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THE ROLE OF HLA-A*02 AND BIOMARKERS IN SOLID MALIGNANT TUMOURS

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The role of HLA-A*02 and biomarkers in solid malignant tumours
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

HLA-A*02 genotype generates significantly worse outcome in patients with metastatic serous ovarian cancer and malignant melanoma. HLA-A*02 is common in the Swedish population (about 60 %), but has decreasing frequency in European countries further south. Mortality in the aforementioned tumours correlates with both the latitude and the corresponding HLA-A * 02 frequency.

This thesis is based on the original findings that a combination of HLA-A*02 genotype, ovarian cancer of serous histology and advanced surgical stage has an extremely dismal prognosis. This combination of factors was named “worst prognosis group”.

The aim of the thesis was to elucidate the frequency and distribution of different HLA genotypes in patients with ovarian carcinoma and cutaneous malignant melanoma. We then investigated the correlation between HLA-A*02 genotype and prognosis in these tumour diseases as well as expression of immunological biomarkers for classical and non-classical MHC class I, β 2-mikroglobulin and infiltrating immune cells.

Ovarian cancer symptoms are discrete and about 75% of patients are detected late, when the tumour has already spread. Cutaneous malignant melanoma on the other hand, is often discovered early since it is visible on the skin and the disease progression more easily recorded.

The studies confirmed previous finding that HLA-A*02 is associated with a significantly shortened survival in patients with metastatic tumours of serous ovarian carcinoma or malignant melanoma. There is a high over-representation of HLA-A*02 in patients with advanced ovarian carcinoma. Especially a segregation of the ancestral haplotype 62.1 (HLA-A*02, -B*15, -Cw*3, -DRB1*04) was identified. This haplotype was also present in the group of patients with malignant melanoma. The ancestral haplotype 62.1 seems to give an initial enhanced protection against cancer cells and the time to recurrence is significantly extended. After metastases, however, the disease progression is significantly shorter compared to patients with other haplotypes. The results indicate that the AHH 62.1 might be involved in immune selection, possibly through a specific immunological mechanism called "elimination-equilibrium- escape" mechanism.

Down- regulation of MHC or up-regulation of HLA-G or aberrant expression of HLA-E has been described as tumour escape mechanisms and linked to poor prognosis in other studies. These findings were confirmed in our cohorts, but only in patients with HLA-A*02 genotype and not in the others. In multivariate analysis HLA-A*02 was the second most important prognostic factor, only surgical stage had a higher hazard ratio.

Our results demonstrate that a multifactorial approach, considering tumour characteristics and biomarkers as well as the patient's genetics, can outline subgroups of patients. This may provide improved opportunities to tailor targeted therapies.

LIST OF SCIENTIFIC PAPERS

- I. Gamzatova, Z. Villabona, L. van der Zanden, H. Haasnoot, G W. **Andersson, E.** Kiessling, R. Seliger, B. Kanter, L. Dalianis, T. Bergfeldt, K and Masucci, G V.
Analysis of HLA class I-II haplotype frequency and segregation in a cohort of patients with advanced stage ovarian cancer. *Tissue Antigens*. 2007 Sep; 70(3): 205-13.
- II. Helgadottir, H. **Andersson, E.** Villabona, L. Kanter, L. van der Zanden, H. Haasnoot, G W. Seliger, B. Bergfeldt, K. Hansson, J. Ragnarsson-Olding, B. Kiessling, R and Masucci, G V.
The common Scandinavian human leucocyte antigen ancestral haplotype 62.1 as prognostic factor in patients with advanced malignant melanoma. *Cancer Immunol Immunother* 2009 Oct; 58(10): 1599-608.
- III. **Andersson, E.** Villabona, L. Bergfeldt, K. Carlson, J W. Ferrone, S. Kiessling, R. Seliger, B and Masucci, G V.
Correlation of HLA-A*02 genotype and HLA class I antigen down-regulation with the prognosis of epithelial ovarian cancer. *Cancer Immunol Immunother*. 2012 Aug; 61(8): 1243-53.
- IV. **Andersson, E.** Poschke, I. Villabona, L. Carlson, J W. Lundqvist, A. Kiessling, R. Seliger, B and Masucci, G V.
Non-classical HLA-class I expression in serous ovarian carcinoma: Correlation with the HLA-genotype, tumour infiltrating immune cells and prognosis. *Oncoimmunology* 2015 sept; *Online*

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LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
APM	Antigen Presenting Machinery
β 2-m	Beta 2-microglobulin
CD	Cluster of Differentiation
CTL	Cytotoxic T Lymphocytes
DNA	DeoxyriboNucleic Acid
ER	Endoplasmic Reticulum
EOC	Epithelial Ovarian Carcinoma
FFPE	Formalin Fixed Paraffin Embedded
Genotype	A combination of alleles in the genome
Haplotype	A cluster of specific alleles within a genomic section likely inherited in its entirety
HC	Heavy Chain
HLA	Human Leukocyte Antigen
HR	Hazard Ratio
IFN γ	Interferon gamma
IL	Interleukin
KIR	Killer cell Immunoglobulin-like Receptor
LD	Linkage Disequilibrium
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
mRNA	Messenger RiboNucleic Acid
NK	Natural Killer
OS	Overall Survival
Phenotype	A product of the genotype, i.e., proteins, cells or whole organisms produced according to the code in DNA
PCR	Polymerase Chain Reaction
SBT	Sequenced Based Typing
SSP	Sequence-Specific Primer typing
SSO	Sequence-Specific Oligonucleotide typing
TCR	T-Cell Receptor

TIL	Tumour Infiltrating Lymphocyte
TNM	Classification based on Tumour stage, lymph Node involvement and distant Metastasis
UICC	Union Internationale Contre le Cancer
WHO	World Health Organization
WP	Worst Prognosis group

1 INTRODUCTION

In the new era of individualized cancer therapy precise selection will become crucial, i.e. selection of therapy in combination with selection of patients.

This will demand a multi factorial view including tumour histology regarding type, grade and stage, expression of biomarkers, tumour mutational status and tumour microenvironment as well as the genetics of the individual patient.

Tumour samples obtained during surgical removal are, up to this date, evaluated with focus on the histopathological characteristics and tumour staging according to the AJCC/UICC-TNM classification. TNM is an abbreviation of the combinational data on primary tumour extent (T), number of metastasis in regional lymph nodes (N), and distant metastases (M). Even though the prognostic information is limited and gives no predictive information on response to treatment, the TNM constitutes the basis for decisions made about treatments offered to the patient. The outcome of patients with the same TNM score and treatment varies significantly. Emerging evidence emphasize the significance of the tumour microenvironment (Broussard and Disis 2011). Recent studies have shown that the immune contexture, based on computerized quantification of lymphocytes in the tumour core and tumour invasive margin, significantly predict risk of relapse, with a prognostic value that complement or may even exceed that of the TNM-classification (Galon *et al.* 2006) (Pages *et al.* 2010) (Mlecnik *et al.* 2011). These data are currently being validated in a multicentre study.

In this thesis we have focused on patients with ovarian carcinoma and malignant melanoma. We added information on Human Leukocyte Antigen (HLA) genotype in addition to established prognostic and predictive factors. We were able to identify groups of patients by their prognostic outcome. We found that HLA-A*02 haplotype is an independent factor for poor prognosis. Furthermore, HLA-A*02 genotype plays a crucial role in defining the prognostic significance of several other tumour related prognostic markers.

Molecular methods are constantly improving and becoming less expensive. In combination with the computerised world it is now possible to collect an enormous amount of clinical and genetic data from patients and tumours and identify the key factors in the networks of factors interacting and characterizing different diseases. We show that HLA-A*02 is one of these important connecting nodes in the networks of factors defining prognosis in high grade serous carcinoma and malignant melanoma and possibly other neoplastic diseases as well. HLA genotyping in combination with established prognostic biomarkers might in the future enable a better selection of cancer patients for therapy.

2 BIOMARKERS

Everything that can be measured or detected in the body that provides information on the health status of an individual can be called a biological marker (biomarker). Biomarkers are indicative of physiological processes and are fundamental for the diagnosis and monitoring of disease. Examples of biomarkers include a spectrum from measurements of blood pressure or glucose in urine to sequencing of DNA for gene mutations or immunohistochemical staining of tissue for analysis of protein expression. Biomarkers are the foundation of modern health care and the development of new drugs.

3 TUMOUR IMMUNOLOGY

The immune system protects the body against bacteria and viruses, but already in the late fifties Burnet proposed the hypothesis of immune surveillance against malignant cells (Burnet 1957). This hypothesis was soon discarded, probably because the relationship between the immune system and neoplastic cells is very complex and due to the fact that tumours do occur despite a functioning immune system. However, more recent data have given reason to reconsider the idea and appreciate that the immune system influence the way tumours grow and develop. This is known as immune editing, which in theory consists of three steps: elimination, equilibrium and escape (Dunn *et al.* 2006).

3.1 CANCER IMMUNOEDITING

3.2 ELIMINATION

Transformed cells in the body are recognized and killed by the innate and adaptive immune system.

3.3 EQUILIBRIUM

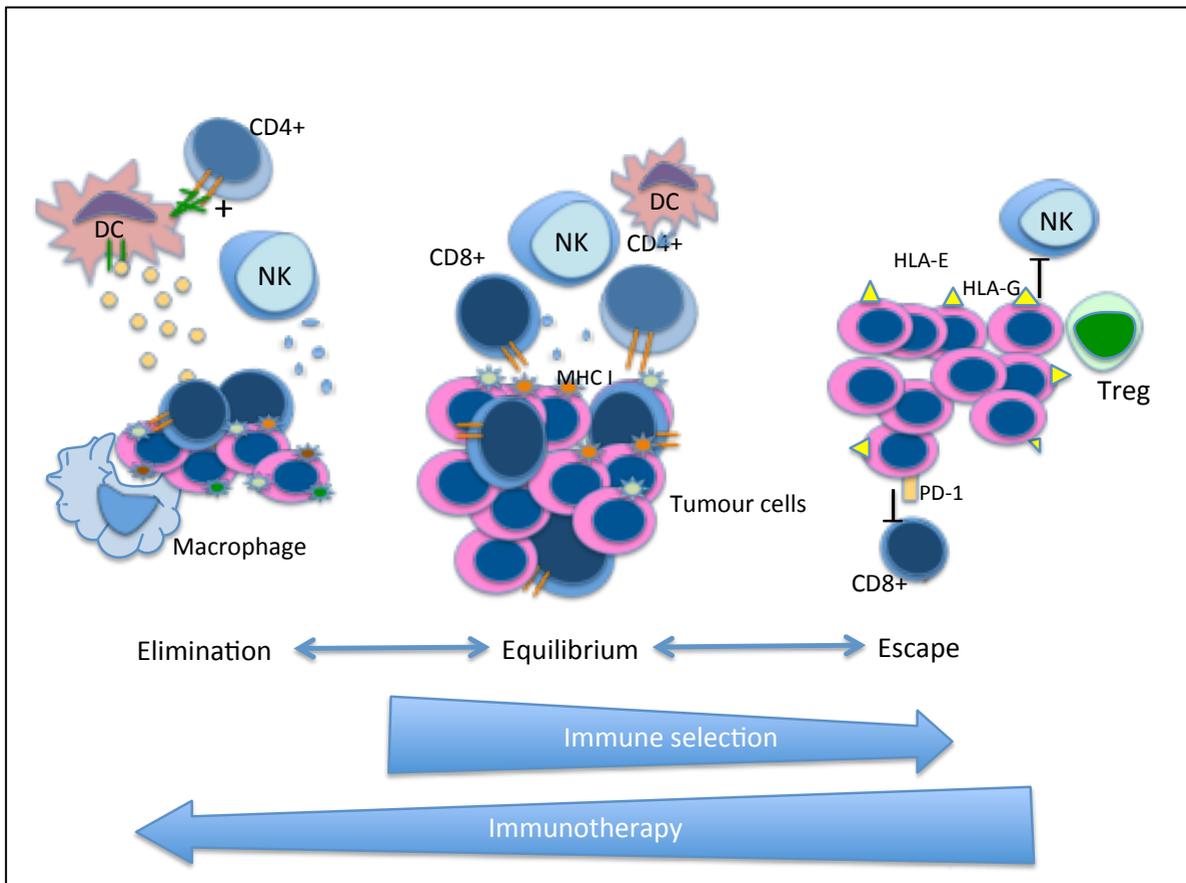
If cancer cells are not immediately eliminated they continue to grow in balance with suppression by the immune system. During equilibrium, the immune system controls the growth but is not able to completely eradicate the malignant cells. This phase can persist for a long period but can result in an immune selection of less immunogenic cells that resist immune elimination and enter the escape phase.

3.4 ESCAPE

Escaping cancer cells might eventually disrupt the equilibrium balance and establish a clinically detectable tumour able to spread further.

There seems to be three main mechanisms for cancer cells to evade the immune system. Tumour cells can selectively down-regulate their expression of major histocompatibility complex (MHC) on the cell membrane surface allowing them to escape immune surveillance detection. Another strategy is to interfere with the abilities of T-cells to kill and induce tolerance. This can be achieved by shedding tumour specific proteins, often in large amounts

and during a longer period of time. Lastly, some tumour cells may express molecules on their surface that bind to immune cells and inhibit their function. This is known as tumour induced immunosuppression.



There is a balance in the interaction between immunosurveillance and escape mechanisms. Immune cells will attack cells expressing non-self antigens or failing to present self-antigens. Tumour cells can down-regulate MHC I expression, induce tolerance in various manners and secrete inhibitory molecules. Immunotherapy aims to shift the balance in favour of elimination of tumour cells. A strong immunosurveillance can on the other hand enhance immune selection of aggressive malignant clones.

3.5 THE ALTERED- SELF CONCEPT

Doherty and Zinkernagel presented a hypothesis, known as the altered-self concept, that was really ground breaking at the time, published in Nature in 1974 (Zinkernagel and Doherty 1974a, Zinkernagel and Doherty 1974b). They were awarded the Nobel Prize in 1996 for their work on cytotoxic T-cell lymphocytes (CTL) and MHC.

By showing that T-cells are activated by a double signal based on the immune cells ability to distinguish between foreign and self-antigens, they could explain the specificity of immune

surveillance. They demonstrated that MHC must be of a compatible haplotype to be recognized by T-cells and that antigens must be presented in the context of MHC. A cell with MHC presenting a self-antigen is spared while a foreign antigen, like a virus, will trigger the cytotoxic T-cell to destruct the cell. Antigens that are not in the context of MHC are simply ignored by T-cells, but will on the other hand be neutralized by antibodies produced by B-cells.

3.6 TUMOUR ANTIGEN

A tumour antigen can be defined as an antigen recognized by tumour specific T-cells. There are different pathways for a self-antigen to become a tumour antigen, the most important being mutation of genes or post-translational modification of proteins. Both ways result in a tumour antigen presentation through classical MHC class I on the surface of tumour cells that can trigger T-cell response (Restifo *et al.* 2012). Over-expression of normal self-antigens on the cell surface in abnormal high levels might also be considered as a tumour specific antigen since therapeutic molecules can target it (Pardoll 2015).

3.7 MHC EXPRESSION AND TUMOUR ESCAPE

One of the most described tumour immune escape mechanisms is defects in the MHC class I antigen-processing machinery (APM) or even complete haplotype loss. Insufficient MHC presentation makes the cell invisible to cytotoxic T-lymphocytes (CTL) and several clinical studies have documented a link to clinical disease progression (Nano *et al.* 2009). Many studies describe that MHC expression can be restored with interferon-gamma (IFN- γ) suggesting that deregulation is more common than structural deficiencies (Masucci *et al.* 2010). Tumours may also utilize many different inhibitory factors that reduce immune activity. These mechanisms are normally preventing autoimmunity in immune active situations (Chen and Mellman 2013).

4 HLA AND MHC

HLA and MHC are terms that are used for both the gene and the corresponding product, i.e. the protein. Both the classical MHC class I and MHC class II are antigen presenting molecules that trigger the immune response, while the non-classical MHC class I are tolerogenic molecules that inhibit immune response (Carosella *et al.* 2003). The classical MHC class I and MHC class II are often referred to as groups, the former including HLA-A, -B and -C products and the later HLA-DR, -DQ and -DP products. The non-classical MHC class I molecules are distinguished due to their individual specific functions and termed by the genetic origin as HLA-G or -E.

5 THE ANTIGEN PROCESSING MACHINERY AND MHC EXPRESSION

All classes of MHC are peptide-presenting molecules expressed on nucleated cells surfaces. Peptides can be both intracellularly and extracellularly derived. All peptides are trimmed by the antigen processing machinery before being loaded onto the MHC molecule and presented as antigens (Seliger 2014).

Intracellular peptides are processed by proteasomes and presented primarily by MHC class I molecules (encoded by HLA-A, -B and -C genes) recognized by CD8 positive (CD8+) CTLs. Extracellular peptides derived from i.e. bacteria are first phagocytised by antigen- presenting cells like macrophages and dendritic cells, before being degraded and presented by MHC class II molecules (encoded by HLA-DR, -DQ and -DP genes) to CD4 positive (CD4+) CTLs (Vyas *et al.* 2008, Restifo *et al.* 2012, Mahdi 2013). The non-classical MHC class I/HLA-E (HLA-E) present antigens derived from conserved peptides of the other MHC class I molecules except non-classical MHC class I/HLA-F and are recognized by NK cells (Rouas-Freiss *et al.* 2005). The non-classical MHC class I/HLA-G (HLA-G) present peptides equivalent to classical MHC class I, but inhibit immune response upon presentation (Hoare *et al.* 2008).

The most important molecules involved in the antigen presenting machinery are; chaperones, calnexin, calreticulin, ERp57 and tapasin (TPN) (Seliger 2014).

5.1 MHC CLASS I

All nucleated cells in the human body present endogenous peptides in the context of MHC to circulating T-cells. Healthy cells recognized as “self” are spared while those that are recognized as foreign are attacked by cytotoxic T-cells. The MHC expression at the cell surface is actually a trimeric complex: MHC, β 2-microglobulin (β 2-m) and the presented peptide. MHC represents the heavy chain (HC) and β 2-m the light chain. Proper installation of the presented peptide is required for expression on the cells’ surface (Seliger 2014).

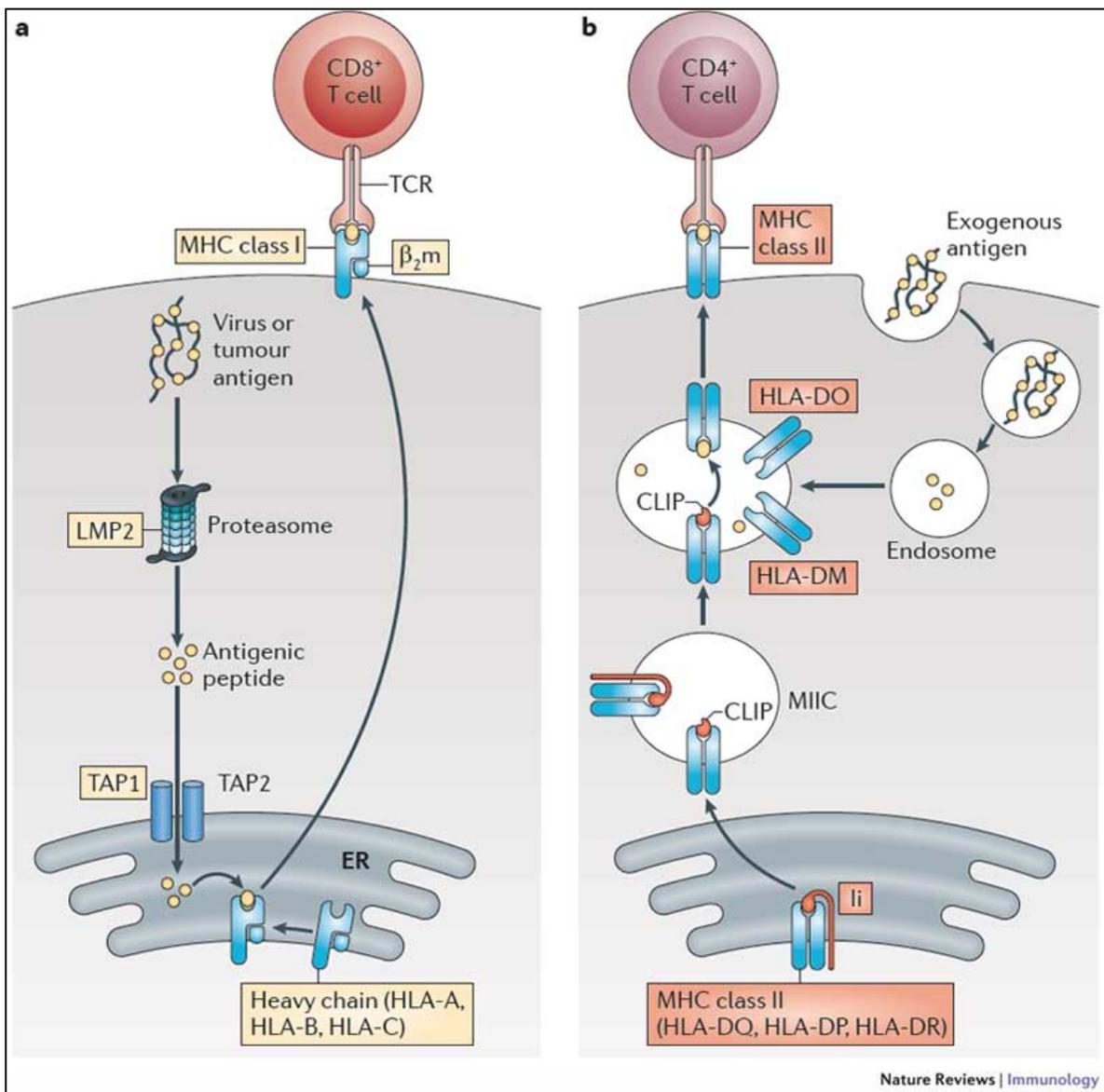
Proteins are cleaved into 9-11 amino acid long peptides by proteasomes and amino peptidases in the cytosol. The peptides are translocated via transporter- associated with antigen processing complex (TAP) to the endoplasmic reticulum (ER), where they are further trimmed by proteasomes like LMP2, before assembled with the HC / β 2-m heterodimer. The now trimeric HC/ β 2-m/peptide complex is transported via the Golgi apparatus to the cell surface where it is recognized by T-cells (Kobayashi and van den Elsen 2012).

However, the trimeric complex can escape from the ER and disassemble. HC can also spontaneously dissociate from β 2-m and appear as a free form on the tumour cell surface with an open conformation. The equilibrium between β 2-m free and β 2-m associated MHC- HC expression on the cell surface can be modified by the activated T-lymphocytes (Vyas *et al.* 2008).

5.2 MHC CLASS II

The MHC class II molecule is also a heterodimer like MHC class I, but consists of two homogenous heavy chains, alpha and beta. Both genes are located in the Class II region (Kobayashi and van den Elsen 2012)

The synthesis of MHC class II occurs in the ER where the alpha and beta chains are produced. The naive heterodimer forms a complex with an invariant chain (Ii) that blocks the peptide-binding groove from being loaded with endogenous peptides. When the naïve complex is fused with an endosome containing extracellular peptides, the invariant chain is broken down. Only a small fragment, CLIP is left in the binding cleft. CLIP is replaced with extracellular derived peptides by the HLA-DM molecule. The now mature and stable MHC class II/exogenous peptide complex is then presented on the cell surface. The extracellular proteins presented by MHC class II are mainly 15-24 amino acids long, exogenously derived pathogens, like bacteria. The MHC Class II molecules interact and mature the CD4+ T-cells into T helper cells that will help to trigger an immune response, especially activation of B-cells and antibody production (Kobayashi and van den Elsen 2012).



Reproduced with permission from the publisher (Kobayashi and van den Elsen 2012).

a) MHC class I is a heterodimer of a heavy chain (HC) and a light chain (β₂-m) and is expressed on all nucleated cells mainly presenting endogenous proteins.

b) MHC class II is a heterodimer of two homogenous peptides, an alpha and beta chain, both of which are encoded in the HLA region. MHC class II is mostly expressed on professional immune cells and presents exogenous proteins from bacteria and viruses. Recently one has become to understand that there is a lot of cross talk between the two classes.

5.3 NON-CLASSICAL MHC CLASS I

The non-classical MHC class I genes includes HLA-G, -E and -F. They are located close to the HLA-A locus on the telomeric end of the chromosome. HLA-G and -E are rather well studied and are both tolerogenic molecules (Rouas-Freiss *et al.* 2005).

HLA-G

HLA-G is recognised by the receptors ILT-2, ILT-4 and KIR2DL4. When HLA-G is expressed it stops proliferation of immune cells and inhibits cytotoxic activity of especially NK cells, but it can also transform CD4+ and CD8+ T-cells into suppressor T-cells. HLA-G presents endogenous peptides and share several binding pockets structures with the classical MHC class I of HLA-A*02 origin. It has been shown that many HLA-A*02 restricted peptides also bind to HLA-G (Diehl *et al.* 1996).

HLA-G is expressed in foetal cytotrophoblasts to protect the foetus from the mother's immune response (Crisa *et al.* 1997). Failure to express HLA-G during pregnancy leads to spontaneous abortion. There are also some other immune privileged tissues that express HLA-G, namely thymus, cornea, pancreas and erythroid and endothelial precursor cells (Carosella *et al.* 2003, McCormick *et al.* 2009). Its expression has further been demonstrated in inflammatory and viral diseases, transplants and in tumours (Rebmann *et al.* 2014, Rouas-Freiss *et al.* 2014). HLA-G expression is induced in inflammatory settings by environmental factors like hormones, stress proteins and cytokines like GM-CSF, interferons and interleukins (Carosella *et al.* 2003).

There are seven different isoforms of HLA-G. Four are membrane-bound (HLA-G1-4) and three are soluble (HLA-G5-7). HLA-G can thus be expressed on cell's surfaces like the classical MHC class I, but it can also be secreted or incorporated into exosomes (Riteau *et al.* 2003). HLA-G molecules can also be transferred from the cell where it was produced, into the cell membranes of other cells in the microenvironment through trogocytosis (Joly and Hudrisier 2003). Recipient cells can be both surrounding tumour cells and immune cells like NK or T-cells. Even though only as few as ten per cents of cells express HLA-G it can thus extend its regulation of immune cells in multiple ways and completely change a hostile immune microenvironment into a tolerogenic milieu (Carosella *et al.* 2003, Carosella *et al.* 2008). Expression of HLA-G in most solid tumours has been linked to a worse prognosis (Davidson *et al.* 2005, El-Chennawi *et al.* 2005, Hansel *et al.* 2005, Rouas-Freiss *et al.* 2005, Adithi *et al.* 2006, Barrier *et al.* 2006, Kleinberg *et al.* 2006, Ye *et al.* 2007). On the opposite a better prognosis has been observed in haematological malignancies indicating that the immune suppressive properties of HLA-G functions in a negative feedback loop on the malignant immune-cell clone and might stop proliferation (Rouas-Freiss *et al.* 2014).

HLA-E

HLA-E is recognised by the CD94-NKG2 receptor, which is expressed on NK cells. It is also tolerogenic and signals the NK cell to stop cytotoxic lysis (Lee *et al.* 1998, Tomasec *et al.* 2000). HLA-E present antigens derived from conserved peptides of the other MHC class I molecules except HLA-F. The stability of the HLA-E expression is dependent on the origin of the presented peptide (Braud *et al.* 1998, Hoare *et al.* 2008) and especially peptides derived

from HLA-G (Clements *et al.* 2005) but also HLA-A2 (Braud *et al.* 1997) can enhance surface expression stability of HLA-E and the inhibition of NK cytotoxicity (Marchesi *et al.* 2013). Even very small changes in the peptide conformation will affect the recognition by the NK cell receptors and possibly change the effector cell function. The association of HLA-E/ β 2-m is weaker than for classical MHC class I. HLA-E will thus favour down regulation, due to higher availability of both degraded MHC class I peptides and free β 2-m (Marin *et al.* 2003). HLA-E expression is found in most human cells (Wei and Orr 1990) but is rather weak in healthy tissue in contrast to tumours where it is often up regulated.

6 RECEPTORS INVOLVED IN THE MHC I- II MACHINERY

6.1 T-CELL RECEPTORS

T-cell receptors (TCR) are activating receptors found on the surface of T-cells. MHC class I mature naive CD8⁺ T-cells into cytotoxic T-cells, whereas MHC class II matures naive CD4⁺ cells into T-helper cells.

The TCR is promiscuous and can recognize many different antigens, but many antigens can also be recognized by different TCRs.

On the T-cell there is also an inhibitory receptor, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). This receptor signals the T-cell to inactivate upon stimulation by specific molecules on dendritic cells and is important for the regulation of the immune system (Chen and Mellman 2013). Ipilimumab is an agent that targets CTLA-4, reverting the inhibition of the immune system (Tarhini *et al.* 2010).

6.2 LEUKOCYTE IMMUNOGLOBULIN-LIKE RECEPTORS

The leukocyte immunoglobulin-like receptors (LILR) are MHC receptors expressed by myeloid cells, especially monocytes. They have immune- modulatory effects on a wide range of immune cells (Rouas-Freiss *et al.* 2014). The gene clusters for these receptors are found in chromosomal region 19q13.4 (HUGO gene nomenclature committee).

There are both activating and inhibitory LIL-receptors, LILRA and LILRB. They bind both classical and non- classical MHC class I molecules, but with different affinity strength depending on HLA allele. Especially HLA-G has a very high affinity to the inhibitory molecule. Viruses like HIV and CMV can exploit LILRs to reduce immune surveillance and it is reasonable to believe that they are modulated in tumour immune escape mechanisms (Marchesi *et al.* 2013, Rouas-Freiss *et al.* 2014).

The activating receptors consist of three forms: LILRA1-3. LILRA1 is found on monocytes, LILRA2 on all cells derived from myeloblasts and LILRA3 is a soluble form secreted by macrophages.

The inhibitory LILRBs are termed immunoglobulin like transcript receptor 2 (ILT2) and 4 (ILT4). They are present on natural killer (NK), T-, B- and dendritic cells as well as neutrophils. They mediate negative signalling that counteracts immune activation. ILT2 binds

β 2-m associated HLA-G (Allan *et al.* 2000). ILT4 binds β 2-m-free HLA-G molecules (Carosella *et al.* 2008).

6.3 KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTOR

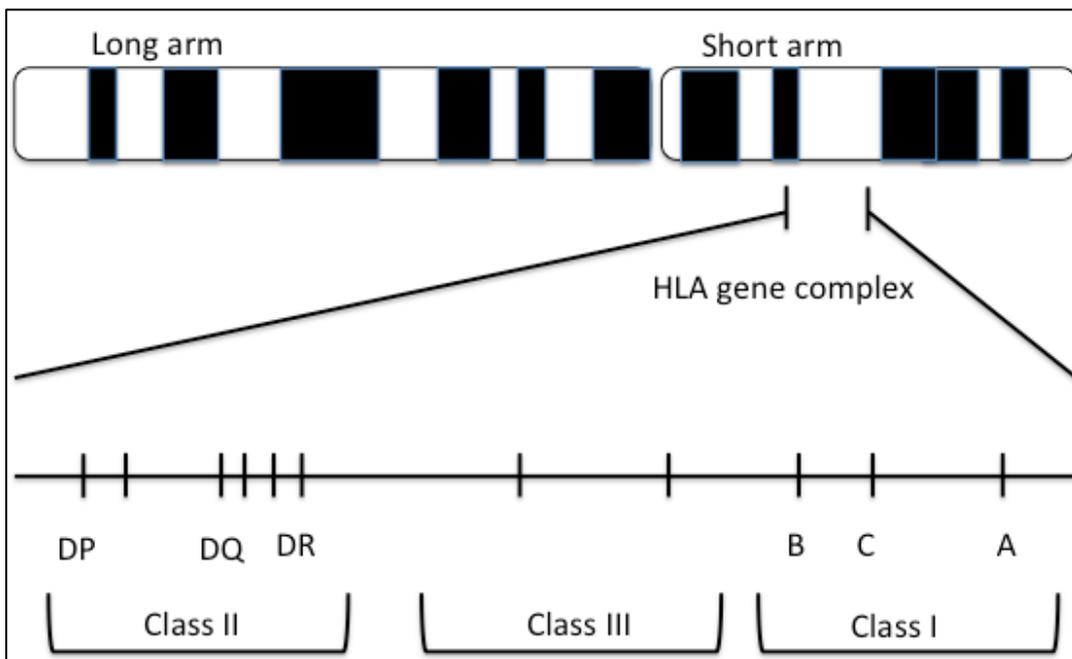
Cells with intact MHC class I expression are spared by the NK cells through recognition by the killer cell immunoglobulin-like receptor (KIR). The NK cell is activated when the MHC class I expression on infected cells or tumour cells is reduced. One KIR, the KIR2DL4 receptor, resides in the endosome and is only known to recognize HLA-G, acting as inhibitor upon HLA-G exposure (Carosella *et al.* 2008). KIR genes are located on chromosome 19. The expression of natural killer group 2 receptor (NKG2) in heterodimerization with cluster of differentiation 94 (CD94), is specific for HLA-E. The heterodimer is expressed by 50% of NK- cells and CTLs and HLA-E positive cells are thus protected from both NK and CTL cells (Vivier 2006).

7 THE HUMAN LEUKOCYTE ANTIGEN

The HLA locus is located on the short arm of chromosome 6 (6p21.3) and spans 3.6 Mb to 7.6 Mb with the telomeric repeats. It is an extensively studied region of the genome. As many as 28 % of the genes in the adjacent regions are involved in diverse aspects of the immunological response mechanism and the genetic region is linked to many autoimmune diseases (Masucci *et al.* 2010).

The HLA class I region contains loci for both classical HLA-A, -B and -C and non-classical HLA- E, -F and -G genes. The HLA class II describes the HLA-DP, HLA-DQ and HLA-DR determinants. HLA class III and the complement components C2, C4 and Factor B are located in a region between the DR and B loci (Mahdi 2013).

The HLA locus is the most polymorphic region in the human genome. Variations are predominantly restricted to the exons 2 and 3, which code for the peptide-binding domain of the protein. The heterogeneity of the HLA locus is considered to be beneficial for survival of the human species (Masucci *et al.* 2010)



Modified gene map of Chromosome 6

7.1 ANCESTRAL HAPLOTYPES

Within the HLA-region, areas with high linkage disequilibrium (LD) are interspersed with regions of low LD. The high LD regions stick together and form so-called haplotype blocks, the low LD regions correspond to recombination hotspots.

So-called ancestral haplotypes are highly preserved haplotype blocks found in large human populations. A specific genomic length with a specific content of alleles characterizes them. Our ancestors' migration across the world has divided these haplotypes uneven. The most common ancestral haplotypes in Sweden are 62.1 (B15-CW3-DRB1*04), 60.1 (B40 -Cw3) and 8.1 (B8 Cw7-DRB1*03) (Masucci *et al.* 2010).

7.2 HLA-A*02

HLA genotypes vary among different ethnic populations over the world. HLA-A*02 is one of the most frequent HLA-A alleles in all ethnic populations (Middleton *et al.* 2000) and the most common haplotype in Sweden with 58 % compared to an average of 41.3 % worldwide. The different distribution of HLA genotypes over the world can be tracked back to the migration of human populations (Degli-Esposti *et al.* 1992). Our group have noticed a correlation between HLA-A*02 gene frequency and the geographical latitude, with decreasing HLA-A*02 prevalence in the more southern latitude countries of Europe (De Petris *et al.* 2004).

It is reasonably to believe that HLA-A*02 is so widely present in human populations due to evolutionary benefits. Its promiscuity allows presentation of a wide range of antigenic epitopes, which can provide immunological survival advantages (Browning and Krausa 1996).

Many peptide vaccines are HLA-A*02 restricted. The downside of this is that HLA-A*02 losses are frequent in tumours (Norell *et al.* 2006).

There are 456 different HLA-A*02 alleles according to the allele Search Tool from European Molecular Biology Laboratory 2013. These genetic differences affect the peptide-binding groove of the MHC and influence the binding affinity of peptides. Thus, it influences the repertoire of peptides that are presented for CTL recognition. The HLA-A*02 allele variants will also affect the CTL response regarding magnitude and specificity. The distribution of different HLA-A*02 alleles in ethnic populations are the basis for ethnicity-specific HLA-A*02 restricted T-cell responses in various diseases (Chen *et al.* 2012).

HLA-A*02:01 is the most common HLA-A*02 variant and is present in 95 % of HLA-A*02 positive individuals in Caucasian populations. HLA-A*02:03, A*02:06 and A*02:02 are predominant in Asia and A*02:02 in Africans (Chen *et al.* 2012). It is believed that HLA-A*02:01, A*02:02, and A*02:05 are the oldest alleles from which the other subtypes arose (Browning and Krausa 1996). HLA-A*02:01 is frequently used in studies on HLA-A*02-restricted CTL responses.

The HLA-A*02 genotype has been associated with a number of diseases like rheumatoid and psoriatic arthritis, Alzheimer's disease, vitiligo, diabetes mellitus and Hashimoto's thyroiditis (Chen *et al.* 2012). It has also been associated with spontaneous abortion in infertile couples where HLA-A*02 is thought to induce increased maternal immune response to the foetus (Komlos *et al.* 2007). On the other hand HLA-A*02 seems to have a protective role in multiple sclerosis.

7.3 METHODS OF TESTING HLA

HLA typing is performed routinely before organ transplantation and was originally made using serology with antibodies against MHC-A HC (Mahdi 2013).

Serotyping is generally enough to prevent transplant rejection or graft versus host disease, but the ability to identify different HLA types by this method is limited to the availability of sera containing the various MHC specificities. Today, molecular methods are used for DNA analysis. The most common methods are sequence-based typing (SBT), sequence-specific primer typing (SSP), and sequence-specific oligonucleotide typing (SSO) (Mahdi 2013). The HLA genes are highly polymorphic, but the majority of the sequence variations are contained within exons 2 and 3, which is why most molecular tests focus on identifying polymorphisms in these regions. DNA probes or primers artificially synthesized to match specific nucleotide sequence polymorphisms unique to a particular HLA type are used (Fung and Benson 2015).

Sequenced based typing (SBT) is generally performed, via the traditional Sanger nucleotide termination method or via next-generation sequencing methods. In sequence-specific primer typing (SSP) specific DNA amplification products are produced via the use of heat-stable DNA polymerase and thereafter detected with HLA type specific DNA primers in multiple wells to determine the presence or absence of specific DNA products. Sequence-specific oligonucleotide typing (SSO) can be used to detect more broadly, exon- and locus-specific

DNA amplification products with SSO probes that grossly determine HLA type. If more high-resolution typing is required, exon 4 testing may be added (Mahdi 2013).

Since the methods for HLA typing are DNA based, non-fragmentized DNA is required. This is why fresh blood samples are preferred. However, blood samples are not always available, especially not in retrospective studies where many of the patients are no longer alive. Our group have therefore developed a novel method for complete HLA-A typing of DNA derived from formalin-fixed paraffin embedded tissue (Villabona *et al.* 2014).

8 PHARMACOGENOMICS AND EPIGENETICS

8.1 PHARMACOGENOMICS

Some genes are known to associate with particular responses to medications and other therapies, which is why patients might need pre-screening prior to treatment. One example is HIV-infected patients with HLA-B*57:01. They have a high risk of adverse events if treated with abacavir. By pre-screening for this specific gene before medication, these patients can be excluded from a potential hazardous treatment (Fung and Benson 2015).

HLA seems to predict the response of immunological treatments, especially vaccine or cytokine therapies. However, statistical data has not been strong enough to justify HLA typing of cancer patients to be performed routinely (Fung and Benson 2015).

8.2 HLA ASSOCIATION WITH DISEASE

The immune system plays a crucial part in many diseases and it is not surprising that specific HLA types, with their particular polymorphisms, have been associated with many different diseases, especially autoimmune disorders (Goris and Liston 2012).

8.3 HLA ASSOCIATION WITH CANCER

Many genetic alterations have been linked to the risk or prognosis of malignant diseases. Recently, the role of HLA in cancer has also gained more interest.

An Asian study on nasopharyngeal carcinoma and HLA found a two-fold increase in individuals with HLA-A*02:07 and a 50% reduction in carriers with HLA-B*46:01 who were negative for HLA-A*02:07 (Yu *et al.* 2009).

A Swedish study could show a poorer prognosis in HLA-A*02 positive patients with HPV-induced oropharyngeal carcinoma than in patients with HPV-negative cancers (Tertipis *et al.* 2014).

We have evidence that HLA-A*02 genotype is a strong prognostic factor linked to the aggressiveness of ovarian cancer of serous histology, prostate cancer and malignant melanoma, but we have no indication that this genotype would increase the risk of these cancers diseases (De Petris *et al.* 2004).

The poorly understood relevance of HLA in malignancies might, at least partly, be due to the different variations of HLA genotypes as well as different allelic subtypes, within populations in different parts of the world, but probably also limited access to relevant clinical data. Since the HLA-locus is a region with high incidence of polymorphism and linkage disequilibrium, one might also postulate a genetic linkage to possible onco- or tumour suppressor genes influencing disease progression. Especially the non-coding sequences present near the HLA locus on chromosome 6 have gained interest recently. For example the genes for TNF- α and components of the complement system exist in this region and some are within conserved blocks (Windsor *et al.* 2005).

8.4 GENES ON CHROMOSOME 6 ASSOCIATED WITH CANCER.

One might speculate that HLA-A*02, or perhaps genes closely connected to the HLA-A locus on chromosome 6, are related to other known or unknown molecules involved in tumour proliferation, metastatic potential, tumour cell migration or factors involved in immune-escape mechanisms. For example, FISH analysis regarding chromosomal imbalances on 6p25, centromere 6 and 6q23 can identify malignant melanomas with metastatic potential (North *et al.* 2011).

Some genes of special interest on chromosome 6 are **p21**, also known as cyclin-dependent kinase inhibitor 1 (CDKN1A) on 6p21, and transcription factor activator protein-2 alpha (**TFAAP2- α**) on 6p24. Both are in close proximity to the HLA-A locus. One interesting observation is that metastatic melanoma cell lines do not express TFAAP2- α , whereas non-metastatic cell lines express high levels of TFAAP2- α (Ruiz *et al.* 2001). p21 is regulated by TFAAP2- α , but also by the suppressor protein tp53, which is commonly mutated in cancer. Both p21 and TFAAP2- α are involved in cell proliferation and progression of different types of cancers. Failure to produce p21 contributes to uncontrolled cell proliferation. tp53 is one of the substrates of the mitogen activated protein kinase 14 (**MAPK14**). MAPK14 is involved in cell proliferation, differentiation and transcription where it plays an important role in senescence. It is activated by environmental stress and proinflammatory cytokines and is important for stress related cell regulation. (Boutros *et al.* 2008, Mombach *et al.* 2014). Other genes of interest are interferon gamma receptor (**IFN- γ R**), target for IFN- γ , and tumour necrosis factor (**TNF**). Both are cytokines involved in immune regulation. Up regulation of IFN- γ in tumours prior to immune therapy has been linked to better immune response (Galon *et al.* 2015).

Insulin-like growth factor 2 receptor (**IGF2R**) is the target for IGF2, a growth factor normally active during the growth of the foetus and much less active in adult tissues. IGF2R also functions as receptor for granzyme B, which is essential for cytotoxic T-cell-induced apoptosis (Brown *et al.* 2008).

Different growth factors and growth factors receptors that can affect the tumour microenvironment are also potential candidates. Interesting examples are vascular endothelial growth factor (**VEGF**), fibroblast growth factor receptor (**FGFR**) and connective tissue growth factor (**CTGF**) (Zhu *et al.* 2015).

8.5 HLA AND EPIGENETICS

High copy numbers of HLA genes are found in many tumours. The consequence of this increase is not fully understood, but it is known that if a gene is amplified, the original function might be lost or even counteracted on an epigenetic level. Amplification may therefore affect prognosis in multiple ways, which is why the same haplotype may be associated with good prognosis in one malignancy, but poor prognosis in another.

Data supporting the significance of epigenetic mechanisms was given in a recent paper on patients with ovarian carcinoma of serous histology and high malignancy grade (Wang *et al.* 2014). Hypo- methylation of chromosome 6 in position p21.3 (HLA locus) was correlated to longer disease free period.

On an epigenetic level one may also speculate that microRNAs (miRNA) correlate with specific HLA haplotypes. miRNAs are small pieces of RNA that do not themselves encode for any protein but which regulate the transcription of other genes, usually located on another chromosome. miRNAs play a role in the initiation of tumorigenesis, but the mechanisms are not fully understood. Recent findings suggest miRNAs as interesting targets for immune modulatory drugs (Jeker and Marone 2015).

9 TUMOUR MICROENVIRONMENT

In histological analysis of tumours there is a broad variation regarding presence of tumour infiltrating lymphocytes (TIL) and desmoplastic stromal response. Immune cells can be seen in infiltrates of varying density both in the tumour core and in the invasive margin. Sometimes lymphoid islets, representing lymphoid hyperplasia, are seen in the tumour periphery. A classification based on the immune cell type, amount and location has been shown to correlate that of the risk of relapse (Galon *et al.* 2006). The prognostic value of such a clarification was found to be superior to that of the classic TNM classification which motivated an editorial with the title "TNM Staging in colorectal cancer: T is the T cell and M is for memory" together with a publication of Mlecnik *et al.* in *Journal of Clinical Oncology* (Broussard and Disis 2011, Mlecnik *et al.* 2011). The immune system has gained a lot of attention in recent years, both for biological understanding of cancer development and prognosis and as promising target in clinical management of cancer (Chen and Mellman 2013).

9.1 IMMUNE CELLS

All blood cells originate from multipotential hematopoietic stem cells in the bone marrow. These stem cells give rise to two main lineages in the haematopoiesis: the myelocytopoiesis and the lymphocytopoiesis. The first cell in the myelocytopoiesis is the common myeloid progenitor cell from which the myeloblasts and its descendants monocytes and granulocytes origin. The erythrocytopoiesis and thrombocytes are other lineages from the common myeloid progenitor cell. The common lymphoid progenitor cell can mature into natural killer (NK) cells as well as lymphocytes, both B- and T-cells (B.Young and J.W.Heath, *Functional Histology*, 4th ed).

Granulocytes

Neutrophils, basophils and eosinophils are leukocytes with intracytoplasmic granules. Neutrophils are the major effector cells in acute inflammation. Basophils secrete histamine that causes increased capillary permeability and an enhanced inflammatory environment. Mast cells are similar to basophils, but mature in peripheral tissue, often skin or gastrointestinal mucosa. Eosinophils are specialized at killing parasites, but they are also involved in destruction of tumour cells (B. Young and J.W. Heath, Functional Histology, 4th ed).

Myeloid-derived suppressor cells

Myeloid derived suppressor cells (MDSC) are of myeloid origin. Under normal conditions the immature myeloid cells differentiate into dendritic cells, neutrophils or macrophages, but under immunogenic circumstances, like infection or cancer, the differentiation shifts towards the suppressive population. MDSCs accumulate in peripheral blood or tissue where they execute immune suppressive effects, mainly by inhibiting T cell and NK cell activity and proliferation. They also have additional non-immunological functions such as promoting tumour angiogenesis and metastasis (Jiang *et al.* 2014) which is why they are interesting therapeutic targets.

Monocytes

Monocytes are also myeloid derived cells. They migrate from the blood stream to tissues where they mature into macrophages or dendritic cells depending on location,

Macrophages and dendritic cells.

Macrophages or dendritic cells are tissue phagocytic cells responsible for local clearance. They can be activated both by the innate and adaptive immune system. In essence, they are divided into two polarized groups, classically activated M1 and alternatively activated M2. Macrophages that infiltrate solid tumours are referred to as tumour associated macrophages (TAM). M1 macrophages are involved in the early elimination of tumour cells. When M1 cells phagocytize tumour cell debris they mature into antigen-presenting cells (APC) that present tumour antigens in the context of both MHC class I and II to naïve T- cells. They thus initiate the inflammatory response and promote the adaptive immune response, but also NK cells which counteract tumour formation. M2 macrophages also phagocytize debris but stimulate tumour growth since they promote wound healing by producing growth factors. Furthermore, some of these stimulate angiogenesis. In early tumour stage, M1 macrophages dominate while in later tumour stage a switch to M2 cells has been described (Mantovani and Sica 2010, Movahedi *et al.* 2010, Sica and Mantovani 2012, Marchesi *et al.* 2013).

T-cells

T-cells are lymphocytes originating from the common lymphoid progenitor cell. They are distinguished by the presence of a T-cell receptor (TCR) and can be subdivided into several subpopulations, the most important being T helper, T cytotoxic, T memory and T regulatory cells. Naïve T-cells, but also CD4⁺ helper and CD8⁺ cytotoxic T-cells express CD45RA⁺. Memory T-cells on the other hand will lose CD45RA⁺ and gain CD45RO⁺ expression after antigen exposure and maturation (Mahnke *et al.* 2013).

Naïve T-cells activated by antigens are induced to proliferate and differentiate. Depending on the specific trigger-antigen and temporal differentiation stages of a combination of effector cytokines like IL-2, IFN- γ and TNF, the inflammatory milieu is formed (Franciszkievicz *et al.* 2012).

CD4+ T helper cells

T helper cells express the CD4 glycoprotein and are known to regulate the magnitude and persistence of the immune system through cytokine production. Type 1 (Th1) CD4+ T-cells produce IFN- γ and stimulate expansion of antigen-specific cytotoxic CD8+ T-cells. They also contribute to the maturation of B-cells (Zhu *et al.* 2010). Type 2 (Th2) CD4+ T-cells produce IL-4 and -13. They do not participate in tumour suppression. CD4+ T-cells are themselves activated by APC expressing antigens in the context of MHC class II.

CD8+ T cytotoxic cells

T-cells expressing CD8 activate their cytotoxic effects after recognition of antigens presented together with MHC class I. They have been considered to be the main cytolysis producing cells with the greatest killing ability. They excrete cytolytic molecules such as granzyme B and perforin that kill target cells (Mahnke *et al.* 2013, Kared *et al.* 2014).

In recent years, a more complex picture has emerged due to overlapping features between the CD4+ and the CD8+ T-cells. For example it has been demonstrated that CD4+ T-cells can express cytolytic molecules (Zaunders *et al.* 2004) and that CD8+ T-cells may stimulate B-lymphocytes and dendritic cells (Frentsch *et al.* 2013).

Memory T-cells

Most of the activated effector T-cells that have fulfilled antigen clearance will die, but a small proportion (less than 5 per cent) develop into long-lived memory T-cells, recognized by the presence of CD45RO+. Different subtypes of memory T-cells produce different cocktails of cytokines. Some of them lack the immediate killing activity whereas others, like effector memory T-cells, are capable of rapid cytotoxicity. All memory T-cells can home to lymphoid tissues where they rest until activated (Mahnke *et al.* 2013).

Regulatory T-cells

Regulatory T-cells (Tregs) are CD4+ expressing lymphocytes that also express IL-2 receptor (the alpha chain CD25) and fork head family transcriptional regulator box P3 (FOXP3). They constitute 5-10 % of the peripheral CD4+ lymphocytes. Tregs have limited proliferative ability and are prone to apoptosis. If the tumour microenvironment is dominated by Tregs they can suppress the proliferation and the antitumour effector function of cytotoxic T-cells by producing TGF- β and IL-10. Increased numbers of Tregs have been detected both systemically in peripheral blood of cancer patients and locally in for example late stage ovarian cancer (Woo *et al.* 2001).

This suppressive action is critical in healthy individuals in order to prevent the activation of auto reactive T-cells and thus limiting autoimmune damage during an immune response. Once stimulated, Tregs act in an antigen-non-specific fashion. Apart from Tregs there are several

other types of T-cells that also can gain suppressive capacity during an immune response. Approaches to eliminate immunosuppressive Treg cells before immunotherapy are currently being tested (Finn 2008).

Lymphokine activated killer cells

Lymphokine activated killer cells (LAK- cells) are lymphocytes activated particularly by IL-2. They mature into cytotoxic effector cells that effectively can lyse tumour cells. They are not dependent on HLA expression, can lyse cells resistant to NK cells and do not display activity against normal cells.

B-cells

B-cells are the other primary immune cells of the adaptive immune system. When activated they start to produce antibodies against antigens on the tumour-cell surfaces, making these tumour cells easy targets for phagocytes and activation of the complement system.

Natural killer cells

Natural killer cells (NK) belong to the first line of defence of the innate immune system. They induce apoptosis in target cells by releasing granzyme. NK cells are tightly regulated in order to suppress their cytolytic activity to prevent autoimmune diseases. They do not need antibodies or activation through MHC presentation to recognize cells under stress and can thus provide rapid killing upon activation. They are identified by the presence of CD56 and the absence of CD3. There are two sub-populations with high (bright) and low density (dim) of CD56 and with or without expression of CD16. Especially CD56 dim NK cells are effective killers, whereas CD56 bright secrete cytokines (Hanna and Mandelboim 2007). NK cells discriminate between self- cells and transformed self- cells through the activating receptor NKG2D (Bonavida 2014).

9.2 CYTOKINES

Cytokines are important molecules in a complex signalling system between cells. They are produced by many different cell types and are the fundament in recruitment and regulation of immune cells. Tumour cells, stromal cells and immune cells can secrete cytokines and thus orchestrate the tumour microenvironment. The balance between stimulatory and inhibitory signals that regulate the immune system is important for an adequate response and to prevent autoimmune overreaction that might lead to tissue damage. However, tumour cells can exploit these inhibitory signals in their favour to escape immune surveillance (Chen and Mellman 2013).

Tumour necrosis factor

Tumour necrosis factor (TNF) is a proinflammatory cytokine involved in cancer antigen presentation mainly by dendritic cells. TNF is also a pyrogen that mediates cell lysis and enhances endothelium permeability (Chen and Mellman 2013).

Interleukin 2

Interleukin 2 (IL-2) is another early expressed cytokine in the inflammatory situation and

drives NK and T-cell priming and activation. The IL-2 receptor (CD25) is expressed on antigen activated, antigen-specific CD8+ T-cells (Yu *et al.* 2009).

Interferon gamma

IFN- γ production rises in the later stages of immune response. IFN- γ activates macrophages and stimulates the cytolytic activity of NK cells.

IFN- γ can elevate or restore the expression of MHC class I and II molecules. IFN- γ is thus important for an effective immune response. Clinical trials on prostate cancer patients treated with HER2/neu vaccine, found that high levels of IFN- γ prior to vaccination, correlated to strong immunological responses and also clinical benefits (Perez *et al.* 2014)

Transforming growth factors-beta

Transforming growth factors-beta (TGF- β) is a late expressed cytokine functioning as potent regulator of cell growth, differentiation and migration. Thus, it counteracts killing of target cells. Cancer cells may start to overexpress TGF- β to evade immune attack. It promotes metastasis and angiogenesis (Bierie and Moses 2006). The level of expression appears to correlate to poor differentiation in tumours. TGF- β induces differentiation of T-cells into Tregs. Patients with high levels of TGF- β at baseline, before tumour vaccination, show decreased immunological response and a shorter overall survival (OS) (Perez *et al.* 2014).

Granulocyte-macrophage colony-stimulating factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF and M-CSF) induce stem cells to differentiate into granulocytes and monocytes. It is presently used to prevent neutropenia in cancer patients receiving chemotherapy.

Macrophages developed in the presence of GM-CSF differentiate into M1 while M-CSF potentiates M2 polarization (Hamilton *et al.* 2014).

CYTOKINES	Time expressed in immune response	Function	Immune stimulator/suppressor
TNF	Expressed early in cancer antigen presentation	Mediates cell lysis and enhance endothelium permeability Cancer antigen presentation	Stimulator
IL-2	Expressed early	T-cell priming and activation. Prolong survival of antigen specific T- cells	Stimulator
IFN- γ	Expressed late	Activate macrophages and NK cells. Enhance/restores MHC expression	Stimulator
TGF-β	Expressed late	Promotes tumour cell growth and metastasis	Inhibitor
GM-CSF M-CSF	Expressed continuously	Stimulate proliferation of monocytes and granulocytes	Stimulator

Schematic table over the most important cytokines regarding time point during the inflammatory response and function in the immune system.

10 IMMUNOTHERAPY

“Biologic therapy differs conceptually from surgery, radiation therapy or chemotherapy because it acts, not by directly attacking the tumour, but by stimulating the natural host defence mechanism to mediate cancer regression”(Rosenberg 1992).

When a tumour is clinically detected, tumour cells have already broken the balance of equilibrium and developed immunosuppressive mechanisms. Immunotherapy may tip the balance back in favour of immunostimulation and tumour elimination. However, immunotherapies are counteracted by standard cancer treatments like chemotherapy and radiation treatments that have immunosuppressive effects. Despite this, many studies show that those receiving immunotherapy respond better to second-line chemotherapy (Finn 2008).

Monoclonal antibodies

Targeted therapy with monoclonal antibodies (mAb) exploits the mutational differences between normal cells and tumour cells. Cancer cells present tumour antigens together with the MHC on the cell surface or overexpress specific receptors like Her 2-neu or VEGFR. Engineered mAbs can target these molecules with the purpose of enhancing the immune response towards the target cell or to block receptors and thus inhibit down streams intracellular signals (Finn 2008).

mAbs can also be delivered in combination with drugs or radiation particles. The cancer cells engulf the mAbs together with the toxic particles and are thus killed from within. If instead a radiation particle is used, low doses of radiation will be delivered over a long period of time directly to cancer cells and kill them while leaving the majority of surrounding cells undamaged. mAbs combined with immune stimulatory cytokines will help induce immune response in the tumour microenvironment. New challenges include finding new targets and producing antibodies with new or enhanced effector functions, probably in combination with other immune modulatory drugs (Chen and Mellman 2013).

Checkpoint inhibitors

Immune checkpoints refer to specific receptors on immune cells that inhibit the cytotoxic activity and induce tolerance. Tumours can take advantage of these pathways to shut off cytotoxic attacks. Therapies with checkpoint inhibitors aim to block these receptors and restore immune activity. CTLA 4 is such an inhibitory receptor on cytotoxic T-cells and an antibody against it (ipilimumab) is already approved for clinical use. PD-1 is another inhibitory receptor expressed on several immune cells including T-, B- and NK- cells. Therapeutically antibodies have been developed for both PD-1 and its ligand PD-L1 (Yu *et al.* 2009). Another molecule that also can be included as a checkpoint is HLA-G (Carosella *et al.* 2015).

Non-specific immune stimulation or cytokine immunotherapy

Since cytokines are the messengers of the immune system, the idea behind cytokine immunotherapy is to introduce large amounts of cytokines to promote a non-specific immune

response towards cancer cells. The most common administered cytokines are interleukins, interferons and GM-CSF. Interleukins, especially IL-2 regulates activation and differentiation of T-cells. Interferons, such as IFN- α , boost the cytolytic ability of NK cells. GM-CSF stimulates the growth of both blood and immune cells in the bone marrow (Pardoll 2015).

Modified bacteria

An old kind of immunotherapy still in use today is the altered tuberculosis vaccine Bacillus Calmette-Guerin (BCG) that can be administered into the urinary bladder to treat early urothelial carcinoma or to prevent recurrence. The bacteria cause inflammation that guides the immune response to the site of the tumour and destroy the cancer cells (Mellman *et al.* 2011).

Toll-like receptor agonists

Toll-like receptors (TLR) are found on the surface of most immune cells and recognize polysaccharide structures on microorganisms. This is how for example macrophages initially detect possible damaging agents like bacteria or parasites. Toll-like receptor agonists thus aim to activate the non-specific immune system (Vyas *et al.* 2008).

Cancer vaccines

Cancer vaccines can be given prophylactically to prevent the appearance of cancers induced by viruses or as therapy to treat existing cancers. The therapeutic cancer vaccinations include several types; autologous tumour cell vaccines, peptide vaccines, dendritic (APC) cell vaccines and vector based vaccines (Chen and Mellman 2013).

Autologous vaccines are made from extracts from the patients own tumour cells. The cells are treated with irradiation to reduce viability and modified to make the antigens more susceptible to immune attack before infused back into the patient.

Peptide vaccines are small fragments of proteins expressed only on tumour cells and therefore specific for a certain type of cancer. They are not patient specific, but often HLA-A*02 restricted (Norell *et al.* 2006).

Dendritic cell vaccines are custom made for the individual patient. White blood cells are extracted from the patient's blood and matured into dendritic cells in a lab. Thereafter they are exposed to the patient's cancer cells *in vitro* so that they present tumour specific antigens before being injected back into the patient. In the body they activate the T-cells that will now recognize and destroy cancer cells expressing those antigens (Poschke *et al.* 2014).

Vector based vaccines are made from altered viruses or bacteria that no longer cause disease. Instead they are genetically modified to target specific cancer cells and create an immune response, both innate and adaptive. Oncolytic virus immunotherapy targets specific tumour cells, infects them and replicates continuously within the cell until it ruptures, releasing cancer-fighting viruses and immune-stimulating antigens. If the virus contains the gene for GM-CSF, this protein is also released and boosts the immune system (Mellman *et al.* 2011) Adoptive T-cell transfer focuses on manipulating the body's own T-cells to attack cancer cells. Autologous T-cells with specificity for a tumour antigen are harvested, grown and expanded *in vitro* before intravenous administration back into the patient. If the cells are collected from the actual tumour they are only multiplied, but if they are taken from the blood

stream they need modification to recognize specific cancer cells. Sometimes special receptors like chimeric antigen receptors (CARs) are added. CARs are artificial receptors that will activate the T-cell upon recognition of a tumour cell and make it more aggressive and more long-lived (Mellman *et al.* 2011).

11 TUMOUR TYPES IN THIS THESIS

11.1 OVARIAN CANCER

Epithelial ovarian cancer (EOC) accounts for about 3 per cent of cancers in women and according to the cancer registry (2013) in Sweden, approximately 750 women are diagnosed with the disease every year. Unfortunately, the lack of early symptoms and screening tests results in late detection of the disease, and a majority of the patients are diagnosed in advanced stages (Seidman *et al.* 2004)

Ovarian cancer consists of a group of tumours with different histological characteristics, the most important being serous, endometrioid, mucinous and clear cell carcinoma. However, more recent molecular genetic studies have led to the development of a new approach to the pathogenesis of ovarian cancer where tumours are classified by mutational characteristics rather than morphology. The findings indicate that EOC can be divided into two main categories, type I and II. Type II tumours include serous high-grade, high-grade endometrioid, undifferentiated carcinoma and carcinosarcoma, while type I includes the remaining, more low-grade tumour types (Kurman and Shih Ie 2011).

High-grade serous cancer is thus a type II EOC. It is the most common type accounting for approximately 45-55% of EOC cases in Sweden and 75% are diagnosed at a late stage, causing a high mortality rate. Many patients are unresectable at diagnosis or require aggressive abdominal surgery and many receive multiple chemotherapeutic cycles, although most patients relapse. Expected 5 year survival is less than 50% (Agarwal *et al.* 2006). Thus, development of new therapies for serous EOC is urgently needed.

11.2 MALIGNANT MELANOMA

Cutaneous malignant melanoma is less prevalent than non-melanoma malignancies of the skin but the deadliest. The incidence is correlated to decrease in latitude, but is generally increasing with an average of 4 % per year, according to the WHO. In Sweden, almost 3350 individuals, with equal distribution of women and men, are diagnosed with the disease every year and malignant melanoma constitutes almost 6% of malignant tumours according to the Swedish cancer registry (2013).

The main risk factors are genetic, mainly fair-skinned individuals with a large number of atypical nevi, or behavioural risks like increased UV exposure.

When melanoma has metastasized, the mortality rate is high. Local lymph node metastases provide a 5-year survival of 45% and drops to only 10% for distant metastases. The clinical outcome, however, is inconsistent. Strong evidence suggests the existence of a spontaneous

anti-tumour T cell response, clinically observed as halos and regression (Speeckaert *et al.* 2011). There is a great need for better prognostic markers.

12 DIFFERENT SURVIVAL RATES IN CANCER DISEASES IN DIFFERENT COUNTRIES

According to the World Health Organization (WHO fact sheet No 297, updated Feb. 2015) “Cancers figure among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths”. The numbers are expected to rise by as much as 70 % over the next two decades. The Swedish Cancer Registry recorded more than 61 000 new malignant tumours in 2013.

Behavioural and dietary risks, namely tobacco and alcohol use, unhealthy diet, increasing body mass index (BMI) and physical inactivity, account for one third of cancer related deaths, one fifth is believed to be caused by viral infections such as HBV/HCV, HIV and HPV, especially in low- and middle -income countries. Ageing, environmental factors and genetics are of course also fundamental risk factors.

Cancer is not equally distributed over the world, approximately 70 % of cancer related deaths occur in Africa, Asia and Central and South America. This is probably due to insufficient health care and not developed screening programs in combination with behavioural and dietary risk factors. However, taking all this into account, survival rates in different countries still varies (WHO fact sheet No 297, updated Feb. 2015).

In our previous works we have found that patients with malignant solid tumours have an increased vulnerability if they carry the HLA-A * 02-gene subtype. HLA-A * 02 is common in the Swedish population (60 %) and falls in frequency in countries with decreasing latitude. Ovarian cancer and malignant melanoma patients with HLA-A * 02 gene have a poorer prognosis compared to the patients that do not have this gene. This prognostic information is only secondary to clinical stage (De Petris *et al.* 2004).

13 AIMS OF THE THESIS

The overall premise of this work is the notion that HLA-A*02 genotype influences the prognosis in several common malignant tumours. Although the underlying mechanism itself might not be elucidated the findings supports the endeavour to identify subgroups of patients for better and individualized medical treatment.

The specific aim of each paper was:

Paper I: To analyse a cohort of patients with ovarian carcinoma for HLA genotype (HLA-A, HLA-B, HLA-Cw, HLA-DRB1) and determine the frequencies of distinct haplotypes.

Paper II: To analyse a cohort of stage III–IV malignant melanoma patients regarding HLA haplotype and correlation to prognosis.

Paper III: To investigate the possible correlation of HLA-A*02 genotype and MHC class I expression and prognosis in ovarian cancer.

Paper IV: To study the correlation of HLA-A*02 genotype, non-classical and classical HLA class I expression, infiltrating lymphocytes and prognosis in ovarian cancer.

14 MATERIALS AND METHODS

14.1 PATIENTS

These studies are based on the observation that HLA-A*02 genotype was overrepresented in a patient cohort with advanced ovarian cancer that was screened in the purpose of recruiting to vaccine immunotherapy in the Stockholm County.

All 32 participants in **study I** were initially considered for recruitment to an HLA-A*02-restricted peptide vaccination trial. The main inclusion criterion was ovarian cancer and most of them had received more than two lines of chemotherapy. Hence, patients with advanced stages were overrepresented compared to the normal distribution of ovarian cancer patients referred to the Oncology department at Radiumhemmet, Karolinska University Hospital. Seventy-five per cent was diagnosed with serous histology. The full HLA haplotype was analysed on peripheral blood samples and medical records were reviewed for clinical data.

Study II included 91 patients with metastatic malignant melanoma who were initially considered for immunotherapy trials at the oncology department at Radiumhemmet, Karolinska University Hospital. Patients were recruited between 2001 and 2007. They all had a full HLA haplotype analysis performed on peripheral blood. Clinical records were reviewed with regard to age, gender, and tumour stage, site of metastasis and received treatment.

In **study III** we initially considered 182 patients for inclusion. They were all referred to the Oncology department at Radiumhemmet, Karolinska University Hospital between 1995 and 2004. Histological slides were reviewed but only 162 patients had available FFPE blocks of tumour tissue for additional immunohistochemical analysis. DNA analysis for HLA genotyping was performed on peripheral blood from living patients and on FFPE material from deceased patients. Medical records were scrutinized for clinical data. Distribution regarding stage and tumour histology types was in concordance with figures from the FIGO report for European countries. Forty-seven patients with HLA-A*02 genotype had serous adenocarcinoma and advanced surgical stage and were grouped together as a defined group with particular poor prognosis. FFPE tumour blocks were sectioned for immunohistochemical analysis.

In **study IV** we extracted 72 patients from study III. We included all patients with serous ovarian cancer in advanced surgical stage and then grouped them by HLA-A genotype into HLA-A*02 genotype (worst prognosis) or HLA-A of other genotypes. FFPE tumour blocks were sectioned for additional immunohistochemical analysis. An additional eight patients with available frozen ascites were included for a matched primary tumour and metastasis comparison.

Paper	Cohort	Data collected	Number of patients in the study
I	Karolinska University Hospital		32 patients with advanced or relapsing ovarian cancer recruited for vaccination study
II	Sweden	Sept 2001-Feb 2007	91 patients in advanced stage malignant melanoma recruited for immunotherapy
III	Karolinska University Hospital	1995-2004	182 patients with ovarian carcinoma, all stages
IV	Karolinska University Hospital	1995-2004	72 patients with serous adenocarcinoma and advanced surgical stage + 8 patients with matched samples

Table over included patients in the different studies.

14.2 CONTROL GROUP FOR HLA HAPLOTYPE

In **study I and II** we used the healthy Swedish bone marrow donors (HSD) registry (also known as Tobias register) as control. The registry comprised 40,192 individuals with 22,916 females (57 %) and 17,276 males (43 %) at the time. Fifty-eight per cent express the genotype HLA-A*02. All HSD are typed for HLA- A, B, and DRB1 but only a few (350) for HLA-Cw. The early entries in the registry (1990-1998) were serologically defined but later cases typed by genomic analysis comparably to the patients in our studies. The coordinating centre of the Bone Marrow Donors Worldwide Registries (Leiden, The Netherlands) provided us with the data.

For all patients in **studies I-IV** an attempt was made to obtain the original H&E-stained slides of tumour(s) from the Pathology department involved, as well as the original pathology report. When slides could not be found in archives new sections were prepared from paraffin blocks if possible. Several consecutively cut sections were prepared for immunohistochemical staining on chosen blocks. If blocks were not available, patients were excluded from immunohistochemical analyses.

14.3 HLA-GENOTYPING

DNA was extracted from peripheral blood from consented patients or from archived FFPE material from deceased patients. On DNA extracted from peripheral blood we performed a complete HLA allele analysis. However, DNA from FFPE material is too fragmented for complete analysis, but we could use primers specific for HLA-A*02. The analyses were performed by co-author Villabona.

14.4 COLLECTION OF ASCITIC FLUID FOR METASTATIC CELLS

In **study IV** we analysed collected ascites from 8 patients with advanced ovarian carcinoma of serous histology. The ascites had been collected during initial debulking surgery and cryopreserved. Immunophenotyping of thawed tumour cells was performed by flow cytometry. The analysis was performed by co-author Poschke. A cell pellet from each sample was prepared as FFPE blocks for confirming IHC analysis. We also collected corresponding FFPE solid tumours for immunohistochemical comparison. In available cases, we collected tumour tissue from both primary sites, defined as the site with the greatest tumour mass and from distant lesions fulfilling the criteria of at least stage III disease.

14.5 TUMOUR TISSUE

Histological slides were reviewed and one FFPE block from each case was chosen. Sections were cut 4 μ m thick and mounted onto super frost slides.

The sections were prepared and stained according to protocols described in study III. We used IgG1, IgG2a mouse mAb or simply omitted the primary antibody for negative controls.

14.6 ANTIBODIES USED IN THE DIFFERENT STUDIES

Paper III: HC-A, HC-10 and L368 for IHC.

Paper IV: HLA-G, HLA-E, CD8 and FOXP3 for IHC.

W6/32, BB7.2, EpCAM, HLA-E and HLA-G mAbs for flow cytometry.

Antibodies for classical MHC class I HC and β 2m: HC-A, HC-10 and L368

The MHC molecule is typically presented on the cell surface as a trimeric complex together with β 2-m and a peptide. The full trimeric complex is recognized with the mAb W6/32, but this does not work in FFPE tissues. Both HC-A and HC10 recognize the β 2m-free HC of MHC. The mAb HC-A recognizes most products of HLA-A (excluding -A*24), but also HLA-B*73:01 and HLA-G. The mAb HC-10, recognizes most HC products of HLA-B (excluding -B*57:02, -B*58:04 and -B*73) but also HLA-A*03, -A*10, -A*28, -A*29, -A*30, -A*31, -A*32, -A*33. By combining these antibodies we could cover products from most HLA haplotypes, except from HLA-A*24. HLA-A*24 is a common genotype in some Asian countries but very rare in the Swedish population. Especially considering that most humans are heterozygotes the risk of having false negative results is very low.

The mAb L368 is specific for β 2-m. We investigated this to see if there was any significant

difference in expression of MHC and β 2-m that would imply that the HC was not properly presented on the cell surface.

To ensure detection of any significant differences we scored the percentage of stained malignant cells in semi quantitative intervals as follows: 0 (0 %), 1 (1–25 %), 2 (26– 50 %), 3 (51–75 %) or 4 (76–100 %). Staining intensity was scored as weak, moderate or intense. The localisation of positive staining was recorded as membranous and/or cytoplasmic.

Antibodies for non-classical MHC class I: MEM-E/02 and MEM-G/1

The monoclonal antibodies MEM-E/02 and MEM-G/1 are specific and recognize the HC of HLA-E and HLA-G of human origin respectively.

HLA-G expression was evaluated as positive or negative. Most of the positive cases were only weakly positive and stained only a small fraction of tumour cells.

HLA-E is normally expressed in most human tissues and was therefore evaluated as absent, less intense than, equal to or more intense than normal tissue using score 0-3. Score 2 was regarded as normal expression whereas 0,1 and 3 were regarded as aberrant. Since all lesions showed a very heterogeneously staining pattern each slide was given two scores, the first for the dominating cell population and the second for the subordinate cell population.

Antibodies for immune cells: CD8 and FOXP3

The mAb Anti-CD8 (Clone C8/144B (Dako)) reacts with the cytoplasmic domain of the α -chain of the CD 8 molecule. It is expressed by cytotoxic T cells, thymocytes and NK- cells. Quantification of intratumoural CD8+ cells were counted in high power fields and divided into four scoring groups; 0=0 T cells, 1= \leq 5 T cells, 2=6-19 T cells and 3= \geq 20 T cells.

Anti FOXP3 is a specific marker of natural T regulatory cells (nTregs). nTregs were recorded as present or absent.

Antibodies for flow cytometry analysis: W6/32, BB7.2, Ep.CAM, HLA-E and HLA-G.

Flow cytometry analysis offers the possibility to use different and complementary antibodies together. Especially the W6/32 antibody (Fitc, BD) recognizing the trimeric HLA/ β 2m/peptide complex can be used, but also mAb BB7.2 (Fitc, BD) which is specific for MHC HC of HLA-A*02 genotype (Hilton and Parham 2013).

EpCAM (EBA-1, PerCP-Cy5.5, BD) is expressed in most epithelial cells and malignancies of epithelial origin. It was used to determine the amount of living epithelial (cancer) cells in ascites samples.

HLA-E (3D12HLA-E, PE, eBioscience) and HLA-G (87G, PerCP-eFluor710, eBioscience) are specific for HLA-E and –G expression respectively.

14.7 STATISTICAL ANALYSIS

Survival. Study II, III and IV

The χ^2 test was used to establish the significance of differences in discrete categorical

variables or factors in patient characteristics. Primary end point was defined as time to death from any cause. Survival time was calculated from the date of first diagnosis to the date of death from any cause or last clinical follow-up.

In **study II** the overall survival was split into two time variables; time from primary diagnosis to first metastasis, versus time from first metastasis to end point.

Kaplan–Meier method was used for cumulative survival rates estimation. The log-rank test was used for comparisons to detect univariate differences between groups.

Univariate Cox regression analyses were performed for each prognostic factor. Results from the Cox regression models were presented as Hazard Ratios (HR) together with 95 % confidence intervals (CI). P values from the regression model refer to the Wald test.

Multivariate Cox regression analyses were performed including binary coding of all factors. P values <0.05 were considered statistically significant. Interactions were evaluated by including product terms in the model.

We used StatView™ for Windows, SAS Institute Inc. Version 5.0.1 and STATISTICAL™ StatSoft Inc. Version 7.

HLA gene and haplotype frequency calculation. Study I and II

The frequencies of HLA class I and II genotype was calculated by dividing the number of alleles for each HLA type with the number of total patients. Homozygotes were counted once. Haplotype frequency was determined by the maximum likelihood method.

Fisher's exact test was used to calculate the statistical significance of the frequencies of individual HLA alleles or haplotypes compared to HSD.

Two-tailed P values were utilized to detect positive and negative associations. P value <0.05, adjusted for the Bonferroni correction was regarded as significant, but also non-significant P values were recorded.

15 RESULTS

15.1 RESULTS PAPER I

Genotyping for HLA-A, -B, -Cw and DRB1 was performed on the 32 included patients. We found an increased frequency of HLA-A*01, HLA-A*02 and HLA-B*08 but decreased frequency of HLA-A*03 compared to controls. HLA-A2 homozygotes were twofold higher in patients. Combinations of A*02-B*05, A*02-B*15, A*02-DRN1*03, A*02-DRB1*04, A*02-B*15-Cw*3 and A*02-B*08-DRB1*03 had odd ratio as well as the level of the lower confidence interval above 1 and significant P-value, but only when considered as single, non-corrected analysis. HLA-B*15 and HLA-Cw*3 were only detected in HLA-A*02 positive patients. The multi-gene haplotype HLA-A*02, -B*15, -Cw*3, -DRB1*04 was segregated. This is a known ancestral haplotype called 62.1.

15.2 RESULTS PAPER II

In this cohort of 91 patients with malignant melanoma in stage III or IV we found that the presence of the AHH 62.1 (HLA-A*02, -B*15, -Cw*3, -DRB1*04) was associated with prolonged relapse free- period from the date of the primary diagnosis, but also a significantly shorter survival time after the appearance of the first metastasis. There was no significant difference in overall survival. The findings indicate that AHH 62.1 promotes strong anticancer immunity, but also that a selection of malignant clones that eventually will escape the immune surveillance might take place.

15.3 RESULTS PAPER III

We confirmed that down-regulation of MHC class I antigen in tumour tissue correlated with worse prognosis in this cohort of 162 patients with ovarian carcinoma. We also demonstrated a significant higher frequency of MHC down-regulations in patients with HLA-A*02 genotype and serous ovarian cancer in advanced stages, i.e. the group that we have previously identified as "worst prognosis group (WP)". The characteristics and poor prognosis of this group was validated in this study. When we performed a multivariate Cox-analysis, the WP group had a significant higher HR compared to loss of MHC class I HC expression. Only clinical stage had higher HR.

15.4 RESULTS PAPER IV

In this cohort of selected patients with serous ovarian cancer in advanced stages we could show that patients expressing HLA-G and/or aberrant expression of HLA-E had a significantly worse prognosis if they carried the HLA-A*02 genotype but not otherwise. Foci with expression of HLA-G correlated with loss of classical MHC class I and aberrant expression of HLA-E in the same area. This combination was significantly correlated to worse prognosis.

Despite the limited number of matched cases we found a trend that the expression of HLA-G is more frequently observed in ascites or metastatic tumour lesions compared to tumour tissue from primary site. Expression of HLA-G showed inverse correlation to tumour infiltrating lymphocytes.

16 POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund

Maligna tumörsjukdomar drabbar ca 14 miljoner människor och orsakar drygt 8 miljoner dödsfall per år i världen. Äggstockscancer och malignt melanom i huden hör till de tumörformerna med högst dödlighet och är i stort behov av nya behandlingsmetoder. Individanpassad medicinsk behandling syftar till att anpassa val av behandling till rätt patienter, samt att ge behandling vid lämpligast möjliga tidpunkt. För detta krävs hänsynstagande till både patientens grundhälsa samt information om patientens genetiska profil och tumörens specifika egenskaper som kan mätas som biologiska markörer i blod eller vävnad. Idag är det rutin att screena efter en rad olika mutationer vid tumörsjukdomar. Ofta finns det en direkt korrelation mellan sjukdom och en muterad gen som avgör om patienten kommer ha nytta av ett visst läkemedel eller inte.

HLA är en grupp gener som återfinns på kromosom 6. Generna kodar för en molekyl på cellernas yta som kallas MHC. Genom MHC kan kroppens vita blodkroppar känna av om en cell har blivit infekterad och skall avlägsnats, eller om den tillhör kroppen och skall bibehållas. Eftersom tumörer har förändrade proteiner, kan de vita blodkropparna upptäcka dessa med hjälp av MHC. De kommer då uppfatta dessa celler som främmande i kroppen och attackera dem. Tyvärr kan tumörceller använda sig av många olika försvarsmekanismer mot immunförsvaret. När tumörceller lyckas överlista immunförsvaret kan de växa till sig obehindrat och sprida dottertumörer till andra lokaler i kroppen. Detta kallas tumour escape. MHC är således helt avgörande för människans immunförsvaret och överlevnad.

MHC på cellens yta är en kombination av proteiner från olika HLA-gener och variationen är stor mellan människor i olika världsdelar, vilket sannolikt beror på olika överlevnadsvinster beroende på miljö. Även om HLA varierar stort mellan individer och befolkningsgrupper så finns det delar av generna som är starkt sammanknutna och ärvs i grupp, så kallade ancestrala haplotyper (AHH). Dessa kan spåras långt tillbaka i människans historia och har bidragit mycket till kunskapen om människans vandring mellan världsdelar.

Man har sett att olika HLA typer är kopplade till olika sjukdomar. Till exempel är HLA-B*27 känd för att öka risken för Bechterews sjukdom. HLA-A*02 är främst kopplad till olika autoimmuna sjukdomar som t.ex. diabetes.

Den här avhandlingen baseras på tidigare upptäckt att HLA-A*02 innebär en markant sämre prognos för patienter med spridd äggstockscancer av serös typ, samt prostatacancer och malignt melanom i huden. HLA-A*02 är vanlig i den svenska befolkningen (ca 60 %) men ses mindre frekvent i europeiska länder söderut. Vi har tidigare visat att dödligheten i ovannämnda tumörsjukdomar är kopplad till förekomsten av HLA-A*02 i befolkningen i olika länder. Speciellt har vi kunnat visa att patienter med spridd, serös äggstockscancer har en extremt dålig prognos om de bär HLA-A*02 genen. Patienter med denna olyckliga kombination av faktorer har vi kallat ”worst prognosis group”.

Syftet med den här avhandlingen var att undersöka hur HLA genotypen förhåller sig till prognos och andra kända biologiska markörer såsom uttryck av MHC på cellernas yta samt närvaro av tumörinfiltrerande lymfocyter hos patienter med äggstockscancer och malignt melanom i huden.

Undersökta tumörtyper.

Äggstockscancer utgör ca 3 procent av tumörsjukdomar hos kvinnor. Äggstockscancer ger sena symtom och ca 75 % upptäcks först när tumören är spridd varför dödligheten är relativt hög. Det finns egentligen flera olika typer av cancer som kan uppstå i äggstockarna. Den vanligaste kallas högradig serös cancer (ca 45-55 %) och är en aggressiv variant. Om tumören är spridd vid diagnos kan patienten inte alltid opereras, eller så krävs mycket omfattande kirurgi som i sig är en risk. Många patienter behöver dessutom upprepade cellgiftsbehandlingar. De flesta patienterna i denna grupp har en förväntad femårsöverlevnad under 50 %.

Malignt melanom i huden utgör ca 6 % av maligna tumördiagnoser per år. Det är den dödligaste formen av elakartade tumörer i huden, men upptäcks ofta relativt tidigt eftersom den ses som en avvikande fläck. Om tumören är liten och opereras bort tidigt är prognosen god, men om patienten hunnit få spridning av sjukdomen är 5-årsöverlevnaden mycket sämre, 45 % vid spridning till närliggande lymfkörtlar och endast 10 % vid spridning till andra organ.

Delarbete I

Arbetet syftade till att analysera förekomsten av olika HLA typer i en grupp på 32 patienter med spridd äggstockscancer. Med PCR analyserades DNA-sekvenser för HLA-A, -B, -Cw och DRB1. För jämförelse användes HLA frekvensen hos svenska benmärgsdonatorer. Hos patienterna såg vi fler HLA-A*01, HLA-A*02 och HLA-B*08 men färre HLA-A*03 jämfört med kontrollgruppen. Särskilt såg vi en fördubbling av patienter som hade ärvt HLA-A*02 från båda föräldrarna (homozygoter) i patientgruppen, det vill säga, vi hade en hög överrepresentation av främst HLA-A*02. Vi kunde även se att kombinationen HLA-A*02, -B*15, -Cw*3, -DRB1*04 var segregerad. Detta är en känd AHH, dvs kombination av gener som ärvs i grupp, som även benämns 62.1. Den är vanlig i Sverige och är sedan tidigare associerad med autoimmuna sjukdomar.

Delarbete II

Det intressanta med melanom i huden är att man har god uppfattning om tidsförloppet i sjukdomen eftersom sjukdomen upptäcks tidigt och hela sjukdomsförloppet dokumenteras väl. Vi analyserade frekvensen av olika HLA genotyper hos 91 patienter med malignt melanom och spridd sjukdom. Vi kunde se att AHH 62.1 var kopplat till förkortad överlevnad efter första metastas, men förlängd tid mellan första diagnos och första tecken på spridning av sjukdomen. Resultaten tyder på att AHH 62.1 skulle kunna vara kopplat till den tumörimmunologiska "elimination-equilibrium-escape" mekanismen. Om man initialt har en stark immunologisk kontroll kan tumören kontrolleras under lång tid, men tills slut lyckas några tumörceller mutera till att bli extremt effektiva på att smita undan och lura

immunförsvaret. De kan då växa till helt obehindrat vilket leder till ett mycket aggressivt sjukdomsförlopp. Det här fenomenet kallas immune selection.

Delarbete III

För att undersöka en möjlig mekanism bakom den dåliga prognosen kopplad till HLA-A*02, ville vi undersöka uttrycket av HLA-genens produkt, dvs. proteinuttrycket av MHC på tumörcellernas yta. Man har sett i andra studier att prognosen i olika tumörsjukdomar försämras om MHC inte uttrycks som det ska. I teorin beror det på att tumörcellerna blir ”osynliga” för immunförsvaret.

Vi undersökte 162 kvinnor med äggstockscancer. HLA genotypen analyserades på DNA från blodprov eller DNA från tumören, beroende på vad vi kunde få tillgång till.

Vid immunfärgning på tumörerna såg vi att nedsatt uttryck av MHC var kopplat till sämre prognos. Vårt fynd stämde således väl med det som är beskrivet i andra studier. Vi kunde dock även konstatera att nedsatt MHC uttryck var vanligare hos patienter med HLA-A*02 genotyp och serös ovarialcancer i avancerat stadium, dvs. den grupp som vi tidigare identifierat som "worst prognosis group".

När vi jämförde de olika prognostiska markörerna såg vi att nedsatt MHC uttryck endast kunde kopplas till försämrad prognos hos patienterna i ”worst prognosis group”. Det visade sig dock också att alla patienterna som tillhörde "worst prognosis group" hade mycket sämre prognos än de andra grupperna, oavsett om de hade uttryck av MHC eller inte. Resultatet tyder på att mekanismen bakom HLA-A*02s prognostiska egenskap inte bara är kopplat till uttrycket av MHC-proteinet utan att andra okända faktorer också är inblandade.

Delarbete IV

I detta arbete valde vi endast ut patienter med serös äggstockscancer och spridd sjukdom, dvs ”worst prognosis group”. Sjuttio-två patienter samt åtta patienter där vi hade tillgång till ascites-material, inkluderades. Vi ville undersöka om det finns en korrelation mellan HLA-A*02 och uttryck en grupp så kallade icke klassiska MHC, HLA-G och -E. Dessa HLA typer bidrar normalt till att skydda kroppens celler från immunförsvaret. HLA-G ses normalt i moderkakan under graviditet för att skydda fostret från moderns immunförsvaret, medan HLA-E finns på de flesta celler i kroppen och skyddar mot angrepp från NK-celler. Både HLA-G och -E är således molekyler som dämpar immunförsvaret. Därför tittade vi även på om det fanns kopplingar till förekomsten av vita blodkroppar inom tumören.

Vi kunde se att både uttryck av HLA-G och onormalt uttryck av HLA-E innebar en klart sämre prognos, men igen bara hos patienter med HLA-A*02 genotyp. De patienter med annan HLA genotyp hade bättre överlevnad oavsett om de hade uttryck av de immundämpande HLA-proteinerna eller inte.

Vi hade bara åtta patienter där vi kunde analysera både huvudtumör och spridda tumörceller, men kunde ändå se en trend att uttryck av HLA-G var vanligare i spridda tumörceller än i huvudtumörer.

Till sist kunde vi visa att tumörer med HLA-G uttryck hade jämförelsevis mindre förekomst av vita blodkroppar.

Sammanfattningsvis har vi visat att genen HLA-A*02 är kopplad till en klart försämrad överlevnad hos patienter med spridd tumörsjukdom av serös äggstockscancer eller malignt melanom i huden. I patientgruppen med malignt melanom kunde vi även se att HLA-A*02 verkar ge ett initialt förstärkt skydd mot cancerceller, eftersom dessa patienter hade förlängd tid mellan primärdiagnos och första metastas jämfört med andra HLA haplotyper, Vidare har vi sett att andra kända prognostiska markörer, som t.ex. nedsatt uttryck av MHC, eller ökat uttryck av HLA-G eller förändrat uttryck av HLA-E är kopplat till sämre prognos, men bara i den grupp patienter som är bärare av HLA-A*02-genen. Kopplingen mellan olika molekyler och gener i tumörer är komplex och det är svårt att ge en fulltäckande förklaringsmodell. Vad vi har påvisat är dock vikten av ett multifaktoriellt synsätt. Om man samlar så mycket information som möjligt om både tumör och biomarkörer, men även om patientens unika genotyp, ökar möjligheterna att verkligen kunna individanpassa den medicinska behandlingen.

17 GENERAL DISCUSSION

This project is based on three important new original findings. First an increased frequency of HLA-A*02 among patients with metastatic ovarian cancer treated at the Department of Oncology Radiumhemmet, second, the higher frequency of HLA-A*02 among normal Swedish population compared to the other European countries and thirdly a significant correlation of the HLA-A*02 frequency to the degree of latitude in Europe and a correlation to age adjusted mortality rates in ovarian cancer, prostate cancer and malignant melanoma in 16 European countries.

In this thesis we have investigated known prognostic factors in relationship to HLA-genotype and correlated to clinical outcome.

In **paper I** we could confirm an unusual overrepresentation of HLA-A*02 in the patient cohort and we could furthermore demonstrate a segregation of the ancestral haplotype 62.1 (HLA-A*02, -B*15, -Cw*3, -DRB1*04). AHH 62.1 is a common haplotype in Sweden and known to be associated with autoimmune diseases. However it seems paradoxical that a haplotype with strong immunogenicity should be correlated to a shorter survival when one should have expected a relatively stronger anti-tumour control.

Our findings in **paper II** indicate that AHH 62.1 indeed seems to initially exercise a strong anti-tumour control but also that a selection of malignant clones, able to escape immune surveillance, might take place. The time frame of the cancer-immunity cycle is shifted compared to patients with other haplotypes even if the overall survival did not differ between groups. The results support that different HLA haplotypes affect the tumour microenvironment and that the threshold in the "elimination-equilibrium-escape" mechanism is reached at different times. An initially effective elimination is more likely to select particularly aggressive tumour clones compared to more moderate immunity.

Paper II give valuable information since patients with malignant melanoma have a disease progression that is relatively easy to monitor clinically. In ovarian cancer patients we lack this information since the disease is often "silent" in the beginning. When patients finally seek medical care, most of them are already in advanced stages. We therefore have no data on for how long the disease has occurred, it might very well be that patients with extraordinary short survival (our worst prognosis group) have had a very long elimination-equilibrium period, but we only see their escape phase. If HLA haplotypes affect the balance in the cancer-immunity cycle, HLA typing may provide valuable clues in the choice of immunotherapy. A fundamentally strong immune system might be best complemented with checkpoint inhibitors and drugs restoring MHC expression or bi-specific antigens guiding the immune cells to the escaping clones, whereas weaker immune systems may benefit more from a combination of both drugs promoting stimulatory factors and checkpoint inhibitors.

One frequently described escape mechanism is loss or down regulation of MHC expression, making the tumour cell invisible for T-cell recognition. HLA-A*02 allele loss is also frequently described in tumours (Norell et al. 2006). In **paper III** we confirmed the

correlation of MHC down-regulation with worse prognosis but we also demonstrated that the WP group had a significant higher HR compared to MHC class I HC down-regulation in multivariate analysis. Only clinical stage had higher HR. In fact, loss of MHC class I expression had no significant impact on survival in patients who did not fulfil the criteria for worst prognosis grouping. Our results indicate that the prognostic impact of HLA-A*02 is reasonably not only correlated to the expression of its protein products and that genetic or mRNA factors also may have to be taken into consideration.

In **paper IV** we introduced the analysis of HLA-G and -E. The rationale behind this was that both genes are found close to the class I region on chromosome 6 and they provide additional escape mechanism frequently described in tumours. We could not find any correlation between HLA-A*02 genotype and expression of HLA-G, it was equally distributed between patients with or without HLA-A*02 genotype. However, HLA-G expression had an impact on survival only in the HLA-A*02 population and the same applied to HLA-E malfunctions. Furthermore, in the HLA-A*02 population, we found that the small foci with HLA-G expression was consistent with loss of the classical MHC class I HC expression and aberrant HLA-E expression in the same tumour area in most cases. These patients had a very poor prognosis of approximately 18 months. The results indicate that the combination of HLA-A*02 genotype and malfunction of the non-classical HLA class I induces tumour escape.

Immunohistochemistry

Analysis on FFPE tissue reveals a lot of interesting and important findings. However, the picture is often complex and it can be hard to discriminate what is prognostically important and what is not. Many tumours show a heterogeneous pattern and sometimes several levels of differentiation grade or even a mixture of tumour types. In immunohistochemical staining this may result in a heterogeneous staining pattern as well. In most cases a percentage cut off is agreed upon, an academically compromise that only partly reflects the true biology. All new antibodies therefore need waste numbers of test staining on different samples to build up experience for a good interpretation. When dealing with comparable new antibodies, especially those not yet in clinical use, one must always remember that new insights in the future may lead to different scoring methods.

HLA genotype analysis.

Each HLA allelic variant has its unique peptide binding capacity, which is reflected in both the specificity and functionality. For truly personal treatment one might therefore need to consider high resolution allele typing.

It would have been preferably to have the full haplotype of every patient included in the studies, however, from deceased patient only FFPE tissue from archives were available and due to DNA fragmentation we could only perform HLA-genotyping with primers specific for HLA-A*02. However, our studies include Swedish patients and approximately 95 % of HLA-A*02 individuals in Caucasian population are of HLA-A*02:01 allelic type.

18 FUTURE PERSPECTIVES

Most patients today are still diagnosed and treated according to standardized protocols but the trend towards individualized treatment is obvious. More biomarkers are used in order to better predict response to treatment and the emerging field of immunotherapy is advancing rapidly. The Food and Drug Administration (FDA) have already approved many new regimes and many more are in clinical trials.

Our research validates the hypothesis that HLA typing provides better access to prediction of outcome in patients with advanced ovarian cancer and malignant melanoma. Our results indicate that HLA-A*02 plays a fundamental role in the tumour surveillance, equilibrium and escape mechanism. It is of course of great importance to investigate if these findings holds true in other types of cancers.

We also have indications that HLA-A*02 prolong the equilibrium phase period but that this ends with a very short escape period. These results emphasise the need for better tests for early diagnose to have a chance for treatment before the tumour has spread. It may also give guidance in the choice of immune therapy.

In all our studies we have not been able to elucidate the mechanism behind the poor prognosis linked to HLA-A*02, but what we have seen is that the prognostic significance of the known prognostic factors that we introduce only can be confirmed in patients with HLA-A*02 genotype. This underlines the need of a multifactorial approach with special attention paid to the complexity of tumours and the genetics of the patient.

It is clear that tumours show a broad spectrum of mutations and many of the known onco- or suppressor genes have obvious direct consequences. However, if one considers that multiple genetic mutations in a single tumour may have effects that either reinforce or counteract each other the picture becomes very complex. Many studies published, including those in this thesis, analyse a handful of biomarkers at a time, with the result that a biomarker can provide an outcome in one study but a completely different outcome in another study model.

Mathematical calculations may take into account such effects and can confidently describe the whole network of mutations and their relative effects on the phenotype of tumours. A report in Nature Genetics in 2014 (AlQuraishi *et al.* 2014) describes just such a mathematical model but there are many more examples outside the medical science. By storing large amounts of data one can construct interesting maps over factorial relationships. With the right questions and the right point of view, one can clarify relationships that from the beginning are not so obvious.

A large number of clinical trials, even though initially promising, are closed due to small effect or major side effects. A better characterisation of included patients might improve results and offer a better fit between medicine and patients.

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