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Novel mechanisms regulating CCRL2 expression and function

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Dedico questa tesi a mia nonna Graziella.

Dedico questa tesi anche a tutti coloro che fanno quotidianamente a pugni con la salute mentale.

ABSTRACT

CCRL2 is a seven transmembrane domain receptor, structurally and functionally related to the atypical chemokine receptors family. However, the only recognized ligand for this receptor is the chemotactic protein chemerin. To date, two main functions were described to be associated to CCRL2. The first one is to act as presenting molecule when expressed on the surface of epithelial barrier cells, where it can concentrate chemerin, thus creating a membrane-bound chemerin gradient able to regulate the migration of adjacent leukocytes expressing the chemerin functional receptor Chemerin1. On the other hand, CCRL2 has been shown to regulate leukocytes migration through the interaction with conventional chemokine receptors. Indeed, by forming heterodimers, CCRL2 can regulate the functionality of the prototypical neutrophil chemokine receptor CXCR2. CCRL2 functionality was also investigated *in-vivo* in models of inflammatory diseases and carcinogenesis, providing important insights of its role in different pathological conditions.

Part I

Recently, a novel possible function for CCRL2 was described: the interaction with TLR4. This interaction is functional to induce the retention of TLR4 on the plasma membrane of tumor associated macrophages, thus promoting the maintenance of a pro-inflammatory TLR4-mediated signaling, preventing the switch towards a M2-like anti-inflammatory phenotype. Thus, TAMs could retain a pro-inflammatory anti-tumoral M1-like phenotype.

The first part of this thesis will elucidate the functional relationship between CCRL2 and other TLRs, specifically TLR7. The functional interaction between CCRL2 and TLR7 was investigated by using an *in vitro* model of bone-marrow derived dendritic cells (BMDCs) generated by $CD34^+$ hematopoietic bone marrow precursors, obtained from WT and CCRL2 knockout (KO) mice. We focused our attention on dendritic cells (DCs) as they are the main antigen

presenting cells, playing a crucial role in bridging innate and adaptive immune responses. WT and CCRL2 KO BMDCs were then stimulated with TLR7 agonist R848, with the purpose of performing a phenotypical and functional characterization. Our results displayed an impaired secretion of pro-inflammatory cytokines in CCRL2 KO DCs after TLR7 triggering, suggesting CCRL2 may be involved in the regulation of TLR7 mediated pro-inflammatory signaling. Moreover, on the one hand, flow cytometry experiments showed that the impaired pro-inflammatory secretion was not due to a defective maturation of CCRL2 KO cells. On the other hand, qPCR and Western Blot experiments demonstrated that CCRL2 does not affect TLR7 expression. Thus, given the crucial role of NF-κB in the production of pro-inflammatory cytokines, NF-κB translocation to the nucleus was assessed via Western Blot. The translocation of NF-κB to the nucleus was found impaired in CCRL2 KO BMDCs compared to WT BMDCs. These results suggest CCRL2 may play a crucial role in the NF-κB phosphorylation, thus impairing the pro-inflammatory response in DCs and affecting the secretion of pro-inflammatory cytokines in the supernatant.

Part II

Colorectal cancer (CRC) is the fourth most deadly and the third most common cancer worldwide with approximately 900’000 diagnosis per year, accounting around 10% of cancer-related deaths worldwide. Despite the role of CCRL2 in carcinogenesis is still under investigation, in a murine model of lung cancer it was demonstrated that its ablation reduced the recruitment of several myeloid cells subsets and NK cells in the tumor microenvironment, thus positioning CCRL2 as a tumor-suppressor gene. Inflammation represents one of the key drivers of CRC and preliminary data, generated in our laboratory, related to AOM/DSS murine model of inflammation-associated carcinogenesis, have

shown that CCRL2 KO mice are more susceptible to the development of intestinal polyps in comparison to WT control animals. Moreover, previous results suggest a correlation between low CCRL2 expression and poorer survival rate in patients. Taken together, these findings suggest that CCRL2 may play a role as a novel tumor-suppressing gene, whose silencing promotes carcinogenesis.

One of the most relevant mechanisms of tumor-suppressor gene silencing is represented by DNA methylation. On this basis the aim of the second part of this thesis was to investigate CCRL2 methylation-mediated silencing in CRC. The model in which we decided to investigate CCRL2 methylation in CRC were human CRC cell lines that expressed (RKO) or did not express (SW480) CCRL2. The level of methylation of CCRL2 was investigated by two different approaches: an indirect method consisting in the treatment with a demethylating agent, 5'-Aza-2'-deoxicytidine (5-AZA-dC) and a direct method based on the immunoprecipitation of methylated DNA (MeDIP). Our results showed high levels of methylated DNA in the transcription factors binding site (TFBS) region of CCRL2 putative promoter in SW480 cells. In contrast, RKO cells, which were found to express CCRL2 in normal conditions, showed low levels of methylated DNA in the TFBS region. In fact, the treatment with 5-AZA-dC, led to an increase of CCRL2 expression in SW480 cells, but not in RKO cells.

Moreover, we set the basis for the analysis of histone modifications in CCRL2 TFBS region by chromatin immunoprecipitation (ChIP), targeting the tri-methylated fourth lysine residue of H3 histone (H3K4me3), a modification associated to transcriptional activation. In accordance with the results obtained by MeDIP, SW480 cells showed lower levels of H3K4me3 compared to RKO cells. Thus, our results support the hypothesis of CCRL2 epigenetic silencing in the context of CRC and lay the foundation to investigate the role of CCRL2 as a novel tumor-suppressor gene.

INDEX

1. General introduction: the atypical receptor CCRL2 1

1.1. Chemokines and chemokines receptors **2**

1.2. Chemokines receptors: classification and functionality **5**

1.2.1. Features and functionality of GPCRs **6**

1.2.2. Features and functionality of ACKRs **7**

1.3. CCRL2: the atypical among the atypicals **11**

1.3.1. Structure and expression of CCRL2 **11**

1.3.2. CCRL2 functionality **13**

1.3.3. CCRL2 creates a chemerin gradients for leukocytes expressing its functional receptor (Chemerin1) **14**

1.3.4. CCRL2 can form heterodimers with other chemokine receptors **16**

1.3.5. CCRL2 and its functional partnership with Toll-like Receptors **16**

Part I: The role of CCRL2 in the regulation of TLR7 mediated murine Dendritic Cells activation 18

2. Introduction (Part I) 19

2.1. Dendritic cells (DCs) **19**

2.2. Classification of DCs subsets **19**

2.3. DCs activation and immune response **20**

2.4. Toll-like Receptors (TLRs) **22**

2.4.1. General features of TLRs **22**

2.4.2. TLR7 activation **24**

2.4.3. Potential roles of TLR7/8 triggering in humans **26**

3. Aim (Part I) 28

4. Materials and methods (Part I) 30

4.1. Animals **31**

4.2. $CD34^+$ cells isolation **31**

4.3. Bone Marrow-derived Dendritic Cells (BMDCs) culture and differentiation from $CD34^+$ cells **32**

4.4. Flow cytometry analysis **32**

4.5. BMDCs stimulation (kinetic ELISA, kinetic qPCR and kinetic Western Blot) **33**

4.6. RNA isolation and qPCR **33**

4.7. Cytokines measurement **35**

4.8. SDS-PAGE and Western Blot **35**

4.9. Statistical analysis **36**

5. Results (Part I) 37

5.1. Assessment of basal expression of TLRs in bone marrow-derived DCs (BMDCs) **38**

5.2. Pro-inflammatory cytokines production is impaired in CCRL2 KO BMDCs stimulated with R848 (TLR7 agonist) **39**

5.3. Maturation of BMDCs stimulated with TLRs agonists **40**

5.4. Assessment of the kinetics of cytokine expression and secretion in WT and CCRL2 KO BMDCs stimulated with R848 **42**

5.5. Upregulation of TLR7 expression levels after R848 stimulation **44**

5.6. Reduced translocation of NF-kB transcription factor in the nucleus in CCRL2 KO BMDCs **46**

6. Discussion (Part I) 49

Part II: study of CCRL2 gene methylation in Colorectal Cancer (CRC) **55**

7. Introduction (Part II) **56**

7.1. Colorectal Cancer (CRC) **56**

7.2. Chromosomal Instability (CIN) **59**

7.3. Microsatellite Instability (MSI) **59**

7.4. CpG island methylator phenotype (CIMP) **60**

7.5. Histone modifications **62**

7.5.1. The role of histone methylation in CRC **63**

7.5.2. The role of histone acetylation in CRC **67**

7.6. The relationship between histone and DNA methylation **69**

7.7. The role of 5-AZA-dC in the therapy of cancer and in the study of CCRL2 regulation **70**

7.8. The role of inflammation in colorectal tumors **71**

7.9. The role of CCRL2 in cancer and colorectal inflammation **73**

8. Aim (Part II) **76**

9. Materials and methods (Part II) **78**

9.1. Cell cultures and treatments **79**

9.2. Flow cytometry and immunofluorescence analysis **79**

9.3. Immunohistochemistry **80**

9.4. RNA isolation and qPCR **81**

9.5. MeDIP (Methylated DNA Immunoprecipitation) **82**

9.6. ChIP (Chromatin Immunoprecipitation) **82**

9.7. Statistical analysis **84**

10. Results (Part II) 85

10.1. Analysis of CCRL2 expression in biopsies from healthy and tumor tissue from CRC patients **86**

10.2. Analysis of CCRL2 expression in CRC cell lines **87**

10.3. Study of CCRL2 methylation in SW480 and RKO cell lines **88**

10.3.1. Indirect study of CCRL2 methylation: treatment of the cells with 5-AZA-2'-Deoxycytidine (5-AZA-dC) **88**

10.3.2. Study of the methylation of CCRL2 with direct methods: the Research of CpG islands within CCRL2 genomic locus **92**

10.3.3. Choice, design and validation of the primers for MeDIP **93**

10.3.4. The methylation of control genes was assessed by MeDIP to validate the protocol for the investigation of CCRL2 methylation in CRC cell lines (RKO and SW4080). **95**

10.3.5. Analysis of CCRL2 methylation by MeDIP in CRC cell lines (RKO and SW4080) **96**

10.4. Analysis of the methylation of H3 histone in the promoter of CCRL2 in CRC cell lines **98**

11. Discussion (Part II) 100

REFERENCES 104

ACKNOWLEDGEMENTS 129

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1.GENERAL INTRODUCTION: THE ATYPICAL RECEPTOR CCRL2

1.1. Chemokines and chemokine receptors

Chemokines are the main regulators of leukocyte migration, a key process for both innate and adaptive immunity, governing their recruitment, migration, localization and distribution, which are crucial for immune cell priming, effector functions, memory responses and immune regulation [1]. "Chemokines" is a word made up from the terms "**chemo**tactic" and "cyto**kines**" and they are a large family of secreted cytokines that signal within the cell through G protein-coupled seven transmembrane chemokine receptors expressed on the cell surface. Several types of migratory behaviors can be stimulated by chemokines (chemotaxis hapotaxis, chemokinesis and haptokinesis) and, moreover, they can induce cell arrest and adhesion [1].

Besides migration, chemokines also influence leukocyte survival and effector functions such as proliferation, differentiation, cytokines production and degranulation both in homeostatic and pathological conditions [1]. Chemokines indeed are not only produced in response to inflammatory conditions (infections, autoimmune diseases, cancer, etc.) but they are also pivotal to immune surveillance in homeostatic conditions, which requires constitutive expression of chemokines to guarantee the regular trafficking of leukocytes from the bone marrow, lymph nodes and thymus into and out of peripheral tissues [1]. For instance, CCL19, CCL21 and CXCL13 are constitutively expressed in secondary lymphoid organs and define the spatial interaction of dendritic cells, T cells and B cells within the lymph nodes. The chemoattractant receptors are additionally responsible for lymphocyte recirculation and homing into lymphoid organs and for the organization of functional lymphoid microenvironments during lymphoid organogenesis [1,2]. CXCL12 is also a constitutively expressed chemokine, with a crucial role in hematopoiesis is the fundamental signal that maintains $CXCR4^{+}$ HSCs within niches. Thus, cells

migration in and out of the BM follows opposite chemokine signals and different expression levels of their cognate chemokine receptors [2–4].

Chemokines are involved in several inflammatory diseases: when a damage or an infection occurs, the rapid recruitment of cells belonging to the innate immunity is essential to confine and kill pathogens, to avoid microbial dissemination, start the inflammatory process and lead to the repair of the damaged tissue. A wide range of chemokines can be secreted in response to several infectious and inflammatory stimuli [1]. This redundancy is aimed to fulfil the wide range of biological functions they own besides leukocyte migration, since they can also influence immune cell survival and effector functions such as proliferation, differentiation, cytokine production and degranulation both in homeostatic and pathological conditions [1].

Chemokines are a super-family made up of around 50 endogenous ligands, which is based on the structure, characterized by a single polypeptide chain of 70-100 amino acids in length (8 – 12 KDa) [5–8] and conserved couples of cysteine residues linked by disulphide bonds: a central three stranded β-sheet, a C-terminal α-helix and a short N terminus that plays a pivotal role in receptor activation, shaping together the characteristic “chemokine fold” [1,9]. Based on the cysteine residue number and location, four chemokine subfamilies have been identified (Figure 1). The largest family of chemokines has the first couple of cysteines (out of four in total) adjacent to each other (CC chemokines). The CX3C chemokines instead have three amino acids between the two cysteines, CXC have only one amino acid and, if there’s a single couple of cysteines, the chemokine is called XC. Nevertheless, another class of chemokines (XC) identified in zebrafish [10], lacks one of the two N terminus cysteines but maintains the third and fourth ones, but there is no evidence that this is present also in mammals [11]. Originally, chemokines were named after their specific function, but later in 2000 a new systematic nomenclature strategy that includes a subfamily designation (CXC, CXCR, CX3C or XC) followed by the

letter “L” (ligand) and a number according to when the coding gene was identified, was introduced [12].

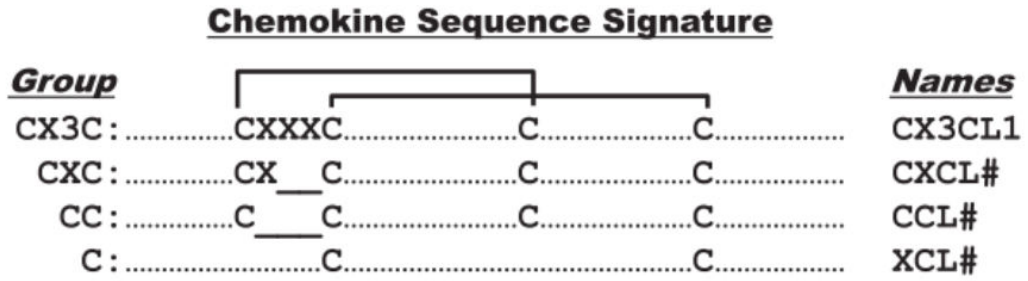

***Figure 1**. Classification of chemokines according to their primary structure* [13]

Chemokine-binding receptors are coupled with G proteins and featured by a seven transmembrane domains structure and are categorized as CCR, CXCR, CX3CR and XCR according to the class of chemokines they bind (“R” stands for “receptor”).

Chemokines can also be classified according to their production and role in homeostatic conditions (such as CXCL12, CXCL13 and CCL21) and inflammatory conditions (such as CCL2, CCL5 and CXCL8) [6]. In the first case, chemokines are secreted constitutively in organs like the bone marrow (BM), lymph nodes (LNs) and thymus, assuming a role in normal leukocyte recruitment and distribution. In the second case, chemokines are released by specific cell types in inflammatory and infectious conditions, thus creating a complex pattern of chemokines expression related to several inflammatory diseases both in human and in mice [6].

1.2. Chemokine receptors: classification and functionality

Surface receptors for chemokines represent the largest branch of the γ subfamily of group-A seven transmembrane domain receptors (7TMRs) [13] and allow chemokines to exert their biological function on the target cells. Chemokine receptors (CKRs) mediate several functions, such as immune surveillance, by regulating cell migration, adhesion, growth and survival. Different types of leukocytes express different arrays of chemokine receptors making them able to respond to specific ligands. The ligand-receptor interaction is often promiscuous, since each chemokine can bind more than one receptor and vice versa. In Figure 2, chemokines and the receptors (typical and atypical) are summarized.

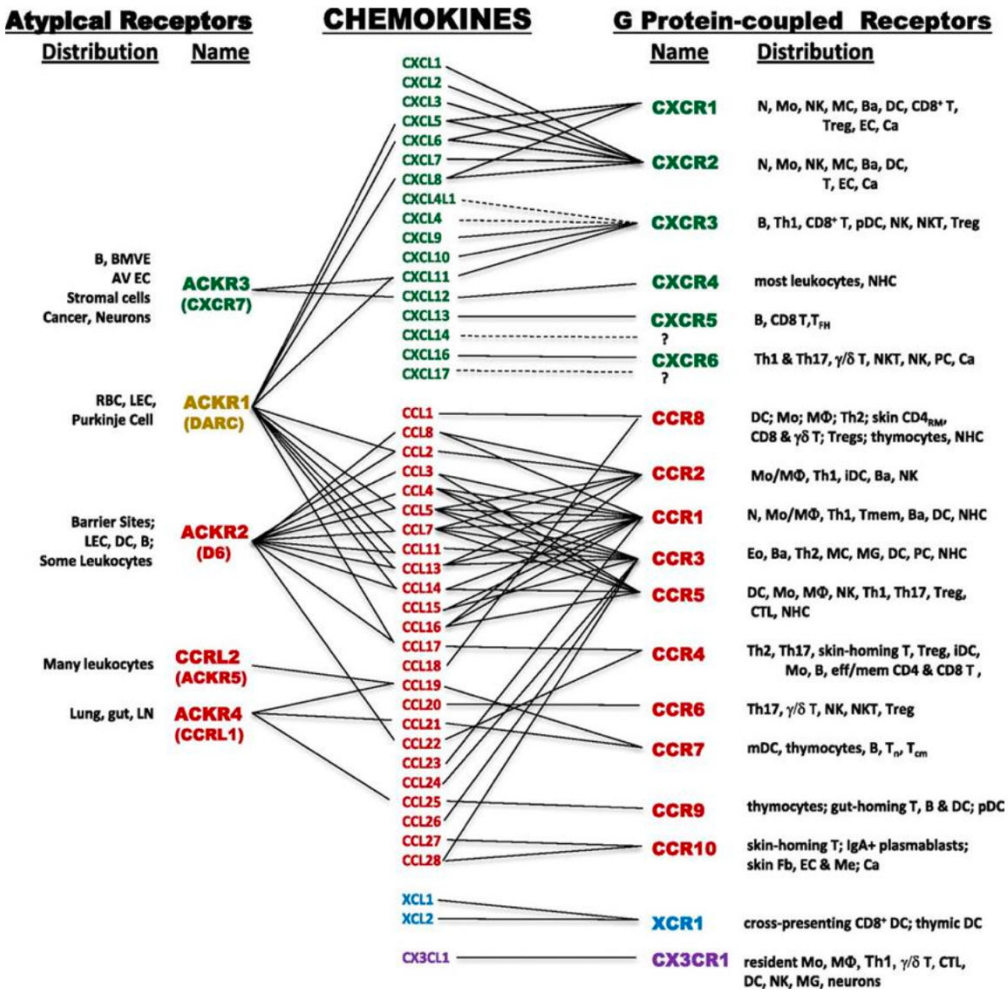

***Figure 2**: A summary of the binding profile of typical and atypical chemokine receptors* [13]

The fact that a single chemokine can interact with multiple receptors and vice versa is evolutionally aimed at the contrast of exogenous pathogens through the enhancement and amplification of the recruitment of leukocytes during infections [1]. Mammalian coding sequences for chemokines receptors are around 20 and were discovered after their ligands (chemokines). Moreover, each one of them is selective for a chemokine subfamily and for this reason they are classified based on the subfamily of chemokines to which their ligands belong. For example, chemokine receptors are named CCR, CXCR, CX3R, and XCR followed by an identifying number [14]. Chemokine receptors are classified in two main groups: G-coupled Chemokine Receptors (GPCRs) or Conventional Chemokine Receptors (cCKRs) and Atypical Chemokine Receptors (ACKRs). Moreover, for years it was though that cCKRs functionally and physically existed as monomers, but nowadays, thanks to studies exploiting Fluorescence and Bioluminescence resonance energy transfer *in vitro* (FRET and BRET) it is well known that they can function as homodimers, heterodimers or even higher order oligomers [15] as a mechanism of regulation of leukocyte migration.

1.2.1. Features and functionality of GPCRs

G-coupled Chemokine Receptors (GPCRs), that signal through the activation of the associated G-protein, are also known as Conventional Chemokine Receptors (cCKRs).

Currently, 18 cCKRs have been described, including 10 CCRs, 6 CXCRs and a single CX3CR and XCR. Receptor specificity is redundant and complex: different chemokines interact with multiple cCKRs, and a cCKR can bind different chemokines. Anyway, all the chemokines that interact with a cCKR may bind it with different affinities, thus differentiating the activated pathways depending on the affinity of the specific ligand [1,16]. Nevertheless, cases in which a cCKR binds a non-chemokine ligand are also described: for example, β-defensins can

activate CCR6 [17] and the “alarmin” HMGB1 (High Mobility Group box 1 protein) is emerging as a CXCR4 ligand [1,18].

cCKRs share a conservate DRYLAIV motif (Asp-Arg-Tyr-Leu-Ala-Ile-Val), positioned in the second intracellular loop. This motif is pivotal in the binding of G proteins, initiating the activation of intracellular signaling [13,19]. In their inactive state CKRs are coupled to a heterotrimeric G protein composed of α, β and γ subunits [20]. After the interaction with their ligand, cCKRs undergo conformational changes that lead to the exchange of GDP for GTP at the α subunit, causing the separation of the α subunit from βγ dimers [13]. The α subunit activates Phospholypase C (PLC), an enzyme associated to the cell membrane. PLC triggers a cascade of messengers that causes the release of intracellular calcium (Ca^{2+}), that regulates several cell responses that converge in cell polarization, integrin activation and chemotactic migration [12]. The Gβγ subunits can regulate a number of effectors, including phospholipase (PLC) β2/3, phosphatidylinositide 3 (PI3K)γ, ion channels, G protein coupled receptor kinases (GRK), extracellular signal-regulated kinases (ERK1/2) and some adenylyl cyclases (Figure 3). Downstream, low molecular weight GTP-binding proteins (for example Rac, Rho, Cdc42) can be activated to mediate the final effects of receptor activation, such as actin polymerization, adhesion and membrane protrusion [21]. To start moving actively, cells must acquire a precise polarization, resulting in an elongated shape featured by a larger front and a smaller and narrower back. This is due to the formation of a F-actin rich lamellipodia at the leading edge and actomyosin structure in the back to allow cell locomotion [21].

1.2.2. Features and functionality of ACKRs

Atypical Chemokine Receptors (ACKRs) regulate the chemokine system and leukocyte migration, modulating chemokine gradients through the removal

from the environment of their own ligand without an associated G-protein signaling [22]. They are characterized by a high grade of homology with conventional chemokine receptors, indeed ACKRs can bind chemokines [23]. ACKRs shares a series of features that can be summarized as follows [22]:

- As cCKRs they are seven-transmembrane spanning structures.
- Demonstrated affinity to multiple chemokines (Figure 2).
- The structural key difference with cCKRs is the lack of a conserved DRYLAIV motif, responsible for the G-protein mediated signaling [23].
- Uncoupling from conventional signaling pathways triggered by cCKRs that lead to directed cell migration.
- A role in the regulation of the bioavailability of chemokines [22].
- ACKRs are predominantly expressed by endothelial cells or stromal cells [24–29] although they are also expressed by leukocytes [30–34].

The original functional paradigm regarding ACKRs described them as “silent” receptors, given their impossibility to signal through a canonical pathway after binding their ligand [35–39]. Nevertheless, some of them are now well known and characterized for their “scavenger” or “decoy” functions [40]. In fact, some ACKRs are responsible for scavenging, sequestration or transport of chemokines across endothelial cells to shape the bioavailability of chemokines for migrating cells [22]. ACKRs are generally linked to endocytic machinery via GPCR kinases (GRKs) and β-arrestins [40] (Figure 3). Following the internalization from plasma membrane, vesicles may deliver their cargo to different destinations: degradation in the lysosomal compartment [41] or, for what concerns polarized cells, re-transportation on the opposite side of the cell monolayer [23]. These receptors are no longer considered “silent” since despite they do not mediate $G\alpha_i$ signal transduction, they typically recruit β-arrestins, that tightly links to the endocytic machinery or means they are constitutively recycling [42–45]. ACKRs can also mediate the triggering of signaling pathways of β-arrestins, such as

MAPK related downstreams [46]. This is the case of ACKR2 that following the interaction with its ligand leads to the rearrangement of microtubules, required for the efficient return of ACKR2 to the surface membrane with consequences on its scavenging functions [47] or AKT phosphorylation [48]. Also, β-arrestin-independent internalization routes are emerging [43,45,49–52] but investigations are still ongoing. However, the main recognized mechanism of internalization involves the clathrin recruitment with β-arrestins 1/2 as adaptor [53]. This mechanism has been well characterized for ACKR2 which, through endosomal vesicle recycling, progressively regulates the gradient of chemokines [54].

The nomenclature "ACKR #" for this group of chemokine receptors was recently approved by the Union of Pharmachology and Human Genome Organization Gene Nomenclature Committee [55]. In humans six receptors are included in this family: ACKR1/DARC, ACKR2/D6, ACKR3/CXCR7, ACKR4/CCRL1, ACKR5/CCRL2 and ACKR6/PITPNM3 [22,56]. Le Mercier et al. in 2021 published a paper in which it is shown that GPR182 is a new atypical chemokine receptor for CXCL10, CXCL12 and CXCL13. GPR182 appears to be involved in the regulation of hematopoietic stem cell homeostasis where it acts like a scavenger in a ligand independent manner [22,57]. ACKR1 and ACKR2 bind to a range of CC- and CXC-mostly inflammatory chemokines [58]. ACKR1 is the most promiscuous chemokine receptor, which binds over 20 chemokines and is expressed by erythrocytes and endothelial cells [23]. For example, it recognizes the potent neutrophil chemoattractant CXCL8, but it was found it also binds CXCL12. Therefore, ACKR1 may have a role in regulating CXCL12 activity, associated to leukocytes mobilization from the bone marrow, development and recruitment during immune surveillance and inflammation [22]. However, the complete range of ACKR1 functionality still has to be elucidated. ACKR2 has scavenging activity on most pro-inflammatory CC chemokines [59–61], despite recently it was demonstrated that its range of interaction is extended to CXCL- chemokines, in

particular CXCL10 [62]. ACKR2 is expressed by lymphatic endothelial cells but also by some leukocyte subtype such as DCs and innate-like B cells [30]. CXCL10 is also a ligand for CXCR3, a major contributor to T cell recruitment and localization to inflammatory sites [63]. ACKR3 can bind CXCL12 with a greater affinity in comparison to its conventional receptor CXCR4 and also CXCL11 [59,64], and is the only ACKR which ablation was found incompatible with life in mice [65–67]. Homeostatic chemokines CCL19, CCL21 and CCL25 show high affinity for ACKR4 [54], thus it was postulated that ACKR4 would regulate migratory responses driven through CCR7 (typically activated by CCL19 and CCL21) and CCR9 (activated by CCL25). ACKR4 therefore plays a crucial role in the regulation of leukocytes migration [68–71], for DC recruitment into afferent lymphatic (CCR7) [72–74], for T cell entry, localization and retention in secondary lymphoid tissues (CCR7) [75,76], for leukocyte migration to the intestine (CCR9) [77] and for immune responses in the follicular microenvironment [78,79]. ACKR6 only has one identified ligand, that is CCL18, and it belongs to the family of Phosphatidyl-Inositol Transfer Proteins (PITPs). Finally, ACKR5/CCRL2 only has one confirmed ligand: a chemoattract protein chemerin [80].

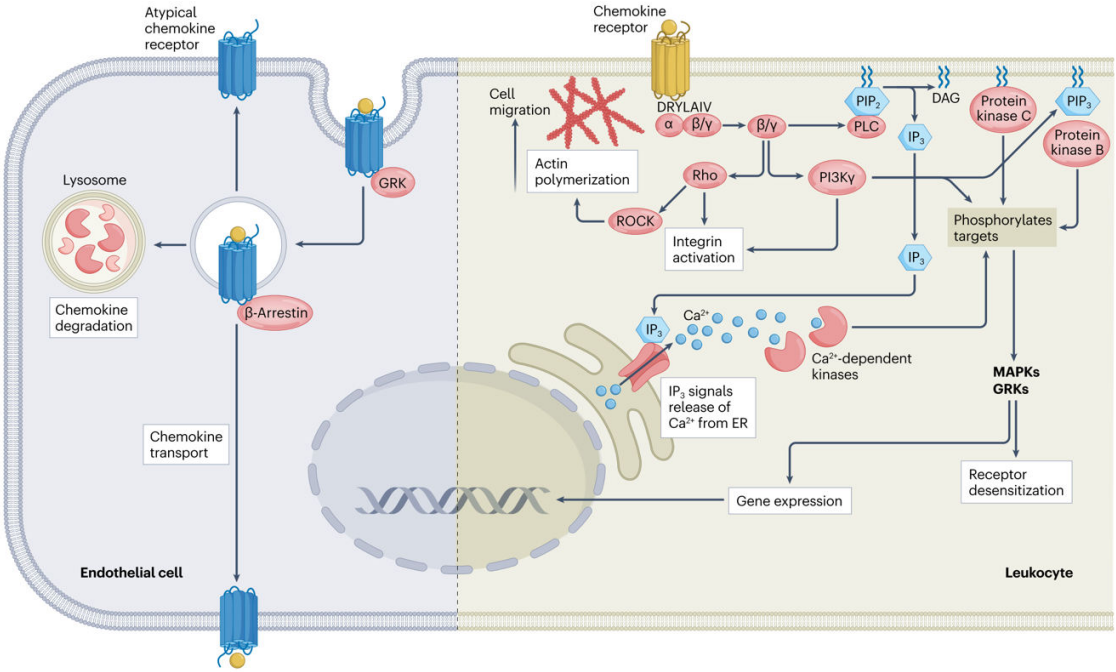

***Figure 3**: Typical and atypical chemokine receptors signaling pathways following the binding of their ligands* [22]

It is important to say that CCRL2 classification as an atypical chemokine receptor is still pending, since it never confirmed its ability to bind chemokines. Chemerin is a protein belonging to the family of adipokines which inactive precursor (pro-chemerin) can be found in the blood of healthy donors but is significantly upregulated in several pathological conditions such as infections, metabolic disorders and autoimmune diseases such as psoriasis, lupus erythematosus, rheumatoid arthritis and Chron disease [81].

1.3. CCRL2: the atypical among the atypicals

In 1998 Fan et al. [82] first described and characterized a new human gene featured by a high homology with chemokine receptors that was called "Human Chemokine Receptor" (HCR). Later, Shimada et al. identified in mouse a novel chemokine receptor displaying structural similarities with ACKRs and subsequently assessed 40% homology with the sequence of CCR1, CCR2, CCR3 and CCR5 [56] called L-CCR (LPS inducible C-C chemokine receptor). L-CCR was found upregulated by LPS in RAW264 cell line (a murine macrophage cell line) [83]. Nowadays this gene is known as CCRL2 (Chemokine CC-motif Receptor Like 2).

1.3.1. Structure and expression of CCRL2

CCRL2 is a seven transmembrane receptor indicated as a member of the ACKR family, localized in the 3p21-23 region on chromosome 3, within the cluster of chemokine receptors coding sequences, aside the genes coding for CCR1, CCR5, CCR8 and CCR10 [84]. L-CCR (Lipopolysaccharide-inducible CC chemokine receptor), how the murine ortholog was originally named, was localized on chromosome 9 [83]. In humans, two different CCRL2 splice variants are expressed: CCRL2A and CCRL2B (also known as CRAM-A and CRAM-B, where

CRAM stands for Chemokine Receptor for activated macrophage) [56,85]. On the other hand, murine CCRL2 consists of a single variant corresponding to CCRL2B. The hypothesis is that the two human isoforms may be differentially expressed and regulated: for instance, CCRL2A expression is limited to pre-B cells, while other stages of the maturation of B cells mainly express CCRL2B [86]. Moreover, CCRL2A isoform can be specifically upregulated in certain pathological conditions, such as in breast cancer by IFNγ [87]: these findings suggest the two isoforms may own different biological roles and significance, but investigations are still ongoing.

CCRL2 is expressed by cells belonging to both hematopoietic and non-hematopoietic compartment. For what concerns the hematopoietic cells, in humans, both CCRL2A mRNA and protein is expressed in monocytes, macrophages, neutrophils, $CD4^+$ and $CD8^+$ T lymphocytes, B cells, monocytes-derived dendritic cells (moDCs), murine bone marrow-derived dendritic cells (BMDCs) and $CD34^+$ cells [56,86,88–93]. In most cases, CCRL2 expression is triggered by proinflammatory stimuli: it was shown that both CCRL2 mRNA and protein are upregulated in murine BMDMs activated with LPS (TLR4 ligand)[94], Poly (I:C) (TLR3 agonist) and CD40L [95]. Moreover, also in human neutrophils the expression of CCRL2 increases following proinflammatory stimuli such as LPS or TNFα alone, in combination with IFNγ or GMCSF [90] and in neutrophils isolated from inflamed joints of Rheumatoid Arthritis patients [91]. A similar regulation of CCRL2 expression was shown also in murine neutrophils [94]. Nevertheless, in murine mast cells, CCRL2 was found to be constitutively expressed but further upregulated *in vitro* in BM-derived cells [80]. Also microglia was demonstrated to express CCRL2 both *in vivo* and *in vitro* in inflammatory conditions [96]. For what concerns the non-hematopoietic compartment, CCRL2 mRNA was detected in inflamed bronchial epithelium [97], in hepatic stellate cells [98], in adipocytes [99], in skin [100], astrocytes[101] and in different cancer tissues including breast [87] and prostate cancers [102]. Moreover, a single cell RNA

sequencing (scRNA-seq) study in mice identified CCRL2 as the hallmark of general alveolar lung capillary endothelial cells [103]. Proinflammatory stimuli (e.g. LPS, TNFα, IFNγ) also upregulates CCRL2 in primary human endothelial cells [104]. In freshly isolated lung endothelial cells CCRL2 was found constitutively expressed, while in mouse liver it was found upregulated by proinflammatory stimuli [104]. CCRL2 upregulation was also demonstrated *in vitro* in lymphatic endothelial cells stimulated with retinoic acid [105]. In general, the organ specific regulation of CCRL2 may suggest specific functional properties according to different anatomical districts.

1.3.2. CCRL2 functionality

Just like the other ACKRs, CCRL2 is unable to activate conventional G protein dependent signaling and to activate cell directional migration, given the absence of the canonical high conserved DRYLAIV motif. In fact, in the domain of the third transmembrane twist domain, CCRL2 displays an altered amino acids sequence in comparison to cCKRs (QRYLVFL) [106]. In the past, studies suggested several ligands for CCRL2, like CCL2, CCL5, CCL7, CCL8 [92,107] or CCL19 [108], but these findings were not confirmed by later papers [80,109,110]. For this reason, its classification as ACKR still remains unclear. So far, the only commonly accepted ligand for CCRL2 is not a proper chemokine, but a non-chemokine pleiotropic chemotactic protein named chemerin [80], a ligand shared with other two receptors [111] (Chemerin1, also called ChemR23 in humans and formerly known as CMKLR1) and Chemerin2 (formerly known as GPR1) [80,110,112–114]. When chemerin interact with CCRL2, no calcium mobilization, ligand internalization or scavenging is triggered [80,106,110]. However, it was demonstrated that CCRL2 plays a role in the modulation of IL8/CXCR2 [94], ERK/MAPK and AKT pathways [115]. This atypical behavior makes CCRL2 a unique member of the non-signaling GPCR chemotactic receptor family.

So far, from a functional point of view, CCRL2 was demonstrated to play a double role in leukocyte trafficking through two main mechanisms: the creation of a chemotactic chemerin gradient attracting leukocytes expressing Chemerin1 and the heterodimerization with other chemokine receptors regulating leukocyte migration [116] (Figure 4).

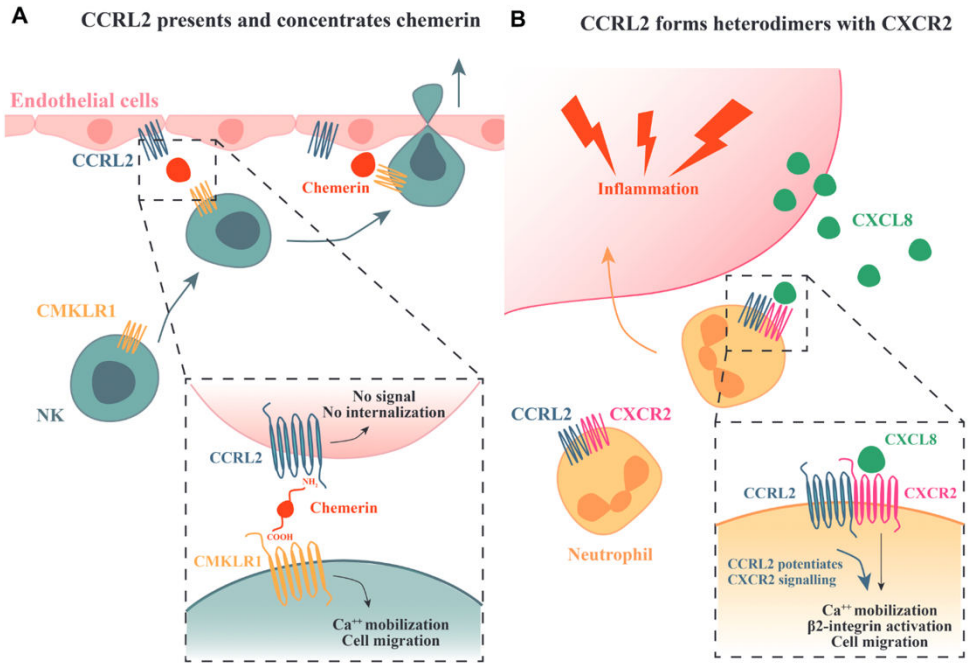

***Figure 4**: The two main mechanisms through which CCRL2 regulates leukocyte trafficking: (A) the creation of a chemotactic chemerin gradient for leukocytes expressing the functional receptor for chemerin and (B) the heterodimerization with other chemokine receptors* [116].

1.3.3. CCRL2 creates a chemerin gradients for leukocytes expressing its functional receptor (Chemerin1)

When CCRL2 is expressed on the surface of endothelial or epithelial barrier cells in proinflammatory conditions, it creates a membrane-bound chemotactic gradient, locally concentrating chemerin for leukocytes expressing the functional chemerin receptor Chemerin 1 [80,104,105,112] (Figure 4A). CCRL2 binds chemerin at the N-terminus leaving the C-terminal portion of the peptide

sequence accessible for the interaction with Chemerin1 [80,104]. Through this mechanism, CCRL2 can promote *in vivo* the recruitment of Chemerin1-expressing cells, such as monocytes, macrophages, dendritic cells, plasmacytoid dendritic cells and NK cells [80,95,104,105,117–121]. This role for CCRL2 was also investigated using CCRL2 knockout (KO) mice tested in several experimental models of inflammatory diseases [94,95,105,122]. For instance, in a model of ovalbumin (OVA) induced airways hypersensitivity, the genetic ablation of CCRL2 caused defective trafficking of antigen-loaded DCs from lungs to mediastinal LNs [95]. Recently, it was also described a possible CCRL2 role in the resolution of the inflammation in the pathological context of the chronic phase of MOG-induced experimental autoimmune encephalitis (EAE), a mouse model that resembles the inflammatory process typical of multiple sclerosis in the central nervous system at the peak of clinical exacerbation of the disease [122]. In this paper it is shown that CCRL2 KO mice had increased mortality and severity of clinical scores in comparison to WT control animals, suggesting a protective role against the exacerbation of the disease for CCRL2. In fact, in CCRL2 KO as compared to WT animals, the histopathological analysis revealed wider demyelination areas and hyperactivation of microglia with unbalanced M1/M2 polarization in macrophages [122], which is a crucial topic in the context of tissue repair and resolution of the inflammation.

Moreover, the polymorphism CCRL2-167F was shown to be associated to a more rapid development of pneumocystis pneumonia (PCP) in HIV patients, probably affecting PCP via immune regulation and induction of the inflammatory process, given its role in leukocyte trafficking [123].

CCRL2 involvement was also observed in the context of *in vitro* experiment on human cancer cell lines, such as prostate and breast cancer, glioblastoma, colorectal cancer (CRC) and in patients with CRC liver metastasis [102,124–126]. CCRL2 involvement was also investigated in different murine models of lung cancer, in which it was observed it plays a crucial role in the recruitment of NK

cells at the tumor site [121]. However, the investigation concerning the role of CCRL2 in cancer is still ongoing and will be further elucidated in the second part of this thesis.

1.3.4. CCRL2 can form heterodimers with other chemokine receptors

CCRL2 is also recognized to play another key role in leukocyte migration through the heterodimerization with other chemokine receptors [116] (Figure 4B). In paragraph 1.2. it was already explained that cCKRs exist in homodimers, heterodimers or even higher orders molecular structures, as demonstrated in studies concerning the crystal structures of chemokine receptors [127]. In general heterodimers can also be formed by a cCKR and an ACKR, as in the case of ACKR3 and CXCR4 [128,129]. The proposed implication of the interaction between CCRL2 and other cCKRs such as CXCR2 is the modulation of the intracellular signaling in response to the cCKR ligand [94]. CCRL2 dimerization with CXCR2 has been described as functional to the regulation of leukocyte migration in a model of inflammatory pathological condition like Rheumatoid Arthritis (RA), where the adoptive transfer of CCRL2 in CCRL2 deficient neutrophils neutralized the protection against the exacerbation of the disease. For this reason, CCRL2 ablation was proposed as a protective factor in RA, given its implication in the recruitment of neutrophils in the joints [94].

1.3.5. CCRL2 and its functional partnership with Toll-like Receptors

A novel functional interaction of CCRL2 with receptors belonging to the toll-like receptors (TLR) family was first proposed by Wei Yin et al. in 2021 [93]. The authors suggested that CCRL2 may mediate a protective effect in a murine melanoma model by heterodimerizing with TLR4. This interaction is associated to a protective role of CCRL2 against tumor growth. In fact, the authors show

that tumors grow faster in CCRL2 KO mice subcutaneously injected with a melanoma cell line compared to WT. CCRL2 in fact may retain TLR4 on the plasmatic membrane of tumor infiltrating macrophages. The maintenance of TLR4 expression on the surface of macrophages indeed enhances the pro-inflammatory Myd88 – NF-κB pathway triggered by tumor-associated TLR4 ligands. This leads to the contrast of the immunosuppressive stimuli in the TME that polarize macrophages towards a M2-like phenotype [130–134], thus maintaining them into a more M1-like pro-inflammatory state, predominant at sites of early oncogenesis. However, the possible roles of CCRL2 in the regulation of the functionality of other TLRs, are still under investigation.

PART I: THE ROLE OF CCRL2 IN THE REGULATION OF TLR7 MEDIATED MURINE DENDRITIC CELLS ACTIVATION

2. Introduction (Part I)

2.1. Dendritic cells (DCs)

Dendritic cells were first observed in human skin by Paul Langerhans in 1868 and identified as neuron cells. A century later, Steinman and Cohn observed these cells in murine spleens and named them “dendritic cells” because of their stellate morphology [135]. DCs are antigen presenting cells (APCs) playing a crucial role in bridging innate and adaptive immune responses [136]. Immature DCs resides in peripheral tissues, where they serve as sentinels. After the antigen uptake, they undergo a process of maturation and start migrating to secondary lymphoid organs. In these sites, they present the antigens to naïve T lymphocytes, regulating antigen-specific immune responses and the mechanisms of tolerance [137].

2.2. Classification of DCs subsets

Given their primary role as APCs, DCs express high levels of major histocompatibility complex II (MHC-II) and lack the typical lineage marker CD3 (T cells), CD19 and CD20 (B cells) and CD56 (NK cells). For these reasons, human DCs are typically referred as HLA-DR^{+} lineage^{-} cells [138].

Different subsets of DCs have been identified and they are classified according to their ontogeny, anatomical location, migration patterns, cytokines secretion and immunological roles, both in humans and in mice [139–141].

The high heterogeneity of the DCs subsets is thought to represent an evolutionary adaptation to provide to the immune system the capability to face different forms of pathogens through the specific fine-tuned and coordinated activation of both adaptive and innate responses [142]. DCs are usually classified in conventional dendritic cells (cDCs), also named myeloid DCs, that include cDC1 and cDC2, and in plasmacytoid dendritic cells (pDCs). cDC1 are specialized

in antigen processing and cross-presentation, being mainly involved in the clearance of tumors and infected cells through the activation of $CD8^+$ T lymphocytes that recognize antigens loaded on MHC-I complexes [143]. cDC2 on the other hand are mostly efficient in the presentation of antigens loaded on MHC-II complexes to $CD4^+$ T cells, regulating Th1, Th2 and Th17 polarizations [144]. cDC1 in mice are identified by surface markers CD11c and MHC-II [145] (more specifically $B220^-$ Sirp-α^- MHC-II^+ $CD11c^+$ $CD11b^{lo}$ $CD24^{hi}$) [146] while in humans they are identified for being $BDCA3^+$ $Gr1^-$ $CD11c^+$ $CD11b^{low/-}$ $XCR1^+$ [146]. cDC2 in mice are characterized by the presence of CD172α and CD11b [147] (more specifically $B220^-$ Sirp-α^+ MHC-II^+ $CD11c^+$ $CD11b^+$ $CD24^{lo}$) [146] while in humans they are identified for being $Gr1^-$ $CD11c^+CD11b^+$ $XCR1^-$ [146]. pDCs, typically expressing CD123, BDCA2, B220 and ILT7 [148] in humans, represent a subset with poor APC behavior that can circulate in the blood and accumulate in lymphoid tissues. In mice they are characterized for being $CD11c^{low/+}$ MHC-II^{low} $B220^+$ $CD11b^{-/low}$ Siglec-H^+CCR9^+ [146]. pDCs play a key role in antiviral response, being the major producers of type I interferons (IFN-I) [149]. Another subset of DCs is represented by monocyte-derived DCs (moDCs), described as a subset of DCs that differentiate from monocytes in inflammatory conditions. In fact, they are also known as inflammatory DCs and are involved in inflammatory diseases such as asthma, rheumatoid arthritis [150], psoriasis, Chron's disease [141,151] and SLE [152].

2.3. DCs activation and immune response

Immature myeloid DCs (mDCs) are phagocytes constantly patrolling the surrounding environment. DCs express multiple pathogen recognition receptors (PRRs), including the TLRs and C-type Lectins receptors (CLRs) on the cell surface, and the intracytoplasmic NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) [148], through which DCs sense both pathogen associated

molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs), such as dying cells [153].

PRRs don't mediate a targeted response but are fundamental to mediate a first wide response after the recognition of molecular patterns (PAMPs) often shared by different classes of microbes [154]. PRRs signal transduction leads to the activation of gene expression for the secretion of a wide range of molecules, including type I IFNs, pro-inflammatory cytokines such as TNFα, IL6 and IL12, chemokines like CXCL9/10 and cell adhesion molecules, that mediate early immune response and the recruitment of the innate immunity [155].

The molecules recognized by PRRs are often fundamental for microbial survival, with the advantage of minimizing the possibilities that the pathogen may escape immune surveillance by losing the targeted molecules [154]. For example, it was recently demonstrated that cDCs and pDCs play a central role in the immune response against SARS-CoV2 infections after their activation mediated by the recognition of the single stranded RNA (ssRNA) viral genome by TLR7 and 8 both *in-vitro* and *in-vivo* [156]. Finally, one of the most powerful and characterized PAMPs is lipopolysaccharide (LPS), which triggers TLR4 and is a main element of the outer membrane of gram-negative bacteria, essential for their survival [157].

DAMPs are endogenous molecules released following cell damage or death caused by infections, chemical injury, toxins, mechanical trauma and burns. These molecules are not released by apoptotic cells, thus avoiding unnecessary immune activation [158]. For this reason, the triggering of PRRs by self-molecules can unleash the outbreak of autoimmune conditions [159].

After DCs are triggered by PRR activation, they undergo a complex process of maturation and shift from antigen-capturing cells to antigen presenting cells. Along with maturation, DCs start upregulating MHC-I, MHC-II and co-stimulatory molecules such as CD40 and CD86 [160]. Moreover, they also secrete

pro-inflammatory cytokines and modify chemokine receptors on the surface. By expressing CCR7, DCs gain access to the lymph-node following CCL19 and CCL21 gradient, where naive T cells await their priming [161]. Once DCs reached the T cells area in the lymph-node, DCs present the antigens, loaded on MHC-I or MHC-II, to naïve $CD4^+$ or $CD8^+$ T cells [137].

2.4. Toll-like Receptors (TLRs)

Toll-like receptors (TLRs) are an evolutionarily conserved family of pattern recognition receptors (PRRs) found on the cell surface and in the endosomes of various innate immune and tissue cells that play a pivotal role in innate immunity activation.

2.4.1. General features of TLRs

The Toll gene was initially identified for its role in the development of the dorsal-ventral axis in Drosophila melanogaster. Subsequently, the Toll protein was also recognized for its involvement in the antimicrobial responses of this organism [162]. Following this discovery, mammalian homologues were identified and termed Toll-like receptors. In mammals, 13 TLRs (TLR1-13) have been identified, although humans express only 10 of these (TLR1-10) [162]. TLRs are type I integral membrane receptors characterized by N-terminal extracellular leucine-rich repeats (LRRs) or ectodomain (ECD), that mediates PAMP recognition [163], a transmembrane domain, and a C-terminal cytoplasmic domain [164]. The LRR domain is essential for ligand recognition, while the cytoplasmic domain, contains the Toll/IL-1 receptor (TIR) domain, responsible for the activation of the signaling pathways [164]. In addition, TLRs 7-9 contain in their LRR domain a distinctive inserted loop region, known as Z-loop, composed of approximately 30 amino acid residues. When pathogen-derived

and/or self-derived nucleic acids reach the endosomes, they get through enzymatic digestion to yield shorter fragments and mononucleotides that can activate nucleic acid-sensing TLRs [164]. In their inactive state, TLRs are monomeric; however, binding of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) induces their homo- or hetero-dimerization, resulting in interactions between the extracellular domains that form a characteristic 'm' shaped structure. This conformation allows the intracellular TIR domains of the two receptors to interact and recruit various adaptor proteins, such as MyD88, Mal, TRIF, and TRAM, to start signal transduction [164,165].

In particular TLR3, TLR7, TLR8 and TLR9 are nucleic acid-sensing TLRs, with the aim of triggering innate immunity against viral and intracellular bacterial infections. TLR3 is activated by double stranded (ds) DNA, a viral replication intermediate, TLR7 and 8, closely related to each other, recognize single stranded (ss) RNA and finally TLR9 is triggered by unmethylated DNA CpG motifs [166,167]. Nucleic acid-sensing TLRs are strategically located into the endosome to easily activate anti-viral defense. In fact, many viruses enter cells via endocytosis, where they are taken up into endosomal compartments. As the viral particles are processed within the endosomes, their nucleic acids are exposed [163]. For this reason, the endosome is an ideal location for detecting viral components that would otherwise be hidden. These receptors may also be activated after the uptake of apoptotic bodies from infected cells, clathrin-dependent endocytosis and the formation of complexes with antimicrobial peptide LL-37 [163]. However, nucleic acids can also be “self” and the recognition of such self-molecules by TLRs may trigger the ignition of sterile inflammation and autoimmune conditions. Accordingly, these TLRs are expressed on the surface of the endosomal membranes [168], to limit the self-recognition of nucleic acids that may be released in the extracellular space [169]. Historically, murine TLR8 is thought to be non-functional, since TLR7 KO mice did not

respond to the stimulations with TLR7/8 agonist R848, or other TLR8 ssRNA ligands [170]. The activation of monomeric TLR7-9 starts with the cleavage of the Z-loop that generates inactive dimers. The binding of enzymatically processed nucleic acids induces their shift into the active state [164].

2.4.2. TLR7 activation

In humans TLR7 is primarily expressed in plasmacytoid dendritic cells (pDCs), but it was also observed in other dendritic cells subtypes, B cells, monocytes, and macrophages [170]. Research on TLR7 crystal structures has shown that this receptor can bind to single-stranded RNA (ssRNA) degradation products at two distinct sites. The first binding site is located at the top of the dimerization interface, between the two TLR7 monomers, and has been shown to accommodate guanosine (G), forming multiple interactions with both the guanine base and ribose [171]. The second site is also within the dimerization interface, situated between the concave surface of the leucine-rich repeat (LRR) domain and the Z-loop (Figure 5A). This site binds uridine-rich oligoribonucleotides in three specific positions, which have been recently detailed [172]:

- The first position shows a preference for uridine (U) over cytidine (C), adenosine (A), or guanosine (G).

- The second position can exclusively bind uridine (U).

- The third position favors uridine (U) and cytidine (C) over adenosine (A) and guanosine (G).

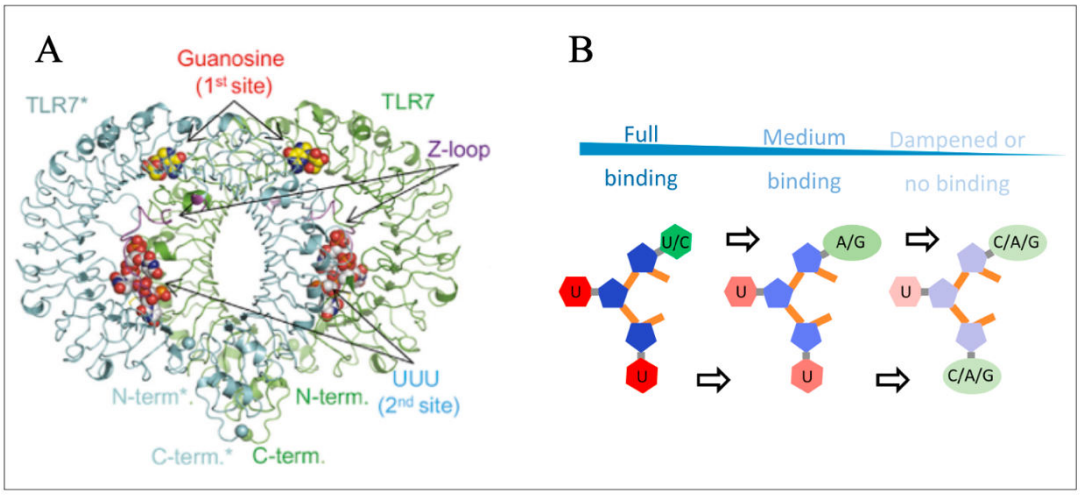

Figure 5: TLR7 structure and activation mechanism. *(A) TLR7 dimer crystal structure (front view). Each monomer is respectively in green and light blue. Guanosine and polyU are positioned in the first and second binding sites, respectively* [171]*. Different nucleosidic combinations that can accommodate in TLR7 second binding site generating full, medium, or dampened binding* [164]*.*

A schematic representation of TLR7 binding preferences for ssRNA sequences is depicted in figure 5B. Upon ligand binding, TLR7 dimerization allows the bridging of the key adaptor protein MyD88, which subsequently recruits the interleukin-1 receptor-associated kinase (IRAK) [173]. Phosphorylation of IRAK allows it to associate with the TNF receptor-associated factor 6 (TRAF6), forming an IRAK/TRAF6 complex that activates nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), resulting in the production of pro-inflammatory cytokines like TNFα, IL6 and IL12. This complex also activates interferon regulatory factor 7 (IRF7), which regulates the production of type I interferons in pDCs [174], with a key role in both antiviral immune responses and the development of nucleic acid-driven autoimmune diseases, known as type I interferonopathies [175] (Figure 6).

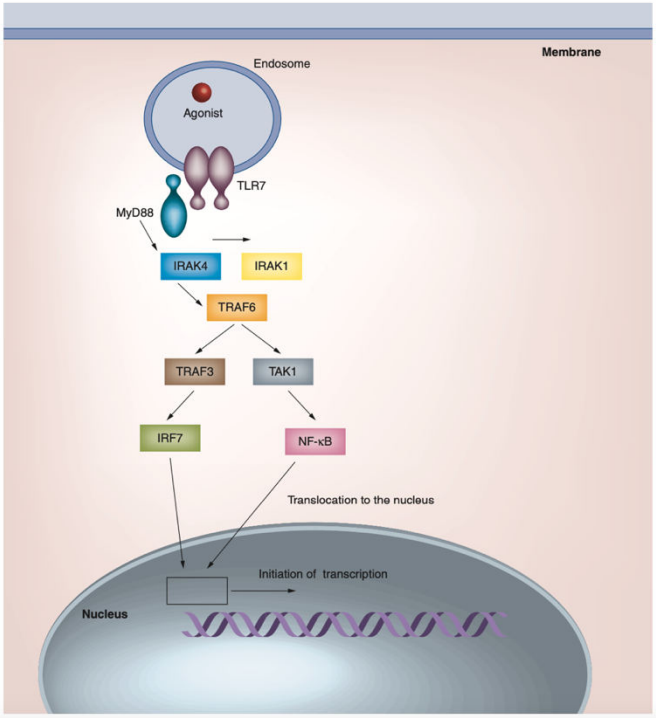

***Figure 6**: overview of the main elements of TLR7 signaling leading to pro-inflammatory response* [176]

2.4.3. Potential roles of TLR7/8 triggering in humans

TLR7 can also be triggered by synthetic ligands, such as R848 (also known as Resiquimod), specific for both TLR7 and TLR8, or Imiquimod (TLR7 specific). These agonists have been exploited for anti-viral therapy, as an adjuvant in vaccines formulation and in the therapeutic strategy for the treatment of cancer [177] with the aim of further sensitize cancer cells to conventional anti-cancer therapies such as chemotherapy and radiation.

For what concerns TLR7/8 agonists exploited as vaccine adjuvants, R848 was also tested on African Green Monkey (AGM) model of neonatal vaccination, leading to a 10-fold increase in anti-influenza virus IgG and IgM levels, $IFN\gamma^{+}$ influenza-specific T cells and to a significant decrease of viral load in the trachea. This improved response was due to an increased total number of DCs in the lymph nodes and showing increased expression of the maturation markers [178].

TLR7/8 agonists like R848 are also promising tools for the treatment of chronic infectious diseases like Hepatitis C (HCV), Hepatitis B (HBV) and HIV with the goal of improving patients' compliance and minimizing side effects of anti-viral therapy. In fact, the treatment with R848 was demonstrated to elicit type I IFNs through TLR7 (and TLR8 in humans) triggering, thus inducing viral RNA degradation and activation of protein kinase R, which blocks translation of viral mRNAs [179].

TLR7/8 agonists were the first TLR agonists approved for cancer treatment [180]. Cancer immunotherapeutic agents are used to trigger a robust primary and secondary antitumor immune response by enhancing immune mechanisms that during disease progression are evaded, thus inhibiting tumor growth and subsequent metastasis. For instance, in a murine model of pancreatic ductal adenocarcinoma (PDAC), it was demonstrated that R848 is able to modulate the tumor microenvironment (TME) through the activation of immune cells. In fact, the combined treatment with stereotactic body radiation therapy (SBRT) and R848 delivery, results in an increased infiltration of $CD8^+$ T cells, and NK cells, as well as a decreased regulatory T cell infiltration likely due to the enhanced activation and maturation of tumor infiltrating DCs. This reduced tumor growth and significantly decreased the incidence of liver metastasis [181]. Additionally, R848 was found able to alter the cytokine/chemokine profile, increasing antitumor cytokines like IFN-γ and granzyme B, and proinflammatory cytokines such as TNFα, CCL2, CCL5, and CXCL10, while decreasing suppressive cytokines like IL-4 and IL-10 [181]. Moreover, in a murine model of lung cancer, it was found that TLR7 activation by R848 alone boosts immune activation, inducing DCs activation, maturation and pro-inflammatory cytokines secretion, thus promoting $CD8^+$ T and NK cells activation [182].

3.AIM (PART I)

To date, two main functions have been described to be associated to the atypical receptor CCRL2. The first one is to act as chemerin presenting molecule on barrier cells to regulate the migration of adjacent leukocytes expressing the chemerin functional receptor Chemerin1 [116]. On the other hand, CCRL2 has been shown to regulate leukocytes migration through the interaction with conventional chemokine receptors. Indeed, by forming heterodimers, CCRL2 can regulate the functionality of the prototypical neutrophil chemokine receptors, such as CXCR2 [94,116]. In 2021 Yin et al. published a study introducing a novel possible function of the atypical chemokine receptor CCRL2 [93]: the interaction with TLRs, in particular, with TLR4. This interaction is functional to induce the retention of TLR4 on the plasmatic membrane of tumor associated macrophages, thus promoting the maintenance of a pro-inflammatory signaling mediated by TLR4 activation, preventing macrophages to switch towards a M2-like anti-inflammatory phenotype. Thus, TAMs could retain a pro-inflammatory anti-tumoral M1-like phenotype and promote the activation of $CD8^+$ cytotoxic T cells against tumor antigens.

Our aim for this first part of the thesis was to further investigate the functional relationship between CCRL2 and other TLRs. Our experimental setting was aimed at comparing the interaction of CCRL2 with a plasma membrane-expressed TLR, such as TLR4 and an endosomal membrane expressed one, such as TLR7. Specifically, the functional interaction between CCRL2 and TLR7 was investigated by using an *in vitro* model of bone marrow-derived dendritic cells (BMDCs). WT and CCRL2 KO BMDCs were generated by $CD34^+$ hematopoietic bone marrow precursors and stimulated with TLR7 agonist R848. The phenotypical and functional characterization of WT and CCRL2 KO DCs was performed to investigate the biological relevance of CCRL2/TLR7 interaction in dendritic cells.

4.MATERIALS and METHODS (PART I)

4.1. Animals

Wild Type (WT) and CCRL2 knockout C57BL/6 mice were used for the experiments. Mice were aged (8-12 weeks) and sex matched. Procedures involving animals handling were conformed to institutional guidelines in compliance with national (D.L. N. 26, 4-3-2014, D.M. 5-8-2021) and international (Directive 2010/63/EU revising Directive 85/609/EEC, September 22, 2010) law and policies.

4.2. $CD34^+$ cells isolation

Three/four WT and three/four CCRL2 KO mice (age-matched and sex-matched) were used to obtain $CD34^+$ cells. Mice were sacrificed by cervical dislocation and both femur and tibia were harvested and kept in cold sterile PBS. In sterile condition, BM was flushed using for each bone 1 mL of RPMI (GIBCO™) added with L-Glutamine (G) (EuroClone™), Penicillin/Streptomicin solution (P/S) (EuroClone ™) and 2 mM EDTA (ethylenediamide tetraacetic acid) (Invitrogen™ by Life Technologies™). Red blood cells lysis was performed through ACK lysis buffer (GIBCO®): cells flushed from each mouse were resuspended in ACK lysis buffer, kept for 5 minutes in ice and 7 ml of RPMI 10% FBS were added to stop the lysis process. After centrifugation, cells were pulled together according to their genotype (WT and CCRL2 KO separately) in a final volume of 5 ml of sterile PBS. Cells were filtered with a 30 µm seize (Miltenyi Biotech™). Cells were counted and processed for positive selection of $CD34^+$ cells. The cell suspension was incubated with 500 µL of MEC14.7 hybridoma per 15 million cells. Subsequently the cells were incubated with anti-Rat IgG MicroBeads (Miltenyi Biotech™): 20 µL of beads + 80 µL of MACS buffer (PBS, 1% FBS, 2 mM EDTA) for 10^7 cells. After incubation cell suspensions were applied to MS columns (Miltenyi Biotech®) for the positive selection of $CD34^+$ cells according to the manufacturer's protocol.

4.3. Bone Marrow-derived Dendritic Cells (BMDCs) culture and differentiation from CD34$^+$ cells

After column purification, CD34$^+$ cells were counted and resuspended at a concentration of 0,2*10^6 cells/mL in RPMI 5% FBS added with β-mercaptoethanol for cell culture (Invitrogen™ by Life Technologies™) and seeded in 6-well plates in the presence of GM-CSF 40 ng/ml (Peprotech™) and FLT3-L 100 ng/ml (Peprotech™) for 8 days. The cells were split 1:3 every 3 days and growth factors were added freshly.

4.4. Flow cytometry analysis

After the differentiation, Bone Marrow-derived Dendritic cells (BMDCs) were blocked with CD16/32 (clone 2.4G2) for 15 minutes at 4°C and stained with Live/Dead® (VioBlue) (ThermoFIsher Scientific™) diluted 1:1000 in PBS for 15 minutes in the dark at 4°C. Live/Dead staining is performed to exclude dead cells from the assessment of the differentiation. Subsequently, DCs were stained with antibodies showed in table 1 for 15 minutes at 4°C for the assessment of the differentiation.

Antigen	Fluorophore conjugation	Dilution	Brand	CAT
MHC-II	FITC	1:100	Miltenyi Biotech®	130-123-666
CD11c	APC	1:100	Miltenyi Biotech®	130-110-702
CD40	PE	1:100	Miltenyi Biotech®	130-116-110
CD86	APC	1:100	Biolegend®	105012

***Table 1**. List of the antibodies used to assess the differentiation of BMDCs.*

Following the stimulation with TLR agonists murine DCs were CD16/32 blocked (clone 2.4G2) for 15 minutes at 4°C and stained with Live/Dead® (VioBlue)

diluted 1:1000 in PBS for 15 minutes in the dark at 4°C. Subsequently BMDCs were stained with antibodies showed in table 1 for other 15 minutes.

4.5. BMDCs stimulation (kinetic ELISA, kinetic qPCR and kinetic Western Blot)

BMDCs were resuspended 2 million/mL in RPMI 5% FBS added with β-mercaptoethanol and stimulated with different TLR agonists for the o/n stimulation experiments:

- R848 (Invivogen™), 500 ng/ml and 1µg/ml
- LPS (Sigma™), 10 ng/ml and 100 ng/ml
- CpG-2216 (Invivogen™), 2 µg/ml and 6 µg/ml

For the kinetic experiments aimed at the quantification of cytokines released in the supernatant and their expression at mRNA level, cells were stimulated with 1 µg/mL R848 for 2, 4, 8 and 24 hours. For kinetic experiments aimed at western blot analysis (paragraph 4.8) cells were stimulated backward with 1 µg/mL R848 for 15, 30 and 60 minutes, to use a single non-stimulated negative control. Cells were then lysed in NP40 buffer.

4.6. RNA isolation and qPCR

RNA was extracted from BMDCs using QIAzol™ Lysis Reagent (Qiagen ™) and the treatment with DNAse I was performed according to the manufacturer's protocol. Reverse transcription was performed with 0,5 µg of RNA using random hexamers and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV RT) (ThermoFisher Scientific®).

The SsoAdvanced Universal SYBR Green Supermix (Biorad®) was used according to the manufacturer's instructions. Reactions were run in duplicate

on a StepOne Plus Real-Time PCR System (Applied Biosystems®) and analyzed by StepOne Plus Software (Applied Biosystems®, version 2.3). Sequences of gene-specific primers are listed in table 2. Gene expression was normalized based on mouse RPL32 mRNA content ($2^{-\Delta CT}$ method).

Gene name	Species	Primer Sequence (5'→3')	
RPL32	mouse	Forward	GCTGCCATCTGTTTTACGG
		Reverse	TGACTGGTGCCTGATGAACT
TLR1	mouse	Forward	CTGTCTTCAGATGCTTACCTCC
		Reverse	GGATGCTACATTGAGTTCCTGC
TLR2	mouse	Forward	CAGCTCACCGATGAAGAAGCTG
		Reverse	GAACTCTACCTCCGACAGTTCC
TLR3	mouse	Forward	CGAACCTGACAGAACTCGATC
		Reverse	TTGGAGGTTCTCCAGTTGGAC
TLR4	mouse	Forward	CACGTCCATCGGTTGATCTTGG
		Reverse	AGTCCTTCCATGATAGAGGGTTC
TLR5	mouse	Forward	TGCTAACAAGCTGACCGTGCTC
		Reverse	TTCACAGTTGCAGACGAACTCG
TLR6	mouse	Forward	GAGCTGAGAGTTCTGAGACTC
		Reverse	CCGATTGTGTGAGACATCCAG
TLR7	mouse	Forward	TGATCCTGGCCTATCTCTGAC
		Reverse	CGTGTCCACATCGAAAACAC
TLR9	mouse	Forward	CATCACCTGAGCCATCTGGAAG
		Reverse	GAGGTTGACCAGACCTTGGAAC
TNFα	mouse	Forward	TGCCTATGTCTCAGCCTCTTC
		Reverse	GAGGCCATTTGGGAACTTCT
IL6	mouse	Forward	GCTACCAAACTGGATATAATCAGGA
		Reverse	CCAGGTAGCTATGGTACTCCAGAA
IL12-p40	mouse	Forward	AAGGAACAGTGGGTGTCCAG
		Reverse	GTTAGCTTCTGAGGACACATCTTG
CXCL9	mouse	Forward	TCTCGGACTTCACTCCAACACA
		Reverse	ACTCCACACTGCTCCAGGAAGA
IP-10/CXCL10	mouse	Forward	CGTCATTTTCTGCCTCATCCTG
		Reverse	CCGTCATCGATATGGATGCAGT

***Table 2**. List of the primers used to perform qPCR on RNA extracted from BMDCs.*

4.7. Cytokines measurement

Following R848 stimulation, the supernatants were collected and stored at -20°C until usage. Cytokines were assayed using the ELISA kits reported in table 3.

Target	Species	Brand	CAT
TNFα	mouse	R&D® DuoSet™	**DY410**
IL6	mouse	R&D® DuoSet™	**DY406**

***Table 3**. List of the ELISA kits used for the quantification of pro-inflammatory cytokines in the supernatant.*

4.8. SDS-PAGE and Western Blot

Following the stimulation indicated in point 5, BMDCs were washed in cold PBS and lysed in L1 buffer (50 mM Tris-HCl; pH 8.0; 2 mM EDTA; 0,1% NP-40, 10% glycerol) supplemented with protease inhibitors to separate cytoplasmatic proteins. Nuclear pellets were washed twice with L1 buffer and then lysed in NP-40 lysis buffer (50 mM Tris-HCl; pH 8.0; 1 mM EDTA; 0,1% NP-40; 10% glycerol) supplemented with inhibitors (1 mM Na_3OV_4; 1 mM NaF; 1 mM PMSF; 2 mM DTT and protease inhibitors cocktail; all from Millipore Sigma®).

Alternatively for the total lysis of BMDCs aimed at the evaluation of TLR7 levels in basal and R848 overnight stimulated DCs, cells were washed twice with cold PBS and then lysed in NP-40 lysis buffer supplemented with inhibitors.

As indicated, equal amounts (assessed via Bradford's method for protein quantification) of the obtained extracts were analyzed through SDS-PAGE and western blot using the antibodies indicated in table 4. When necessary, membranes were stripped through a 0,2 M NaOH solution.

Antibody target	Blocking	Dilution	Brand	Incubation	CAT
mTLR7	5% BSA in TBST	1:1000	Cell Signaling Technology®	o/n 4°C on a shaker	82658
NF-kB (p65)	1% BSA in TBST	1:1000	Santa Cruz Biotechnology®	o/n 4°C on a shaker	sc-372
B-Actin	1% BSA in TBST	1:1000	Santa Cruz Biotechnology®	o/n 4°C on a shaker	sc-47778
Lamin B	1% BSA in TBST	1:1000	Santa Cruz Biotechnology®	o/n 4°C on a shaker	sc-6216
Donkey-anti-goat-HRP	1% BSA in TBST	1:5000	Santa Cruz Biotechnology®	1 hour RT on a shaker	sc-2058
Donkey-anti-rabbit-HRP	1% BSA in TBST	1:5000	Santa Cruz Biotechnology®	1 hour RT on a shaker	sc-2077

***Table 4**. List of the antibodies used to perform immunostaining on BMDCs protein extract.*

4.9. Statistical analysis

Statistical analyses were performed by using paired or unpaired Student's t test. Results were analyzed by GraphPad PRISM 9.0 software.

5.RESULTS (PART I)

5.1. Assessment of basal expression of TLRs in bone marrow-derived DCs (BMDCs)

In this first part of the thesis, our general aim was to extend the knowledge related to the data published by Yin et al., which showed the interaction between CCRL2 and TLR4 in tumor-associated macrophages [93]. Indeed, we extend the investigation to other immune cells, namely dendritic cells, which are responsible for initiating all antigen-specific immune responses. The TLR expression profile, at basal level, was investigated in BMDC generated by WT and CCRL2 KO mice. As shown in Figure 7, among plasma membrane expressed TLRs, TLR2 and TLR4 showed the highest levels of expression, while endosomal TLRs resulted only slightly expressed by qPCR. No difference in the expression levels of TLRs was observed between WT and CCRL2 deficient *in vitro*-generated BMDCs.

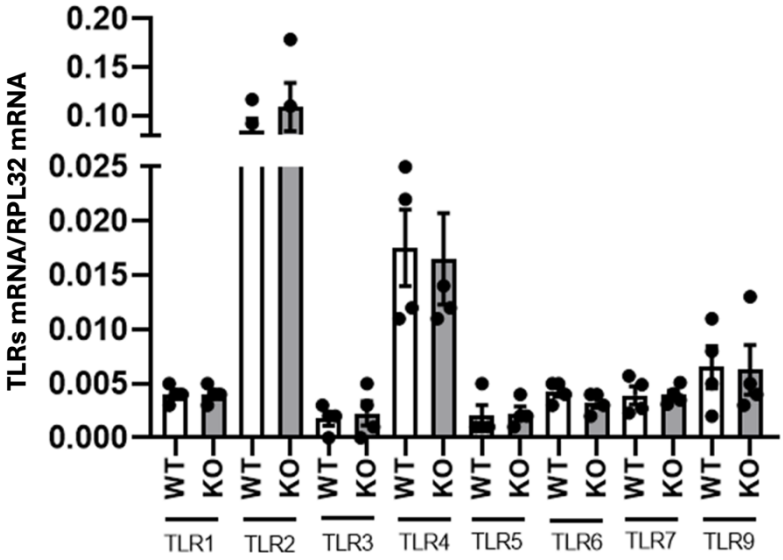

Figure 7: TLRs basal levels in BMDCs. Non-stimulated BMDCs were lysed in QIAzol®, RNA was extracted and cDNA was obtained as reported in the materials and methods section, I. mRNA levels were evaluated by qPCR. The results from the experiments are reported as mean ± SEM (n=4) of TLRs relative to housekeeping gene RPL32.

5.2. Pro-inflammatory cytokines production is impaired in CCRL2 KO BMDCs stimulated with R848 (TLR7 agonist)

DCs play important roles in inflammation and in innate and adaptive immune responses to pathogens and non-self substances. DCs produce a variety of mediators including cytokines and chemokines that may either up- or down-regulate immune and inflammatory responses. To evaluate the functional profile in response to different TLR agonists, *in vitro* generated BMDCs from WT and CCRL2 KO animals were treated with LPS (TLR4 agonist), R848 (TLR7 agonist) and CpG A (TLR9 agonist).

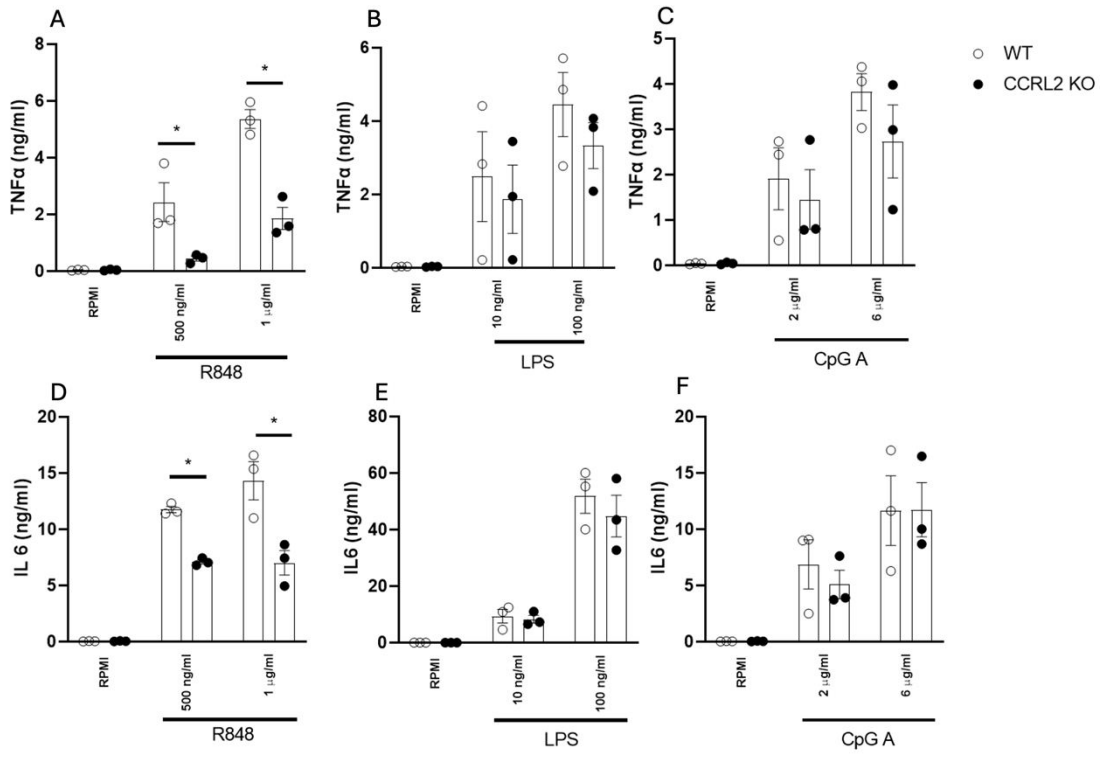

Figure 8. Functional effects of the stimulation of WT and CCRL2 KO BMDCs with different TLR agonists (R848, LPS, CpG A) in terms of pro-inflammatory cytokines production. WT and CCRL2 KO BMDCs were stimulated with two concentrations of R848 (500 ng/ml and 1µg/ml) (A, D), LPS (10 ng/ml and 1 µg/ml) (B, E) and CpG A (2 µg/ml and 6 µg/ml) (C, F) to assess the production of pro-inflammatory cytokines TNFα (A-C) and IL6 (D-F). All the results are expressed as mean ± SEM (n=3) of cytokine concentration detected in the supernatant by ELISA assay (ng/ml). *p<0.05 WT vs CCRL2 KO using Student's t-test.

Figure 8 shows the production of TNF-α and IL6 in the two DC strains stimulated with two concentrations of LPS (10-100 ng/ml), R848 (500ng and 1μg/ml), and CpG (2-6 μg/ml). The production of both TNFα and IL6 in BMDCs stimulated with LPS (TLR4 agonist) and CpG A (TLR9 agonist) resulted increased with the higher concentration of the agonist, but no differences were observed between WT and CCRL2 KO BMDCs. In contrast, the production of both TNF-α and IL6 was significantly impaired in CCRL2 KO BMDCs stimulated with the lower and higher R848 concentration. These results suggest that CCRL2 might be involved in pro-inflammatory response following TLR7 triggering.

5.3. Maturation of BMDCs stimulated with TLRs agonists

At immature stage, DCs patrol the organism to detect the presence of pathogens. Upon activation, mature DCs reach the lymph nodes to activate antigen-specific T lymphocytes and thus initiate an adaptative immune response to control the pathogens. Being DC maturation a crucial process in the regulation of DC functional activity, we investigated the expression of maturation markers in WT and CCRL2 KO BMDCs upon stimulation with TLR agonists.

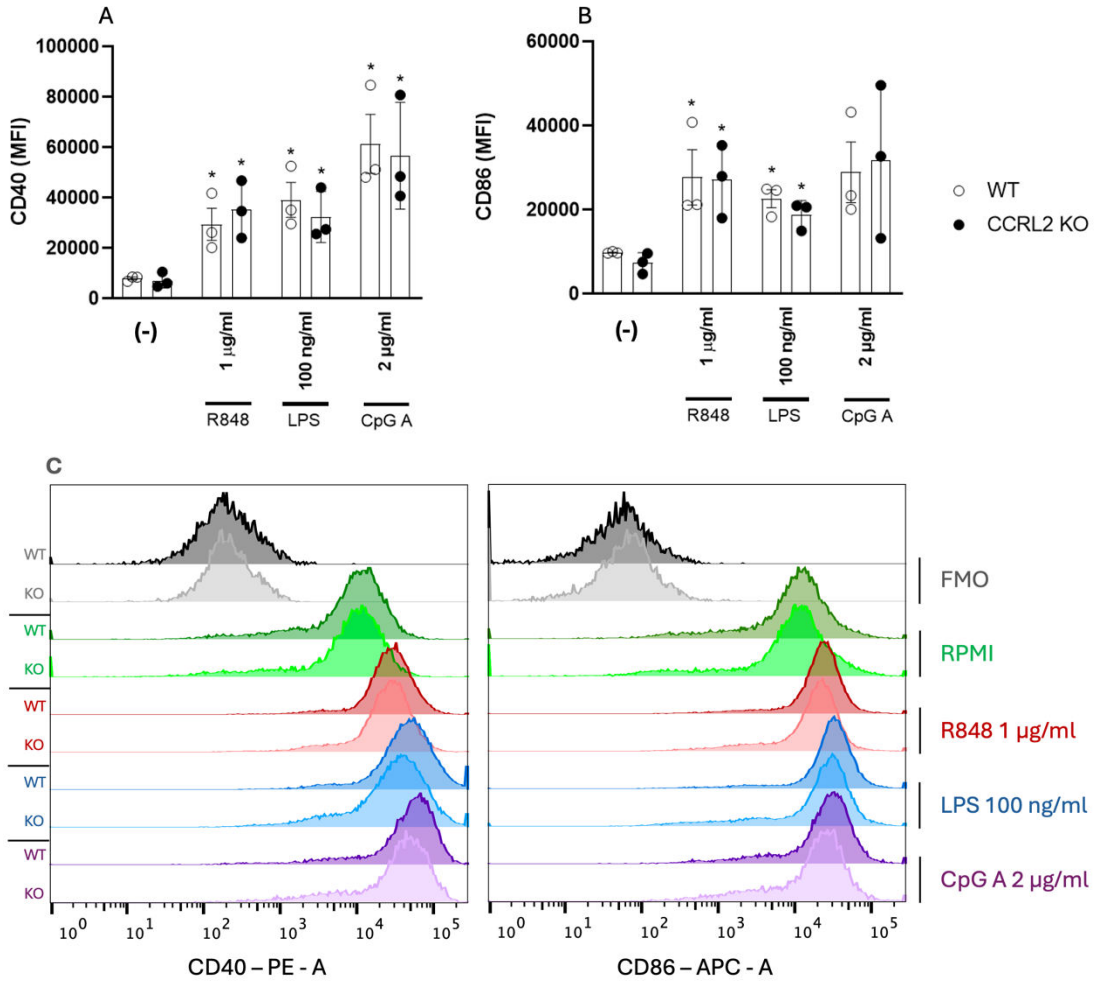

Figure 9. Maturation of WT and CCRL2 KO BMDCs stimulated with different TLR agonists (R848, LPS and CpG A). WT and CCRL2 KO BMDCs were stimulated with R848 (1 µg/ml), LPS (100 ng/ml) and CpG A (6 µg/ml). After 24 hours of stimulation, they were collected and we performed staining with anti-CD40-PE and anti-CD86-APC antibodies to assess maturation. (A) The results for CD40 upregulation are expressed in mean ± SEM (n=3) of CD40-PE MFI. (B) The results for CD86 upregulation are expressed in mean ± SEM (n=3) of CD86-APC MFI. *p<0.05 R848 vs (-) using Student's t-test. (C) A representative FACS experiment showing the upregulation of DCs maturation markers after the stimulation of WT and CCRL2 KO BMDCs,

As shown in Figure 9A, BMDCs stimulated with R848 (1 µg/ml), LPS (100 ng/ml) or CpG A (6 µg/ml), upregulated a typical maturation marker, namely CD40. However, no differences were observed between WT and CCRL2 KO BMDCs compared with unstimulated DCs. Also, CD86, another typical maturation marker for DCs, resulted significantly upregulated upon R848 and LPS stimulation compared with immature DCs. Furthermore, TLR9 triggering with CpG A even though not significantly, however, showed a tendence to increased

expression levels in both the DC strains (Figure 9B). A representative FACS experiment showing the upregulation of maturation markers after WT and CCRL2 KO BMDCs stimulation with TLR agonists is showed (Figure 9C).

In conclusion, the triggering of TLR7 determines an impairment of pro-inflammatory cytokines secretion in CCRL2 KO BMDCs but does not affect their maturation process. Taken together, these data suggest CCRL2 might be involved in the pro-inflammatory downstream signaling following TLR7 triggering

5.4. Assessment of the kinetics of cytokine expression and secretion in WT and CCRL2 KO BMDCs stimulated with R848

To investigate the mechanism underlying the observed differences in TNFα and IL6 production, upon R848 stimulation, in WT versus CCRL2 KO BMDCs, kinetic experiments were performed to assess both levels of mRNA expression and secreted proteins of pro-inflammatory cytokines. WT versus CCRL2 KO BMDCs were treated with a R848 at the concentration of 1 μg/ml. First, the mRNA expression levels of TNFα and IL6 (Figure 10 A, B) showed a tendence to reduced levels in CCRL2 KO BMDCs at all the time points investigated, without reaching a statistical significance. We then extended the investigation to other cytokines and chemokines known to be expressed by activated DCs. As shown in Figure 10, the mRNA expression levels of IL12 (C), CXCL9 (D), and CXCL10 (E) mirrored the same behaviour, showing decreased levels in CCRL2 KO DCs, but not in a statistically significant way.

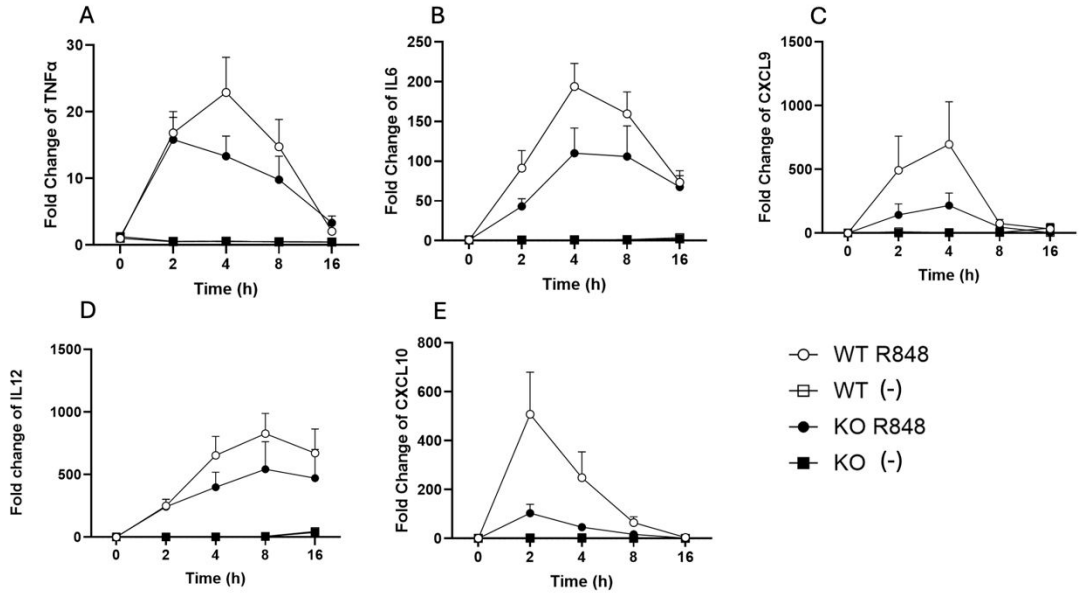

Figure 10. Assessment by qPCR of the levels of expression of pro-inflammatory cytokines after R848 stimulation in a kinetic experiment. BMDCs were stimulated as reported in the materials and methods section I with R848 1 µg/ml, lysed in QIAzol® at the established timepoint. RNA was extracted and cDNA was obtained as reported in the materials and methods section I. Results are expressed in mean ± SEM (n=4) of TNFα (A), IL6 (B), CXCL9 (C), IL12 (D) and CXCL10 (E) fold of induction. R848 = treated with R848 1 µg/ml; (-) = untreated control/RPMI.

Then, we performed a kinetic study of protein secretion, focusing on TNFα and IL6 levels, upon TLR7 triggering. As shown in Figure 11A, TNFα production was significantly reduced in CCRL2 KO BMDCs in comparison to WT cells at 4h, 8h and 16h after stimulation with R848. Similarly, IL6 production was impaired at all the time points, reaching statistical significance only at 16h (Figure 11B).

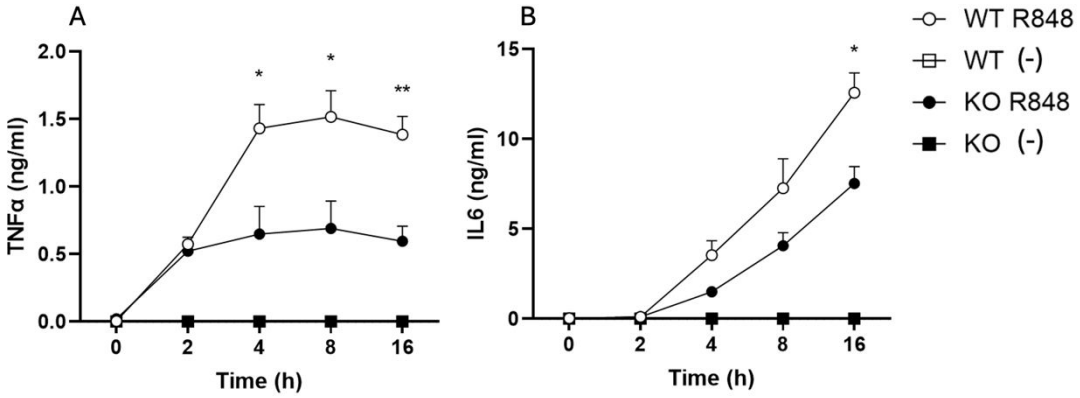

Figure 11. Assessment of the secretion of TNFα and IL6 after R848 stimulation in a kinetic experiment. BMDCs were stimulated as reported in the materials and methods section (I) with R848 1 µg/ml and the supernatant was collected at the established timepoint after centrifugation as reported in the materials and methods section I. Results are expressed in mean ± SEM (n=4) of TNFα (A) and IL6 (B) concentration detected in the supernatant by ELISA assay (ng/ml). *p<0.05 WT vs CCRL2 KO using Student's t-test.

Therefore, these data confirm that an impairment in the production of pro-inflammatory cytokine, at both mRNA and protein levels, may occur in CCRL2 KO mature BMDCs suggesting a possible role of CCRL2 in the regulation of TLR7 signaling.

5.5. Upregulation of TLR7 expression levels after R848 stimulation

To further investigate the mechanism underlying the impairment of pro-inflammatory response observed in CCRL2 KO BMDCs after TLR7 triggering with R848, we investigated the potential role of CCRL2 in the regulation of TLR7 expression. For this reason, we assessed the expression of TLR7 both at mRNA level and protein level, in basal and stimulated conditions in WT and CCRL2 KO BMDCs.

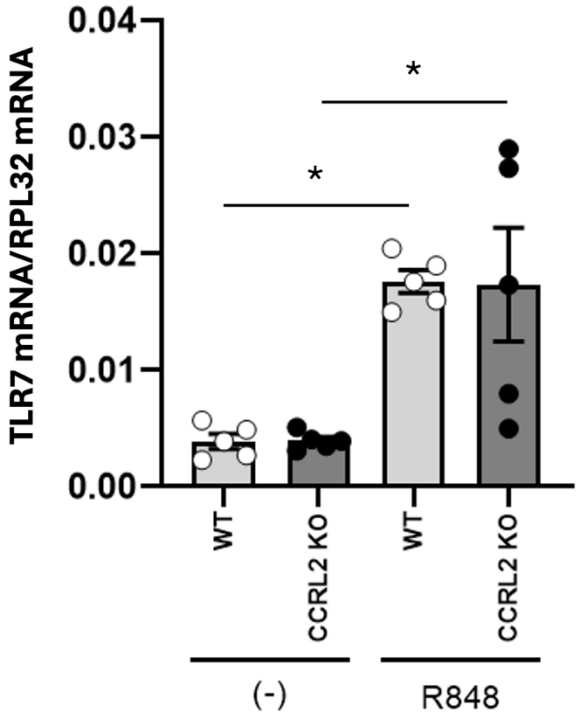

Figure 12. Assessment of TLR7 expression at the mRNA level. BMDCs were stimulated with 1 µg/ml of R848 or not stimulated. After o/n incubation cells were lysed in QIAzol®. mRNA levels were evaluated by qPCR. The results from the experiments are reported as mean ± SEM (n=5) of TLR7 relative to housekeeping gene RPL32. *p<0.05 (-) vs R848 using Student's t-test.

As shown in Figure 12, TLR7 expression increased after the stimulation with R848 both in WT and CCRL2 KO BMDCs. Nevertheless, no difference was observed in TLR7 levels of expression in WT and CCRL2 KO DCs both at basal level and after the stimulation with R848.

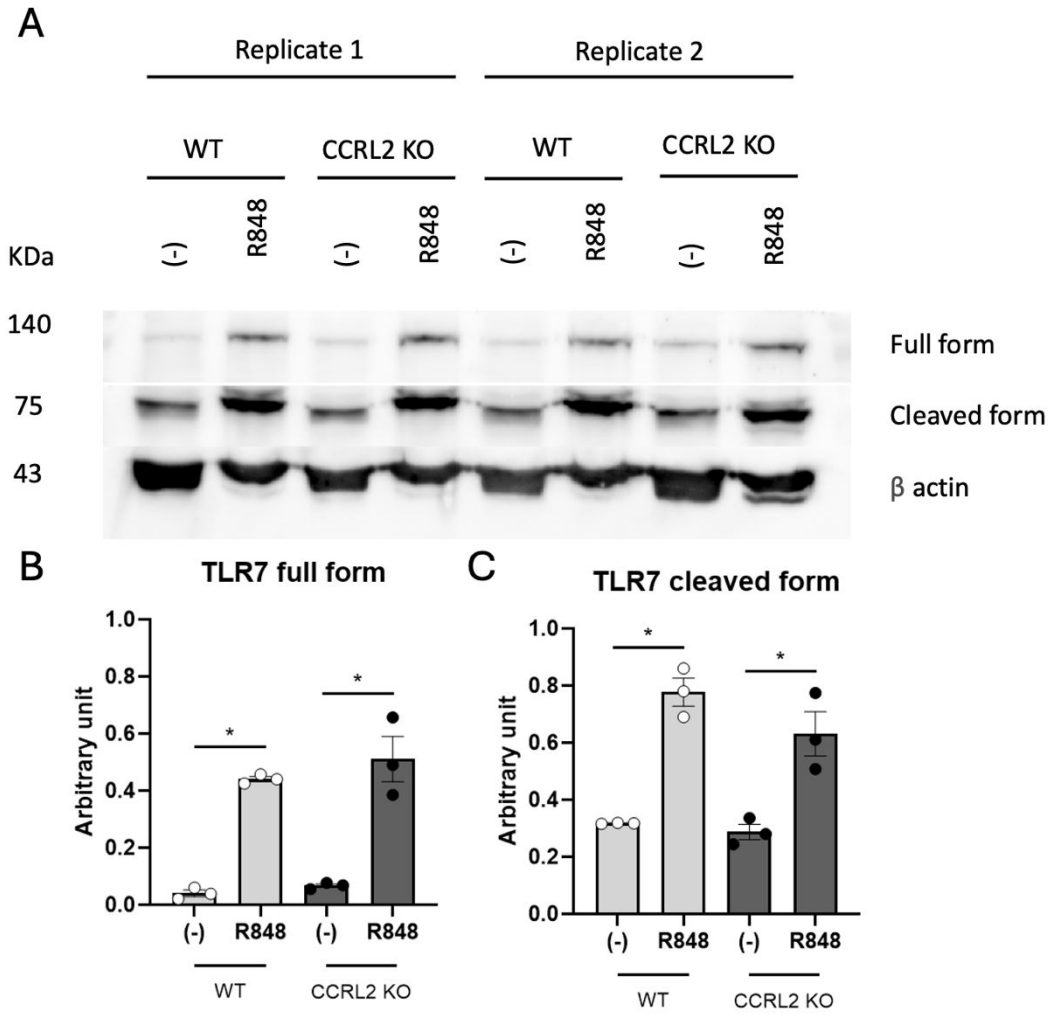

Figure 13. Evaluation of the expression of TLR7 at the protein level. BMDCs were stimulated with 1µg/ml of R848 or not stimulated. After o/n incubation cells were lysed in NP40 buffer with proteases inhibitors and later protein quantification was performed using Bradford's method. Two experimental replicates are shown. (A) two representative replicates of the Western Blot experiment to assess TLR7 protein levels. The two TLR7 forms (full and cleaved) were detected. (B) Densitometric analysis of TLR7 full form. *p<0.05 (-) vs R848 using Student's t-test. (C) Densitometric analysis of TLR7 cleaved form. *p<0.05 (-) vs R848 using Student's t-test. The results in figure B and C are reported as mean ± SEM (n=3) of the arbitrary unit obtained as TLR7 area/β actin area through ImageJ analysis.

We next investigated TLR7 expression at protein levels both at basal conditions and after the stimulation with R848 by Western Blot. Figure 13A shows two independent replicates of TLR7 protein, both in its full and in its cleaved form.

TLR7 triggers antiviral immune response through its capacity to recognize ssRNA. Like all endosomal TLRs, prior to gaining the capacity to bind and respond to ligands, TLR7 must be proteolytically cleaved [183]. The cleavage events are a complex process and are thought to involve a combination of

asparagine endopeptidase (AEP) and/or multiple cathepsins, that lead to the production of two fragments, where the C-terminal one is later involved in homodimerization and signal transduction [184].

TLR7 expression was higher in BMDCs stimulated with R848 in comparison with unstimulated BMDCs both for what concerns its full (Figure 13B) and cleaved form (Figure 13C). Nevertheless, consistently with the results obtained by qPCR (Figure 12), no difference in TLR7 expression was observed between WT and CCRL2 KO BMDCs.

5.6. Reduced translocation of NF-κB transcription factor in the nucleus in CCRL2 KO BMDCs

Given the comparable levels of expression of TLR7 between WT and CCRL2 KO BMDCs, both at basal level and after stimulation with R848, to explain the observation of impaired production of proinflammatory by CCRL2 KO BMDCs, we investigated the potential involvement of CCRL2 in TLR7 signaling pathways. For this reason, the activation of the master regulator of inflammation, namely the transcriptional factor NF-κB, was investigated by evaluating its translocation to the nucleus in WT and CCRL2 KO BMDCs after TLR7 triggering with R848. Specifically, an antibody against the p65 subunit (65 KDa) of NF-κB was used. The chosen housekeeping gene for normalization was Lamin B (67 KDa).

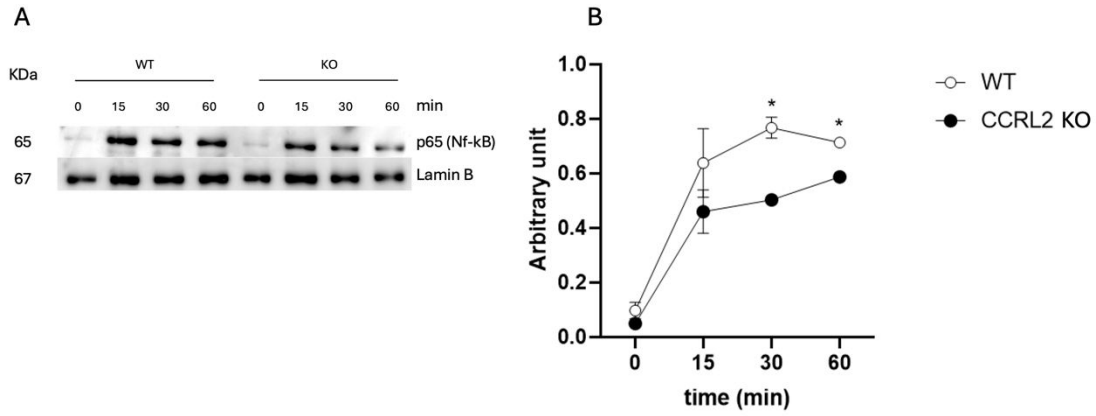

Figure 14. NF-kB translocation to the nucleus in WT and CCRL2 KO BMDCs stimulated with R848. WT and CCRL2 KO BMDCs were stimulated at the same time with 1 µg/ml of R848 for 15, 30 and 60 minutes or not stimulated. A single control was used since stimulation was performed backwards. Later protein extract from the nuclei was obtained as reported in the materials and methods section I. (A) a representative western blot experiment of p65 translocated to the nucleus at different timepoints in WT and CCRL2 KO BMDCs. (B) Densitometric analysis of the western blot experiments. The results from the experiments are reported as mean ± SEM (n=3) of the arbitrary unit obtained as p65 area/Lamin B area through ImageJ analysis. *p<0.05 WT vs CCRL2 KO using Student's t-test.

A reduced translocation of p65 subunit to the nucleus was observed in CCRL2 KO in comparison to WT BMDCs at 30 minutes and 60 minutes after R848 stimulation (Figure 14). Figure 14A shows a representative Western Blot experiment in which the signal from translocated p65 is reduced in CCRL2 KO DCs, while Figure 14B illustrates the densitometric quantification of specific signal in three independent experiments. These results suggest that CCRL2 can have a role in the regulation of the signaling pathway of TLR7 upon R848 triggering. Further studies are needed to better understand the mechanisms of interaction between CCRL2 and TLR7 and the potential impact in *in vivo* setting, taking advantage of genetic models of receptor depletion.

6.DISCUSSION (PART I)

This part of thesis work is focused on the study of the potential role of CCRL2 in TLR-mediated dendritic cell activation. CCRL2 is a seven transmembrane receptor highly homologous to chemokine receptors. However, it shares many characteristics with the ACKRs, including the lack of certain consensus sequences and the inability to induce functional responses [116]. Originally described as a chemokine receptor [107], the ability of CCRL2 to bind chemokines was disputed [106,110]. CCRL2 binds the chemoattractant protein chemerin in the absence of any known intracellular signaling and activation of chemotactic function [80].

To date, two main functional properties have been associated to this atypical receptor: the capability to concentrate and present chemerin to leukocytes expressing the functional receptor Chemerin1, and the ability to form heterodimers with other chemokine receptors, such as CXCR2 [94]. Both these functionalities are involved in the regulation of leukocyte migration. In 2021, Yin et al. published a study in which a novel role for the atypical receptor CCRL2 was described. In particular, it was proposed that CCRL2 could interact and modulate the function of TLR4 by positively regulating its expression on the plasma membrane of tumor infiltrating macrophages, thus promoting an M1-like pro-inflammatory phenotype [93]. The potential role of CCRL2 in anti-tumor responses was related to the indirect capacity to promote the activation of $CD8^+$ cytotoxic T lymphocytes, through the retention of macrophages into a M1-like anti-tumor phenotype [93].

The upregulated expression of CCRL2 in LPS-treated murine macrophage cell line RAW264 cells represents one of the first report describing this receptor, which suggested a potential involvement of CCRL2 in inflammatory responses [83]. However, macrophages do not represent the only immune cell type responsive to LPS able to upregulate CCRL2 expression. Indeed, our group showed the regulation of CCRL2 expression on dendritic cells in *in vitro* and *in*

vivo settings and its role in the functional activity of dendritic cells in a model of airway hyperresponsiveness [95].

Based on the recent report by Yin et al., describing the interaction of CCRL2 with TLR4 in macrophages, we decided to extend our study to the potential interaction of CCRL2 with other TLRs. Taking advantage of the genetic deletion of CCRL2 model, both plasma membrane-expressed TLRs as well as endosomal-expressed ones were investigated in this thesis work. The cellular model we focused on were dendritic cells, the most professional antigen presenting cells, which play a crucial role in bridging innate and adaptive immune responses and are involved in the pathogenesis of different pathological conditions, such as inflammation, autoimmunity and cancer [136,185].

First, WT and CCRL2 KO BMDCs were generated and their phenotypical and functional characterizations were performed. While Yin and colleagues observed that the lack of CCRL2 caused lower levels of surface TLR4 in macrophages, on the contrary, no significant differences were observed in the expression levels of TLR4 neither of the other TLRs in CCRL2 KO versus WT DCs. Furthermore, we investigated the triggering of different TLRs by cognate agonists, namely LPS, a TLR4 agonist, representative of membrane expressed TLRs, and R848 and CpG A, respectively TLR7 and TLR9 agonists, representative of endosomal TLRs. No differences were observed, in terms of mRNA and protein expression of the main proinflammatory cytokines TNFα and IL6 upon TLR4 and TLR9 engagement. On the contrary, we found that CCRL2 deficiency induced a functional impairment in TNFα and IL6 expression and secretion only after TLR7 stimulation.

A defective production of TNFα and IFNα was previously found associated to the impairment of maturation in pDCs stimulated with TLR7 and TLR9 agonists from patients affected by Systemic Lupus Erythematosus (SLE) and treated with hydroxychloroquine [186]. For this reason, we decided to assess WT and CCRL2 KO DC maturation evaluating CD40 and CD86 maturation markers in LPS, CpG

and R848 stimulated cells. In contrast with previous work, our results suggest that the triggering of TLR4, TLR7 and TLR9 does not have any effect on the maturation of CCRL2 KO as well as WT BMDCs suggesting that the difference in pro-inflammatory response following TLR7 triggering is not due to a defective maturation process. Therefore, these results suggest that CCRL2 does not have any role in the maturation of DCs. To test the possibility that CCRL2 may affect the kinetics of expression or secretion of pro-inflammatory cytokines after TLR7 triggering, causing a delayed secretion of TNFα and IL6 in the supernatant, a kinetic experiment was performed. A reduced concentration of TNFα released in the medium was observed after 4, 8 and 16 hours after the stimulation with R848 in CCRL2 deficient DCs, but the curves followed the same pattern, excluding impaired kinetics of release. Also, IL6 production was found impaired only 16 hours after the stimulation, but still excluding delayed kinetics of release. For this reason, we can conclude CCRL2 affects the production of pro-inflammatory cytokines in the supernatant but does not affect their kinetics of release.

To assess if CCRL2 may be involved in the regulation of TLR7 expression, as a mechanism underlying the impaired pro-inflammatory cytokine production, the expression levels of TLR7 were assessed both in immature and in R848-stimulated WT and CCRL2 KO BMDCs, both at the mRNA and protein level. However, we did not find any difference in TLR7 expression levels in the two groups, suggesting that the defective pro-inflammatory response observed in CCRL2-lacking DCs, is not related to the regulation of TLR7 expression.

It is known that the production of pro-inflammatory cytokines like TNFα and IL6 after TLR7 triggering is dependent from the activation of the NF-κB pathway [173]. For this reason, NF-κB translocation to the nucleus was assessed via western blot, in a kinetic experiment targeting p65. The translocation of NF-κB to the nucleus was found impaired in CCRL2 KO BMDCs compared to WT cells. These results suggest that CCRL2 might play a crucial role in the NF-κB

phosphorylation, thus impairing the pro-inflammatory response in DCs and affecting the secretion of pro-inflammatory cytokines in the supernatant.

Based to recent work reporting that CCRL2 can be critical for the activation of ERK, p38/MAPK and AKT pathways in the immune response to *Mycobacterium Tuberculosis* (MTB) [115], the specific mechanism underlying the impaired functional response upon TLR7 triggering, will be assessed by kinetic experiments aimed at the evaluation of phospho-ERK1/2, phospho-p38 and phospho-AKT in the cytoplasm of WT and CCRL2 KO BMDCs after TLR7 stimulation.

A specific subset of DCs, plasmacytoid dendritic cells (pDCs), is known to express the highest levels of TLR7 among DCs [170] and to be able to secrete huge amounts of type I IFNs, especially IFNα [187]. These features make pDCs crucial for antitumoral [188] and antiviral responses [156,189]. Nevertheless, the triggering of TLR7 in pDCs is also involved in the pathogenesis of autoimmune diseases like psoriasis and SLE [152,190]. It has been demonstrated a pivotal role for CCRL2 in orchestrating the migration of DC subsets [95] and the importance of chemerin binding sharing between CCRL2 and CMKLR1 in the recruitment of pDCs to non-lymphoid tissues during pathological conditions such as autoimmune, allergic and infectious diseases as well as in tumors [191–193]. In this scenario, the study of the role of CCRL2 in the modulation of TLR7-mediated immune response in pDCs represents one of the topics worth to be investigated in the future. For these reasons, in the next future, *in vitro* experiments on pDCs isolated from the spleen of WT and CCRL2 KO mice to assess pro-inflammatory cytokines and type-I IFN production after the stimulation with R848 will be performed. The role of CCRL2 in TLR7 mediated pro-inflammatory response will also be investigated with *in vivo* experiments in which R848 will be intravenously injected in WT and CCRL2 KO mice, with the aim of assessing the levels of type-I IFNs. Moreover, to elucidate the specific mechanism of functional interaction between CCRL2 and TLR7, co-immunoprecipitation

experiments will be performed to test if it might be based on a physical interaction between them, as already hypothesized for CCRL2 and TLR4 [93]. Furthermore, the hypothesis of a physical interaction between CCRL2 and TLR7 will be investigated by proximity-ligand assay and FRET.

PART II: STUDY OF CCRL2 GENE METHYLATION IN COLORECTAL CANCER (CRC)

7. Introduction (Part II)

7.1. Colorectal cancer (CRC)

Colorectal cancer (CRC) is the fourth most deadly and the third most common cancer worldwide with approximately 900'000 diagnosis per year [194], two million new cases per year and almost one million deaths per year, accounting around 10% of cancer-related deaths worldwide [195].

The development of CRC is a multifactor multistep process that starts when normal epithelium in the gut accumulates mutations causing the formation of aberrant crypt foci (ACF) able to set the ideal environment for tumor growth. In particular, the disease begins as a benign adenomatous polyp [196], thus developing into an advanced carcinoma with high grade dysplasia and finally progresses into an invasive cancer, with possible generation of metastasis [197] (figure 15).

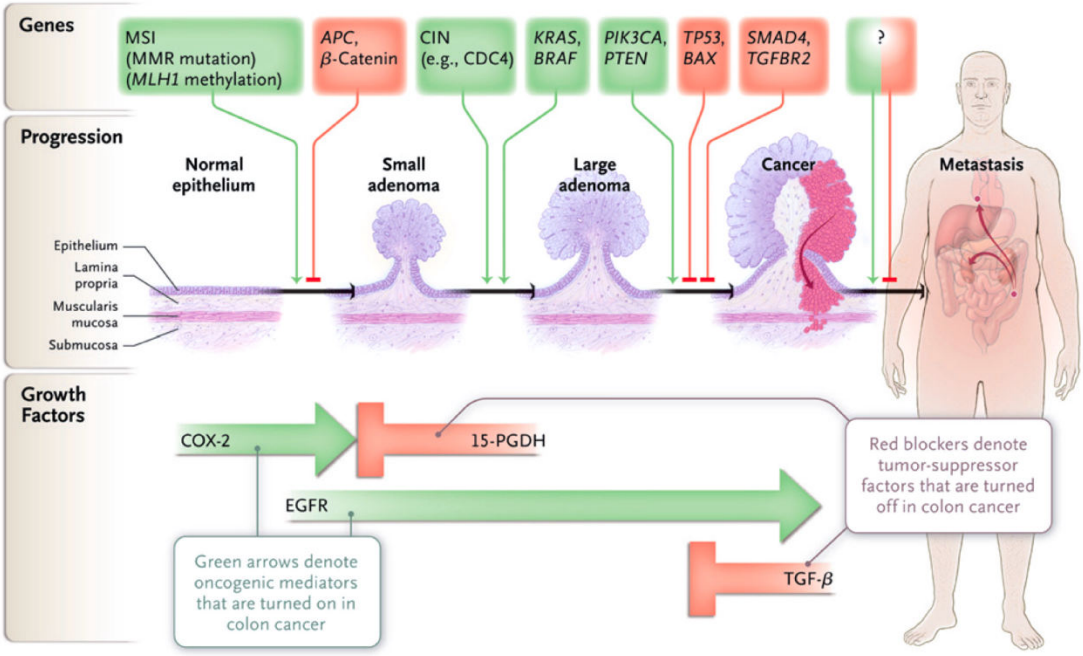

***Figure 15**: Genes and factors that drives the development of CRC through its stages of development* [196]

CRC development can be described by four main stages according to Duke's staging [198] which classifies the severity of the tumor according to histopathology traits and aids in estimating the risk of disease relapse [199] (figure

16): in the A stage cancer is in the inner lining of the bowel, or it is slightly growing into the muscle layer but it is still localized within the bowel. In the B stage, cancer has grown through the muscle layer of the bowel, so it reached different tissues to those in which it originated, but it is still confined in the organ without involving lymph nodes. In the C stage, cancer cells spread to at least one lymph node close to the bowel. In the D stage, cancer cells metastasize to at least another part of the body, frequently the liver, lungs or bones. In the number staging system, this is the same as stage 4, in fact it is also called advanced bowel cancer. The A and B stage are generally treated with efficacy through surgical excision, and more than 73% of C stage colorectal cancers are efficiently treated with the addition of chemotherapy, while the D stage is not curable in most cases [196], associated to a general overall survival of less than 15% [199].

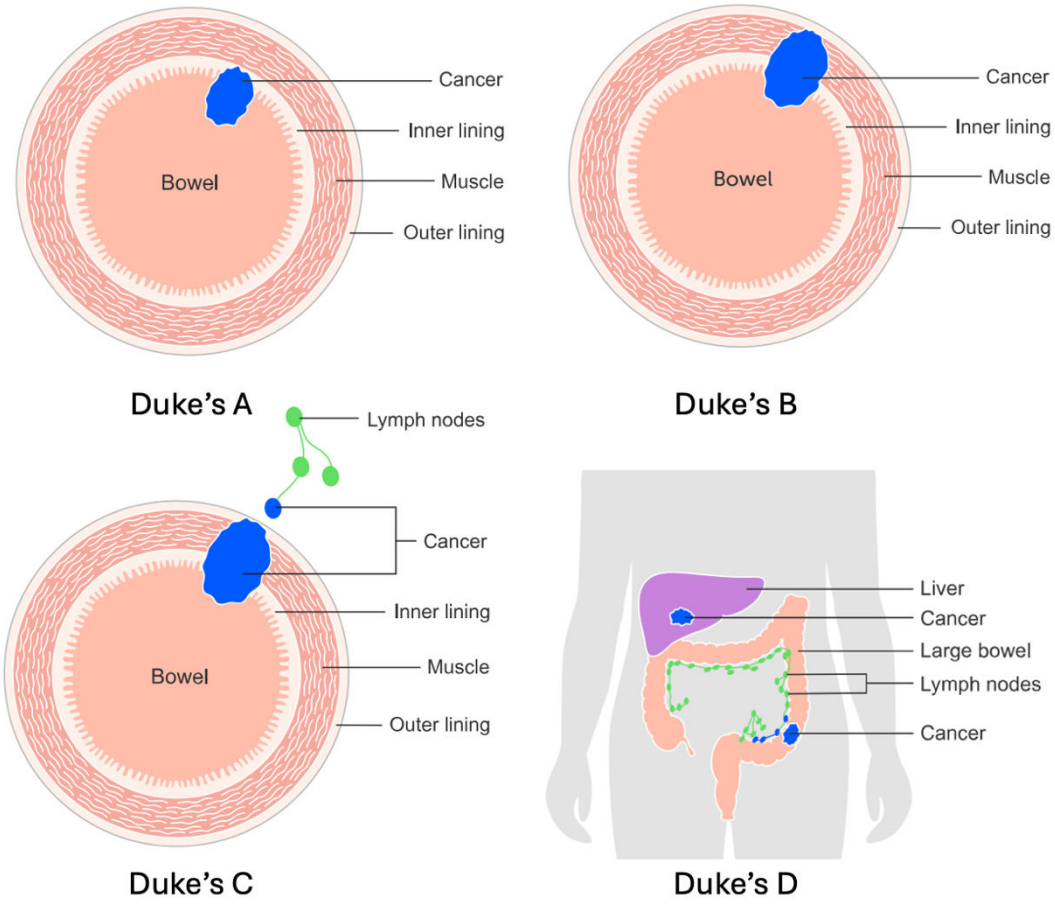

***Figure 16**: Representation of CRC development according to Duke's staging* [198]

CRC can be classified as hereditary or sporadic: several cases are associated with a genetic predisposition, but the majority result from sporadic mutations in the genes *APC* (80-82% cases)[200], *KRAS*, *BRAF* and *TP53*, which trigger uncontrolled cell proliferation and tumor formation [201]. In fact, more than 90% of cases are recognized as sporadic, not being related to genetic inheritance of CRC driving mutations, while only around 2-8 % of cases are related to genetic inheritance and predisposition [196,202]. The most common forms of hereditary CRC are Familial Adenomatous Polyposis (FAP) syndromes and Hereditary Nonpolyposis Colorectal Cancer (HNPCC).

FAP is a hereditary and autosomal dominant cancer syndrome characterized by the presence of hundreds to thousands of synchronous polyps in the gastrointestinal tract, especially in the large intestine that usually occurs in over-40 patients [203,204], which is due to a germline mutation in the Adenomatous Polyposis Coli (*APC*) gene [205]. A polyp can eventually progress into CRC over an estimated period of 10-15 years [194]. The *APC* gene was first discovered in the 1970s, located on the chromosomal region 5q21 and is a tumor-suppressor gene involved in the regulation of β-catenin in all tissues as part of the WNT signaling pathway, as it prevents constant activation of β-catenin that would bring uncontrolled cellular proliferation [206]. Inactivating mutations of the *APC* gene determines the translocation and accumulation of β-catenin to the nucleus with consequent transcription of some proto-oncogenes, like c-myc and cicline D1, that play a central role in cancer development [207]. *APC* regulates spindle microtubules and is required to detect misaligned chromosomes during mitosis [208].

HNPCC, also known as Lynch syndrome, is a hereditary cancer disease involving germline mutations occurring in genes encoding for proteins involved in mis-match repair (MMR), in particular *MSH2* and *MLH1* [209], thus causing microsatellite instability [197].

Sporadic CRC includes a heterogeneous group of pathologies that arises from the accumulation of different genetic and epigenetic alterations [210], that enhance and perpetrate genetic instability leading to progressive accumulation of these alterations, by four main mechanisms:

- *Chromosomal Instability* (CIN)
- *Microsatellite Instability* (MSI)
- *CpG Island Methylation pathway* (CIMP)
- *Histone modifications*

The current level of the understanding of epigenomic alterations in CRC is less than that of gene mutations, for this reason a full understanding of their role still has to be clearly elucidated, and investigations are still ongoing.

7.2. Chromosomal Instability (CIN)

CIN, defined as the presence of structural aberrations or changes in chromosome copy number, is the most common form of genomic instability in CRC, found in more than 85% of cases [211] and leads to many changes in the number and chromosome structure due to defects in chromatin cohesion in human cells [212]. In fact, loss of function of tumor-suppressor genes, including *APC*, whose normal function is to oppose tumorigenesis, has been implicated in the development of CIN [208]. Cin causes the accumulation of mutations also in other tumor suppressor genes (*TP53*, *SMAD4*), but also in oncogenes like PI3KCA, thus leading to an accumulation of tumor-driving conditions [197].

7.3. Microsatellite Instability (MSI)

Patients affected by CRC often display inactivity of genes involved in mis-match repair (MMR). This inactivation can be inherited (as in the case of hereditary non-polyposis cancer) or acquired [211]. The loss of the capability to repair mis-

match damages due to somatic mutation or aberrant methylation is associated to the so-called microsatellite instability [213]. As a consequence, the loss of MMR genes function and resulting inability to repair strand slippage within nucleotide repeats changes the size of microsatellites [196].

Microsatellites are mononucleotide, dinucleotide or trinucleotide repeats found throughout the entire genome. Their repetitive nature makes them vulnerable to transcription errors during DNA replication. These nucleotides repeats assume remarkable relevance if the microsatellite lies within the coding region of a gene as it may lead to altered gene functionality or a change in the associated protein [214]. Somatic inactivation of MMR genes is found in approximately 15% of cases of sporadic CRC. There are associations with older age, female sex and proximal distribution of these tumors. Germline mutations of MMR genes are responsible for HNPCC which carries a lifetime risk of CRC of about 80% [196]. Moreover, tumor-driving mutations associated to MMR genes are generally autosomal dominant [196]. The main MMR genes in which loss of function mutations were found are *MLH1*, *MSH2*, *MSH6* and *PMS2* [214,215]. Also germline mutations of the *EPCAM* gene have been identified as a novel cause of HNPCC, where the disruption of the 3' of *EPCAM* leads to epigenetic silencing of the near *MHS2* MMR gene [216].

7.4. CpG island methylator phenotype (CIMP)

Epigenetic is defined as a set of inheritable modification of genes expression that are not related to the alteration of genetic sequences [217]. The main mechanisms of epigenetic regulation are DNA methylation and histone modifications (methylation, acetylation and phosphorylation).

DNA methylation is a process involved in gene silencing, thus involved in the regulation of gene expression, X chromosome inactivation and silencing of centromeric regions. 5-methyl cytosine (5mC) is the most widespread type of

DNA methylation in eukaryotes [218] and is the only form of direct nucleotide methylation in mammals. The 5mC modification occurs within the so-called CpG islands where the regions of DNA are rich in CpG dinucleotides. This process includes the transfer of a methyl (CH3-) group from S-adenosyl methionine (SAM) to the fifth carbon position of cytosine, catalyzed by the DNA methyltransferase (DNMT) family (*DNMT1* and *DNMT3*), thus forming 5mC [219,220]. *DNMT1* is the most abundant and plays a role of maintenance of CpG islands methylation, while *DNMT3a* and *DNMT3b* are de-novo methyltransferases [221]. The hypermethylation of DNA is due to the overexpression and impaired recruitment of one or more members of the DNMT family [222]. 60–70% of promoters include CpG islands. In most normal cells, CpG sites are unmethylated in the promoters of expressed genes [223]. However, CpG dinucleotides outside of the promoter region often exhibit tissue-specific methylation states, and frequently participate to the regulation of gene expression. The methylation of cytosine doesn't compromise neither the pairing of nucleotides nor the tridimensional structure of the double helix, given the methyl group is positioned in the major groove. There are also proteins that binds cytosines with high affinity able to recruit histone deacetylases, histone methylases and chromatin remodeling complexes, leading to downregulation of transcription and heterochromatin formation [224]. On the other hand, other transcription factors can bind only non-methylated DNA so, in this case, DNA methylation limits the interaction with the transcription factors and as a consequence downregulates gene expression [224]. Some studies have illustrated that CpG islands exist in about 40–60% of tumor suppressor gene promoters, typically spanning 200–2000 base pairs (bp). Aberrant methylation of CpG islands is associated with epigenetic transcriptional silencing of tumor suppressor genes, playing a crucial role in the early stages of CRC pathogenesis [225–229]. This phenomenon in CRC is defined as aberrant mechanism of methylation of CpG Island (CIMP) and is observed in

about 15% of CRCs, most of which show loss of MLH1 expression thus further promoting MMR deficiency and microsatellite instability [196,230]. Two main classes of CIMP were identified: “high-CIMP” and “low-CIMP”. High-CIMP subgroup brings to the formation of a wide extended adenoma, in particular in proximal colon (right colon). It is more frequent in elder women and a good prognosis [231], given it is associated to a lower risk of metastasis development.[225]. High-CIMP cancers are mostly associated in 70-80% of cases to the methylation of MLH1 and to BRAF mutations, a protein kinase involved in RAS/RAF/MEK/ERK signaling pathway. The low-CIMP subgroup is generally associated to the mutation of KRAS and, in contrast to high-CIMP CRC it is both more frequent in men and independent from the MSI [210]. *KRAS* epigenetic alteration was found in 37% of CRCs and leads to the impairment of pathways regulating cell growth, differentiation, apoptosis and proliferation [218].

Other phenotypes related to DNA methylation involves involve for instance epigenetic mutations in TP53 gene and its loss of function [232].

7.5. Histone modifications

Histone modifications represent another crucial epigenetic mechanism of regulation of gene expression. In fact, they have the role to allow the access to the DNA by proteins like transcription factors and chromatin remodeling proteins. Chromatin consists of protein-DNA complexes, where octamers composed of core histones H2A, H2B, H3, and H4 are surrounded by 147 bp of DNA wrapped 1.6 times around the core, thus forming the nucleosome, the fundamental unit of chromatin. H1 histone is not directly part of the nucleosome but it is involved in the process of chromatin compartmentalization [218] (Figure 17). Aberrant histone modifications play a key regulatory mechanism in the development of several types of cancers, including chemotherapy resistance. A minimum of eight different types of

histone modifications have been identified, with common modifications including ubiquitination, phosphorylation, methylation, acetylation, and ADP-ribosylation [233]. Histone complexes determine the grade of condensation of genomic DNA, and post-translational modifications on histones directly influence the expression of oncogenes or tumor suppressor genes by altering the electrostatic charge of the DNA-binding histone tails, thus indirectly promoting cancer onset and progression [234]. For this reason, targeted therapeutic drugs directed at methyltransferases, deacetylases, and related enzymes may become crucial components of future treatments.

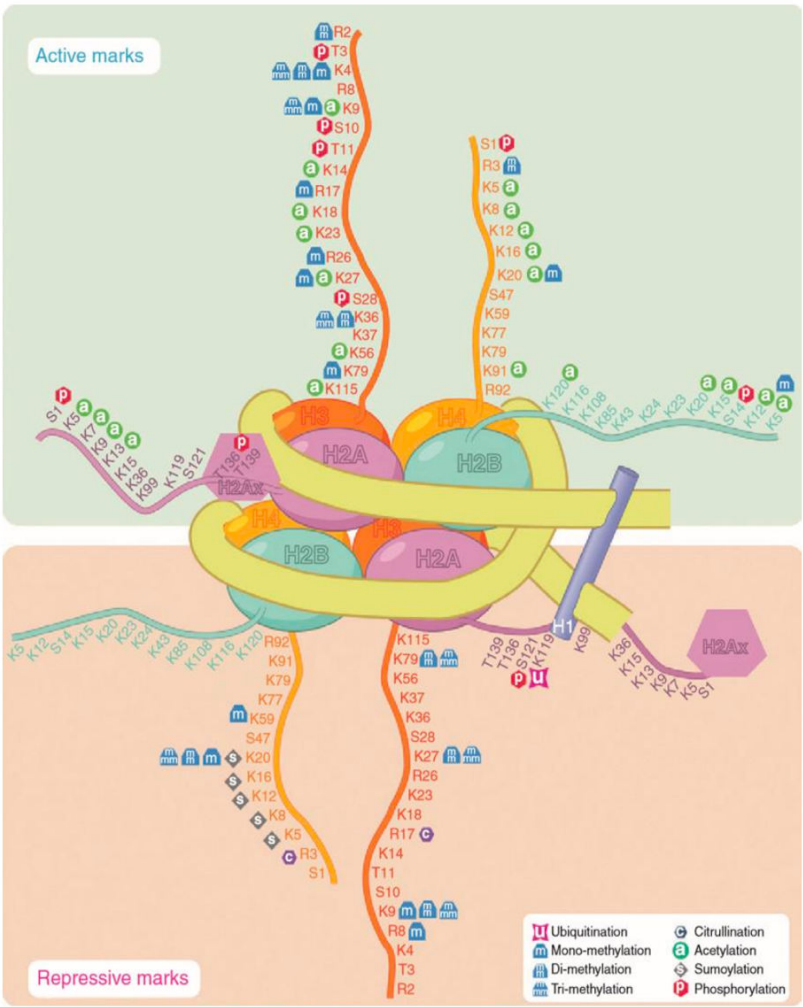

***Figure 17**: Scheme of the nucleosome. DNA wraps the octamer composed of histones H2A, H2B, H3 and H4. Different amino acids in the histone tails are shown associated to their covalent specific modification. Lysine (K), Arginine (R), Serine (S), Threonine (T).* [235]

The impairment of histone modifications in cancer are due to mutations of regulatory enzymes: these enzymes are classified in *writers* and *erasers*. The former catalyze post-transcriptional modifications, the latter remove them [236]. This means that a gain of function mutation of a writer gene may lead to a general increase of the associated histone modification, while a loss of function mutation may determine a global decrease of that modification. In general, the most studied histone modifications, associated to epigenetic profile impairment with a role in CRC are methylation, acetylation and phosphorylation (Figure 18).

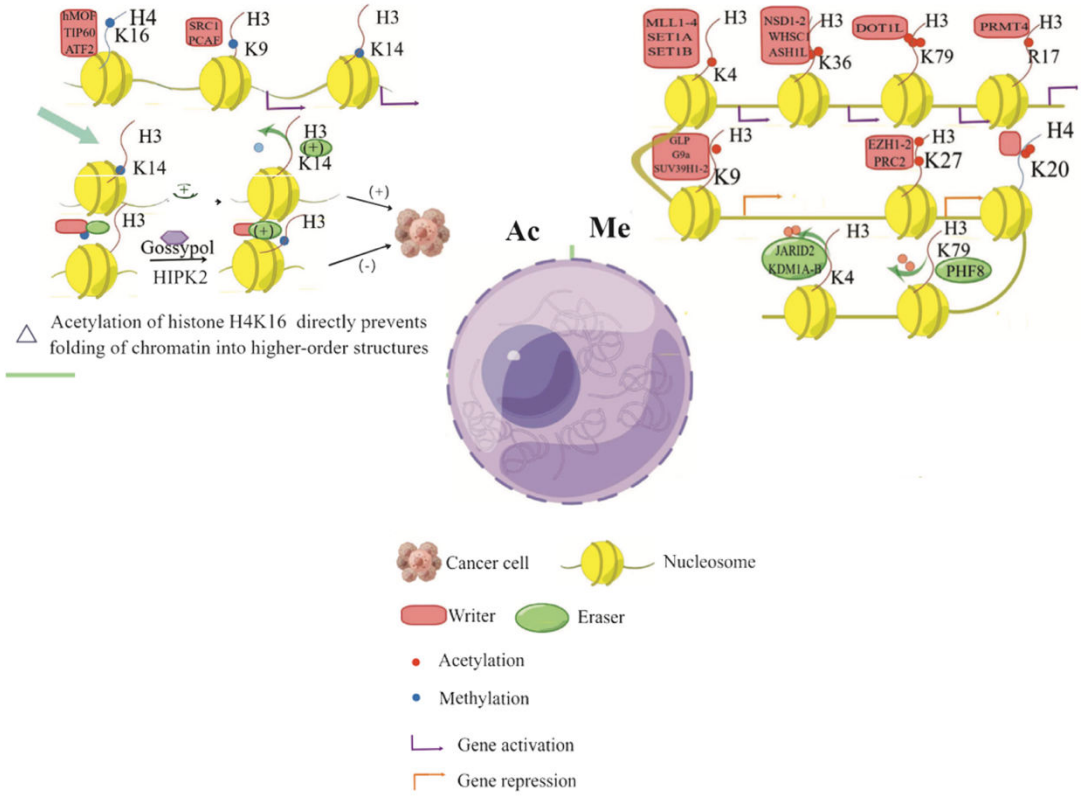

Figure 18*: the three most common forms of histone modifications in colorectal cancer: methylation, acetylation and phosphorylation* [237].

The impairment of histone modifications in cancer are due to mutations of regulatory enzymes: these enzymes are classified in *writers* and *erasers*. The former catalyze post-transcriptional modifications, the latter remove them [236].

This means that a gain of function mutation of a writer gene may lead to a general increase of the associated histone modification, while a loss of function mutation may determine a global decrease of that modification. In general, the most studied histone modifications, associated to epigenetic profile impairment with a role in CRC are methylation and acetylation (Figure 8).

7.5.1. The role of histone methylation in CRC

In general histone methylation dysregulation may cause transcriptional activation of multiple oncogenes, promoting cancer cell signaling for active cancer cell proliferation and malignant transformation [238]. Methylation typically occurs when histone methyltransferases (HMTs, Protein Arginin Methyltrasnferase, PRMT and Lysine Methyltransferase, KMT) catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to the lysine (Lys) and arginine (Arg) residues on the tails of histones H3 and H4 [218] that are the two most methylated histones [235]. These modifications are reversible thanks to the action of demethylases (HDMTs), such as lysine demethylases (KDM) [239]. For what concerns methylation, lysins can be mono-, di- or tri- methylated. The consequences of its methylation are various, while the consequences of the modification of methylation of the Arginines are usually associated to the functionality of tumor-suppressor genes. According to the specific amino acid that underwent methylation, it may serve as a marker for transcriptionally active euchromatin or transcriptionally inactive heterochromatin [240]. For instance, H3K9 (lysine 9 on H3 histone), H4K20 and H3K27 methylations are involved in the formation of heterochromatin, while H3K4, H3K36 and H3K79 are involved for euchromatin [235].

To date, many alterations of ectopic expression of histone methylation and demethylases have been widely described in colorectal cancer. For instance, to report few examples, it was demonstrated that increased global levels of di-

methylated lysine 9 on histone H3 (H3K9me2) in the nuclei of adenocarcinoma more than that in adenoma, suggesting that aberration of the global H3K9me2 level is an important epigenetic event in colorectal tumorigenesis and carcinogenesis involved with gene regulation in neoplastic cells through chromatin remodeling [241]. Moreover, it was reported that the methylation level of trimethylated lysine 9 on histone 3 (H3K9me3) was especially upregulated in invasive regions of colorectal cancer tissues and H3K9 trimethylation was also positively related to lymph node metastasis. in addition, high expression of H3K9 methyltransferase SUV39H1 facilitated the development of CRC which resulted in a poor survival rate in a mouse model [242]. In addition, it was showed that decrease methylation of H3K27me2 in liver metastasis in comparison with primary tumors, whereas the expression of H3K36me2 was reversed. They also demonstrated that the expression level of H3K37me2 is positively associated with tumor size and poorer survival rates, and it could serve as an independent prognostic factor for CRC patients with metachronous liver metastasis [243].

Many studies have also demonstrated that the alteration of HATS and HDMTS is widely present in CRC [244].

Here we report some examples. It was demonstrated that reduction of histone methyltransferase SEDT2 facilitated the CRC development by affecting alternative splicing [245]. Moreover, histone H3K27 methyltransferase EZH2 expression was upregulated in CRC, which was predicted shorter survival and advanced stage implying that it could use to an indicator of clinical outcome in CRC patients [246,247]. Elevate expression of Lysine-specific demethylase (LSD1) observed in colon cancer tissues, and high expression level of LSD1 was strongly correlated with advanced TNM stages and distant metastasis [248]. Conversely, hypomethylation of DNA may activate oncogene transcription, consequently promoting the development of carcinogenesis and tumor. A potential mechanism is the activation of Long Interspersed Nucleotide Element (LINE-1), which occupies about 18% of the human genome [249]. *LINE- 1*, a non-

long terminal repeat retrotransposon, may cause transcriptional disruption and genomic instability. In normal tissues, *LINE-1* is typically found to be highly methylated and inactive [218].

Nevertheless, demethylating agents are employed in CRC therapy. To provide a broader platform for cancer treatment, small molecule inhibitors targeting either histone methyltransferases or demethylases are used for the therapy of CRC, therefore histone modifying enzymes also serve as a drug target [239]. For instance, a study reported that LSD1 inhibitors CBB1003 suppress CRC cell growth through down-regulating LGR5 levels and inactivates the Wnt/β-catenin pathway [250]. Recently, in vitro study shows that JIB-04, a novel histone demethylase inhibitors targets colorectal cancer stem cells (CSC), was able to repress CSC growth, invasion, and migration to fight against colorectal cancer [251].

Also 5-Aza-2'-deoxicytidine (5-AZA-dC) is part of the demethylating agents employed in cancer therapy and it is a S-phase specific drug causing irreversible inactivation of methyltransferases [252].

7.5.2. The role of histone acetylation in CRC

Histone acetylation, like histone methylation, is a reversible and dynamic process achieved by the action of histone acetyltransferases (HATs) and deacetylases (HDACs) [253]. The identified HATs include P300/CBP, GNAT, MYST, P160, PCAF, TAFII230 families [254]. In the higher eukaryotes, HDACs can be classified into four groups on the basis of their homology with the original yeast enzyme sequence. Class I HDACs, including HDAC1, 2, 3, 8; Class II HDACs composed of HDAC4, 5, 6, 7, 9, 10; Class III HDACs, also known as Sirtuins, comprised of SIRT1–7; Class IV HDACs, including HDAC11 [255]. Among these HDACs Class I, II and IV are zinc-dependent, while Class III are NAD^+-dependent.

HATs transfer the acetyl group from acetyl coenzyme A to the terminal of histone amino acid and relax the structure of chromatin thanks to the electric charge, which promotes transcription by means of increasing the accessibility of DNA. On the contrary, HDACs remove the terminal acetyl group of histone lysine making the structure of chromatin more compact, resulting in the inhibition of transcription [256]. For these reasons, hyperacetylation generally leads to the increased expression of the gene which is related to the activation of gene transcription, while hypoacetylation means repression of gene expression.

Up to now, many evidences indicate that histone acetylation finely regulates a wide range of cell functions, such as cell differentiation, assembly of the nucleosome, the change of chromatin structure and the stability of gene expression [257]. it's not surprising that abnormal regulation of histone acetylation is relevant to set a predisposition of developing colorectal cancer and this process is modulated by a range of deregulated enzymes related to acetylation and deacetylation of histones. For example, the acetylation of H3K27 was found significantly increased in CRC samples in comparison to healthy controls' tissue [258]. It was also reported that acetylation of H3K12ac and H3K18ac is significantly increased in moderate to well differentiated CRC, whereas decreased in poorly differentiated CRC. High levels of HDAC2 in adenocarcinoma compared with those in adenoma were also observed, suggesting that the expression of HDAC2 is closely related to the progression from adenoma to adenocarcinoma [259] and has also been found up-regulated in CRC cell lines as compared with their corresponding normal colonic epithelial cells [260]. Furthermore, several evidences suggest that global histone acetylation positively correlates with tumor stage, lymph metastasis, poor survival, poor prognosis, histological subtype and cancer recurrence [261,262]. Moreover, HDAC1 was shown to higher in CRC tissues than in normal tissues and low expression of it indicated better overall survival (OS) [263].

Nevertheless, HDACs inhibitors are involved in the therapy of colorectal cancer. The importance of their role is given by the biological processes they contribute to regulate, such as tumor cell cycle arrest, inhibition of tumor growth, differentiation, apoptosis and angiogenesis [239]. Therefore, various histone deacetylase inhibitors (HDIs) underwent clinical trials with the aim of treating several types of cancer, including CRC. For example, trichostatin A (TSA) suppress the growth of CRC cells in vivo by inducing cell cycle arrest and apoptosis through the modulation of JAK2/STAT3 signaling [264]. Moreover, SAHA, known as suberoylanilide hydroxamic acid, inhibits the growth of CRC via decreasing the expression of histone deacetylases, cyclin D1 and survivin [265]. In general, it was observed that HDACs inhibitors as monotherapy were initially incorporated into the clinical exhibits limited effectiveness, development of drug resistance as well as adverse effect in the treatment of cancer [266,267]. For this reason, the tendency to apply them in corporation with different types of anti-cancer drugs is increasing. Several studies show the combined therapies may amplify the anti-tumor effect to suppress refractory tumors [268]. The limitations of HDACs inhibitors in the treatment of cancer is the lack of specificity, thus, the development of effective and specific epigenetic drugs against CRC and provide an optimized therapeutic regimen for CRC patients is critical.

7.6. The relationship between histone and DNA methylation

DNA methylation and histone modifications are not biologically separated events, but fine-tuned dynamic processes strictly connected to each other [222]. In particular, proteins owning a methyl-CpG-binding-domain (MBD) and DNMT1, are able to recruit HDACs at the methylated promoters where they deacetylate histones, keeping chromatin in a structural state where gene expression is repressed [224]. Moreover, the state of methylation of CpG islands

has a role also in guiding histone methylation [269]. In origin it was believed that histone modifications were secondary and less important in comparison to DNA methylation [224]. Despite this, a study performed on fungi, specifically on a mold called *Neurospora Crassa*, showed that mutations in the gene coding for a H3K9 specific histone methyltransferase causes the loss of methylated cytosines too, providing the first evidence that histones methylation can lead DNA methylation [270]. Nevertheless, as was first pointed out in plants, the methylation of CpG islands depends on histone H3 methyltransferase [271]. Therefore, it was suggested that histones modifications, like methylation and acetylation, are necessary to induce or maintain DNA methylation, probably also through the recruitment of MBD proteins [224]. On the basis of these observations, it is possible to make the hypothesis that histones modifications represent a primary event of gene silencing, while hypermethylation of CpG islands, as a subsequent event, may be useful for the maintenance of genic inactivation.

7.7. The role of 5-AZA-dC in the therapy of cancer and in the study of CCRL2 regulation

The aspecific demethylating effects of 5-AZA-dC can lead to the reactivation of abnormally silenced tumor-suppressor genes, thus contributing to the induction of differentiation or senescence, growth inhibition and loss of cologenicity [272]. In particular, it was demonstrated to be effective in patients with chronic myeloid leukemia [273] and myelodysplastic syndrome [274]. For what concerns solid tumors, it was proved effective in reducing cologenicity and cancer growth in a mouse model of mammary tumor [275]. In solid tumors such as NSCLC effectiveness was visible at high dosages and probably limited due to the delayed action of 5-AZA-dC since the cell kinetics of most human solid tumors is different than that of leukemia because solid tumors in general have

long cell cycle times and 5-AZA-dC is a S-phase specific drug, so the efficacy of the treatment is bound also to its duration and overlap with the specific duration of cancer cell cycle [272]. Research on the effectiveness of 5-AZA-dC in solid tumors is still ongoing, in fact clinical trials in groups of patients affected by solid tumors whose disease is resistant to standard therapy or for which it is not available [276].

5-AZA-dC is important also for its role in the study of the regulation of CCRL2 expression and its roles in cancer biology, as well as of the mechanisms of resistance to DNMT inhibitors in neoplastic diseases. For instance in a recent study, Karantanos et al. used 5-AZA-dC to study the role of CCRL2 in the regulation of pathways associated with poor response or resistance to DNMT inhibitors in myelodysplastic syndrome and secondary acute myeloid leukemia (sAML), finding that CCRL2 promotes resistance to DNMT inhibitors, given that CCRL2 is upregulated in MDS and sAML cells and its knockdown enhances the sensitivity of these cells to 5-AZA-dC. These findings therefore suggest that targeting CCRL2 could improve therapeutic outcomes in MDS and sAML [277]. Moreover, another recent study by Sozio et al., explored the role of the CCRL2/chemerin/Chemerin1 axis in the recruitment of natural killer (NK) cells to the lungs in a mouse model of lung cancer. The research demonstrates 5-AZA-dC can enhance the expression of CCRL2 leading to increased NK cell infiltration and reduced tumor progression [103].

7.8. The role of inflammation in colorectal tumors

It is well known how persistent chronic inflammation is another key driver of the pathogenesis of several tumors, including CRC [278,279]. For instance, chronic inflammation in the gut typical of the Inflammatory Bowel Disease (IBD), in particular ulcerative colitis, can lead to Colitis-Associated Cancer (CAC), a subtype of CRC. The incidence of CRC among IBD patients in fact (i.e. CAC) was

reported to be up to 60% higher than the general population [280]. Inflammation is driven by soluble factors like cytokines and chemokines that may also be produced by cancer cells but also from immune cells (neutrophils, natural killer cells, macrophages, lymphocytes dendritic cells) recruited within the tumor [281]. Pro-inflammatory cytokines promote cancer growth, differentiation and survival [278]. The cells composing the TME also produce oxygen reactive species (ROS), that further enhance the development of the tumor through the accumulation of genetic and epigenetic alterations in epithelial cells [282]. The major part of cytokines promoting tumor growth are triggered by the NF-κB pathway, which aberrant activation was found in more than 50% of CRC cases [283]. In the context of the gut, loss of NF-κB signaling in the epithelial compartment demonstrated increased susceptibility to inflammation but protection from tumorigenesis, through increased apoptosis [284]. On the other hand, constitutively active IKKβ (a kinase in the NF-κB signaling pathway) in the epithelial compartment led to prolonged activation of NF-κB and accelerated development of colorectal tumors with increased DNA damage, associated with a high production of ROS [285]. The most relevant pro-inflammatory cytokines involved in the development of colorectal tumors are TNFα, IL1β and IL6, which also plays a key role in STAT3 activation. IL-6 is frequently found in the tumor milieu of both mouse and human CRC and is associated with poorer survival [278]. IL-6 induces Th17 cell differentiation and regulates STAT3 signaling to induce survival and proliferation of cancer cells [286]. In fact, *in vivo* studies demonstrated that the inhibition of STAT3 signaling reduced tumor burden in murine CRC models [287]. The most upregulated cytokine in the TME of colorectal tumors is CXCL8/IL8, that through the interaction with its receptor CXCR2, enhances the proliferation of cancer cells via autocrine activation and promotes angiogenesis and neutrophils recruitment within the tumor [288]. Chemokines play a crucial role both in the physiological and pathological context. Chemokines in fact provide a fundamental contribution to the

recruitment of leukocytes to the site of inflammation, protagonists of the pathogenesis of CRC [289]. In fact, tumors constitutively produce pro-inflammatory chemokines that increase the immune infiltrate [290]. Inflammation also plays a crucial role on tumor initiating cells (TICs), a sub-population of the bulk tumor that harbors "stem-like" characteristics and are thought to confer resistance to therapies [278]. Mounting evidence suggests functional roles for inflammation in driving TIC phenotypes in CRC, likely via convergence with canonical stem cell pathways (i.e. Wnt signaling). Several *in vitro* and *in vivo* results have demonstrated that CRC TICs express high levels of phosphorylated STAT3 and are sensitive to STAT3 inhibition [291]. Consistently with data in normal intestine suggesting IL22 enhances epithelial cells growth in a STAT3-dependent manner, it was found that IL22 produced by $CD4^+$ T cells leads to the induction of STAT3, conferring CRC cells "stemness" [292]. Similarly, high levels of NF-κB have been described in cells with a TIC phenotype and studies suggest that NF-κB enhances Wnt activation and tumor initiating capacity [293].

7.9. The role of CCRL2 in cancer and colorectal inflammation

Despite the investigations concerning the role of CCRL2 in cancer is still ongoing, what it is known so far is that in non-small-cell lung cancer (NSCLC) patients, high expression of CCRL2 is associated to a positive overall survival and better clinical outcome [121,294]. Even in the case of CRC, a higher expression of CCRL2 is associated to a better overall survival and in comparison to cancer tissue, CCRL2 is more expressed in healthy controls [295].

Reduced levels of CCRL2 have been associated to increased tumor growth in urethane-induced lung carcinogenesis and in a genetic model of Kras/Tp53 – driven (KrasG12D=C/p53LoxP) lung tumor [121].

Accordingly, CCRL2 KO mice were observed to be more prone for tumor growth in comparison to controls after the orthotopic injection of a tumor cell line obtained by the KrasG12D=C/p53LoxP mouse model. The ablation of CCRL2 influenced leukocytes' recruitment in the tumor microenvironment (TME). In particular it was found a reduced presence of some myeloid cells subsets such as macrophages, monocytes, neutrophils and a very consistent reduction of the frequency of NK cells in the lungs. Murine NK cells do not express CCRL2, but it was found expressed in $CD31^+$ cells: this led to the hypothesis that CCRL2 on the surface of lung endothelial cells, may act as a chemerin-concentrating receptor regulating the recruitment of NK cells expressing chemerin's functional receptor, Chemerin 1, thus shaping the characteristics of the TME [121].

CCRL2 was also observed in prostate cancer, but in this case its specific role is still under investigation, both for what concerns tumor progression and immune cells recruitment. However, in the case of prostate cancer, CCRL2 expression is significantly stronger in epithelial cells of cancerous acini than in adjacent benign acini [102].

A study by Karantanos et al. it also pointed out how CCRL2 is upregulated in primitive cells from patients with secondary acute myeloid leukemia (sAML) and CCRL2 knockdown in two sAML cell lines (MDS92 and MDS-L) suppressed cell growth and cologenicity both *in vivo* and *in vitro* [296]. CCRL2 was also demonstrated to potentiate JAK2/STAT interaction. This atypical receptor is therefore also a MDS/sAML cell growth mediator, potentially through JAK2/STAT signaling.

In the case of CRC, CCRL2 has been identified as playing a significant role also for what concerns the metastatic potential, particularly to the liver. In studies involving both rat and human CRC samples, CCRL2 expression was found to be associated with liver metastasis. Specifically, CCRL2, along with CCR1, was

upregulated during the colonization process in the liver, with CCRL2 being significantly upregulated compared to control cells. Additionally, also human CRC samples examined were positive for CCRL2 expression. This suggests that CCRL2 may be instrumental in the metastatic process, particularly in liver colonization, making it a potential target for therapeutic intervention in CRC [297].

CCRL2 was also demonstrated to play a role in colorectal inflammation in murine models resembling human IBD. For instance, a DSS-induced [1] colitis model [298] was used to study the expression of CCRL2 in IBD, finding that it was transcriptionally upregulated in the distal colon and cecum, suggesting that chemerin/CCRL2 axis may play a role in IBD [299]. Furthermore, CCRL2 knockout (KO) mice showed a more rapid weight loss in comparison to WT controls in a DSS-induced colitis model [300]. It was also demonstrated that CCRL2 expression is upregulated in rat models of CIM CRC, especially in the early phases of liver colonization [126].

[1] dextrane sulfate sodium (DSS) is a non-genotoxic pro-inflammatory agent used to model acute and chronic colitis in rodents.

8.AIM (PART II)

Chemically induced CRC models (CIMs) are widely used for the research on CRC and they consist in the intraperitoneal administration of known carcinogens like azoxymethane (AOM), paired with oral administration of DSS in the water. Previous studies conducted in our laboratory show that CCRL2 KO mice are more susceptible to the development of intestinal polyps in comparison to WT control animals by using an AOM/DSS model of inflammation-associated carcinogenesis. Moreover, low CCRL2 expression is associated with a poorer survival rate in CRC patients [295], suggesting that CCRL2 may represent a regulator of gut inflammation and plays a role in CRC pathogenesis.

This second part of the thesis aims to investigate the epigenetic modifications that affect CCRL2 expression in CRC. Specifically, we investigated whether the CCRL2 gene is methylated in CRC cell lines by using two different strategies: an indirect approach, using 5-Aza-2'-deoxicytidine (5-AZA-dC), a demethylating agent, and a direct technique, namely methylated DNA immunoprecipitation (MeDIP).

9.MATERIALS and METHODS (PART II)

9.1. Cell cultures and treatments

Human colorectal cancer (CRC) cell lines were used, specifically RKO, SW480, Caco-2, COIL O-205, HCT116, HT29, LoVo, SW620. Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), along with L-glutamine and antibiotics (100 U/ml penicillin and streptomycin).

Where indicated, CRC cell lines were pre-treated for different time points with increasing concentration (0.1, 1, 10 μM) of the methylation inhibitor 5-Aza-2'-deoxycytidine (5-AZA-dC) (Merck™), with the medium being replaced every 24 hours. CRC cells were stimulated with pro-inflammatory mix, TNFα (Peprotech™) 20 ng/ml, IFNγ (Miltenyi™) 50 ng/ml and LPS (Sigma-Aldrich™) 1 μg/ml, for 24 hours.

9.2. Flow cytometry and immunofluorescence analysis

RKO and SW480 cells were labeled for 30 minutes at 4°C with an anti-human CCRL2 antibody (Biolegend®), followed by an Alexa488-conjugated secondary antibody (Molecular Probes® by Invitrogen™) for an additional 30 minutes at 4°C.

To observe human CCRL2 receptor expression through microscopy, CRC cells previously were seeded onto slides using a cytospin. Subsequently, the cells were fixed with 100% ice-cold methanol for 10 minutes at -20°C, stained with DAPI (Invitrogen™), and mounted with a coverslip using prolong antifase mountant (Invitrogen™). Analysis was performed using a ZEISS® Axiobserver™ Z1 inverted fluorescence microscope equipped with the Apotome™ system and a plan-apochromat 100x/1.4 NA oil objective.

9.3. Immunohistochemistry

Immunohistochemistry was performed as previously described [301], serial 4 µm formalin-fixed and paraffin-embedded healthy and tumor sections from colon of patients affected by CRC were immunostained with a primary antibody against human CCRL2 (Sigma-Aldrich™). The sections were labeled by the avidin-biotin peroxidase procedure with a commercial immunoperoxidase kit (VectaStain Standard Elite®, Vector Laboratories™). Immunoreactivity was revealed by incubation of the section with 3,3'-diaminobenzidine (Vector Laboratories™). Sections were counterstained with Mayer's hematoxylin. Patients were enrolled by the Oncology Department of "Spedali Civili di Brescia".

9.4. RNA isolation and qPCR

RNA was extracted using QIAzol™ Lysis Reagent (Qiagen ™) and treated with the DNAse I according to the manufacturer's protocol. Reverse transcription was performed on 0.5 µg of RNA using random hexamers and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV RT) (ThermoFisher Scientific®).

The SsoAdvanced Universal SYBR Green Supermix (Biorad®) was used according to the manufacturer's instructions. Reactions were run in duplicate on a StepOne Plus Real-Time PCR System (Applied Biosystems®) and analyzed by StepOne Plus Software (Applied Biosystems®, version 2.3). Sequences of gene-specific primers are listed in table 6. Gene expression was normalized based on GAPDH mRNA content ($2^{-\Delta CT}$ method).

Gene name	Species	Primer Sequence (5'→3')	
GAPDH	human	Forward	CCCACTTTTTCCCTCTTCTTG
		Reverse	CAGTGTGCCTTTCATTCCATC
CCRL2	human	Forward	AAGGAGGGCATCCACTGTC
		Reverse	CAGCGTGTAATTGGCCATC

***Table 6**. List of the primers used to perform qPCR on the RNA extracted from the cell lines*

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9.5. MeDIP (Methylated DNA Immuno Precipitation)

MeDIP is an immunoprecipitation lab technique that allows to investigate DNA methylation exploiting an antibody that recognizes 5-methyl cytosines [302]. DNA extraction was performed with the Tissue Genomic Extraction Kit (Fisher Molecular Biology™) according to the manufacturer's instructions.

- Dilute the obtained DNA at 20 µg of DNA in 300 µl of Tris/EDTA (TE).
- Sonicate each sample 2 times for 15 seconds keeping them 30 seconds in ice between each sonication to perform DNA fragmentation.
- Load 12.5 µl of sonicated DNA on 1.5% agarose gel (w/v) to verify DNA fragmentation. Fragments are expected between 300 and 1000 bp, with an average dimension around 500 bp.
- Dilute 4 µg of sonicated DNA in 450 µl of TE in DNA LoBind tubes.
- To perform DNA denaturation, incubate the tubes at 98 °C for 10 minutes on a shaker at 1000 rpm and then keep the tubes in ice for 10 minutes.
- Add 51 µl of IP buffer 10X (100 mM Na-phosphate pH 7.0, 1.4 M NaCl, 0.5% TritonX 100).
- Add 5 µg of anti-methyl cytosine antibody (Millipore™) and incubate at 4°C for 2 hours and 30 minutes in rotation.
- Add 50 µl of Dynabeads protein G and incubate for 1 hour at 4 °C in rotation.
- Wash two times with 800 µl of IP buffer.
- Spin the tubes, put them in the magnetic rack for 1 minute, discard the supernatant and resuspend with 250 µl of proteinase K Digestion Buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% SDS), vortex and add 3.5 µl of proteinase K (20 mg/ml) (Sigma-Aldrich™).

- Incubate the samples at 50 °C overnight on a shaker at 800 rpm.
- Spin the tubes, put them in the magnetic rack for 1 minute.
- Transfer the supernatant (immune precipitate, IP) in a new tube.

For DNA purification the QIAquick PCR purification kit (Qiagen®) was used, according to the manufacturer's protocol.

The analysis of the results of the MeDIP after Real Time qPCR on both input and IP was obtained as a difference (**Δ**CT) between the CT of the IP and the CT of the input. Then we calculated the percentage of the input as

$(\frac{1}{2}^{\Delta CT}) * 100$, to normalize each sample to its input.

qPCR was performed on the immunoprecipitated and on the input (24 ng of sonicated DNA). Sequences of gene-specific primers are listed in table 7.

Gene name	Species	Primer Sequence (5'→3')
GAPDH	human	Forward CCCACTTTTTCCCTCTTCTTG Reverse CAGTGTGCCTTTCATTCCATC
CCRL2 (irrelevant region before the promoter)	human	Forward CCCTCCCACCTCATACTTCTGAG (-1555 nucleotides from the ATG) Reverse GAGGCGACATCAAGTTGGGTGGG (-1095 nucleotides from the ATG)
CCRL2 (putative promoter)	human	Forward CAGCGGGTGGGTTTCTGCGAACAC (-735 nucleotides from the ATG) Reverse CTGAAGCGTCTGAAAGCAACCGG (-424 nucleotides from the ATG)
H19	human	Forward GAGCCGCACCAGATCTTCAG Reverse TTGGTGGAACACACTGTGATCA

***Table 7**. List of the primers used to perform qPCR on the immunoprecipitated and on the input.*

9.6. ChIP (Chromatin Immuno Precipitation)

ChIP is an immunoprecipitation technique that allows the study of protein-DNA interactions, specifically regulatory proteins associated with particular genomic regions, such as transcription factors on promoters. It also enables the study of epigenetic modifications of histones.

The protocol is as follows:

- Add 40% formaldehyde directly to the culture medium to achieve a final concentration of 1%. Incubate the formaldehyde for 10 minutes. This step is used to fix the cells.
- To stop fixation, add 1 M Tris-HCl pH 7.5 to reach a final concentration of 125 mM, place the plates on ice, and perform 3 washes of 10 minutes each with 13 ml of cold PBS.
- Scrape the cells from the plate with 2 ml of PBS (repeat once more) and collect them in a tube.
- Centrifuge at 4°C for 10 minutes at 1200 rpm.
- Discard the supernatant and lyse the cell pellet with 1 ml of cold L1 Buffer (50 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 0.1% NP40, 10% glycerol) containing inhibitors (protease inhibitor cocktail 1000X, 0.1 M PMSF, 200 mM Na_3VO_4, 200 mM NaF, and 100 mM DTT).
- Incubate on ice for 5 minutes.
- Centrifuge at 4°C for 5 minutes at 3000 rpm, discard the supernatant (cytoplasmic lysate), and lyse the nuclear pellet with 600 µl of L2 Buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1% SDS) containing inhibitors.
- Sonicate each sample 3 times for 12 seconds, with 10-second intervals between each sonication, on ice to fragment the chromatin.
- Centrifuge at 8°C for 20 minutes at 13000 rpm to remove nuclear debris.
- Collect 50 µl of the supernatant to check the sonication efficiency on a 1.5% agarose gel (after reversing crosslinking at 65°C for a minimum of 4 hours and extracting the DNA). The process is successful when the chromatin fragments are around 500 bp in size.
- Transfer 120 µl of the supernatant into new DNA lo-bind tubes and add 9 volumes of "Dilution Buffer" (DB: 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.5% NP40).
- Add 3 µl of anti-Trimethyl Histone H3 antibody (rabbit monoclonal) (Millipore™) and incubate overnight under rotation at 4°C.

- Add 50 µl of Dynabeads, pre-washed with "Washing Buffer" (WB: 20 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1% SDS, 1% NP40, 500 mM NaCl), and incubate at 4°C for 1 hour under rotation.
- Spin the tubes, place them in a magnetic rack for 1 minute, and collect 120 µl of supernatant, which will serve as input (non-immunoprecipitated) for PCR normalization.
- Discard the supernatant and perform 3 washes with 800 µl of WB containing 1 mM PMSF, rotating at 4°C for 20 minutes.
- Perform an additional 3 washes of 5 minutes each with 1X TE buffer on ice.
- Spin the tubes, place them in a magnetic rack for 1 minute, discard the supernatant, and resuspend the pellet in 120 µl of "Elution Buffer" (1X TE, 2% SDS) for 30 minutes at 37°C under agitation to elute the antigen-antibody complexes.
- Spin the tubes, place them in a magnetic rack for 1 minute, and transfer the supernatant (immunoprecipitate) into a new siliconized tube.
- Incubate both input and immunoprecipitated samples at 65°C for a minimum of 4 hours to reverse the crosslinking.
- Purify the DNA using the QIAquick® kit (Qiagen ™).
- Perform qPCR on both input and immunoprecipitated samples after purification, using the same primers as those employed for MeDIP. The results are analyzed using the same method as for MeDIP.

9.7. Statistical analysis

Statistical analyses were performed by using paired or unpaired Student's t test and ordinary one-way ANOVA, as appropriate. Results were analyzed by GraphPad PRISM 9.0 software.

10.RESULTS (PART II)

10.1. Analysis of CCRL2 expression in biopsies from healthy and tumor tissue from CRC patients

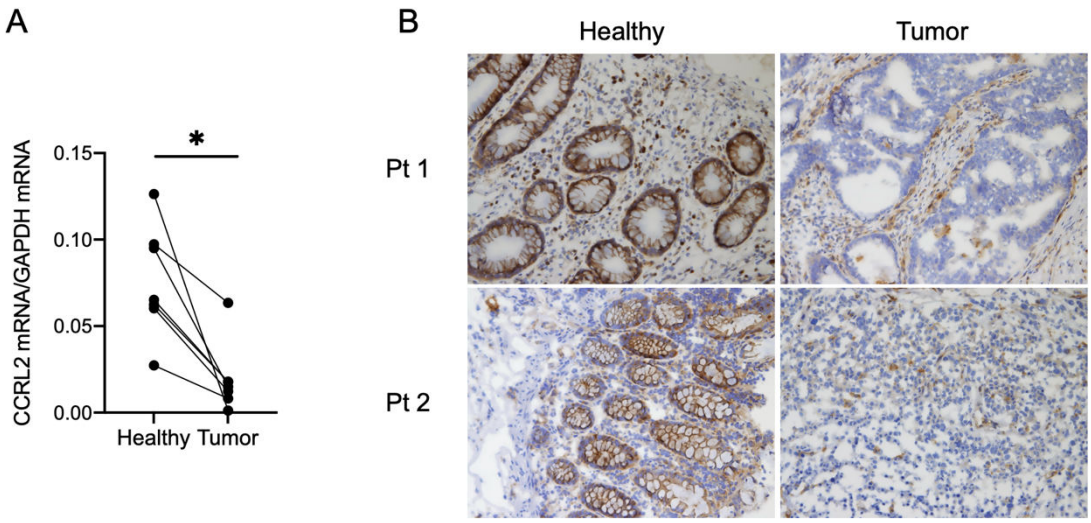

Figure 19. Assessment of CCRL2 expression in biopsies from healthy and tumor tissue from CRC patients. (A) The evaluation of CCRL2 transcript expression was performed by qPCR, and the results are expressed as mean ± SEM (n=7) of CCRL2 mRNA expression relative to the housekeeping gene GAPDH mRNA. *p<0.05 (-). Healthy vs tumor using Student's t-test. (B) Representative IHC comparing CCRL2 expression in biopsies from healthy and tumor tissue.

In Figure 19A we can observe the difference of CCRL2 expression between epithelial colorectal samples from healthy and tumor biopsies from patients affected by CRC. In particular, the expression of CCRL2 is significantly reduced in CRC patients in comparison to healthy controls, consistently with previous studies demonstrating a lower CCRL2 expression in CRC tissue in comparison to healthy tissue [295]. In figure 19B we can see two representative experiments of immunohistochemistry. In both patients the comparison between a tumor tissue section and a healthy section it is possible to observe how CCRL2 is widely expressed in healthy tissue compared to tumor tissue.

10.2. Analysis of CCRL2 expression in CRC cell lines

In this phase, to study the regulation mechanisms of CCRL2 gene in the context of CRC, CCRL2 levels of expression were tested by qPCR in different CRC cell lines (Figure 20A). Thus, a CCRL2-high and a CCRL2-low cell line were chosen, respectively RKO and SW480 (Figure 20A). The results obtained by qPCR in these two cell lines were also confirmed at the protein level through flow cytometry analysis (Figure 20B) and immunofluorescence (Figure 20C).

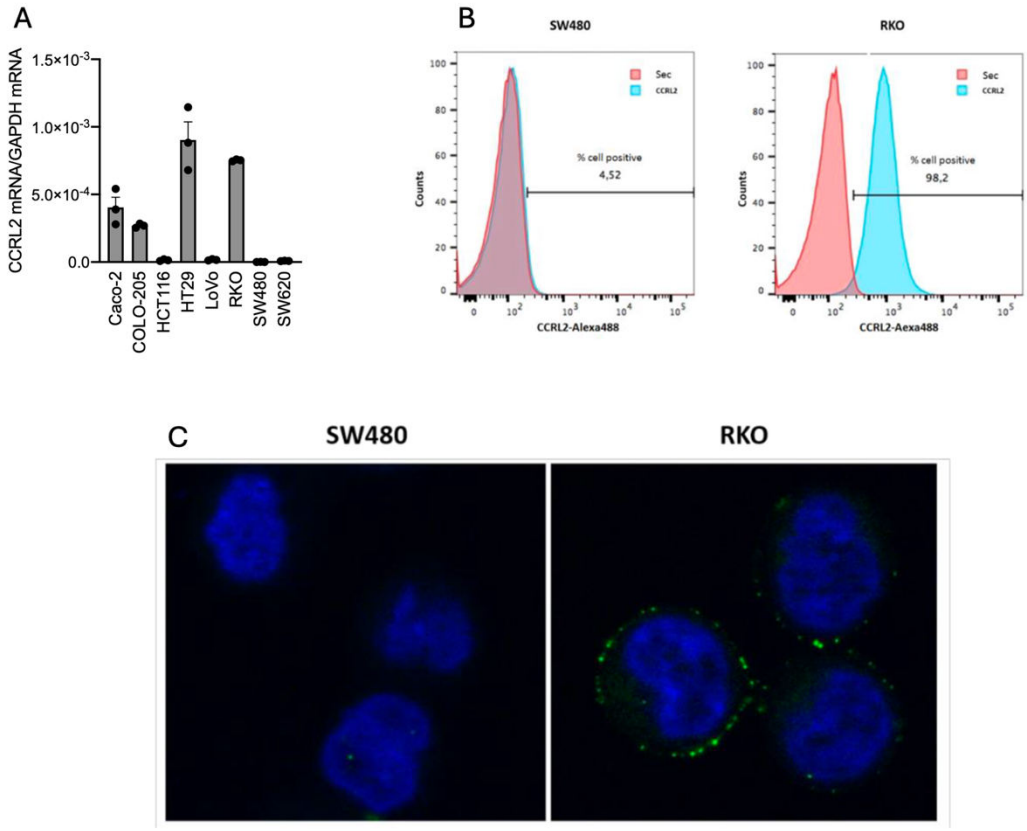

Figure 20. Analysis of CCRL2 expression in CRC cell lines. (A) The evaluation of CCRL2 transcript expression was performed using qPCR, and the results are expressed as mean ± SEM (n=3) of CCRL2 mRNA expression relative to the housekeeping gene GAPDH mRNA. (B) CCRL2 expression was analyzed by flow cytometry using an anti-human CCRL2 antibody, followed by an Alexa488-conjugated secondary antibody. The SW480 tumor cell line is shown on the left, while the RKO tumor cell line is shown on the right. (C) Evaluation of CCRL2 expression by immunofluorescence. DAPI was used after the staining with the anti-human CCRL2 antibody to visualize the nuclei.

10.3. Study of CCRL2 methylation in SW480 and RKO cell lines

Following the analysis of CCRL2 expression in the two different CRC cell lines, we sought to investigate whether CCRL2 was silenced in the SW480 line as a result of epigenetic modifications. Since the most common epigenetic modification observed in tumors, which is associated with gene silencing, is DNA methylation, we focused on studying the methylation of the CCRL2 gene by comparing the two CRC lines.

The analysis of CCRL2 gene methylation was performed using two distinct experimental approaches: initially, an indirect technique was employed, involving the use of a DNA methyltransferase inhibitor; subsequently, the methylation level of CCRL2 was directly studied using Methylated DNA Immuno-precipitation (MeDIP).

10.3.1. Indirect study of CCRL2 methylation: treatment of the cells with 5-AZA-2'-Deoxycytidine (5-AZA-dC)

5-AZAcitidine (AZAcitidine) is a chemical analog of cytidine, a nucleoside present in both DNA and RNA. 5-AZAcitidine and its derivative 5-AZA-2'-deoxycytidine (decitabine, 5-AZA-dC) are drugs with antineoplastic activity, as they inhibit the action of DNMTs, leading to DNA hypomethylation. The fundamental difference between the two nucleosides is that AZAcitidine incorporates into both DNA and RNA, whereas 5-AZA-dC integrates exclusively into the double-stranded DNA helix. DNA methylation happens at the C5 position of the cytosine ring in CG dinucleotides. In 5-AZA-dC, the C5 position is replaced by N (nitrogen) [303]. Thus, 5-AZA-dC cannot be methylated when incorporated into DNA.

5-AZA-dC enters the cells through membrane transporters, such as ENT-1, which is part of the family of transporters responsible for the uniport of nucleosides [304]. After absorption, 5-AZA-dC needs to be activated and converted into the nucleotide 5-AZA-dC-5'-triphosphate by deoxycytidine kinase, for this reason 5-AZA-dC is a *pro-drug* [272]. Following conversion, the analogous nucleotides can be used as substrates for DNA polymerases leading DNA replication and thus be incorporated into the DNA in place of cytidine (both methylated and unmethylated). Once incorporated into the DNA, the analogous nucleotides interact with DNMTs, inducing their degradation and consequently permanently blocking the DNA methylation process [252] (Figure 21).

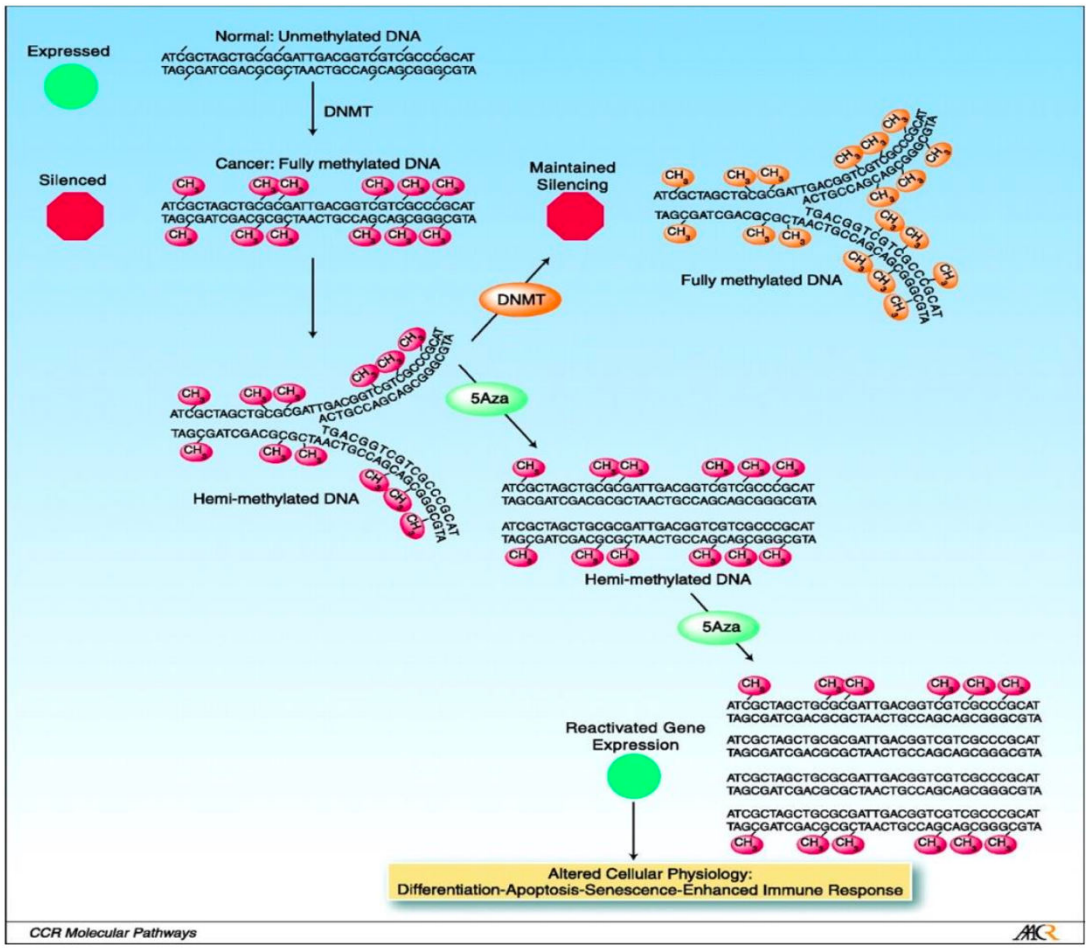

***Figure 21**: the mechanism of action of 5-AZA-dC [305].*

To assess whether the reduced expression of CCRL2 in the SW480 cell line is due to CCRL2 gene methylation, the cells were treated with the demethylating

agent 5-AZA-dC (1 µM) for 24, 48, and 72 hours, replacing the medium every 24 hours. At the end of each experimental time point, RNA was extracted, and CCRL2 expression was evaluated by qPCR. The result shown in figure 22A shows that in the control cell line expressing CCRL2, RKO cells, CCRL2 transcript levels do not change between the untreated and demethylating agent-treated samples at any of the analyzed time points. In contrast, for what concerns the SW480 tumor cell line, it is possible to observe an increase in CCRL2 expression after the treatment with 5-AZA-dC. Moreover, CCRL2 expression increases with the duration of treatment, suggesting that the demethylating agent integrates into the DNA double helix, reducing its methylation status with each replication cycle, allowing increased CCRL2 expression. Additionally, a dose-response curve for the treatment with the demethylating agent was performed in the SW480 CRC cell line. As shown in figure 22B, CCRL2 expression increases in a dose-dependent manner, with statistical significance at the higher concentrations (1 and 10 µM) of 5-AZA-dC. Since neither dose had any effect on the RKO cell line (data not shown) and both induced similar levels of CCRL2 expression, it was decided to continue the experiments with the 1 µM dose of 5-AZA-dC.

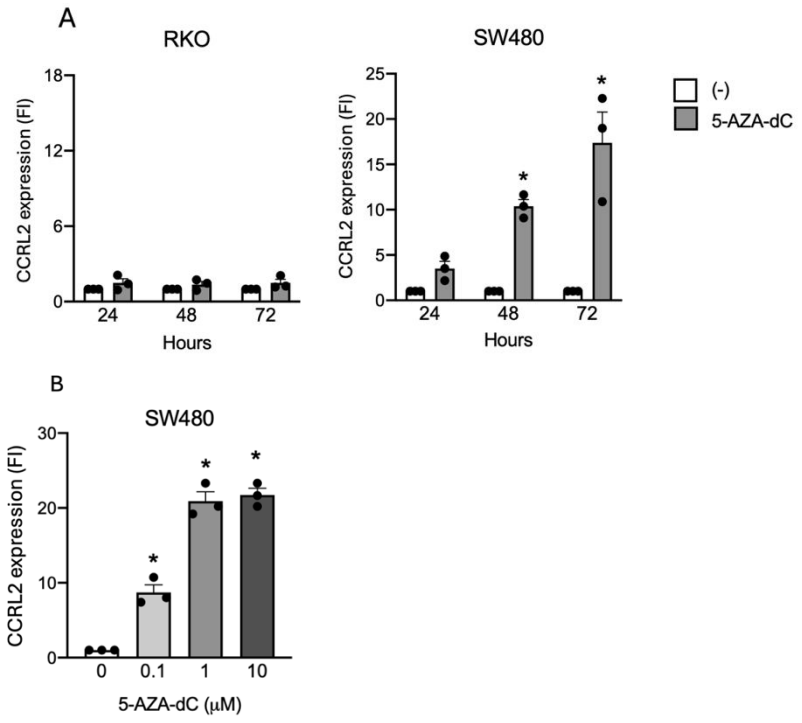

Figure 22. Effect of 5-AZA-dC on CCRL2 expression in the two CRC cell lines. *The CRC cell lines were treated with the demethylating agent 5-AZA-dC (1 µM) for the indicated time points. Subsequently, the cells were collected, and mRNA levels were evaluated by qPCR. (A) The results are expressed as mean ± SEM (n=3) of CCRL2 fold of induction; *p<0.05 (-) vs. 5-AZA-dC using Student's t-test. (B) Dose-response curve of 5-AZA-dC in the SW480 cancer cell line. SW480 cells were treated with increasing doses of 5-AZA-dC for 72 hours. At the end of the treatment, CCRL2 expression was evaluated using qPCR. The results are expressed as mean ± SEM (n=3); *p<0.05 vs. (-) using the one-way Dunnett's ANOVA test. FI = Fold of Induction.*

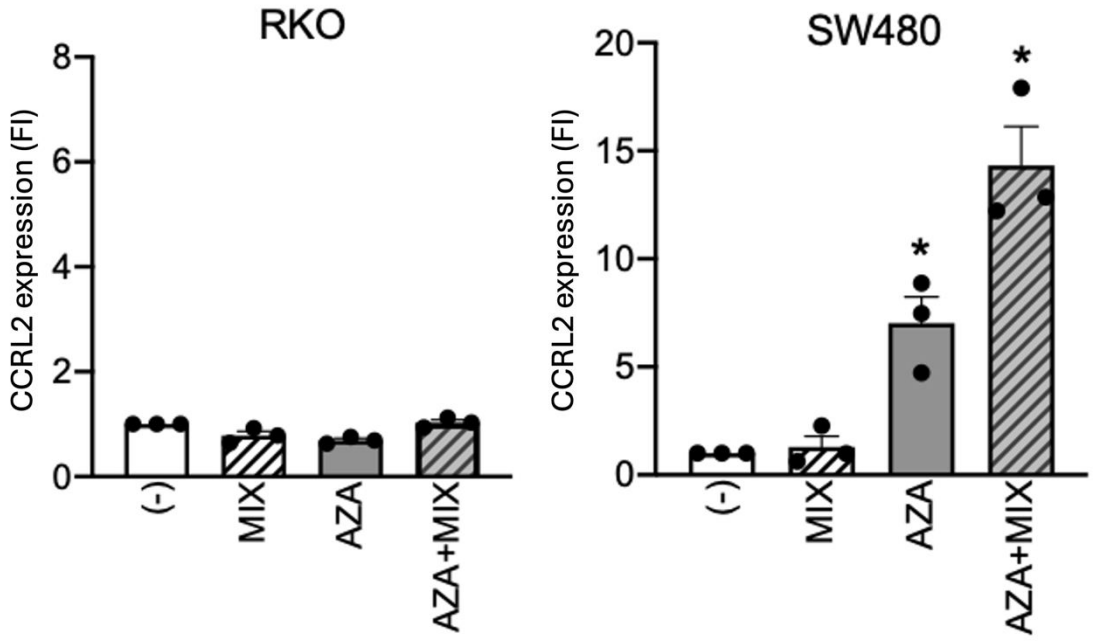

Figure 23. comparison of the effect of 5-AZA-dc alone or in tandem with the pro-inflammatory mix in the two CRC cell lines*. RKO and SW480 cells were stimulated for 24h with 5-AZA-dc alone or in combination with the pro-inflammatory mix (TNFα + IFNγ + LPS). CCRL2 expression was evaluated by qPCR. *p<0.05 AZA vs. AZA + MIX using the one-way Dunnett's ANOVA test. FI = Fold of Induction.*

Furthermore, CCRL2 expression in RKO cells does not increase both after the stimulation with 5-AZA-dC and with 5-AZA-dC and the pro-inflammatory mix, which reproduces an inflammatory context (Figure 23). In fact, RKO cells already express CCRL2 in basal conditions (paragraph 10.2, figure 20A and 20C). In contrast, for what concerns SW480 cells, the expression of CCRL2 increases after the stimulation with 5-AZA-dC, but it significantly increases more after the stimulation with both 5-AZA-dC and the pro-inflammatory mix, in accordance with previous studies describing CCRL2 as a gene upregulated in pro-inflammatory conditions [93,116].

These results support the hypothesis of the existence of an epigenetic DNA methylation mechanism controlling CCRL2 expression in CRC. To confirm this result, a direct analysis of DNA methylation was performed.

10.3.2. Study of the methylation of CCRL2 with direct methods: the Research of CpG islands within CCRL2 genomic locus

In tumors, an aberrant methylation of DNA at CpG islands of tumor-suppressor genes is often observed, causing its silencing. Therefore, our first step was to assess the presence of CpG islands within CCRL2 genomic locus, through the website "CpG island finder". It was found that within the region of CCRL2 included from 2500 bp before the ATG to 1300 bp after the ATG, CpG islands were not found (figure 24).

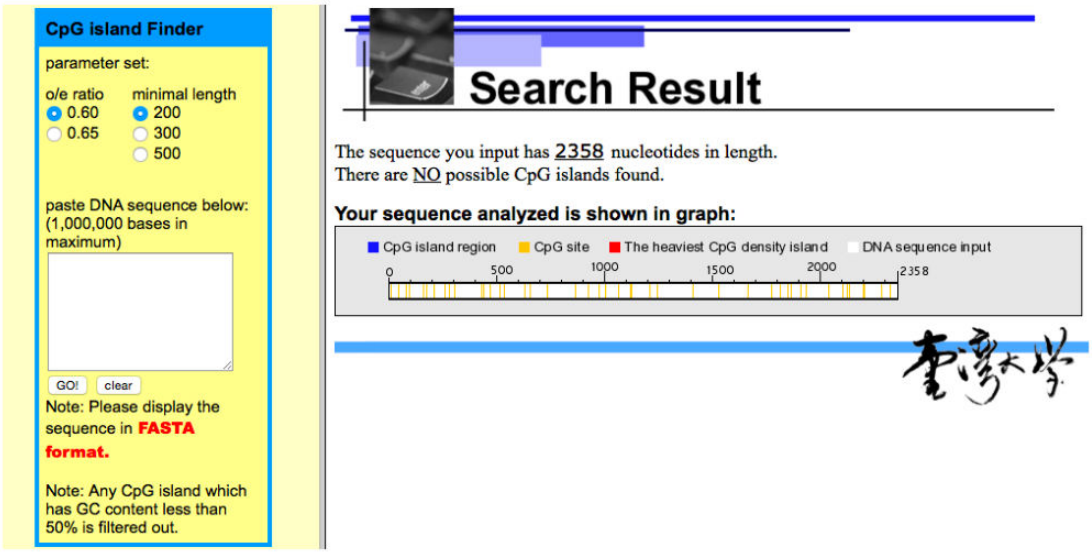

Figure 24. Assessment of the presence of CpG islands within the sequence of CCRL2*. The research was performed through the website "CpG island finder".*

This result led us to choose techniques based on enrichment by affinity for what concerns the study of DNA methylation, excluding the possibility of using

MIRA [2] (Methylated CpG Islands recovery assay), thus preferring MeDIP (Methylated DNA Immunoprecipitation) to perform such analysis.

10.3.3. Choice, design and validation of the primers for MeDIP

Given the lack of CpG islands in the region before the ATG of CCRL2, it was decided to search for CG rich regions for the design and positioning of primers to use for MeDIP. As it is possible to observe from the analysis of the sequence (Figure 25), CG dinucleotides (highlighted in blue) are present but there are no regions with a high CG density. Thus, it was decided to start the study of CCRL2 methylation from the analysis of the promoter region. Since CCRL2 promoter is still not described in literature, "UCSC genome browser" was used to search for a region for transcription factors binding sites for transcription factors (TFBS), which will represent our promoter candidate (highlighted in green). The sequences of the primers that were designed are reported in the materials and methods. The sequences of the TFBS targeted by our primers are highlighted in purple (500 bp before the ATG), while the sequences targeted by our primers for the irrelevant region are highlighted in red (800 bp before the appointed TFBS sequence). From the sonication DNA fragments around 500 bp are obtained, so we were sure that amplifications did not overlap.

[2] MIRA is a technique based on proteins with high affinity for methylated cytosines, while MeDIP exploits specific antibodies against 5-methylcytosine [309].

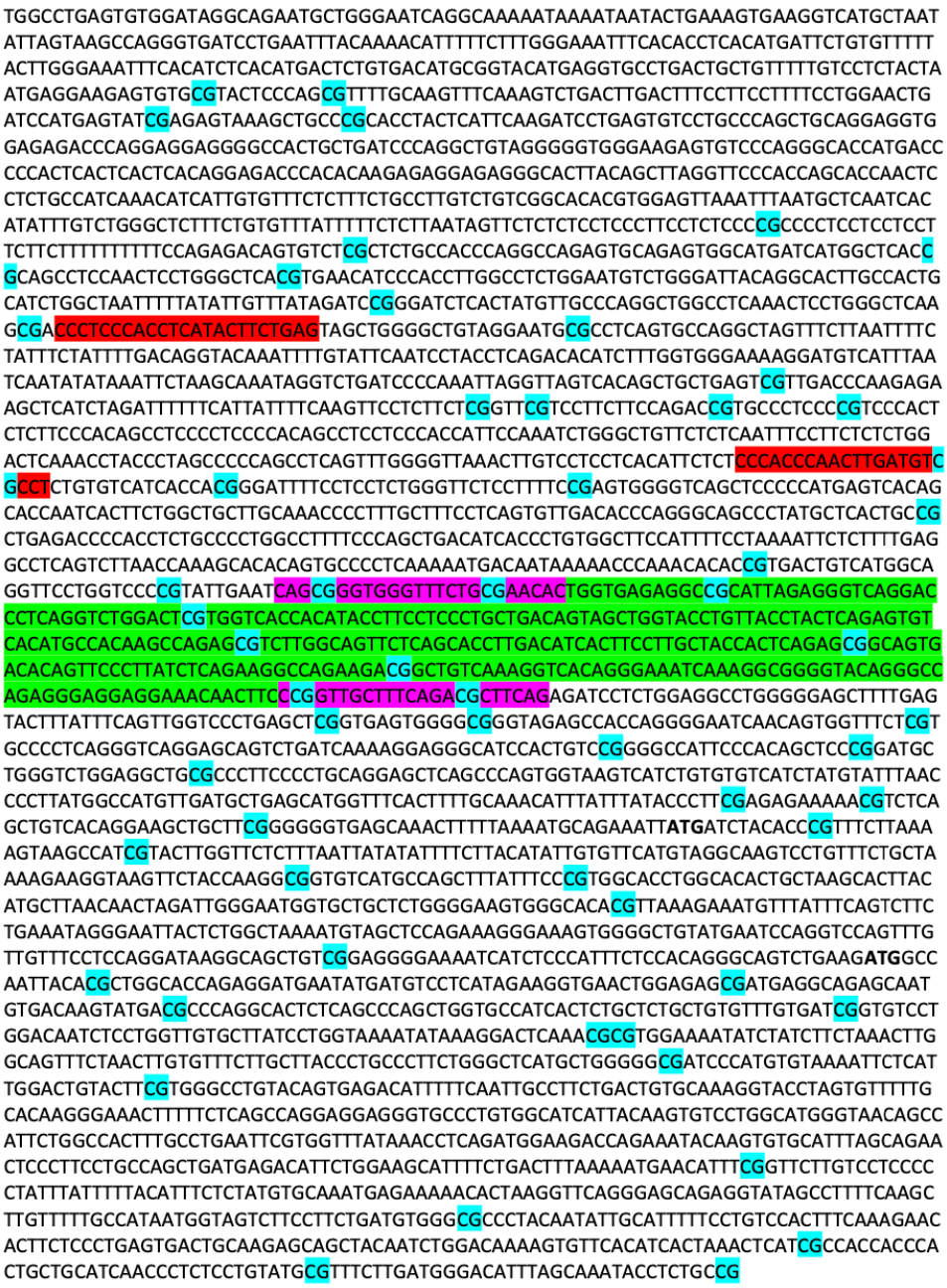

Figure 25*. the analyzed sequences related to CCRL2. CG dinucleotide: blue; CCRL2 putative promoter: green; TFBS primers: purple; irrelevant region primers: red.*

Primer validation was performed using a curve of sonicated genomic DNA, assessing a comparable efficiency (data not shown). After primer validation, the analysis of DNA methylation through the MeDIP technique was performed.

10.3.4. The methylation of control genes was assessed by MeDIP to validate the protocol for the investigation of CCRL2 methylation in CRC cell lines (RKO and SW4080).

To study the methylation of the two CRC cell lines, the extracted and sonicated DNA underwent immunoprecipitation for 5-methylcytosines, and the validation of successful immunoprecipitation was carried out through the analysis of control genes, GAPDH and H19. GAPDH, a housekeeping gene, is actively transcribed and translated, thus serving as our negative control for methylation. In contrast, the H19 gene is under epigenetic control through the phenomenon of imprinting. Genomic imprinting is the primary mechanism regulating monoallelic gene expression in mammals and is caused by gamete-specific DNA methylation of specific regulatory elements called imprinting control regions (ICRs) [306]. More specifically, H19 is a paternally imprinted gene that undergoes epigenetic silencing via permanent methylation, making it our ideal positive control for methylation.

The result shown in Figure 26 is consistent with our expectations: the GAPDH gene is not methylated, and, as shown in the graph, the levels of methylated DNA are almost negligible in both cell lines. In contrast, H19 exhibits high levels of methylated DNA in both RKO and SW480 cells.

The results obtained from these two control genes confirm the proper setup of the immunoprecipitation technique used.

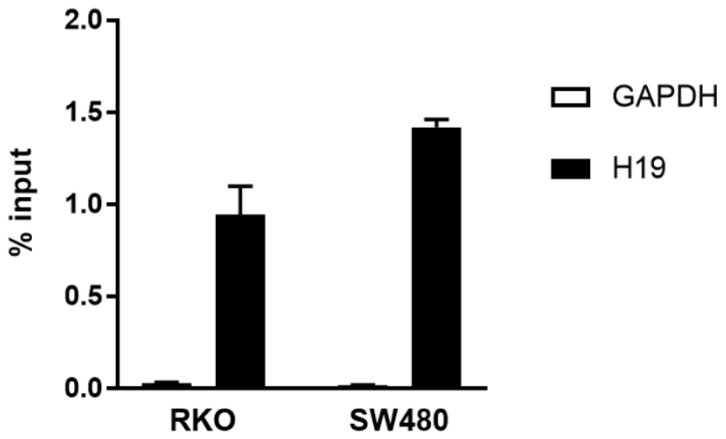

Figure 26. Validation of MeDIP with control genes*. The DNA from the two CRC cell lines underwent immunoprecipitation using a specific antibody against 5-methylcytosines, and the methylation status of the control genes GAPDH and H19 was assessed through qPCR. The results are shown as mean ± SEM (n=3) and expressed as a percentage of the input.*

10.3.5. Analysis of CCRL2 methylation by MeDIP in CRC cell lines (RKO and SW4080)

Based on the results obtained with the control genes, we analyzed the methylation of CCRL2 putative promoter in the two CRC cell lines. SW480 cell line shows high levels of methylated DNA in the TFBS region in comparison to RKO cells. In contrast, the levels of methylation in the irrelevant region of CCRL2 gene as well as in GAPDH are comparable (Figure 27).

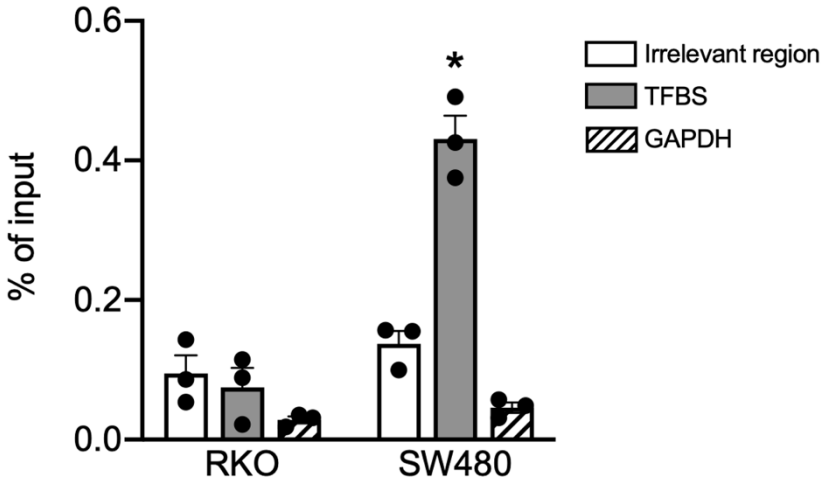

Figure 27. Analysis of methylated DNA levels in the CCRL2 promoter in two CRC cell lines*. The DNA from RKO and SW480 cells was subjected to immunoprecipitation using a specific antibody for 5-*

*methylcytosines, and the methylation status of the CCRL2 promoter (TFBS, black bars) and the "irrelevant" region (white bars) was assessed via qPCR. GAPDH represents the negative control. Results are presented as mean ± SEM (n=3) and expressed as a percentage of input; *p<0.05 vs. respective irrelevant region using Student's t-test.*

Moreover, the treatment with 5-AZA-dC in SW480 cell line significantly reduced the levels of methylated DNA in the TFBS region, confirming the methylation status of this region in normal conditions (Figure 28). In contrast, for what concerns the irrelevant region and GAPDH, there are not significant differences between treated and untreated SW480 cells.

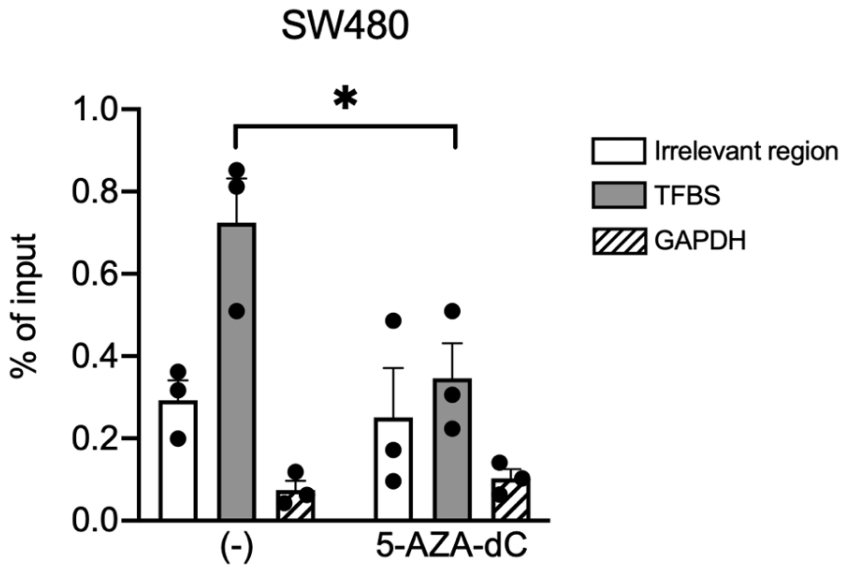

***Figure 28. Effect of 5-AZA-dC on the methylation status of the CCRL2 promoter in SW480 CRC cell line**. SW480 cells were treated with 5-AZA-dC for 72 hours prior to DNA extraction. The DNA was then immunoprecipitated, and the methylation status of the CCRL2 promoter (TFBS, black bars) and the "irrelevant" region (white bars) was assessed through qPCR. GAPDH represents the negative control. The results are presented as mean ± SEM (n=3) and expressed as a percentage of input; *p<0.05 vs. (-) using Student's t-test.*

These results suggest a strict relation between the levels of expression and the methylation status of the promoter. Therefore, it is possible to conclude that CCRL2 is silenced in SW480 cell line through an epigenetic mechanism of methylation of the promoter.

10.4. Analysis of the methylation of H3 histone in the promoter of CCRL2 in CRC cell lines

The levels of acetylation and methylation of histones in the regulatory regions of DNA, in particular those the promoter of a specific gene, are significant to investigate the activation state of that gene. To further validate the results obtained with MeDIP, it was decided to analyze the tri-methylation of the H3 histone in its 4th lysine residue (H3K4me3), a modification associated to transcriptional activation [307]. Trimethylation of H3K4 is frequently lacking in CRC in comparison to healthy controls [308]. using the immunoprecipitation of chromatin (ChIP). Both cell lines show high levels of HEK4me3 in GAPDH gene (positive control), while SW480 cells has a lower level of H3K4me3 in comparison to RKO in the region of CCRL2 putative promoter. In contrast, both cell lines show low H3K4me3 levels in the irrelevant region and in CCRL2 exon (negative controls).

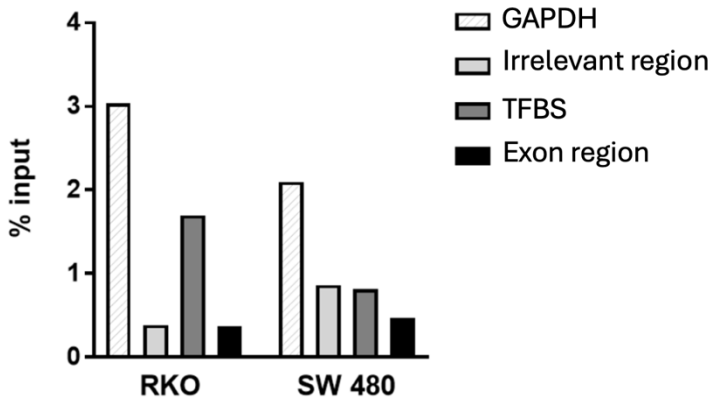

Figure 29. Analysis of H3K4me3 levels in two CRC cell lines*. The nuclear fraction from RKO and SW480 cells was immunoprecipitated using an anti-H3K4me3 antibody and analyzed via Real-Time qPCR. The results are expressed as a percentage of input and correspond to a preliminary and representative replicate.*

In accordance with our previous results obtained through MeDIP, in fact, RKO cells, which expressed CCRL2, shows high levels of H3K4me3, confirm a transcriptionally active state of CCRL2 promoter. In contrast, SW480 cells, that do not express CCRL2, show low levels of H3K4me3 and high levels of

methylation of CCRL2 in comparison to RKO cells (Figure 29). However these are preliminary results, that will be integrated with future experiments.

11.DISCUSSION (PART II)

This second part of the thesis rises from previous data obtained in our laboratory, which suggested a protective role of the atypical receptor CCRL2 in a DSS mouse model of inflammation-induced colon carcinogenesis. Indeed, CCRL2 knockout mice are more susceptible to the development of intestinal polyps in comparison to control animals. This protective role seems to be confirmed in humans, as colorectal cancer (CRC) patients with reduced expression of the CCRL2 gene exhibit a poorer survival curve [295]. These findings suggest that CCRL2 could function as a novel tumor suppressor gene, where its silencing facilitates tumorigenic transformation.

One of the most critical mechanisms for the silencing of tumor suppressor genes is DNA methylation, often associated also with post-translational modifications of histones. The experiments in this thesis were designed to identify and characterize a potential regulation of CCRL2 expression in CRC dependent on DNA methylation. Two different experimental approaches were used: an indirect method involving treatment with the demethylating agent 5-Aza-2'-deoxicytidine (5-AZA-dC), and a direct method involving DNA methylation immunoprecipitation (MeDIP), both of which confirmed an inverse correlation between CCRL2 expression and DNA methylation, suggesting the existence of an epigenetic silencing mechanism of CCRL2 in CRC.

To perform this analysis, we were required to make strategic decisions based on the characteristics of the gene in question. First, an analysis of the genomic region surrounding CCRL2 did not reveal the presence of any CpG island, which is generally considered indicative of regulation through DNA methylation. While it is now clear that the presence of a CpG island is not a strict requirement, the proportion of genes regulated by methylation is generally higher among those with CpG islands. Moreover, the absence of CpG islands makes direct methylation studies more difficult, as it determines the lack of a

reference region for the analysis. For these reasons, it was decided to approach the challenge through an indirect methodology (treatment of cells with 5-AZA-dC) that, despite not proving regulation through DNA methylation, provided strong evidence in its favor.

On this basis, a direct analysis of the regulation of CCRL2 methylation through affinity-based enrichment techniques was performed, excluding the MIRA method due to its efficiency only when the region of interest contains at least five CpG repeats, making it more suitable for studying genes with CpG islands.

Once MeDIP was chosen as our method of choice and validated on control genes, we faced the challenge of choosing the specific genomic region to analyze. We decided to proceed focusing on the promoter region, as it is the regulatory region par excellence, and on an upstream region considered as a potential "irrelevant" control. A high level of methylation in the CCRL2 putative promoter region of SW480 cells (the cell line that does not express CCRL2 in normal conditions) was demonstrated, while no such methylation was seen in RKO cells (the cell line that expresses CCRL2 in normal conditions). Conversely, the upstream irrelevant region of the promoter showed no variation in either CRC cell line. Soon, these results will be completed and integrated by designing primers for the amplification of contiguous regions of the CCRL2 locus, allowing us to "scan" the region for additional segments that may be subject to DNA methylation and, consequently, owning a potential regulatory function.

The analysis of DNA methylation will also be complemented by the study of histone modifications using ChIP (Chromatin Immunoprecipitation). To this regard, we have already obtained preliminary data about the trimethylation of histone H3 at lysine 4 (H3K4me3), a modification associated with transcriptional activation, obtaining results entirely consistent with those of

the MeDIP. Future studies will investigate the effect of the demethylating agent 5-AZA-dC on the opening of the CCRL2 promoter by analyzing, in addition to H3K4me3 levels, changes in histone H3 and H4 acetylation and polymerase recruitment at the promoter site. Furthermore, the characterization of the presence of transcription factors involved in CCRL2 transcriptional regulation under different transcriptional conditions will be performed.

Overall, these results provide solid evidence of the epigenetic silencing of CCRL2 in CRC and fits into the broader framework emerging in our laboratory, which positions CCRL2 as a novel and promising tumor suppressor gene. This paves the way for the potential future application of CCRL2 as a therapeutic target in CRC and strengthen its role as biomarker.

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Giovanni

I can read your mind
"She's having the time of her life"
There in her glittering prime
The lights refract sequined stars off her silhouette every night
I can show you lies (one, two, three, four)
'Cause I'm a real tough kid, I can handle my shit
They said, "Babe, you gotta fake it till you make it" and I did
Lights, camera, bitch smile, even when you wanna die
He said he'd love me all his life
But that life was too short
Breaking down, I hit the floor
All the pieces of me shattered as the crowd was chanting "More"
I was grinning like I'm winning, I was hitting my marks
'Cause I can do it with a broken heart (one, two, three, four)
I'm so depressed, I act like it's my birthday every day
I'm so obsessed with him but he avoids me like the plague
I cry a lot but I am so productive, it's an art
You know you're good when you can even do it
With a broken heart
I can hold my breath
I've been doing it since he left
I keep finding his things in drawers
Crucial evidence, I didn't imagine the whole thing
I'm sure I can pass this test (one, two, three, four)
'Cause I'm a real tough kid, I can handle my shit
They said, "Babe, you gotta fake it till you make it" and I did
Lights, camera, bitch smile, in stilettos for miles
He said he'd love me for all time
But that time was quite short
Breaking down, I hit the floor
All the pieces of me shattered as the crowd was chanting "More"
I was grinning like I'm winning, I was hitting my marks
'Cause I can do it with a broken heart (one, two, three)
I'm so depressed, I act like it's my birthday every day
I'm so obsessed with him but he avoids me like the plague (he avoids me)
I cry a lot but I am so productive, it's an art
You know you're good when you can even do it
With a broken heart
You know you're good when you can even do it
With a broken heart
You know you're good, I'm good
'Cause I'm miserable
And nobody even knows
Try and come for my job

Taylor Swift – The Tortured Poets Department (2024) – I Can Do It With A Broken Heart