to CD8+ , CD16+ or MPO+ markers, no further impact by IL-17+ cells on survival was observed (Figure IV.4). Thus, CRC- infiltrating IL-17+ cells are not per se predictive of clinical outcome, but are associated to the presence of prognostically relevant immune cell subsets.

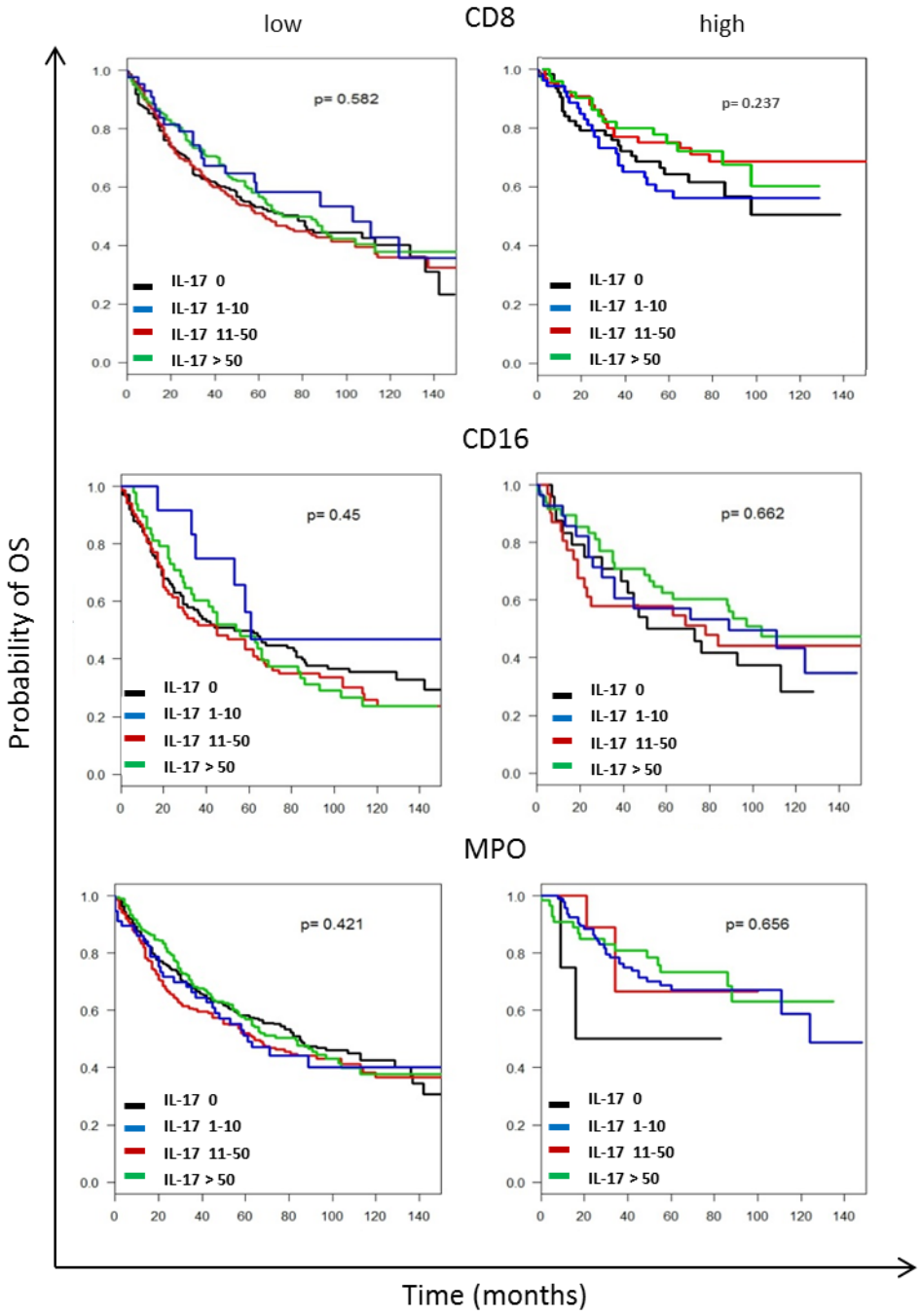


Figure IV.4. Clinical impact of IL-17+ cells on CRC infiltration by CD8+, CD16+ or MPO+ cells. Kaplan-Meyer curves illustrating overall survival probability according to IL-17+ cell density in CRC cases characterized by low or high infiltration of CD8+, CD16+ or MPO + cells.

3 CRC-infiltrating IL-17+ cells consist of polyfunctional Th17 cells

To investigate the molecular background underlying the association between IL-17-producing cells and CD8+ T cells and neutrophils, we first evaluated the cell source of IL-17 within CRC tissues. Upon PMA/Ionomycin restimulation, the phenotype of IL-17-producing cells in CRC or normal colonic tissues, and in PBMC from patients or healthy donors, was assessed by flow cytometry (Figure IV.5). No significant difference in percentages of IL-17+ cells was observed between PBMC from healthy donors and CRC patients. Instead, frequencies of IL-17+ cells were higher in tissue samples than in PBMC ($p<0.003$ versus normal colonic mucosa, $p<0.0001$ versus CRC tissues), and were significantly increased in tumors as compared to control tissues ($p<0.0001$) (Figure IV.5A). In all populations, IL-17 production was exclusively observed in CD3+ T cells, whose large majority expressed CD4, but not CD8 molecule (Figure IV.5B,C and data not shown). Further phenotypic characterization of tumor infiltrating IL-17+ cells revealed that up to 30% of CD4+ IL-17+ cells also expressed the Tregs marker Foxp3 (Figure IV.5B,C). The presence of additional cell populations of the innate immunity potentially producing IL-17, including γδT, NK, invariant NKT, lymphoid tissue inducer (LTi)-like cells and neutrophils, was also evaluated upon staining with specific antibodies including -γδ TCR, -CD56/-CD16, -Vα24–Jα18 TCR, –CD161/-CD127 and -CD66b, respectively. Large proportions of γδT cells and neutrophils (up to 24±5% and 13±9%, respectively) were found within CRC infiltrates. Only a minor fraction (<1%) of γδT cells, however, showed IL-17-production capacity, whereas no IL-17-producing neutrophils were observed. CRC-infiltrating NK, NKT and LTi-like cells were detected in limited numbers and did not include significant fractions of IL-17+ cells.

In addition, a vast majority of tumor infiltrating IL-17+ cells expressed the chemokine receptor CCR6 and the T cell memory marker CD45RO, whereas only few cells expressed HLA-DR (Figure IV.5B). Thus, IL-17 producing cells within CRC tissues consisted almost exclusively of memory Th17.

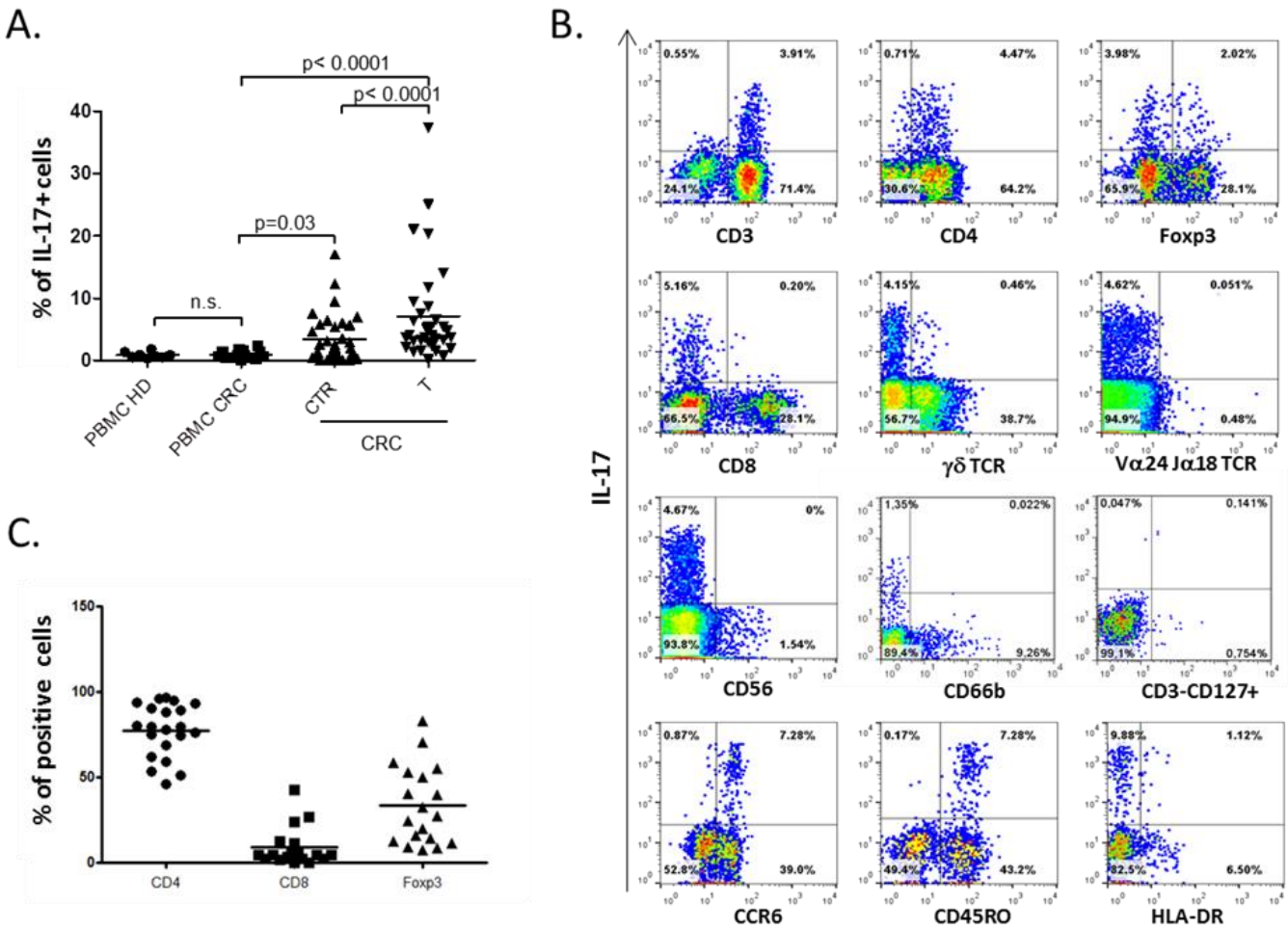


Figure IV.5. CRC-infiltrating IL-17+ cells mainly consist of Th17 cells. PBMC from healthy donors or CRC patients, and single cell suspensions obtained from freshly excised clinical specimens of CRC and corresponding tumor-free colonic mucosa were incubated with PMA/Ionomycin for 5 hours. Surface staining for specific cell population markers and intracellular staining for Foxp3 and cytokines was then performed. **A.** Frequencies of IL-17+ cells on gated CD3+ T cells obtained from PBMC from healthy donors (PBMC HD, n=8) or CRC patients (PBMC CRC, n=14), and single cell suspensions obtained from freshly excised clinical specimens of CRC (T, n=34) and corresponding tumor-free colonic mucosa (Ctr, n= 34). Means are indicated by bars. **B.** Representative flow cytometric analysis of CRC infiltrates stained for IL-17 and cell specific markers. **C.** Frequencies of CRC infiltrating CD4+, CD8+ or Foxp3+ cells within IL-17+ cells (n= 21). Means are indicated by bars.

Moreover, when we evaluated the potential ability of CRC infiltrating Th17 cells to express other effector cytokines in addition to IL-17, we found that a significant fraction of CD4+ IL-17+ cells also expressed TNF-α, IL-21, IL-22, and GM-CSF, and to a lower extent, IFN-γ, and IL-8 (Figure IV.6A,B). The presence of Th17 cells expressing the same panel of cytokines was also evaluated in tumor free colonic tissues and in PBMC from patients or healthy donors. Frequencies of IL-17+ cells expressing TNF-α, IL-21, IL-22, GM-CSF, and IL-8, but not IFN-γ, were higher in CRC as compared to control tissues or PBMC (Figure IV.6C).

To further address whether the release of the described cytokines was originated from the same Th17 subset, we expanded CRC-derived Th17 cell clones from sorted CXCR3-CCR4+CCR6+ infiltrating CD4+ T cells. Importantly, Th17 clones also released TNF-α, IL-21, IL-22, GM-CSF, IFN-γ and IL-8, in addition to IL-17 (Figure IV.7), thus indicating that Th17 within CRC tissues consist of polyfunctional effector cells.

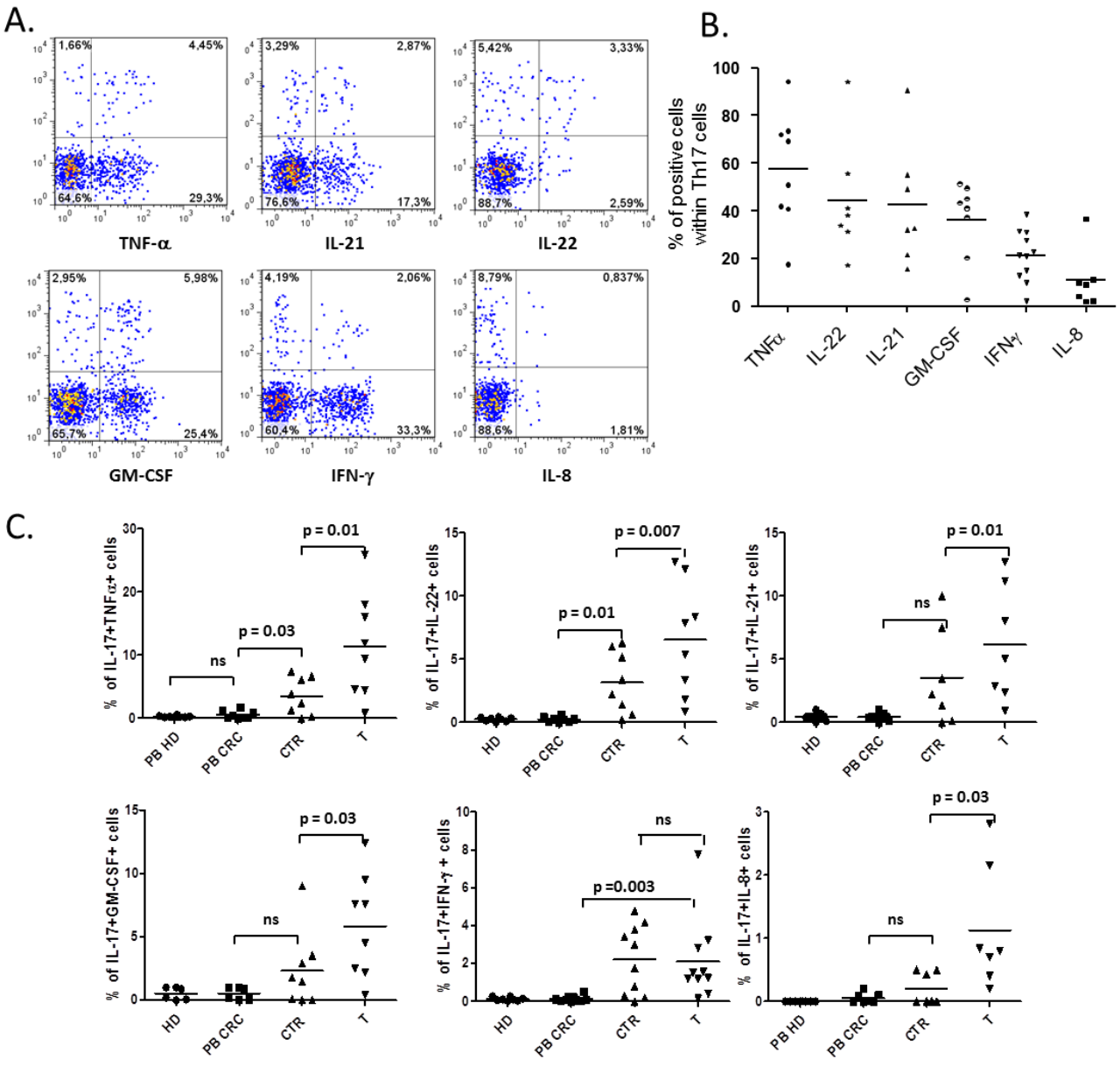


Figure IV.6. CRC infiltrating Th17 consist of polyfunctional effector cells. **A.** Representative flow cytometric analysis of intracellular cytokine staining in CRC infiltrates. **B.** Frequencies of cells positive for the indicated cytokines gated on CD3+CD4+IL-17+ T cells (n≤11). Means are indicated by bars. **C.** Frequencies of cells positive for the indicated cytokines gated on CD3+CD4+ cells, obtained from PBMC from healthy donors (PBMC HD, n<8) or CRC patients (PBMC CRC, n<8), and single cell suspensions obtained from freshly excised clinical specimens of CRC (T, n<8) and corresponding tumor-free colonic mucosa (Ctr, n<8). Means are indicated by bars.

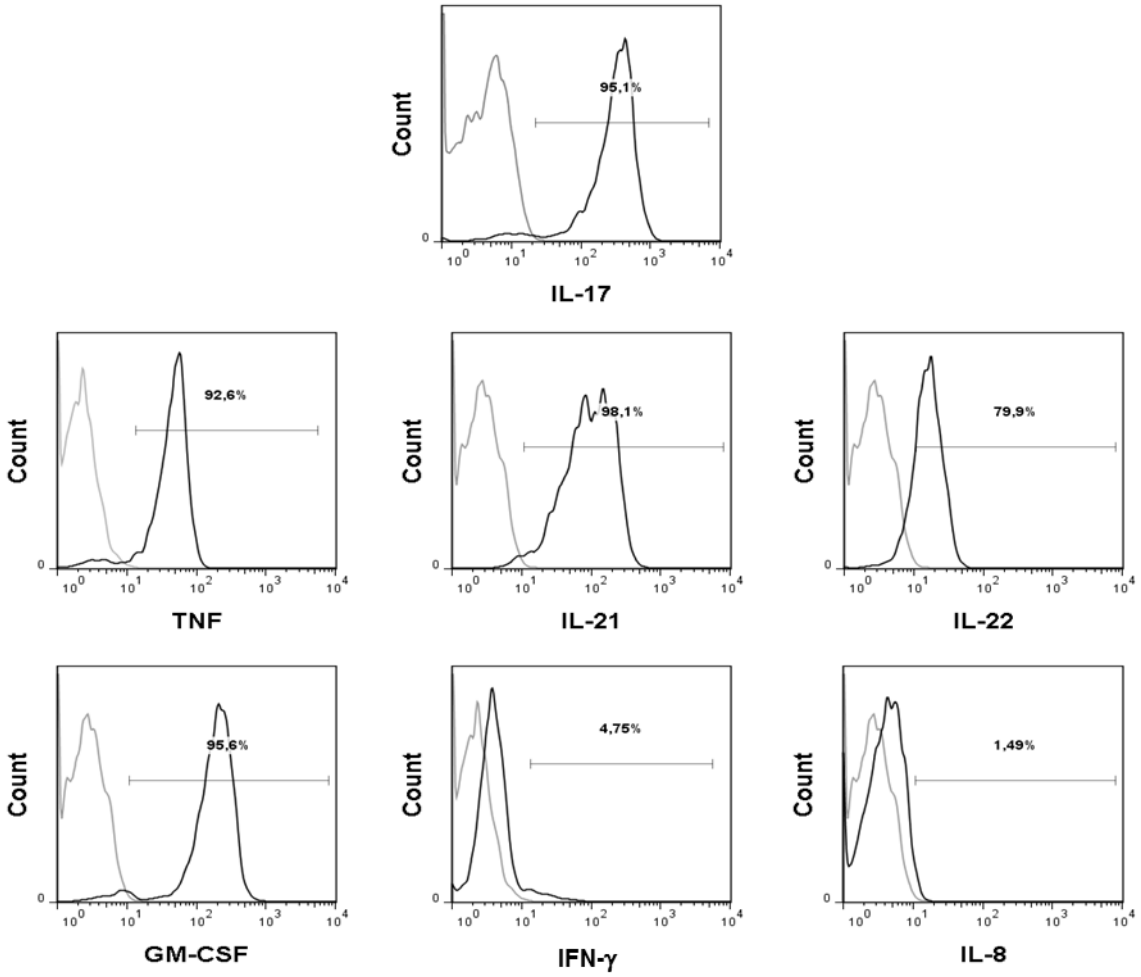


Figure IV.7. Polyfunctional CRC-derived Th17 clones. Representative flow cytometric analysis of a Th17 clone expanded from CRC-infiltrating CD4+ T cells. Cells were stimulated with PMA/Ionomycin/Brefeldin A for 5 hours and intracellular staining was performed. Staining with cytokine-specific antibodies (black histograms) and matched isotype controls (grey histograms) are shown. Frequencies of positive cells are indicated.

4 Tumor-infiltrating Th17 cells are capable to recruit and activate neutrophils

The results of the phenotypic analysis of IL-17+ cells ruled out that neutrophils or CD8+ cells are the IL-17 producers. As a possible alternative explanation of their association with IL-17, we investigated whether Th17 cells contribute to the recruitment of these cell populations into tumor tissues.

Indeed, although IL-17 cannot act directly on neutrophils and CD8+ T cells, due to the lack of expression of functional IL-17 receptors on these cell subsets [25, 134], Th17 cells have been shown to attract neutrophils through IL-8 production [25].

Consistent with these previous findings, neutrophils exhibited a vigorous migration towards supernatants of CRC-derived Th17 clones, in an IL-8-dependent manner, as indicated by the inhibition obtained upon addition of IL-8-neutralizing antibodies (Figure IV.8A). Interestingly, the exposure of neutrophils to Th17 supernatants, but not to IL-17, resulted in a significantly higher MPO release (Figure IV.8B). Thus, tumor infiltrating Th17-derived cytokines might directly promote neutrophil recruitment and activation into CRC tissues.

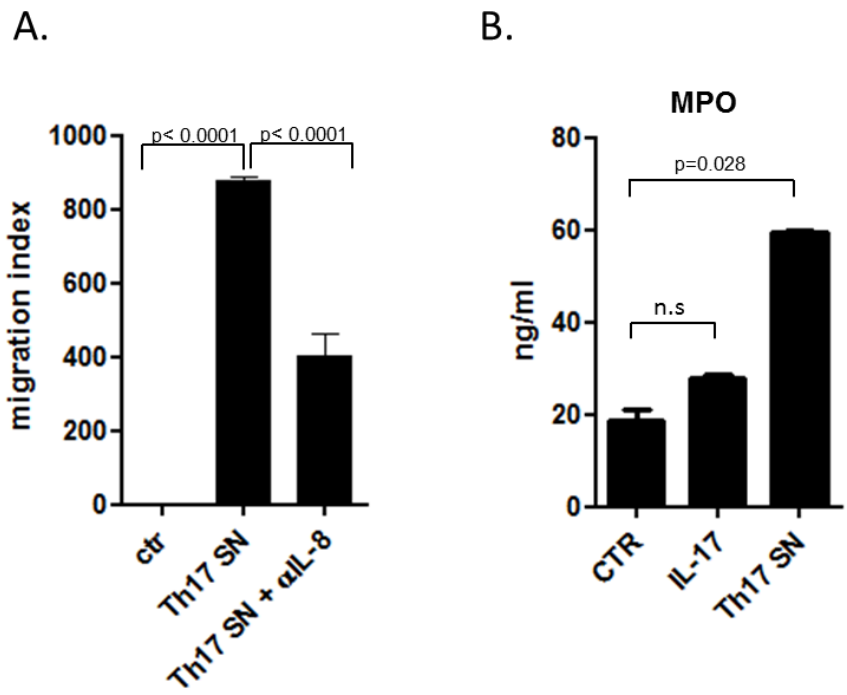


Figure IV.8. CRC-infiltrating Th17 cells favor recruitment and activation of neutrophils. A. Migration of neutrophils, purified from blood of healthy donors, towards control medium (Ctr), supernatants of Th17 clones expanded from CRC infiltrating cells (Th17 SN), or Th17 supernatants pre-treated with anti-IL-8 antibodies (Th17 SN+αIL-8), was evaluated after 90 minutes by flow cytometry. Means ± SD from experimental triplicates are depicted. One representative experiment out of two is shown. **B** MPO release by neutrophils exposed to control medium (Ctr), rIL-17 (50 ng/ml) or Th17 supernatants (Th17 SN), was assessed after 4 hours by ELISA. Means ± SD are depicted. One representative experiment out of three is shown.

5 Th17 cells favor recruitment of CD8+ T cells by triggering chemokine release from endothelial cells

In order to identify the chemotactic factors possibly involved in the recruitment of CD8+ T cells into CRC tissues, we first analyzed the chemokine receptor profiles on CD8+ T cells from peripheral blood and tumor tissues of CRC patients (Figure IV.9A). In both, expression of CCR3, CCR5, CCR6, CXCR3, and CXCR4 was observed on a fraction of CD8+ T cells.

Expression of cognate chemokines to these receptors, i.e. CCL3, CCL5, CCL20, CXCL9, CXCL10 and CXCL12, was detected in whole CRC tissues (Figure IV.9B).

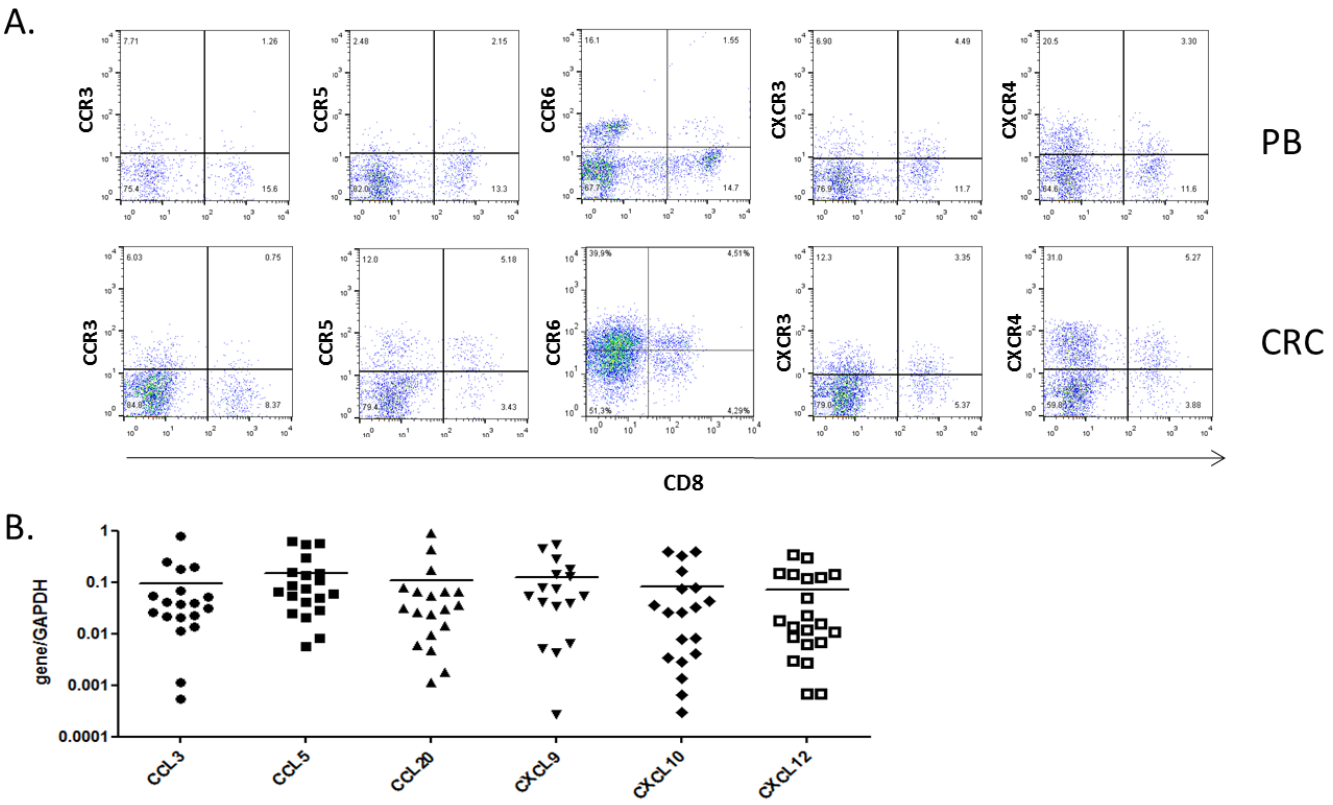


Figure IV.9. Chemokine and chemokine receptor expression in CRC tissues. A. Peripheral blood (PB) and tumor cell suspensions (CRC) from CRC patients (n=4) were surface stained for CD8 in combination with the indicated chemokine receptors. A representative flow cytometric analysis is shown. **B**. The expression of genes encoding for the indicated chemokines was assessed on CRC samples (n=21) by quantitative PCR.

Importantly, upon sorting of tumor, mesenchymal and endothelial cells (EC), from CRC specimens, the expression of these chemokine genes was preferentially detected within the endothelial compartment (Figure IV.10A).

To understand the role potentially played by Th17 cells in the induction of chemokine production by tumor-associated EC, we exposed an EC cell line to recombinant IL-17 or Th17 supernatants *in vitro*. No major effects on chemokine release were detected upon treatment with IL-17 alone. However, upon exposure to Th17 supernatants, a remarkable increase in CCL5 ($p<0.0001$) and CXCL10 ($p<0.0001$) production was observed. In contrast, CCL20, which was already detectable in significant amounts in Th17 supernatants, did not further increase (Figure IV.10B). Finally, no CCL3 and CXCL12 production was detected.

To verify whether Th17-mediated chemokine release by EC was sufficient to promote T cell recruitment, we tested the ability of CD8+ T cells to migrate towards culture medium of EC exposed to recombinant IL-17 or Th17 supernatants. Strikingly, conditioning by Th17 cells significantly boosted the capacity of EC to attract CD8+ T cells in vitro ($p<0.0001$) (Figure IV.10C).

Thus, by triggering chemokine release from tumor-associated EC, Th17 cells may favor the recruitment of CD8+ T cells into CRC tissues.

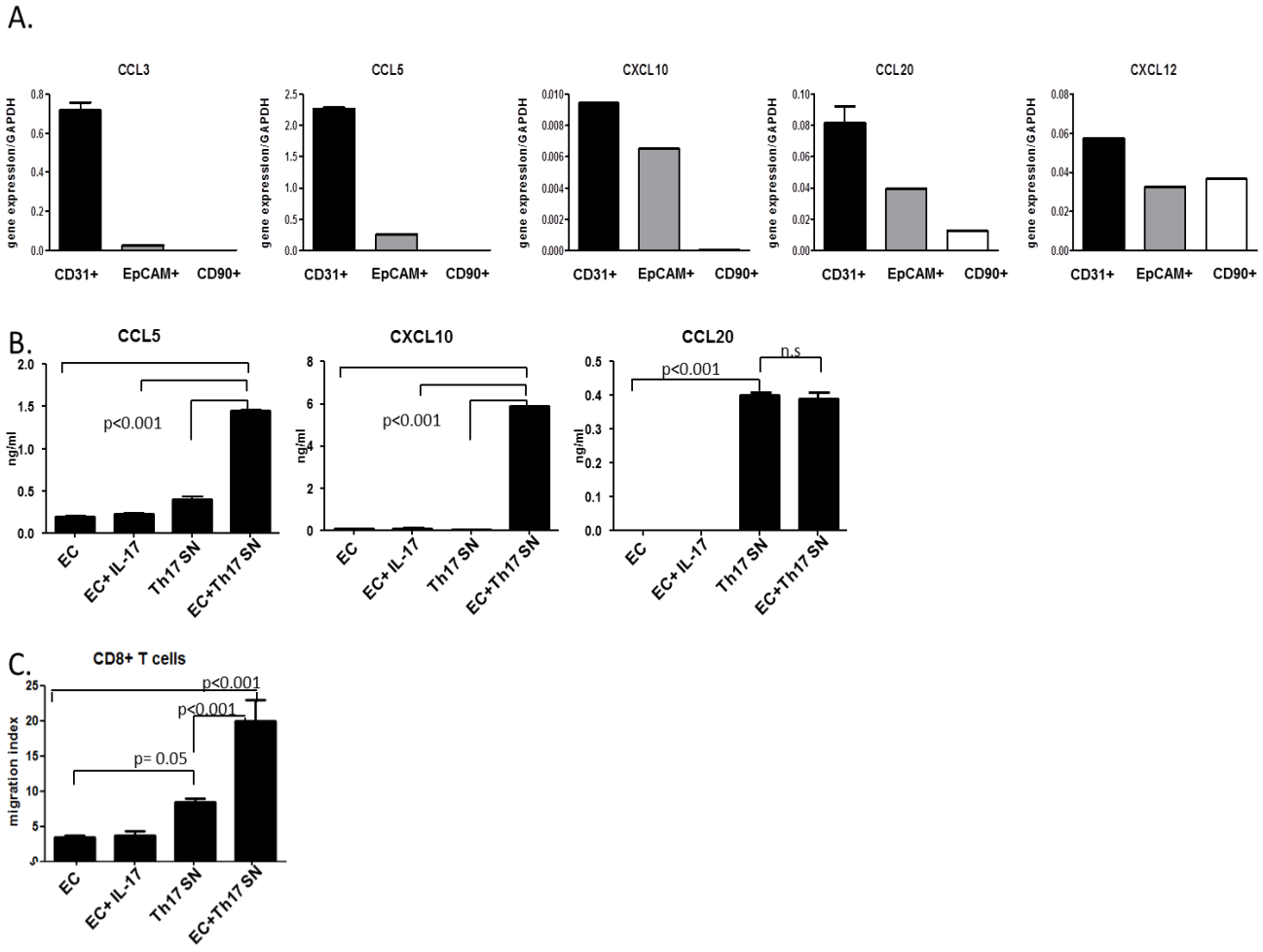


Figure IV.10. CRC infiltrating Th17 cells activate tumor-associated EC to release cytokine capable of recruiting CD8+ T cells. A. Tumor, endothelial and stromal cells were sorted from cell suspensions of CRC specimens by flow cytometry, based on EpCAM, CD31 and CD90 expression, respectively. mRNA levels of the indicated chemokine genes were assessed by quantitative PCR, relative to GAPDH. Results from one out of 4 samples analyzed are show. **B.** Chemokine release by HMEC cells untreated (EC) or exposed to rIL-17 (50 ng/ml) (EC+IL-17) or Th17 clone supernatants (EC +Th17 SN) for an overnight period, was measured by ELISA. Chemokine contents in Th17 SN were also assessed as control. Means ± SD are depicted. One representative experiment out of three is shown. **C.** Migration of CD8+ T cells, purified from PBMC of healthy donors, towards supernatants of HMEC untreated (EC), or exposed to rIL-17 (50 ng/ml) (EC+IL17) or Th17 supernatants (EC+Th17), was assessed after 90 minutes of incubation by flow cytometry. Migration towards Th17 supernatants (Th17 SN) was also assessed as control. Means ± SD are depicted. One representative experiment out of three is shown.

6 Th17 cells directly attract CD8+ T cells into tumor tissues

In the migration assays described above, we unexpectedly observed that Th17 supernatants significantly induced CD8+ T cells recruitment also in the absence of stromal cells (Figures IV.9C). This result was confirmed using supernatants derived from different Th17 clones (Figures IV.10A, $p< 0.05$). This finding suggested that Th17 cells might also release chemoattractants directly acting on CD8+ T cells.

Indeed, significant amounts of CCL5 and CCL20 were detected in supernatants from different Th17 clones (Figures IV.10B and II.11B). Although CCL5 was released in comparable amount also by CRC-derived Th1 cells, CCL20 was found to be preferentially produced by Th17 cells (Figure IV.11B). Importantly, depletion of CCL20 markedly reduced Th17-driven migration of CD8+ T cells ($p<0.0001$), whereas depletion of CCL5 exhibited only a marginal effect (Figure IV.11C). Thus, Th17 cells directly target CD8+ T cells through CCL20 production.

We then assessed whether the direct effect of Th17-derived chemokines may be relevant for CD8+ T cell recruitment into tumor tissues. In order to evaluate the capacity of Th17 to drive T cell migration in the absence of EC, we though to engineer a tridimensional tumor like tissue by taking advantage of a perfused bioreactor system, previously developed for in vitro culture of cartilage and bone tissue[135]. In this system, human CRC cells from established cell lines can be homogeneously seeded on a collagen scaffold, through an alternate perfusion of the culture medium. This allows the generation of a tridimensional tumor tissue whose architecture resembles that of primary tumors (Hirt et al., manuscript submitted).

We first generated a tumor like tissue by culturing HT29 cells in the bioreactor for 7 days (Figure IV.12A, B). Then, to mimic tumor infiltration by T cells, Th17 cells from CRC-derived clones were added into the system under perfusion, allowing their localization in

proximity of the tumor nests (Figure IV.12C). Intratumoral Th17 cells were subsequently activated, in order to trigger chemokine release, or left untreated (Figure IV.13A). Perfusion was then stopped and CD8+ T cells were applied into the system. After an overnight period, tumor infiltration by CD8+ T cells was evaluated by immunofluorescence staining and, upon tissue digestion, by flow cytometry (Figure IV.13B,C). Strikingly, frequencies of CD8+ T cells were significantly higher in the presence of activated Th17 cells (p= 0.023). Immunofluorescence analysis confirmed the presence of CD8+ T cells into the tumor.

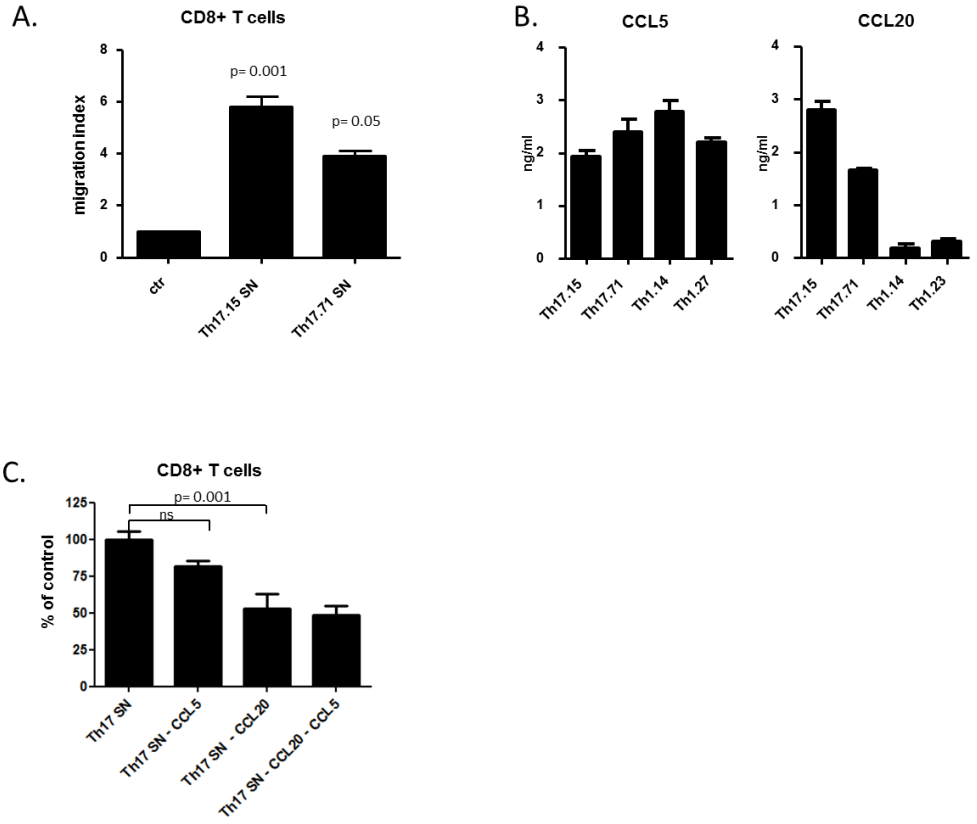


Figure IV.11. CRC infiltrating Th17 cells directly attract CD8+ T cells in vitro. A. Migration of CD8+ T cells, purified from PBMC of healthy donors, towards control medium (Ctr) or supernatants of two different Th17 clones expanded from CRC infiltrating T cells (Th17.15 and Th17.71). Means ± SD from experimental triplicates are depicted. **B.** Supernatants from clones Th17.15, Th17.71, Th1.14, and Th.27 were collected after overnight stimulation with plate bound anti-CD3 and soluble CD28 antibodies. CCL5 and CCL20 contents were measured by ELISA.
C. Percentages of inhibition of CD8+ T cell migration upon depletion of CCL5 (Th17–CCL5), CCL20 (Th17 SN – CCL20) or both (Th17 SN – CCL5-CCL20) from Th17 supernatants. Means ± SD from experimental triplicates are depicted. One representative experiment out of two is shown.

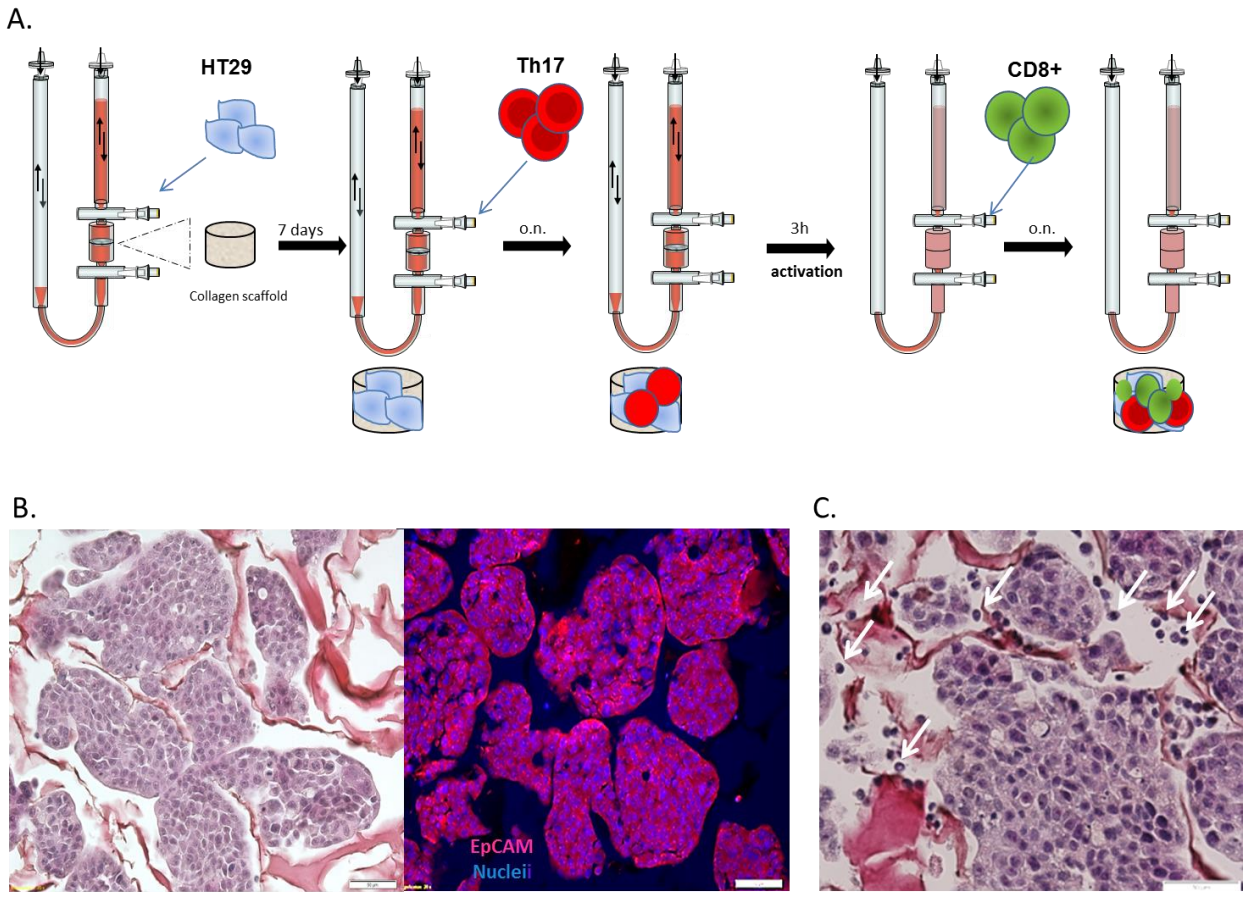


Figure IV.12. Engineering of tumor like tissue. A. Experimental protocol: HT29 cells were injected into the bioreactor and perfused through a collagen scaffold for 7 days. Th17 cells were then seeded for an overnight period (o.n.) and subsequently activated by adding Cytostim to the perfusion medium. Three hours following Th17 activation, perfusion was stopped and CD8+ T cells were added into the system. After an overnight period the tissue was removed and processed. **B**. H&E staining (left panel) and immunofluorescence analysis (right panel, EpCAM red, DAPI blue) of engineered tumor tissue at day 7. **C.** HT29 cells were cultured on a collagen scaffold in a perfused bioreactor up to 7 days, and were then perfused with medium containing Th17 cells. After 24 hours scaffolds were removed and tumor infiltrating Th17 cells (indicated by arrows) were visualized by H&E staining.

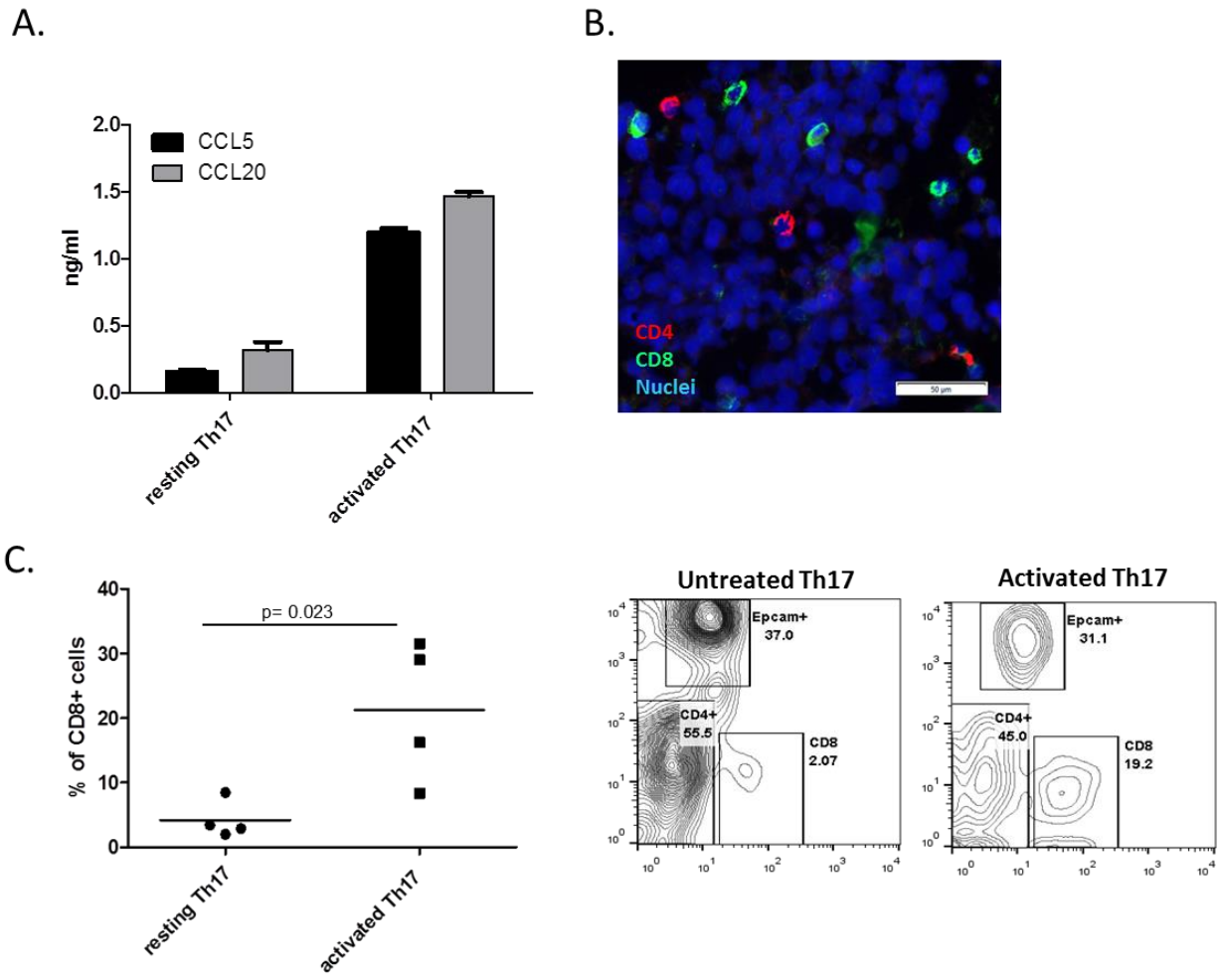


Figure IV.13. CRC infiltrating Th17 cells directly attract CD8+ T cells into tumor tissues. Th17 cells infiltrating engineered tumor like tissues were activated by Cytostim or left untreated **A.** Culture media were collected 20 hours later and chemokine contents were assessed by ELISA. **B, C.** Three hours following Th17 activation, perfusion was stopped and CD8+ T cells were added into the bioreactor. After an overnight period, scaffolds were removed and tumor infiltration by CD8+ T cells was evaluated by immunofluorescence analysis, upon staining with CD4 and CD8-specific antibodies (**B**) and by flow cytometry, upon staining of single cell suspensions with EpCAM-, CD4- and CD8-specific antibodies (**C**). Percentages of CD8+ cells in tumor tissues infiltrated by untreated or activated Th17 cells are reported (**C** left panel; dots represent experimental replicates, **C** right panel: contour plots of one representative experiment out of three).

V. DISCUSSION AND FUTURE PERSPECTIVES

1 Discussion

Tumor infiltration by specific immune cell subsets has been recognized as a critical factor in determining patients clinical outcome [1]. Whereas the anti-tumorigenic activity of infiltrating cytotoxic CD8+ T cells and INF-γ producing Th1 cells is well documented, the role of IL-17 and Th17 cells in cancer remains controversial [1, 2, 4]. IL-17 and Th17 cells have been described, in different human malignancies, as active players in both pro-tumorigenic and anti-tumorigenic processes [107, 123]. Our work extensively explored the impact and biological activity of IL-17 producing cells in human CRC. In particular, we have investigated the clinical relevance of tumor infiltrating IL-17-producing cells in more than 1000 CRC cases, and characterized in depth their phenotype and functional properties.

Our data indicate that in CRC*:* ***a)*** infiltration by IL-17+ cells is not predictive of patient survival, but ***(b)*** is associated to the density of CD8+ T cells and CD16+ MPO+ neutrophils, both predictive of positive clinical outcome; ***c)*** polyfunctional Th17 cells are the major source of IL-17, ***d)*** tumor derived Th17 cells are capable to recruit CD8+ T cells and neutrophils in vitro, either directly, through CCL20 and IL-8 respectively, or indirectly by triggering chemokine release by tumor-associated endothelium.

Thus, ***e)*** altogether our findings suggest that tumor infiltrating Th17 cells, by contributing to the recruitment of immune cells associated to favorable prognosis, might play a positive role in CRC beyond IL-17 production.

a) Prognostic significance of infiltrating IL-17 + cells in CRC

In previous reports the expression of IL-17 has been shown to predict unfavorable CRC clinical outcome [6, 7]. In particular, in a cohort including 99 CRC cases, the presence of IL-17 has been found to negatively correlate with patient disease free survival, and to weaken the beneficial effect of tumor infiltration by CD8+ T cells [7]. Similarly, a minor work by Liu and colleagues, has reported that IL-17 was an indicator of poor prognosis in a small group of patient (n=52) [6]. In our work, we have investigated the clinical relevance of tumor infiltrating IL-17-producing cells in more than 1000 CRC. We performed IL-17 staining by using either the antibody used by Liu et al [7] (training cohort, n=1148) or that used by Tosolini et al [6] (validation cohort, n=649). We confirmed that numbers of IL-17+ cells were higher in tumor than in colonic mucosa samples and were significantly enriched in early stage tumors, as previously described [136-138]. However, in contrast to previous reports, we found that the presence of IL-17 within the tumor was not *per se* a prognostic indicator. Nevertheless, few differences in the protocol used in our work as compared to others must be remarked. IL-17 positivity was assessed by three independent observers, whereas in the study reported by Tosolini et al. the TMA staining was analyzed and quantified by image software [7]. Moreover, we have evaluated the prognostic relevance of IL-17 expression on overall survival, instead of disease-free survival [7]. However, in a group of cases for which more clinical information were available (n=446), we did not observe any association of IL-17 with the occurrence of local recurrence or distant metastasis. Thus, the discrepancy in the prognostic impact of IL-17+ cells between our and previous studies most likely relies on the different numbers of cases evaluated.

b) **Association of infiltrating IL-17+ cells with other CRC infiltrating immune cells**

Upon the evaluation of the TMA, we interestingly found that infiltration by IL-17+ cells was associated to that of CD8+ T and MPO+CD16+ myeloid cells, both predictive of favorable clinical outcome in CRC [7-10].

Indeed, the density of cytotoxic T cells within the tumor has been well recognized as a strong positive prognostic factor [1]; concordantly, the infiltration of CD8+ T cells was also significantly associated with prolonged survival in our group of patients [10]. In addition, in a previous work we have shown that high densities of CD16+CD33+HLA-DR- myeloid cells predicted patient survival [9]. Based on the phenotypic profile we have then hypothesized that neutrophils could be the myeloid cells identified as positive prognostic factor. In a subsequent study we found that MPO+ neutrophils were indeed an indicator of prolonged survival time in the same cohort (see appendix, page 88, Droeser et al.) [8]. Importantly, the positive impact played by infiltrating CD8+ T cells and MPO+ neutrophils was not affected by the presence of IL-17. Indeed, the infiltration by IL-17+ cells did not influence patients survival also upon stratification of CRC cases according to the infiltration by CD8+, CD16+ or MPO+ cells.

c) **Phenotypical characterization of infiltrating IL-17-producing cells**

The presence of CD4+ lymphocytes producing IL-17 (Th17 cells) within CRC tissues has been previously report [4]. However, their phenotype and cytokine profiles were not fully characterized. We then characterized in depth the phenotype of IL-17-producing cells within CRC tissues. In line with previous findings, we found that, upon PMA/Ionomycin restimulation, IL-17 production

was exclusively observed in CD3+ T cells, whose large majority expressed CD4 molecule. Furthermore, CD4+IL-17+ cells infiltrating CRC resembled the classical phenotype of the memory Th17 subset expressing CD45RO and CCR6. In addition, a fraction of CD4+IL-17+ cells also expressed the Tregs marker Foxp3. Interestingly, *ex vivo* stimulated Th17 cells and *in vitro* expanded CRC-derived Th17 clones were capable to release a large spectrum of pro-inflammatory cytokines and chemokines. Thus, the majority of IL-17 producing cells within CRC tissues consisted of polyfunctional memory Th17.

Unexpectedly, however, a recent study from Wu and colleagues has reported that innate γδT17 cells are the major cellular source of IL-17 in human colorectal cancer [139]. Intriguingly, we have found that γδT cells were present within the CRC infiltrates, but only a minor fraction showed IL-17-production capacity.

Although Wu's results and ours may appear contradictory, it should be remarked that these two studies were conducted in groups of patients belonging to different ethnical groups and geographical areas (i.e. Switzerland and China). This may be of relevance when considering immune cell populations, such as γδT and Th17 cells, which are modulated by defined species of microbiota [62, 140]. Indeed, it has been recently recognized that the composition of the gut flora varies between individuals belonging to different populations [141], and might influence the differentiation of γδT and Th17 cells.

Thus, the discrepancy between the phenotype of IL-17-producing cells described in Wu's work, or ours may relate to the presence, within the patients evaluated, of distinct gut microbial species possibly driving preferential expansion of IL-17 producing -γδT or Th17 cells, respectively.

d) Th17 cells trigger neutrophils and CD8+ T cells recruitment

Since we excluded that CD8+ T cells or neutrophils were responsible of IL-17 production in CRC tissue, we alternatively hypothesized that the association between IL-17 and this two cell populations, might relate to the ability of Th17 cells to recruit them into tumor sites. Peripheral blood Th17 cells have been shown to directly attract neutrophils, through IL-8 production [25]. In line with these findings we found that CRC infiltrating Th17 cells also induced IL-8-dependent neutrophil migration *in vitro*. Interestingly, exposure of neutrophils to Th17 supernatants resulted in higher secretion of MPO, a lysosomal enzyme catalyzing the production of reactive oxygen species. Although the molecular mechanism underlying the favorable prognostic significance of MPO+ neutrophils in CRC remains to be understood, Th17 cells might contribute to their recruitment and their potential cytotoxic effects. In addition, we have found that CRC-derived Th17 cells activate tumor-associated EC to release chemokines, such as CCL5 and CXCL10, capable of attracting CD8+ T cells. Notably, IL-17 alone was not sufficient to trigger EC activation, but the secretion of additional Th17 cytokines was required, as previously reported [40]. Most importantly, we documented for the first time the ability of tumor infiltrating Th17 to directly recruit CD8+ T cells through own production of CCL20, whose receptor CCR6 is expressed on a fraction of circulating and tumor infiltrating CD8+ T cells. This data underline the multiple effects mediated by the Th17 cell subset, which are not simply mirrored by IL-17 activity. Consistent with our observation, an association between tumor infiltration by Th17 and that by cytotoxic CD8+ T cells has been previously reported in a melanoma mouse model. Adoptively transferred tumor-specific Th17 cells have been shown to recruit cytotoxic CD8+ T cells ultimately mediating

tumor eradication [43, 142]. These *in vivo* models however, would have not been able to discriminate between direct and indirect effects of Th17 on CD8+ T cell recruitment. Therefore, to assess the relevance of the direct Th17-mediated effect on cytotoxic cells, we took advantage of a perfused bioreactor system allowing engineering of a tridimensional tumor tissue infiltrated by Th17 cells, in the absence of EC. Strikingly, activation of Th17 cells dramatically enhanced the recruitment of CD8+ T cells into the tumor. Altogether, these results suggest that Th17 cells may promote tumor infiltration by CD8+ T cells by acting on a double axis: on the one hand, they might favor EC activation leading to CD8+ T cell recruitment from the blood stream, on the other they could directly guide the positioning of CD8+ T cells into the tumor nests.

Thus, our findings indicate that by favoring recruitment of effector cells into the tumor site, polyfunctional Th17 cells might indirectly contribute to a beneficial immune response in CRC.

e) **Th17 cells play a positive role in CRC**

Altogether our findings unrevealed a positive role played by tumor infiltrating Th17 cells in CRC and underline their pleiotropic effects beyond IL-17 production. However, the fact that Th17 cells might play a positive role in CRC and the concomitant lack of positive impact of tumor infiltrating IL-17+ cells appears contradictory. In the light of this scenario, it should be taken into account that: *i)* the capacity of Th17 to recruit clinically relevant cell populations into tumor tissues relies on their production of multiple cytokines and chemokines, not on IL-17 only; *ii)* the positive effect played by Th17 cells could be

counterbalanced by the detrimental impact of the known proangiogenic activity of IL-17 [32, 90, 96, 124, 142]; *iii)* CRC infiltrating IL-17+ cells include a fraction of Foxp3+ cells, possibly endowed with immunosuppressive activity [40, 136]; *iv)* cell populations other than Th17 may also contribute to the recruitment of beneficial immunocompetent cells into tumor tissues. Indeed, Th1 cells secrete IFN-γ and TNF, capable of activating chemokine release by EC [143]. Furthermore, we found that CRC infiltrating Th1 clones also secrete CCL5 and CXCL10. However, no IL-8 or CCL20 production was observed. It is therefore possible that different T helper subsets may favor tumor infiltration by distinct immunocompetent cell populations. Th17 cells may be critical, in particular, for the recruitment of MPO+ neutrophils and CCR6-expressing CD8+ T cells. Notably, within PBMC, CCR6 expression identifies a subset of early effector memory T cells, characterized by high IFN-γ and TNF production capacity, and gut homing receptor expression [37]. We also observed a large fraction of CCR6+ cells within CD8+ T cells infiltrating tumors as well as control tissues. Further investigations are warranted, however, to elucidate the potential effector function of this cell subset in mucosal and anti-tumor immunity. In addition, a positive role played by Tregs in CRC has been described. Indeed, at contrary with the majority of cancer, in CRC high level of Foxp3+ cells correlates with prolonged survival [120, 121]. Hence, characterizing in depth the suppressive or inflammatory activity possibly acquired by tumor infiltrating IL-17 producing-Foxp3+ cells could better elucidate the role of this population in CRC.

2 Future perspectives

Antigenic specificities of CRC infiltrating Th17 cells remain to be investigated.

It is possible that Th17 recognize tumor-associated antigens. Indeed, in a parallel study we have recently found that HLA class II antigens are expressed by about 23% of CRCs [144] and their expression is upregulated in CRC cell lines cultured in the presence of IFN-γ. Of note high expression of HLA class II antigens on CRC is predictive of improved survival [144]. Thus, by presenting HLA class II restricted tumor-associated antigens, CRC cells could activate specific Th17 cells eventually recruiting CD8+ T cells.

On the other hand, a fraction of Th17 cells may be directed against bacterial antigens derived from the gut flora. Mouse models have shown that microbiota are required for the accumulation of Th17 in the lamina propria. Moreover, colonization with the commensal SFB has been reported to be sufficient for the induction of Th17 in the intestine [67]. Accordingly, it has been recently shown that SFB specific T cells mostly consist of Th17 [145], thus suggesting that the quality of T cell responses directed against commensal -derived antigens is determined by the bacterial type. Although SFB has not been found within the human microbiome, it is likely that other commensal species might similarly promote the differentiation of specific Th17 cells.

In human, differences in gut microbiota have been described in a variety of chronic disorders, including inflammatory bowel diseases, allergy and obesity [146]. However, whether the different flora composition affects the pathogenesis or it is only a consequence of these disorders, remains a matter of investigation.

More recently, defined bacteria species, including *Fusobacterium nucleatum* and several *Bacteroides* species, have been found to be enriched in human CRC samples [147, 148]. However their precise role in CRC pathogenesis remains to be evaluated.

Most importantly, whether these microbiota might directly modulate the anti-tumor immune response, and consequently, impact tumor progression has not been investigated so far. It is intriguing to speculate that specific commensal bacteria present at the tumor site, may activate specific Th17 cells, thus possibly promoting recruitment of CD8+ T cells contributing to a more benign patients clinical outcome.

The comparative analysis of the microbiome in tumors characterized by a different immuno-contexture may provide information regarding the role of the gut flora in shaping immune responses developing in CRC.

Furthermore, the analysis of T cell receptor repertoires of infiltrating lymphocytes and of their reactivity to tumor-associated or microbial antigens might shed light on the potential interplay between tumor- versus bacteria-specific T cell responses occurring within CRC microenviroment.

The implementation of these studies may eventually provide the rationale for developing new potential therapeutic approaches aiming at expanding defined T cell mediated responses upon modulation of the gut flora.

VI. Bibliography

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VII. Appendix

VIII. Acknowledgments

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IX. Curriculum Vitae

Curriculum Vitae

FRANCESCA AMICARELLA

PERSONAL INFORMATION

Name: Francesca Amicarella

Nationality: Italian

Date of birth: 14/12/1983

Address: Breisacherstrasse 6, 4057 Basel, Switzerland

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WORK EXPERIENCE

February 2010-July 2014: PhD student
Research topic: Immunobiology of interleukin-17 in human colorectal cancer.
Department of Biomedicine and Institute of Surgical Research and Hospital Management, Cancer immunotherapy group, University of Basel, Basel, Switzerland.

March 2008 – May 2009: Research technician
4-Antibody AG, Basel Switzerland.
October 2005 - November 2007: Mater thesis training period
Main topic: Characterization of human Th17 cells.
Supervisors: Sergio Romagnani,Francesco Annunziato, Lorenzo Cosmi and Francesco Liotta.
Università degli studi di Firenze, Firenze, Italy.

EDUCATION

December 2007: Master degree in Medical Biotechnology
Degree thesis entitled *"Phenotypic and Functional features of human Th17 cells.* (final mark: 110+lode/110**),**
Supervisors: Sergio Romagnani, Francesco Annunziato, Lorenzo Cosmi and Francesco Liotta.
Università degli studi di Firenze, Italy.

April 2006: Bachelor degree in Biotechnology
Degree thesis entitled *"Detections of CD14+CD34low stem cell in peripheral blood from healthy donors of different age".* (Final Mark 108/110)
Supervisors: Francesco Annunziato and Francesco Liotta.
Università degli studi di Firenze, Italy.

July 2004: Diploma of laboratory technician of genomic analysis
GENOLAB. Università degli studi di Firenze, Italy.

July 2002: Scientific Diploma
Liceo Scientifico "Andrea Bafile" L'Aquila, Italy

LABORATORY SKILLS

- Cell culture (2D/3D), expansion and cloning (stroma cells, endothelial cells, T-cells, B-cells, monocyte/macrophage, murine tumors cell lines)
- Immunomagnetic cell sorting
- Multiparameter flow cytometry
- Migration assay
- ELISA test
- Preparation of single-cell suspension form human clinical samples
- Generation of human T-cell clones
- Immunofluorescence
- DNA analysis with restriction endonucleases
- DNA cloning in plasmidic vectors
- Extraction of plasmidic DNA from bacteria
- RNA and DNA electrophoresis in agarose gel
- DNA purification from agarose gel
- Amplification of DNA sequences by PCR
- Bacterial transformation with plasmic vectors

MEMBERSHIPS

Member of American Association for Cancer Research

GRANTS

2014 Krebs Liga Beider Basel (KLBB). **Amicarella F**. (main applicant), Iezzi G.
Project: Role of IL-17-producing T-helper cells in human colorectal cancer. CHF 6`000

PUBLICATIONS

Ectosomes released by platelets induce the differentiation of naïve CD4+ T cells into T regulatory cells
Amicarella F, Sadallah S, Eken C, Iezzi G. and. Schifferli JA . *JTH*. 2014

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Clinical impact of programmed cell death ligand 1 expression in colorectal cancer

Droeser RA, Hirt C, Viehl CT, Frey DM, Nebiker C, Huber X, Zlobec I, Eppenberger-Castori S, Tzankov A, Rosso R, Zuber M, Muraro MG, **Amicarella F**, Cremonesi E, Heberer M, **Iezzi G**, Lugli A, Terracciano L, Sconocchia G, Oertli D, Spagnoli GC, Tornillo L. *Eur J Cancer.*2013.

Submitted:

Tumor infiltrating Th17 cells contribute to the recruitment of immune cells associated to favorable prognosis in human colorectal cancer

Francesca Amicarella, Manuele Giuseppe Muraro, Christian Hirt, Eleonora Cremonesi, Valentina Mele, Valeria Governa, Junyi Han, Xaver Huber, Raoul A. Droeser, Markus Zuber, Michel Adamina, Raffaele Rosso, Alessandro Lugli, Inti Zlobec, Luigi Terracciano, Elisabetta Padovan, Paul Zajac, Serenella Eppenberger-Castori, Francesca Trapani, Daniel Oertli and Giandomenica Iezzi.

PARTECIPATION AT CONFERENCE AND SYMPOSIA:

Tumor infiltrating Th-17 cells promotes the recruitment of cytotoxic CD8+ T cells into human colorectal cancer. Amicarella Francesca, Trapani Francesca, Cremonesi Eleonora, Muraro Manuele Giuseppe, Mele Valentina, Elisabetta Padovan, Adamina Michel, Zuber Markus, Huber Xaver, Oertli Daniel, Raoul Droeser, Lugli Alessandro, Zlobec Inti' Terracciano Luigi M., Zajac Paul, Spagnoli Giulio C. , Eppenberger Serenella, and Iezzi Giandomenica***. Basel Onco-Day*** *13 June 2013.* ***Oral presentation***

Tumor infiltrating Th-17 cells promotes the recruitment of cytotoxic CD8+ T cells into human colorectal cancer. Amicarella Francesca, Trapani Francesca, Cremonesi Eleonora, Muraro Manuele Giuseppe, Mele Valentina, Elisabetta Padovan, Adamina Michel, Zuber Markus, Huber Xaver, Oertli Daniel, Raoul Droeser, Lugli Alessandro, Zlobec Inti' Terracciano Luigi M., Zajac Paul, Spagnoli Giulio C. , Eppenberger Serenella, and Iezzi Giandomenica***. WIRM-VIII****, Davos 13-16 March 2013.* ***Oral presentation***

IL-17 production by tumor-infiltrating Th17 cells favors inflammation and promotes survival in human colorectal cancer. Francesca Amicarella, Michel Adamina, Markus Zuber, Xaver Huber,Daniel Oertli, Alessandro Lugli , Inti Zlobec, Francesca Trapani, Serenella Eppenberger, Luigi M. Terracciano, Paul Zajac, Giulio C. Spagnoli1 and Giandomenica Iezzi. ***AACR Annual Meeting****, Chicago, IL, 1-4 April 2012 (poster presentation)*

Prevalence, phenotype and prognostic significance of IL-17-producing cells infiltrating human colorectal cancers. F. Amicarella, I. Zlobec, M. G. Muraro, J. Han1, X. Huber, M. Zuber, D. Oertli, A. Lugli, G.C. Spagnoli and G. Iezzi. ***WIRM-VI****, Davos 18-21 March 2012 (poster presentation)*

Prevalence, phenotype and prognostic significance of IL-17-producing cells infiltrating human colorectal cancers. F. Amicarella, I. Zlobec, M. G. Muraro, J. Han1, X. Huber, M. Zuber, D. Oertli, A. Lugli, G.C. Spagnoli and G. Iezzi. ***45th Congress of the European Society for Surgical Research*** *(ESSR), Geneva (Switzerland), 9-12 June 2010* ***Oral presentation***

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IL-17 promotes the differentiation of human T-helper 17 cells by enhancing IL-1b and IL-23 secretion from activated monocytes. Xaver Huber, **Francesca Amicarella**, Nermin Rafaat, Raoul Droeser, Giulio C. Spagnoli and Giandomenica Iezzi. ***ECI,*** *13-16 September Berlin 2009 (poster presentation)*

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Italian: native
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Spanish: basic