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Immune Cell Infiltration and Prognosis in Colorectal Cancer

Agnes Ling

Department of Medical Biosciences, Pathology
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Magic is just science that we don´t understand yet

– Arthur C Clarke

Table of Contents

Abstract

Background: Colorectal cancer (CRC) is globally the second most common form of cancer among women, and third in men. It is also one of the most common causes of cancer-related death in high-income countries. Surgical resection is the basis for curative therapy but still almost half of the patients die from metastatic disease. It is therefore imperative to strive on in the search for more efficient strategies to improve patient survival. The success scores for accurate prediction of patient prognosis remain discouraging and novel markers to identify high-risk patients are called for.

The tumour immune response has proven critical to prognosis in CRC. A high amount of tumour infiltrating lymphocytes have in studies been found to significantly improve patient outcome. The opposite has been seen in patients with sparsely infiltrated tumours. Findings in this area have driven forth the design of the Immunoscore® system, which may be implemented in clinic as a complement to the TNM staging system. Ongoing research is also focusing on which immune evading mechanisms CRC might deploy in order to progress and metastasize.

Aim: To study immune cell infiltration in relation to prognosis in CRC. More specifically the aim has been to investigate the prognostic importance of different subsets of immune cells infiltrating the tumour, not only according to quantity but also to intratumoural subsite (tumour invasive front, tumour centre and within the tumour epithelium). The tumour immune response was also evaluated in different molecular subgroups of CRC. Another part of this thesis concerns possible molecular mechanisms involved in tumour immune escape in CRC.

Methods: CRC cases in the Colorectal Cancer in Umeå Study (CRUMS) were evaluated using immunohistochemistry, gene expression analyses as well as methylation analyses. Cytokine and chemokine expression was evaluated in CRC tumour tissues and one CRC cell line (Caco2) and derivatives using semi-quantitative real-time PCR. Methylation was analysed using methylation-specific pyrosequencing.

Results: We found high quantities of both cytotoxic T cells (CTLs) as well as of regulatory T cells (Tregs) to associate with a better patient outcome. The infiltration of CTLs within the tumour epithelium provided the strongest prognostic information, whilst Tregs withheld the strongest association to prognosis at the tumour invasive front and tumour centre. We could further show that a high Th1 lymphocyte infiltration was strongly associated with a better prognosis in patients with CRC, independently of intratumoural subsite. Another

finding was that the extent of Th1 infiltration and patient outcome differed in different molecular subgroups of CRC. We also found down-regulation of TAP1, a protein involved in antigen presentation by MHC class I, to be significantly associated with low infiltration of various subtypes of immune cells. Down-regulation of TAP1 was also correlated to poor prognosis in patients with early stages of CRC. Furthermore, we found TAP1 expression to be inversely correlated with methylation at sites close to the *TAP1* promoter region.

Conclusion: Tumour infiltrating T lymphocytes have a significant positive impact on prognosis in CRC patients. Different subsets of T lymphocytes vary in their dependency on intratumoural subsite, in to what extent they exert their prognostic influence. We moreover found varying Th1 lymphocyte infiltration rates as well as prognostic impact thereof, in different molecular subgroups of CRC. Our results also show down-regulation of TAP1 to be a mechanism of tumour immune escape in CRC. Further findings suggest methylation of the *TAP1* gene to be a putative mechanism for TAP1 downregulation.

Abbreviations

AJCC	American Joint Committee on Cancer
APCs	Antigen presenting cells
APM	Antigen-processing machinery
CRC	Colorectal cancer
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CMS	Consensus molecular subtype
CRUMS	Colorectal Cancer in Umeå Study
CTL	Cytotoxic lymphocyte
CTLA-4	CTL associated protein-4
FFPE	Formalin–fixed paraffin-embedded
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
IFN γ	Interferon γ
IRS	Immunoreactive score
MHC	Major histocompatibility complex
MSI	Microsatellite instability
MSS	Microsatellite stability
NK	Natural killer
PD-1	Programmed cell death receptor 1
TAP	Transporter associated with antigen processing
Th1/2	T helper 1/2
TNM	Tumour node metastasis
Treg	Regulatory T lymphocyte
TAMs	Tumour associated macrophages
TCGA	The Cancer Genome Atlas
TGFβ	Transforming growth factor β
UICC	Union for International Cancer control

Original papers

I. Ling A, Edin S, Wikberg ML, Öberg Å, Palmqvist R. The intratumoural subsite and relation of $CD8^+$ and $FOXP3^+$ T lymphocytes in colorectal cancer provide important prognostic clues. Br J Cancer. 2014 110(10):2551-9

II. Ling A, Lundberg IV, Eklöf V, Wikberg ML, Öberg Å, Edin S, Palmqvist R. The infiltration, and prognostic importance, of Th1 lymphocytes vary in molecular subgroups of colorectal cancer. J Pathol Clin Res. 2016 2(1):21-31

III. Ling A, Löfgren-Burström A, Larsson P, Li X, Wikberg ML, Öberg Å, Stenling R, Edin S, Palmqvist R. TAP1 down-regulation elicits immune escape and poor prognosis in colorectal cancer. Oncoimmunology 2017 7;6(11):e1356143

Populärvetenskaplig sammanfattning

Varje år drabbas cirka 6000 personer i Sverige av tjock- och ändtarmscancer och globalt sett är det en av de tre vanligaste cancerformerna. Trots förbättrad behandling och en förbättrad diagnostik så är det fortsatt många som dör i spridd sjukdom. Det finns ingen entydig enda riskfaktor kopplad till utvecklingen av tjock- och ändtarmscancer men det finns en tydlig koppling till flera livsstilsfaktorer. Tjock- och ändtarmscancer är vanligast i de rikare delarna av världen och antalet insjuknande i den här cancerformen ses öka i de länder som haft en kraftig ekonomisk utveckling. Det har även visat sig att när människor flyttar från ett land med låg förekomst av tjock- och ändtarmscancer till ett land med hög förekomst, så ökar deras risk för att drabbas. Till riskfaktorer räknas övervikt, låg fysiskt aktivitet, rökning och konsumtion av rött/processat kött. Som vid många andra cancerformer är hög ålder en betydande riskfaktor och medelåldern vid insjuknande är 70 år.

Den i första hand botande behandlingen är kirurgisk, med strävan att avlägsna tumören i sin helhet. Flertalet ändtarmscancerpatienter erhåller även strålbehandling innan operation. Beslut om vilken behandling en patient ska få, om till exempel det kirurgiska ingreppet ska följas av cytostatikabehandling, fattas i huvudsak utifrån cancerstadium. Cancerstadium graderas utifrån hur djupt tumören invaderar tarmväggen, om det finns spridning till lymfkörtlar och eller andra organ. Stadierna sträcker sig från I-IV och ju högre stadium desto mer avancerad sjukdom och sämre prognos. Det har emellertid visat sig att ytterligare faktorer spelar in för prognosen vid tjock- och ändtarmscancer. Förutom en ökad kunskap och förståelse för olika molekylära egenskaper hos den här tumörformen, så har immunförsvaret visat sig spela en stor roll för prognosen. Ju mer aktivt immunsvar kring tumören, desto bättre överlevnad hos patienten.

I det här avhandlingsarbetet är syftet att undersöka om och hur olika typer av tumörinfiltrerande T-celler påverkar överlevnaden hos tjock- och ändtarmscancerpatienter. I studierna undersöks både hur antalet infiltrerande immunceller, liksom deras lokalisation inom tumören och deras förhållande till varandra, påverkar prognosen. Dessutom studeras eventuella skillnader mellan olika molekylära undergrupper av tjock- och ändtarmscancer. Vidare så undersöks möjliga mekanismer med vilka tumören försöker undkomma upptäckt av immunförsvaret.

Det undersökta materialet i avhandlingens arbeten består huvudsakligen av tumörvävnad från över 400 tjock- och ändtarmscancerpatienter. Vävnaden ingår i en kohort; Colorectal Cancer in Umeå Study (CRUMS) med material insamlat vid Norrlands Universitetssjukhus mellan åren 1995 och 2003. För varje

patientfall finns en riklig mängd information kring både människa och tumörvävnad.

Det vi fann under dessa studier är att tumörinfiltrationen av T-celler spelar en signifikant roll för överlevnaden vid tjock- och ändtarmscancer. Ju fler infiltrerande immunceller desto bättre överlevnad. Vi kunde också se att typen av immunceller, deras förhållande till varandra och var någonstans i och kring tumören de befann sig, spelade en viktig roll. Infiltrationen av så kallade Th1 lymfocyter och deras prognostiska betydelse skilde sig dessutom åt mellan olika molekylära undergrupper av tjock- och ändtarmscancer. Vidare såg vi att tumörer som nedreglerat/uttrycker mindre av ett protein vid namn TAP1, var mindre infiltrerade av immunceller. Därför tror vi detta kan vara ett sätt genom vilket cancern söker undvika upptäckt av immunförsvaret.

Introduction

Incidence and etiology

Globally, colorectal cancer (CRC) is the second most commonly diagnosed form of cancer among women and the third among men. It is also the third and fourth leading cause of cancer death in women and men, respectively[1]. There are geographical differences and the highest incidence is seen in Japan and also Europe, Oceania and North America. The lowest incidence is found in Africa, Latin America, the Caribbean and some Asian countries[2]. CRC is associated with a western lifestyle and known risk factors are smoking, physical inactivity, a high BMI and the consumption of red/processed meat.

Trends show CRC incidence to be increasing in many historically low incidence countries, whilst it is decreasing in some of the high-income countries. The increase in formerly low incidence countries might be due to rapidly changing life styles with an increase of risk factors. The decrease in the United States of America is believed to be at least partly attributed to an implemented screening and reduced risk factors such as smoking[3].

Staging and prognosis

Prognosis corresponds closely to disease progression and tumour stage is classified according to the American Joint Committee on Cancer (AJCC) and Union for International Cancer control (UICC) TNM staging system[4]. (T) concerns primary tumour extent ranging from a tumour still being confined to the submucosa (T1), not invading through the thick muscular layer of the colon (muscularis propria) to the highest grade (T4) describing a tumour perforating visceral peritoneum or invading adjacent tissues and organs[5] (*Figure 1*). (N) describes lymph node involvement and (M) distant metastasis. The stages ranging from I to IV are based on TNM, where stage I tumours are T1 or T2 without lymph node metastasis (N0) or distant metastasis (M0). Stage II tumours have invaded through the muscularis propria (T3 or T4) but are N0M0. Stage III describes tumours of any T with lymph node metastasis and stage IV are tumours with distant metastases, regardless of T- and N-stage (*Figure 2*). Whilst the prognosis is very good in patients with a stage I tumour, 5-year survival reaching over 90%, it worsens with a deepened invasion, lymph node involvement, vascular invasion, perineural growth and metastatic spread[6, 7].

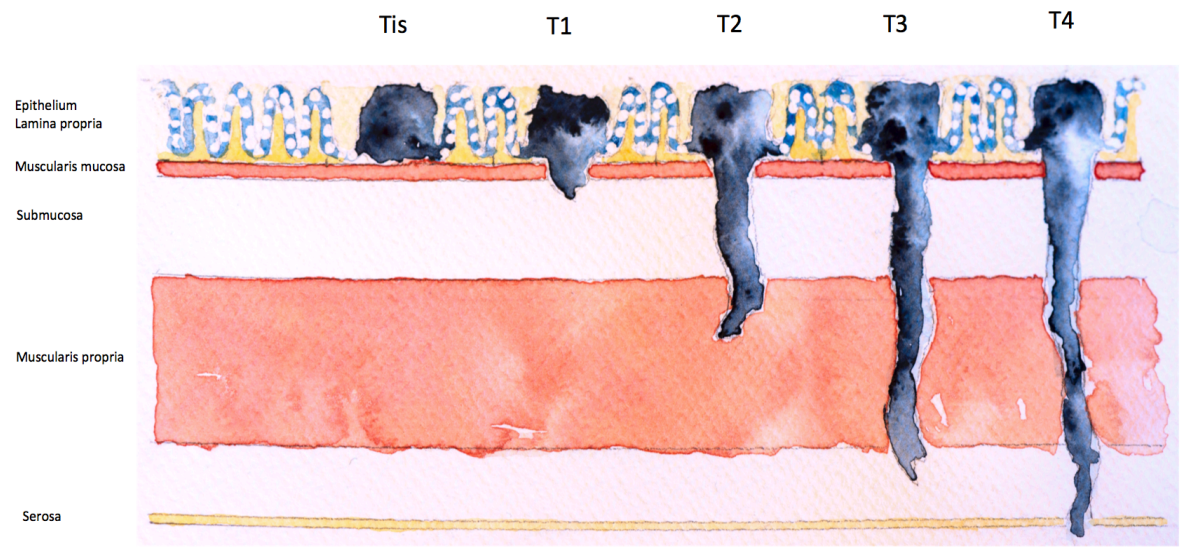

Figure 1. T in TNM-staging concerns invasion depth of the primary colorectal tumour. Tis (in situ): the tumour is still confined to the epithelium or lamina propria, T1:The tumour has invaded through the muscularis mucosa in to the submucosa, T2:The tumour has grown into the circular and longitudinal muscular layers. T3: The tumour has grown through the muscular layers and in T4: the tumour perforates the visceral peritoneum or has invaded adjacent tissues and organs

The vast heterogeneity of CRCs, with tumours displaying a plethora of different molecular traits, also signify prognostic differences. The outcome of CRC patients of the same TNM stage may differ substantially and the success scores for accurate prediction of patient prognosis, only based on AJCC/UICC TNM staging remain somewhat discouraging.

In addition to the TNM staging system, the state of the tumour immune response has been found critical for prognosis in CRC[8, 9], and the implementation of an Immunoscore® in clinical practice is in progress, work initiated by J Galon et al.[10]. This will be discussed more closely below.

T-Primary tumour
Tx Primary tumour cannot be assessed
T0 No evidence of primary tumour
Tis Carcinoma in situ: intraepithelial or invasion of lamina propria
T1 Tumour invades submucosa
T2 Tumour invades muscularis propria
T3 Tumour invades subserosa or into non-peritonealised pericolic or perirectal tissues
T4 Tumour perforates visceral peritoneum and/or directly invades other organs or structures
T4a Tumour perforates visceral peritoneum
T4b Tumour directly invades other organs or structures

N-regional lymph nodes
Nx Regional lymph nodes cannot be assessed
N0 No regional lymph-node metastasis
N1 Metastasis in 1-3 regional lymph nodes
N1a Metastasis in 1 regional lymph-node
N1b Metastasis in 2-3 regional lymph nodes
N1c Tumour deposit(s), i.e. satellites, in the subserosa, or in non-peritonealised pericolic or perirectal soft tissue without regional lymph-node metastasis
N2 Metastasis in 4 or more regional lymph nodes
N2a Metastasis in 4 to 6 regional lymph nodes
N2b Metastasis in 7 or more regional lymph nodes

M- Distant metastasis
M0 No distant metastasis
M1 Distant metastasis
M1a Metastasis confined to one organ
M1b Metastasis in more than one organ or the peritoneum

Stage	T	N	M
Stage 0	Tis	No	Mo
Stage I	T1, T2	No	Mo
Stage II	T3, T4	No	Mo
Stage IIA	T3	No	Mo
Stage IIB	T4a	No	Mo
Stage IIC	T4b	No	Mo
Stage III	Any T	N1,N2	Mo
Stage IIIA	T1,T2	N1	Mo
	T1	N2a	Mo
Stage IIIB	T3, T4a	N1	Mo
	T2, T3	N2a	Mo
	T1, T2	N2b	Mo
Stage IIIC	T4a	N2a	Mo
	T3, T4a	N2b	Mo
	T4b	N1, N2	Mo
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b

Figure 2. Stage grouping based on TNM,

Treatment

In CRC, curative therapy is based on surgical resection. More locally advanced rectal cancers often receive preoperative radiation, sometimes combined with chemotherapy. Adjuvant treatment with chemotherapy after surgery is offered to stage III CRC patients and to patients with a high-risk stage II tumour. High-risk tumours are defined by factors such as locally advanced invasion (a high T stage), intravascular and perineural growth, and high grade tumours (poorly differentiated). Patients with distant metastases are offered chemotherapy in both curative and palliative settings and can be treated with metastatic surgery (mainly liver and lung metastases). 5-fluoruracil (5-FU) is the cornerstone of chemotherapy in CRC, used either as a single drug or more often in combination with other drugs (e.g. leucovorin, oxaliplatin and irinotecan). In addition, some patients with advanced disease receive antibody-based targeted drugs such as angiogenesis blocking, anti-EGFR and multi-target tyrosine kinase blocking agents[11, 12] . Before anti-EGFR therapy, predictive molecular pathology testing of tumour tissue is suggested. Patients with mutations

in *KRAS*, *NRAS* and *BRAF* are excluded due to low response to anti-EGFR therapy[13].

Tumourigenesis and molecular subgroups of CRC

CRC develops through different molecular pathways of which three; the chromosomal instability (CIN), the microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP) pathway, have been well described[14] (*Figure 3*).

Chromosomal instability (CIN)

Almost thirty years ago a model was presented by Vogelstein and Fearon over the tumourigenesis of CRC[15]. The model suggests that colorectal carcinomas develop from preexisting adenomas through the accumulation of mutational changes. This model describes initiation, promotion and progression through the loss of certain chromosomal regions. Mutational activation of oncogenes coupled with the more extensive inactivation of tumour suppressor genes, starting out with the inactivation of the adenomatous polyposis coli (APC) gene. This leads to hyperproliferation of the epithelium, followed by additional mutations in for example the *RAS* gene - associated with growth of the adenoma, genetic alterations, mainly deletions leading to further growth and progression. The loss/inactivation of the tumour suppressor gene *p53* is believed to push forward the transition from adenoma to carcinoma.

This, by now well known, pathway of tumourigenesis in CRC is called the chromosomal instability (CIN) or MSS (microsatellite stable) pathway and describes chromosomal instability and loss of heterozygocity (LOH). In addition, tumours of the CIN pathway are often found to be *KRAS* mutated; a known poor prognostic factor[16, 17]. CIN is the underlying cause of the majority of CRCs[18].

Microsatellite instability (MSI), CIMP and BRAF mutation

Further understanding has been reached concerning other molecular mechanisms taking part in the malignification of the colonic mucosa, such as epigenetic alterations[19]. Only a couple of years after Vogelstein and Fearon presented this ground breaking model, another pathway was described, soon to be called the Microsatellite Instability Pathway (MSI). A research team, led by Manuel Perucho, was comparing DNA from normal colonic mucosa with CRC tissue and they found 12 % of the tumours with regions that were not deleted but shortened, containing simple repetitive sequences[20]. The same year, this discovery was paralleled by the work of other research teams[21, 22], where Thibodeau et al. were to coin the concept of microsatellite instability. MSI tumours display DNA mismatch repair (MMR) deficiency and the tumourigenesis

is driven forward by an accumulation of insertion or deletion mutations due to inactivation of the mismatch repair genes which are responsible of controlling and correcting errors emerging during DNA replication[23-26]. A common cause of deficient mismatch repair is the lost expression of MLH1 and PMS2 due to methylation of the *MLH1* promoter[27]. MSI tumours also show distinctive phenotypic features, mostly developing in the proximal colon, frequently being richly infiltrated by lymphocytes, poorly differentiated, often with a mucinous or signet ring like appearance and being less prone to metastasize[28-31].

Whilst 85% of CRC tumours are Microsatellite stable, MSS, the remaining 15% are MSI, with about 3 % of these being associated with Lynch syndrome, also known as Hereditary non polyposis CRC Syndrome [22, 32-35].

CIMP (CpG island methylator phenotype) is yet another alternative pathway of tumourigenesis[36] and is driven rather by epigenetic than genetic events, showing hypermethylation in specific promoter regions. The alterations leading to inactivation of tumour suppressor genes[37]. An association has been found between *BRAF* mutation, CIMP-status and MSI tumours, with studies showing up to 50% of *BRAF* mutated CRC tumours to also be MSI. All three characteristics come under the hypermutated tumour cluster[38-40]. *BRAF* mutations are generally considered poor prognostic factors, primarily in MSS tumours[41]. In MSI tumours however, the prognostic impact of *BRAF* mutation isn´t as readily settled. There are studies showing worsening prognostic effect of a mutated *BRAF,* others finding it to improve patient outcome and yet other studies describing no significant influence on prognosis[42-45].

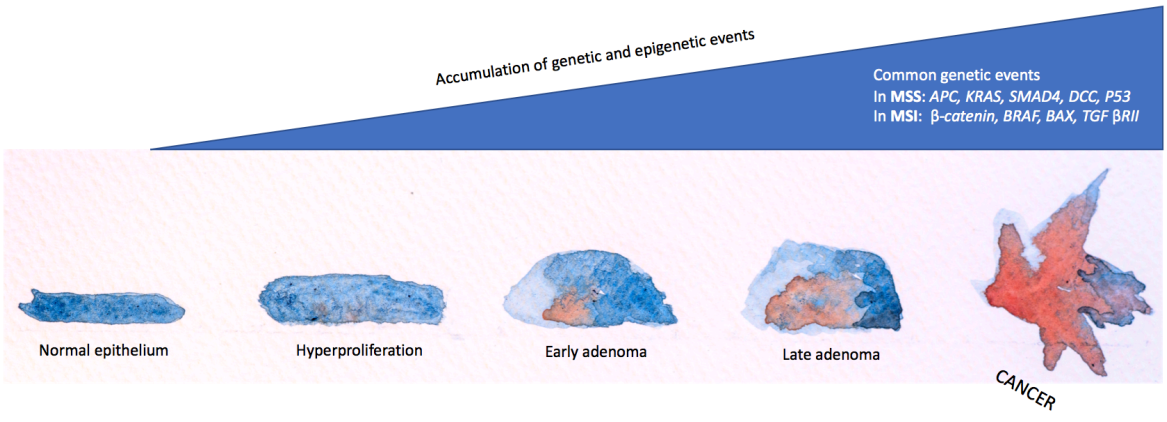

Figure 3. Genetic and epigenetic events drive the progression in to invasive CRC

Consensus Molecular Subtypes

The understanding of colorectal tumour subsets is constantly growing and the heterogeneity of this disease is underlined by expanding data showing further

subclassifications according to molecular characteristics. Studies have also shown how tumours respond differently to treatment depending on their molecular traits[46-48]. The term consensus molecular subtypes (CMS) of CRC is gaining growing recognition, further subdividing colorectal tumours into four distinctive groups defined by different gene-expression traits. In the first cluster; CMS1, the tumours are characterised by hypermethylation, hypermutation and enrichment for *BRAF* v600E. Most MSI tumours fall into this group. They are also more intensely infiltrated by immune cells, particularly $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ T helper 1 (Th1) cells and natural killer (NK) cells. MSS tumours mainly fall into the other three categories with the majority represented by CMS2 with marked WNT and MYC signalling activation. CMS3 describes tumours with pronounced metabolic dysregulation and finally CMS4 with evident transforming growth factor (TGF)-β activation, angiogenesis and stromal invasion[49]. CMS4 tumours are associated with a higher risk of metastases and a worse prognosis. In the CMS4 tumours another type of immune profile is seen compared to the one in CMS1. Here, a more "inflamed" type is described by a pro-metastatic environment with immune-suppressive factors such as TGF-β and other chemokines related to carcinogenic enhancement[50]. While the adaptive immune system is more activated in the CMS1 cluster, the CMS4 immune microenvironment is more polarized towards innate immunity[51].

The innate and the adaptive immune system

The immune response is traditionally categorised in terms of the innate and the adaptive immune response. Here, in these studies, the focus lies within the adaptive immune system but a brief comment on the innate immune system is in order.

Innate immune response

The innate part of the immune system is the most ancient and constitutes the front line of host defence. Its components are complex comprising the barriers to infection through, for example the skin epithelia, but it also includes antimicrobial peptides and cellular elements (e.g. neutrophils, eosinophils, mast cells, macrophages and dendritic cells). The innate immune system furthermore instructs the response of the adaptive immune system. Components of the innate immune system has, much like the adaptive immune system, been shown to play a double-edged role in tumour immunology[52-54].

Adaptive immune response

The adaptive immune response is, compared to the innate, highly specific for foreign antigens and it also has the capacity to remember. This memory provides the host a means to increase the resistance to future "reinfections" with the same

pathogen. The adaptive immune response mainly constitutes the clonal expansion of T- and B lymphocytes holding an impressive variety of receptors with the potential to recognize a theoretically limitless amount of pathogens[55].

The T lymphocytes of the adaptive immune response present a diversity of subgroups. $CD4^+$ T helper cells play a great part in conducting the appropriate response to the threat at hand. After the naïve Th lymphocytes have been activated they polarize and differentiate into either Th1 or Th2 effector cells[56], where Th1 cells mainly produce IFN-γ and IL-2 and are vital in resisting intracellular pathogens. Th2 cells on the other hand, with the production of e.g. IL-4 and IL-5, protect the body from extracellular pathogens[57]. Further subgroups of T helper cells have been described, amongst them the elusive Th17 lymphocytes. In contrast with the mutually exclusive lineages of Th1 and Th2 cells, Th17 cells show a greater plasticity[58].

Th1 cells take part in the activation of the effector CTLs, as well as in the recruiting of other effector cells, in concert with the antigen presenting cells (APCs), e.g. dendritic cells[59, 60]. In studies on antitumour effects of the adaptive immune system, Th1 cells are believed to have an effect both through the increased density of activated CTLs, as by direct actions with the production of IFN-γ and TNF-α [61, 62]. CTLs have the capacity to specifically kill affected cells. They are selected in the thymus to recognize and react to foreign (non-self, viral or mutated) molecules presented on the major histocompatibility complex (MHC) class I of all (nucleated) cells[59]. APCs are responsible for capturing foreign antigen, process it and then present it on MHC class II. By this they co-stimulate the T-cell response[63]. When naïve CD8 T cells are activated they quickly proliferate and evolve into effector cells upon which the killing of target cells ensues[64, 65]. CTLs can kill target cells by two different pathways: The granule exocytosis pathway, using perforin to make a pore in the cell membrane and injecting granzyme B, or the pathway by which FasL is upregulated on the target cell initiating programmed cell death. These pathways are both activated by signals from the T cell receptor (TCR) and they both lead to apoptotic cell death[66].

The universe is constantly striving for balance and so is the immune system. Th1 cells inhibit Th2 proliferation by secreting IFN-γ, and Th2 cells, by the production of the cytokine IL-4, suppress the development and IFN-γ secretion of Th1 cells[67, 68]. While Th1 and Th2 (and Th17 lymphocytes) are essential in their role as protectors against intracellular and extracellular pathogens, they also have the potential of causing autoimmune and inflammatory disease[69, 70].

And this is where the $FOXP3^+$ regulatory T cells (Tregs) step in, inducing tolerance and limiting the effects of Th lymphocytes[71]. By this, they can hinder the adaptive immune response from running amok. Tregs represent a suppressive

$CD4^+$ subpopulation of T cells and their dysfunction, e. g. due to mutations of the *FoxP3* gene, has been found associated to immunopathology, allergy and autoimmune diseases[72]. In tumour immunology they are believed, and in studies shown, to dampen the tumour immune response, thus decreasing its effectiveness[73-75]. The role of Tregs in tumour immunity is however not fully elucidated, especially so in CRC[76, 77]. Tregs show plasticity equal to the before mentioned Th17 lymphocytes and seem to adapt to the environment at hand[78]. Studies have shown Tregs converted to IL-17 producing Th17 cells but also to IFN-γ producing Th1 cells[79]. Since no Tregs produce the pro-inflammatory pro-angiogenic cytokine IL-17 in the thymus, this ability is believed to be generated in the periphery[80]. In tumour immunology, Tregs have been shown to cluster with the Th2 response[81].

The activity of T cells is further balanced through the expression of different immune checkpoint molecules such as CTLA-4 (CTL associated protein-4) and PD-1 (programmed cell death receptor 1) as well as the cytokine TGF-β[82, 83].

The Janus-faced tumour associated inflammation

The impact of the inflammatory infiltrate surrounding the tumour is double-edged, to say the least. In this thesis, the focus lies mainly with the potentially beneficial workings of the adaptive tumour immune response. The tumour associated immune reaction can however also be described in far darker terms. Balkwill et al. phrased it as follows; "If genetic damage is the match that lights the fire" of cancer, some types of inflammation may provide the "fuel that feeds the flames"[84]. There are agents of the immune system acting protumourigenic by excreting for example growth- and angiogenic factors and in helping remodeling the surrounding stroma, thus enabling invasion and metastases[85]. The inflammatory events around a tumour have in several aspects similarities with wound healing and tumours have been described as wounds that do not heal, leading to a persisting potentially harmful chronic inflammation[86].

In cancer research, a link between inflammation and the development of cancer was suspected already in the 19th century. The famous allfather of modern pathology; Rudolf Virshow, noted that solid tumours often arose in chronically inflamed sites[84, 87]. A strong association has been found between different kinds of tumours and chronic inflammation. In the area of the colorectum, patients with inflammatory bowel disease (IBD) have a much higher risk of developing cancer due to the chronic inflammatory state[88, 89]. Furthermore; a regular use of Non-steroid Anti-Inflammatory Drugs (NSAIDs) has been shown to reduce the risk of developing CRC and, in CRC patients; to reduce the overall mortality[90, 91].

Whether the tumour immune infiltrate is to have detrimental or beneficial effects on patient outcome thus depends on several factors. The complexities and the plasticity of different immune cells shun away from any effort to define them in to black and white descriptions. The polarization of tumour associated macrophages (TAMs) being an example of that. TAMs have a tumour preventing (M1) subtype and a tumour promoting (M2) subtype. Macrophages play an important role in both innate and adaptive immune responses and they can change phenotype according to their environment[92]. Whilst M1 macrophages focus on host defence taking both bactericidal and tumouricidal actions, M2 acts in immune regulation, tissue remodelling and tumour progression[93]. M2 macrophages predominate in a chronic inflammatory setting and if a tumour is to be considered a wound that will not heal, the TAMs are suggested to mainly bear M2 characteristics and be associated with a worse prognosis[94]. In CRC however; TAMs are associated with improved patient survival and the impact of M1 macrophages have been found to outshine the effects of their M2 counterparts[95-97].

In general, M1 macrophages are involved in the Th1 response together with the CTLs, and M2 macrophages in the immune modulating Th2 response together with Tregs (*Figure 4*).

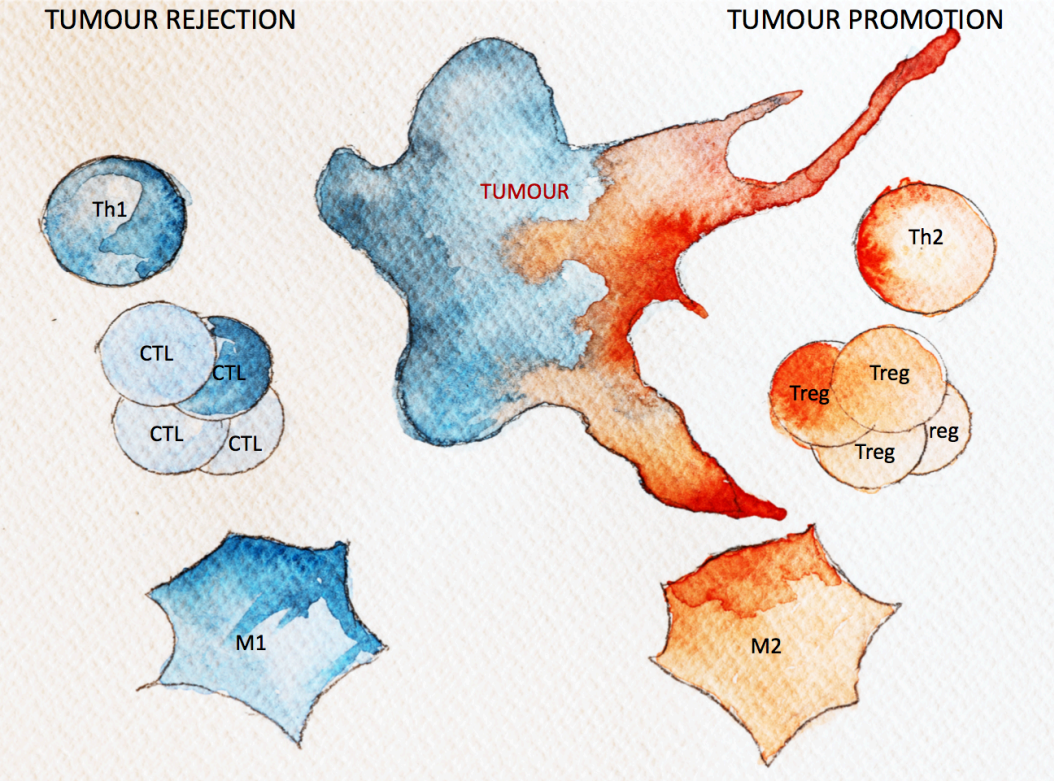

Figure 4. The opposing roles of different immune cell subsets in tumour progression.

Tumour immunology and prognosis

The prognostic importance of immune cell infiltration is, as described above, multifaceted and depends on several factors such as immune cell composition, the microenvironment of the tumour, as well as the profile of expressed cytokines and chemokines[85, 98-100]. On the one hand, inflammation can support tumour development and progression, but on the other, the tumour infiltrating lymphocytes have shown significant suppression of tumour growth[101, 102].

In CRC, the adaptive immune response has proven to be a distinctive variable bearing prognostic information, in addition to the TNM staging system[8, 102]. Studies have shown tumour immune cell infiltration to harbour strong prognostic clues, approximating and perhaps even exceeding the prognostic predictability of TNM [8]. Since the definition of the Immunoscore®, by Galon et al[10], its prognostic potential has been evaluated in a large international multicentre study. The Immunoscore® is founded on a digital-based quantification of total T cell (CD3) and cytotoxic T cell (CD8) count at the tumour invasive margin and in the tumour centre. After some adjustments, this scoring system has been evaluated in internationally assembled cohorts of stage I-III CRC patients. The results showed that the Immunoscore® had the highest relative impact on prognosis compared to other known clinical parameters, including the TNM classification system[103].

One implication of the Immunoscore®, would be the possibility to find stage II colon cancer patients, the majority of which receive surgical resection as sole intervention, with a scarce amount of tumour infiltrating T lymphocytes and thus being in need of more vigorous treatment.

So there is a side to the immune system capable of hampering tumour progression and therefor tumours often hone their immune evading strategies. In the updated version of Hallmarks of cancer by Hanahan and Weinberg; tumour immune evasion was one of the added hallmarks[100]. “Cancer immunoediting” is a term describing how the immune system eliminates and also shapes the malignant tumour[104]. In this immunoediting, three phases have been described namely: Elimination, equilibrium and escape.

The *elimination*, as proposed by Dunn et al. and others[105, 106], is initiated by the recognition of tumour cells by cells of the innate immune system and also to some extent their killing of tumour cells. This is then followed by maturation and migration of APCs. After this, tumour antigen specific T lymphocytes proliferate and cytotoxic mechanisms are activated leading to the elimination of tumour cells.

All would be well if the story ended here, but then follows the phases of immunoediting. This is where the immune response selects for tumour cells with reduced immunogenicity and a resistant tumour variant is formed, an *equilibrium* reached. The next step is *escape,* where the immune response has led to a selection of tumour cell variants able to evade immune detection and elimination, leading to an uncontrolled growth of the tumour. The escape mechanisms have been described as follows:

Resistance to cell death, the induction of immunological ignorance and tolerance; through immunosuppressive inflammatory cells and loss of tumour antigen recognition, either by alterations of tumour or of effector cells[104]. An example of the former mechanism, also seen in CRC, is the down-regulation of the MHC class I[107-109]. MHC class I or human leukocyte antigen I (HLA I), as it is also called in humans, presents molecules and peptides generated by the intracellular ubiquitin-proteasome pathway. Normal peptides presented by MHC class I on the cell do not elicit an immune response, whilst mutated proteins (e.g. from tumour cells) or non-self-proteins from intracellular pathogens, do. They trigger an adaptive immune response through binding to the T-cell receptor of CTLs. Hence, down-regulation of the MHC class I molecules has been shown as a means of tumour immune escape in several kinds of cancers[110-112], and has in previous studies been linked to a worsen prognosis[113]. However; all healthy nucleated cells express MHC class I molecules and not expressing them alarms the innate immune system. The MHC class I molecules also serve as ligands of inhibitory killer cell immunoglobulin like receptors (KIRs) on NK cells and in the absence of a MHC molecule - and thus an inhibitory signal- the cytotoxic mechanism of NK cells is activated[114-117].

Immunotherapy

And here, in a thesis on tumour immunology, one can´t desist from addressing, however briefly, the subject of immunotherapy.

In 2013, Science declared Immunotherapy as the breakthrough of the year[118], and on the first of October this year the Nobel Committee announced two researchers in the field of immunotherapy as the winners of the Nobel prize in physiology/medicine; namely Tasuko Honjo at the Kyoto University and James P Allison at the University of Texas MD Anderson Cancer Center. They have both played a crucial role in the development of efficient immune-checkpoint inhibitors. Therapy targeting CTLA-4 and PD-1 are the most clinically relevant immune-blocking agents to date. By blocking these “T-cell breaks”, the tumour immune response can be significantly fortified, and immune-checkpoint inhibitors have shown effect in several cancer types, first and foremost in melanoma patients[119]. When it comes to CRC, checkpoint blockade has shown

significant effects only in MSI tumours, a finding thought to be explained by the high tumour mutational burden in this variant of CRC[120]. Immunotherapy is however a vast field and various methods for enhancing the tumour immune response are continuously investigated. Methods reaching from adoptive T-cell transfer; where by *ex vivo* expansion, an army of tumour-specific CTLs is raised, to the engineering of genetically modified T cells[121].

Aims of this thesis

This thesis is in the pursuit of an expanded understanding on how tumour infiltrating immune cells may affect CRC patient outcome. Herein is furthermore investigated, which mechanisms the colorectal tumour might deploy in order to evade the tumour immune response.

The thesis consists of three papers with the following specific aims:

Paper I

- To investigate the relation of patient prognosis to the infiltration of CTLs and Tregs in CRC tumours. The potential prognostic influence related not only according to quantity, but also to the relationship between the two subsets and their subsite within the tumour.

- To evaluate if CTL and Treg infiltration can be linked to certain molecular subtypes of CRC

Paper II

- To investigate the prognostic impact of tumour infiltrating Th1 lymphocytes at different intratumoural subsites in CRC patients.

- To evaluate whether Th1 infiltration differs in molecular subgroups of CRC.

Paper III

- To investigate possible molecular mechanisms involved in tumour immune escape in CRC.

Materials and Methods

Study population

Patients included in study I, II and III were from the Colorectal Cancer in Umeå Study (CRUMS)[122]. This cohort comprises consecutively collected tumour tissue from CRC patients who underwent surgical tumour resection between 1995 and 2003 at the Umeå University Hospital in Sweden.

Formalin–fixed paraffin-embedded (FFPE) tissue was sampled from all patients and pathological variables were characterised by one pathologist. MSI screening status, CIMP status, and *BRAF* and *KRAS* mutational status has previously been analysed in this cohort[43, 122]. MSI screening status was assessed by immunohistochemical analysis with a positive MSI screening status describing tissue samples with tumour cells lacking nuclear staining for one or more of the proteins MLH1, MSH2, MSH6 and PMS2. CIMP status was determined by evaluation of hypermethylation of an eight-gene panel (*CDKN2A, MLH1, CACNA1G, NEUROG1, RUNX3, SOCS1, IGF2* and *CRABP1*) by the Methyl Light method (quantitative real-time PCR) with previously described primer and probe sequences; CIMP-negative tumours, 0 genes; CIMP-low tumours, 1-5 genes; and CIMP high tumours, 6-8 genes. $BRAF^{V600E}$ mutation was determined by the Taqman allelic discrimination assay, described by Benlloch et al.[123]. *KRAS* mutational status was analysed performing sequencing of codon 12 and 13 as has been previously described[43].

By reviewing the patient records and the Swedish population registry during autumn 2012, updated clinical data, including survival data was obtained (by a surgeon). The median follow-up time was 113 months. Exclusion criteria included unavailable or insufficient tumour sample or lack of clinical information. Some patients were excluded due to death by perioperative complications (death within 30 days of and due to operation).

Immunohistochemical evaluations

Immunohistochemistry is widely used throughout the world, in both clinical and research settings. It is a technique based on the use of antibodies, which can detect a wide range of antigens/proteins. The protein of interest can be visualized through conjugation of the antibody to an enzyme, which enables a colour producing reaction.

In these studies, we used immunohistochemistry to evaluate the quantity and distribution of different subsets of T lymphocytes (paper I and II) as well as of the expression of the protein TAP1 (paper III).

In the preparation process, 4 µm FFPE tissue sections were dried, de-waxed and then rehydrated. Staining was performed with a Ventana Benchmark Ultra staining machine. Visualization was achieved using the iVIEW DAB Detection kit and tissue architecture was unveiled by a counterstain with haematoxylin.

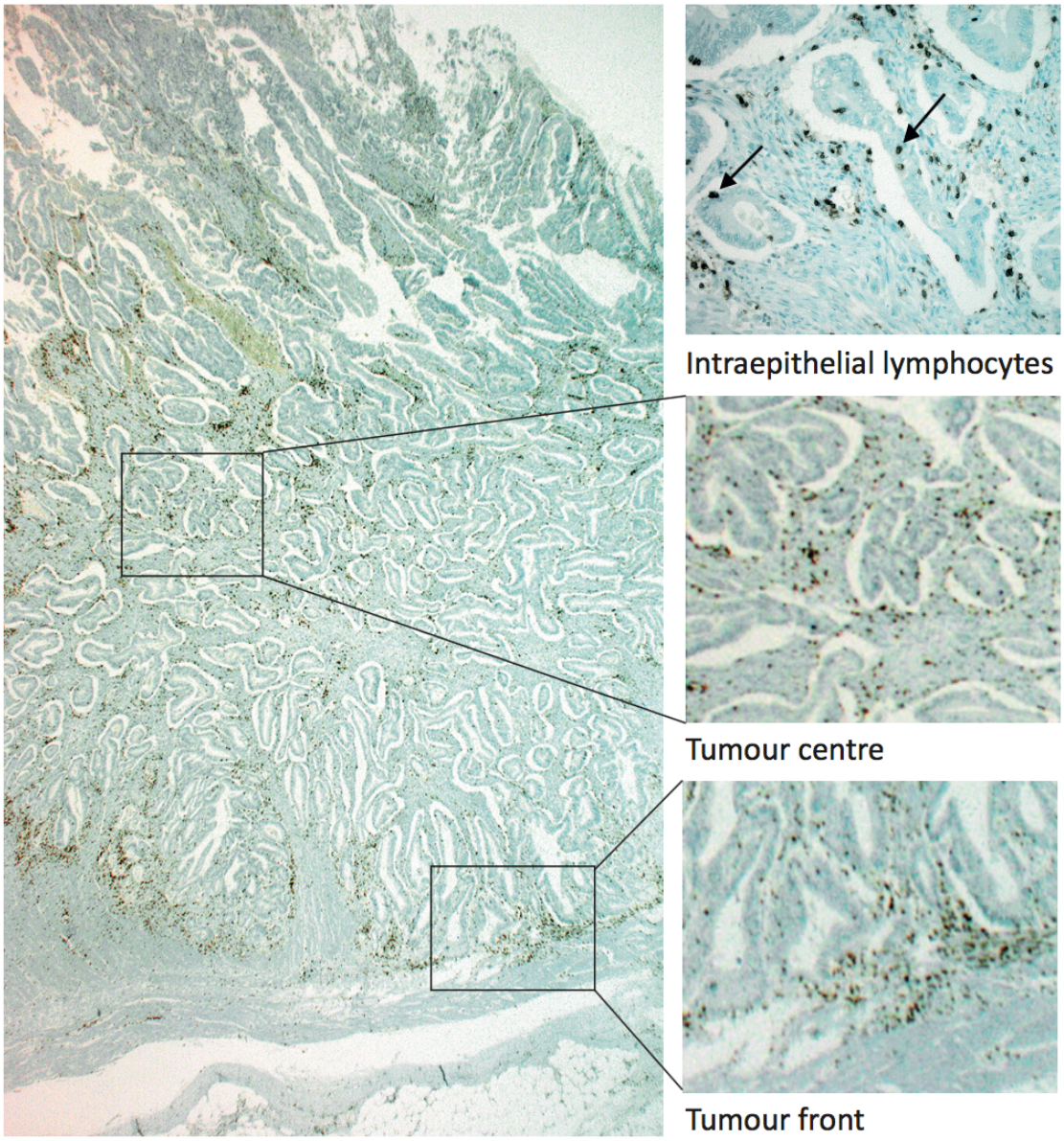

Figure 5. Immunohistochemical staining illustrating lymphocytic reaction at different intratumoural subsites.

The immunohistochemical staining for different subsets of T lymphocytes was evaluated with light microscopy as the most representative area at different intratumoural subsites: the invasive tumour front, the centre of the tumour and within the tumour epithelium (*Figure 5*). TAP1 expression in tumour cells was evaluated at the tumour invasive front and the tumour centre. In paper I and II, the specimens were evaluated twice by the same observer under the supervision

of an experienced pathologist, and discordant cases were reviewed a third time, followed by a conclusive judgement. A subset of the specimens (n=50) in paper I were also examined by a an experienced gastro-pathologist and an inter-observer agreement was assessed (kappa-values 0.66-0.87). In paper III, all specimens were evaluated by two observers, of whom one was an experienced gastro-pathologist (kappa-values 0.538-0.574). In cases of discrepant scoring, a third estimation was made by both observers followed by a conclusive judgement.

In paper I and II, T lymphocyte subsets were semi-quantitatively scored as 1-4, ranging from no/sporadic to highly abundant, according to Dahlin et al.[122]. A total score was also obtained for each case, adding together the scores from each subsite. The total score was then divided into three groups, according to S Ogino and A Dahlin[122, 124]. In paper I, CD8 was used as a marker for CTLs and FOXP3 for Tregs.

T-bet was the marker used for Th1-lymphocytes evaluated in paper II. In using a semi-quantitative instead of a computed quantitative method we have the disadvantage of observer variability. On the other hand, the semi-quantitative evaluation of whole slides further enables the observer to identify different tumour compartments, to exclude necrotic areas and to easier assess the often heterogenetic dispersion of immune cells.

In paper III, TAP1 expression was evaluated according to the Immunoreactive score (IRS)[125]. IRS is a semi-quantitative assessment of expression determined by multiplying the staining intensity in 4 gradations (from 0=no to 3=strong intensity) with the percentage of positive cells in 5 gradations (from 0=0% to 4≥80%). The resulting IRS score (1-12) was further divided into groups of low (IRS≤6) or high (IRS>6) according to Kasajima et al.[126]. Expression of MHC class I and TAP2 was also evaluated according to the IRS in a subset of 22 CRUMS patients, selected to have either low or high expression of TAP1. TAP1-expression scores were further studied in relation to the infiltration rate of different subsets of immune cells. The latter having been analysed in previous studies, in the same cohort[52, 95, 122, 127, 128].

Cell culture

Here we used the colon cancer cell line Caco2 (ATCC) which, with its derivatives, were grown in Dulbecco´s modified Eagle´s medium with glutaMAX supplemented with 10% fetal bovine serum (Gibco) and maintained at 37°C in an atmosphere of 5% CO_2. The stable transfectants expressing mutant *BRAF* (Caco2-*BRAF*^{V600E}) or mutant *KRAS* (Caco2-*KRAS*^{G12V}) have earlier been described by Lundberg et al[129].

Gene expression analysis

In paper II, cytokine and chemokine expression was analysed on both tumour tissue and a CRC cell line, by semi-quantitative real-time PCR.

In paper III, gene expression analysis was performed on isolated tumour tissue from 20 patients from the CRUMS cohort. The included patients were selected to be stage II, MSS, CIMP negative and *BRAF* wild type. We further used data on infiltration of the general T lymphocyte marker CD3, which had previously been assessed in this cohort[122]. We chose 10 tumours with low CD3 infiltration and 10 tumours with high CD3 infiltration. FFPE tumour specimens were cut into 4 µm sections and stained with hematoxylin. By using Laser Microdissection and Pressure catapulting (LMPC), tumour tissue was selectively cut out using the PALM MicroBeam Laser Capture Microdissection System (Zeiss). Captured tissues were collected and RNA was extracted using The High Pure RNA Paraffin Kit (Roche). Gene expression was analysed by the Whole-Genome Gene Expression DASL HT Assay (Illumina). Labeled cRNA was hybridized to the human HT-12 v4 expression BeadChip. Microarrays were scanned using the Illumina HiScan® System and the data analysis performed using the GenomeStudio software. The differential expression was analysed using cubic spline normalization and the Illumina custom error model without FDR (False discovery rate) correction.

DNA Methylation Analyses

DNA methylation is an epigenetic mechanism by which a methyl (CH_3) group is added to DNA leading to an alteration (mostly loss) of the function of and/or the expression of the genes. Often the methyl group is bound to a CpG site (C=cytosine, p=preceding, G=guanine) in the DNA nucleotide sequence. CpG sites are scattered throughout the genome, or clustered in CpG islands in the promoters of important genes. DNA methylation plays an important role for a normal cell to maintain genomic stability and tissue-specific gene expression. However, epigenetic alterations such as promoter DNA hypermethylation has been found to be a potential key event in cancer development, leading to inappropriate silencing of gene expression[130, 131].

In paper III, methylation specific pyrosequencing assays were performed on DNA isolated from FFPE tumour tissue of 22 CRUMS patients which had either low (IRS≤6) or high (IRS>6) TAP1 expression. DNA was extracted employing the Illustra Nucleon Genomic Extraction Kit (GE Healthcare) and it was bisulfite treated with the Epitec Fast Bisulfite Kit (Qiagen). The methylation specific PCRs were performed using the PyroMark® PCR Kit (Qiagen). The PCR was run in an ABI Veriti thermal cycler. DNA methylation in the *TAP1* region was determined

by pyrosequencing using the PyroMark Q24 Advanced System (Qiagen). Data was analysed using the PyroMark Q24.1.0.10 Sofware (Qiagen) and methylation percentage (mC/mC+C) of each CpG site was calculated.

External reference data

Reference methylation and expression data in paper III was obtained from The Cancer Genome Atlas (TCGA) Research Network[132]. It was comprised of 291 primary colon adenocarcinoma samples for which both RNA-sequencing expression data and array methylation data were available.

Statistics

In order to determine statistical significances, $\chi 2$ tests were used for cross–tabulations and the exact linear-by-linear association test was used to evaluate linear relationships. To test correlations between categorical variables the Spearman´s rank correlation test was used and we used the Mann-Whitney U-test for differences in continuous variables between groups.

Cancer-specific survival was assessed using the Kaplan-Meier survival analysis and comparisons between outcomes in different groups were performed with the log-rank test. Multivariable survival analyses were performed using Cox proportional hazard models. The statistical analyses were performed using PASW statistics (SPSS Inc.). $P<0.05$ was considered statistically significant.

Ethical approval

An informed consent was retrieved from the patients and the handling of patient data as well as tissue samples was approved by the research ethical committee at Umeå University Hospital (Regional Ethical Review Board in Umeå, Sweden) and in accordance with the Helsinki declaration.

Results and discussion

Almost a century has passed since MacCarty et al. showed a correlation between immune cell infiltration and prognosis in cancer patients[133]. The association between immune system activity and prognosis in CRC was described by Svennevig et al. four decades ago[101], and in the first decade of this millennia studies revealed tumour infiltrating lymphocytes to significantly correlate to prognosis in CRC patients[8, 9, 98].

Here, we examine the potential prognostic impact of different subsets of tumour infiltrating T lymphocytes, not only according to functional orientation and quantity, but also to their intratumoural subsite. In addition, we correlate the infiltration of these T lymphocyte subsets to molecular subgroups of CRC. We further investigate potential mechanisms of immune evasion in CRC.

Paper I

The intratumoural subsite and relation of $CD8^+$ and $FOXP3^+$ T lymphocytes in CRC provide important prognostic clues

In this study, we analyse the tumour infiltration rate of CTLs ($CD8^+$) and their alleged counterpart; Tregs ($FOXP3^+$). Combining immunohistochemical analyses of density, different intratumoural subsites, relation between the two T cell subsets, and tumour molecular characteristics, we try to decipher how these variables might affect prognosis in CRC patients.

A total of 426 CRC patients from the CRUMS cohort were included in this study. We found the frequencies of infiltrating $CD8^+$ and $FOXP3^+$ cells to be noticeably and positively correlated. The amount of $FOXP3^+$ cells was generally lower than that of $CD8^+$ cells. Intraepithelial $FOXP3^+$ cells were only sporadically seen. Colon carcinomas were separated from rectal cancers due to the reducing effect radiation therapy exerts on T cell infiltration in many patients of the latter group.

In univariate analyses, a high amount of infiltrating $CD8^+$ cells was found significantly associated with improved survival in colon cancer patients. In multivariable analysis, adjusting for stage, age, sex and localisation (proximal *vs* distal), the prognostic significance for CD8 total score, tumour invasive front and centre was lost. The prognostic effect of intraepithelial infiltration of CD8 cells did however remain significant

When comparing infiltration of FOXP3+ cells in the tumour invasive front to that in the centre, no prognostic discrepancy was found between intratumoural subsites. A high infiltration rate, irrespective of subsite, showed a significant association with a better prognosis. The prognostic effect of FOXP3+ cell infiltration stayed significant in multivariable analysis, for total score, at the tumour invasive front and in the centre. When comparing different relations between CD8+ and FOXP3+ cells, to prognosis, the results were as follows: The association of a high amount of intraepithelial CD8+ cells with a good overall survival, was unaffected by FOXP3+ density at the tumour front. In colon cancer patients with low infiltration of intraepithelial CD8+ cells, a better outcome was however found in patients with a high infiltration of FOXP3+ cells compared with those having a low infiltration rate of both subsets.

In molecular subgroups of CRC, defined by MSI screening and CIMP status, the evaluation of lymphocyte infiltration showed significances for CD8+ cell infiltration at different subsites in association to MSI screening status. MSI tumours more often being highly infiltrated by intraepithelial CD8+ lymphocytes. FOXP3 expression as a total score was significantly associated with MSI screening status but not when related to different subsites. Neither CD8+ nor FOXP3+ infiltration was found correlated to CIMP status.

Infiltration of intraepithelial CD8+ cells was found to be a significant prognostic factor in MSS but not in MSI cases. FOXP3+ infiltration at the tumour front on the other hand, was significant for prognosis in both MSI and MSS, where the patients with tumours poorly infiltrated by FOXP3+ cells presented the worst outcome. In survival analyses of colon cancer subgroups according to CIMP status, high amounts of infiltrating CD8+ and FOXP3+ cells were found to be associated with a better prognosis. This was especially the case in CIMP-negative and CIMP-low groups.

When adjusting for MSI and CIMP status in a multivariable model, the positive prognostic impact stayed significant for a high infiltration rate of FOXP3+ cells at the tumour invasive front and of intraepithelial CD8+ cells.

Interpretation paper I

The results of this study further underline the prognostic influence of tumour infiltrating immune cells. In addition, we could see that the intratumoural subsite of and the relation between CTLs and Tregs made a difference. We saw that a high infiltration rate of one subset strongly associates with a high infiltration of the other subset. Findings in line with other studies[134, 135]. In survival analysis, a high amount of intraepithelial CTLs was a beneficiary prognostic factor regardless of Treg infiltration. The prognostic influence of a low infiltration rate of intraepithelial CTLs on the other hand, was dependent on Treg infiltration, patient outcome improving with a higher amount of the latter.

When it comes to the intratumoural subsite, the prognostic effect of CTLs only stayed significant for the intraepithelial subsite, in multivariable analysis. This could perhaps be explained by their activity in direct contact with tumour cells. Through stimulation of the T cell receptor (TCR), their effector mechanism is activated and they can kill the tumour cell either by granule exocytosis or through the death-receptor pathway[136]. CTLs located in immediate adjacency to the tumour cells are thus more likely tumour specific and more likely activated. This theory on the prognostic importance of CTL sublocalization is endorsed by other, earlier studies[8, 137].

The immune modulating Tregs have been associated with hampering effects on the anti-tumour immune response in some cancers[138-141], and in the development of new immunotherapies the inhibition or depletion of Tregs in combination with effector T cell activation have been evaluated[142, 143].

The role of Tregs in tumour immunity is however not so easily decided. In this study we show a high infiltration of $FOXP3^+$ cells to significantly associate with an improved outcome in CRC patients. Furthermore, the prognosis worsens with reduced amounts of infiltrating Tregs. These findings reflect the results from studies by Salama et al.[144], Ladoire et al.[145] and Frey et al.[134]. So is there a difference between Tregs of the colon and Tregs in other tissues? Are they even regulatory or may the tumour infiltrating $FOXP3^+$ cells actually represent conventional T cells transiently expressing FOXP3 upon TCR activation? Martin et al. proved this possible in 2010[146]. Saito et al. showed functional and phenotypical heterogeneity among $FOXP3^+$ T cells with non-suppressive subpopulations[147]. Subpopulations of $FOXP3^+$ cells have also been shown to secrete pro-inflammatory cytokines IL-2 and IFN-γ[148]. There are however other studies reaching the conclusion that CRC-derived $FOXP3^+$ cells really do represent Tregs with suppressive functions[149, 150].

In general there have been uncertainties concerning what marker best identifies Tregs. Markers associated to Tregs such as CD25, and CTLA-4 are upregulated

on both CD4+ and CD8+ cells and thus not specific enough[78]. FOXP3 has, as mentioned before, been found transiently expressed in effector T cells, although at lower levels and unstably so[151-153]. Other studies have however found strong correlations between FOXP3 expression and immune suppressive Treg functions, also describing FOXP3 to be predominantly restricted to Tregs both in the thymus and the periphery, and that FOXP3 seems to be required for the development of Tregs[154-156].

Staying with the notion that the FOXP3 expressing lymphocytes in this study really represent regulatory T cells, could the explanation then be found in the organ specific traits of the colon? Being a barrier organ, it is in constant contact with foreign antigens. Might Tregs to some extent block tumour promoting inflammation in the intestinal environment, thus exerting their protective effect[145]? The beneficial effect of tumour infiltrating Tregs has been found in other parts of the gastrointestinal tract and the potentially protective workings of Tregs due to dampening of tumour promoting inflammation, was suggested by Haas et al. when they and others found high amounts of Tregs to associate with better outcomes, in gastric cancer patients[157, 158]. The intestines are rich in bacteria which can activate inflammatory reactions and the expression of tumour promoting cytokines[159]. High amounts of Tregs, suppressing microbe-induced inflammation, could thereby imply a protection to both the development and the progression of a tumour in the in the colorectal epithelium. A recent study on urinary bladder cancer did for example find regulatory T cells to take part in the suppression of the pro-invasive factor matrix metalloproteinase 2 (MMP2)[160]. Another aspect is the possibility that a high infiltration rate of $FOXP3^+$ cells is an indication of an active potent immune response. A synergistic effect might also be expected by the expression by both subsets, CTLs as well as Tregs, of ligands enabling tumour infiltration and T-cell extravasation [161, 162].

Immune cell infiltration was also evaluated in CRC subgroups defined by MSI screening and CIMP status. Our results indicate that *the prognostic* importance of lymphocyte infiltration is probably independent of these different molecular characteristics, even though MSI tumours are generally more intensely infiltrated. These findings are in line with other studies showing the immune response to be a stronger predictor of patient outcome than MSI-status[103, 163, 164]

In summary, high amounts of both tumour infiltrating CTLs as well as Tregs are associated with a better outcome in CRC. Additional prognostic information can be reached by analysing the intratumoural subsite of, and the relation between, these two T cell subsets.

Paper II

The infiltration and prognostic importance of Th1 lymphocytes vary in molecular subgroups of CRC

Here we investigate the prognostic impact, in CRC patients, of tumour infiltrating Th1 lymphocytes; a T cell subset bearing important functions in supporting the activity of CTLs. T cell densities were assessed by the immunohistochemical evaluation of the Th1 marker T-bet. Prognostic evaluations were also performed in molecular subgroups of CRC defined by MSI status, CIMP status and *BRAF* and *KRAS* mutational status. Altogether, 418 CRC patients from the CRUMS cohort were included. Tbet$^+$ expression was furthermore compared with the infiltration of previously analysed pan T lymphocytes (CD3$^+$)[122] CTLs (CD8$^+$), Tregs (FOXP3$^+$)[127] as well as the macrophage subsets M1 and M2 (NOS2$^+$ and CD163$^+$, respectively)[95]. Infiltration of T-bet$^+$ Th1 lymphocytes was found to be strongly and positively correlated to the expression of these markers.

The infiltration rate of T-bet$^+$ lymphocytes was correlated to clinicopathological variables using the total score, since no extra information was acquired by relating these parameters to T-bet$^+$ lymphocyte infiltration at the different intratumoural subsites. Significant results were seen for an increase of T-bet$^+$ cells in the right colon, and a decrease in preoperatively irradiated rectal tumours. We also found a strong inverse association between T-bet expression and tumour stage as well as lymphovascular invasion.

The infiltration of T-bet$^+$ cells was further investigated in relation to molecular parameters in CRC. A high infiltration was significantly associated with tumours classified as MSI, CIMP high or *BRAF* mutated. *KRAS* mutant tumours, in contrast, were less infiltrated by T-bet$^+$ cells. Another finding was that highly infiltrated MSI tumours often were *BRAF* mutated.

In survival analysis, we found increased infiltration of T-bet$^+$ cells to be significantly associated with an improved prognosis in CRC patients. The prognostic importance of T-bet$^+$ cell infiltration stayed significant in multivariable analysis adjusting for stage, age, sex, localisation and preoperative radiation. We further found that patients with *BRAF* mutated tumours with a low infiltration rate of T-bet$^+$ cells had an especially poor prognosis. When inserting MSI screening status, CIMP status and *BRAF* and *KRAS* mutation status into the multivariable model, the prognostic significance of T-bet infiltration was however found to be independent of these molecular traits.

The discrepancy in T-bet infiltration between *KRAS* mutated and *BRAF* mutated tumours lead us to perform a cytokine- and chemokine *in vitro* expression analysis on a CRC cell line. We sought to investigate whether these two distinct mutational statuses somehow differently affect the expression of T lymphocyte recruiting, polarizing and regulating cytokines and chemokines. The *BRAF* mutated CRC cell line showed a significantly higher level of the Th1 attracting chemokine CXCL10, compared to both the wild type as well as the *KRAS* mutated cell line. The *BRAF* mutated cells were also found, in comparison, to express reduced levels of the chemokine CCL22 and the cytokine TGF-β. CCL2 and TGF-β being associated with stimulation of the Th2/Treg axis.

In order to confirm these in *vitro* findings, a cytokine- and chemokine expression analysis was performed also on tumour tissue from 12 CRC patients. We here analysed the expression of CXCL10, CCL22 and TGF-β by semi-quantitative real-time PCR in tumour specimens carrying either oncogenic mutations in *BRAF* or *KRAS*. The level of expressed CXCL10 was found to be significantly higher in *BRAF* mutated tumours compared to those with *KRAS* mutation. A tendency towards lower CCL22 and TGF-β expression, although without significant values, was noted in *BRAF* mutated tumours.

Interpretation paper II

Th1 lymphocytes and their tumour infiltration have previously been shown to be an important prognostic factor in CRC[81]. Here we found patient prognosis to improve with an increased Th1 cell infiltration and we also found that the intratumoural subsite was of lesser importance. This diverges from what we saw in paper I, studying CTL and Treg infiltration, where only the intraepithelial CTLs were found to have significant impact on prognosis. This might be explained by the function of Th1 cells, as understood thus far. While CTLs perform their effect in immediate contact with tumour cells, Th1 lymphocytes excrete cytokines stimulating the recruitment and activation of CTLs[165], hence being able to exert their influence from a distance. By this, one could surmise Th1 to be a suitable marker to score in CRC tumours, since its prognostic influence remains the same regardless of intratumoural subsite. We also found that Th1 infiltration and patient outcome differed according to molecular subgroups. An increased infiltration of Th1 lymphocytes was seen in MSI, CIMP high and *BRAF* mutated CRC tumours and it was also in these subgroups that we found the most pronounced prognostic effect. Infiltration of the Th1 lymphocyte subset has been shown elevated in MSI tumours in previous studies by Boissère-Michot et al.[166]. The increased T lymphocyte infiltration often seen in MSI tumours have been linked to the generally improved survival in this group of patients[138, 167].

Whilst *BRAF* mutated tumours were more infiltrated than their wild type counterparts, the opposite was seen for *KRAS* mutated tumours. This could partly be explained by our results on cytokine- and chemokine expression in *BRAF* and *KRAS* mutated CRC cell lines and tumour specimens. Here we found *BRAF* mutated, in comparison to *KRAS* mutated, tumour cells and tissue having higher expression levels of the Th1-attracting chemokine CXCL10 and reduced amounts of CCL2 and TGFB1 which stimulate Th2/Treg recruitment and polarization. Even though an interesting finding, the analyses were performed only on a small subset of the tumours and need to be verified using larger patient cohorts. Stratifying for MSI screening status we found that MSI tumours highly infiltrated by Th1 lymphocytes often were *BRAF* mutated thus suggesting this mutation to possibly contribute to the prognostic importance of MSI in CRC. Previous studies have shown the close association between MSI and *BRAF* mutation[38, 39, 45], and furthermore, this study shows a particularly poor prognosis in patients with CIMP-high or *BRAF* mutated tumours sparsely infiltrated by Th1 lymphocytes.

Paper III

TAP1 down-regulation elicits immune escape and poor prognosis in CRC

Here we investigate possible mechanisms in CRC, of tumour immune evasion. The study was initiated by selecting tissue from 20 patients of the CRUMS cohort. Ten of which had previously been assessed as highly infiltrated by $CD3^+$ T cells and the other 10 with a low amount of infiltrating $CD3^+$ T cells. By LMPC, tumour tissue could selectively be cut out for a whole genome expression array analysis. The tissue yield was low, as was the number of detected genes, likely due to low RNA input levels as well as poor quality, perhaps explained by old FFPE tissue. From this initial analysis, we did however find that (out) of the differentially expressed genes, many were involved in antigen presentation and immune modulation. We chose to proceed with *TAP1*, a component of the MHC class I antigen-processing machinery (APM), which was expressed to a lower extent in the CD3 low tumours.

Our next step was to evaluate 436 patients from the CRUMS cohort for TAP1 expression, in the tumour centre and tumour invasive front, by immunohistochemical analysis. Using the before mentioned IRS score we could divide tumours in to either TAP1 high or TAP1 low. When correlating TAP1 expression to clinicopathological characteristics we found an inverse association to tumour stage, with TAP1 expression decreasing with higher tumour stages. A significant correlation was also seen between a low TAP1 expression in the tumour front and perineural invasion. No significant correlations were found

when relating TAP1 expression to molecular subtypes such as MSI and CIMP screening status, or *BRAF* and *KRAS* mutation.

When proceeding to analyse immune cell infiltration and TAP1 expression in all the included cohort tumours we found a significant correlation to most previously assessed immune cell subsets. A low TAP1 expression was significantly correlated to a lower infiltration of both CTLs ($CD8^+$), Tregs ($FOXP3^+$), Th1 ($T\text{-}bet^+$), M1 macrophages ($NOS2^+$) and M2 macrophages ($CD163^+$). No correlation was however seen to neutrophil ($CD66b^+$)[52] infiltration.

TAP1 expression was further evaluated in relation to the expression of other components of the APM. This was performed by immunohistochemical staining of TAP2 and MHC class I (HLA-A, -B, and –C) in tumour tissue from 22 of the CRUMS patients, scored as either TAP1 low (n=12) or high (n=10). TAP2 and MHC I expression was assessed according to IRS and classified as either high or low. A significant positive correlation between the three components was found.

In univariate survival analysis, a high expression of TAP1 was found to significantly associate with an improved prognosis. A significance that stayed through multivariable analysis adjusting for sex, localisation and tumour grade. It was however lost in multivariable analysis stratified by stage. Still, TAP1 expression in the tumour front was found significantly associated to prognosis in patients with CRC tumours of stage I and II.

From here, we sought to investigate possible mechanisms of TAP1 down-regulation. A known epigenetic alteration controlling gene expression is methylation[168, 169]. Considering this we turned to the TCGA colon adenocarcinoma (COAD) dataset, which include methylation status for several (88) CpG sites associated with the *TAP1* gene. In the analysis of this data, we reached a decision to focus on CpG sites closest to the *TAP1* promoter, since they held the strongest correlation to TAP1 expression. Using methylation-specific pyrosequencing, we found that for the tumours with a lessened expression of TAP1 (n=12) all the selected CpG sites displayed higher levels of methylation compared to the TAP1 high tumours (n=10).

Interpretation paper III

In this paper, we show how TAP1, a component of the APM, is more often down-regulated in tumours with low infiltration of lymphocytes, as well as macrophages. These findings suggest that TAP1 downregulation and thus, supposedly, alterations of the surface expression of the MHC class I molecules, might be a tumour strategy for immune evasion. Furthermore, in this study we find methylation to be a possible regulating mechanism of TAP1 expression.

We cannot with complete certainty explain how the down-regulation of TAP1 effects immune cell infiltration but with the help of literature concerning the function of this protein we may at least speculate.

A schematic illustration of antigen processing and presentation by MHC class I can be found in *figure 6*. TAP1 forms an antigen processing heterodimer with TAP2. Through this dimer, peptides from degraded cytoplasmic proteins are translocated over the endoplasmic reticulum (ER) membrane. The TAP-associated peptide is then loaded into the MHC class I complex, which is subsequently transported to the surface. If TAP1 is lacking one could assume the entire antigen presenting machinery to be affected. When analysing MHC class I and TAP2 expression in a subset of tumours from the cohort, we saw that a down-regulated TAP1 expression correlated with decreased levels of both TAP2 and MHC class I. This implies that immune escape in tumours with down-regulated TAP1 expression, partly may lie in a loss of other components of the APM. There were however cases with intact MHC class I expression even though TAP1 was down-regulated. A finding inviting other potential scenarios, such as TAP1 down-regulation leading to the presentation of empty MHC class I molecules. A means as of which the tumour, hypothetically, might avoid both CTL and NK cell recognition. This theory has been proposed also by Leone et al.[114].

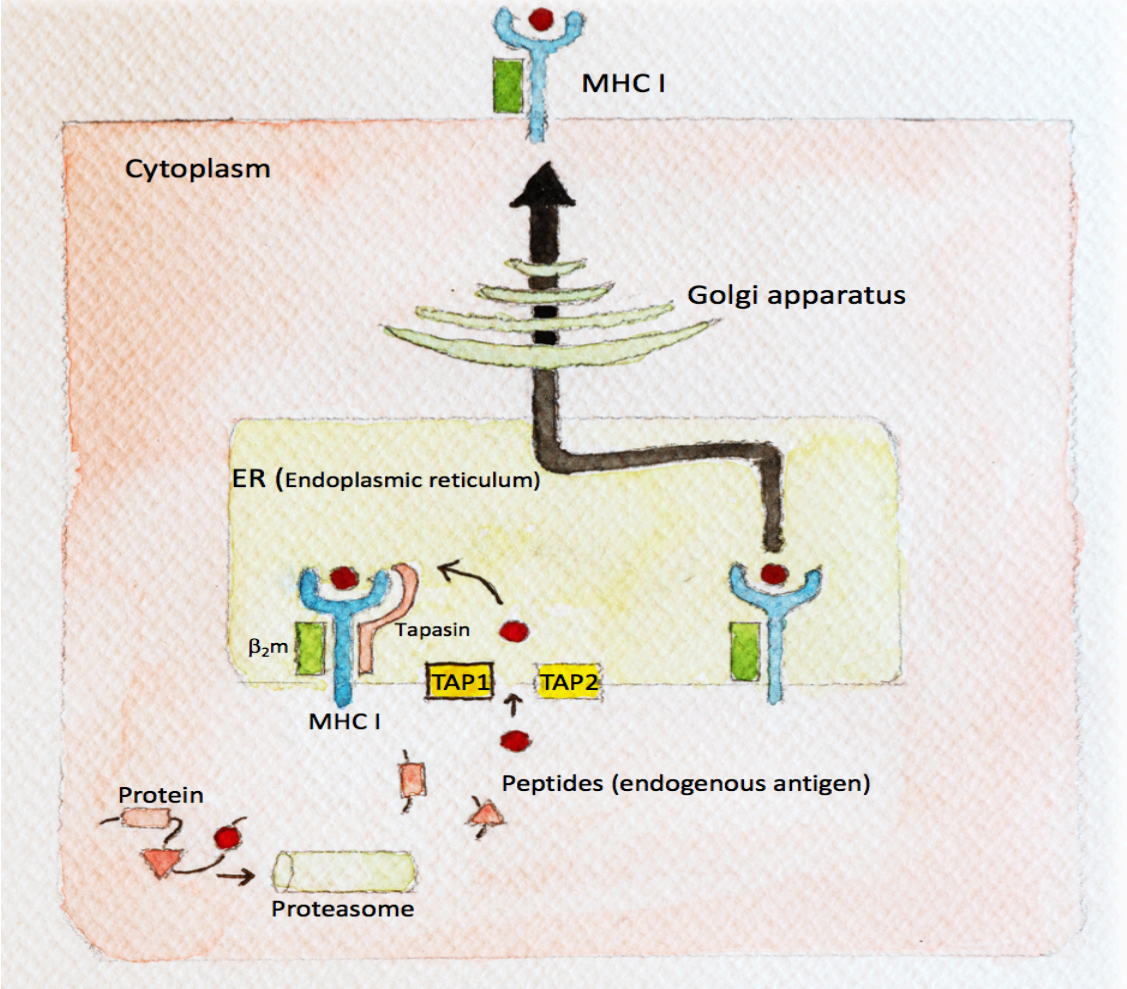

Figure 6. The MHC class I antigen presenting process, illustrating the location of TAP1.

TAP1 down-regulation in association with a decreased infiltration by immune cells has previously been shown by Kasajima et al.[126]. In their study, they also demonstrate a strong correlation between TAP1 expression and the expression of MHC class I, as well as TAP2. Compared to our study, they did however not see a significant correlation to prognosis. The down-regulation of TAP1 has been shown a poor prognostic factor in other cancers[170-172]. The results for previous studies on MHC class I expression and prognosis in CRC has thus far been inconclusive[173-176]. In this study, we find TAP1 expression to be significantly correlated to prognosis in patients with tumours of lower stages (stage I and II). A finding that implies TAP1 down-regulation to be a potential marker for Stage I and II CRC patients in need of extended treatment aside from surgical resection.

Our study also investigates possible mechanisms of TAP1 down-regulation. The silencing of TAP1 through epigenetic regulation due to hypermethylation has been shown in other cancer types[177, 178]. In line with this, we found methylation of CpG sites close to the *TAP1* promoter, to be a putative explanation. These findings are however based on analyses of a small fraction of patients and need verification in a larger study set.

In conclusion, the results from this study suggest that hypermethylation and hence down-regulation of TAP1 expression, is a tumour immune-evasion mechanism. TAP1 down-regulation also being correlated to a poorer prognosis in patients with stage I-II CRC tumours.

Conclusions

Paper I

Investigating seemingly opposing subsets of tumour infiltrating T lymphocytes, we found both regulatory T cells and cytotoxic T cells to correlate with prognosis in patients with CRC. The higher the infiltration of $CD8^+$ and $FOXP3^+$ T cells in CRC tumours, the better the patient outcome. Tregs thus, and somewhat surprisingly, not being the culprit it has been believed and shown to be in other cancer types. We could also see that the relation between these two lymphocyte subsets as well as their intratumoural subsite carry important prognostic information.

Paper II

Focusing on the tumour infiltration by Th1 lymphocytes (T-bet) we show how they affect prognosis in CRC patients. A high amount of Th1 cells improving patient outcome, regardless of intratumoural subsite. We furthermore found a discrepancy in Th1 lymphocyte infiltration between *KRAS* and *BRAF* mutated tumours. *BRAF* mutated tumours to a greater extent showing a high infiltration of Th1 lymphocytes compared to *BRAF* wild type tumours whilst the opposite was seen for *KRAS* mutated tumours. In cytokine- and chemokine expression analyses in both CRC cell-lines and tumour tissue we found *BRAF* mutated CRC tumours to express higher amounts of the Th1-attracting chemokine CCL10, and lower levels of the Th2 polarizing CCL22 and TGF-β, compared to *KRAS* mutated CRCs. This could possibly and partly explain the differences in Th1 infiltration rate between these mutational subgroups. An especially poor prognosis was seen in patients with *BRAF* mutated tumours with a scarce amount of infiltrating Th1 lymphocytes.

T-bet, as a marker for Th1 lymphocytes, might be a valuable marker in the clinical setting, in identifying patients with a particularly poor prognosis, who are in need of more vigorous treatment.

Paper III

Here we found that down-regulation of TAP1, a component of the antigen presenting machinery, may be a mechanism of tumour immune escape in CRC. A low TAP1-expression level significantly correlated with a low tumour immune cell infiltration. Furthermore suggested by our results was that the down-regulation might be due to methylation of CpG sites close to the *TAP1* promoter. We could also see that TAP1-expression may be a prognostic factor in stage I and II CRC patients.

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