Mismatch repair and MUTYH deficient colorectal cancers : at the crossroad of genomic stability and immune escape

Cunha Carvalho de Miranda, N.F. da

General Introduction

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Chapter 1

1. COLORECTAL CANCER

1.1. Epidemiology and etiology of colorectal cancer

Colorectal cancer is the third most frequently diagnosed cancer and the fourth most common cause of cancer-related deaths worldwide. Its incidence is estimated at 17.2 per 100,000 individuals, although higher prevalence is observed in the so-called "developed countries" (1). The overall five-year survival rate for colorectal cancer is higher than 50% but the individual patient prognosis is highly dependent on tumor-staging at diagnosis (2, 3). For instance, patients affected by localized lesions (stages 0 and I) present five-year survival rates higher than 90% (2). The majority of colorectal cancers (approximately 95%) arise in a sporadic context, while autosomal dominant and recessive cancer syndromes are responsible for the remainder (Figure 1). Nevertheless, familial aggregation of colorectal cancers, not explained by known cancer syndromes, is observed in approximately one-third of the so-called "sporadic cases" (4). The identification of genetic predisposition factors in these families constitutes one of the major remaining challenges in colorectal cancer research (5, 6). Tobacco smoking, high intake of saturated fat and red meat, and alcohol consumption constitute major environmental factors that have been associated with an increased risk for colorectal cancer (7).

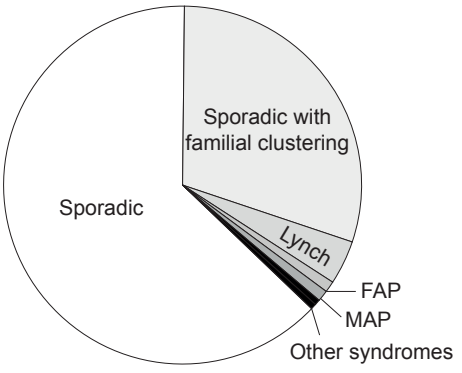

Figure 1. Spectrum of colorectal cancers according to their etiology (FAP - Familial Adenomatous Polyposis syndrome; MAP - MUTYH-associated polyposis syndrome).

1.2. Genetics of oncogenesis

Colorectal cancer is a heterogeneous disease where different oncogenic pathways can support cancer development (8, 9). Classically, colorectal cancers have been divided according to the type of genetic instability that is observed in tumors (Figure 2). Extensive accumulation of nucleotide insertions and deletions at DNA microsatellite sequences (short nucleotide repeats) are observed in 15-20% of colorectal cancers. Such phenotype, denominated microsatellite instability-high (MSI-H), is caused by a defective DNA mismatch repair system (10, 11). Almost invariably, MSI-H sporadic colorectal cancers display DNA hypermethylation of the *MLH1* gene promoter, thereby silencing its expression, as well as widespread methylation of gene promoters throughout the genome (10, 11).

Lynch syndrome, previously denominated hereditary non-polyposis colorectal cancer (HNPCC) syndrome, is the hereditary counterpart of MSI-H colorectal cancers and affects carriers of germline mutations in mismatch repair genes, where *MLH1*, *MSH2*, *MSH6,* and *PMS2* are most commonly affected. Lynch syndrome is an autosomal dominant genetic condition where one defective allele of a mismatch repair gene is inherited. Cancer development in carriers generally involves the somatic inactivation of the second copy of the gene (10, 11). The mismatch repair system is a caretaker of the genome that is essential for the repair of nucleotide mismatches and small base insertions and deletions (12). Microsatellite DNA sequences are hotspots for the accumulation of mutations, resulting from the frequent slippage of DNA polymerases at these sites (13). This is proposed to result

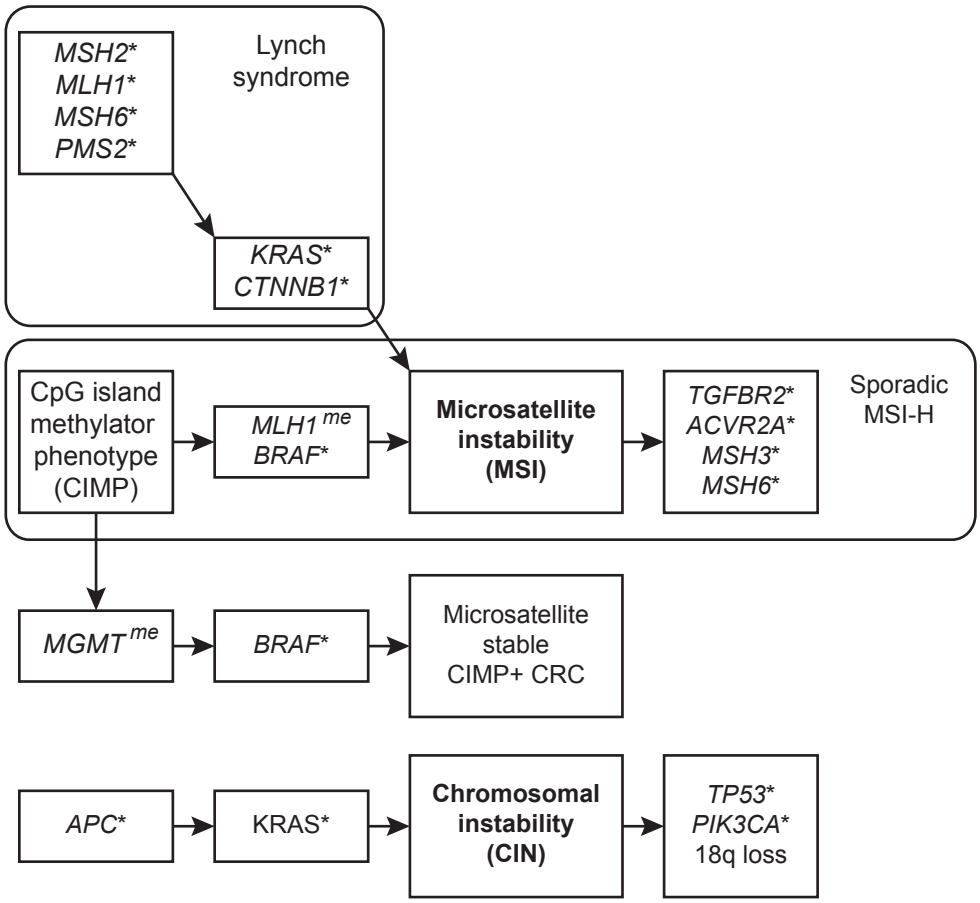

Figure 2. Simplified scheme representing the most frequent (epi-) genetic alterations occurring during colorectal carcinogenesis in different genetic pathways. (* - mutations; me - methylation; CRC - colorectal cancer).

from the formation of loop DNA structures in single stranded microsatellites and from inefficient proofreading exonuclease activity by the DNA polymerase (14, 15). Large chromosomal aberrations are rare in MSI-H colorectal cancers and their cells generally possess peridiploid DNA contents, similar to the one of a healthy somatic cell (16). MSI-H colorectal cancers develop more frequently in the colon ascendens and are further characterized by a poorly differentiated and mucinous histology and a dense intraepithelial, lymphocytic infiltrate (8, 17, 18).

Most colorectal cancers (80-85%) are mismatch repair proficient and do not display microsatellite instability (MSI). Instead, the majority of microsatellite stable colorectal cancers present gross chromosomal aberrations that translate into aneuploid DNA contents in tumor cells (19). Recurrent chromosomal aberrations in colorectal cancer include gains of chromosomes 7, 8q, 13, and 20q and losses of 4q, 8p, and 18q (20-22). The generation of chromosomal instability (CIN) has been associated with the loss of function of the *Adenomatous Polyposis Coli* (*APC*) gene, a classical tumor suppressor in colorectal cancer (23). Truncating mutations in *APC* occur in the majority of colorectal cancers with CIN and are considered to be one of the

initiating events in colorectal tumorigenesis (24, 25). APC is part of a protein complex that controls the availability of β-catenin, a key signal transducer of the canonical Wnt signaling (26). Loss of APC promotes the stabilization and nuclear accumulation of β-catenin that, upon association with specific transcription factors, activates the transcription of proto-oncogenes such as *MYC* and *CCND1* (26, 27). APC defects were also shown to disturb kinetochore function and chromosomal segregation during mitosis, thereby supporting APC's role in the propagation of CIN (23). Activation of Wnt signaling has also been suggested to promote the so-called stemness of cancer cells that, thereby, can overcome replicative senescence (28, 29). Germline mutations in *APC* cause familial adenomatous polyposis (FAP), an autosomal dominant disease that is responsible for less than 1% of all colorectal cancers (30). Although common, truncating somatic mutations in *APC* are less frequent in MSI-H colorectal cancers when compared to tumors with CIN (31). Interestingly, MSI-H colorectal cancers, particularly the ones associated with Lynch syndrome, display relatively frequent mutations in the β-catenin gene (*CTNNB1*) (31, 32). Such mutations were suggested to increase the stability of β-catenin and, thereby, to produce an effect similar to the loss of APC (33). Although alterations in the Wnt signaling pathway constitute a hallmark in colorectal cancer development, biallelic inactivation of *APC* or activating mutations in *CTNNB1* are only present in approximately 80% of tumors. The comprehensive characterization of the genomic landscape of colorectal cancers identified less frequent mutation targets such as *SOX9*, *TCF7L2*, *AXIN2*, *FBXW7*, *ARID1A,* and *FAM123B*, which, cumulatively, might explain Wnt activation in the remaining proportion of cases (24).

Another form of (epi-) genetic instability can be recognized in a subset of colorectal cancers and it refers to the widespread methylation of CpG islands at gene promoters (34). The CpG island methylator (CIMP) and the MSI phenotypes are largely overlapping but CIMP-positive, MSI-negative tumors still account for approximately 8% of colorectal cancers (35). Of note, in a sporadic context, CpG methylation changes, often accompanied by mutations in *BRAF*, are considered to precede the onset of MSI (36). Furthermore, CIN can accompany CIMP in a substantial proportion of cases (37, 38). Interestingly, a fraction of colorectal cancers simultaneously lack CIN, MSI, and CIMP (37). The type of genetic instability observed in different tumors has been shown to correlate with the patients' survival and response to therapy. An improved patient prognosis has been associated with the MSI phenotype, while worse patient survival was reported for CIMP-positive tumors that lacked MSI (37, 39). Paradoxically, MSI colorectal cancers appear to be less sensitive to fluorouracil (5-FU), the standard chemotherapeutic adjuvant in colorectal cancer therapy (40, 41).

In addition to FAP and Lynch syndrome, a number of other cancer syndromes are responsible for the onset of colorectal cancer in a hereditary setting. MUTYH-associated polyposis (MAP) constitutes the only known colorectal cancer syndrome that is inherited in a recessive manner. It is caused by germline mutations in the gene that encodes for the MUTYH DNA glycosylase (42). Although MAP patients display a milder phenotype than FAP patients, most carriers of biallelic mutations in *MUTYH* develop numerous polyps at a young age that, eventually, progress to malignant lesions (42). The MUTYH protein is part of the DNA base-excision repair pathway and is involved in the repair of one of the most common forms of oxidative damage, the oxidation of guanine to 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG). In the absence of MUTYH, cells display a distinctive mutation signature that is characterized by the abundance of G:C

to T:A transversions, which results from the mispairing of 8-oxoG with adenines during replication (43). Similar to MSI-H cancers, MAP patients were reported to have better overall survival than sporadic colorectal cancer patients (44). Furthermore, MAP tumors most often develop in the proximal colon and frequently present mucinous histologies and high amounts of intraepithelial lymphocytic infiltrate (45).

A number of human syndromes have been associated with the finding of hamartomatous and/or hyperplastic polyps in affected individuals (46). However, for several of those, the "a priori" risk for colorectal cancer development is unknown. On the other hand, the risk for colorectal cancer has been clearly assessed in the autosomal dominant Peutz-Jeghers syndrome (PJS) and in the juvenile polyposis syndrome (JPS), which are caused by germline mutations in the *STK11/LKB1* gene and the *BMPR1A* or *SMAD4* genes, respectively (47). Although mutations in *BMPR1A* and *SMAD4* explain a considerable proportion of juvenile polyposis cases, the genetic basis of disease is still elusive in a large number of patients. It was recently established that patients suffering from the PTEN hamartoma tumor syndrome, comprising Cowden and Bannayan-Riley-Rubalcaba syndromes, are also at increased risk for colorectal cancer (48). Finally, a recent whole genome sequencing approach identified high-penetrance variants that confer susceptibility to colorectal polyposis and cancer in the *POLE* and *POLD1* genes (49). Those variants were located in the proofreading domains of the polymerases and compromised the repair of mismatches introduced during DNA replication.

1.3. Mutation landscape of MSI-H and MAP colorectal cancers

MSI-H colorectal cancers are notorious for the accumulation of insertions and deletions at microsatellite DNA sequences throughout the genome. Accordingly, genes containing microsatellite repeats within their coding regions are often targeted by mutations in those tumors. The *TGFBR2* and *ACVR2A* genes, which contain ten and eight adenine microsatellites, respectively, are found mutated in the majority of MSI-H colorectal cancers (50-52). Interestingly, the *MSH3* and *MSH6* mismatch repair genes also carry microsatellite sequences within their sequences that are targeted by MSI (24). This secondary targeting of mismatch repair genes constitutes an additional source of DNA repair deficiency that contributes to the high mutation load of MSI-H cancers (53-55). Similar to the inactivation of the *APC* gene, the constitutional activation of the MAPK signaling pathway is one of the primary events in colorectal cancer tumorigenesis. It occurs mainly through the establishment of activating mutations in the KRAS GTPase or the BRAF serine/threonine kinase (Figure 2), in a mutually exclusive manner (56). Mutations in the *BRAF* gene are most frequent in sporadic MSI-H cancers and absent in Lynch-associated cancers (57, 58). In turn, mutations in the *KRAS* gene are more common in colorectal cancers with CIN and the ones developing in patients with Lynch syndrome (Figure 2) (59-61). As discussed previously, in addition to their hypermutated genomes, most MSI-H sporadic cancers display a methylator phenotype, responsible for the altered expression of a myriad of genes (Figure 2) (24).

The majority of MAP carcinomas display mutations in the *APC* and *KRAS* genes that are postulated to derive directly from the MUTYH-associated, base-excision repair deficiency (62). G>T transversions at GAA triplets are frequent in *APC* and nearly all *KRAS* mutations found in MAP carcinomas are restricted to a c.34 G>T transversion, an uncommon substitution in the remaining spectrum of colorectal cancers. Mutations in *TP53* and *SMAD4* are also encountered in a substantial proportion of MAP carcinomas

but are not restricted to G>T transversions, suggesting that they might occur at a later stage in tumorigenesis (45). In agreement with the fact that the base-excision repair system is not directly involved in the repair of small insertions and deletions, MSI is rarely observed in MAP carcinomas (45, 62). Interestingly, MAP tumors display a distinctive form of chromosomal instability characterized by the widespread presence of chromosomal copy-neutral loss of heterozygosity (63).

2. TGF-β SIGNALING PATHWAY: A MULTIFACETED REGULATOR OF CARCINOGENESIS

The transforming growth factor-β (TGF-β) signaling pathway regulates cell proliferation, differentiation, apoptosis and migration (64). Abnormalities in this pathway compromise tissue homeostasis and may support carcinogenesis (65, 66). Signal transduction is initiated with the binding of a TGF-β ligand to the TGF-β type 2 transmembrane serine/threonine kinase receptor TGFβR2, which becomes activated and phosphorylates the type 1 TGF-β serine/threonine kinase receptor TGFβR1 (67). A type 3 TGF-β receptor (TGFβR3) facilitates the interaction between TGF-β ligands and the serine/threonine kinase receptors (Figure 3) (68). Upon activation, TGFβR1 phosphorylates a receptor-regulated Smad (Smad2, Smad3) that forms a heterocomplex with the co-Smad, Smad4, in the cytoplasm (Figure 3) (69, 70). This complex translocates to the nucleus where it modulates the expression of gene targets together with additional transcription factors (Figure 3) (71). Alternative ligands (e.g. Activins) and receptors (e.g. ACVR2A, ALK4) can also convey TGF-β signaling to Smad2 and Smad3 (70). The bone morphogenetic protein (BMP) pathway operates in an analogous way to TGF-β but makes use of different ligands, receptors, and intracellular Smad proteins, except for Smad4, which operates as a co-Smad in both the TGF-β and BMP pathways (69).

As discussed previously, the *TGFBR2* and *ACVR2A* genes are fated to mutate in MSI-H colorectal cancers due to the presence of microsatellite repeats within their protein-

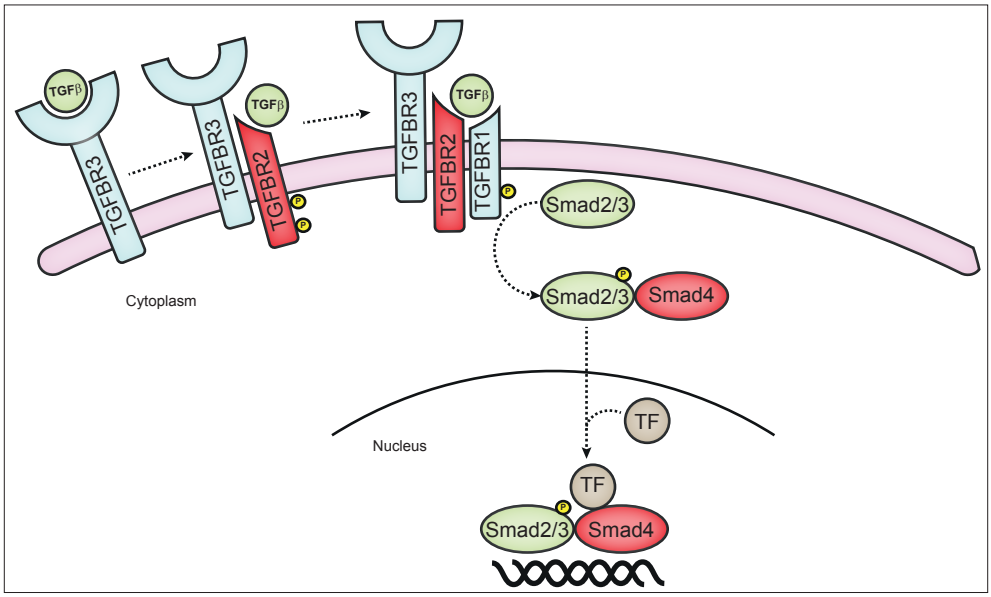

Figure 3. The TGFβ signaling pathway (adapted from Meulmeester et al. (72)). The molecules more often affected by mutations in colorectal cancer are depicted in red. (TF - transcription factor).

coding sequences (50-52). Mutations often target both alleles and result in frameshifted, early-truncated proteins that are unable to transduce TGF-β signaling. Mutations in TGF-β receptor genes are uncommon in microsatellite stable tumors that instead target the Smad proteins, most often Smad4. The *SMAD4* gene is found mutated in up to 15% of microsatellite stable colorectal cancers and its locus (18q21.1) is targeted by loss of heterozygosity in the majority of CIN colorectal cancers (21, 73, 74). Since the *SMAD2* gene is located in the same chromosomal region, it is also affected by loss of heterozygosity. Mutations in *SMAD2* and *SMAD3* occur in a minority of colorectal cancers (75).

Disruption of the TGF-β pathway leads to the decreased expression of TGF-β target genes such as the cell cycle regulators *CDKN1A* (p21) and *CDKN2B* (p15), thereby providing a growth advantage to tumor cells (76, 77). Accordingly, loss of SMAD4 expression has been associated with advanced disease stages and poor prognosis in colorectal cancer patients (78, 79). On the other hand, a dual role has been attributed to TGF-β in the sense that activation of this pathway might also promote malignant behavior. High levels of TGF-β ligand at primary tumors were correlated with metastatic disease and tumor recurrence in colorectal cancer (80, 81). Furthermore, TGF-β production by cancer cells was shown to dampen anti-tumor immune responses and to promote the colonization of tumor metastasis through its activity on stromal cells (82, 83). By acquiring defects in TGF-β signalling mediators, tumor cells can modulate their microenvironment through TGF-β production without suffering from its growth suppressive effects.

3. TUMOR IMMUNOLOGY

3.1. The immune system: unable but equipped?

Cancer development is accompanied by massive changes at cellular and tissue level that, theoretically, could be detected and dealt with by the immune system. Nevertheless, reports on immune system-mediated, spontaneous tumor rejections, in humans, are scarce. As most cancer-related deaths occur after reproductive age, and are thus not involved in natural selection, the contribution of this disease for the shaping of the immune system is considered to be limited. Nevertheless, both the innate and adaptive immune systems are equipped with mechanisms to detect and eliminate anomalous cells. Moreover, the increased risk for malignancies in patients receiving immune suppressants partially supports a role for the immune system as a tumor suppressor (84). It should be noted, however, that the use of immune suppressants also impairs the clearing of infections by oncogenic viruses and thus, the increased cancer risk observed in these patients is not exclusively attributable to an impaired anti-tumor immune response.

The genetic and epigenetic alterations that occur in cancer cells lead to changes in their protein repertoire that include the production of mutated proteins and the abrogation of proteins that would normally be expressed in their non-transformed counterparts. The former may constitute novel antigens for which central T cell tolerance was not imposed (85). They could trigger anti-tumor immune responses that would eventually lead to the destruction of cancer cells ("non-self recognition") (86, 87). The activation of anti-tumor immune responses requires the uptake of tumor antigens by professional antigen presenting cells such as dendritic cells (Figure 4). The high turnover of cancer tissues guarantees an abundant source of tumor antigens but

also of molecular "danger signals" that are essential for dendritic cell activation (88). Upon activation, dendritic cells migrate to tumor-draining lymph nodes where they induce proliferation and activation of antigen-specific CD4+ and CD8+ T cells (Figure 4). The activation of CD8+ T cells occurs through the presentation of antigens by Human Leukocyte Antigen (HLA) class I molecules (cross-presentation) while antigen presentation to CD4+ T cells is mediated by HLA class II molecules (89). Once activated, CD8+ T cells acquire cytotoxic capacity and the ability to eliminate cancer cells that express the same tumor antigen that led to their activation. The killing of tumor cells may occur through the release of lytic granules by cytotoxic CD8+ T cells (CTLs) that contain the pore-forming protein Perforin and Granzyme A and B proteases but also by the Fas-FasL cell death pathway, provided that tumor cells express the Fas receptor (90, 91). Expression of HLA class I molecules by tumor cells is an essential condition for the recognition of tumor antigens by CTLs (92, 93).

In addition to their role in presenting "non-self" antigens to immune cells, HLA class I molecules are fundamental for recognition of the "self". Their absence from the cell surface ("missing-self") evokes the action of natural killer (NK) cells, another lymphocyte with cytotoxic potential (94, 95). HLA class I molecules constitute ligands for NK cell receptors that contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) at their cytoplasmic tail. These motifs become phosphorylated upon HLA class I binding and subsequently suppress NK cell activation (96). In addition, activating signals are also required to trigger NK cell-mediated cytotoxicity, generally transduced by the NKG2D receptor at NK cells. Those signals are provided by ligands such as MICA, MICB or ULBP, which are upregulated in target cells as a consequence of cellular stress (97-99). It is considered that the balance between inhibitory and activation signals provided by target cells ultimately determines the action of NK cells.

The combination of T cell-mediated recognition of tumor antigens and the detection of anomalous cells by NK lymphocytes could constitute effective anti-tumor barriers. In support of this, recognition of tumor antigens by autologous T cells has been widely demonstrated in cancer patients (100, 101), while a variety of tumors have been shown to express NK cell-activating ligands (102, 103). Thus, not surprisingly, the presence of tumor-infiltrating immune cells constitutes a relevant prognostic indicator in cancer patients (104, 105). Remarkably, Galon and colleagues discovered that qualitative and quantitative profiles of immune cell infiltration in colorectal cancer were better prognostic indicators than tumor staging, although a potential overrepresentation of MSI-H tumors in the study cohort was not accounted for (106). The presence of high numbers of tumor-infiltrating lymphocytes has generally been associated with improved clinical outcomes in colorectal cancer patients (107). Reports were most concordant when analyzing specifically the infiltration by cytotoxic T cells expressing granzyme B (108, 109), suggesting a major role for antigen-driven anti-tumor immune response. The potential role of other T cell subsets such as regulatory T cells (Tregs) in the progression of colorectal cancer has not been clearly established (107). As for NK cells, they are relatively infrequent in colorectal cancer tissues but the expression of NKG2D ligands in cancer cells has been associated with improved patient prognosis (103, 110). NK cells appear to be particularly important in controlling tumor metastases by eliminating circulating tumor cells in the blood stream (111, 112).

3.2. Immune escape: too fast, too furious

The mechanisms underlying cancer cell

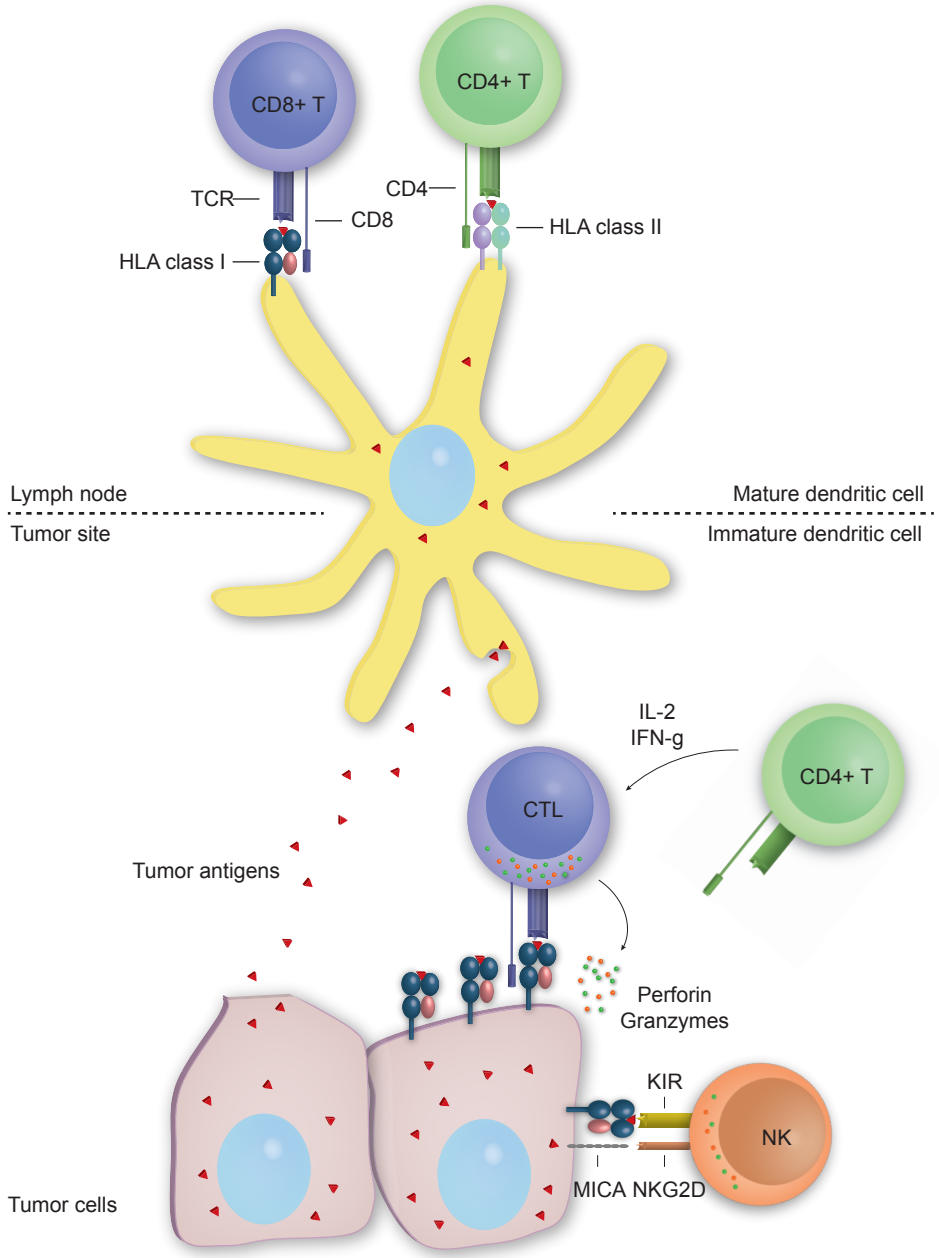

Figure 4. Dendritic cells play a central role in mediating anti-tumor immune responses. The high cellular turnover of tumor tissues guarantees an abundant source of tumor antigens for dendritic cells. After picking up the antigens and transporting them to the draining lymph nodes, dendritic cells activate naïve CD4+ and CD8+ T cells through the presentation of HLA/antigen complexes. CD8+ T cell activation occurs through HLA class I while CD4+ T cell activation is mediated by HLA class II. Additional co-stimulatory signals are required for T cell activation and are provided by the interaction between B7 ligands present on antigen presenting cells and the CD28 receptor on T cells. Once activated, CD8+ T cells gain cytotoxic capacity and the ability to eliminate target cells that present their specific antigen.

resilience to the action of the immune system, also in the context of immunotherapy, have been a major object of study for tumor immunologists throughout the years. Anti-tumor immune responses constitute strong vectors of selection that contribute towards the shaping of clonal evolution in cancer. One of the most common and functionally interpretable immune evasive mechanisms is the loss of HLA class I expression by tumors. By losing HLA class I expression, cancer cells are excused from presenting tumor antigens to CTLs, thereby avoiding detection and destruction (113). Furthermore, loss of HLA class I is also expected to result in failure of therapeutic approaches based on CD8+ T cell recognition such as vaccination with tumor antigens or adoptive transfer of autologous T cells (114). On the other hand, abrogation of HLA class I expression would support NK cell-mediated recognition in the presence of activating signals and thus, further escape mechanisms are expected to accompany HLA class I loss (115, 116). Cellular stress, such as the one derived from DNA damage, was shown to result in increased expression of NK cell activating ligands (117). Therefore, and providing that DNA damage response mechanisms are in place, tumor cells should upregulate the expression of NK cell activating ligands as a consequence of their chronic exposure to replicative damage and/or chromosomal instability. Not surprisingly, a considerable proportion of human cancers lack NKG2D ligands and are, thereby, resistant to NK cell-mediated lysis, even when HLA class I expression is abrogated (103, 118, 119). Absent or low expression of NKG2D ligands has been generally correlated with increased malignant behavior of tumors (103, 120, 121), but conflicting findings underline the complexity of anti-tumor immunity (122, 123). An additional escape mechanism to NK cells is provided by the release of soluble forms of NKG2D ligands by tumor cells, which induce the internalization and destruction of NKG2D, thereby impairing NK cell function (124). Furthermore, acquired expression of the non-classical HLA-G antigen and the loss of HLA class II expression have also been reported in cancer cells (125, 126). As a corollary of the accumulated evidence on the selection of immune evasive traits during cancer progression, Douglas Hanahan and Robert A. Weinberg have recently acknowledged immune escape as a hallmark of cancer in an updated version of their seminal review (127). The aforementioned phenotypes are conceptually concordant with Darwinian models of evolution that imply the elimination of "less-fit" tumor clones (128). The generation of clonal diversity is fundamental for the emergence of immune escape phenotypes and other traits. The latter is assured by the impairment of DNA repair and damage response mechanisms that support the accelerated evolutionary process that accompanies tumorigenesis. Nevertheless, there is a high probability that carcinogenic processes not always lead to the generation of tumor variants with immune evasive properties and that, occasionally, tumors are indeed swiftly eliminated by the immune system in asymptomatic individuals.

3.3. HLA genes and the HLA class I antigen presenting pathway

The HLA system is the human counterpart of the major histocompatibility complex (MHC), a unifying feature in vertebrate organisms that plays a key role in the immune system. The MHC class I and class II loci are comprised of the most polymorphic genes known in vertebrates in spite of the strong selective pressure imposed by the evolutionary "arms race" between hosts and pathogens. Instead, a positive selection is in place for the maintenance of MHC variability, derived from the fact that the immune system has to deal with a myriad of pathogens (129).

The HLA class I molecule is a heterodimer

consisting of a variable heavy chain (α) and the non-polymorphic β2-microglobulin molecule, encoded by a gene located on chromosome 15. Three loci encode for different HLA class I heavy-chains on chromosome 6p: *HLA-A*, *HLA-B* and *HLA-C*. These are generally defined as the classical HLA class I genes for which more than 5000 alleles are currently known (130). The *HLA-E*, *HLA-F* and *HLA-G* genes comprise the non-classical HLA class I genes, which are considerably less polymorphic than the classical HLA class I genes. Their proteins are not involved in general antigen presentation but, instead, they bind peptides derived from the classical HLA class I molecules themselves (131). Similar to the classical HLA class I molecules, they can also modulate NK cell activity (132). HLA class II molecules are composed of two variable chains (α and β), encoded by six main HLA class II genes also located on chromosome 6p: *HLA-DPA1*, *HLA-DPB1*, *HLA-DQA1*, *HLA-DQB1*, *HLA-DRA* and *HLA-DRB* (130).

Each HLA class I (classical) or class II allelic variant is able to present a pool of peptides that display affinity to that specific allele. This affinity is defined by anchor residues located at the extremities of a peptide sequence that directly bind the HLA molecule. Since the sequences within anchor residues are relatively free to vary, each HLA class I molecule can present a broad range of peptides (133). Cross-presentation-apart, the peptides presented in the context of HLA class I are derived from endogenous proteins that have either reached the end of their functional life or resulted from defective transcription or translation (Figure 5). Those peptides are generated in the cytosol by the 26S proteasome which is composed of a 20S core barrel protein complex with protease activity, sandwiched by two 19S caps (134). The LMP2 (PSMB9), LMP7 (PSMB8) and LMP10 (PSMB10) proteins are core subunits of the 20S complex in the immunoproteosome, a modified

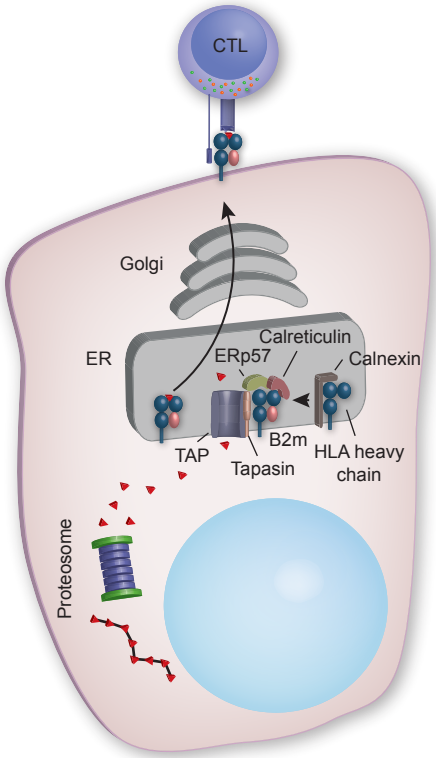

Figure 5. HLA class I antigen processing machinery.

proteasome form that is particularly effective in generating peptides for HLA class I in the presence of inflammatory signals (135). Recently, the cytosolic endopeptidases nardilysin and TOP were also shown to complement the proteosome's activity and to be essential for the generation of specific CTL epitopes (136). In order to be loaded onto HLA class I molecules, peptides must be transported by the transporter associated with antigen processing (TAP) proteins TAP1 and TAP2 into the lumen of the endoplasmic reticulum (Figure 5). The TAP proteins associate with HLA class I molecules through their interaction with Tapasin, a HLA class I chaperone. Additional chaperones such as Calnexin, Calreticulin and ERp57 are involved in stabilizing HLA class I and in assisting the loading of peptides onto this molecule (137). Often, peptides require further trimming in the endoplasmic

reticulum before loading onto HLA class I. This task is performed by the ERAP1 and ERAP2 aminopeptidases (138). Since the peptides generated by the 26S proteasome are highly unstable in the cytosol, only a small fraction of those reach the cell surface in complex with HLA class I (Figure 5) (139). The HLA class II antigen presenting pathway deals with peptides derived from exogenous proteins that are processed by endocytic pathways (134).

3.4. The HLA system: around the dogmas

Traditionally, HLA class I molecules have been considered to be expressed on nearly every nucleated cell of the human body, except for few "immune privileged" sites (e.g. brain, cornea, liver, and testis). However, a number of studies support that additional tissues present non-detectable or reduced HLA class I expression (115, 140, 141). We (de Miranda and Morreau, unpublished), and others (142), have also observed that HLA class I expression is often higher in colorectal cancers than in the normal mucosa, which might derive from an overall increase in protein expression in tumors or from a natural response to cellular stress. Therefore, in certain contexts, the lack of HLA class I expression in tumors might not represent a loss but rather the inability of tumor cells to induce HLA class I expression. Nevertheless, since normal colorectal tissue consistently displays immune-reactivity to anti-HLA class I antibodies, we refer to HLA class I loss throughout the thesis.

In the opposite direction, there is a generalized misconception that HLA class II expression is restricted to antigen-presenting cells such as B cells, dendritic cells and macrophages. On the contrary, HLA class II expression can be induced in a variety of cells including epithelial cells, endothelial cells, and fibroblasts in the presence of inflammatory signals (143, 144). Additionally, a variety of tumors have been shown to acquire expression of HLA class II molecules during tumorigenesis (126, 145, 146). These observations are of great relevance as HLA class II molecules are known to mediate the presentation of tumor-specific antigens (147, 148). Local activation of CD4+ T cells at tumor sites might support a more effective CTL response (149) but also the triggering of Th1 and Th2 inflammatory responses that engage macrophages and eosinophils, respectively (150). Furthermore, a subset of CD4+ T cells appears to possess cytotoxic capacity and the ability to eliminate target cells presenting tumor antigens in a MHC class II context (151, 152).

4. OUTLINE OF THE THESIS

In this thesis, we compiled five studies where we report some of the genetic and molecular alterations that accompany the tumorigenesis of mismatch repair and MUTYH deficient colorectal cancers, with particular focus on immune escape mechanisms.

Both sporadic and hereditary mismatch repair deficient colorectal cancers are characterized by the presence of a conspicuous intraepithelial lymphocytic infiltrate, indicative of an anti-tumor immune response. We hypothesized that those tumors would be particularly prone to adopt immune evasive strategies, such as the loss of HLA class I expression, in order to escape from immune cell-mediated recognition and destruction. In **chapter 2**, we studied the expression of HLA class I, and associated antigen processing machinery molecules, in a well-characterized set of sporadic and Lynch colorectal cancers. We compared the frequencies of HLA class I loss between mismatch repair-deficient and proficient colorectal cancers and dissected the molecular mechanisms that underlie HLA class I defects in sporadic and hereditary mismatch repair deficient tumors.

Following the discovery of the MUTYH-

associated polyposis (MAP) syndrome and the reported histopathological similarities with mismatch repair deficient tumors, we speculated that MAP colorectal cancers might also present a high frequency of HLA class I alterations. In **chapter 3**, we characterized the expression of HLA class I and associated antigen processing machinery molecules in a cohort of MAP colorectal cancers and compared our findings to the ones reported in chapter 2.

The outgrowth of tumor clones lacking HLA class I expression is likely to result from the immune system-mediated destruction of HLA class I-positive cancer cells. In addition to providing an effective immune escape mechanism from cytotoxic T cells, the loss of this essential immune recognition molecule may also alter the capacity of cancer cells to invade surrounding tissues or to disseminate at distance (metastases). **In chapter 4**, we investigated a potential correlation between the type and density of lymphocytic infiltration in Lynch colorectal cancers with their HLA class I phenotype and clinicopathological stage. By relating the density of intraepithelial lymphocytic infiltrate with distinct HLA class I phenotypes we sought to establish a link between the agent of selection and the selected traits, respectively.

In chapter 5, we have studied one of the most common genetic alterations found in MSI-H colorectal cancers: the accumulation of frameshift mutations in the *TGFBR2* gene. TGFβR2 is a fundamental receptor for the transduction of TGF-β signaling in cells. Despite the fact that biallelic truncating mutations in *TGFBR2* occur in the majority of MSI-H cancers, some studies have reported that TGF-β signaling is still active in these tumors. We have attempted to replicate the latter findings in a cohort of MSI-H tumors and in a panel of colorectal cancer cell lines. Furthermore, we provide a mechanistic explanation for the retained sensitivity to TGF-β observed in *TGFBR2* mutants.

The alterations observed in HLA class I expression and in TGF-β pathway components in colorectal cancers are tightly connected to the role of the microenvironment in selecting the "most fit" tumor phenotypes. In colorectal tumors that develop in a background of mismatch or base-excision repair deficiency, the relation between tumor genotypes, phenotypes, and the environmental agents of natural selection is particularly evident and fascinating. These relations are discussed in a review paper that comprises **chapter 6**.

A few concluding remarks and future perspectives are presented in **chapter 7**.

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