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Molecular pathology of mismatch repair deficient tumours with emphasis on immune escape mechanisms

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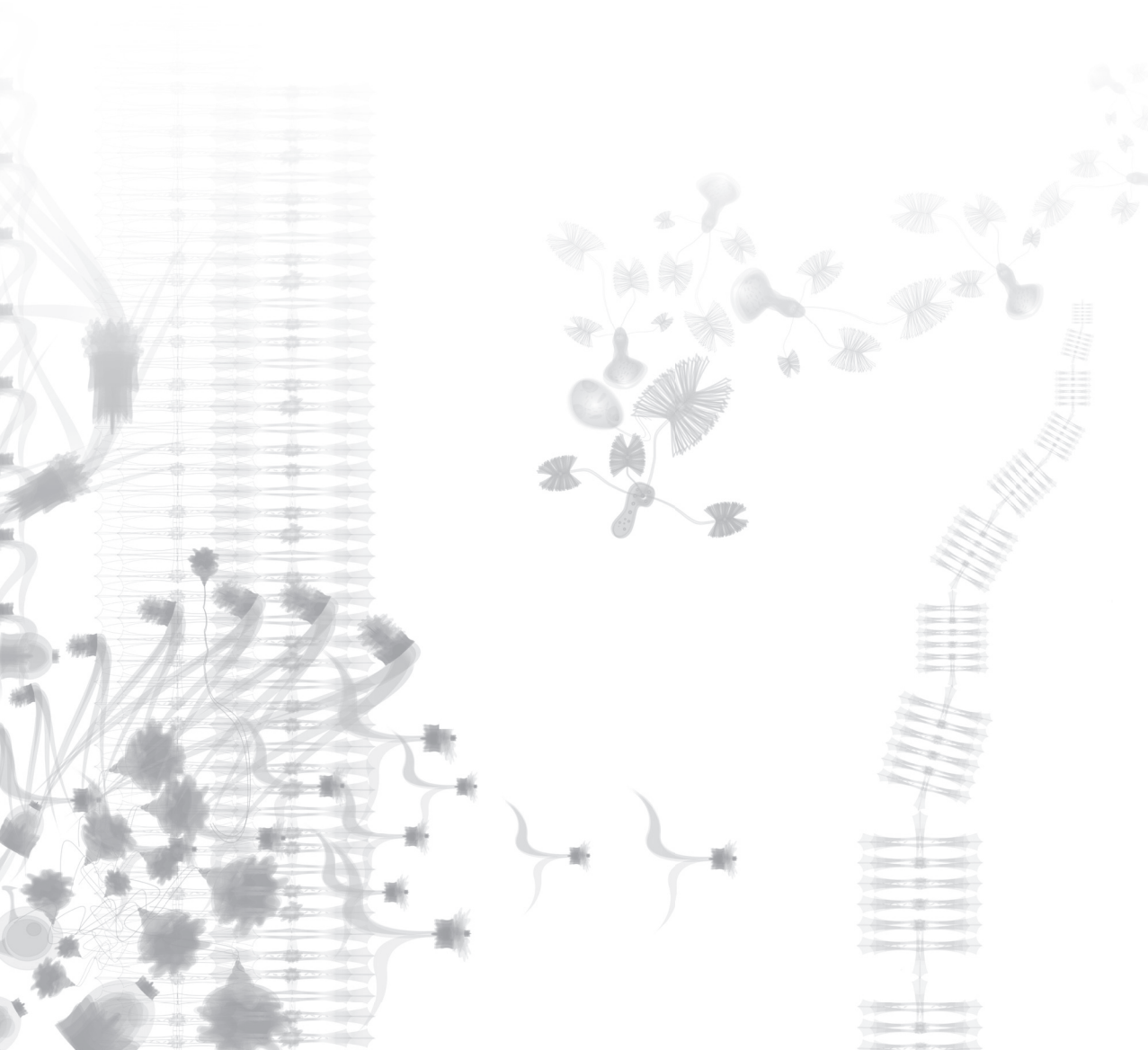
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General Introduction



The accumulation of genetic defects is a hallmark of cancer, and its detailed characterization has illuminated potential therapeutic targets and will keep on providing those. Yet, the identification of distinct colon tumour entities makes it necessary to adjust therapeutic strategies. One promising therapeutic strategy is the employment of the adaptive immune system in eradicating host tumour cells. However, its feasibility in eradicating human cancer is not well known as tumour escape mechanisms have not been studied in detail. In this chapter, current concepts of colon tumour development and tumour immunology are discussed and an outline of this thesis is given.

1. COLON TUMOUR DEVELOPMENT

1.1. A roadmap of cancer

Despite the huge heterogeneity of cancer, even between tumours from the same histological subtype, it has been proposed that there are only six general features any cell needs to acquire in order to become a cancer cell [1]. Like a roadmap that includes inevitable roadblocks. These features or roadblocks are: insensitivity to anti-growth signals, self-sufficiency in growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

The roadmap does go with a manual. There are many different ways in disrupting cell signalling pathways in order to get to the same point; some are shortcuts, others require multiple steps just to cross one roadblock (see figure 1). Which steps may be taken will be explained in paragraph 1.2. And there is no necessary order of the roadblocks to be taken. The final course is tumour specific and probably depends on the cell's constitutive biology as well as its micro-environment. For details, see also paragraph 1.3.

For colorectal cancer, one possible route which partly contains the roadblocks mentioned above has already been mapped (see figure 1) [2]. This route parallels the development of distinct histological features of the majority of colon tumours: the progression from normal epithelium - to aberrant crypt foci - to polyps (early, intermediate and late adenoma)

- to carcinoma. That unique feature makes colon cancer nearly an *in vivo* cancer model of its own, which is probably why the route of colon cancer has been studied so well.

Many of the roadblocks were originally identified by *in vitro* cell culture experiments and animal models. Meanwhile most have been confirmed in human tumour tissues. However, the roadmap still has its blind spots, for instance on micro-environment interactions, and future research likely will identify other roadblocks to be cleared by developing tumours including those of the colon. In this thesis we focus on the need for tumour cells to evade recognition and destruction by the immune system.

1.2. Genetic instability and clonal selection: how to cross roadblocks?

Tumours may be described as accumulations of cells exhibiting acquired disruptions of cell signalling pathways leading to loss of (normal) growth control. Both qualitative as well as quantitative changes in the protein components may account for the compromised functionality of signal transduction pathways in cancer cells. These changes may result from alterations at the genomic, transcriptional, translational or post-translational level. We will focus on those at the genomic and transcriptional level.

Genomic alterations comprise various types of irreversible changes in the content and organization of the genetic information of cells. For instance numerical and structural chromosome

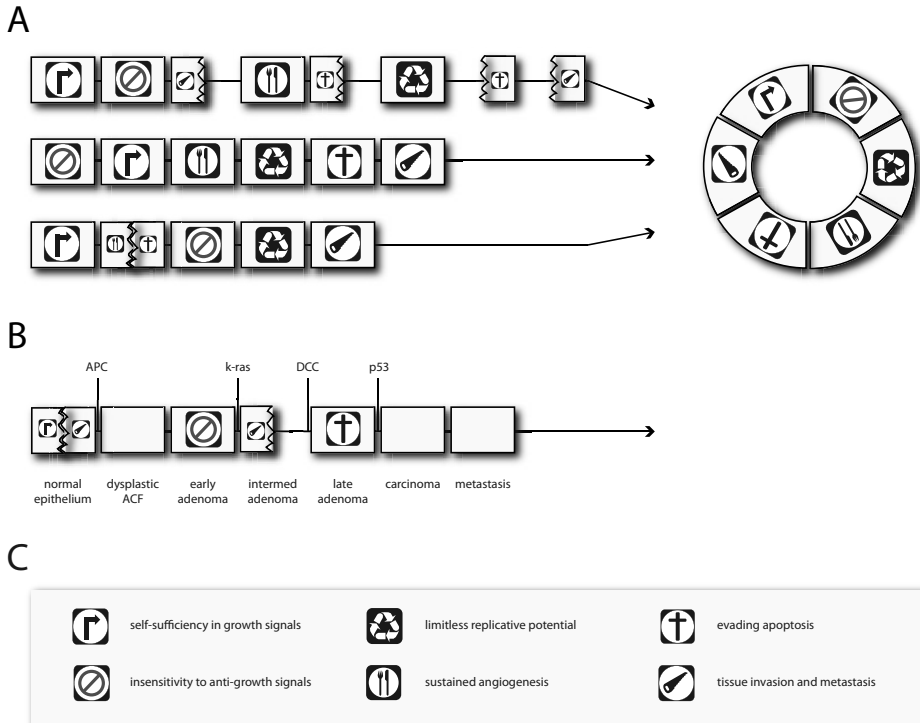


Figure 1. The roadmap to colon cancer.

The six features of cancer cell biology shown in C, need to be acquired. However, the order of acquisition may differ between different tumours, as illustrated in A. The seven-step model of colorectal cancer nicely fits to this model, as shown in B. Adapted from [1] and [2].

alterations have been describes almost a century ago. Only much later alterations at the single gene level were identified and genes altered in cancer were classified in two main categories: oncogenes and tumour suppressor genes [3, 4].

Distinct types of genetic alterations have been identified in cancers. Normally, these alterations would activate specific error detection systems that would either repair them or, if unsuccessful, arrest further cell cycle progression. Classically, they can be divided into 4 categories: subtle sequence changes, alterations in chromosome or chromosome fragment number, chromosome translocations, and gene amplification [5]. In colon cancer, the former two have been most frequently studied. Put simply,

genetic instability in colon cancer may manifest itself as either subtle single gene changes or as large chromosomal aberrations. Grossly, the former case has been associated with microsatellite instability (MIN), the latter case is also denoted as chromosomal instability (CIN). MIN tumour cell DNA content usually is conserved in the peri-diploid state, whereas in CIN tumours, tumour cells' DNA content is altered leading to a state of aneuploidy [2]. Interestingly, in colon cancer, these two manifestations seem to (at least partly) exclude each other.

The level of gene transcription is influenced by quantity of transcription factors and micro RNAs, or by altered DNA accessibility for these. Accessibility is determined by the state of DNA

hyper- and hypomethylation, and histone modifications [6]. The epigenetic state can be passed on to daughter cells. The epigenetic state can be passed on to daughter cells and the accumulation of both genetic and epigenetic changes is a typical characteristic of tumour development.

As cancer is not the plan of an evil genius but rather the unfortunate outcome of chance events cells do not have a will of their own, nor do tumours wish to become destroyers of their host; they are simply the survivors of a Darwinian micro-evolution [7]. The acquisition of necessary features therefore are the result of a multistep progression process, in which – like in macro-evolution – mutations may provide a selective growth advantage leading to clonal expansion of daughter cells and to clonal divergence. Furthermore, a tumour comprises in fact a collection of heterogeneous cell populations, but may in the long run be overgrown by a population that is best adapted to its micro-environment [8].

Is has been a debate for decades whether genetic instability is the cause or effect of neoplastic transformation, and the same discussion may be repeated for epigenetic mechanisms [9]. Do tumours need increased mutability as a driving force, or is it merely an accumulation of normally occurring errors derived by clonal expansion? Is genetic instability a state or rate? Mathematical models showed that the number of mutations found in tumours is too great to be explained by basal mutation rate [10]. However these models did not take into account the effect of clonal selection and expansion, nor the fact that cell turnover exceeds tumour growth. Such models show that an increased mutation rate may merely be a side effect, not a necessity [11]. It has also been argued that the level of aneuploidy itself contributes to malignant transformation. By increasing the expression of thousands of genes at once, this might lead to

the necessary qualitative changes of cell physiology and metabolism [12]. However, as mentioned above, not all neoplastic cells are aneuploid. Finally, it is stated that increasing genetic instability would be detrimental for a tumour, as the chances of deleterious mutations would exceed the chance of growth advantage. Yet, evolutionary models have shown exactly the opposite. Enlarging its diversity a cell population increases its chance of surviving micro-environmental changes and genetic instability achieves just that [13]. Besides, clones giving birth to non viable progenitors will be overgrown by more successful clones. Darwinian selection results automatically in a ‘just right’ rate of instability [14]. So as the state or rate question remains, we conclude that tumours have two ‘vehicles’ available during their roadtrip, genetic instability and clonal expansion, and they probably use both.

1.3. Tumour development and metastasis: which order of appearance?

Clinically, metastases are considered the end stage of cancer. Dissemination increases the tumour chances to survive medical therapy annihilation and therefore commonly causes cancer patients’ death. However as discussed in paragraph 1.2, tumours can not anticipate to a changing environment. Therefore metastasis should be considered as a side effect of the micro-evolutionary process by which tumour cells acquire adaptations to the local micro-environment and vice versa. So what determines metastatic progression and when is it acquired? Different theories exist, which we will discuss hereinafter.

- The progression model. Ever since Nowell’s theory of step-wise evolution, this classical model foresees a step by step progression driven by clonal expansion of random mutants, which was elegantly implemented

in the multi-step model of colon cancer (see paragraph 1.1), where metastasis confines the last step [2, 7]. Considering tumour heterogeneity, the subpopulation with metastatic capacity would actually not need to contain the majority of tumour cells; in fact, one may expect it not to be so, since the cells tends to disseminate and the acquisition of metastatic prone mutations would not likely lead to growth advantages at the primary site.

- Cancer stem cells. Tumours comprise heterogeneous cell populations and may contain a subpopulation of stem cells that - in contrast to other populations - has unlimited replicative potential. Only these cells would be capable of forming new tumours, even at distant locations, as they can self-renew infinitely [15].
- Alternative pathways. As addressed in paragraph 1.1, the roads toward the acquisition of proliferative benefits are many. Thus, there are multiple alternative genetic paths that lead to tumour formation [16]. According to this model, some combinations of mutations yield a high tendency to metastasize, whereas other combinations would not. This implies that metastatic potential may already be defined early during tumorigenesis and does not result from mutations or epigenetic changes involving specific genes.
- Genetic predisposition model. This model pleads for the contribution of the allelic composition of the host genome [17, 18]. Subtle changes in gene functions, already present before tumour formation, may determine the metastatic potential. Interestingly, this may also predict that the micro-environment, both at primary and distant sites, fills a principal part in tumour development.

We may conclude that we still have insufficient insight in the factors driving the metastatic

process. This is illustrated by the fact that despite huge efforts so far only few 'metastasis' genes have been identified. Furthermore, it remains puzzling that disseminating tumour cells can be detected even at early stages of tumour development which does not necessarily predict distant metastasis [15, 19, 20]. Finally, it is remarkable that metastatic potential may be predicted by the gene expression profile of bulk tumour tissue including multiple heterogeneous tumour subsets and a significant share of tumour stroma [21, 22].

2. MULTIPLE COLON CANCER SYNDROMES AND ROADMAPS

The identification of hereditary cancer syndromes has contributed largely to the identification of tumour suppressor genes. Based on Knudson's two hit model, carriers of heterozygous germline mutations have such a raised risk of developing cancer that it is inherited in an autosomal dominant way [23]. Another hereditary colon cancer syndrome that has been identified, the *MUTYH*-associated polyposis, is inherited in an autosomal recessive way. Interestingly, genetic and genealogical studies have revealed that the increased risk only applies for specific tissues, or even specific tumour types. This issue remains a puzzle. The syndromes associated with increased risk of colon tumours are displayed in table 1.

Sporadically developing colon tumours, i.e. non-syndrome associated (constituting almost 95% of all colon tumours), also bear mutations of the colon cancer syndromes associated genes, and their development may parallel the hereditary counterpart in some way. Classically, they have been assigned to the tumorigenic pathway of either of the two most commonly studied colon cancer syndromes: familial adenomatous

Neoplastic lesions	Inheritance	Syndrome	OMIM	Genes responsible
colorectal cancer without polyps	AD	Lynch syndrome or Hereditary non-polyposis colorectal cancer (HNPCC)	#120435	<i>hMLH1, hMLH2, hMSH6, hPMS2</i>
colorectal cancer with adenomatous polyps	AD	Familial adenomatous polyposis (FAP)	#175100	<i>APC</i>
colorectal cancer with hamartomatous/mixed/hyperplastic polyps	AD	Birt-Hogg-Dube syndrome*	#135150	<i>FLCN</i>
	AD	Cowden disease (CD)*	#158350	<i>PTEN</i>
	AD	Hereditary mixed polyposis syndrome 1*	#601228	<i>#15q15.2-q22.1</i>
	AD	Hyperplastic polyposis syndrome*	unassigned	
	AD	Juvenile polyposis (JPS)	#174900	<i>SMAD4, BMPR1A</i>
	AD	Juvenile polyposis/hereditary hemorrhagic telangiectasia syndrome (JPHT)*	#175050	<i>SMAD4</i>
	AD	Peutz Jegher's syndrome (PJS)	#175200	<i>STK11</i>
colorectal cancer with adenomatous, serrated adenoma and hyperplastic polyps	AR	MUTYH-associated polyposis (MAP)	#608456	<i>MUTYH</i>

Table 1. Clinical syndromes with an increased risk of colorectal cancer. Adapted from [24]. OMIM annotation as used and explained on <http://www.ncbi.nlm.nih.gov/omim>. * colorectal cancer risk not clear

polyposis (FAP) and Lynch syndrome (previously depicted hereditary non-polyposis colorectal cancer). The former, FAP, is characterized by mutations of *APC* [2]. Normally, APC protein captures free cytoplasmic β -catenin in order to have it destroyed. β -catenin is involved in at least two distinct cellular processes: cellular adhesion (through E-cadherin, and important in the normal crypt organization of the colon epithelium), and the Wnt signalling pathway leading to transcription of, amongst others, cell cycle promoters *c-myc* and *cyclin D1* [25, 26]. Both processes may be involved in the APC gatekeeper function in

colon tumours. Patients develop hundreds of adenomatous polyps, of which some may develop into the carcinoma stage. The Lynch syndrome is caused by germline mutations of members of the DNA mismatch repair family *hMLH1*, *hMSH2*, *hMSH6*, and *PMS2*. Mutation carriers also have an increased risk of developing endometrial carcinoma and other lesions, but do not develop many polyps. Due to mismatch repair defects these tumours are characterized by a large accumulation of subtle DNA sequence changes, preferably of microsatellite repeats, referred to as microsatellite instability (MSI). Approximately 60% of

sporadically developed tumours develop from a polyp and bear somatic mutations of *APC*. However, in 15% of consecutive series of colorectal cancer MSI is observed, mainly due to epigenetic knock-out of the mismatch repair gene *hMLH1* [27].

During embryogenesis, the mid-gut develops into the proximal colon (cecum, ascending colon and two third of the transverse colon), whereas the hindgut develops to the distal colon (one third of transverse colon, descending colon, and rectum). These separate embryological origins have distinct blood- and lymph supply and drainage, e.g. the micro-vascular volume is greater in the proximal colon. The regions are exposed to different dietary and digestive constituents, pH conditions and microbial colonization [28]. Therefore, one may suggest that tumours arising from the proximal (also called right-sided) colon differ from those of the distal colon. This is partly reflected in molecular tumour development. MSI tumours primarily develop in the proximal colon. Furthermore, especially within the proximal colon distinct subsets of carcinomas have been identified based on differences in morphology, microsatellite instability, (underlying germline) mismatch repair gene mutations, high or low frequent CpG island hypermethylation, and *KRAS* and *BRAF* mutations [29].

The identification of multiple pathways of development pleads for distinct tumour entities even though they arise from the same organ and cell type. Indeed, subsets have been associated with distinct clinical features such as dissemination and chemotherapy response [30]. Unraveling these differences is necessary to direct future therapeutic strategies of colon cancer effectively.

3. TUMOUR IMMUNOLOGY

3.1. Immunosurveillance

Neoplasms develop from autologous cells and therefore intuitively one may think these would not evoke an immune response. Yet, the accumulation of genetic aberrations during tumour development leads to numerous tumour specific antigens, which are potential targets of the adaptive immune system [31]. In fact, tumour immune control has already been proposed over a century ago [32] and has later been introduced as the immunosurveillance hypothesis [33]. Initially, the hypothesis could only be supported by studies on virally induced or allograft tumour development in immune compromised organisms. It was not until the 1990s that increased risk ratios of developing sporadic tumours in immune compromised patients were shown to exist, although colorectal cancer is not typically frequent in such patients [34, 35]. Meanwhile, evidence for immunosurveillance has been backed up by the numerous tumour escape mechanisms that have been identified [36] as more knowledge is gained about the mechanisms leading to proper adaptive (anti-tumour) immune responses, which will be discussed in the following paragraphs.

3.2. Colon cancer immunity

A current model of colon cancer immunity is depicted in figure 2. It shows the key steps in the generation of a functional or non-functional immune responses to tumours. We will briefly discuss it here, and then explore several steps in more detail in the following paragraphs.

Tumour antigens are picked up from dying or dead tumour cells by dendritic cells. These sentinels (as one might consider them) will sequentially bring the antigens to nearby lymph nodes where they can activate cytotoxic T cells assisted by helper T cells through a process

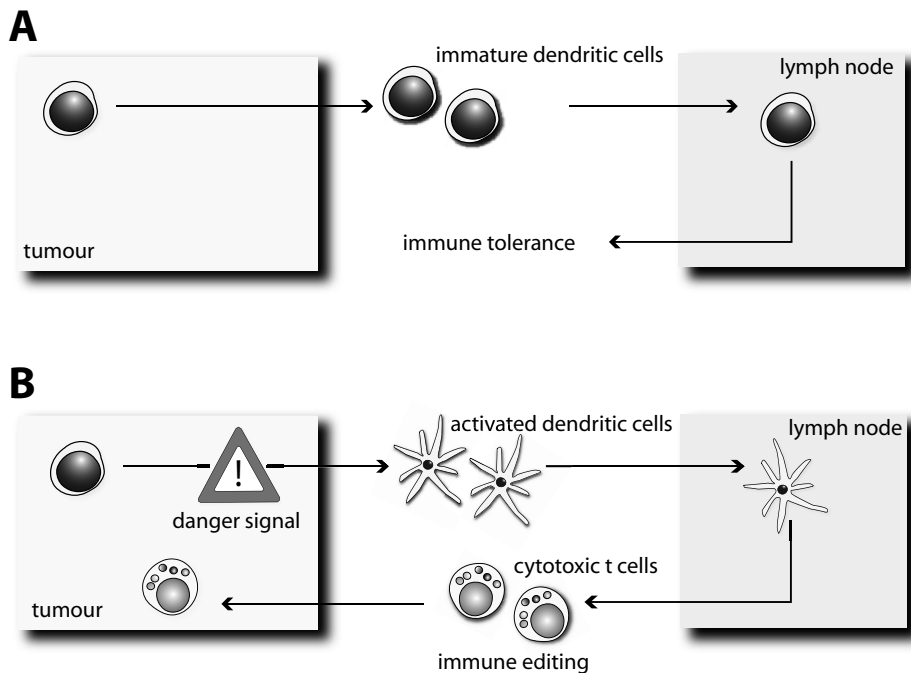


Figure 2. Model of cancer immunity. A. Tumour antigen pick up in the absence of danger signal leads to immune tolerance. B. In the presence of danger signal, activated dendritic cells will home to draining lymph nodes and there lead to activation of cytotoxic T cells, which will invade and attack the tumour. Adapted from [42].

called cross-presentation [37]. However, in order to do so, the sentinels must be awakened to an active state first, while searching the debris. Such awakening is caused by the local inflammatory environment, which is effectuated by the innate immune system; this is called the danger signal [38-40]. Without it, dendritic cells will only educate T cells to tolerate tumour antigens, leading to immune tolerance [41]. When tumour specific killer T cells are activated, they will migrate to, infiltrate and eventually kill tumour cells. They thereby represent clear selective forces of tumour development, a process referred to as immune editing [34]. The outcome of such is either tumour rejection or immune escape.

It is noteworthy to mention that consecutively disseminating tumour cells may encounter quite different immune responses as they

enter a completely different arena: the lymph and blood. However, this is beyond the scope of this introduction.

3.3. Innate immune system and danger signal: an inflammation battlefield

Inflammation is the early physiological response to tissue damage. It is the first action of war. Furthermore, it may determine following attack modes, viz. the nature of adaptive immune responses. Therefore it is also referred to as the danger signal. If performed effectively inflammation leads to tissue repair, growth and remodelling. Therefore a complex interplay of multiple units of the innate immune system army is needed. The units are directed towards the battlefield by signal molecules, including

cytokines, chemokines, growth factors and proteases [43, 44].

At the front line are the neutrophils (who get there by regulating the expression of consecutively selectins, integrins, cell-adhesion molecules, and, finally, proteases). Once arrived they will start 'firing' a considerable set of cytokines and chemokines. Eventually, this will recruit downstream effector cells and dictate the nature of an immune reaction.

The second movement involves the mononuclear phagocytes. Entering battle as monocytes they differentiate into either mature macrophages or immature dendritic cells (DCs). Mature macrophages produce the main body of growth factors and cytokines (the acute phase) and remodel the extracellular matrix by cleaning up (phagocytosis of dead cells and production of proteolytic enzymes) and by rebuilding (production of matrix components and promoting angiogenesis and lymphangiogenesis). Dendritic cells pick up antigens from the debris through various mechanisms and are essential in directing adaptive immune responses as discussed in paragraph 3.4.

Then the mast cells arrive which have stored various inflammatory mediators including histamine, cytokines and lipid mediators such as prostaglandins and leukotrien. Once the mast cells are activated through the complement system or by binding to immunoglobulin E-antigen complexes these factors are secreted leading to clinical inflammation features, e.g. the increase of plasma into the tissue and consequent drainage of tissue fluids into lymph nodes.

Finally, fibroblasts take care of collagen deposition, epithelial cells reepithelialise while anti-inflammatory cytokines slowly evaporate the fog of war.

Normally, the intricately linked processes eventually fade out once the damage is repaired.

Tumours however can be considered as chronic tissue damage like wounds that do not heal [45]. Paradoxically, as already known for over a century, chronic inflammation, f.e. inflammatory bowel disease, can also lead to neoplastic conversion [46, 47]. Growth factors resulting from inflammation actually stimulate tumour growth (and even sometimes suppress immunity). Furthermore, tumours also produce chemokines and cytokines, thereby directing inflammation themselves [43, 47]. Thus, inflammation actually serves a dual role in cancer: signalling danger and activating the immune system on the one hand, on the other hand growth stimulation and tissue remodeling [43, 48].

3.4. Cross-presentation: intelligence at the battlefield

The regional lymph nodes are 'command centres' of the adaptive immune response. It is here that molecular information about the 'enemy', antigen, is delivered by antigen presenting cells. This is called cross-presentation. Occasionally a 'deserter' of the enemy troops may enter the 'camp' by itself which could also supply the lymph node with information. However, it has been shown that this process is highly inefficient and does not lead to proper immune response [38, 49]. There are actually three cell types that are able to pick up and show foreign antigens to effector cells, macrophages, B cells and DCs, but only the latter are able to pick up intracellular antigens and are able to activate naïve T cells.

As they are summoned at the site of inflammation, immature DCs start collecting antigens through various mechanisms: through opsonisation of apoptotic bodies, through receptors of (tumour) heat shock proteins that bound to (tumour) intracellular antigens, through receptors of immunoglobins bound to antigens, or by pinocytosis [50]. During the collection of

antigens, dendritic cells develop into mature dendritic cells. This educational process, directed by earlier mentioned danger signals and possibly other factors, is important for the outcome of cross-presentation later on.

Once mature and forced by the increasing lymph flow, DCs migrate to local lymph nodes where they spread throughout the cortex. There they will encounter naïve T cells who continuously pass by through high endothelial venules (HEVs). The first encounter is managed by transient binding of cell adhesion molecules. Now, T cells have time to sample a large number of antigens as they are presented on the DC surface by human leukocyte antigen family molecules (HLA, which are responsible for antigen presentation at every immune cell encounter, and will be discussed extensively in the next paragraphs). If a T cell recognizes an antigen presented (by binding of its unique T cell receptor to the HLA:antigen complex), it leads to stabilisation of the cell adhesion up to days, which gives the DC an opportunity to educate the naïve T cell about the nature of the antigen, as itself was taught during maturation. This is called co-stimulation and numerous factors are involved in this co-stimulatory dialogue, including CD4/CD8, B7:CD28, CD40:CD40L, 4-1BBL:CD137 and ICOS:ICOSL [44]. If successful it leads to clonal expansion (proliferation and differentiation) of the T cell, called cross-priming. If co-stimulation is poor or absent, it leads to T cell anergy (inactivation), hence to immune tolerance of the specific antigen which is known as cross-tolerance.

3.5. Adaptive cell-mediated immunity: the T cell army

The T cell arm of adaptive immunity is specialised in antigen specificity, which is a key feature in distinguishing tumour cells from normal cells. It basically encompasses two distinct 'soldiers': CD8 and CD4. Both are activated by

DC cross-priming. They differ in communicating antigen specificity through a different class of HLA molecules, being HLA class I for CD8, and HLA class II for CD4. HLA class I molecules present intracellular antigens, HLA class II molecules present extracellular antigens; CD8 and CD4 are the T cell receptor co-receptors for HLA class I and class II respectively. Since virtually all somatic cells express HLA class I molecules, the CD8 T cells are able to target these, and hence become - once activated - armed cytotoxic effector cells (CTL). To support the 'search and destroy' missions CD4 T cells are required. These provide regulatory signals to prime and orchestrate the immune response, and become, once activated, so-called helper cells (Th). Basically, T helper cells do so by either of two strategies, Th1 or Th2. During co-stimulation naïve CD4 T cells either develop into Th1 or Th2 cells. Th1 cells enforce man-to-man battle, cell-mediated immunity by priming CTLs and macrophages. Th2 cells command the 'artillery', viz. humoral immunity by activating plasma cells and granulocytes. Th1 and Th2 cells enforce their strategy by producing a variety of cytokines. Simultaneously, they inhibit one another. Hence, once again, an adequate, full scale adaptive cell mediated immune attack is dependent on proper DC 'intelligence': cross-presentation, which on its turn is dependent on the danger signal.

3.6. Immune editing: tumour attack and escape

Once a proper cell-mediated immune response is generated, armed effector T cells will engage the enemy back at the battlefield. First, they initiate attack by antigen-nonspecific cell-adhesion of LFA-1: ICAM-1/ICAM-2 [44]. If specific tumour-antigen is sensed, this adhesion is enforced and remained long enough for the T cell to reorient its cytoskeleton around in order to release induced effector molecules by exocytosis of

lytic granules. The CD8 CTL effector molecules encompass perforin (perforating the target cell membrane), granzymes (proteases that trigger apoptosis), and Fas ligand (targeting the 'death receptor', Fas) [51, 52]. Furthermore, CTLs secrete cytokines IFN- γ , TNF- α , and TNF- β which among other things activate macrophages and can even be cytotoxic directly. This scenario described above appears to apply to colon tumours as colon tumour-antigen-specific CD8 CTLs have been identified [53, 54], as well as CTLs infiltrating colon tumours [55], which have been found to produce effector molecules [56].

So now that we know how tumour cells are besieged, we can also figure out the ways of tumours to try to escape this: 1) tumour cells may shield by down regulation of the receptors for effector molecules, including Fas and IFN γ receptor [57, 58] or block, e.g. expression of PI-9 which subsequently inactivates granzyme B [59], 2) tumour cells may fight back: expressing Fas ligand themselves [60], produce immunosuppressive cytokines [61], or even manipulate the tumour stroma to make it impenetrable for CTLs [62, 63], 3) tumour cells may hide: they may shed (tumour) antigen presentation, by altering expression of HLA:antigen complexes. This would seem an effective strategy, for it guarantees escape from any CTL damage. There are different ways to do this, which is explained in the next chapter.

3.7. Manipulating HLA class I expression: the art of hiding

Human leukocyte antigens are the human counterpart of the major histocompatibility complex, which was originally discovered by tumour allograft studies in mice. Antigen presentation, as executed by these molecules, is now considered to play a key role in the formation and execution of adaptive immunity as it makes up the immunologic language to communicate self from non self. As mentioned in paragraph 3.5, two classes

of HLA molecules are distinguished: HLA class I antigens are expressed on nearly all body cells, HLA class II antigens primarily on lymphocytes and mononuclear phagocytes. HLA class I molecules are made up of a highly polymorphic heavy or α chain and a conserved light or β chain called β 2-microglobulin (β 2m, encoded on chromosome 15q21). The heavy chain contains two peptide binding domains enabling it to present a nonamer peptide which constitutes the actual antigen [64]. The repertoire of peptides potentially presented by HLA complexes is limited and is determined by the organisms set of class I heavy chains, which is encoded by a cluster of 6 genes all located within the gene-dense chromosome region 6p21.3, denoted as *HLA A* to *G*. Of these, *HLA A*, *B*, and *C* are called the classical HLA class I antigens, as they are the most polymorphic with 124, 258, and 74 different alleles distinguished [65]. *HLA E*, *F*, and *G*, the non-classical HLA class I antigens, vary much less with 5, 1, and 14 different alleles respectively, and are considered less important in antigen presentation as they mainly present antigens derived from HLA class I heavy chains themselves.

Before the HLA:antigen complex is transported to the cell surface, antigen – primarily derived from endogenous, intracellular proteins – needs to be processed, and loaded on HLA molecule, which is assembled in the endoplasmic reticulum (see figure 3). Antigen processing is part of cellular 'housekeeping'; i.e. removal of redundant proteins, which is executed by the proteasome, a multi-enzyme complex, composed of a 20S complex, which is sandwiched by two 19S regulatory complexes. As characterized in yeast, the 20S comprises 28 subunits organized into 4 stacked rings. The outer rings are formed by seven homologous polypeptides termed α subunits, the inner rings are formed by 7 β subunits, 3 of which perform the actual proteolytic activities [66, 67]. A subset of catalytic β

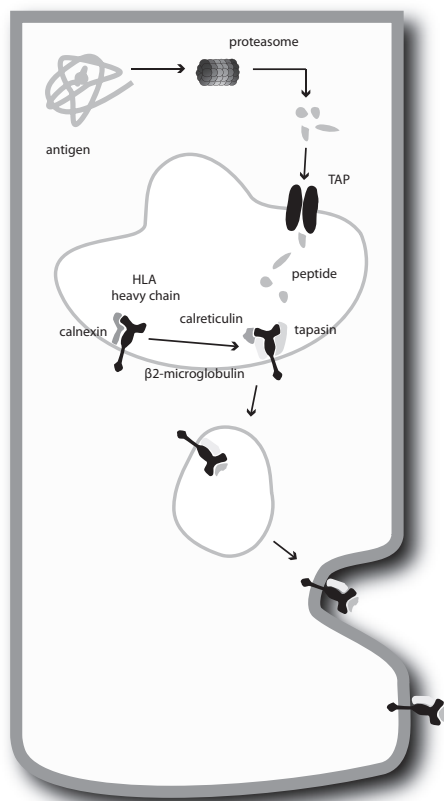


Figure 3. Antigen processing and HLA class I assembly. Adapted from [68].

subunits called the large multifunctional peptidases LMP2, LMP7, and LMP10 (MECL1) are encoded in the same chromosome region as the genes encoding for *HLA*. They replace the constitutive subunits X (MB1), Y (delta), and Z after exposure to IFN γ , which enhances production of peptides capable of associating with HLA class I molecules or, alternatively, increases the variety of peptides that can be produced within a cell [67]. The peptides are then transported into the endoplasmic reticulum by TAP1-TAP2 heterodimers, loaded on class I molecules. Consecutively, the secondary and tertiary structure of this HLA:antigen complex is obtained with help from chaperones calnexin, calreticulin, tapasin, and

ERp57. Through the Golgi complex, the newly assembled HLA:antigen complexes are then transported to the cell surface [64].

If we restrict ourselves to the classical HLA I antigens, every individual carries two copies of the *HLA* genes leading to a phenotype of maximally six different alleles. In order to escape the presentation of a certain set of antigens, many phenotypic alterations are possible, but they have been classified into 5 types of altered *HLA class I* expression [69, 70]. Alterations of HLA phenotype in colorectal cancer have been reported in up to 87% [71], but because detection of the type of alteration strongly depends on laboratory techniques used, reports on frequencies of alterations detected seem to be incomplete (see chapter 5). Here, we will solely discuss the molecular mechanisms leading to these alterations.

- Loss of all HLA class I antigens. Multiple mechanisms may lead to a total loss of HLA I molecules on the surface, although they appear to be rather complex. Theoretically, it may be the result of the accumulation of mutations in all single HLA alleles or the deletion of the entire chromosomal HLA region of both chromosomes. More efficient would it be to get rid of the β 2-m light chain by mutation or deletion, but this would probably still require mutation of both copies. Transcriptional silencing of the β 2-m promoter or the entire *HLA* region might be an alternative mechanism, although this has not been observed yet.
- Haplotype loss. Loss of a single copy of the *HLA* region on 6p21.3 leads to loss of a haplotype. Chromosomal aberrations causing loss of heterozygosity are frequent in tumours and lead to such an altered phenotype.
- Locus down regulation. Down regulation of for instance both *HLA A* copies would need disturbance of transcriptional regulation, by manipulation of either genetic promoter

elements, or the transcription factors. There are some differences in regulatory elements and factors between the different *HLA* loci [72].

- Allelic loss. Mutation of a single *HLA* allele.
- Composite phenotypes. Although originally designated as the waste bin of unexplained phenotypic alterations not matching one of above, composite phenotypes can now be considered as the dominant type of alteration in colon cancer (see chapter 5 and 6). Immune editing may require loss of some alleles but retention of others, complex phenotypes may be evolved during tumour development and appear not to be as random as expected (see chapter 5 and 6). Furthermore, disturbances in antigen processing and HLA assembly may lead to down regulation of some, but not all *HLA* alleles (see chapter 6).

4. OUTLINE OF THIS THESIS

In this thesis, we present our studies on the nature and distribution of HLA class I aberrations in colon cancer in relation to underlying genetic background.

First, we studied the feasibility of the use of both immunohistochemistry of mismatch repair genes and microsatellite instability analysis in Lynch syndrome-associated endometrial and colon tumours. As described in **chapter 2**, this is an effective approach in order to predict germline mutations in Lynch syndrome patients. We applied the same strategy this time for large series of hereditary colon tumours in **chapter 3**. Furthermore, we extended the approach on a large series of hereditary colon tumours by constructing tissue micro-arrays and analysed its reliability. Now, we acquired a powerful tool to study large series of colon tumour with preservation of essential information of molecular subtype.

Focussing on mismatch repair deficient colon tumours, we identified frequent mutations within the untranslated part of the *IFNGR1* gene, coding for the IFN γ receptor. This cytokine is important for CTLs in order to effectively kill target cells, in part by boosting tumour cell HLA expression. In the current literature, conclusions regarding the importance of such mutations is often based on the frequency of its observation. However, in **chapter 4** we show that this is not always the case. Studying functional consequences remains necessary. Mismatch repair deficient colon tumours retain their sensitivity to IFN γ .

In **chapter 5**, we focus on HLA expression in colon tumours. Using an elaborate flow cytometry technique enabled us to study allele specific quantitative expression of freshly isolated single tumour cells. Doing so, we gained intriguing insights regarding the frequency and nature of HLA class I alterations: a) our study showed that complete eradication of HLA antigens is less common than previously assumed, but b) we identified two different patterns of HLA alterations, c) the patterns are related to distinct tumour subsets: mismatch repair deficient tumours and tumours from the proximal colon (i.e. proximal to the splenic flexure). Both entities showed frequent HLA alterations, which may cause them insensitive to immunotherapy approaches. Subsequently, we constructed tissue arrays to study large series of both sporadic and hereditary mismatch repair deficient tumours and tumours from the proximal colon, which, as explained in **chapter 6**, confirmed our findings above. Furthermore, we comprehensively screened for genetic mutations contributing to the alterations of HLA phenotype which we identified in half of the cases. Interestingly, different mechanisms were identified and related to the three subtypes, underlining the difference in tumour development and behaviour of colon tumour subsets.

In **chapter 7**, we conclude with a few general remarks concerning the topics covered in this thesis.

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